TOPICS IN GENETIC ANALYSIS

G. PONTECORVO
Topics in Genetic Analysis

Selected Papers of G. Pontecorvo Raman Professor, 1982-83

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FOREWORD

Prof. Guido Pontecorvo visited India for three months during December 1982-February 1983, as Raman Professor of the Indian Academy of Sciences. Prof. Pontecorvo and Mrs. Pontecorvo spent much of this period in Bombay (the Tata Institute of Fundamental Research) and in Bangalore (the Indian Institute of Science). He also visited and lectured at Universities and Institutions in Delhi, Poona, Hyderabad, Calcutta, Madras, Ahmednagar, Patna and other cities. The Academy takes great pleasure in bringing out this selection of papers by one of its distinguished Honorary Fellows. It also includes his Gandhi Memorial Lecture which he delivered at the Raman Research Institute, Bangalore.


S. RAMASESHAN
President
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Prof. G. Pontecorvo
Guido Pontecorvo was born in Pisa (Italy) in the year 1907. He studied classics and graduated from the University of Pisa in Agricultural Science, summa cum laude, in 1928; two years later, he entered the Agricultural Advisory Service in the city of Florence as an animal breeder. Fascism in Italy and the gathering clouds of war in Europe forced Pontecorvo to emigrate to Scotland. In 1938 he joined a select band of students who had gathered at the University of Edinburgh around the great American geneticist H.J. Muller.

Pontecorvo took his Ph.D. from the Institute of Animal Genetics at Edinburgh and joined the Zoology Department at Glasgow University in 1941 on a Carnegie grant. Two years later he was appointed lecturer at the University of Edinburgh. In 1945, Pontecorvo returned to Glasgow. His earlier researches were concerned with animal breeding and radiation effects and genetics of speciation with Drosophila. At Glasgow Pontecorvo began his work with Aspergillus nidulans a line of research which was to earn him a place of his own in the annals of genetics. He was successively appointed Lecturer, Reader and then Professor of Genetics as the first occupant of the Chair in genetics at Glasgow. In 1956 he was appointed Director of the Institute of Genetics and Head of the Unit of Somatic Cell Genetics created by the Medical Research Council. In 1968, Pontecorvo moved to London to join the staff of the Imperial Cancer Fund Laboratories at Lincolns Inn Fields. He continues to work there after his formal retirement in 1980.

The years in Glasgow were very fertile. The Genetics Department was located in the old medical school, a somewhat dilapidated and morose structure, in which Pontecorvo’s group occupied a few rooms. It was, in the fifties, a highly international group with research scholars and visitors drawn from many parts of the world, attracted by Pontecorvo’s ideas and reputation. There was a continuous stream of visitors. Some came to talk about their own work, others to learn the intricacies of mitotic genetics or to apply its methods to mammalian cell genetics. There were animated conversations on the mutation theory, gene regulation and recombination and, above all, on DNA and the newly arrived idea of the genetic code. Pontecorvo is a fervent believer in working with one’s own hands. His professorial office was a small desk in his one-room laboratory. Sir Michael Stoker records that when he offered Pontecorvo a position at the Imperial Cancer Fund, Pontecorvo’s answer was that he would come providing his laboratory was small enough and there were no assistants.

Among Pontecorvo’s varied contributions to genetics, the two that stand out as most consequential are his discovery of the parasexual cycle in fungi; this led him to develop methods of genetic analysis that were the forerunners of modern somatic cell genetics, an approach to genetics of higher animals that has revolutionised our knowledge of human genetics. The other is his work on intragenic recombination and the seminal paper in 1952 on the organisation of the genetic material. This paper outlined a new theory of the gene, a year before the discovery of DNA structure by Watson and Crick and the revision of the gene concept by Seymour Benzer.

First about the parasexual cycle; classical genetics was restricted to organisms with a biparental sexual cycle. The development of microbial genetics, in the forties, brought into prominence new and unconventional genetic systems of fungi, bacteria and viruses which greatly extended the scope and power of genetic analysis. The
ground for this new flowering of genetics in the fifties was laid by the work of Beadle and Tatum on *Neurospora crassa*. Pontecorvo entered the field of biochemical genetics with a homothallic fungus, *Aspergillus nidulans*. The life cycle of *Aspergillus* lends itself to conventional sexual genetics, provided appropriately marked strains can be crossed. To do this Pontecorvo made use of "forced heterokaryosis". Working with heterokaryons Roper and Pontecorvo discovered spontaneous diploidisation of nuclei during vegetative growth and the formation of diploid heterozygous conidia. Pontecorvo realised the importance of this discovery in that, it freed genetic analysis from the restriction of an obligatory sexual cycle. His colleagues and he demonstrated mitotic crossing-over and segregation in diploid mycelia and worked out the modalities of gene mapping through mitotic crossing-over and haploidisation. Crossing-over in somatic cells had been studied by Curt Stern in *Drosophila*. Pontecorvo foresaw that mitotic segregation could be harnessed to extend genetics to asexual species. This was a decisive step in the development of somatic genetics. In the later fifties, Pontecorvo himself switched to working with human cell cultures. His ideas thus played a critical role in the rapid development of human genetics through the parasexual cycle. In 1975, Pontecorvo applied to human cells the method of fusion by polyethylene glycol, and a technique for selectively eliminating chromosomes from fused cells by irradiation and other radiomimetic treatments.

A second development of considerable importance in modern genetics to which Pontecorvo made an important contribution concerns our understanding of the nature of gene. The picture of gene that was nearly universally accepted before the forties was that of "beads strung along a thread". In practice a gene was recognised by different sorts of operations; tests of allelism depend upon function, mutations indicate the parts of a gene that can undergo a stable change, crossing-over indicates separation of mutable loci. It was tacitly assumed that these different operations referred to the same entity, "the particulate gene". A chink in this simplistic picture appeared when E.B. Lewis, in 1945, found that the mutations of the *bithorax* locus of *Drosophila*, although allelic from a functional point of view, could, nevertheless, recombine with each other. Recombination between alleles, in classical genetics, was a paradox. Lewis himself believed, as it indeed turned out to be the case, that the bithorax locus was a string of functionally related genes. He termed the apparent allelism of such genes "pseudoallelism". Pontecorvo saw, in this phenomenon, the possibility of a radically different interpretation. He assumed that allelic recombination was a much more general phenomenon, a universal property of all genes. In a seminal paper entitled "Genetic formulation of gene structure and gene action" published in 1952, in *Advances in Enzymology*, Pontecorvo pointed out that different operational definitions of a gene need not refer to the same entity and the gene as a unit of function could be a segment of the chromosome stretched over a considerable length. Crossing-over could occur within this length. The picture of genes as a linear array of recombinable units was vindicated a year later by the structure of DNA proposed by Watson and Crick and by the brilliant work of Seymour Benzer on the rII genes of Phase T4 that was to follow.

In his Jessup lectures entitled "Trends in Genetic Analysis", Pontecorvo traces several of his insights into the nature of the genetic material to his teacher H.J. Muller. Indeed he has expressed the opinion on several occasions, including
his Raman lectures on the Development of Genetic Ideas, that Muller's contributions to the evolution of the gene theory have not been adequately appreciated. Muller had an equal regard for his pupil. Prof. S.P. Ray Chowdhury, a contemporary of Pontecorvo at Edinburgh, once told me the following anecdote. Muller, it seems, had the habit of supervising his students rather closely, urging them to organize their work into well-spelled projects and encouraging them to mutually criticize each other's plans. Someone in the group once asked him why he did not do this to Pontecorvo. Muller replied "Guido? He does not need this; he has his own ideas."

Pontecorvo has received many distinctions and honours. He was Jessup Lecturer, Columbia University (1956); Messenger Lecturer, Cornell University (1958); Leeuwenhoek Lecturer, Royal Society (1960); Royal Society Visiting Professor at Rio de Janeiro (1967) and Shiraz (1974); Visiting Professor at Middlebury College and Washington State University, and Guest Professor of Academia dei Lincei, Pisa (1975-80). In 1982-83, he was Raman Professor at the Indian Academy of Sciences and delivered the Gandhi Memorial Lecture. In 1984, he gave the Jean Weigle Memorial Lecture at the California Institute of Technology. Pontecorvo has received the E.C. Hansen Medal of the Carlsberg Foundation, the Darwin Medal of the Royal Society and the Honorary Compano d'oro of the University of Pisa. He is a Fellow of the Royal Society of London, of the Royal Society of Edinburgh, a foreign member of the American Academy of Arts and Sciences, Danish Royal Society of Science and Letters, U.S. National Academy of Sciences, the Indian Academy of Sciences and the Indian National Science Academy. He is a former President of the British Genetical Society and a former Vice-President of the Institute of Biology.

He was awarded honorary degrees by many universities including the L.L.D. of the University of Glasgow, but above all these honours and recognitions is the admiration and affection in which Pontecorvo is held by his many friends, colleagues and students in the community of geneticists. Known to them as Ponte or simply Guido, he is loved for his personal warmth and uncompromising scientific spirit. A man of strong likes and dislikes, Pontecorvo has no use for the pompous and the pretentious.

Sir Michael Stoker, President of Clare Hall, Cambridge, and a long time friend and colleague remembers his days in Glasgow in the following words: "Some of us came to know Ponte in Glasgow. There, a group of virologists found that their neighbour was one of the world's leading geneticists, full of friendly wisdom and encouragement, providing you did not call him Professor Pontecorvo. It was an exciting time, frequently enlivened by his regular battles with the University establishment. My own attempts to moderate his colourful and libellous letters to a succession of Vice Chancellors were not always successful". The present author can recall one of these enlivening episodes. The Vice Chancellor was to bring an important visitor Quentin Hogg (now) Lord Hailsham, then Minister of Science in the British Government to see the Genetics Department. Ponte walked into the small and crowded room in which several of us worked to announce the impending visit. One of the students, rather pertly, asked Ponte what he would like us to say to the Minister. Pat came the answer "Make as much heat and smoke and steam
as you can. The Minister, in any case, will not understand anything. At least the Vice Chancellor should see the terrible conditions in which we work”.

In his youth in Pisa, Pontecorvo was one of a small group of avid mountaineers. This group included the physicists Enrico Fermi, Emilio Segre and Franco Rosetti. The love of mountains and flowers, then acquired, has remained with Pontecorvo, a life-long passion. His expertise in alpine plants is no less than his knowledge of genetics. Currently, Ponte is writing a book on Plants at High Altitudes.

Obaid Siddiqi
Tata Institute of Fundamental Research
LIST OF PAPERS REPRODUCED

I. Mutation and Recombination


II. Somatic Cell Genetics


III. Essays


THE INDUCTION OF CHROMOSOME LOSSES IN DROSOPHILA SPERM AND THEIR LINEAR DEPENDENCE ON DOSAGES OF IRRADIATION

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(With one Text-figure)

I. THE PROBLEM

The latest works on the induction of gross chromosome rearrangements by radiation—genetical ones by Muller and co-workers (1938, 1939, 1940) including Belgovsky (1937) on Drosophila and cytological ones by Sax (1938, 1939, 1940) and Fabérgé (1940) on Tradescantia microspores, to quote only those giving the most decisive evidence in this regard—leave no doubt that the process of structural change occurs in two distinct stages. These are (a) primary changes, produced by individual ionizations, and (b) combinations of the primary effects. It is inferred that these two stages consist, respectively, of (a) breakage of the chromonemata at at least two points, and (b) fusion of the broken ends two by two. Presumably this fusion may occur either in the original way (restitution), in which case the effect of the breakage is only temporary, or in a new way, giving a viable or inviable rearrangement. There is evidence that an individual successful ion or pair of ions produced by high-energy radiation—no matter of what wave-length within the range from γ-rays of radium to soft X-rays—is responsible for the production of a break. One of the aims of the present paper is to present further data in support of this proposition.

One of the ways whereby the mechanism of structural change has been investigated, particularly in Drosophila, is to study the curve relating the frequency of induced rearrangements to the dosage of irradiation. This curve results from an interaction between several factors: (1) the primary effects of irradiation (presumably the individual breaks), (2) the many possible ways in which the affected chromosomes can join with each other, and (3) effects of differential viability, some of which are dependent, and some not, on the type of rejoining.

In Drosophila it was found, for instance, that between 1000 and 4000 or 5000 r. the frequency varies directly as an exponent of the dose, the exponent being about 1.5 (Muller, 1936, 1938, 1939; Belgovsky, 1938;
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Bauer et al. 1938; Bauer, 1939). The reason of this exponent being 1.5 instead of 2.0, as had earlier been expected on the conception of the mechanism of structural change above outlined, remained obscure until certain valuable contributions to the solution of the problem were presented by Stadler (1936), Haldane (1935—written communication to Muller) and Catcheside (1938). The latter worked out theoretical tables giving the results expected from the interaction of the different effects already described, when certain limiting assumptions were made to simplify the problem. These tables, extended and generalized by Muller and the writer (unpublished, referred to by Muller, 1940), show that, if each break is independently produced by one ionization, the curve relating the dosage with the frequency of obtained rearrangements among total surviving individuals should be an S-shaped one. In its lowest part, that is, nearest the origin, the curve should show the frequency varying with a power of the dosage that approaches 2, and this power should gradually diminish to 1 and even less as the dosage increases. Thus the power, approximately 1.5, experimentally found between 1000 and 4000 or 5000 r., and the highest power, approaching 2, found at very low dosages (Muller, 1940), become explainable.

Catcheside’s calculations were based on three assumptions which admittedly do not hold: (1) that only one break per chromosome is produced; (2) that fusion between broken ends is random; (3) that no broken end fails to rejoin either in the original way or with a broken end from a different break. In the above-mentioned calculations by Muller and the author it was shown that the removal of limitations (1) and (2) would not substantially affect the shape of the theoretical curve. As for assumption (3) few pertinent data have hitherto been available. One of the aims of the present investigation concerns itself with this point.

There is genetical and cytological evidence (Muller et al. 1937; Muller et al. 1938; Belgovsky & Muller, 1938; Belgovsky, 1937; Raffel, 1939) that in Drosophila, in contrast with the case of maize (Stadler, 1939; McClintock, 1939), chromosomes without telomeres are generally not capable of functioning indefinitely through a period in which a number of cell divisions occur. And various cases that had been taken to be terminal deficiencies of the X were shown actually to be minute intercalary deletions or other minute rearrangements, the very terminal bands being still present. Some further evidence (genetical) of the same kind has been found by the writer (as yet unpublished).

If then chromosomes without a permanent telomere are practically never found in Drosophila, this means either that a broken end never
fails to rejoin, either in its original way or with a broken end derived from another end, or else that the two parts produced by a break, one acentric and the other monocentric, must, when not rejoined in one of these ways, become lost by some mechanism or be lethal to the zygote.

The cytological and genetical works of McClintock (1938a, b) on maize, and the cytological work of Carlson (1939) on Cortophaga are suggestive of one possible mechanism whereby such loss may occur. That is, when the acentric and the monocentric parts of a broken and as yet un-rejoined chromosome undergo mitotic division, the resulting sister chromatids may fuse at their homologous points of breakage and give a V-shaped acentric fragment of double size and a dicentric chromosome, respectively. The acentric fragment may be transmitted for a few cell generations, but sooner or later becomes lost in the cytoplasm. The dicentric chromosome results in the formation of a temporary or permanent anaphase bridge.

McClintock (1938b) had already proved that when there is a ring-shaped dicentric chromosome in maize repeated breakages of the bridges occur at the anaphases of successive mitoses, followed each time by fusion of the non-homologous broken ends, so that the dicentric ring changes in size; it tends ultimately to be lost. Other evidence by the same author showed that small-sized dicentric ring chromosomes are very easily lost through lagging at anaphase. On McClintock’s interpretation, if the movement towards opposite poles is initiated by forces acting at the centromere region but continued by other forces exerted on the chromosome when they are some distance away from the equatorial plate, these small dicentric rings may be too small to reach this distance. The final process of loss of an initially large dicentric ring may be, then, of the same kind.

Some such process as this has not yet been followed through successive cell generations in other material for non-ring dicentric chromosomes; it is therefore a working hypothesis to assume that, in its main lines, it may take place in Drosophila both with ring and non-ring chromosomes. In the non-ring, there would be this difference: the fusion could not occur until later, after chromosome division, and would then join homologous broken ends of sister chromatid fragments. Further, the possibility must be considered that in Drosophila breakage of a dicentric chromosome does not occur, but that, as in the case of McClintock’s small rings, the dicentric fails to be included in either daughter nucleus.

Now, if the ends derived from breaks produced by irradiation in the chromosomes of Drosophila sperm sometimes fail to rejoin in time, a
fusion of sister chromatids at the point of breakage may occur at the first
splitting following irradiation,¹ and either cause immediate loss or start
the process of repeated breakage at anaphase. In either case, on this
assumption, the formation of a sister-chromatid dicentric is the first step
in the process of loss. As these losses should be produced by single
breaks, in contrast with all gross rearrangements, which are supposed to
require two or more breaks, the frequency-dosage relationship of the
former should be different from that of the latter. If the “primary
changes” already mentioned are really the breakages, then these losses,
being due to single breakage, should vary in frequency no more rapidly
than the first power of the dose.

The finding of such a result—contrasting so much with that found for
gross rearrangements—would thus help to fill the gaps in the structure of
evidence in support of the “breakage first” theory of chromosome re-
arrangements. Some evidence to this effect has recently been presented
by Muller (1939, 1940), but, as he stated at the time, that work was only
preliminary and a more systematic and extensive attack (that to be
reported herein) was already in progress under his direction.

The group of problems which it was proposed to investigate in our
present work was accordingly the following: (a) whether or not irradia-
tion of *Drosophila* sperm results in chromosome losses which under
suitable conditions are non-lethal to the zygote and which may be
attributed to broken ends that fail to rejoin either in the original way or
with ends from other breaks; (b) if the answer be affirmative, what is the
frequency-dosage relationship for these losses and what light may it
throw on the validity of our interpretation that the losses are due to
single breaks; and (c) what light may it throw on the interpretation of the
shape of the frequency-dosage curve for gross rearrangements.

On the technical side the problem was therefore that of detecting
losses of whole chromosomes, such as might be produced when a dicentric
chromosome arises by fusion of the broken ends of sister chromatids.
Here a special method, involving the use of the *sc. Y* chromosome (vide
infra), was devised by Muller; this represented a technical advance over
his earlier described method (1939, 1940), in which a dominant variegated
allele of brown had been used for disclosing the presence of an ordinary
Y-chromosome.

¹ The assumption (Patterson, 1933; Mather, 1937), made to explain “fractional”
rearrangements, that the chromosomes are in part or all split in the *Drosophila* sperm is, as
Muller (1939, 1940) points out, no longer necessary, since evidence has been obtained that
rearrangements are not completed until after fertilization.
II. Technique adopted for detection of losses of X and Y

Among the possible methods for genetically detecting the loss of a chromosome, the first that comes to the mind, so far as the X and Y are concerned, is the utilization of the mechanism of sex determination. Any method of this kind, however, presents several practical difficulties, one of which is the great number of cultures required to obtain statistically sound results, and another the fact that effects simulating losses, and distinguishable from them only with difficulty, are produced in several ways.

It was therefore thought that some way of distinguishing attached-X females carrying a Y from those not carrying it would be more promising. If a potential loss of the X-chromosome can be induced in an X-bearing sperm and this happens to fertilize an X.X egg, the resulting zygote will be a perfectly viable X.X/O female, in general phenotypically indistinguishable from an X.X/Y one. The same result will be obtained if loss of the Y-chromosome is induced in a Y-bearing sperm. Hence the possibility of detecting losses of X or Y chromosomes induced in the sperm depends upon suitable methods of distinguishing, by inspection, X.X/O from ordinary X.X/Y females.

An excellent opportunity in this direction was provided by the chromosome designated as sc. Y^L (Crew & Lamy, 1940). This chromosome had originally been described as rod-shaped, but co-operative investigations (Pontecorvo, 1940a) showed it to be V-shaped: one of the two arms is the Y^L and the other is even longer than the Y^L. This latter (longer) arm is provisionally described as composed, starting from the centromere, of the main part of the proximal region of the Y^S, including its main block and probably bb^+ but excluding some or all of the more distally located fertility genes (Neuhaus, 1939), then of a large part of the proximal heterochromatic region of the X (including block A and bb^+), and finally of the left tip of a sc^S1 chromosome, extending from its original left break to the end, and thus including the genes for sc^S1, ac^+ and y^+. The presence of this distal X-chromosome component, carrying y^+ and the other markers mentioned, makes the sc. Y^L valuable for our purpose. In fact, a cross of males with the sc. Y^L to y= females will give daughters all of which ordinarily have normal grey body colour, the yellow of their attached-X’s being “covered” by its wild-type allelomorph carried in the

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1 In the notation used above, and throughout this paper, a full stop indicates attachment of the two parts shown on either side of the stop. When the two parts are alike (homozygous) the sign = will be used after the stop; thus y yf. = means attached-X’s homozygous for y yf (see Muller, 1934).
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The component of the paternal sc \( Y^L \). Except for mutations or deletions of \( y^+ \), only females of type \( X^-.X/0 \), originating when the paternal sc \( Y^L \) or \( X \) is "lost," will show the yellow body colour. The \( P_1 \) males used must of course contain, for fertility, a \( Y^S \) (short arm of the \( Y \)) also; it is convenient to have this attached to their \( X \)-chromosome, to the right of the centromere; thus their composition is \( X^-.Y^S/sc.^+Y^L \).

The preliminary tests by Muller (op. cit.)—with the method based on the distinction of \( X^-.X/0 \) from \( X^-.X/Y \) females by means of the effect of the extra \( Y \) in suppressing the eye-colour variegation caused by a dominant allele of \( bw \)—had shown that the frequency of induced non-lethal losses of \( X \) and \( Y \) was rather low (of the order of 2% with 4000 r. in his material). It was therefore necessary to have a stock with a very low spontaneous frequency both of those events which lead to a loss and of those which give the same phenotypical effects as a loss, namely: (a) non-disjunction in the males; (b) mutation to yellow in the sc component of the \( sc^+.Y^L \); (c) release into circulation in the stock of an extra \( Y \), or of parts of \( Y \), not having the "marked" sc component.

The stock first tested had its males of composition \( y^+.X.Y^S/sc.^+Y^L \) and females \( y^+.w.f.^-.sc.^+.Y^L \). In mass cultures of this stock some phenotypically \( y \) males, \( y^+.w.f. \) females and wild-type females appeared. The phenotypically \( y \) males and \( y^+.w.f. \) females proved in different cases to be of non-disjunctional origin, in which case they carried no \( Y \), or to carry a whole \( Y \), a \( Y^S \) or a \( Y^L \) without the sc component.

The presence of a whole \( Y \) in circulation in the stock could easily be explained by a crossing-over in the male between the \( sc^+.Y^L \) and the \( X^-.Y^S \), giving an \( X \) with a sc duplication, on the one hand, and a whole \( Y \) on the other hand. The presence of a \( Y^L \) deprived of the \( y^+ \) marker could also be explained easily, as a spontaneous "mutation" to \( y \) (or better, a minute rearrangement) in the sc component of the \( sc^+.Y^L \). The rate of this spontaneous "mutation" has been measured by Belgovsky (1938, 1939) and by Muller & Makki (1938; Muller, 1939, 1940) as some 1 in 10,000 in an inverted \( X \)-chromosome (\( sc^8 \)) in which, as in the \( sc^+.Y^L \), the \( y^+ \) locus is very close to a portion of heterochromatic region that had originally been proximal in location. The manner of origination of the \( Y^S \), however, was not so obvious until the following fact was observed by Miss Lamy and analysed by the writer (Lamy & Pontecorvo, 1940): a part of the phenotypically wild-type females proved to be of composition \( y^+.=/sc.^+Y^L \), namely, newly originated \( X^-.X \) females with their attached-\( X \)'s of the same type as the paternal \( X \). A fact of the same kind had already been briefly reported by Neuhaus (1936). The origination of these \( X^-.X \) females...
can be explained by a crossing-over between different arms of sister chromatids in the paternal $X.Y^S$ chromosome; its complementary product must be a free double $Y^S (Y^S. =)$. This would account also for the presence of the $Y^S$ found genetically to exist in the stock.

Incidentally, this mode of new origination of attached-$X$'s, which has been successively detected in several different $X.Y^S$ and $X.Y^L$ stocks, can be of great practical use for the building of any kind of $X.X$ stock. Moreover, if this may be a method of spontaneous origination of attached-$X$'s, then these chromosomes are not necessarily isochromosomes (Darlington, 1939). Perhaps investigations in this direction would afford a test for deciding between such an origin for these cases and that proposed by Darlington (misdivision of the centromere).

The following measurements of the primary frequency with which the yellow exceptional females arise (some 1.5%) showed that it was far too high for making the above stock suitable for our purpose:

**Table 1. Primary exceptional flies in the stock of composition**

<table>
<thead>
<tr>
<th>Ordinary females phenotype: $w^f$</th>
<th>Exceptions (phenotypes)</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$w^f \varphi \varphi$</td>
<td>$w^f \varphi \delta$</td>
</tr>
<tr>
<td>1255</td>
<td>1</td>
<td>18</td>
</tr>
</tbody>
</table>

A new stock, built up with the same $y.w^f. =/sc. Y^L$ females but using males with an $X.Y^S$ chromosome of different origin, marked by the recessive forked, gave the following satisfactory results (Table 2):

**Table 2. Primary exceptional flies in the stock of composition**

<table>
<thead>
<tr>
<th>Ordinary females phenotype: $w^f$</th>
<th>Exceptions, classified phenotypically:</th>
<th>No. of flies</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Females $y.w^f$</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>Females $f$</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>Males $w^f$</td>
<td>1 (detachment of the $X.X$)</td>
</tr>
</tbody>
</table>

The different behaviour of the two stocks as regards non-disjunction is probably not caused by the $sc. Y^L$ but by some peculiarity of the $X.Y^S$, resident in its proximal regions. As the above results show, this second stock was found to be suitable for the proposed experiment.

To avoid the dissemination of whole $Y$-chromosomes or of either of the arms of the $Y$ originating in the manner already explained, the stock was maintained by the inbreeding of individual pairs of brothers and sisters, with continual elimination of families in which an undue proportion of exceptions appeared.
III. Experimental set-up, and $F_1$ results

X-rayed males of the stock previously described—$fX.Y^S/sc.Y^L$ were crossed to $ywf./sc.Y^L$ females, and the exceptional (yellow) females appearing in $F_1$ were recorded.

The following technique was adopted: the males collected from inbred pair matings, when 1–3 days old, were thoroughly mixed and divided into three groups. The males of one group, used for control, were mated individually with two virgin females. The other males were X-rayed at room temperature (about 16° C.) with a broad-focus water-cooled Coolidge tube (tungsten target) operated at 8 mA. and 70 kV.; a 0.5 mm. Al. filter was used. The intensity on the flies was about 350 r. per min. Two doses were given, namely, 1000 and 4000 r. The relative values of the two doses (1:4) in this experiment have considerable accuracy, owing to the technique described below. The same cannot be said of the absolute values.

For the high dose the males were exposed to the irradiation for about 10 min. For the low dose, the males were divided into four equal groups and each group irradiated together with the males of the high dose, but for a length of time such as to give only one-quarter of the high dose. That is, after each quarter (1000 r.) of the high-dose treatment had been given, one of the four groups of low-dose males was removed and another one substituted. This method, first adopted by Offermann in 1934 (unpublished), has the advantage of equalizing to a considerable extent variations in the conditions of treatment that might otherwise affect the high- and low-dose groups differentially.

Immediately after treatment, the males were mated individually in vials with virgin females, six females for each male of the high-dose group and three for each male of the low-dose group. After 4½ days the males were discarded and the females of each vial transferred to fresh vials. The cultures were maintained at a constant temperature of 24 ± 0.5° C. and were examined when almost all the pupae had hatched; at this time, practically all of the imago progeny were still alive.

The method of individual matings was adopted so as to make possible the detection of male parents carrying an unmarked $Y^S$ or other part of a $Y$, instead of, or in addition to, a $sc.Y^L$. For the presence of such parts would have given results in the next generation simulating loss. No cases of this kind occurred, however, in the 1020 individual matings of the above experiment. Males with such aberrations are to be expected with an incidence of some 3 in 10,000, provided the stock is always multiplied,
as it actually was, by inbred pair matings, so as to exclude secondary occurrences.

The count of \( F_1 \) females from this experiment is shown in Table 3. Among the eighty-six exceptional flies found in the \( F_1 \) from the males

<table>
<thead>
<tr>
<th>Dose in r.</th>
<th>Ordinary ( \Omega \Omega ) (phenotype ( w_f ))</th>
<th>Exceptional ( \Omega \Omega ) (phenotype ( y w_f ))</th>
<th>% exceptional</th>
</tr>
</thead>
<tbody>
<tr>
<td>4000</td>
<td>4,987</td>
<td>84 + 2(\dagger)*</td>
<td>1-70 ±0-18</td>
</tr>
<tr>
<td>1000</td>
<td>10,427</td>
<td>56</td>
<td>0-54 ±0-07</td>
</tr>
<tr>
<td>Control</td>
<td>7,071</td>
<td>5</td>
<td>0-07 ±0-03</td>
</tr>
<tr>
<td>Total</td>
<td>22,485</td>
<td>146</td>
<td>—</td>
</tr>
</tbody>
</table>

* Two cases of "fractionals".

treated with the high dosage, two were mosaics of the "fractional" type (Muller, 1927). As the results obtained in genetic analyses of these and many other similar "fractionals" found in a parallel series of experiments throw an interesting light on the mechanism of induction of structural changes, a detailed discussion of the problem will be given elsewhere. It is sufficient here to point out that these fractionals are not counted as exceptional units, but as halves.

IV. Tests of the exceptional flies

The exceptional flies are detected by their yellow body colour; this character, in the regular flies, is "covered" by the wild-type allele carried in the \( sc \) component of the \( sc. Y^L \).

In addition to the complete loss of the \( X. Y^S \) or \( sc. Y^L \), several other ways are conceivable whereby the same phenotypical effect can be produced. These are:

(a) presence of a normal \( Y \), or of one of its two arms, carrying no marker;

(b) minute deletion or other minute rearrangement in the \( sc \) arm of the \( sc. Y^L \) chromosome, producing a loss or change to yellow of its wild-type allelomorph; actual gene mutation of the \( y^+ \) gene in this chromosome to \( y \)—a much rarer phenomenon—may also be included here;

(c) large deletion of the \( f X. Y^S \) chromosome, with left break to the left of \( y^+ \) and right break between \( w^+ \) and the centromere;

(d) large deletion of the \( f X. Y^S \) chromosome, producing a ring-shaped chromosome with left break between \( w^+ \) and the centromere and right break anywhere in the \( Y^S \) arm, but proximal in respect of at least one of its fertility genes, or producing a sterility position effect in it;
(e) deletion of the \( \text{sc.} \ Y^L \) chromosome, producing a ring-shaped chromosome with one break in the \( \text{sc} \) arm, proximal in respect of \( y^+ \) or producing "mutation" of it to yellow, and the other break anywhere in the \( Y^L \) arm but proximal in respect of at least one of its fertility genes or producing a sterility "mutation" in it;

(f) "mutation" (minute rearrangement of some kind or actual gene mutation) to \( y \) in the \( \text{sc} \) arm of the \( \text{sc.} \ Y^L \) and simultaneous sterility "mutation" in the \( Y^L \);

(g) translocation of the \( fX.Y^S \) or \( \text{sc.} \ Y^L \) to the fourth chromosome with acentric-dicentric reunion of the broken ends and loss of both chromosomes;

(h) complex rearrangements resulting from combinations of the already described simple ones.

Cases (a) are consequences of crossing-over in the male and are therefore independent of the X-ray treatment. The control series were carried out largely for the purpose of checking this occurrence.

The occurrence of the other types had to be ascertained by tests carried out on the exceptional flies themselves. The following technique was adopted:

In cases (b) and (c) the \( Y^L \) arm of the \( \text{sc.} \ Y^L \) chromosome, or the \( Y^S \) arm of the \( fX.Y^S \) chromosome, respectively, remains unaffected. An \( X.X \) exceptional female carrying either will transmit it to her sons. If these sons carry, attached to their \( X \)-chromosome of paternal origin, the complementary arm of the \( Y \), they will have the whole set of fertility genes and will be fertile (Stern, 1929; Neuhaus, 1939). Therefore by mating each exceptional female to \( X.Y^S \) and \( X.Y^L \) males successively and testing the sons of each for fertility, it can be ascertained whether or not she carried one of the arms of the \( Y \), and, in case she did, to identify it (Muller, 1939, 1940).

In cases (d) and (e) the fertility genes—several of which have been shown (Neuhaus, 1939) to be located in the distal portions of each arm of the \( Y \)—are affected and therefore none of the preceding tests is suitable. However, the presence of \( bb^+ \) in the proximal regions both of the \( X.Y^S \) and of the \( \text{sc.} \ Y^L \) suggested a test based on the detection of this gene. Males of a \( bb \)-deficient stock, \( y \ \text{sc}^4L \ \text{In-S sc}^{S_1}R \), obtained by Muller by crossing-over between the \( \text{sc}^4 \) and the \( \text{sc}^{S_4} \) inverted chromosomes, are viable only if the deficiency of \( bb^+ \) in the \( X \) is "covered" by its presence in the \( Y \) or elsewhere. From the cross of a male of such type with an exceptional nullo-\( Y \) attached-\( X \) female, no sons are produced except for a few fertile males resulting from non-disjunction of the \( X \) and \( Y \) in the
father. If, however, the exceptional female tested carries a $Y^S$, a deleted $fX.Y^S$ or a deleted $sc.Y^L$ in which there is an unaffected $bb^+$ gene, a greater number of males will be produced, and these will be sterile. In an $X.Y^S$ chromosome there are two loci containing $bb^+$, both proximally placed. In a $sc.Y^L$ chromosome at least one locus with $bb^+$ is surely present in the proximal part of the $sc$-containing arm, while no such locus, or one with only a very weak $bb$ allele, is present in its $Y^L$ arm, as results obtained in our own investigations have shown. If a ring is formed in the $X.Y^S$ or in the $sc.Y^L$ as a result of two breaks occurring on opposite sides of the centromere, followed by joining of the broken ends, there is a good chance for one of the breaks to have been distal in respect of a locus containing $bb^+$. This chance must be very high for rings formed from the $X.Y^S$, because of the two $bb^+$-containing loci originally present, one in each arm, in proximal positions. When the ring is formed in the $sc.Y^L$, having a $bb^+$-containing locus or loci in only one of its arms, the chance of this locus being included is lower.

Case (f)—a mutation in one of the fertility genes carried in the long arm of the $sc.Y^L$ chromosome coinciding with a $y$ "mutation" in its $sc$ component is also distinguishable from complete loss by the test for the presence of $bb^+$.

Case (g)—an aneucentric (acentric-dicentric) translocation of the $X.Y^S$ or the $sc.Y^L$ with the fourth chromosome—would produce, through elimination of both the chromosomes concerned, an exceptional (yellow) fly which would also be haplo-IV and easily recognizable as such. No exception of this kind occurred in the experiment here reported.

All the induced rearrangements described require one (case b) or two ionizations (cases c, d, e, f, g). The occurrence of cases due to three ionizations must certainly be less frequent. Most of them, however, could be distinguished from losses—with the same limitations as for the rings—by means of the test for $bb^+$.

All the types from (a) to (f), and in addition some of the more complex types of exceptional flies carrying different parts of the $sc.Y^L$ or $X.Y^S$ chromosomes, actually occurred as a consequence of X-ray treatment. Fig. 1 shows, for instance, the metaphase plate of a ring resulting from one break in each arm of the $sc.Y^L$.

Summing up the method: three tests had to be carried out with any given exceptional (yellow) female before it could be ascertained to which type she belonged. These were:

(1) Test for the presence of the whole set of fertility genes carried by the $Y^L$. 

11
(2) Test for the presence of the whole set of fertility genes carried by the $Y^S$.

(3) Test for the presence of $bb^+$. Exceptional females giving negative results with all the three tests can be considered as being nullo-$Y$ females in the great majority of cases. The same results are, to be sure, given when a ring chromosome not including $bb^+$ is carried by the female as when a female is nullo-$Y$. But such cases are probably a small fraction of the total, judging by the small number of rings that do contain $bb^+$. For the tests of earlier workers on deleted $X$-chromosomes (Muller & Painter, 1929; Muller & Gershenson, unpublished) have shown that in a large proportion of deleted $X$'s having one break in the heterochromatic region the locus of $bb$ has not been removed.

Practically, the three tests were carried out as follows:

The exceptional flies, when collected, were already inseminated by their $fX.Y^S/sc.Y^L$ brothers. A period of 4–5 days laying (each female being kept individually) was sufficient to give rise to enough offspring for the first test. The sons coming from any given culture of this "brood" were mated in mass (5–10 males with 5–10 females) to their sisters. In case they proved to be fertile, the test was verified by mating 5–10 such males individually, each male with 2–3 virgin females, so as to exclude cases of fertility of males due to the occasional origination of males by non-disjunction. A positive result from this test (i.e. fertility of many of the sons) was considered as a proof that the exceptional fly had carried the whole set of fertility genes of the $Y^L$. 

Fig. 1. A ring-shaped chromosome resulting from one break in each arm of the sc. $Y^L$. Mitotic metaphase plate of a female carrying attached-X's, a normal $Y$ and the ring. $a$, camera lucida drawing ($\times 3500$) of same plate as in microphotograph $b$ ($\times 800$).
The same exceptional female, discharged of a part of the sperm received in the first type of mating, was then mated to males of the composition $X.Y^L/Y^S$. The two progenies were easily distinguishable because the daughters from the first type of mating, carrying the paternal $sc.Y^L$, were phenotypically $y^+w_f$ and the sons $f$, while the daughters of the second type of mating were $y.w_f$ and the sons wild-type. The sons derived from this second type of mating were tested for fertility in the manner already described for the ones from the first mating. A positive result of this test was considered as proof that the exceptional fly had carried the whole set of fertility genes of the $Y^S$.

After the second type of mating, the fly was transferred to fresh food for 4-5 days and then transferred again and mated, this time with $y.sc^4.L.in-S.sc^{51}.R$ ($bb$-deficient) males, marked in one of their second chromosomes by $Cy$ and $L^4$. If no yellow scute sons appeared in the progeny, but many females, about half of them $Cy.L^4$, the result of the test for the presence of $bb^+$ was considered as negative. Usually, however, even when a deleted chromosome carrying $bb^+$ was not present in the exceptional fly, a few sons appeared in consequence of the comparatively high frequency of primary non-disjunction (at least 5%) occurring in the males of the $y.sc^4.L.in-S.sc^{51}.R$ stock. In this case these sons were tested individually for fertility (presence of a whole $Y$) to confirm their non-disjunctional origin. When, on the other hand, many males appeared in the progeny and on being tested individually proved in great majority to be sterile, the result of the test was considered positive, and the conclusion was drawn that the exceptional fly carried a deleted, probably ring-shaped, $sc.Y^L$ or $X.Y^S$.

The impossibility of carrying out the above triple test, with its various ramifications, on all the exceptional flies is obvious. Each fly, already some days old when collected, must give progeny with three different types of males. Often the fly died before giving the whole series of progeny. The tests which were carried through were, however, sufficiently numerous to show the trend of the results, and to allow certain definite conclusions to be drawn. The results of these tests are summarized in Table 4.

In this table only those flies are recorded which gave at least the first two kinds of progeny, whereby the presence of the whole set of fertility genes of each arm of the $Y$ is detected. The third kind of progeny—from $bb$-deficient males—was obtained with about half of the flies which had already passed through the other two tests.

The results indicate that the majority of the exceptional females—
Chromosome Losses in Drosophila Sperm

roughly two-thirds in the treated series—are nullo-Y. Furthermore, the proportion of exceptions which are nullo-Y is, in first approximation, of the same order for the two doses (although with larger numbers we should expect the excess of various kinds of incomplete chromosomes formed at the higher dose, as compared with the lower, to become significant).

Table 4. Data on percentage of exceptional \(F_1\) females which constitute cases of complete loss of \(X\). \(Y^S\) or of \(sc. Y^L\)

<table>
<thead>
<tr>
<th>Treatment</th>
<th>4000 r.</th>
<th>1000 r.</th>
<th>Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>A. Exceptional (\Phi) tested for the fertility genes in both arms of the (Y)</td>
<td>43</td>
<td>35</td>
<td>4</td>
</tr>
<tr>
<td>(a) Found to carry the whole set of fertility genes of (Y^L) only</td>
<td>9</td>
<td>3</td>
<td>2</td>
</tr>
<tr>
<td>(b) Found to carry the whole set of fertility genes of (Y^S) only</td>
<td>3</td>
<td>5</td>
<td>0</td>
</tr>
<tr>
<td>(c) Found to carry both the sets of fertility genes of (Y^L) and (Y^S)</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>B. Total in category A found to carry the whole sets of fertility genes of one or both arms of (Y)</td>
<td>12</td>
<td>8</td>
<td>2</td>
</tr>
<tr>
<td>C. Remainder, carrying neither whole (Y^L) nor whole (Y^S)</td>
<td>31</td>
<td>27</td>
<td>2</td>
</tr>
<tr>
<td>D. Exceptional flies in category C tested for presence of (bb^+)</td>
<td>19</td>
<td>12</td>
<td>2</td>
</tr>
<tr>
<td>E. Flies of category D found to carry a deleted (X). (Y^S) or (sc. Y^L) with (bb^+)</td>
<td>2</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>F. Remainder of category D, not carrying (bb^+)</td>
<td>17</td>
<td>11</td>
<td>2</td>
</tr>
<tr>
<td>G. Proportion of exceptional (\Phi) carrying no whole arm of (Y) ((C/A))</td>
<td>(\frac{4}{5} = 72 \pm 7%)</td>
<td>(\frac{3}{5} = 77 \pm 7%)</td>
<td>(1 = 50 \pm 25%)</td>
</tr>
<tr>
<td>H. Proportion of preceding carrying no deletion with (bb^+) locus ((F/D))</td>
<td>(\frac{4}{9} = 89 \pm 6%)</td>
<td>(\frac{3}{9} = 92 \pm 8%)</td>
<td>(1 = 100 \pm 7%)</td>
</tr>
<tr>
<td>I. Percentage of exceptional (\Phi) representing complete loss of (X). (Y^S) or of (sc. Y^L) ((G \times H))</td>
<td>64 (\pm 7%)</td>
<td>71 (\pm 9%)</td>
<td>50 (\pm 25%)</td>
</tr>
</tbody>
</table>

The conclusion can be drawn that complete losses of the \(X\)- and \(Y\)-chromosomes are in fact induced by irradiation of the mature sperm. Moreover, those losses here demonstrated are not lethal to the zygote. Whether the latter characteristic holds for all the induced losses of \(X\)- and \(Y\)-chromosomes or only for a part of them—those which can be detected by the present method—will be discussed later.

V. THE FREQUENCY-DOSAGE RELATIONSHIP

The data summarized in Table 3 give a frequency of \(1.70 \pm 0.18\%\) exceptions at the higher dosage, of \(0.54 \pm 0.07\) at the lower dosage, and of \(0.07 \pm 0.03\) in the controls, the dosage ratios among the three series being \(4:1:0\). The tests of the exceptional flies (Table 4) have shown that the proportion of losses of the whole \(X\). \(Y^S\) or \(sc. Y^L\) chromosome among
them is $64 \pm 7$, $71 \pm 9$ and $50 \pm 26\%$, respectively, for the three series. We have then the data for determining three points of the curve relating the dosage of irradiation with the frequency of losses, namely for those points corresponding to the abscissae 0, 1000 and 4000 r. This makes it possible to test whether or not, at a first approximation, this relationship can be represented by a linear equation (straight line).

The experimental evidence in *Drosophila* shows that the frequencies both of induced "point" mutations and of minute rearrangements increase, within the dosages here used, approximately in linear manner, that is, as the first power of the dosage. The frequency of gross rearrangements, on the other hand, increases, within the same range, as an exponent of the dosage higher than 1 and lower than 2: approximately as the $1.5$ power (see review of the matter by Timoféeff-Ressovsky for "point" mutations and by Muller for rearrangements at the Seventh International Congress of Genetics, 1939).

The present data on the frequency of exceptional flies at the three above-mentioned doses can be interpolated by a linear equation, as shown in Table 5. The agreement between the data found and those

<table>
<thead>
<tr>
<th>Dose</th>
<th>Cases of loss of whole $X_Y^S$ or $sc. Y^L$ among exceptional flies</th>
<th>Frequency of losses</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Exceptional flies found %</td>
<td>Found (B×C) %</td>
</tr>
<tr>
<td>0</td>
<td>0.07 ±0.03</td>
<td>$0.035 \pm 0.03$</td>
</tr>
<tr>
<td>0</td>
<td>50 ±25</td>
<td>$0.38 \pm 0.07$</td>
</tr>
<tr>
<td>0</td>
<td>$1.70 \pm 0.18$</td>
<td>$1.09 \pm 0.16$</td>
</tr>
<tr>
<td>1000 r.</td>
<td>$0.38 \pm 0.07$</td>
<td>$0.334$</td>
</tr>
<tr>
<td>4000 r.</td>
<td>$1.09 \pm 0.16$</td>
<td>$1.105$</td>
</tr>
</tbody>
</table>

*Interpolating equation $Y_d = \bar{y} + 0.257 (d - \bar{d})$, where $Y_d =$ percentage frequency at dosage $d$ (in 1000 r.).

expected is very good, being $\chi^2 = 0.04$, a value which, for one degree of freedom, is exceeded by chance in more than 80% of cases. The slight departure from linearity is, in any case, in the direction of an exponent lower than 1. And the departure in this direction would have been even greater if there had been a more distinct excess of incomplete chromosomes among the exceptions at the higher dose, as a greater body of data would have been expected to show.

At the same time, the possibility had to be tested that a curve in which the frequency of losses varied as the $1.5$ power of the dose, like the curve for gross rearrangements, would also fit the data sufficiently well. The expected value for 1000 r. may be calculated on this assumption from the 4000 r. value, as the latter is affected by a proportionately lesser
error. Allowing for the controls, this expected value for 1000 r. is
0·13 ± 0·02, as compared with the obtained value of 0·38 ± 0·07. The
difference, 0·22 ± 0·07, is some three times its standard error. Thus the
frequency-dosage relationship is suitably expressed by a linear equation
and cannot be expressed by an equation having the exponent 1·5. It may
be concluded that, within the range of X-ray dosages used in this experi¬
ment, the frequency of losses appears to be, in first approximation, a
linear function of the dosage of irradiation, and that it does not vary as
rapidly with dosage as the frequency of gross structural changes does.

It must be observed that the way in which the frequency of losses has
been expressed here is an empirical one and does not represent the actual
frequency per treated sperm. In fact, when a non-lethal loss is induced in
an X-bearing sperm fertilizing an X . X egg, a viable exception of com¬
position X . X/O results from a zygote which otherwise would have been an
inviable triplo-X. The frequency of losses per treated sperm is therefore
only half that given in the above table, namely 0·17 % with 1000 r. and
0·55 % with 4000 r.

VI. Discussion

The results reported above show (a) that, in agreement with Muller's
preliminary evidence, losses of the whole X- or Y-chromosome are
actually produced by irradiation of the mature sperm and that they are,
in part at least, non-lethal to the zygote; (b) that between 0 and 4000 r.
their frequency increases—at a first approximation—as the first power of
the dosage and certainly less rapidly than the ca. 1·5 power characteristic
of gross rearrangements involving two or more breaks.

As for the frequency-dosage relationship, although there is no sig¬
ificant departure from linearity, the evidence indicates that, if any
exists, it is in the direction of an exponent even lower than 1. It is to be
expected that a refinement of the experiment would show this slight
departure from linearity to be real, for such a departure would arise from
(a) the fact that the basically linear curve would tend to "saturation",
i.e. at higher dosages the coincidence of two or more breaks, each leading
to loss, becomes greater; and more especially from (b) the fact that, with
an increased number of breaks per nucleus, greater opportunity would be
given for a break to take part in a lethal or non-lethal gross rearrangement
(especially a translocation) instead of in a loss.

A most interesting preliminary report by Bauer (1939 b) has just
appeared in which the induction of losses of the Xc² ring chromosome was
investigated by measuring the amount of change caused in the sex-ratio
when irradiated males bearing the ring were crossed to females with normal X’s. The results, expressed as the ratio of males minus females to males (all corrected for controls), when plotted against dosage gave “an oversaturated one-hit curve, which results from the difference between a one-hit and a two-hit curve. The former suggests the proportionality to the dosage of the production of dicentric double rings, the latter corresponds to the two-hit curve of the lethal (aneucentric) Y-translocations.”

In an experiment by the writer (1940b), in which the frequency of losses of X-chromosomes of different lengths and structures were investigated by the earlier method of Muller (1939, 1940), involving the difference in expression of dominant alleles of brown shown by X.X/Y and X.X/O females, viable losses of the Xc^2 chromosome proved to be induced, at 4000 r., with a frequency¹ significantly greater than 2.8 ± 0.5% per treated X-bearing sperm. This figure represents only real losses; therefore it is not masked, as Bauer’s is, by other effects, such as an excess of dominant lethal gene and chromosome mutations arising in the Xc^2 as compared with the Y, and “conversion” of females into males by deletion of the X.

Now the graph published by Bauer shows a deficit of some 40% in the number of females as compared with that of males for the same dose, 4000 r., and recent tests by Muller and the writer (unpublished) confirm this result. On the other hand, the frequency of viable losses of Xc^2, found in our experiment cited above, when calculated in a way comparable to Bauer’s, turns out to be about 5%. Thus the remainder of the deficit of females, 35 out of the 40%, must be ascribed almost entirely (that is, except for the “conversions” due to deletion above mentioned) to an excess of dominant lethals arising in the Xc^2-bearing sperm, associated either with losses of the X or with gene mutations or minute or gross structural changes of it, as compared with those arising in the Y-bearing sperm. When a non-ring X-chromosome is used, some 10% of euchromatic translocations, mostly two-break ones, involving the X are obtained with 4000 r. Both these and the complementary aneucentric translocations are lethal in the case of an Xc^2 chromosome, but in the

¹ The frequency of losses with the Xc^2 chromosome proved to be more than twice as high as that with a rod-shaped X. This is in agreement with expectancy on the basis of the proposed mechanism for the origin of losses of the rod-shaped X—breakage followed by fusion of sister chromatid fragments, producing a dicentric—because in a ring chromosome two, out of the three possible ways in which the broken ends of chromatid fragments may rejoin two by two, lead to formation of a dicentric, while in a rod-shaped chromosome only one out of these three possible ways gives this result. The matter is explained in detail in the other paper (being prepared for the Press).
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case of the Y (which is involved in translocations with roughly the same frequency as the X) only the latter would be lethal, leaving a difference of 10%. A further group of some 10% can be ascribed to dominant lethals (gene mutations and minute and gross rearrangements other than aneucentric translocations) arising in the X, as was shown by Muller (1928) and confirmed by data of several authors (Barth, 1929; Gowen & Gay, 1933; and preliminary results recently obtained by Muller and the writer). The remainder of the deficit—apparently some 15% at least—is evidently produced by lethal losses of the Xc² or, to be more exact, by the difference between lethal losses of the Xc² and lethal losses of the Y, should the latter losses exist at all.

From the above rough estimates the lethal and non-lethal losses of the Xc² appear to occur in a ratio of about 3:1, and losses of both kinds together to account for at least one-half of the change in the sex ratio observed by Bauer with the 4000 r. dosage. The other half, then, is constituted of a mixture of other "one hit" effects and of "two or more hit" effects. The proportions of losses, of other "one hit" and of "two or more hit" effects change with the dosage, the frequencies of the former two being preponderant at low doses and increasing linearly while that of the latter increases as a power of the dosage that is at first 2 and gradually diminishes to 1.5 and less as the dosage rises. It seems therefore that an exact allowance for all these different effects is necessary before conclusions concerning the dosage-frequency relation for losses can be drawn from Bauer's results. However, a combined use of Bauer's method and that adopted here may afford a more nearly adequate means of approach than either of the two alone. For with the former, all losses—lethal and non-lethal—are scored, but there are other overlapping effects, difficult to allow for, while with the latter there is not such an interference of effects, but there is the drawback that only non-lethal losses are detected.

The linear proportionality to dosage found in the present work for non-lethal losses was expected on the basis of the loss mechanism postulated; in fact, if losses are produced by single breaks, and single breaks by single successful ionizations, the frequency-dosage relationship expected is, at a first approximation, that of a one-hit curve, i.e. a straight line. This result argues strongly, from a new angle, for the "breakage first" theory of structural changes, according to which a power of the dosage higher than the first is expected for expressing the frequency of gross rearrangements (two or more breaks) and a power equal to or lower than the first for single-break events, as losses are supposed to be. The possibility cannot yet be excluded, however, that the mechanism of losses may
be entirely different from that proposed in this paper, as for instance by some action of the radiation on the centromere or on achromatic constituents of the mitotic apparatus. Parallel experiments to clarify this point are in progress.

The fact that lethal and non-lethal losses together proved to constitute a considerable portion of structural changes shows that also assumption 3 (p. 198)—made when calculating the frequency-dosage curve for gross rearrangements—does not hold (provided the mechanism of losses here advocated is correct). However, as the frequency of losses proved to be roughly a linear function of the dosage, allowance for it would not substantially alter the shape of the theoretical curve, although it would of course affect its absolute values.

Summary


2. Losses of X and Y are found to be produced by X-irradiating mature sperm, and those found by the method used are non-lethal to the zygote.

3. Between 0 and 4000 r. the frequency of the above losses is, in first approximation, a linear function of the dosage, and it certainly varies less rapidly than the 1.5 power of the latter—the power found, for these dosages, in the case of gross rearrangements.

4. The mechanism of origination of these losses is interpreted as being through the occurrence of single breaks followed by lateral fusion of sister chromatid fragments, leading to formation of a dicentric chromosome which is either lost in the first change or becomes subjected to repeated breakage at successive anaphases and is ultimately lost.

5. It is suggested, as a result of comparisons of the frequency of the losses found with the amount of change of the sex ratio caused by the same dosage, that a major part of the losses are lethal to the zygote.

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REFERENCES


VIABILITY INTERACTIONS BETWEEN CHROMOSOMES OF DROSOPHILA MELANOGASTER AND DROSOPHILA SIMULANS

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(With One Text-figure)

Although Drosophila melanogaster and D. simulans are the two species the genetics of which is best known, they have been but little utilized for the genetic analysis of the process of speciation on account of the complete sterility of their hybrids. However, a method for circumventing this sterility, at least in part, has been recently devised (Muller & Pontecorvo, 1940), thus opening new possibilities for such an analysis.

The 'levels of divergence' (Spencer, 1940) in the process of speciation so far open to genetic approach, in the genus Drosophila as well as in other organisms, have been those in which at least the hybrids of one sex are partially fertile. It is now possible to attack a new level, that at which the sterility of the hybrids is complete.

The morphological differences between the wild types of D. melanogaster and D. simulans are less conspicuous than those between most mutant stocks within either species. The mitotic chromosome complements are almost identical. The salivary chromosomes show only minor differentiations (Bonnier, 1924; Sturtevant & Plunkett, 1926; Patau, 1935; Kerkis, 1936; Horton, 1939; Slizynski, 1941). Many mutants are known that behave as alleломorphs in the hybrids (Sturtevant, 1920, 1921a, 1929).

'Biological' isolation (Timofeef-Ressovsky, 1940) is complete through preferential mating and the invariable sterility of the hybrids. It is likely that before their recent introduction into North America the two species had distinct geographical areas.

However similar D. melanogaster and D. simulans appear to be the developmental paths must be different (cf. Waddington, 1940; Haldane, 1941) as shown by their hybrids. In fact, certain classes of hybrids are inviable, all other are sterile and show morphological abnormalities such as erratic absence of bristles and cross-veins, incomplete banding of sternites, rough eyes, etc. (Sturtevant, 1920 et seq.).

Because of their bearing on the mechanism of speciation, on the one hand, and of gene action on the other, investigations are in progress on the genetic and developmental basis of these hybrid effects. In the present paper some results on the origin of the inviability of certain classes of hybrids are reported.

Problem

As first observed by Sturtevant (1920) only female, or non-disjunctional male, imagines arise from crosses between D. melanogaster females with free X's, and simulans males; and only male imagines from crosses between melanogaster females with attached X's, and simulans males. These unisexual progenies are due to the invariable death, before the imago stage, of the individuals of the missing sex. According to Kerkis (1933) death occurs at the latest in the early pupal stage.

From the reciprocal cross between simulans females with free X's and melanogaster males imagines of both sexes are obtained, but the viability of the regular (Xm/Xs)
Viability interactions between chromosomes

females* is erratically variable and generally very low. These females have the same chromosomal constitution as those, perfectly viable, from the cross melanogaster ♂ × simulans ♀; therefore the difference in viability must be due to either a maternal or a cytoplasmic effect. The situation may be summarized by saying that the presence of a simulans X-chromosome is indispensable for the viability of the hybrids; but the additional presence of a melanogaster X-chromosome reduces the viability when the combination is made in simulans cytoplasm (Sturtevant, 1929).

The situations described by Federley (1929) and Bytinski-Salz & Günther (1930) for certain crosses between moths are substantially the same, when the difference in the heterogametic sex is considered.

In a preliminary work by Muller & Pontecorvo (1940) the inviability of hybrids carrying no simulans X-chromosome was found to be determined by interactions between the X-, II- and III-chromosomes of the two species.

Using the method which is now reported in fuller detail, they succeeded in obtaining imagines with combinations of chromosomes of the two species such as those which would arise from a backcross of the F₁ hybrids to the melanogaster parent, should sterility not make this backcross impossible.

Among the artificially produced recombinants, henceforth called ‘partial hybrids’, those carrying no simulans X were found to be able to survive when either one or both large autosomes were purely of melanogaster origin. A single partial hybrid was even fertile: a female with X-, II- and III-chromosome pairs of melanogaster origin but with the Y and one of chromosome IV of simulans origin. From this fertile female the Y’s and IV’s were introduced in an otherwise purely melanogaster genotype, and the genetical behaviour of these two foreign chromosomes was investigated (Muller & Pontecorvo, 1941). Unfortunately, their work was suddenly interrupted before completion.

The present paper reports new results obtained on a larger scale. The problem investigated is that of specifying the interactions between the chromosomes of the two species which determine the viability effects.

Methods

Segregation in triploid females gives origin to eggs ranging from a completely haploid to a completely diploid set of chromosomes. When fertilized by a normal haploid sperm, eggs which carry one large autosome in diploid, and the other in haploid, condition produce trisomic embryos that never complete development. On the other hand, by X-raying the sperm, losses of one or more paternal chromosomes are induced (Muller, 1940 a; Pontecorvo, 1940, 1941, 1942; Pontecorvo & Muller, 1941) by simple breakage followed by union of sister chromatids and elimination of the dicentric chromatid during cleavage (for discussion of the mechanism see Pontecorvo, 1942). Embryos originated by the coincidence of the appropriate type of segregation in the egg and the complementary type of loss of one or more paternal chromosomes were found to be viable, no doubt because they have a normal diploid complement.

For instance, an egg carrying chromosome II in diploid condition when fertilized by a sperm in which chromosome II has been broken and successively eliminated may give rise to a viable diploid fly in which the chromosome II pair is entirely of maternal origin.

* Throughout the present paper the suffixes m or s to the symbol of a chromosome will be used to indicate its melanogaster or simulans origin, respectively.
If the egg carries both major autosomes in diploid condition and both paternal autosomes are lost, a fly may arise with both pairs of purely maternal origin. ‘Marking’ of the autosomes makes it possible to identify flies in which some such process has taken place.

This method was first successfully used in crosses between *melanogaster* triploids and irradiated *melanogaster* males (Pontecorvo, 1940; Pontecorvo & Müller, 1941). Then it was applied to the interspecific cross, using instead irradiated *simulans* males and the same *melanogaster* triploids (Muller & Pontecorvo, 1940). The *simulans* males were of the wild type, and the *melanogaster* triploids were made homozygous for brown (bw; II; 104-5) and ebony (e; III; 70-7) and heterozygous for eyeless (ey; IV; 0-2). They carried attached-X’s homozygous for yellow (y; I; 0-0) and a free X with the scute S1 inversions (In(I) scS1) which effectively prevent crossing-over along the whole X. In addition many carried an extra Y-chromosome. Any possible combination of paternal and maternal chromosomes X, II, and III from this cross was obviously identifiable by inspection.

The only previous published attempt to cross *simulans* males with *melanogaster* triploids is that by Schultz & Dobzhansky (1933), who found that mating occurs with much greater difficulty than with *melanogaster* diploids. They obtained only a single fertile culture out of about 100 made. Muller & Pontecorvo were more lucky in their preliminary attempt, about a third of the cultures then made being successful even though the males had been heavily irradiated.

At the beginning of the present work it was found that the age of the *melanogaster* females, both diploids and triploids, when first put with the *simulans* males is a very important factor for securing interspecific mating: females 1 or at most 2 days old mate fairly easily, especially if the *simulans* males have been collected young and kept for a few days separate from females. With females, especially triploids, more than 2 days old fertile cultures are obtained only erratically.

Accordingly a new routine, giving almost 100 % fertile cultures (Table 1), was adopted: wild-type, or *forked*, *simulans* males several days old were irradiated and kept at low temperature (12-15° C.). Each day a number of newly hatched *melanogaster* triploid virgins of the same stock previously used were collected and immediately mated with an equal number of irradiated males. Cultures, made in 2 x 10 cm. vials with 10-20 pairs each, were kept at 18 ± 2° C., transferring the pairs two or three times at 5–7 days’ interval. Four series were carried out with the following conditions of irradiation: oil-cooled Coolidge tube, tungsten target, beeswax back scatter, 200 kV., 15 mA., 3 mm. Al. filter distance 20 cm.; intensity about 900 r./min.; total dosage 4000–4500 r.

**Results**

The difficulty in obtaining large numbers of progeny from a cross like this is easily understood: only 10 % of eggs from triploids and only 10 % of X-bearing sperms, treated with our dosages, give viable hybrids, among which only about 3 % are ‘partial hybrids’. Thus ‘partial hybrids’ constitute some 1:3000 of the eggs laid.

The results obtained are given in Tables 1 and 2.

**Diploid** flies showing either or both maternal autosomal recessives are practically always ‘partial hybrids’, i.e. flies in which the corresponding paternal (*simulans*) autosome or autosomes, has been lost, in other words, in which the pair, or pairs, concerned is of purely maternal (*melanogaster*) origin. A single case was obtained of a diploid fly, showing the brown character, as a consequence of a mutation, minute deletion or position effect.
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in the paternal II
. In addition, a triploid fly showing the brown character was obtained. Its phenotype was unmistakably aneuploid: almost certainly a deleted II was present. Both these cases are omitted from the tables.

Table 2 gives the details of the partial hybrids obtained. Those obtained in the preliminary experiment by Muller & Pontecorvo are also tabulated. For the purpose of the present investigation both sets of data can be utilized, as the experimental conditions were practically the same. The chromosomal constitutions of each phenotype are shown in Table 3 and, graphically, in Fig. 1. Only one constitution is compatible with fertility, i.e. that of y; bw; e females.

It must be observed that among both full and partial hybrids the individuals of each particular phenotype show a very great variability in their fitness: some die shortly after eclosion, others are apparently of normal vigour.

Inspection of Tables 1 and 2 shows:

(a) That among full hybrids, i.e. those having hybrid constitution of both major autosomes, and, therefore, bw; e+ phenotype, the only classes present are those carrying X\(_y\): yellow females, yellow intersexes and scute S1 males, all produced in great numbers when triploids of this stock are crossed to melanogaster males (Table 4), are here totally absent.

Table 1. \(F_1\) from the cross of irradiated (4000-4500 r.) simulans wild-type or forked males with melanogaster triploids of composition \(\gamma/sc^{S1}; bw; e; (ey/+)\)

<table>
<thead>
<tr>
<th>Cultures</th>
<th>Total</th>
<th>Fertile</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>no.</td>
<td>no.</td>
</tr>
<tr>
<td>Series</td>
<td>no.</td>
<td>no.</td>
</tr>
<tr>
<td>1</td>
<td>14</td>
<td>14</td>
</tr>
<tr>
<td>2</td>
<td>19</td>
<td>19</td>
</tr>
<tr>
<td>3</td>
<td>14</td>
<td>13</td>
</tr>
<tr>
<td>4</td>
<td>11</td>
<td>11</td>
</tr>
<tr>
<td>Totals</td>
<td>58</td>
<td>57</td>
</tr>
</tbody>
</table>

* The classification of grey females as diploids or triploids is not entirely reliable.

Table 2. Recombinants (‘partial hybrids’) classified according to the phenotype

<table>
<thead>
<tr>
<th>Males</th>
<th>Muller &amp; Pontecorvo* no.</th>
<th>New series no.</th>
<th>Together no.</th>
</tr>
</thead>
<tbody>
<tr>
<td>(sc^{S1}; bw)</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>(sc^{S1}; e)</td>
<td>2</td>
<td>8</td>
<td>10</td>
</tr>
<tr>
<td>(sc^{S1}; bw; e)</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>(bw)</td>
<td>1</td>
<td>—</td>
<td>1</td>
</tr>
<tr>
<td>(e)</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>(bw; e)</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td>3</td>
<td>10</td>
<td>13</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Females</th>
<th>Muller &amp; Pontecorvo* no.</th>
<th>New series no.</th>
<th>Together no.</th>
</tr>
</thead>
<tbody>
<tr>
<td>(bw)</td>
<td>2</td>
<td>6</td>
<td>8</td>
</tr>
<tr>
<td>(e)</td>
<td>1</td>
<td>2</td>
<td>3</td>
</tr>
<tr>
<td>(bw; e)</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>(y; bw)</td>
<td>2</td>
<td>2</td>
<td>4</td>
</tr>
<tr>
<td>(y; e)</td>
<td>4</td>
<td>3</td>
<td>7</td>
</tr>
<tr>
<td>(y; bw; e)</td>
<td>1</td>
<td>—</td>
<td>1</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td>10</td>
<td>13</td>
<td>23</td>
</tr>
</tbody>
</table>

* The full hybrids \((bw^+; e^+)\) obtained in this series numbered about 350.
(b) That not all classes of partial hybrid are represented, and that, among those represented, the numbers differ from class to class.

Table 3. Recombinants obtained from irradiated (4000-4500 r.) melanogaster and simulans males, respectively, crossed to melanogaster triploids of composition \( \text{y/}sc^{51}; \text{bw}; \text{e} \); (ey/+)

<table>
<thead>
<tr>
<th>Types of segregation, each giving origin to two types of eggs*</th>
<th>Type of sperm fertilizing the egg</th>
<th>Autosome of sperm undergoing loss</th>
<th>Phenotype of resulting recombinant no.</th>
<th>Recombinants obtained from cross to irradiated triploids</th>
</tr>
</thead>
<tbody>
<tr>
<td>(1) ( \text{y/} )bw ( \text{e} ) ( \text{y}/ )bw ( \text{sc}^{51} )bw ( \text{e} ) ( \text{e} )</td>
<td>( Y )</td>
<td>II</td>
<td>( \text{y; bw} ) ?</td>
<td>22</td>
</tr>
<tr>
<td>(2) ( \text{y/} )bw ( \text{e} ) ( \text{y}/ )bw ( \text{sc}^{51} )bw ( \text{e} ) ( \text{e} )</td>
<td>( X )</td>
<td>III</td>
<td>( \text{e} ) ?</td>
<td>27</td>
</tr>
<tr>
<td></td>
<td>( Y )</td>
<td>III</td>
<td>( \text{sc; e} ) ?</td>
<td>11</td>
</tr>
<tr>
<td>(3) ( \text{y/} )bw ( \text{e} ) ( \text{y}/ )bw ( \text{sc}^{51} )bw ( \text{e} ) ( \text{e} )</td>
<td>( Y )</td>
<td>II, III</td>
<td>( \text{y; bw; e} ) ?</td>
<td>1</td>
</tr>
<tr>
<td>(4) ( \text{y/} )bw ( \text{e} ) ( \text{y}/ )bw ( \text{sc}^{51} )bw ( \text{e} ) ( \text{e} )</td>
<td>( X )</td>
<td>II, III</td>
<td>( \text{bw} ) ?</td>
<td>17</td>
</tr>
<tr>
<td></td>
<td>( Y )</td>
<td>II</td>
<td>( \text{sc; bw} ) ?</td>
<td>20</td>
</tr>
<tr>
<td>(5) ( \text{y/} )bw ( \text{e} ) ( \text{y}/ )bw ( \text{sc}^{51} )bw ( \text{e} ) ( \text{e} )</td>
<td>( X )</td>
<td>III</td>
<td>( \text{e} ) ?</td>
<td>12</td>
</tr>
<tr>
<td>(6) ( \text{y/} )bw ( \text{e} ) ( \text{y}/ )bw ( \text{sc}^{51} )bw ( \text{e} ) ( \text{e} )</td>
<td>( X )</td>
<td>II</td>
<td>( \text{bw} ) ?</td>
<td>10</td>
</tr>
<tr>
<td>(7) ( \text{y/} )bw ( \text{e} ) ( \text{y}/ )bw ( \text{sc}^{51} )bw ( \text{e} ) ( \text{e} )</td>
<td>( X )</td>
<td>II, III</td>
<td>( \text{bw; e} ) ?</td>
<td>1</td>
</tr>
</tbody>
</table>

* The only chromosomes considered are X, II and III, each being indicated by their markers, yellow (yy) attached-X's chromosomes, scute (sc^{51}) free X-chromosome, brown (bw) II chromosome, and ebony (e) III chromosome. The two products of each type of segregation are separated by a line. Segregation of the Y-chromosome, present in about 60 % of the triploids used, is most frequently as indicated for types (5), (6) and, probably, (7), whilst for the other types no data are available.

† The normal (\( \text{bw} \); e\( ^{+} \)) offspring of this cross, was represented by 8883 flies of which 8106 are classified in detail in Table 4; the remainder were obtained in later series where the normal progeny was not classified in detail.

The invariable inviability of full hybrids lacking X, is once more confirmed, also for hybrid intersexes carrying two doses of melanogaster autosomes (cf. Schultz & Dobzhansky, 1933).

The numbers of partial hybrids obtainable with the technique adopted depend on two factors: (1) the random coincidence of a determinate type of segregation in oogenesis with the complementary loss of one or more paternal chromosomes (see Table 3); and (2) the viability of the different types of embryos resulting from each of the preceding combinations.
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The data obtained by crossing triploids of the same stock used here to irradiated *melanogaster* males (Pontecorvo, 1940, 1942 and unpublished: Pontecorvo & Muller, 1941) offer a means of estimating the first of these factors and therefore of deducing the second.

**Table 4. Imagines obtained from the crosses to irradiated *melanogaster* and simulans males, respectively, compared as to their distribution among the different classes**

<table>
<thead>
<tr>
<th>Obtained from cross to:</th>
<th>Melanogaster 3♂</th>
<th>Simulans 3♂</th>
<th>Expected no.</th>
</tr>
</thead>
<tbody>
<tr>
<td>A Normal imagines (bw⁺; e⁺)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Grey diploid ♀♀</td>
<td>1892</td>
<td>427</td>
<td>181</td>
</tr>
<tr>
<td>Grey triploid ♀♀♀</td>
<td>1211</td>
<td>198</td>
<td>116</td>
</tr>
<tr>
<td>Patroclinous ♀♂♂</td>
<td>440</td>
<td>63</td>
<td>42</td>
</tr>
<tr>
<td>Grey intersexes</td>
<td>1151</td>
<td>97</td>
<td>110</td>
</tr>
<tr>
<td>Yellow diploid ♀♀♀</td>
<td>1770</td>
<td>0</td>
<td>169</td>
</tr>
<tr>
<td>Scute S1 ♀♂♂</td>
<td>1065</td>
<td>0</td>
<td>102</td>
</tr>
<tr>
<td>Yellow intersexes</td>
<td>777</td>
<td>0</td>
<td>55</td>
</tr>
<tr>
<td>Total</td>
<td>8106*</td>
<td>775</td>
<td>775</td>
</tr>
</tbody>
</table>

χ² = 723, P < 0.01, n = 6.

**Table 3 shows in the first column the different types of segregation giving origin to eggs, with one or both major autosomes in diploid condition. According to whether eggs of each of the proper types are fertilized by an X- or a Y-bearing sperm in which the loss of the required autosome (or autosomes) has been induced, an imago of a determinate phenotype may arise. The numbers obtained of each phenotype in the crosses to irradiated *melanogaster* and *simulans* males, respectively, are shown side by side in the two right-hand columns.**

The results with *melanogaster* males† show (Tables 3 and 4) that:

† It will be noted (Table 3) that, from both the intra- and interspecific crosses, imagines showing both autosomal recessives are obtained in very small numbers. This is not due to an extremely low frequency of segregations 3, 4 and 7 which are required to compensate for the loss of both paternal autosomes: in fact normal (bw⁺; e⁺) diploids, triploids and intersexes are produced just by such segregations. It is therefore due to the rarity of simultaneous loss of both autosomes. The frequency of 6tc; e imagines is barely more than expected for the random coincidence of the loss of each autosome independently produced. With the heavy dose of irradiation given, a very high proportion of chromosomes II and III in the sperms surely undergoes an aneucentric translocation. The fact that these translocations do not lead to numerous imagines of hw; e phenotype means, that the embryo is not able to eliminate dicentric chromosomes produced by translocations as it does with dicentric chromatids produced by simple breaks. This holds also for translocations between the X, or Y, and II, or III (see for discussion Pontecorvo, 1942).
Significant differences exist between the numbers of imagines in the classes of 'normal', $bw^+$; $e^+$, progeny (Table 4) originated from each type of segregation of the major chromosomes: grey diploid $\varnothing\varnothing$, triploids, $sc^{51}$ males and yellow intersexes, all of them from one type of segregation, are represented by 1892, 1211, 1065 and 577 individuals, respectively, instead of by roughly equal numbers; yellow diploid $\varnothing\varnothing$, and grey intersexes, originated from another type of segregation, are represented by 1770 and 1151 imagines. The lower number of $sc^{51}$ males, as compared with grey females, is, no doubt, due to the effect of the $sc^{51}$ mutant. As for the differences between the other classes, part at least are due to the lower viability of hypopl都喜欢 for chromosome IV as compared with hyperpluids. In fact at least 50%, and probably many more, triploids and intersexes are diplo-IV whilst many diploids are triplo-IV.

Among segregations giving origin to 'recombinants', $bw$ or $e$ (Table 3), types 1 and 5 in which two chromosomes II go to the most crowded pole are as frequent as types 2 and 6 in which two chromosomes III go to the most crowded pole (compare $y$; $bw$ with $bw\varnothing\varnothing$ and $y$; $e$ with $e\varnothing\varnothing$). In addition, losses of chromosome II occur with a frequency not significantly different from that of losses of chromosome III (compare $y$; $bw$ with $e\varnothing\varnothing$ and $y$; $e$ with $bw\varnothing\varnothing$). However, $sc^{51}$; $e\varnothing\varnothing$ are definitely scarcer than would be expected from these two conclusions. Independent evidence shows that the scarcity of $sc^{51}$; $e\varnothing\varnothing$ is due to a viability interaction between the $sc^{51}$ chromosome and the mutant ebony, or some other closely linked gene. This interaction has partly become suppressed in the triploid stock used by modifiers accumulated in chromosomes other than $\Phi$ and III.

We can now compare the results of the inter- with those of the intraspecific cross. The problem is whether or not the numbers in each class of 'normal' ($bw^+$; $e^+$) and of 'recombinant' ($bw$ and/or $e$) imagines from the cross to simulans males are in proportions significantly different from those obtained from the cross to melanogaster males. In Table 4 the two series of data are compared. The values of $\chi^2$ show that both in the case of 'normal' as well as of 'recombinant' progeny the outcome of the intraspecific cross is unquestionably different from that of the interspecific cross.

Let us analyse how this difference arises.

In the first place it is obvious that, among full hybrids, the total absence of imagines of the three classes without yellow $\varnothing\varnothing$, scute $S\bar{1}\bar{3}\bar{3}$, yellow intersexes—makes up for the greatest part of $\chi^2$. Limiting the analysis to the four classes of viable hybrids only, $\chi^2=92-2$, a still highly significant value for 3 degrees of freedom. Hence the four classes of viable hybrids are in different proportions than the corresponding classes from the melanogaster cross. In particular, triploids, patroclinous $\bar{3}\bar{3}$ and grey intersexes are, as compared with grey diploid $\varnothing\varnothing$, scarcer among the hybrids than among the melanogaster progeny.

Secondly, the comparison between 'partial' hybrids from the interspecific cross and 'recombinants' from the intraspecific cross gives the highly significant value of $\chi^2=42-8$, for 11 degrees of freedom (Table 4). Substantial contributions to this $\chi^2$ come from the excess among partial hybrids of $sc^{51}$; $e\varnothing\varnothing$ and the defect of $e\varnothing\varnothing$ and $\bar{3}\bar{3}$ and $sc^{51}$; $bw\bar{3}\bar{3}$.

If both full and partial hybrids are in proportions different from those of the corresponding classes of the melanogaster cross the question then arises of whether these differences originate from differential viability.

There can be no doubt that this is so in the case of full hybrids, but it seems reasonably certain that this is so also in the case of partial hybrids. In fact those classes of partial
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hybrids which are represented by higher numbers of individuals—i.e. bw ♀ (8); y; e ♀ (7); sc^51; e ♂ (10)—show that in a hybrid embryo losses of a simulans chromosome II occur as frequently as losses of a simulans chromosome III. It has been already pointed out that the same holds for losses of melanogaster autosomes in a melanogaster embryo. Then, if there is no differential course in the process itself of loss the discrepancies in the proportions of imagines from the two crosses must arise from differential survival.

We reach, therefore, the conclusion that both full and partial hybrids carrying different combinations of chromosomes from the two species have different viabilities.

With the technique adopted the composition of the hybrids as for the Y- and IV-chromosome escapes identification. An attempt to make the triploid stock homozygous also for a recessive in chromosome IV, eyeless, had to be discarded because of low viability. However, the results obtained by Muller & Pontecorvo (1940, 1941) from the breeding of the single fertile partial hybrid and some of the data reported here show that both the Y- and IV-chromosomes are almost surely not responsible for any of the viability effects observed, with the exception of those probably arising from hypoploidy for chromosome IV, which will be discussed later.

In fact the fertile partial hybrid mentioned above was an attached-X female having all chromosomes of melanogaster origin except the Y and one of chromosome IV. Crossed to melanogaster males this female gave viable sons all carrying the simulans Y, and about half carrying also a simulans IV.

Furthermore, among partial hybrids those having the following phenotypes usually carry a simulans Y (Table 3): females: y; bw, y; e and y; bw; e; males: sc^51; e and sc^51; bw; e. Partial hybrids of these phenotypes may, of course, be lacking the simulans Y if produced by a non-disjunctional sperm or by an X- or Y-bearing sperm in which the loss of the X, or the Y, has been induced at the same time as the loss of one or both autosomes. The frequency of these coincidences (see footnote, p. 56) is sufficiently low, however, to allow the conclusion that practically all the partial hybrids of the above classes actually carry the Y^.

This seems to dispose of Morgan’s (1929) suggestion that the lethality of hybrids lacking X^ might result not from the lack of X^ but from the presence of Y^, It seems highly improbable that Y^ could have a lethal action in hybrids with one set of autosomes of its own species, whilst giving no effect in (partial) hybrids with part or all chromosomes of the foreign species.

As for the simulans chromosome IV, its usual presence in single dose in full, as well as in partial, hybrids suggests that it does not produce any relevant dominant effect on viability in the two extreme cases of combinations with all other chromosome pairs of purely melanogaster origin (y; bw; e; females, and sc^51; bw; e males) and of combinations with all chromosome pairs of hybrid constitution (grey diploid females). Furthermore (Muller & Pontecorvo, 1941), even imagines carrying IV^ in homozygous condition and all other chromosomes of melanogaster origin are still fairly viable. It seems very unlikely that IV^ could act differentially when in hybrid condition in the other types of partial hybrids.

It may be concluded with reasonable safety that the fact of not being able to detect the individual composition of each partial hybrid as far as concerns the Y- and IV-chromosomes is not a serious limitation in the interpretation of our results.
Discussion

(a) Types and number of viability interactions

The results show that hybrids carrying different combinations of the major chromosomes of the two species have different viabilities. In Fig. 1 these are plotted against the chromosome combinations. The units of measurement are arbitrary, viability being inferred by the proportions of hybrid imagines in each class, as compared with the corresponding proportions in the melanogaster cross.

Before discussing the possible mechanism underlying the differential viability the following limitations must be pointed out.

First, only combinations considered are normal hybrids with one or two melanogaster sets and partial hybrids with combinations as those which would result from a backcross to melanogaster. Reciprocal combinations would require reciprocal crosses the technical means for which are unobtainable under the present circumstances: simulans triploids are available only in Russian laboratories; the claret mutant of D. simulans which causes abnormal segregation in oogenesis (Sturtevant, 1929) is not available in the British Isles. As for combinations of one autosome pair from one species with another from the other species, no method of achieving this has yet been devised.

Secondly, the partial hybrids obtained are recombinants between whole chromosomes as, obviously, no crossing-over can possibly occur. Therefore we are concerned here only with interchromosomal, and not also with intrachromosomal, recombinants as would be obtained in a real backcross. This simplifies the position but limits the scope of the analysis.

Thirdly, our partial hybrids are produced from pure melanogaster eggs and not from hybrid eggs as would be the case with a real backcross. This too, probably, simplifies the position. In fact cytoplasmic or maternal effects may reasonably be excluded here: the

<table>
<thead>
<tr>
<th>Females</th>
<th>Males</th>
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<tr>
<td>X</td>
<td>II</td>
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<tr>
<td>&gt;</td>
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<td>&gt;</td>
<td>e</td>
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<tr>
<td>&gt;</td>
<td>bw; e</td>
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Fig. 1. Viability, from 0 to 100, of each type of full and partial hybrid, relative to the viability of the corresponding melanogaster types. The simulans chromosomes are in solid line, the melanogaster chromosomes in outline. Only the X-, II- and III-chromosomes are shown. All types carry one chromosome IV from simulans. The XX females and intersexes, and the males with melanogaster X carry a simulans Y; the males with simulans X carry a melanogaster Y, present in the majority of triploids of the stock used.
pure *melanogaster* egg-plasm does not give any marked viability effect in the ordinary hybrids; it seems unlikely that it would affect selectively the viability of partial hybrids, the chromosomal constitution of which is more nearly that of pure *melanogaster*. Cytoplasmic or maternal effects, however, are to be expected for some of the reciprocal combinations made in pure *simulans* egg-plasm, as one at least is already known (Sturtevant, 1929) to operate in full hybrids from *simulans* females.

If we may exclude, for combinations made in *melanogaster* egg-plasm, an extra-chromosomal origin of the viability effects, the question then arises of which chromosomal interactions are operative.

Some relations clearly stand out (Table 4 and Fig. 1):

1. **Complete lethality** when $X_m$ in hemi- or homozygous condition, i.e. acting as a recessive, is present with both $II_a$ and $III_a$ in hybrid condition, i.e. acting as dominants, even against a double set of *melanogaster* autosomes (full hybrids without $X_g$; $\delta\delta$, $\varnothing\varnothing$ and intersexes).

2. **High viability** when $X_g$ in hemi- or homozygous condition, i.e. as a recessive, is present with $II_a$ in hybrid condition, i.e. as a dominant ($se^{51}$; $e\delta\delta$ and $e\varnothing\varnothing$ partial-hybrids).

3. **High viability** when $X_g$, as a dominant, is present with $III_a$, as a recessive ($bw$ $\varnothing\varnothing$ and $\delta\delta$ partial hybrids).

4. **Reduced viability** when $X_g$, as a dominant, is present with $III_a$, as a recessive ($e\varnothing\varnothing$ and $\delta\delta$ partial hybrids).

Other relations, as those revealed by the differential viability of full hybrids, will be discussed later.

First, we have the complete lethality of combinations without $X_g$ when both large autosomes are in hybrid condition as contrasted with the good viability of the same combinations when only one, no matter which, large autosome pair is of purely *melanogaster* origin. The lethal effect arises from the interaction of $X_g$, acting as a recessive, with both $II_a$ and $III_a$ acting as dominants. Each of these two interactions probably modifies one or more developmental processes in some way that is little harmful in itself but incompatible with the other. In fact the lethal effect cannot be accounted for by simply additive results of two independent interactions, one between $X_g$ and $II_a$, and the other between $X_g$ and $III_a$. The data show the first to have no appreciable effect on viability ($y$; $e\varnothing\varnothing$ and $se^{51}$; $e\delta\delta$), the second to have only a limited effect, if any, in the females ($y$; $bw$) and a more drastic one in the males ($se^{51}$; $bw$): a specific action of the $se^{51}$ chromosome is the only available explanation of the difference between the two sexes.

Secondly, there is the low viability of partial hybrids carrying $X_g$ but no $III_a$ ($e\varnothing\varnothing$ and $\delta\delta$). This reveals a third interaction between $X_g$ acting as a dominant, and $III_a$, acting as a recessive.

We must now consider the group of viability effects differentiating the four classes of viable full hybrids (Table 4).
by themselves, are in the right direction: both \(bw\) and \(e\ \varnothing\) are relatively scarcer than \(bw\) and \(e\ \varnothing\).

The scarcity of hybrid \(\varnothing\) reveals, then, an interaction between \(X_g\), acting as a recessive, and one or more \(melanogaster\) autosomes acting as dominants.

In the second place, hybrid triploids and intersexes are relatively less viable than hybrid diploid females, their proportions being about\(3/4\) and \(1/3\), respectively, of those obtained in the \(melanogaster\) cross. It has already been pointed out that hypoploidy for chromosome IV accounts for the scarcity of triploids and intersexes even among the pure \(melanogaster\); for the intersexes of course this acts in addition to the upset balance between \(X\)-chromosome and autosomes which must determine some viability effect too.

We see now that triploids and intersexes are less viable than diploids also among hybrids but much more so than among pure \(melanogaster\).

From the present results and from the breeding of the single fertile partial hybrid (Muller & Pontecorvo, 1940, 1941) we know that:

(a) IV\(_g\) in hybrid condition does not affect viability in any appreciable measure when in combination either with all other chromosome pairs of pure \(melanogaster\) origin, or with all other chromosome pairs of hybrid composition.

(b) IV\(_g\) in homozgygous condition reduces considerably the viability when in combination with all other chromosome pairs of pure \(melanogaster\) origin.

The low viability of hybrid triploids and intersexes would follow from these facts should the interaction between IV\(_g\) and one or more \(melanogaster\) major chromosome, indicated by (b), be capable of partial expression in the particular conditions of ‘dosage’ occurring in hybrid diplo-IV triploids and intersexes. If this assumption is correct the interaction is between IV\(_g\) and an unidentified \(melanogaster\) autosome both acting as not completely recessives.

Thus, two interactions at least are revealed by the data on full hybrids: one, between \(X_g\)—recessive—and an undetermined \(melanogaster\) autosome—dominant—accounts for the lower viability of males as compared with females; the other, between IV\(_g\) and an undetermined \(melanogaster\) autosome—both partially recessives—accounts for the reduced viability of triploids and intersexes.

To summarize, we have first found, from the results with partial hybrids, at least three viability interactions. From the results with full hybrids we find now at least two others. In all, then, at least five viability interactions have been detected between the chromosomes of the two species.

These are only a very minor part of the interactions which may, and indeed certainly do, exist. In fact we have dealt here only with those affecting viability. There must be many others with more specific effects: some, affecting the onset and course of meiosis, and others affecting the process of bristle formation, have already been detected in this same material and will be the subject of a separate report. Furthermore, our recombinants can only reveal interactions characterized by certain dominance relations; and, for example, Muller & Pontecorvo (1941) have already shown that even the small chromosome IV is responsible for a whole series of viability, fertility and other interactions detectable only in partial hybrids homozygous for IV\(_g\). Finally, interactions having their origin in different sections of homologous chromosomes of the two species escape detection with the present technique. It may safely be concluded that the number of interactions operating is surely of a different order of magnitude than that found here.
Viability interactions between chromosomes

(b) Mechanism of the viability interactions

The mechanism underlying the viability interactions, being wholly, or practically so, of chromosomal origin, must lie either in structural changes or in complementary mutation (or combinations of mutations) differentiating the two species.

It would not be difficult to imagine, and indeed not impossible to build up experimentally, two stocks of *D. melanogaster*, or *simulans*, each homozygous for a series of minute translocations of the insertional type which, crossed with each other, would imitate at least the most striking viability effects resulting from the interspecific cross.

The visible differences so far detected in the structure of the salivary gland chromosomes of the two species (Patau, 1935; Kerkis, 1936; Horton, 1939; Slizynski, 1941) are:

1. One extra band in *X*, in *II L*, *II R* and *IV L*. In addition, terminal differences of a disputable nature between *IV R* of the two species.

2. One large inversion (about 8 divisions) and one small (10 bands) in *III R*; one small inversion (10 bands) in *IV R*; two small inversions in *X* and one in *II R*.

3. Seven regions in *X*, five in *II L* and two in *II R* in which synapsis is irregular.

Thus the only visible structural changes which may be of the nature of translocations are those under (1). Chromosome *III* not being involved, they cannot account for all our viability interactions.

If translocations have to be the cause then, we must think in terms of extremely minute ones escaping cytological detection. Indeed, in our hybrids the slight viability effects when the interactions operate singly, and the advanced stage of development at which death occurs when they operate jointly, already restrict the types of translocations possibly involved to precisely those of minute size.

Koller (1932) pointed out, and Sturtevant & Beadle (1936), Dobzhansky (1937), Darlington (1939) and Muller (1939, 1940b) developed, that the lethality of part of the progeny from heterozygotes for large translocations, pericentric inversions and shifts must hinder their establishment in the process of divergence. It is a fact that paracentric inversions are found far more frequently than the former types of structural changes in natural populations and in closely related systematic groups. However, this argument does not hold for structural changes so minute that they but little affect the viability and fertility of hyper- or hypoploid individuals (Muller, 1940b). As recently developed by Wright (1941), the chance of fixation of these minute changes, in the extreme case of the unbalanced individuals being at no disadvantage, may even be greater than that of an indifferent mutation.

In our case the translocations, of a very minute size, could have occurred in successive steps each of which, producing but slight reduction of viability in the aneuploid condition, would have not been seriously hampered during the preliminary-heterozygous phase.

We must conclude that, though unlikely, the possibility that minute translocations cause part of the viability interactions cannot altogether be discarded.

Alternatively let us consider whether or not the mechanism of interacting, or 'complementary', mutations (Dobzhansky, 1937; Muller, 1939, 1940) offers a better interpretation of the facts.

The minimum of five viability interactions observed in our material could be brought about by pairs, or triplets, of genes represented by different allelomorphs in the *melano-
G. Pontecorvo

... and simulans chromosomes, the members of each pair, or triplet, being located in non-homologous chromosomes.

For the first two interactions, which cause the lethality of full hybrids lacking \( X_g \), we may assume three genes in \( X, \) II and III, respectively, each represented by a recessive allelomorph in the melanogaster and a dominant in the simulans chromosome. The melanogaster allelomorphs act as a lethal \( (l_m) \) in \( X_m \), and two specific suppressors \( (su^m) \), in II\(_m\) and III\(_m\), respectively. As for the third interaction, which causes the low viability of partial hybrids, we may postulate a recessive semilethal \( (l_s) \) in \( X_m \) and a recessive suppressor of it \( (su^s) \) in any of the simulans autosomes, e.g. III\(_s\). Finally, for the interactions affecting the viability of hybrid triploids and intersexes, we may postulate a partially dominant semilethal \( (l_d) \) in IV\(_s\) and a partially dominant suppressor of it \( (su^{d}) \) in any of the simulans autosomes, e.g. II\(_s\). Indicating with + the dominant (or almost dominant) allelomorphs the composition of a hybrid female would be:

\[
\begin{array}{cccc}
\text{Simulans} & + & + & l_s \\
\text{Melanogaster} & l_m & su^s & + \\
\end{array}
\]

As pointed out by Muller (1940a) in the process of divergence ‘suppressor’ mutations of the kind suggested here must become established first. In other words, a mutation which is indifferent, or even slightly disadvantageous when first established, may become indispensable when other changes in the remainder of the genotype have occurred.

Conclusions

The preceding is obviously only a way of representing each of our interactions in terms of the minimum possible number (nine) of genes located in non-homologous chromosomes. We do not know how many are actually involved. We can, however, discuss whether it is more likely that the interactions are caused by a small number, at least nine, of ‘main’ mutational steps or by great numbers of mutations. In the latter case, each of the observed viability effects would be the resultant of many slight interactions.

In this respect it is significant to recall what has already been pointed out about the variability in the vigour of the imagines of each type of full and partial hybrids. The same striking variability is typical of other characters, such as the development of the gonads, bristles, etc. (cf. Pontecorvo, 1943). Furthermore, a perusal of published data shows the same high variability among the \( F_1 \) of interracial or interspecific crosses in material as varied as Drosophila pseudobscura (Dobzhansky & Boche, 1933), Peromiscus (Sumner, 1932), human races (reported by Muller, 1936), Corvus, Bufo, several echinoderms (reported by P. Hertwig, 1936), to take only a few examples from metazoa. Thus the high variability is at least a not uncommon, and probably a general, feature of \( F_1 \) hybrids; it contrasts strikingly with the usual uniformity of the parental populations.

‘Balanced’ polygenic combinations (Mather, 1941, 1942; Wigan, 1941) offer a scheme which may explain this situation.

The parental populations would be heterogeneous for polygenic combinations subject, within each population, to ‘balance’ against each other by selection: hence the phenotypical uniformity. The polygenic combinations of one population, on the other hand,
Viability interactions between chromosomes

would hardly be expected to be balanced also against those of another population with
which no interbreeding occurs: hence the variability of the hybrids.

In our hybrids the phenotypical disturbances, of viability or other characters, arise
when a particular chromosome of one of the two species is present with another, non-
homologous, chromosome of the other species. Furthermore, it must be remembered, the
two species have almost identical phenotypes.

We can envisage the situation which leads to anyone of the observed interactions
supposing these identical end products to result from the equilibrium between two
reactions each governed by polygenic combinations located in non-homologous chromo-
somes. Each species is heterogeneous for the combinations governing each of the two
reactions, but this heterogeneity is ‘balanced’ by selection so as always to keep each
of the two reactions around a certain average. Every time that, through recombination,
one of the two polygenic combinations far exceeds this average value, or fails short of it,
the end-products will fall out of the adaptive limits, unless a compensatory change happens
in the other (non-homologous) polygenic combination. Such a coincidence must be very
rare for two changes of an extreme nature, but must be very frequent for two changes
just on the borderline of the adaptive limits.

We may think of the differentiation between the two species causing the incapacitatioD-
of their hybrids as having been brought about by a series of successive coincidences of
the latter kind. Namely, while one of the polygenic combinations, through crossing over,
determined a change in a certain direction of the reaction it governs, the other determined
a compensatory change of the other reaction, the equilibrium between the two remaining
about the same. Obviously this ‘equilibrium’ is nothing else than what we have termed
‘interaction’ throughout this paper. The final outcome would be that two non-homologous
chromosomes would carry polygenic combinations physiologically linked to each other,
i.e. no longer interchangeable, unless jointly, with those of the other species.

It is useless to say that the same process can apply to interactions having their origin
in homologous chromosomes: only that our technique did not allow such interactions to
be detected.

The interactions dealt with here are those determining viability effects: as there seems
to be no reason in the present case for an adaptive nature of such effects they must have
arisen, after the isolation—geographical or genetic—of the two species had become
complete, during the inevitable process of further ‘cryptic’ divergence. It will be seen,
however, that the mechanism put forward here when operating, for instance, on mating
behaviour may determine genetic isolation.

Summary

1. Using Muller & Pontecorvo’s (1940) method—irradiated Drosophila simulans ♀♂ x
D. melanogaster ♀♀—a sufficiently large number of artificial recombinants (‘partial hybrids’) were obtained carrying different combinations of chromo-
somes of the two species. The combinations, all but one sterile, are those which would
arise from a backcross of the F₁ hybrids to melanogaster, if the sterility of the F₁ hybrids
did not make this backcross impossible. They are only combinations between whole
chromosomes, not also between sections of homologous chromosomes, and they are made
in pure melanogaster cytoplasm, not in hybrid cytoplasm, as would be the case in a real
backcross.
2. A whole range of viability effects, entirely of chromosomal origin, has been detected in full hybrids (diploid and triploid ♀, ♂, intersexes) as well as in 'partial' hybrids. The viability effects show high degrees of variability between individuals carrying the same chromosomal combinations. This variability is also characteristic of other hybrid effects.

3. The viability effects arise from interactions between non-homologous chromosomes of the two species, the X and the two major autosomes playing a predominant part. The Y-chromosome is certainly not involved, and the IV-chromosome plays only a minor role. The interactions detectable in our combinations are at least five.

4. No obvious connexion has been found between the interactions and the visible structural differentiation of the salivary gland chromosomes of the two species.

5. 'Complementary' mutations may offer an explanation of the origin of the interactions: a minimum of nine mutations—three in X, one in II, two in III, one in IV, one in any one of the autosomes and one in II or III—would be sufficient to account for the effects observed. It is suggested, however, also on the basis of accessory evidence, that pairs of 'polygenic' combinations, located in non-homologous chromosomes, rather than pairs of complementary 'main' mutations are involved.

6. The possible role of interacting polygenic combinations in the process of genetic isolation is adumbrated.

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REFERENCES


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STURTEVANT, A. H. (1921a). Genetic studies on Drosophila simulans: II. Sex-linked group of genes. Genetics, 6, 43-64.


SUMNER, F. B. (1932). Genetic distributional and evolutionary studies of the subspecies of deer mice (Peromyscus). Bibliogr. genet. 9, 1-106.


It has long been known that some chromosomes, or chromosome segments, respond to staining during mitosis or meiosis in a different way from the rest of the set. They are called 'heterochromatic' to distinguish them from the 'euchromatic' rest of the set taken as a standard. The cyclic staining reaction of the chromosomes is generally attributed to synthesis, during prophase, and breakdown, during telophase, of deoxyribonucleic acids—a view recently challenged by Stedman and Stedman, but which will be followed here, the substance actually involved being irrelevant for the present discussion. It is now clear, especially from the work of Darlington and his collaborators, that the visible difference between heterochromatin and euchromatin is a consequence of a difference—referred to as 'alloidy'—in the timing and/or in the amplitude of the above cycle. In heterochromatin the maximum nucleic acid charge attained by euchromatin at metaphase may be attained earlier, later, never or even surpassed. This behaviour has been taken as showing that heterochromatin has a lower reactivity in nucleic acid synthesis, and that, therefore, only when the demand for nucleic acid precursors by the more active euchromatin is low can heterochromatin successfully compete for them. It will be 'seen' that another explanation is also possible.

Let us consider the characteristics and behaviour of heterochromatic segments. In the first place, the same segment, or chromosome, may be visibly allocyclic in one tissue and not in another, or it may have different allocyclic behaviour in different tissues. An example of the former type are the sex-chromosomes of many mammals, strongly allocyclic at meiosis but not so at mitosis; and of the latter the X-chromosome of the Acrididae, in which the cycle is shifted one way in certain divisions of the germ track and the opposite way in others. Thus the allocyclic behaviour is determined by at least two factors: the reactivity of the heterochromatin segment itself and the conditions of the cell. As some of the latter can be controlled, what happens in Nature between tissues has been mimicked experimentally within one tissue. For example, by low-temperature treatment it has been possible to detect as heterochromatin in mitotic or meiotic metaphase segments that under normal conditions could not be identified as such.

Secondly, two or more heterochromatic segments in the same nucleus, or even in the same chromosome, often differ from each other, besides differing from euchromatin, in their nucleic acid cycle. A conspicuous example of this is provided by the pairing and differential segments of mammalian sex-chromosomes: both are heterochromatic but with distinct cycles. In this case it is certain that the different chromosomes with different genetical properties.

Thirdly, heterochromatic segments, or chromosomes, have been identified of any visible length. They may be located in any part of the chromosome set. Of course, the greater their length and the stronger their allocly, the more easily they are detected. Our knowledge of their occurrence in animals and plants is, thus, inevitably a very distorted one.

Fourthly, certain heterochromatic segments in some species have the property of 'non-homologous' association in the prophase of meiosis, or in the polytene chromosomes. This property, however, is of erratic occurrence and tends to occur in groups of related species, rather than at random. This suggests less differentiation than euchromatin. Indeed, this later, is by no means a general characteristic of all heterochromatin.

Finally, since the discovery by Muller and Painter of genetically 'silent' chromosome segments, and their identification by Heitz, the types of proteins in the chromosomes have been identified of any visible length. They may be located in any part of the chromosome set. Of course, the greater their length and the stronger their allocly, the more the same size. The 'inertness' of heterochromatin may mean that either there are actually few, or no, genes in it, or there are a full quota of genes but the developmental effects of mutation or change in quantity in them are difficult to detect. Recent work strongly suggests that the latter alternative is the correct one.

Several trends of thought and lines of research seem to agree in regarding heterochromatin as internally less differentiated than euchromatin. Indeed, this idea was already implicit in Muller's early suggestion that what we now know as heterochromatin originates from mutation towards 'inertness' of genes kept in a permanently heterozygous state, as those of the Y-chromosome, and that, in such situations, 'inertness' has been found to accompany allocyly in all unambiguous cases. This 'inertness' manifests itself in two ways. One is that, per unit length, heterochromatic segments carry fewer, or no, genes detectable by sharply alternative effects of allocation in all unambiguous cases. The other is that deficiencies or duplications for heterochromatic segments have far less harmful effects than deficiencies or duplications for euchromatic segments of the same size. The 'inertness' of heterochromatin may mean that either there are actually few, or no, genes in it, or there are a full quota of genes but the developmental effects of mutation or change in quantity in them are difficult to detect.
specify and low specificity. Further, with his collaborators, he has shown that the heterochromatic 'supernumerary' chromosomes, characteristic of many animals and plants, are by no means inert. These 'supernumeraries', though varying in number between cells of an individual and individuals in a population, tend, as mentioned earlier, towards an equilibrium, which Darlington and his collaborators have shown to be adaptive. Finally, Mather has given a concrete expression to the idea of the uniform structure of heterochromatin by suggesting that the heterochromatin is made up of 'polymers', and by finding genetical evidence which supports this idea. Study of the genetical and cytological properties of mammalian sex-chromosomes also supports Mather's view.

Hence there seems to be a considerable amount of agreement for considering heterochromatin as internally less differentiated than euchromatin. On two points, however, the present trend of thought is not equally definite. One is the connexion between this lesser differentiation and the allocyclic behaviour. The other is the question of whether or not the elements that make up heterochromatin are necessarily different from those that make up euchromatin. These two questions will now be discussed.

Let us first consider what makes the nucleic acid cycle of euchromatic regions appear uniform throughout the nucleus. It is proper to raise this question before discussing what makes heterochromatin differ in this respect, because, in fact, during a brief part of the division cycle euchromatic regions are far from uniform along their length. This part is early prophase, especially of meiosis, when nucleic acid has already condensed along the chromosomes but their lengthwise contraction is barely starting. In this short period the chromosomes are distinctly visible and obviously differ from each other in their nucleic acid charge. Each chromosome shows a distinct reactivity of its own in nucleic acid synthesis; it is 'eneuchromatic' in relation to others, either because its cycle is shifted in time, relative to that of other chromosomes, or because different chromomeres synthesize at different rates or reach different final charges. The giant salivary gland chromosomes of Diptera show this longitudinal differentiation of euchromatic regions even better; their 'bands' present an array of different charges and are intermingled along the chromosomes more or less at random with respect to these charges. As mitosis or meiosis proceeds, the chromosome contract unequally by spirialisation and loss of protein. Consequently it is no longer possible to distinguish the individual chromomere; all that a chromosome segment can show is the aggregate effect of its component chromomeres. In segments with intermingled chromomeres of different charges, the result is apparently uniform within and between segments. The reaction of euchromatic regions, optically uniform as they are after prophase throughout the whole chromosome set, is, thus, the statistical consequence of their being made up of a mixture of highly differentiated chromomeres. The immediate corollary is that a segment in which chromomeres are more uniformly may differ in its allocyclic reaction from the former.

If we accept the view that heterochromatin is made up of elements less differentiated than euchromatin, we have only to assume that these elements are chromomeres, and chromomeres of the same type as those which we find intermingled in euchromatin. The essential difference between the two types of chromatin would then lie exclusively in the linear arrangement of chromomeres with the same nucleic acid cycle; a heterochromatic segment being one with a very high proportion of these similar or identical chromomeres. Segments made up of repeated replicas of a single type-chromomere, or few, will be the extreme terms of a series between euchromatin and heterochromatin. Unfortunately, the only case in which, so far, it has been possible to make out the minute structure of heterochromatin in a suitable stage is that of some Dipterae salivary chromosomes. Here each heterochromatic segment actually consists of chromomeres far more uniform than those of the euchromatin; moreover, in Drosophila, some, but not all, heterochromatin segments in a salivary gland cell agree in having the same types of chromomeres. The latter feature, however, like that of 'non-homologous' association probably connected with it, is certainly not general.

Clearly the preceding suggestion accounts for the allocyclic behaviour of heterochromatin. In fact, the nucleic acid cycle of the type-chromomere, or chromomeres, making up a heterochromatic segment, will but seldom happen to be identical with the nucleic acid cycle of euchromatin, which is an average. More often it will be distinct, being out of phase or in other ways differing from the latter, just as two 'euchromatic' chromomeres may differ from each other. Furthermore, the type-chromomere, or chromomeres, of each heterochromatic segment need not, and often will not, be the same for different segments, thus accounting for the different allocyclic behaviour of two or more segments in a nucleus. On the other hand, it may be inferred that 'non-homologous pairing' occurs just in those species where these type-chromomeres happen to be the same in more than one segment; in other words, in cases where two or more heterochromatic segments have a common origin, as Prokofjeva and Muller suggest for Drosophila. This would also explain why this property appears erratically but tends to occur in several of a group of related species. Thus allocyclic behaviour can be accounted for by assuming a different linear arrangement of similar chromomeres without need of the further assumption of any difference in the nature of the euchromatin, or for euchromatin. Can we, on this simple assumption, also account for the mechanisms in cell physiology that are being attributed to heterochromatin?

The main inference is that heterochromatin acts as a regulator of the nucleoprotein synthesis of the cell and therefore controls the reproduction of the chromosomes. Indeed, the whole of part of this evidence would become decisive only if it were established that euchromatin does not produce the same effects. However, on the basis of the structure of heterochromatin as proposed here, one would expect each heterochromatin segment to exercise both a localized and a general specific effect on the conditions of nucleoprotein synthesis in the nucleus, often different from the non-specific effect (in this respect) of any euchromatic segment of comparable length. It is, therefore, permissible to venture a prediction:
namely, that close investigation will reveal very characteristic differences in the action of different heterochromatic segments in the same species, and between species. It is perhaps due to the fact that so much use has been made of Drosophila for the investigation of heterochromatin that this possibility has been overlooked (but see Demerec*).

This brings us to speculate upon the origin of heterochromatin. It is implicit in the view expressed here that a heterochromatic segment should arise every time that a minute euchromatic region undergoes repeated reduplications in the genotype and the replicas remain adjacent to each other on the chromosome. Models of some such process are known, and there is every reason to believe that, once a first replica has become established, the mechanical and genetical possibilities of its being repeated are enhanced. The finding by Harland that the same function may be performed in one species by a single gene and in a related species by many is perhaps an example of the genetical consequences of this process. There is, thus, plenty of scope for new heterochromatic blocks to be formed, for old ones to be eliminated, and for variations in size. Furthermore, inversions and other appropriate structural changes may break up an originally compact heterochromatic segment into many small ones interspersed among euchromatin (cf. Kaufmann*). Natural selection and accidental variation seem to have a number of possibilities here.

As stressed by Wharton and by White, among others, heterochromatin certainly plays an important part in speciation. This part stands out in terms of Mather’s theory of polygenetic variation. Mather has studied the function of linkage between 'polygenes' (definable perhaps as genes existing in repeated replicas in the genotype) as a basis on which the evolutionary plasticity of a species is founded;
GENETIC SYSTEMS BASED ON HETEROCARYOSIS

G. PONTECORVO

Genetics has been built on the study of species with sexual reproduction. An essential feature of genetic systems based on sexual reproduction is the alternation of meiosis and karyogamy, which has meaning only in relation to heterozygosity. These genetic systems could therefore be called "heterozygotic systems." For obvious reasons of economy, genetic systems could therefore be called "hetero-meaning only in relation to heterozygosity. These alternation of meiosis and karyogamy, which has with sexual reproduction. An essential feature of the gene as the basis of life," it was obvious that Mendelism was not the only way to a definition of the gene. Recently Beadle and Coonradt (1) with their work on Neurospora have given beautiful proof of what had been inferred from studies of somatic mutation in Drosophila, maize, etc.; namely, that even tests of allelism can be carried out without recourse to sexual reproduction. Pontecorvo and Gemmell (8) have confirmed this in a completely agamic species like Penicillium notatum, and other examples will be given here. The view that there cannot be a "genetics" of agamic species seems thus outdated. Classical genetics has taught us concepts and techniques which have some application in the study of any system of heredity and variation involving particulate units, even if not mechanically held together in chromosomes, provided these units have two essential properties of the gene: mutation, and reproduction in the mutated form.

The present paper attempts to describe certain genetic systems not based on karyogamy and meiosis but on segregation and recombination of whole nuclei in multinucleate cells. These systems seem to be universal in the Fungi imperfecti, widespread side by side with sexual reproduction in most other Fungi, and almost certainly occurring in other groups of microorganisms (Myxomycetes, Protozoa, Algae). To make their genetic study more attractive, there is also the likelihood that the mechanisms of heredity and variation in bacteria may become more easily approachable if we understand these. Certain Hyphomycetes, such as Penicillia and Aspergilli, will be taken as models, though the Ascomycetes, such as Neurospora, and the Basidiomycetes like the rusts and smuts—in which standard systems based on sexual reproduction coexist with these systems—have been and will continue to be extremely useful for the study of certain aspects of the problem.

The characteristics of the genetics of Hyphomycetes are as follows. (1) Sexual reproduction, involving fusion of nuclei and meiosis, is absent, or so rare as to play an unimportant part. (2) Most cells of a colony are multinucleate. (3) Pairs of cells occasionally fuse with each other. (4) Following such fusions, one or more nuclei may migrate from one cell into the other. If the nuclei of the cells that fuse are genetically different, one multinucleate cell, and part or all of its descendants, may come to carry nuclei that are not "all alike. The same condition may, of course, arise as a consequence of mutation in one or more of the several nuclei of a cell. This condition is known as "heterocaryosis." Since ample reference to the pioneer work on heterocaryosis, mainly by Hansen, Dodge, and Lindegren, is given in a recent paper by Beadle and Coonradt (1), it will be dispensed with here. The terminology of heterocaryosis has been used in the most disconcerting ways; an attempt at unification is made in an Appendix at the end of this paper.

The analogy between heterozygosis and heterocaryosis is simple. In a heterozygous nucleus, allelomorphic genes are located in homologous chromosomes within that nucleus; in a heterocaryotic cell, the "allelomorphs" are located in different nuclei of that multinucleate cell. Segregation and recombination of the allelomorphs carried in a heterozygous nucleus require meiosis and karyogamy. Segregation and recombination of the "allelomorphs" carried in different nuclei of a heterocaryotic cell require an entirely different mechanism. Of course, in species like Neurospora where heterocaryosis and sexual reproduction coexist the standard mechanism of segregation and recombination may also operate, and in this case we may speak of allelomorphs without quotation marks. This is why species like these have been and will continue to be extremely useful: they bridge the gap between genetic systems of the usual types—"heterozygotic systems"—and genetic systems based exclusively on heterocaryosis —"heterocaryotic systems." In other words, they make it possible to attack with Mendelian techniques certain aspects of heterocaryosis. In exclusively heterocaryotic systems the absence of karyogamy and meiosis prevents the exchange of genes between nuclei. Thus differences between nuclei can arise only through mutation—in its broadest sense of change in quality, quantity, or arrangement of nuclear particles; but differences between cells can arise both through mutation and through recombination of genetically different nuclei.

The experimental study of heterocaryotic systems has barely started. Therefore the present paper can be no more than a statement of some of the fascinating problems they present and an
illustration of certain techniques by which these problems are being tackled. The comparative approach will be extensively used, because from the present knowledge of heterozygotic systems we can make useful predictions open to verification in heterocaryotic systems. Two main fields of comparative investigation are the genetics of metabolism (including growth and development) and the genetics of cell populations. In respect to the former we are faced with the action of genes carried in different nuclei of a cell; in respect to the latter we are faced with cells in which the assortment of nuclei can change in successive cell generations.

**Hyphal Fusions**

In a growing colony of, for example, an Aspergillus or a Penicillium, hyphal anastomoses can readily be observed. They occur between branches of the same homo- or heterocaryotic hypha (Fig. 3) or between homo- or heterocaryotic hyphae of different origin (Fig. 4), which may or may not differ in the kinds of nuclei they carry. Inspection of a few slide cultures can leave no doubt that the frequency of hyphal fusions is very variable. The conditions controlling this frequency are, however, totally obscure. One factor is certainly the frequency of chance contacts between hyphae, which depends on the density of hyphae per unit volume of the medium. Thus if two colonies are grown on solid medium starting from inoculi far apart from each other, very few fusions will be observed when the colonies meet, because of the narrow “man’s-land” between them which is crossed by only a few hyphae. On the other hand, if the two colonies are started close to each other, there is no gap between them, and fusions are abundant. Other factors affecting the frequency of hyphal fusions are the external conditions and, very probably, the genetic constitution of the hyphae that meet. With a number of strains—some X-ray mutants—of assexual species like Penicillium notatum, Aspergillus oryzae, and A. niger, and with the homothallic A. nidulans, interstrain fusions are so frequent as to readily be observed. They occur between branches of the same one. In such cases the conidia may be heterocaryotic (6).

Once a cell, or a hypha, has become heterocaryotic for two or more kinds of nuclei, what happens to the nuclei of the different kinds?

It is necessary first to stress the following points.

1. The number of nuclei in cells of the same kind and in the same strain may vary very considerably, but is never very high; the higher numbers being of the order of tens. Cells of different kinds in the same strain (e.g., conidia as compared with submerged hyphae) may also have quite different numbers. In Penicillium notatum, for instance, the submerged hyphae have up to a dozen nuclei per “cell,” in Aspergillus oryzae the average is probably twice as much or more; the conidia are uninucleate in the former and multinucleate in the latter, but the nuclei are all derived from the same nucleus. In other species the conidia may be uninucleate, with the nuclei not all derived from the same one. In such cases the conidia may be heterocaryotic (6).

2. The nuclei of the same “cell” divide independently of each other (Fig. 5). Contrary to what generally happens in syncytia and in multinucleate cells of higher organisms (e.g., insect spermato-
gonia, pollen grains of Orchidaceae, megakaryocytes of mammals), the nuclei of multinucleate cells in the Fungi imperfecti are therefore not synchronized and may divide at different rates. These two conditions—a variable but small number of nuclei per cell, and independent multiplication of each nucleus—make it conceivable that either or both of two mechanisms operate in determining the fate of the different kinds of nuclei in cells descendant from one that first became heterocaryotic. One assumption, made by most previous authors, is that the proportions of nuclei of different kinds in a cell are the consequence of random distribution at cell division, and that therefore nuclei of one kind may constitute, in different cells, anything from 0 to 100% of the total. The additional inference, drawn by Bendle and Coonradt (1), is that in the case of heterocaryons in which only certain definite proportions between the different nuclei endow the cell with maximal growth rate, those cells which, by chance assortment of nuclei, happen to have these proportions are favored by selection.

An alternative—or more probably a concomitant—mechanism, which has not been considered before, is that nuclei of different kinds may multiply at different rates and that these rates are in certain cases dependent on the proportions of the nuclei of different kinds in the same cell, or in a group of neighboring cells. Investigation of whether either or both of these mechanisms actually operate is at present in progress, the working hypothesis being that they both operate.

That random assortment at cell division does occur seems very probable. In the first place, there is the evidence from species forming multinucleate endogenous spores (6, 3). In these species some of the spores produced by a heterocaryon may be homocaryotic. In the second place, there is the evidence, reported below, from the comparison of heterocaryons that have a higher growth rate than either component homocaryon with heterocaryons that do not have this advantage.

The technique is as follows. By means of irradiation, "morphological" mutants are produced and two kinds of mutant are made use of: those with growth rates equal to the original strain (the "wild type"), and those with distinctively lower growth rates. To obtain mutants with growth rates equal to that of the wild type, the simplest way is to irradiate growing colonies of the wild type and isolate them as sectorial mutants. The shape of a sector (Figs. 1, 6, 7) gives an indication of the ratio of the growth rates of sector and mother colony (cf. 9; for mathematical treatment, 15). On the other hand, to obtain "morphological" mutants with growth rates lower than that of the wild type, the simplest way is to irradiate spores, plate them out, and isolate colonies which show at the same time a suitable morphological change and a reduced growth rate. Of course both types of mutant can also be obtained occasionally as a consequence of spontaneous mutation.

As first shown by Dodge (3), and confirmed by Beadle and Coonradt (1), pairs of morphological mutants often form heterocaryons morphologically different from either mutant but identical to the wild type, in the same way as in higher organisms a heterozygote for two nonallelic recessive genes may be different from either homozygous recessive but identical to the double-homozygous dominant. Thus, for example, a white-spored and a yellow-spored, or two different white-spored, X-ray mutants of Penicillium notatum form heterocaryons with green spores like those of the strain from which they were obtained (Fig. 8). Again, the two X-ray mutants of Aspergillus oryzae shown in Fig. 9—morphologically very different from wild type—form heterocaryons almost identical to the latter. Again, two light-colored "spontaneous" mutants of A. niger form heterocaryons with heads approaching the wild type in color (4). Many other examples could be given. In a culture started from a mixed inoculum of two such morphological mutants it is therefore possible to detect patches of heterocaryotic mycelium if they arise at all. Mutants differing from wild type in the color of the spore are particularly suitable in this respect.

The relevant fact is that from combinations of pairs of morphologically different strains two quite distinct patterns of heterocaryotic mycelium are obtained, according to whether the two strains both had growth rates lower than wild-type, or both—or at least one—had growth rates equal to the wild type. In the first case the heterocaryon very often grows faster than either component and may even grow as fast as the wild type. In a colony started from a mixed inoculum the heterocaryon soon overgrows the two homocaryons (Figs. 9, 11). It is in this respect similar to a "balanced" heterozygote, and the designation "balanced heterocaryon" seems appropriate. In the second case—when the growth rates of both, or at least one, strain are as high as that of the wild type—the heterocaryon is very unlikely to have a growth rate higher than that of wild type and therefore will not overgrow the two homocaryons, or at least not the one that has the wild-type growth rate. In this case a colony started from a mixed point-inoculum on solid medium has a striking appearance. At and around the point of inoculum there is a high proportion of heterocaryotic mycelium. But as the colony expands, it resolves itself into sectors of either component homocaryon, with only a few irregularly shaped strips of heterocaryon, which tend to vanish as they get farther from the center (Fig. 8). If inoculation has been made with a mixed-spore suspension, either on liquid medium or by flooding solid medium (Fig. 10), the pad appears as a mosaic of the two homocaryons dotted by patches of heterocaryon, variable
in size but generally small. Transplantation of one such heterocaryotic patch onto solid medium again produces a colony with a high proportion of heterocaryon at the center and decreasing proportions as the colony grows. A substantial heterocaryotic sector has never been observed in our heterocaryons. Thus a growth rate as high as that of the faster-growing component is not enough to enable a heterocaryon to establish itself as a lasting part of a growing colony. A plausible explanation of this is that the two kinds of nuclei are assorted at random at cell division and cells with nuclei all of one or all of the other kind arise from heterocaryotic cells faster than new heterocaryotic cells can arise from new fusions of homocaryotic ones.

This mechanism may also operate with “balanced heterocaryons,” in which case, of course, if the heterocaryotic cells multiply at a rate sufficiently greater than that of either kind of homocaryotic cell, they can overcome the disadvantage of occasional extinction resulting from random segregation of nuclei. The physiological genetics of heterocaryons (vide infra) suggests, however, that selection in favor of heterocaryotic cells may not be the only process preserving the nuclear equilibria in balanced heterocaryons. An intracellular regulatory mechanism may conceivably exist, acting, for instance, through a differential effect on the rates of multiplication of nuclei of different kinds, wherever in a cell or group of cells their ratios have drifted away from the optimal range. In other words, selection of nuclei may operate as well as selection of cells.

Consider two “biochemical” mutants, A and B, differing from each other because A synthesizes metabolite alpha, but not beta, and B synthesizes beta but not alpha, both substances being diffusible and essential for growth. A will grow only on medium supplying beta, and B on medium supplying alpha; the heterocaryon, however, will grow on a medium supplying neither (cf. 1 for many examples). Suppose that the synthesis of alpha and beta at optimal rates requires not less than a certain proportion of nuclei A and a certain other of nuclei B: optimal synthesis, and therefore optimal growth, including optimal rates of division of the nuclei, will take place in any cell or group of cells where the ratios of A to B nuclei lie within these optima. Suppose now that these limits are exceeded by chance, and a cell contains too high a proportion of B nuclei. In such a cell there will be a temporary abundance of substance beta and a shortage of substance alpha. Nuclei A, which can synthesize alpha, but require beta, will therefore be at an advantage and will multiply faster than nuclei B, thus re-establishing the equilibrium.

Techniques to test this hypothesis are being developed, and they may be useful also for elucidating other problems; for instance, that of how many nuclei migrate through each hyphal anastomosis. They are based on the following reasoning. Experimental results (vide infra) support Beadle and Coonradt’s (1) inference that in certain types of balanced heterocaryon the proportions between the two types of nucleus must be kept fairly constant during growth of the heterocaryotic mycelium. If the ratio for optimal growth were attained only as a consequence of chance assortment of nuclei at cell division, followed by a higher rate of multiplication of those cells that happen to have this ratio, a considerable number of cell divisions should be necessary on the average to produce one such cell after each anastomosis. If, on the other hand, it were attained as a consequence of differential multiplication of the two kinds of nucleus, it should be attained very rapidly, perhaps in the very cells that fused. The patterns of heterocaryotic mycelium obtained—e.g., by flooding solid medium with mixed-spore suspensions—should be different in the two cases, and from their study it might be possible to deduce which is the process actually involved. Several variables can be experimentally controlled: (1) the medium, which probably determines different optimal nuclear ratios; (2) the proportions between spores of the two kinds; and (3) the density of the spore suspension, and therefore the frequency of chance contacts per unit area. These techniques are still in a very tentative state, and I shall therefore refrain from further speculation.

Physiological Genetics of Heterocaryons

It has been pointed out by most previous authors that, from certain points of view, the physiological effects of genes carried in one nucleus, as we know them from classical genetics, are paralleled by those of genes carried in the different nuclei of a heterocaryotic cell. We may thus have dominance in a heterocaryon, just as in a heterozygote, though in the former the “alleles” are carried in different nuclei of a multinucleate cell and in the latter they are carried in the same nucleus.

In two important respects, however, we should expect the physiological genetics of heterocaryons to differ substantially from that of heterozygotes. The first is that in a heterocaryon the different kinds of nucleus can be represented in different proportions from one cell to another, and there is some evidence that these proportions may be controlled, at least selectively, by conditions outside the cell. The second is that, in the case of genes whose action is affected by intranuclear conditions (e.g., position effects), or of genes with localized intranuclear action (e.g., genes affecting specifically the mutation rates of other genes), the parallel between heterocaryons and heterozygotes breaks down.

While dominance in heterocaryons has been amply demonstrated, “autonomous” and “nonautonomous” action of the genes has not hitherto been
Fig. 1. The shape of a "sector" in a colony gives an indication of the ratio ($K$) of the growth rate of the sector to that of the mother colony. Various types of sector obtained after irradiation of a growing colony are shown. The heavy circle indicates the growing edge of the colony at the time of irradiation. (Reproduced from Nature 154.)

Fig. 2. "Autonomous" (left) and "nonautonomous" (right) nuclear action in heterocaryons. Left, the pigment of the uninucleate conidia is determined by the kind of nucleus segregated in each conidium. Right, the pigment is determined by the heterocaryotic conidiophore and not by the kind of nucleus segregated in each conidium.
FIGS. 3-14 (see opposite page for legends).
shown to have its counterpart in them. This can now be demonstrated.

In species like Aspergillus and Penicillia, most of which produce conidia with one nucleus, formation of conidia in a heterocaryon automatically leads to segregation of nuclei of different kinds. This has been shown to be the case for both the balanced and nonbalanced heterocaryons, in Aspergillus niger and A. nidulans (4), in A. oryzae (present paper), and in Penicillium notatum (8); and it holds also for microconidia in Neurospora (11).

The fact that conidia are, in these species, unicellular or at least (e.g., in A. oryzae) carry nuclei all derived from one nucleus, makes it possible to investigate whether the action of certain genes is cell-localized or otherwise. Consider two color mutants; e.g., a yellow-spored and a white-spored, both obtained from a green-spored wild type. The heterocaryon between these two mutants will carry both “white” and “yellow” nuclei in its hyphae and conidiophores but will allot either a “yellow” or a “white” nucleus to each conidium. What color will each conidium develop? From classical genetics we would expect quite different results in different cases. If the “yellow” and “white” were recessive and nonallelomorphic, their dominant alleles being necessary for the production of two diffusible substances, we should expect the color of every conidium to be green, irrespective of which kind of nucleus was segregated into it (“nonautonomous gene action”). If, on the other hand, the two dominant alleles were necessary for the normal performance of two metabolic steps strictly localized within the conidium, we should expect the conidia to be either yellow or white (never green) according to which nucleus they received (“autonomous gene action”). The various other possible combinations of “autonomy” and “dominance” can be easily worked out, and there is no reason to doubt that they may all occur in heterocaryons.

So far, examples of both extreme types of action, autonomous and nonautonomous, with dominance have been found; they are diagrammatically represented in Fig. 2. An example of nonautonomous action is given by heterocaryons between two white-spored X-ray mutants of Penicillium notatum, and between each of them and a yellow-spored mutant. The conidia produced by such heterocaryons are all green, like those of the wild type, though they give origin to either white or yellow colonies (8). The same situation has been described by Gossop, Yuill, and Yuill (4) for heterocaryons between pairs of color mutants of Aspergillus niger.

I am indebted to J. L. and E. Yuill for having called my attention to two examples of the other kind of gene action (autonomous) and for having allowed me to use their remarkable photographs. Heterocaryons between A. nidulans (green spores) and its white-spored mutant “alba” produce heads in which single chains of conidia are either green or white (Fig. 13). Heterocaryons between a green-spored and a light-colored mutant of A. tamarii again produce heads with either green or light-colored chains (Fig. 14). Incidentally, this constitutes a beautiful genetical proof that the conidia in a chain all have nuclei of one kind, presumably derived by repeated division of the nucleus in the sterigma.

As a last remark on the physiological genetics of heterocaryons, it is necessary to stress that in

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**LEGENDS FOR FIGURES 3-14 (see opposite page)**

Figs. 3 and 4. Hyphal anastomoses between (1) branches of the same hypha (Aspergillus oryzae, X-ray mutant 2B) and (4) different hyphae (A. nidulans).

Fig. 5. Penicillium notatum, showing several nuclei per “cell” dividing independently. X 800, Zirkle’s fixing-staining-mounting carmine.

Figs. 6 and 7. Mutant sectors of various shapes arising after irradiation of growing colonies of P. notatum; the sectors originate from the growing edge of the colony at the time of irradiation. The strain isolated from the narrow white sector in 7 has a growth rate identical to that of the parent strain. (Fig. 6 reproduced from Nature 154.)

Fig. 8. Colony arising from mixed point-inoculum on solid medium of two white-spored X-ray mutants of P. notatum. Heterocaryotic patches with green spores, like the wild type, originate at and around the position of inoculation but fade away as the colony grows. Both mutants have growth rates equal to that of the wild type, and the heterocaryon has no greater rate.

Fig. 9. Top, A. oryzae (wild type); left, X-ray mutant 1; right, X-ray mutant 2B; bottom, heterocaryon produced by inoculating mutants 1 and 2B together. All started by point-inoculum at the same time. The heterocaryon has a growth rate almost equal to wild type and much greater than either mutant.

Figs. 10 and 11. The same pairs of mutants as in 8 and 9, respectively, but inoculated by flooding a surface with a thick mixed-spore suspension. The heterocaryotic patches in 10 (darker) arise all over the surface but cannot compete with the two homocaryons; on the other hand the heterocaryotic patches in 11 (fluffy) are rapidly overgrowing the homocaryons.

Fig. 12. Segregation of the two kinds of nuclei in the heterocaryon between A. oryzae mutants 1 and 2B. Flattening of conidia produces colonies of type 1 or 2B. Occasional heterocaryotic colonies arise through new fusions between sporulations lying close to each other on the medium.

Figs. 13 and 14 (courtesy of J. L. and E. Yuill). “Autonomous” nuclear action in heterocaryons. Heterocaryotic head of A. nidulans (green spores) and its mutant alba (white spores), showing chains of green and white spores respectively (13). Similar situation with color mutants of A. tamarii (14).
most Fungi there seems to be a continuity of cytoplasm between cells in a hypha, and it is then possible that nuclei can migrate from one cell to the next. In addition, the cytoplasm is very actively streaming but apparently the nuclei are not carried in the flow (13). All of these details are still controversial. A provisional picture is that of a hypha in which the products of the activity of the various nuclei are readily mixed so that we may perhaps consider it as a single unit. If this picture is broadly correct, we may usefully treat the nuclei within a hypha as constituting a single population.

**Control of Nuclear Ratios**

It was shown by Dodge (3) and amply confirmed by Beadle and Coonradt (1) that when two mutant homocaryotic strains have lower growth rates than the wild type, the heterocaryon between them may have a higher growth rate than either mutant and often as high as that of the wild type. Thus in a growing colony started from mixed inoculum the heterocaryon readily supplants both homocaryons and the heterocaryotypic condition is preserved by selection, either of heterocaryotic hyphae or, as put forward by Coonradt, of nuclei within a hypha—or, more probably, of both. In their remarkable theory of balanced heterocaryons, Beadle and Coonradt assume that in those made up of two components there will be a range of ratios between the numbers of nuclei of each kind within which growth will be maximal, and that selection will keep the ratios within this range. How large this range will be depends on the dominance relations of the two or more pairs of "allelomorphs" involved. To simplify: if nuclei of strain 1 are (AB) and those of strain 2 are (aB), the heterocaryon 1 + 2 will have (AB) (A) (capital letters indicate dominants, and parentheses indicate different nuclei). If both A and B are strongly dominant—i.e., if the metabolic step controlled by each of them is performed in a cell at full rate even if nuclei carrying A (or B) constitute 50% or less of the total—then there may be a wide range of nuclear ratios compatible with a growth rate as high as that of the wild type ("haplo-sufficient" genes of classical genetics). If, on the other hand, both A and B are weakly dominant—i.e., if 50% or more nuclei carrying A (or B) are necessary for full performance of the metabolic step controlled by A (or B)—then a growth rate as high as that of the wild type may never be attained by the heterocaryon, and the maximal growth rate will be compatible with only a narrow range of nuclear ratios.

Beadle and Coonradt, as well as Sansome (12), have sampled the nuclear constitutions of a number of two-component balanced heterocaryons of *Neurospora crassa*. In duplicate tests of three heterocaryons having growth rates as high as the wild type, the ratios of nuclei of one mutant to those of the other were found by the former authors to vary as follows: 1:17.6 and 1:1.6; 1:8 and 1:1.5; 1:2.2 and 5:1. Even though the technique of sampling has many pitfalls, there is no doubt that in these cases the nuclear ratios varied over a wide range, as expected on the basis of the theory. On the other hand, one of our balanced heterocaryons between two X-ray mutant strains of *A. oryzae* seems to satisfy the expectation of the theory for the alternative situation. The heterocaryon (Fig. 9) has a growth rate slightly lower than that of the wild type but much higher than that of either component (strains 1 and 2B). Four heterocaryotic cultures grown at the same time and sampled by plating spore suspensions gave the following ratios of nuclei 1 to 2B: 1:2.7 (130/347); 1:2.9 (483/1388); 1:3.1 (251/777); 1:2.6 (574/1485)—the numbers between parentheses indicating the numbers of colonies in each sample (heterocaryotic colonies were counted as two, one of each kind, since in the majority of cases it is possible to see that they originated from new fusions between sporelings of the two types lying side by side, Fig. 12). It is remarkable that in nonsystematic samplings made earlier the same two strains had consistently given nuclear ratios of about 1:6. Obviously, suggestive as these results are, more work—especially with strictly controlled external conditions—will be necessary before we can consider it as proved that nuclear ratios are selectively controlled (vide infra).

An amplification of the theory of balanced heterocaryons is immediately apparent if we consider balanced heterocaryons made up of more than two components. I am indebted to my colleague J. G. Carr for having pointed it out to me. In the case of two kinds of nuclei, carrying respectively the weak dominants A and B, no ratio of nuclei (aB) to (AB) will give full growth, for the simple reason that it is impossible to have in the same cell more than 50% of nuclei (aB) as well as more than 50% of nuclei (AB). But if we have three kinds of nuclei, (ABC), (aBC), and (AbC), forming, two by two, heterocaryons which for the same reason as before do not show full growth, their three-component heterocaryon may well show full growth. In fact, in such a heterocaryon the A, B, and C "alleles" can each be present in proportions up to 66.6%. *A. fortiori*, a four-component heterocaryon, (AbCD) + (aBcD) + (AbCd) + (aBcD), may show full growth while combinations two by two or three by three of the component strains fail to do so; in this case the A, B, C, and D "alleles" may each be present in proportions up to 75%. Suitable mutant strains for testing these deductions are being prepared. Should the preceding reasoning be correct, the conclusion should be drawn that multiple-component balanced heterocaryons have greater adaptability than those made up of only two components.

So far we have considered nuclear ratios as adaptively regulated on account of the genetical proper-
ties of the nuclei themselves. In other words, if an essential metabolite alpha, necessary for growth, is produced at a certain rate under the control of nuclei A, and another metabolite beta under the control of nuclei B, the ratio of nuclei A to B in a heterocaryon will be regulated in such a way as to give the outputs of alpha and beta that result in maximum growth. However, if alpha and/or beta were diffusible substances which the colony could also obtain from the medium, the adaptive ratios of the two kinds of nuclei would be expected to vary according to the amounts of either or both substances available in the medium. Heterocaryotic systems may thus present us with a novel type of adaptation to external conditions: an adaptation which may even be based on an intracellular regulation of the genotype. Clearly we are not far from some kind of Weismannian segregation of determiners during growth and differentiation, and its theoretical implications are obvious enough. This line of investigation may also link up with the study of bacterial variation in response to environmental conditions.

Population Genetics of Heterocaryotic Systems

It would be futile to embark on a detailed genetic theory of populations in heterocaryotic systems before at least some of the processes discussed so far are more precisely understood. It may be useful, however, to consider the distinctive features of some genetical situations in such populations.

In the first place, as already pointed out, we may be justified in considering a hypha as a mass of cytoplasm with a population of nuclei. Such a population is subject to: (1) variation in numbers; (2) drift—i.e., random variation in the proportions of the different kinds of nucleus; (3) migration—i.e., influx and outflow of nuclei, following hyphal anastomoses; (4) mutation; and (5) selection. Selection may act either on the nuclei themselves as proposed here, or on the hyphae carrying them. There are here all the elements considered by Fisher, Haldane, and Sewall Wright in their work on the genetical theory of populations in the case of heterozygotic systems. No doubt their techniques would be extremely useful to expand researches on the mechanics of spore dispersion, like those reviewed by Gregory (5), so as to include the study of these problems.

Variation in Bacteria and Heterocaryosis

Since the present paper is largely speculative, a little more speculation may be pardoned. Can the study of heterocaryosis help towards the understanding of certain aspects of variations in bacteria? Sectoring, secondary colony formation, mass changes in response to the medium, mutation, etc., are all features common to variation in bacteria and Fungi imperfecti. On the other hand, most bacteria have no visible nuclei. Since the results of irradiation suggest that they have a particulate type of inheritance (cf. 14, 10, 2), each kind of particle could be represented in each bacterium in many replicas, to ensure that it is handed over at cell division despite the absence (?) of a precise mechanism like that of mitosis. It may also be assumed that, similarly to the intracellular regulation of nuclear ratios, postulated in heterocaryons, the different kinds of particle are kept in equilibrium in each cell by differential multiplication, but are also liable to random variation in frequencies at cell division, including occasional complete loss. If, in addition, particles can be exchanged between two bacteria, the whole picture should be similar to that of heterocaryotic systems with the difference that in Fungi the units of segregation and recombination are whole nuclei while in bacteria they might be of a more elementary nature, say genes or groups of genes (2).

The present hypothesis could be tested (as suggested to me by Dr. S. E. Luria) by the technique...
already described for Fungi; i.e., mixed inoculation of pairs of morphological mutants, and search for patches of nonmutant growth. Single cells of this type should segregate for the two components. (Part of this hypothesis is supported by the results of Lederberg reported at this Symposium.)

Note added in proof: The demonstration (Demerec and Latarjet, this Symposium) of the one-hit type of dependence of mutation on dosage of irradiation in E. coli, and the finding (McIlwain, Nature 158: 898-902, 1946) that certain enzymes may be represented by only one or very few molecules per cell in bacteria, are against the hypothesis of each kind of particle being present in a variable but sufficiently high number of replicas. A more likely alternative, pointed out to me by Dr. H. J. Muller, is that the hereditary particles of different kinds in bacteria—and perhaps in viruses as well, in view of Delbrück's and Hershey's results (this Symposium)—are (linearly?) kept together in a way basically similar to that occurring in higher organisms.

CONCLUSIONS

Clearly, heterocaryotic systems call for a novel type of genetics; though, of course, the body of knowledge of classical genetics will make the task enormously easier than would otherwise have been the case. The Fungi imperfecti are probably the most suitable material for starting these investigations, with the additional precious help of species like Neurospora where heterocaryosis and sexual reproduction coexist. The essential feature of heterocaryotic systems is that they are based on mechanisms of segregation and recombination of hereditary particles other than meiosis and karyogamy. In this respect, the choice of the Fungi imperfecti, with their apparently normal types of nuclei, should not close our eyes to the implications of these studies for the attack on heredity and variation in other important groups, particularly the bacteria.

APPENDIX ON TERMINOLOGY

Nucleus with one or more sets of homologous chromosomes: haploid, di-, tri-, tetra-, polyploid.

Cell with one or more nuclei: uni-, bi-, tri-, quadri-, multinucleate.

Tissue, Colony, Individual with one or more cells: uni-, bi-, etc., multicellular.

Nucleus, di-, or polyploid, carrying genetically and/or structurally identical (or different) homologous chromosomes: homozygous, heterozygous.

Cell, bi-, or multinucleate, carrying genetically and/or structurally identical (or different) nuclei: homocaryotic, heterocaryotic.

When there is no danger of confusion, attributes of one structure may be used for a higher structure. For example, a homocaryotic colony is one made up of homocaryotic cells; a heterozygote is a multi-cellular individual with all (or, better, practically all) cells heterozygous, etc. The author favors spelling heterocaryosis with a k (heterokaryosis).

REFERENCES

10. ROFFE, R. R., LINDY, R. L., and SMALE, H. H. Mutation or variation of Escherichia coli with respect to growth requirements. J. Bact. 48: 401-412, 1944.

DISCUSSION

Lederberg: Experiments with heterocaryons in Neurospora bear out most of Dr. Pontecorvo's conclusions. Neurospora heterocaryons, however, seem to be more stable than those in Penicillium or Aspergillus, even where there seems to be no selective advantage in heterocaryosis. Heterocaryons between wild type and most biochemical mutants do not in general segregate out in presence or absence of the growth-factor requirement of the mutant. Heterocaryons involving nuclei carrying two distinct albino genes show pigmented conidia through many transfers, and on plates, with no evidence of spontaneous segregation. By isolating hyphal tips, one occasionally does secure a homocaryotic culture, as evidenced by lack of pigment. This is a rare occurrence, however.
Combinations between two Neurospora species (N. crassa and N. sitophila) may exhibit a different behavior. An albino-p.a.b.-less crassa (1633-15300) and a pyridoxinless sitophila (299) will grow together on minimal medium. The components will segregate out very readily according to whether p.a.b. or pyridoxin is supplied. Even on minimal medium in plates, one finds patches of albino conidia, suggesting a segregation of the crassa nuclei.

WHITE: It seems to me that the analysis of heterocaryotic systems would be greatly facilitated if one could obtain heterocaryons containing visibly different types of nucleus. Since there is no meiosis in the forms you have been working on, I suppose that one can consider their nuclei to be essentially haploid. But it is presumably possible to obtain polyploid nuclei by the use of colchicine, camphor, or some other substance. Would it not be worth while to make heterocaryons containing nuclei of different degrees of ploidy, which would presumably be of visibly different sizes, so that one could determine what happens during hyphal fusion and other phases in the cytology of the Fungi imperfecti?

PONTECORVO: This is a very good suggestion, and has indeed already been considered. Mrs. Sansome has produced by camphor treatment giant conidia in P. notatum; but, as far as I know, it is not yet clear whether the nuclei are distinguishable in size from those of an ordinary strain. The nuclei of these fungi are so small and so erratic in their staining reactions that even an apparently simple comparison between nuclei differing in their volume by a factor of two or four may be a difficult proposition.
The title of the present paper paraphrases that of the stimulating essay by Gulick in these _Advances_ for 1944. Gulick started from the proposition that genes are cell particles of megamolecular size possessed of certain outstanding attributes. This is the “shorthand” picture which has been in the mind of geneticists for many years, the attributes being: (1) _self-reproduction_—a gene takes a specific and indispensable part in the biosynthesis of more of its own kind; (2) _mutation_—a gene may undergo sudden infrequent changes, in some cases reversible by sudden infrequent change, and the changed form if still workable becomes the new self-reproducing model; (3) _specificity of action_—each gene affects the metabolism of the cell in a specific way. Points (1) and (3) may be visualized as two aspects of the same process (e.g., Haldane, 1942; Muller, 1947). The present article will attempt to appraise the limitations and the heuristic value of the picture mentioned above, as it results from genetic work.

Perhaps there is some analogy between the position of the genes in the biological thinking of today and that of enzymes up to Buchner. Then an “organized ferment” could be identified only by comparing tissues or strains for their ability to perform a particular chemical reaction. Some postulated the existence of a substance, others were thinking in terms of a property of the cell as a whole, until Buchner settled the matter in favor of the substance and Sumner crystallized it.
Similarly, "hereditary factors," or genes as we call them now, can be identified only by comparing individuals or strains as to certain biological properties. A recognizable difference in a "character" (a shape, a process, a substance, an enzyme activity, etc.) reappearing among individuals or cells of later generations according to certain defined rules is said to be caused by a difference in one or more genes. In most cases we can trace with certainty the cause of a difference in character back to a region of a chromosome, with an order of definition of a hundred angstroms (Pontecorvo, 1951). We have then no alternative to assuming a difference in the chromosome region. But it should be clear that as yet there is no other means of detecting this difference, apart from its biological effects.

The chemistry of the chromosomes is beginning to be explored. At the same time an increasing number of differences in "character" are becoming expressible in terms of differences of a chemical nature (in a pigment, an antigen, an enzyme, in the rate of a biochemical process, in the permeability of a membrane, etc.). Moreover, the study of the mode of action of radiations and chemical mutagens is attempting to throw some light on mutation, i.e., the process whereby a difference in the chromosome region arises. Yet we are still quite unable to formulate in biochemical or biophysical terms either the nature of the difference in the chromosome which causes the difference in "character" or the causal sequence which links the latter to the former.

Because a great part of the present knowledge on chromosomes comes from the microscopic observation of fixed and stained material, and because the success of early genetics was made possible precisely by picturing them as "strings with beads," there is a certain mental inertia against visualizing them as cyclically changing steady states. This difficulty extends of course to the genes. Yet there is nothing either from cytology or from what is known of the chemistry of chromosomes to suggest that a chromosome is in any way more static than a cell or a whole organism; indeed it is more than possible that the chemical makeup of the chromosome is utterly different at different stages of the cycle. What is permanent is the cyclic process: through innumerable cycles, at a certain point each cycle presents us with a chromosome pattern unchanged in its microscopic details and in its sequence of genes.

This chromosome pattern is part and parcel of the cyclic mechanism since changed patterns, on the rare occasions when they do arise,
are reproduced as changed in successive cycles. In addition, every microscopically visible part of the pattern is "self-reproducing" in the negative sense that if missing it is not replaceable in successive nuclear cycles. It is certain that even parts of the pattern too small to be microscopically visible are still "self-reproducing" in this negative sense: from calculations on the maximum size of a gene, and of the minimum size of the part of a gene capable of undergoing change (vide infra), we may be sure that a length of chromosome shorter than a few thousand angstroms and longer than 100 Å. is still "self-reproducing," but we do not know anything below this lower limit. It is an amusing question whether there is any part of a chromosome which if missing can be replaced by the cell: one of the definitions of a gene could well be the ultimate portion of a chromosome not able to arise de novo.

These introductory remarks call attention to some of the basic problems of genetics: the definition of a gene, its size, and the relations of gene arrangement to gene action. In the present article some of these problems will be considered.

It is my firm conviction that genetics can suggest to biochemistry novel approaches to protein synthesis and to the spatial organization of biochemical processes. New ideas from this angle may only arise, however, if biochemists become clearly aware of the limits within which the concepts of genetics are valid.

II. Definition of Gene

There are various ways in which a gene can be defined; they are consistent with one another at certain levels of genetic analysis, but not at others. It is precisely at the levels at which inconsistencies arise that the interest of biochemistry in genetics and of genetics in biochemistry becomes greater.

In the first place, as mentioned, genes can be defined as the ultimate parts of chromosomes which are still "self-reproducing," i.e., if missing they are not replaced in successive nuclear cycles. The value of this definition—the gene as unit of "self-reproduction"—is doubtful at the present time because we have no means of identifying this ultimate unit, should it exist at all. To be sure, in the case of a virus an experiment by Miller and Stanley (1941) is of the kind which could give a meaning to an analogous definition, i.e., identify the ultimate portion of a virus still capable of self-reproduction. They acetylated
the NH$_2$ and phenolic groups, or formed phenylureido derivatives of the tobacco mosaic protein. Up to 70% NH$_2$ groups and 20–40% of the phenolic groups could be so “covered” without impairing the activity titer of the virus. Cells infected with apparently pure changed virus yielded only virus of the original type. There are clearly plenty of loopholes in this experiment, foremost that in this material each cell could not be infected by a single changed particle and therefore recombination between differently changed particles to yield the original type could have taken place. This recombination is known to occur in bacteriophages (Hershey and Rotman, 1949; Luria, 1947) and probably in other viruses. However, this is an experiment of the kind which might tell eventually which parts of a virus megamolecule are not self-reproducing, and more important which parts are, in the sense that when changed they are copied in the changed form.

This leads to the second definition of a gene: the ultimate part of a chromosome which when changed is reproduced in the changed form in successive nuclear cycles. In genetic jargon a change of this kind is called a “mutation.” This definition of the gene as ultimate unit of mutation implies identity between gene and ultimate “mutable” part of a chromosome. The size of this ultimate part can be estimated by inducing mutations with ionizing radiations and particles (Sec. III).

A third definition is that of the gene as ultimate unit of physiological activity. We may distinguish between two genes if one is responsible for a difference in one property of cells or organisms, and the other is responsible for a difference in another property. This definition implies a one-to-one correspondence between genes and properties and depends on our means of defining and analyzing properties. For instance, human individuals with the gene-determined abnormality, phenylketonuria, have a high level of phenylalanine in their blood and they smell of bitter almonds, besides showing a pleiad of morphological, psychological, and other abnormalities. We know that these two distinct “chemical” properties are the consequences of one and the same biochemical error, namely, the inefficient conversion of phenylalanine into tyrosine (Penrose, 1935). Phenylalanine accumulates in the blood; it is excreted mainly as phenylpyruvic acid and is oxidized in the air to benzaldehyde, hence the smell. If we did not know this causal sequence, from the details of its heredity we might have considered this to be an example of one gene with multiple specificity of action.
One example of the converse situation is the following: several laboratory strains of *Aspergillus nidulans* are known which differ from the wild type in requiring L-arginine for growth (Pontecorvo, 1950). Different genes are found to be involved in different strains. More detailed tests show that there are differences in the details of these nutritional requirements, some strains being able to utilize, besides arginine, ornithine, and others ornithine and proline. Without this further analysis we might have suspected any one of several genes to produce the same effect.

In general, as the above examples show, the one-gene-one property relation works out to hold better the deeper the analysis of the effects of genes goes. The reason is, of course, that the more easily detected effects of a gene are physiologically several orders of integration removed from its primary activity. The point is whether or not this primary activity, which at present we cannot even define let alone identify, is single and specific as Garrod suggested long ago in the same sense as, e.g., the activity of an enzyme is usually single and specific though its effects in the cell and the organism may be multiple and nonspecific.

A fourth, historically the first, definition of a gene is that of ultimate unit in hereditary transmission or more precisely ultimate unit of recombination. Since this definition is based on purely genetic concepts, symbols, and units of measurement I shall deal with it in some detail.

In a higher organism with sexual reproduction one gene may be identified when: (1) individuals, or cells, of two true breeding strains differing in one pair of alternative properties, crossed with one another give origin to a first generation with all individuals, or cells, identical to one of the parents or uniformly different from both, in respect to those properties; and (2) through sexual reproduction this first generation begets a second generation in which the two original types are in 1:3 proportions, or the two original types plus the novel type found in the first generation are in 1:1:2 proportions.

In suitable organisms—in particular in certain microorganisms—the process underlying these statistical results can be followed in individual cells. For instance in yeast if we mate two cells, one from each of two particular strains, one requiring adenine for growth, and the other independent of it, the hybrid cell derived from the fusion of the two is able to grow and multiply in the absence of adenine. Any
one of the daughter cells on appropriate medium may become transformed into an "ascus" and form four ascospores within itself. The four ascospores from a single ascus can be dissected out and each may give origin to a strain: the four strains so established from a single ascus are invariably two adenine-requiring and two nonrequiring. In favorable cases in which the alternative properties are detectable in the spore itself—e.g., differences in ascospore color in *Bombardia* (Zickler, 1934)—one can actually see the results of this "segregation" of the two types, one half of the spores of an ascus being of one color and the other half of the other.

The well-known genetic interpretation of these facts is that the nucleus of the hybrid cell formed by fusion of the nuclei of the two cells, one from the requiring strain and the other from the nonrequirer, carries one of each of two different forms (alleles) of a gene, which we may symbolize in the above example as \( AD \) and \( ad \), or as we say the nucleus is "heterozygous" for these two alleles. When the heterozygous cell, or any of its descendants, undergoes meiosis—\( i.e., \) the special type of division which precedes sporogenesis (analogous to the formation of gametes in higher animals)—the two kinds of alleles are segregated into different nuclei and therefore into different ascospores. The nuclei of the ascospores, and of all the cells derived after germination from each ascospore, have the gene represented only once ("haploid") and therefore carry either one or the other allele. Again, by fusion of two of these haploid cells a cell with a double complement of genes ("diploid") is formed: if the two carried the same allele, the diploid would have the same allele in duplicate ("homozygous") and breed true; if they carried different alleles, the diploid would be "heterozygous" and the alleles again would segregate at the next sporulation.

When we are dealing with more than one difference in alternative hereditary properties—\( e.g., \) the above-mentioned one and requirement for, versus independence of \( p \)-aminobenzoate as a growth factor—and each of these alternative properties behaves in inheritance as exemplified, segregation of one pair of alleles may or may not take place independently of the segregation of the other pair. In yeast in the case of \( AD/ad \) and \( PAB/pab \) (symbols analogous to the previous ones) the two pairs segregate independently, \( i.e., \) the ascospores formed by cells heterozygous for both are in equal proportions of types \( AD \ PAB; \  AD \ pab; \ ad \ PAB \) and \( ad \ pab \). In such a case, a
cross between two haploid strains $AD\ PAB \times ad\ pab$ gives results identical to those of a cross $AD\ pab \times ad\ PAB$. In other cases, however, e.g., $ad/AD$ and requirement versus nonrequirement for inositol ($IN/IN$), the four types are not formed in equal proportions, but two types are produced in greater proportions than the two others. In this case which two classes will be more numerous depends on the way the cross was made. For example, from a cross $AD\ IN \times ad\ in$, in which one parent has both requirements and the other neither, more $AD\ IN$ and $ad\ in$ spores would be produced than $AD\ in$ and $ad\ IN$; but from a cross in the reciprocal way—$AD\ in \times ad\ IN$—more $AD\ in$ and $ad\ IN$ spores would be produced than $AD\ IN$ and $ad\ in$. In other words, the original combinations (“parental”) tend to reappear more frequently than the new combinations (“recombinants”); the proportion of “recombinants” being the same no matter which way a cross is made. In the present example, data published by Lindegren (1949) show that the parental combinations appear in 70% of the spores, i.e., 35% for each parental class, and the “recombinants” in 30%, i.e., 15% for each recombinant class. Two genes the alleles of which do not recombine at random but show this tendency are called “linked.”

Tests of more than two linked genes have led to the fundamental discovery of the additive properties of recombination frequencies, i.e., if genes A and B recombine with 15% frequency, and genes B and C with 5% frequency, genes A and C will recombine with a frequency which approximates either the sum (20%) or the difference (10%) of the other two. This led to the hypothesis of the linear arrangement of genes. As cytology advanced the conclusion that in the chromosomes is the basis of those hereditary differences which behave as exemplified became inescapable. The postulate that in most sexually reproducing organisms genes are present in duplicate in the nuclei at certain stages of the life cycle and singly at others was supported by the fact that chromosomes are in the diploid condition in the former and in the haploid condition in the latter. The genes could be conceived as individual regions or active sites along the length of a chromosome, those behaving as “linked” with one another in inheritance being localized in the same chromosome pair.

The discovery that the giant salivary gland chromosomes of Diptera are substantially bundles of fully extended chromosomes gave a clear visual demonstration that chromosomes are fibrous cell structures highly differentiated along their length and with a pattern of lin-
ear differentiation which is unique for any one region. By removing experimentally parts of chromosomes, or changing their arrangements in relation to the rest, the precise localization of each gene at a particular small region along this pattern was achieved. The distance between two linked genes along the chromosome was found to be highly correlated with the frequency of recombination between them in inheritance.

Furthermore, the study of the details of meiosis showed that the behavior of linked genes was entirely accounted for by the reciprocal exchanges of parts of homologous chromosomes which take place at meiosis. The visual proof that recombination of linked genes ("crossing over") is actually the result of these exchanges clinched the matter (Stern, 1931).

The recapitulation of these simple facts of genetics, with which most of the readers will be quite familiar, is to stress that the definition of one gene on the basis of breeding experiments is quite unequivocal for practical purposes in routine genetic analysis. We say that we are dealing with a difference in one gene when through meiosis a heterozygous diploid produces in equal proportions only two relevant kinds of nuclei: for the enormous majority of cases this definition is quite sufficient. It is, however, an operational definition dependent in extreme cases on the refinement of our technique. How do we know that only two kinds of nuclei are produced? Simply by analyzing a number of products of meiosis and finding among them only these two kinds. We have, however, no evidence that by analyzing more we would not succeed in finding the additional types expected in the case of two or more linked genes.

Suppose, modifying the example given above, that by crossing a strain requiring adenine and inositol (ad in) with one not requiring either (AD IN) only $\frac{1}{1000}$ of the ascospores produced by the heterozygote were of recombinant types (AD in and ad IN): unless we tested a large number of ascospores we would miss these recombinants. We would, then, have no evidence that more than one pair of alleles, i.e., more than one gene, was involved in the difference between the two strains. In short, we can define nonallelism, but we cannot define allelism: between two very closely linked genes (two pairs of alleles) and one gene (one pair of alleles) the distinction becomes a technical one, limited by our ability to analyze large numbers of progeny.

It might be argued that the enzymologist is faced by an analogous
problem when isolating an enzyme. But in chemistry there are
criteria for the definition of one molecular species independent from
that of the inability to purify it further.

In summary, a gene can be defined: (1) as a part of a chromosome
which is the ultimate unit of mutation; (2) as the ultimate factor of
inheritable differences, i.e., as unit of physiological action; and (3)
as the ultimate unit of hereditary recombination. How far are these
three definitions interchangeable?

In routine genetic work—I wish to stress it again—in the great ma¬
jority of cases two genes are clearly distinguishable because: (a) they
mutate independently of one another; (b) their differential effects on
the cell or the organism are distinct; and (c) they undergo recombi¬
nation with an appreciable frequency. The three definitions are,
therefore, practically consistent and they all express different proper¬
ties of one and the same thing. The practical validity of these defini¬
tions is brought home very forcibly by the great predictive value of
genetic theory, unparalleled in any other field of biological research
and approaching that of organic chemistry. Inconsistencies, how¬
ever, arise when we test the three definitions in extreme cases, partic¬
ularly in cases of very close linkage.

Anticipating what will be developed in sections III and IV, incon¬
sistencies of the following kinds are frequent when dealing with very
small sections of a chromosome: (a) joint mutation of two closely
linked genes with distinguishable effects; this is of common occurrence
when mutation is the result of, or correlated with, changes in the rela¬
tive positions of closely linked genes; (b) nondistinguishable effects
of two closely linked genes which mutate independently; (c) insepa¬
rarability by crossing over of two genes which mutate independently
and produce distinguishable effects. Examples of higher orders of in¬
consistency, i.e., cases in which one only of the three definitions is
applicable, are less common, but there are technical difficulties in rec¬
ognizing them clearly.

III. Size of a Gene

The most reliable method of resolving the chromosome into its lin¬
ear array of genes if that of crossing-over. So far, the resolving
power of crossing-over is limited only by the maximum number of
products of meiosis which can be analyzed. There is, thus, no a priori
limit down to interatomic distances. Practically, recombination
frequencies of the order of $10^{-8}$ are at present the lowest measurable, even with microorganisms. By the use of appropriate selective techniques it should be possible, however, to push the resolution one or two orders of magnitude further.

The units used to measure crossing-over express an estimate of the frequency of its occurrence between two genes at meiosis: *i.e.* (for two very closely linked genes), the proportion of products of meiosis showing recombination. The usual unit is the centi-Morgan (cMo), equal to 1% crossing over, with the subunits milli-Morgan (mMo) and micro-Morgan ($\mu$Mo) equal to 1 per thousand and 1 per million crossing-over, respectively.

We may attempt to translate these units into units of length by making some crude assumptions and using available data from *Drosophila*. One is that the frequency of crossing-over per unit length of the chromosome—which a comparison of *averages* over long stretches shows not to vary vastly between chromosomes and regions of chromosomes (see Table I)—remains about the same even over very small regions. Another (Muller, 1935), is that the length of a giant salivary gland chromosome of *Drosophila* is not vastly different from that of the same chromosome in the interphase between two mitotic divisions in somatic cells or in early prophase of meiosis. At these stages the chromosome is supposed to be fully extended, *i.e.*, not spiralized as it is during the later stages of either mitosis or meiosis.

### Table I

**Conversion of Units of Crossing-Over into Units of Length (Salivary Gland Chromosomes of *Drosophila melanogaster*)

<table>
<thead>
<tr>
<th>Regions</th>
<th>Genes considered</th>
<th>Distance between these genes, $\mu$</th>
<th>Crossing-over between these genes, mMo</th>
<th>$\mu$ to 1 mMo*</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Chromosome 1</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1-A1 to 15-F</td>
<td>$y$ and $f$</td>
<td>334</td>
<td>567</td>
<td>0.6</td>
</tr>
<tr>
<td>1-A1 to 3-F1</td>
<td>$y$ and $ec$</td>
<td>49</td>
<td>55</td>
<td>0.9</td>
</tr>
<tr>
<td>7-B3 to 10-A1</td>
<td>$ct$ and $v$</td>
<td>75</td>
<td>13</td>
<td>0.6</td>
</tr>
<tr>
<td><strong>Chromosome 2</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>49-E to 59-E1</td>
<td>$vg$ and $mi$</td>
<td>230</td>
<td>377</td>
<td>0.6</td>
</tr>
</tbody>
</table>

* 1 mMo (milli-Morgan) = 1 per thousand crossing-over.

With these assumptions, the first of which is admittedly doubtful, it is seen (Table I) that our present technical limit of analysis (about
1/100,000 crossing-over) corresponds to a chromosome length of, say, 100 A. That is, crossing-over may resolve two genes even if they are only about 100 A. apart. It will be realized of course that these calculations have at least as much logical weakness as if one attempted to calculate the distance in yards between two stations very close to one another knowing the time taken by a train to cover this distance and the average speed of the train over a line hundreds of miles long.

The following crude calculations will attempt an estimate of (maximum) gene length based on results of crossing-over. Recently Roper (1950, data corrected 1951, personal communication) succeeded in measuring crossing-over two-by-two between three closely linked sites of mutation in *Aspergillus nidulans*. I deliberately call them "sites of mutation" and not "genes" because of the considerations to be made later. Crossing-over between the two outer ones was 1 mMo, and between the middle one and one of the outer ones about 0.4 mMo. 1 mMo is equivalent (Table I) to a length of fully extended *Drosophila* chromosome of about 0.75 μ, and 0.4 mMo is equivalent to 0.3 μ. If it were permissible to apply the values obtained in *Drosophila* to *Aspergillus*, 0.75 μ (or 7.500 A.) would be the length of the whole segment including the middle site, plus any distance on either side; and the length of the middle site could be calculated at most as the difference 0.75 - 0.3 μ, or of the order of 4.500 A. This value is about 3 times greater than the classical estimate of the maximum length of a gene made by Muller and Prokofyeva (1934) and Muller (1935) on a different basis. They localized four genes in a small region of a salivary gland chromosome 0.5 μ in length and deduced that the existence of other genes in it was unlikely. This gave a mean length for each of the four genes of 1250 A. In view of the large number of tacit assumptions made in both types of calculation, but particularly in our own, the two values seem to show sufficient agreement. In particular, in our own example there is no reason to believe that more mutable sites could not be located in the same segment. It is clear, however, that the (maximum) length of a gene estimated from calculations of either of the kinds mentioned here is such that the value of picturing the gene as of megamolecular size is disputable.

Entirely different methods are those for estimating the size of that part or parts of a gene which are responsible for its mutation under the impact of ionizing radiations or particles. The matter has been reviewed repeatedly and the most comprehensive recent accounts are by
Lea (1946) and by Buzzati and Cavalli (1948). Essentially two methods of estimation have been used, both based on the fact that one ionization or one small cluster of ionizations in a reactive part of the cell, probably of the chromosome, may be sufficient to produce a mutation.

One method makes use of the dependence of the frequency of mutation on the dose of irradiation. Since irradiation of the order of $10^7$ to $10^8$ r. is required for an average of one mutation per gene, and one ionization or a small cluster within a reactive volume is assumed to produce mutation, the "molecular weight" of this volume can be calculated at 10,000 to 100,000, equivalent to a diameter, if assumed to be spherical, of 20–60 A. (Lea, 1946, p. 178). The other method is based on the comparison of the relative mutagenic efficiencies of radiations, or particles, with different densities of ionization along their tracks. If one ionization or a small cluster within a reactive volume is sufficient to produce a mutation, radiations or particles forming tracks in which the ionizations are close to one another relative to the size of the target should be less efficient. Comparison of radiations or particles of different types leads to an estimate (Lea, 1946, p. 179) of a target with a diameter of 40–90 A, if assumed to be spherical, i.e., in very good agreement with the previous estimate.

Both these estimates are likely to be in error by defect mainly because of the assumptions: (a) of a spherical target; (b) of a probability of 1 that one ionization within the target will produce a mutation, and of a probability of 0 for ionizations outside; and (c) that all mutations are detected. Lea (1946, p. 180), however, suggested that the correct diameter is not likely to exceed 100 A., even making allowance for the uncertainty of these assumptions.

Lea argued that the target volume can be identified with the gene. The argument was that the analogous study of the effects of irradiation in inactivating viruses has revealed, in the case of small viruses, the identity between target volume and volume of the whole particle, as determined by independent measurements (ultracentrifugation, electron micrography, etc.).

This argument by analogy is the best that one can do: it does not consider the possibility of energy transfer from site of absorption to site of mutation and in any case it resolves itself into a matter of definition, for the considerations which follow.

The estimates of the size of a gene by means of genetic techniques
give *maximum* lengths of the order of thousands of angstroms. The target volume estimates give *minimum* diameters of tens of angstroms. The difference seems to be too great to infer that the two may eventually coincide (see also Frey-Wyssling, 1948, p. 149). A tentative conclusion could be that the gene as a unit of physiological activity is based on a chromosome segment longer, in certain cases at least, than any one part of the chromosome where mutation may occur. This means that either a gene has only one mutable site or unit, constituting a small portion of it (compare with Frey-Wyssling's, 1948, "carrier hypothesis"), or it has several mutable sites, changes at any one of which all produce the same change or similar changes, in physiological action. The examples to be discussed in Section IV support this second possibility. Thus the target volume of one gene may be made up of more than one subunit. It is true that this possibility was rejected by Timofeeff-Ressovsky (1938) on the grounds that it would make reverse mutation necessarily less frequent than direct mutation, while cases supposed to show the opposite were known. It seems to me, however, that even now not one case of reverse mutation has been studied in sufficient detail to give any crucial evidence one way or the other.

From the preceding discussion, and the considerations to be developed in Section IV, a picture of the gene emerges which qualifies the one from which Gulick (1944) started. As units of physiological action, genes—or at least some genes—are likely to have their structural basis over a stretch of chromosome of considerable length, say of the order of 1000 A. The various parts of this length are coordinated in activity and changes in quantity or quality in any one of them and in arrangement between them in a number of ways may constitute a "mutation," i.e., the changed pattern acts as the new template for the successive nuclear cycles and determines a changed activity. Crossing-over may occur within this length of chromosome between the various mutable sites: thus the ultimate unit of crossing-over and the ultimate unit of mutation—which do not need to be the same thing—could be at least in some cases one or two orders of magnitude shorter than the chromosome segment which forms the basis for the unit of physiological action. We must, however, keep the mind open to the possibility of greater variations around this main outline: there may be a whole array of genes varying in complexity from one to several mutable sites.
This brings to the forefront a question implicit in the writings among others of Muller (1947; also Raffel and Muller, 1940) and Goldschmidt (1944, 1946, 1950): has the picture of the gene as a sharply delimited portion of a chromosome—the "corpuscular" gene—still the heuristic value which it had unquestionably in the early days of genetics?

For genes based on more than one unit of crossing-over and/or mutation there are several possible modes of integration of these units. For instance, neighboring genes may share some units; even over a stretch of chromosome shared by two genes, the units of mutation of one may not all be units of mutation for the other; and, to expand an idea of Mather (1948), the integrations of mutational units may not be constant but change as cells differentiate: in other words, the genes need not be the same in differentiated cells, and this without implying change in their mutational units but only in the way these units are integrated. Indeed it is conceivable that simple changes in the degree of chromosome spiralization or folding may make new integrations of the different units of a chromosome possible just because of the change in the spatial relations of these units.

Clearly it is only a matter of taste whether we should now keep the term gene only for the unit of physiological action: personally I should like to do so.

**IV. Positional Aspects of Gene Action**

The genes, as unit of physiological action, emerging from the preceding considerations are obviously not megamolecules. They are processes, or functions, not atomic edifices: and in first approximation their structural basis lies in a more or less clearly delimited chromosome segment of a length tentatively estimated of the order of 1000 A., *i.e.*, too long to be usefully considered as a megamolecule. For convenience of speech we may continue to call gene the structure, when there is no danger of confusion, provided we keep in mind that we are using a figure of speech as when in French we use the same word for tongue and language.

If the gene is a process based on integration between the parts of a small chromosome segment, the spatial relations between these parts and between them and those of neighboring segments must play a decisive role. In the first approximation there must be at least two kinds of spatial relationships, *i.e.*, within and between genes;
though for the reasons discussed before a sharp distinction between the two types may be meaningless in ultimate analysis. In fact, the genetic evidence is fraught with examples of how decisive are spatial relationships, and the significance of them has been emphasized particularly by Goldschmidt (1944 and 1946) of whom many ideas will be recognized in the present paper.

In the first place (review: Lewis, 1950), there is the fact that a change in the linear pattern of the chromosome often goes together with mutation of the genes having their structural basis near the points where the order has been changed, but not of other genes in the changed sequence: thus, if A B C D E F becomes A E D C B F, any one or more of the genes localized near A, E, B, and F may mutate, but usually not the others (in the segment DC). In other words, the mutation is a consequence of changed neighborhood: even the loss of neighbors, such as by deletion of a distal piece of chromosome, may bring about mutation in one or more of the genes near the point of the deletion. Minute rearrangements of chromosome pattern, of a size just visible under the microscope, may also produce mutation in one or more genes included in the minute segment affected or located close to it. Each gene, then, is a localized process not independent from the neighboring genes.

In the second place, there are the examples of even finer integration of chromosome parts which go under the name of "pseudo-allelism." The relevant literature has been discussed recently (Pontecorvo, 1951); two cases will be exemplified here. In Drosophila, Green and Green (1949) have found in a chromosome segment about 2 mMo in "length" three mutational sites, referred to as "lozenge." The mutant alleles at each site determine in the homozygote a reduction in the pigmentation of the eye and other effects of a morphological kind. These mutant alleles are recessive to the normal alleles, i.e., a heterozygote for any one of them has normal pigmentation and normal other characters. In individuals heterozygous for two different mutant alleles the effect is quite different according to whether the mutant alleles are both on the same chromosome and the normal alleles on its homologue, or the mutant and normal alleles are distributed between the two homologues. In the former case the effect is normal, in the latter it is mutant. Thus in a heterozygote the presence of all the normal alleles on the same chromosome is necessary for normal activity. If we symbolize the normal alleles
with capital letters, the mutant alleles with small letters, and use the fraction sign to indicate the distribution of alleles between the two homologous chromosomes, individuals with the arrangements

\[
\frac{LZ_1, LZ_2}{l_2, LZ_2} \quad \text{or} \quad \frac{LZ_1, LZ_2}{LZ_1, l_2} \quad \text{or} \quad \frac{LZ_1, LZ_2}{l_2, l_2}
\]

are normal, but individuals with the arrangement

\[
\frac{LZ_1, l_2}{l_2, LZ_2}
\]

are mutant. The same applies to similar combinations involving the third site.

In \textit{Aspergillus nidulans}, Roper (1950, 1951, and personal communication) has found two very similar cases: in this material, however, there is the hope of being able eventually to analyze the effect of the genes in biochemical terms. Two groups of mutational sites, of two and three sites, referred to as the "paba" and "bi" loci respectively, have been identified. The "paba" loci are in a chromosome segment of less than 0.02 mMo and the "bi" loci, as mentioned before, in a segment of 1 mMo. The effects of the mutant alleles are to make growth dependent on exogenous \( \alpha \)-aminobenzoic acid and biotin, respectively, presumably because of failure of the biosynthesis of these metabolites. In the case of the "bi" loci—which has been studied extensively—the block occurs somewhere between pimelic acid and desthiobiotin. In each case cells heterozygous \textit{at one} of the loci are normal; but cells heterozygous at two loci with arrangements of the type

\[
\frac{Bi_1, bi_2}{bi_1, Bi_2}
\]

are mutant.

Two interpretations of cases of this kind come to the mind: in both spatial relationships play a fundamental part. Taking the example of the "bi" loci, one interpretation is that we are dealing here with three genes, \( Bi_1, Bi_2, Bi_3 \), affecting presumably through the appropriate enzyme activities different consecutive steps in the biosynthesis of biotin: the synthesis proceeds at such a small rate ("millimicro-
\textit{molar}," McIlwain, 1947) or involves such labile or nondiffusible intermediates that the enzymes need to be spatially organized, as along an assembly line, and this spatial organization has its roots in that of the corresponding genes. Indeed it is a working hypothesis
of this kind (Pontecorvo, 1950) which promoted the search for close linkage between genes involved in consecutive steps in a series of reactions of millimicromolar order. This search gave the two positive results mentioned here in the first two cases analyzed. On the basis of this interpretation, the failure of the normal synthesis to proceed in heterozygotes of the type

\[
\begin{align*}
Bi_1 & b_i_2 \\
\bar{b}_i & Bi_2
\end{align*}
\]

would be due to the fact that even though the two normal alleles, \( Bi_1 \) and \( Bi_2 \), are present in one nucleus, because they are one in one chromosome and the other one in the homologous chromosome their spatial relationships are no longer functional.

The other interpretation, which at present seems to be more plausible, is that we are dealing with one gene and the three alleles are due to mutation at three different mutation sites of that gene, in every case the result of mutation being that of inactivating the gene. On this interpretation, in a heterozygote for two different mutant alleles recombination between mutational sites could, and does in fact, occur but the normal action of the gene obviously could not, and does not in fact, occur: of the three sites there are only two still functional in each homologous chromosome, the inactivation of any one is sufficient to render the gene inactive, and two inactive forms of the same gene can hardly be expected to lead to normal activity.

Clearly a crucial experiment to distinguish between the two interpretations is not easy to design. The first interpretation implies that a different step in the biosynthesis of biotin is blocked in each mutant; there is a hope, then, of identifying these different steps biochemically. So far (Roper, personal communication), however, all the very sensitive tests tried, based on complementary feeding between the mutants, have been negative, and for the very reasons for which it is assumed that the three steps must be localized, i.e., rate of the reactions, labile intermediates, etc., it might be an almost impossible task to resolve the over-all reaction into its three or more steps. The second interpretation implies that only the same biochemical step fails in all three mutants, and therefore no biochemical difference should be expected between them. Thus, there is a possibility of finding positive evidence for the first interpretation, but only negative evidence for the second.
Whichever may be the correct interpretation it is clear that with this and similar examples we are faced with a novel genetic fact: three closely linked sites of mutation, perfectly distinguishable by means of crossing-over, with mutant alleles at each site behaving as if they were also alleles at the others.

There is another topological aspect of gene action which must be considered: does the distribution of genes along and among chromosomes suggest any kind of orderly grouping? If we examine the distribution of genes in the genetically best analyzed organisms—Drosophila and maize—among the hundred of genes located the effect of only very few is expressible in biochemical terms. We have therefore to search for indications of grouping between genes the known effects of which are on morphological characters or morphogenetic processes. If we classify the genes acting on one organ or tissue, or acting on one developmental process, we do not find them to be grouped among and along the chromosomes. This is not surprising, of course, because the genes affecting one morphological process may do so through biochemically unrelated channels.

In those microorganisms, Neurospora, Aspergillus, yeast, Escherichia coli, where genetic analysis has gone farthest, several tens of genes the effects of which are known in biochemical terms have been identified and located. Here again, the genes acting on successive steps of a chain of biochemical reactions do not appear to be in any way grouped. The exceptions are cases like that of the bi loci mentioned above, which is by no means unique (see Bonner, 1950). Whether these are examples of close linkage between different genes acting on successive steps in one series of biochemical reaction, or examples of complexity in the chromosomal basis of one gene, is precisely the question discussed above.

In conclusion, for the time being the answer is that if there is any order in the distribution of genes along and among chromosomes it is certainly not obvious. But, as pointed out elsewhere (Pontecorvo, 1950, 1951), the difficulty is that we do not know for which kind of order to look, and any working hypothesis restricting the field of search would be most welcome.

V. Genes and Enzymes

A problem of fundamental interest is that of the relationship between genes and enzymes, and more generally, between individual
genes and individual cell proteins. In a precise form the working hypothesis of "one-gene-one-enzyme" has been formulated only recently by Beadle (1945), mainly on the basis of the work of his school on the genetic control of biosynthetic processes in *Neurospora* (recent reviews: Horowitz, 1950; Horowitz and Mitchell, 1951). In vague terms the idea that genes produce their effects on the cell by somehow determining the potentiality of the cell to develop certain enzyme activities goes back to the earliest attempts to discover in which ways genes act. It is in fact linked to such works as Garrod's (1923) between 1902 and 1923 on the heredity of metabolic errors in man, of Scott-Moncrieff (1937) and others on the genetics of flower pigments, in short to the beginning of "physiological genetics." The "one-gene-one-enzyme" working hypothesis states (Beadle, 1945) that, in the synthesis of a given enzyme, or other specific proteins such as antigens, the final specificity depends on one and only one gene. This hypothesis of course does not imply that the presence of an appropriate allele is sufficient for a cell to synthesize the corresponding enzyme, or other protein, but only that the cell may synthesize it. "Whether or not the enzyme is actually found... is apparently determined by other factors of which the presence of substrate can obviously be one" (Spiegelman, 1950; also Monod, 1950, p. 57).

The main material which led Beadle to the formulation of the one-gene-one-enzyme hypothesis lies in the several hundred nutritional mutant strains studied by his school in the mould *Neurospora*. Of other microorganisms in which genetic analysis is possible analogous mutants have been obtained by Fries (1945) in *Ophiostoma*, by the present writer and his collaborators in *Aspergillus nidulans*, by Lederberg (1947) in the bacterium *Escherichia coli*, by Perkins (1949) in the smut *Ustilago maydis*, and by Pomper (1950) in yeast. The nutritional mutants of these microorganisms, in all sufficiently analyzed cases, work out to be dependent for growth on an exogenous supply of a particular substance because they are unable to carry out a part of a chain of biochemical reactions for the synthesis of that substance (a vitamin, an amino acid, a purine, etc.). Each difference from the normal strain in one of these blocks has usually proved to be determined by a difference in a single gene. A few analyzed cases in which blocks in more than one chain of reactions appeared to be due to one gene have turned out to result from secondary effects. The
conclusion that the one-gene-one-block relationship holds in the
great majority at least of these cases is superficially inescapable. 
Since most of the reactions in question are probably catalyzed by 
enzymes, the hypothesis has gone further to postulate a one-gene-
one-enzyme relationship.

There is no doubt of the value of this hypothesis from the practical 
point of view of having stimulated an impressive amount of work of a 
systematic type for the analysis of sequences of reactions in bio-
synthetic processes. However, this most fruitful work, the value of 
which is easily appreciated by biochemists, has led to the impression 
among them that the working hypothesis is now an ascertained fact 
(e.g., Dounce, 1950, p. 258). It will be clear from the present paper 
that the hypothesis is still an hypothesis, if for no other reasons that 
we cannot say precisely what one gene is. The following paragraphs 
will attempt to discuss some of its limitations, and possibilities for the 
investigation of chromosome structure and gene action, and especially 
for helping toward the chemical formulation of both.

There are three points to consider: (a) whether or not up to the 
present the experimental results lend support to the hypothesis; 
(b) whether or not one could design crucial experiments to support it 
or reject it; and (c) whether or not some modification of the hypoth¬
thesis at the present stage may increase its value.

According to a recent review by Horowitz (1950), out of 484 
nutritional mutants of *Neurospora* tested by the Beadle school, 
405, or 84%, have a requirement for a single growth factor. The 
unpublished results obtained in the writer's laboratory with *Asper¬
gillus nidulans* are even more striking: out of 557 fully tested mutants, 
546, or 98%, require a single growth factor. Fries (1945) with *Ophio-
stoma* obtained 435 (94%) mutant strains, out of 463 tested, respond¬
ing to a single growth factor. In all three cases, of the residue—from 
16% in *Neurospora* to 2% in *Aspergillus*—which do not respond to 
any single growth factor, a proportion will undoubtedly turn out to 
have two requirements due to simultaneous mutation in two genes, 
others will show single requirements for growth factors not used in the 
tests. Of the remainder we might expect some to be examples of a 
situation similar to that found by Bonner (1946) in a mutant requiring 
simultaneously isoleucine and valine: this strain is unable to aminate 
the keto analogue of isoleucine, and there are good reasons to believe 
that this intermediate accumulates and inhibits the amination of the
keto analogue of valine. All told, then, it seems unlikely that more than a very small fraction of the nutritional mutants will work out to be examples of not easily resolvable multiple requirements caused by mutation in a single gene.

In the cases (see Horowitz, 1950, and Horowitz and Mitchell, 1951) in which any one nutritional mutant or group of mutants has been thoroughly investigated, the genetic difference between parent and mutant strain has been found to lie usually in one gene, and the biochemical difference in the failure—complete, partial, conditional, or unconditional—of just one part of one chain of reactions for the synthesis of an essential metabolite. What the one-gene-one-enzyme hypothesis infers is that the failure is in just one reaction in the chain, that the step is under enzymic control and that the enzyme itself is either absent or partially, totally, conditionally, or unconditionally inactive.

In only two published cases so far have direct attempts at identifying the enzyme activity in parent and mutant strains been made and in no case has the isolation of the enzyme been attempted. In a mutant which requires tryptophan, Mitchell and Gordon (unpublished, referred to by Horowitz and Mitchell, 1951) were unable to show any in vitro enzyme activity for the coupling of serine and indole in preparations made by using methods capable of detecting down to 2% of the activity shown by nonmutant strains. On the other hand, Wagner (1949) was able to demonstrate in vitro enzyme activity for the coupling of β-alanine and pantoyl lactone in a mutant requiring pantothenate. The activity in the mutant was detectable only in acetone-dried cell residue, but not in intact cells. Clearly these two results do not point one way or the other: work of this kind is most desirable, and the main difficulty lies in the fact that so little is known of the enzyme systems involved in reactions for the biosynthesis of essential metabolites.

In this respect, one example shows how fruitful joint genetic and biochemical work can be when concentrating on enzyme systems or proteins for which considerable knowledge is already available. The example comes from work on man, and precisely on the genetics of sickle-cell anemia: Pauling, Itano, Singer, and Wells (1949) and Wells and Itano (1950) have shown that the carbon-monoxide-hemoglobin in individuals homozygous for the allele which determines this clinical condition is electrophoretically different from that of
individuals homozygous for the normal allele and, what is more remarkable, the heterozygotes have both kinds of hemoglobin. As stressed by Haldane (1942, p. 59), one necessary, but not sufficient, condition before we can say that some substance is the immediate product of a gene is that whenever a certain allele is present the substance should also be, or at least potentially, present. This is usually fulfilled in the inheritance of cellular antigens in man and birds and now we find it fulfilled in the case of hemoglobin.

In conclusion, in regard to the first point raised, the available evidence from microbial genetics so far does not disprove the one-gene-one-enzyme hypothesis, and neither does evidence from higher organisms as regards other specific proteins.

Granted that the evidence so far accumulated is inadequate to uphold or reject the hypothesis, and further evidence of the same kind would also be inadequate, we have to consider, as Delbrück pointed out (1946), whether crucial experiments can be devised and of which kinds they should be.

One sort of evidence to be looked for is suggested on the one hand by the behavior of multiple alleles in the case of nutritional or fermentation mutants in microorganisms and on the other hand by the fact mentioned above that heterozygotes for genes determining differences in antigens, or other proteins, usually have both kinds of antigen, or other protein.

An example from Houlanhan and Mitchell's (1947) work on the uridine-requiring mutants of \textit{Neurospora} will illustrate the idea. The normal strain does not require uridine for growth; one mutant has no requirement at 25°C. and an absolute requirement at 35°C.; another mutant has a partial requirement at 25°C. and an absolute one at 35°C.; and a third mutant has absolute requirements at both temperatures. Each of these mutants differs from the normal strain in one allele, and the mutant alleles are taken to be all three alleles of one another because genetic tests have failed to find evidence of nonalleomorphism. On the one-gene-one-enzyme hypothesis, the behavior of the normal and the three mutant strains implies quantitative or qualitative differences in one enzyme depending on which allele is present. If qualitative (\textit{e.g.}, different temperature optima, etc.) these differences might be detected after isolation of the enzyme from the normal strain and the two temperature-sensitive mutants; perhaps the enzyme would be absent altogether in the mutant having
absolute requirement at both temperatures. Furthermore, heterozygotes—which can now be produced in filamentous fungi by a technique developed by Roper, 1952—might show the presence of two kinds of enzyme, just as sickle-cell anemia heterozygotes have both kinds of hemoglobin.

Clearly, an investigation of this kind would have a much better hope of success in the case of enzyme systems the knowledge of which is already advanced: and here again is where the genetic-biochemical analysis of biosyntheses is not, at present, the easiest. Perhaps enzymes of the cytochrome system, which are known to be affected by genes in yeast (Chen, Ephrussi, and Hottinguer, 1950; Slonimsky and Ephrussi, 1949), or enzymes involved in carbohydrate metabolism (Winge, 1949, for yeast; Monod, 1950, for *Escherichia coli*; etc.) might constitute a more easily manageable field.

If a clear case were found of multiple alleles in which (a) the isolated enzyme from the normal and some of the mutant types had somewhat different characteristics, and (b) in the heterozygotes two of these slightly different enzymes were present, this would be strong positive evidence for one side of the one-gene-one-enzyme hypothesis. It would show that the characteristics of an enzyme may depend on a gene, within the limits pointed out before, and that these characteristics are a close consequence of gene activity. It would not show, however, that either these characteristics depend on one gene only, or that one gene affects the characteristics of only one enzyme.

On the latter point, however, an ingenious approach suggested by Horowitz (1950) seems to lend support to the idea that a majority of the genes affecting biosyntheses are not involved in more than one metabolic process. First of all, as shown above, the proportion of nutritional mutants of *Aspergillus nidulans*, and, in lesser degree, of *Neurospora* and *Ophiostoma*, which may require more than one growth factor due to mutation in one gene is small, to say the least. This means that a gene, mutation of which determines multiple growth factor requirements *satisfiable from the medium under the experimental conditions used*, is not common. In other words, single-gene control of multiple biosynthetic processes of this kind is rare, or nonexistent.

The next question to consider is what proportion of genes may control simultaneously a biosynthesis, compensable from the medium when blocked, and some other essential metabolic process for which compensation cannot take place. If genes like these existed, strains
carrying mutant alleles for them would not be usually isolated because nonviable with the technique generally adopted. Horowitz (1950) has attempted to calculate the proportion of these hypothetical genes by making use of the temperature-sensitive mutants of Neurospora. There are two types, those which do not require a growth factor at a certain temperature, but require it at another one (higher or lower), and those which do require a growth factor at one temperature and are nonviable, despite the availability of all the usual growth factors, at another one. The latter would be examples of genes controlling simultaneously a growth factor requirement and some process noncompensable from the medium. Making a number of plausible assumptions, Horowitz calculates on the basis of 26 temperature-sensitive mutants of Neurospora (14 of the first type, 12 of the second) that the proportion of these “multivalent” genes is about one in four.

It seems that an approach like this may ultimately lead to crucial information. The main weakness of it at present—apart from the general one of the difficulty of defining one gene—seems to lie in the fact that the tests for the identification of genes in Neurospora are still very crude (as compared with those usable in Drosophila and Aspergillus) and a proportion, perhaps all the supposedly single “multivalent” mutant genes, may work out to have their basis in a chromosome segment in which, or near which, a rearrangement of linear order has occurred with consequent mutational effect over more than one gene. However, a new technique developed by Atwood (1949) now permits in Neurospora “heterokaryons,” the preservation of mutant genes that would otherwise have a lethal effect. There are possibilities of using this technique for a large-scale direct study of the problem of “multivalent” genes. Furthermore, Roper’s (1952) technique which makes it easy to produce strains of filamentous fungi with heterozygous diploid nuclei in their mycelium, now permits preservation of these “lethal” genes in a heterozygous condition and the study of their single or multiple effects.

Finally, we must discuss whether at the present moment some modification of the one-gene-one-enzyme hypothesis might increase its value as a stimulus to research. Two points should be kept in mind. One, repeatedly stressed in previous papers (Pontecorvo, 1950, 1951), is that genetically the chromosome pattern can be an-
alyzed to an extraordinary degree of precision. Biochemistry is clearly in need of something which will permit it to pass from the study of time-sequences of reactions to sequences organized in space as well as in time. The chromosome as an integrated pattern of active points may offer a grip. It is unlikely that a direct physicochemical study of the chromosomes, though most useful, will lead far in this direction. As Luria (1950) pointed out for analogous work on viruses, the virus in its intracellular dynamic state is utterly different from the extracellular particles on which the study is carried out. It is therefore the study of the chromosome as a biochemical assembly line organized in space and time which is more promising. The second point is that configuration, structure, and arrangement are essential features of chromosome activity.

It is tempting to try to modify the one-gene-one-enzyme working hypothesis in order to take these two points into account. Tentatively, I should like to suggest that genes, or at least some genes, do not take part directly or indirectly in the synthesis of enzymes or in determining their ultimate specificity, but rather provide the spatial organization required for certain enzymes or groups of enzymes to function in the cell. The one-gene-one-enzyme relationship is still retained in the sense that the specific organization of one small segment of a chromosome is necessary in order that a specific enzyme may work in the right time and space relationships with other enzymes and substrates. Whether or not a cell in which the small segment has mutated—by any kind of change in structure or chemical constitution—will still show the presence of an enzyme would thus depend on the stability of the enzyme when unable to function, and when therefore readjustments of velocity balances between a number of competing reactions take place in the cell. In this respect the stimulating ideas developed by Weiss (review: 1950) bring the topological aspect into problems of this kind.

If the present suggestion stimulates some research into the relationships between gene distribution along and among chromosomes and enzyme activities, it will have served its purpose.

VI. Crossing-Over

Genetic analysis is essentially based on crossing-over (Sections II and III). It is not generally realized that in addition crossing-over is a process of profound biochemical interest. For the reasons to be
discussed presently, any attempt to investigate in biochemical terms
the synthesis of "self-reproducing" cell structures—be they chromo-
somes, viruses, *Pneumococcus*-transforming principles, or perhaps,
any protein—is bound to be the poorer if it ignores crossing-over.
The fact is that, so far, in all cases in which the required critical ex-
periments have been made, "self-reproducing" cell structures have
turned out to behave in a manner suggestive of a mechanism akin to
that of crossing-over as known in meiosis of higher organisms. These
cases include bacteriophages (Hershey and Rotman, 1949), *Pneumo-
coccus*-transforming principles (Ephrussi-Taylor, 1951), the "nuclear"
apparatus of *Escherichia coli* (Lederberg, 1947) and the nuclear
apparatus of asexual molds (Pontecorvo and Roper, unpublished).

Reduced to its essentials, crossing-over consists of this: when two
identical, or almost so, linearly differentiated "self-reproducing"
structures are duplicated in one cell, any one of the longitudinal bonds
may be established either along each structure or crosswise between
two of them. Consider, for instance, two homologous chromosomes,
1 and 2, the linear specificities along which will be indicated by letters,
capital versus small representing differences as between alleles of one
gene, and the hyphens representing longitudinal bonds:

1. A-b-C-D-e-F
2. A-B-c-D-E-F

It is known from classical genetics that in a cell in meiosis crossing-
over at any one point takes place between two only of the four prod-
ucts of reduplication of these two chromosomes, but all four may
take part in crossing-over two-by-two at different points. It is not
known for certain whether or not the two that cross over must neces-
sarily be non"sister." For example:

<table>
<thead>
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<th>&quot;Mother&quot; chromosome pair</th>
<th>Reduplication</th>
<th>Formation of longitudinal bonds</th>
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<tr>
<td>1. A-b-C-D-e-F</td>
<td>A b C D e F</td>
<td>A-b-C-D-E-F</td>
</tr>
<tr>
<td></td>
<td>A b C D e F</td>
<td>A-b-c-D-E-F</td>
</tr>
<tr>
<td>2. A-B-c-D-E-F</td>
<td>A B c D E F</td>
<td>A-B-C-D-e-F</td>
</tr>
<tr>
<td></td>
<td>A B c D E F</td>
<td>A-B-c-D-e-F</td>
</tr>
</tbody>
</table>
If we try to apply a scheme for protein synthesis, as suggested by Haurowitz (1950), to chromosome reduplication we face the following difficulty. The scheme requires: (a) that the “mother” chromosomes act as templates for the specific adsorption of building blocks (say, amino acids); and (b) that the lengthwise linkages are formed only between these new blocks, the linkages of the “mother” chromosomes remaining as they are. But, as shown above, all four products of reduplication of a chromosome pair take part in crossing-over: indeed we cannot even speak of template and copy, or of mother and daughter chromosomes. Thus Haurowitz’s scheme, without modifications, cannot help as a model of synthesis of chromosomes and, presumably, of other self-reproducing units.

There are a number of details of crossing-over which should be kept in mind when formulating models for protein synthesis: e.g., “interference,” i.e., crossing-over at one point makes another crossing-over nearby less likely; prevention of crossing-over, as it occurs in certain organisms or under certain circumstances; stimulation of crossing-over, etc.

The point is that crossing-over seems to be an ubiquitous associate of “self-reproduction”: it would be surprising if this were merely a fortuitous association. Recently (Pontecorvo and Roper, 1952, and unpublished) diploid nuclei with appropriate genetic markers have been produced artificially in organisms, such as *Aspergillus nidulans* and *A. niger*, in which the nuclei of the vegetative cells (hyphae) are usually haploid. In these diploid nuclei crossing-over occurs regularly in normal mitotic divisions which preserve the diploid condition. We come to realize, thus, that the situation found in higher organisms, where crossing-over is almost exclusively confined to meiosis in the germ cells, may have required the evolution of special mechanisms preventing crossing-over in diploid somatic cells. We may therefore learn something about protein synthesis by investigating both crossing-over and what prevents it.

There is little doubt that a greater familiarity on the part of biochemists with the facts of crossing-over would help toward the design of more comprehensive working hypotheses and experiments on protein synthesis.
Bibliography

**The Genetics of Aspergillus nidulans**

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I. Introduction

In 1945 a search was made for a microorganism suitable for a genetic approach to certain problems of spatial organization of the cell. The outlines of these problems have been given elsewhere (Pontecorvo, 1950, 1952b, 1952c). Tentative work with a number of species of molds led to the conclusion that *Aspergillus nidulans* (Eidam) Winter, a homothallic ascomycete, was on the whole the most suitable. Genetically this species was unexplored: a not surprising fact, since no homothallic fungus had been investigated before, homothallism usually being assumed to be too serious an obstacle in the way of experimental breeding. Henrard (1934), who made an attempt at genetic analysis in *A. nidulans*, concluded: "Malheureusement nous avons affaire à une espèce homothalle, ce qui augmente considérablement la difficulté, à la supposer surmontable. Si nous confrontons deux souches homothalles . . . il y a peu de chance pour que, parmi les fructifications obtenues, il y a des hybrides; et s’il y en a, comment les distinguer?"

The present work shows that these difficulties are unreal and that homothallism may even constitute an advantage in certain technical respects. It is now clear that the innumerable homothallic species of fungi are not necessarily less suitable for genetic investigation than the heterothallic ones. The principles underlying our techniques (Pontecorvo, 1949a, and present paper) together with those underlying Lederberg’s (1947) technique for bacteria, and the recent ones for asexual filamentous fungi (Roper, 1952; Pontecorvo and Roper, 1952) open the way for the genetic investigation of an enormous range of microorganisms with a wide variety of life cycles. In the applied fields of industrial fermentations, "microbial breeding" is now as obvious a possibility as plant breeding in horticulture. The choice fell on *Aspergillus nidulans* because:

1. It lends itself to standard genetic analysis, since it has a normal sexual cycle. In the greatest part of the cycle the nuclei are haploid; the fusion of two haploid nuclei—presumably when the diploid nucleus of the young ascus is formed—altersates with meiosis which restores immediately the haploid condition in the ascospores.

2. It lends itself to the techniques of balanced heterokaryosis (Dodge, 1942; Beadle and Coonradt, 1945; Kniep, 1920; Pontecorvo, 1947) because its hyphae are multinucleate, and hyphal anastomosis followed by migration of nuclei between hyphae occurs readily.

3. Since it forms uninucleate vegetative spores (conidia) of dark-green color, and spore color mutations supply excellent "markers," plating of conidia is equivalent to sampling individual nuclei, a very
convenient fact for the study of a variety of problems: e.g., segregation of nuclei from heterokaryons (Gossop, Yuill, and Yuill, 1940; Pontecorvo, 1947); detection of cytoplasmic inheritance; isolation of artificially induced diploids (Roper, 1952); isolation of mutants; measurement of mutation rates; selection of somatic recombinants (Pontecorvo and Roper, 1952).

4. Its minimal nutritional requirements being as simple as they can be in a fungus—one source of organic carbon, nitrate as source of nitrogen, and inorganic salts—there is ample scope for the study of the genetics of biosyntheses.

5. Since it forms compact colonies on agar, its ascospores or its conidia lend themselves to plating techniques as those used for yeasts and bacteria: 48 hours after plating and incubation at 37°C, the colonies are classifiable as to morphology, color of conidia, etc.

6. Its fruiting bodies—"perithecia" or, more accurately, "cleistothecia"—do not eject the ascospores when ripe: the mature ascospores can therefore be preserved for months or years within the cleistothecia and used when required. This is particularly useful when it is necessary to reinvestigate an old cross.

7. Its asci (eight ascospores) are easy to micromanipulate and lend themselves to tetrad analysis. However, the spores are not arranged in linear order within the ascus; this makes the location of centromeres more laborious (Lindegren, 1949; Whitehouse, 1950) than in forms (e.g., Neurospora) where a linear order occurs. The new technique of centromere location by means of somatic crossing-over in heterozygous diploids (Pontecorvo and Roper, 1952; Pontecorvo, 1952a), however, might replace that of ascus analysis for this purpose.

8. Random samples of mature ascospores from many perithecia are easily taken, and enormous numbers of ascospores can be tested. This means that with selective techniques one can detect and estimate recombination rates as low as $10^{-8}$ (Roper, 1950a, and unpublished).

9. Its conidia are produced in bundles of parallel chains, with all the conidia of each chain deriving their nucleus from a single nucleus in the sterigma. This is most convenient for the study of a number of problems requiring exact knowledge of cell lineages and nuclear lineages. The present work has been substantially the result of team activity. The following members of the staff and present or former research students of the Department of Genetics, University of Glasgow, have contributed to it in various measure: Dr. J. A. Roper, Miss L. M. Hemmons, Mr. K. D. Macdonald, Mr. E. C. Forbes, Mr. A. W. J. Bufton, and Miss O. B. Adam. Some of the sections of the present paper appear under authorship other than that of the senior author or under joint author-
ship. This is an attempt to apportion these contributions where they have been exclusive or predominant.

II. Life Cycle

1. Vegetative Cycle

As in most other filamentous fungi in which sexual reproduction occurs, *Aspergillus nidulans* (Eidam) Winter, an ascomycete (Order Plectascineae, Family Aspergillaceae) shows a vegetative cycle side by side with a sexual cycle (Fig. 1). The species was first described in detail by Eidam (1883); further information is to be found in papers by Dangeard (1907), Schwarz (1928), and Henrard (1934); and an up-to-date review is in Thom and Raper’s *Manual of the Aspergilli* (1945). Schwarz stated, without details, that *A. nidulans* is homothallic; and Henrard, by showing that single ascospores give origin to self-fertile strains, went most of the way toward proving it.

On germination, a uninucleate (Dangeard, 1907; Yuill 1950) haploid vegetative spore (conidium) produces colorless septate hyphae with multinucleate "cells." Some of these (foot cells) differentiate to form

![Diagram of life cycle of *A. nidulans*.](image)
a multinucleate stalk (conidiophore). 100 μ in length and 6 μ in diameter, growing out of the medium and ending in a globose multinucleate vesicle. 10 μ in diameter. The foot cell, the stalk, and the vesicle have a brownish thick cuticle. From the surface of the vesicle, a number of uninucleate elongated buds, 5 μ in length, (primary sterigmata) develop synchronously and, again synchronously, each one of these gives origin to a second series of one, two, or, rarely, more uninucleate secondary sterigmata. How the nuclei of the two or more sister secondary sterigmata are related to the nucleus of the primary sterigma is not known for certain (see Yuill, 1950).

The nucleus in each secondary sterigma divides repeatedly, and at each division one daughter nucleus remains in the proximal part of the sterigma; the distal part with the other daughter nucleus is then constricted out and differentiates into a conidium. This process is repeated many tens of times. The conidia are thus formed in long unbranched chains, with the last formed near the sterigma and the older ones, further away, gradually attaining full size and full green color. All the conidia of a chain derive their nucleus from the nucleus in the sterigma, a deduction familiar to mycologists, which can now be fully confirmed on genetic grounds (section V-1).

In *A. nidulans* the chains produced by one vesicle remain parallel to one another, forming a columnar head of up to 40 μ in diameter and containing up to 100 chains (Fig. 2). It is possible to follow a single
chain of conidia throughout its length under a stereoscopic microscope. In cultures left undisturbed, the length of the chains may become enormous, say 400 μ, this implies that the nucleus in each sterigma divided about 100 times. The conidia are 3 to 3.5 μ in diameter (see table 30). They remain capable of germination for at least one year at room temperature, probably for several. In the wild type their color varies from dark green to dull gray-green according to age, media, and other external conditions. In a liquid medium after 5 hours at 37°C, most conidia have germinated.

One important feature of the vegetative cycle is hyphal anastomosis. It occurs readily between branches of one hypha, between different hyphae of one monosporous mycelium, or between hyphae of two mycelia of the same or of different strains, when their hyphae meet on the surface of the medium. As a consequence of anastomosis between hyphae of genetically different strains, two or more different kinds of nuclei may come to be included within the same cell, which can thus give origin to heterokaryotic mycelium.

Fig. 3. Conidial head of heterokaryon between a white and a green strain; chains of conidia are either white or green.

When a heterokaryotic hypha forms a conidiophore, the vesicle may carry nuclei of more than one kind, but each secondary sterigma, being uninucleate, can carry only one kind of nucleus. All the conidia in one chain will therefore have the same kind of nucleus, but different chains in the same head may differ in this respect. Thus, in a hetero-
karyon, the mechanism of formation of conidia leads inevitably to segregation of the different kinds of nuclei into different conidia. As shown by Gossop, Yuill, and Yuill (1940), this segregation can be verified by inspection when the two kinds of nuclei determine differences in color of the conidia and (Pontecorvo, 1947) the difference is genetically cell-localized (Fig. 3).

2. Sexual Cycle

The sexual cycle is concentrated in specialized organs, the fruiting bodies (cleistothecia or perithecia). There are large blanks in the knowledge of its morphologic details, and even certain details of the nuclear cycle have to be deduced, so far, from the genetic results reported in the present work.

Mature perithecia are present about 8 to 10 days after incubation of cultures at 37°C. They are spherical bodies, mostly 100 to 200 μ in diameter, with an outer coat of yellowish loose hyphae carrying giant cells of unknown function—the "Hülle cells" (Eidam, 1883)—characteristic of the Aspergilli of the nidulans and a few other groups. Inside the coat of Hülle cells there is a shining, hard, dark red-brown wall 1 to 2 μ thick, originating from a single layer of cells, which constitutes the outer casing of the perithecium. This wall does not break at maturity unless crushed rather hard. The mature perithecium is full of asci, from 10 to 100,000 in perithecia of more than 100 μ in diameter, and each ascus of spheroidal shape, about 10 μ in diameter, contains within its colorless thin sheath eight brown-red ascospores, each binucleate (Adam, unpublished) discoidal, (diameter 3.5 × 4 μ) with two characteristic ridges. The mature ascus breaks very easily, liberating its eight ascospores, which germinate as quickly as the conidia.

I have traced back the processes leading to the mature ascus as far as the ascus primordium. The immature perithecium is filled with thick contorted hyphae of such irregular shapes that it is difficult to grasp their details. On these hyphae ("ascogenous hyphae"), the ascus primordia bud out like grapes in a bunch. The primordia are almost spherical, about 6 μ in diameter, with one conspicuous nucleus, which seems to result from fusion of two smaller nuclei (Fig. 4). The nucleus of the ascus primordium undergoes the two meiotic divisions, and the four products of meiosis divide again, giving in all eight haploid nuclei. The content of the ascus is then cut out into eight spores, each with one nucleus, and this nucleus divides again before the spore is fully mature (Adam, unpublished).

As to the nuclear cycle, from the study of meiosis (section II-3), from ascus analysis, and in general from the genetic evidence there is
little doubt that the nucleus of the ascus primordium is diploid \((2n = 8)\),
the two nuclei of the ascospore are haploid and sisters, the eight ascospores of an ascus represent the four products of meiosis in duplicate, and the nucleus of the conidium is haploid. As to the nuclei in the mycelium, a direct cytological examination is of no avail because of the smallness of the chromosomes. But we may take it that from the haploid nuclei of the ascospore the nuclei of the mycelium derive by mitosis, and from these the nuclei of the conidia.

Fig. 4. Four stages in the ascus primordium: (a) two nuclei presumably about to fuse; (b) the nucleus of the zygote; (c) first meiotic metaphase—four structures, one out of focus; (d) first meiotic anaphase.

As to the nuclei in the ascogenous hyphae, the analysis of the asci of individual perithecia produced in a cross shows that the asci of any one perithecium tend to be either all selfed of one parental kind, or all selfed of the other parental kind, or all crossed (section V-4). This last type of perithecium could arise only with one of two mechanisms: (1) two nuclei, one of each kind, fuse at the beginning of the perithecium and give origin to a diploid heterozygous nucleus from which the nuclei of the 10 to 100,000 ascus primordia of that perithecium derive; or (2) two nuclei, one of each kind, enter into conjugate divisions at the beginning of the perithecium, or very early in its development, and fusion between two descendants, one of each lineage, takes place at some later stage, presumably in each ascus primordium.

The first alternative must be rejected if fusion of two nuclei in the ascus primordium does really occur (Fig. 4). On the other hand, conjugate divisions are believed to be widespread in the Ascomycetes (but see Martens, 1946, for a critical discussion). In the absence of crucial evidence, we shall take it, tentatively, that conjugate divisions in the ascogenous hyphae and karyogamy in the ascus primordium occur in \textit{A. nidulans}.
3. Meiosis

Only a very superficial study of meiosis has been carried out, the main point which was urgent to ascertain being the number of chromosomes in *A. nidulans*. After having succeeded in isolating strains with diploid nuclei in their vegetative cells (section VII-1), it also became important to verify cytologically the occurrence of tetraploid meiosis.

The best material is obtained from young perithecia when the first asci with fully colored ascospores begin to appear. This is usually the case after 4 to 6 days of incubation in plate cultures on a complete medium. Older cultures can also be used by selecting immature perithecia when they just show a pink color in their walls.

We have made use extensively of fresh crushed preparations in aceto-lactic-orcein following McClintock (1945). The staining improves on keeping for about two weeks.

The stages which we have been able to recognize with some confidence are shown in Fig. 4a, b, c, and d. Stage a, which we interpret as that of two nuclei about to fuse in the ascus primordium, could be taken, of course, as a division. However, comparison with stage d, which is undoubtedly an anaphase seems to exclude this interpretation. As to stage b, we interpret it as that of the diploid zygote; it shows a nucleus larger and better staining than at any other stage, and the ascus primordium is somewhat larger than in the preceding stage. Stage c is undoubtedly the first metaphase of meiosis, seen in almost polar view. In the many cases in which a count has been possible, we have seen four bivalents in haplo-diploid strains. Two of these are of medium size, one is much larger, and one very small, almost dotlike. We conclude, provisionally, that *A. nidulans* has four chromosome pairs (in the zygote), and of these one is very large, one very small, and two of medium length. Though *A. nidulans* is certainly not easy material for cytological work, it is probably not more difficult than *Neurospora* (McClintock, 1945).

Tetraploid meiosis has been observed in diplo-tetraploid strains, i.e., strains (section VII-1) which presumably have diploid nuclei in their vegetative cells and tetraploid nuclei in most ascus primordia. In these strains, there are plenty of asci in meiosis, but the majority degenerate afterwards. Not more than a few hundred mature asci are present at best in one peritheciun. These asci (section VII-1) are mainly 16-spored, and the ascospores have a germinability of less than 1 to 50. The most striking, though expected, feature of metaphase of meiosis in diplo-tetraploids is that instead of the four bivalents invariably seen in haplo-diploids, bodies varying in number from four to seven have been
observed. No case of eight bodies has been seen, but observation is difficult enough not to give too much weight to this. The bodies are often of complex structure, suggesting multivalent associations.

The observations on diplo-tetraploid strains permit only the conclusion that tetraploid meiosis certainly occurs in a high proportion of their asci.

III. METHODS OF CULTURE

1. Strains

Three original strains of *A. nidulans* have been the starting point of our work. One, kindly supplied by Mr. J. L. Yuill, we call the "wild type" and we designate it "+." It is the green-spored strain to which Yuill (1939, 1950) gives the symbol A69 and Thom and Raper (1945, p. 159), the symbol NRRL 194. A second strain, also received from Mr. J. L. Yuill, is the white-spored mutant "alba" (Yuill, 1939) which arose spontaneously in 1937 from A69. It is given the symbol A70 by Yuill (1950), and NRRL 195 by Thom and Raper. This strain differs by a single known allele (wa) from wild type (section VI-1).

A third strain is a mutant, unable to utilize sulphate as a source of sulphur, obtained by Dr. Hockenhull (1948) from A69 following nitrogen mustard treatment and given by him the symbol Sₜ. This strain again differs by a single known allele (s₂) from wild type (section VI-1).

All other strains produced in our work are spontaneous or induced mutants from these three or recombinants obtained from ascospores in crosses or by mitotic crossing-over from heterozygous diploids. Strains not requiring additional growth factors are kept on slopes of "minimal medium" (see below) at room temperature, subcultured by conidial transfer every 6 to 12 months. They are kept pure and fertile by occasional isolation, with the micromanipulator, of a single ascospore or of a single conidium or by single-colony isolation from plated conidia or ascospores. Most mutant strains requiring additional growth factors are kept on slopes of "complete medium" (see below). Despite the risk of accumulation of further hereditary nutritional differences in the strains kept in this way, the extra cost and labor of keeping them on minimal medium, supplemented only with the specific growth factor(s) required by each mutant would not be justified. Certain nutritional mutants, however, are inhibited by substances present in the complete medium and these must be kept in this way.

2. Media

*Minimal medium*: sodium nitrate, 6 g.; potassium chloride, 0.52 g.; magnesium sulphate (7H₂O), 0.52 g.; potassium di-hydrogen phosphate 1.52 g.; iron and zinc,
traces; dextrose, 10 g.; pH adjusted to 6.5 with sodium hydroxide (before sterilization); distilled water, 1000 ml. All ingredients of analytical reagent standard.

*Sporulation minimal medium*: used for production of abundant perithecia; same as minimal medium but sodium nitrate reduced to 1 g. and dextrose increased to 20 g.

*Complete medium*: It has been repeatedly modified since the beginning of the present work; in its present formula it consists of solution in 1000 ml. tap water as for minimal medium supplemented with: Difco Bacto Peptone, 2.0 g.; yeast extract "Yeastrel", 1.0 g.; casein hydrolyzate, 5 ml.; acid and alkali hydrolyzates of yeast nucleic acid, 3 ml.; acid and alkali hydrolyzates of thymus nucleic acid, 2 ml.; peptic and tryptic casein digest, 5 ml.; hydrolyzed plasma, 3 ml.; hydrolyzed corpuscles, 3 ml.; B vitamin solution, 1 ml.; pH adjusted to 6 ± 0.2.

The various solutions are prepared as follows:

*Casein hydrolyzate*: (according to the method of McIlwain and Hughes, 1944; *Biochem. J.* 38, 187). One liter of solution made from 200 g. B.D.H. light white soluble casein. Kept in the dark over chloroform for up to three months.

*Nucleic acid hydrolyzate* (yeast and thymus): 2 g. nucleic acid in 15 ml. $N\ \text{NaOH}$; 2 g. nucleic acid in 15 ml. $N\ \text{HCl}$. The two mixtures heated at 100° C. for 20 minutes, then mixed, brought to pH 6 and filtered hot. Volume adjusted to 40 ml. and solution kept in dark over chloroform: to be shaken before taking samples.

*Casein digests*: 30 g. B.D.H. light white soluble casein mixed with 250 ml. water and divided into equal portions. Portion 1, brought to pH 8 with NaOH, and 2 g. trypsin added. Incubated at 40°C. over chloroform for 40 hours, then centrifuged and the supernatant boiled, adjusted to pH 6. The sediment mixed with water to a paste, adjusted to pH 1 with HCl, and 1 g. pepsin powder added; incubated at 40° C. for 40 hours, adjusted to pH 6, and boiled. Portion 2 treated in the same way but the order of digestion inverted. All four solutions mixed, brought up to 240 ml. and pH 6, stored in dark over chloroform.

*Hydrolyzed plasma and corpuscles*: Plasma from oxalated horse blood (25 ml.) mixed with 25 ml. 2 $N\ \text{HCl}$, autoclaved at 120° C for 10 minutes, adjusted to pH 7, filtered, brought up to 50 ml. volume, stored in dark over chloroform. Corpuscles from same, treated same way, but pH adjusted to 10, and volume brought up to 75 ml.

*Vitamin solution*: riboflavin, 10 mg.; nicotinamide, 10 mg.; $p$-aminobenzoic acid, 1 mg.; pyridoxin-HCl, 5 mg.; aneurin-HCl, 5 mg.; biotin, 0.02 mg.; Ca-pantothenate, 20 mg.; choline chloride, 20 mg.; irositol, 40 mg.; folic acid, 1 mg.; distilled water, 10 ml. Koch sterilized.

To all media, when required, 1.5% Davies powdered agar is added, and after melting the medium is filtered through asbestos pulp. Sterilization of all usual media is carried out at 10 lb. for 10 minutes.

3. Incubation and Growth Rates

*A. nidulans* grows within a very wide range of temperatures; the optimum (Eidam, 1883) is unusually high, i.e., near 40° C. In our laboratory, cultures are incubated at 36 to 37°C.

On agar minimal medium at 36 to 37°C, colonies of the wild type started from point inoculum of conidia grow in radius at a constant rate of about 5.9 mm. per 24 hours after an initial lag. On complete medium, this constant rate is about 6.5 mm. per 24 hours (Table 1). When well-
TABLE 1

Rates of Colonies of \textit{A. nidulans} on agar at 37°C.

Duplicate petri dishes, with 20 ml. medium, point-inoculated with conidia. Distance in millimeters of growing edge at successive times measured to the nearest millimeter from point of inoculation.

<table>
<thead>
<tr>
<th>Hours after inoculation</th>
<th>48</th>
<th>96</th>
<th>120</th>
<th>144</th>
<th>168</th>
<th>192</th>
<th>216</th>
<th>240</th>
<th>264</th>
<th>288</th>
</tr>
</thead>
<tbody>
<tr>
<td>\textit{Complete Medium}</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dish 1 (mm.)</td>
<td>12</td>
<td>24</td>
<td>30</td>
<td>36</td>
<td>43</td>
<td>49</td>
<td>56</td>
<td>63</td>
<td>70</td>
<td>75</td>
</tr>
<tr>
<td>Dish 2 (mm.)</td>
<td>12</td>
<td>25</td>
<td>32</td>
<td>38</td>
<td>45</td>
<td>52</td>
<td>58</td>
<td>65</td>
<td>72</td>
<td>78</td>
</tr>
<tr>
<td>\textit{Minimal Medium}</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dish 1 (mm.)</td>
<td>7</td>
<td>19</td>
<td>24</td>
<td>30</td>
<td>36</td>
<td>42</td>
<td>47</td>
<td>53</td>
<td>60</td>
<td>66</td>
</tr>
<tr>
<td>Dish 2 (mm.)</td>
<td>8</td>
<td>19</td>
<td>25</td>
<td>30</td>
<td>36</td>
<td>41</td>
<td>47</td>
<td>53</td>
<td>59</td>
<td>66</td>
</tr>
</tbody>
</table>

Separated colonies are wanted (e.g., for isolating from individual colonies), plating of conidia or ascospores should be aimed at not more than 50 colonies per petri dish (Fig. 5). When only counts and/or classification as to conidial color are required, up to 200 colonies per dish are still manageable. Colonies of strains with normal speed of development of conidia can be classified as to colors about 48 hours after plating.

4. Plating

The conidia are non-wettable, and those in a chain are not easily separated. Conidial suspensions for counting and plating are made in

Fig. 5. Colonies from a cross segregating for yellow and green conidia; 48 hours after plating the ascospores.
sterile distilled water or saline containing 1:10,000 calzolene oil as a wetting agent. The conidia are separated from one another by sucking them up and down vigorously for at least 100 times in a Pasteur pipette, with a capillary spout. After counting with a hemocytometer dilutions are made in screw-top containers each with 9 ml. distilled water or saline without calzolene. For serial dilution, 1 ml. suspension is added to the 9 ml. of a container and so on in order to give serial steps by a factor of 1/10. Plating is done by spreading with a glass rod not more than 0.1 ml. suspension over the agar surface of each petri dish.

For the plating of ascospores, if a pooled sample from many perithecia is required, the perithecia are picked with a platinum wire into a test tube of saline-calzolene and sucked up and down repeatedly with a Pasteur pipette in order to remove conidia and Hüille cells. The clean perithecia are then transferred to a new test tube of any liquid medium without wetting agent and crushed against its wall. The ascospores—dark red—do not cluster if the perithecia are ripe. When the ascospores of a single peritheciun are needed, the perithecum is carefully cleaned of mycelium, Hüille cells, and conidia by rolling it with a needle on the surface of agar medium, and it is then crushed into 0.1 to 0.2 ml. of liquid. In fully fertile strains the content of ascospores of one peritheciun varies from about 50,000 to 900,000. Germination of ascospores is almost complete after 6 hours at 37°C.

IV. ISOLATION OF MUTANTS

1. General Methods

The first step in our work was that of securing a good supply of mutants; about 600 are now available. Two of these—the white conidia mutant “alba” differing in one gene (w\textsubscript{a}) from wild type, and a parathiotrophic mutant also differing in one gene (s\textsubscript{a}) from wild type—were supplied by other laboratories. All the others are spontaneous or induced mutants obtained in our laboratory in one or more steps from the wild type or from “alba.” Since the conidia of \textit{A. nidulans} are uninucleate and haploid, the isolation of mutants consists in the isolation of colonies originated from single conidia.

All sorts of “visible” mutants, differing from the wild type in a way detectable by inspection (color of conidia growth habit, secretion of a pigment, secretion of an enzyme, detectable by visible reaction, etc.), arise with the greatest ease, especially after irradiation. We have, however, kept and made use of only the following six including “alba” (see p. 185 and Table 17):
$w_a$ (''alba''): white conidia; incompletely cell-localized effect; spontaneous from wild type (Yuill, 1936).

$y$: yellow conidia; cell-localized effect; X-ray induced from wild type (1947).

$w\ ad\ y$: white conidia; incompletely cell-localized; adenine-requiring; X-ray induced from $y$; simultaneous occurrence of the two mutant alleles $w$ and $ad$, (1947).

$w\ paba, bi$: white conidia; P.A.B.A.- and biotin-requiring; spontaneous from $paba, bi$, (1951).

$bi, pr$: reduced extracellular proteolytic activity (Fig. 6); biotin-requiring; ultraviolet induced from $bi$, (1951).

$paba, y co$: compact colony; yellow conidia; P.A.B.A.-requiring; origin of $co$ unknown; detected 1951 in strain $paba, y$.

Fig. 6. Plate-tests on milk medium of segregants for the 'visible' characters: colors of conidia and proteolysis; the latter revealed by presence or absence of clear ring around the colonies.

These 'visible' mutants are most convenient as markers. With the possible exception of $co$, the analysis of which has not yet been completed, the visible mutant effect is due to one locus in every case. The same locus seems to be involved in all three mutants with white conidia because (a) from more than 2000 ascospores out of zygotes $w_a/w_a$ and from more than 300 $w/w_a$, no colored recombinant was obtained; (b) diploids (section VII-1) heterozygous for each of the three alleles and the wild type allele have colored conidia, whereas heterozygotes $w/w_a$ have white conidia.

"Nutritional" mutants constitute the bulk of those which have been isolated and made use of in our work. Needless to say, of the about 600 available, only a minor fraction, i.e., 27, have been analyzed genetically, and some only in a preliminary way. Nutritional mutants, or auxotrophs, differ from the wild type (prototroph) in being unable to grow
on minimal medium unless a growth factor(s) is added (Beadle and Tatum, 1941).

To obtain auxotrophs in quantity, there are practical difficulties well known in microbial genetics since the pioneer work of Beadle and Tatum. Among the colonies originating from single conidia, the auxotrophs constitute a small proportion of the total, not more than 3% even after the most effective mutagenic treatment. The auxotrophs can only be identified by testing on non-supplemented medium, on which the parent strain grows and they do not. After having been identified as an auxotroph, each strain has to be characterized; i.e., its growth factor(s) requirement must be identified. All this is laborious, and devices to reduce labor are necessary.

In our work it was found that a very considerable economy of labor could be achieved in the characterization of the auxotrophs simply by rationalizing the sequence of successive approximations required. The details are given in Section IV-3. As to the isolation of auxotrophs, one selective technique, based on the differential survival of auxotrophs under specific starvation, was prompted by the work of Fries (1940a,b) with *Ophiostoma*; it has made the isolation of auxotrophs as easy in *A. nidulans* as the penicillin technique (Davis, 1948; Lederberg and Zinder, 1948) has made it in bacteria. The “starvation” technique, as we call it, has provided about 500 of the 600 auxotrophs available; it is dealt with in Section IV-2. A few auxotrophs were obtained by an adaptation of Lederberg and Tatum’s (1946) “delayed enrichment” technique. The bulk of the remainder were obtained by “total isolation,” modifying slightly the adaptation of the original technique of Beadle and Tatum (1941) previously worked out for *Penicillium notatum* (Pontecorvo, 1946).

As mutagenic agents, X-rays (85 kv.) and ultraviolet (90% output in the 2537 A region from Hanovia XI low-pressure mercury lamp) were used. With X-rays, the conidia were treated dry by putting under the beam a piece of agar carrying sporulated mycelium 6 to 10 days old. The suspension of conidia was made after irradiation. A single dose of irradiation—50,000 r.—was used throughout. The viable counts from conidia so treated and plated on complete medium were about 1/200; the proportion of auxotrophs among survivors, pooling all results, was 2.35% (81/3438).

With ultraviolet, the conidia were suspended in 10 ml. saline, the suspension placed in a petri dish 45 cm. distant from the lamp, and the dish rocked gently during irradiation. The time of treatment was kept constant: 8 minutes. However, because of changes in mains voltage and other non-controlled conditions, the actual treatment varied vastly
between experiments, giving survival rates between 5% and 25%. We shall therefore express the dose in terms of viable counts relative to conidia plated (hemocytometer estimate). With ultraviolet the highest proportion of auxotrophs obtained among survivors was 1.25% in two experiments with viable counts of 5%.

**Total isolation.** With this technique the conidia are treated as mentioned and plated on complete medium at such a density as to obtain 20 to 50 colonies per plate. After incubation for 48 hours, isolations are made from each colony well separated from the others, and the tests for identifying the auxotrophs and characterizing their growth-factor requirements are carried out (Section IV-3).

At the beginning of this work, each colony was isolated onto slants of complete medium, and the further tests were carried out from these. Later it was found that Fries’ (1948b) plate tests save much labor. The technique is now as follows:

1. From each colony a small amount of mycelium is spot-transferred onto dishes of minimal medium, 20 isolates per dish.

2. After 48 hours of incubation, the transfers that show much less growth than the majority (usually at the expense of the small amount of complete medium carried over with the inoculum) are "rescued" onto complete medium slants, and from these the further tests are carried out (Fig. 7).

---

**Fig. 7.** Diagrammatic sequence in the routine for characterization of auxotrophs: plate-tests followed by auxanography.
### Table 2

Mutants Obtained by "Total Isolation"

<table>
<thead>
<tr>
<th>Series</th>
<th>Strain treated</th>
<th>Isolates (no.)</th>
<th>Total (no.)</th>
<th>Auxotrophs*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>S</td>
</tr>
<tr>
<td><strong>X-rays, 50,000 r.</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>19-11-46</td>
<td>y</td>
<td>201</td>
<td>7</td>
<td>3.5</td>
</tr>
<tr>
<td>28-1-47</td>
<td>w</td>
<td>338</td>
<td>7</td>
<td>2.1</td>
</tr>
<tr>
<td>28-2-47</td>
<td>+</td>
<td>262</td>
<td>8</td>
<td>3.1</td>
</tr>
<tr>
<td>15-3-48</td>
<td>y</td>
<td>364</td>
<td>8</td>
<td>2.2</td>
</tr>
<tr>
<td>J.A.R.-1</td>
<td>y thi</td>
<td>1372</td>
<td>21</td>
<td>1.5</td>
</tr>
<tr>
<td>J.A.R.-2</td>
<td>bi</td>
<td>671</td>
<td>26</td>
<td>3.9</td>
</tr>
<tr>
<td>S-1, S-2</td>
<td>bi</td>
<td>230</td>
<td>4</td>
<td>1.7</td>
</tr>
<tr>
<td></td>
<td>Total X-rays</td>
<td>3438</td>
<td>81</td>
<td>2.4</td>
</tr>
<tr>
<td><strong>U.V. (in brackets)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>S-3, S-4 (5%)</td>
<td>bi</td>
<td>400</td>
<td>5</td>
<td>1.3</td>
</tr>
<tr>
<td>S-5 (25%)</td>
<td>bi</td>
<td>500</td>
<td>2</td>
<td>0.4</td>
</tr>
<tr>
<td>S-6 (12.5%)</td>
<td>bi</td>
<td>1070</td>
<td>5</td>
<td>0.5</td>
</tr>
<tr>
<td></td>
<td>Total U.V.</td>
<td>1970</td>
<td>12</td>
<td>0.6</td>
</tr>
</tbody>
</table>

* S, unable to utilize sulphate; N, unable to utilize nitrate; A.A., requiring an amino acid; N.A., requiring a purine or pyrimidine; Vit., requiring a vitamin.

Results of series J.A.R.-1 and J.A.R.-2 supplied by Dr. J. A. Roper. Results of series S1-S6 are the "0-hr. controls" of the experiments with starvation (Section IV-2).

Table 2 gives the results of all the series carried out so far by total isolation. The ultraviolet results are the "controls" of the experiments in which "starvation" was used as a selective means (section IV-2). The pooled results from "total isolation" give 93 auxotrophs out of 5408 isolates. Of the 93 auxotrophs, 69 were fully tested (section IV-3), and for 67 a growth factor was identified as capable *singly* to permit growth when added to minimal medium. Only two of the 69 strains required simultaneously more than one growth factor. As to the requirements: 7 strains were unable to utilize sulphate but could grow on more reduced inorganic sulphur compounds, e.g., thiosulphate; 17 were unable to utilize nitrate but could grow on nitrite or ammonium salts; 12 required an amino acid; 11 a purine or pyrimidine; and 22 a vitamin or a mixture of vitamins. The details of the spectrum of mutants will be discussed later (section VI-4).

**Delayed enrichment.** A small-scale attempt at increasing the yield of mutants was made by adapting to *A. nidulans* Lederberg and Tatum's
G. PONTECORVO

"delayed enrichment" technique for bacteria. This is based on the following reasoning: if a mixture of auxotrophic and prototrophic cells is plated on minimal medium, the latter give origin to colonies, but the former do not. After incubation, as soon as prototrophic colonies are barely visible (when they can be marked on the reverse of the petri dish) complete medium is added, and the auxotrophic colonies may then come up. The auxotrophs are therefore identified, because they grow only after addition of complete medium.

With Lederberg and Tatum's technique the plated cells are covered by a layer of agar medium to make it possible to add, after incubation, a further layer of complete medium. With A. nidulans it was found that the hyphae growing vertically reached the surface of the covering layer too soon. This difficulty was overcome by using not a wild-type strain but a strain requiring aneurin and by controlling its growth rate by means of a limiting amount of aneurin in the minimal medium. Irradiated conidia of the aneurin-requiring strain (y thi) were spread over a bottom layer of 5 ml. of agar minimal medium, covered with a second layer of 5 ml. of the same medium and incubated for 24 hours. The barely visible colonies were then spotted, and a further layer, this time of complete medium, was poured on top. After further incubation, all the colonies developed after the addition of complete medium were isolated and tested.

Unfortunately, as already found in the case of Aerobacter aerogenes (Devi, Pontecorvo, and Higginbottom, 1951), only a minor proportion of the delayed colonies turned out to be auxotrophs (i.e., requiring an additional growth factor besides aneurin, which all required). In fact, for irradiation with 50,000 r. the yield of auxotrophs among isolates was raised only from 2.4%, as in "total isolation," to about 5% (Table 3).

<p>| TABLE 3 |</p>
<table>
<thead>
<tr>
<th>Comparison of the Efficiency of Total Isolation, Delayed Enrichment, and Starvation for the Isolation of Auxothrophic Mutants</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>X-rays</strong> (50,000 r.)</td>
</tr>
<tr>
<td><strong>Total isolation</strong></td>
</tr>
<tr>
<td>Isolates</td>
</tr>
<tr>
<td>Mutants</td>
</tr>
<tr>
<td>Per cent</td>
</tr>
</tbody>
</table>

The efficiency of the starvation technique in the experiments tabulated above is considerably lower than the best now attainable, which approaches 60%.
The reason for this is that irradiation produces an enormous scatter in the germination times of the conidia; many delayed colonies are therefore not auxotrophs.

The technique was discontinued.

2. "Starvation" Technique
by K. D. Macdonald and G. Pontecorvo

This technique, prompted by Fries' (1948a; 1948b) work with Ophiostoma, has proved to be of extraordinary efficiency, yielding up to 60% auxotrophs.

In the first place, it was found that the conidia of a biotin-requiring A. nidulans mutant (bi) when plated embedded in (biotin-free) minimal medium died off quickly, after 100 hours less than 1% survived. In the second place, it was found that conidia of strains requiring, besides biotin, another growth factor (adenine or aneurin) died in minimal medium at a slower rate than those of the strain requiring only biotin. These results were in full agreement with Fries' findings.

That Fries' results were paralleled in Aspergillus was shown by appropriate "reconstruction" experiments, in which conidia (green or yellow) of two biotin-requiring strains (bi and ybi) were mixed in known proportions with conidia (white) of a biotin-adenine requirer (w ad bi) or with conidia (yellow) of a biotin-aneurin requirer (y bi thi). The mixed conidial suspensions were spread in a series of dishes over a bottom layer of minimal medium, covered by a second layer of minimal medium, and incubated at 37°C. No colonies, of course, developed after this first incubation, the medium lacking the required growth factors. After different lengths of incubation, different dishes were enriched by the addition of a top layer of complete medium. The number of colonies developed after further incubation indicated the number of conidia still viable at the moment of addition of the complete medium. By making use of the color differences (white, yellow, green) between the strains, the classification of the survivors of each strain could be done by inspection. Figure 8 shows the results of such reconstruction experiments: after about 100 hours "starvation" (i.e., time from inoculation in minimal medium to addition of complete medium) very few of the bi (biotin-requiring, green) or of the ybi (biotin-requiring, yellow) conidia were viable, whereas more than 20% of those of the other two strains were still capable of giving origin to colonies. Thus, selection of the latter was almost 100% effective.

The next step was the investigation of whether double-auxotrophic conidia, arising as a consequence of induced mutation among irradiated
conidia, would behave in the same way as the conidia from double auxotrophic strains--i.e., die more slowly under starvation than those of the parent strain. The conidia of the bi strain were irradiated, plated embedded in minimal medium, and incubated as in the "reconstruction" experiments. Complete medium was added as a top layer to different dishes at different times after inoculation, and a random sample of all

![Graph showing survival rates of conidia over time.](image)

**Fig. 8. Reconstruction experiments:** under starvation the conidia of strains requiring biotin and aneurin or adenine (y bi, thi and w ad, bi,) die off at a slower rate than conidia of strains requiring biotin only (bi, and y bi). The colonies developed thereafter were isolated and tested for growth-factor requirements additional to that for bi, which all had. The results of a number of experiments (Series S-1 to S-5), both with ultraviolet and X-rays, covering periods of starvation from 0 to 160 hours are graphically shown in Fig. 9 and given in detail in Table 4. In each of the curves of Fig. 9, two points are most relevant. One is the percentage of (double) auxotrophs among survivors at 0 time; this percentage represents the relative yield of mutants recoverable without selection, i.e., by "total isolation." The other is the maximum percentage of double auxotrophs among survivors.
Mutants Obtained by Starvation after Irradiation

Series S-1 and S-2: 50,000 r., X-rays. Series S-3 and S-4: U.V., 5% survival.

<table>
<thead>
<tr>
<th>Hours of starvation</th>
<th>Conidia plated *</th>
<th>Colonies</th>
<th>Isolates</th>
<th>Auxotrophs among isolates</th>
<th>Auxotrophs per 10^5 spores plated</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(no.)</td>
<td>(no.)</td>
<td>(no.)</td>
<td>(no.)</td>
<td>(%)</td>
</tr>
<tr>
<td><strong>N-rays, 50,000 r.</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>63,000</td>
<td>335</td>
<td>230</td>
<td>4</td>
<td>1.7</td>
</tr>
<tr>
<td>46</td>
<td>45,000</td>
<td>184</td>
<td>115</td>
<td>2</td>
<td>1.7</td>
</tr>
<tr>
<td>74</td>
<td>45,000</td>
<td>129</td>
<td>88</td>
<td>3</td>
<td>3.4</td>
</tr>
<tr>
<td>96</td>
<td>630,000</td>
<td>165</td>
<td>119</td>
<td>18</td>
<td>15.6</td>
</tr>
<tr>
<td>111</td>
<td>1,010,000</td>
<td>130</td>
<td>93</td>
<td>31</td>
<td>33.3</td>
</tr>
<tr>
<td>117</td>
<td>560,000</td>
<td>44</td>
<td>40</td>
<td>12</td>
<td>30</td>
</tr>
<tr>
<td>123</td>
<td>1,120,000</td>
<td>42</td>
<td>38</td>
<td>11</td>
<td>28.9</td>
</tr>
<tr>
<td>147</td>
<td>2,240,000</td>
<td>57</td>
<td>54</td>
<td>22</td>
<td>40.8</td>
</tr>
<tr>
<td><strong>U.V. (5% survival)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>23,100</td>
<td>1010</td>
<td>400</td>
<td>5</td>
<td>1.25</td>
</tr>
<tr>
<td>114</td>
<td>2,640,000</td>
<td>889</td>
<td>300</td>
<td>56</td>
<td>18.7</td>
</tr>
<tr>
<td>137</td>
<td>1,980,000</td>
<td>191</td>
<td>137</td>
<td>89</td>
<td>65</td>
</tr>
<tr>
<td>143</td>
<td>2,640,000</td>
<td>362</td>
<td>200</td>
<td>91</td>
<td>45.5</td>
</tr>
<tr>
<td>161</td>
<td>2,640,000</td>
<td>254</td>
<td>140</td>
<td>72</td>
<td>51</td>
</tr>
<tr>
<td><strong>U.V. (25% survival)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>3,000</td>
<td>734</td>
<td>500</td>
<td>2</td>
<td>0.4</td>
</tr>
<tr>
<td>96</td>
<td>56,700</td>
<td>348</td>
<td>160</td>
<td>2</td>
<td>1.25</td>
</tr>
<tr>
<td>112</td>
<td>112,800</td>
<td>383</td>
<td>175</td>
<td>12</td>
<td>6.9</td>
</tr>
<tr>
<td>123</td>
<td>56,700</td>
<td>147</td>
<td>123</td>
<td>10</td>
<td>8.1</td>
</tr>
<tr>
<td>136</td>
<td>112,800</td>
<td>104</td>
<td>86</td>
<td>24</td>
<td>27.9</td>
</tr>
<tr>
<td>159</td>
<td>225,000</td>
<td>185</td>
<td>160</td>
<td>35</td>
<td>21.9</td>
</tr>
</tbody>
</table>

* Hemocytometer estimate.

auxotrophs, obtained usually after periods of starvation of over 100 hours. Compared with the former this percentage gives an idea of the enrichment effected by starvation. In the best of the curves of Fig. 9 this enrichment is by a factor of about \( \times 70 \).

It will be noted that the enrichment factor in the case of double auxotrophs produced by irradiation is considerably smaller than in the reconstruction experiments. This is probably the result of the scatter in germination times produced by irradiation, noted already in section IV-1 for the "delayed enrichment" technique.
The qualitative spectrum of mutant types selected by starvation is superficially similar to that of "total isolation" (Table 6). Quantitatively, however, there are some striking differences, such as the high proportions of mutants unable to utilize sulphate ("parathiotrophic") and of those requiring adenine, and the low proportion of mutants requiring a vitamin and of those unable to utilize nitrate ("paraazotrophic"). The proportion of parathiotrophic mutants increases steadily with an increase in time of starvation (Table 5); that of paraazotrophic and of vitamin-requiring mutants shows the opposite trend.

When we compare in detail the qualitative spectra of mutants (Table 6), important differences and similarities appear. For instance, among the arginine-requiring mutants, those responding to arginine only and those responding to arginine or ornithine were not found after starvation, whereas those responding to arginine, ornithine, or proline were abundant. The agreement between total isolation and starvation
TABLE 5

<table>
<thead>
<tr>
<th>Hours of Starvation*</th>
<th>Isolates (no.)</th>
<th>Total tested (no.)</th>
<th>Fully tested</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Total S. N. A.A. N.A. Vit.</td>
</tr>
<tr>
<td>0</td>
<td>5408</td>
<td>93 24</td>
<td>69 (100) 7 (10) 17 (25) 12 (17) 11 (16) 22 (32)</td>
</tr>
<tr>
<td>96-117</td>
<td>887</td>
<td>131 3</td>
<td>128 (100) 71 (65) 0 (0) 9 (7) 37 (29) 11 (9)</td>
</tr>
<tr>
<td>118-139</td>
<td>384</td>
<td>134 0</td>
<td>134 (100) 81 (60) 2 (2) 6 (4) 42 (31) 3 (2)</td>
</tr>
<tr>
<td>140-161</td>
<td>554</td>
<td>220 4</td>
<td>214 (100) 146 (68) 1 (5) 13 (6) 55 (25) 1 (5)</td>
</tr>
</tbody>
</table>

* The 0-hour data are from all the results in Table 2. The other data are from series S-1 and S-2 (X-rays); S-3, S-4, and S-5 (U.V.), omitting the results at 46 and 74 hours in series S-1 because of the small numbers. Figures in brackets are %.

† S, unable to utilize sulphate; N, unable to utilize nitrate; A.A., requiring amino acids; N.A., requiring purines or pyrimidines; Vit., requiring vitamins.

in not yielding certain types of auxotrophs (e.g., tryptophan-, histidine-, guanosine- or inositol-requiring) is certainly remarkable.

After having perfected the starvation technique, we investigated two points by means of it. The first was whether the failure to obtain certain types of auxotrophs both in total isolation and starvation experiments might be due to the presence in the complete medium of substances specifically inhibitory for these mutants. This is the case for histidine-requiring mutants of Neurospora (Lein, Mitchell, and Houlanhan, 1948) and guanosine-requiring ones of Ophiostoma (Fries, 1950). This possibility was probed by adding, after starvation, not complete medium, but minimal medium plus biotin, supplemented with some of the growth factors, mutants for which had not been previously obtained. The second point was whether, as suggested by Fries (1948a), the longer survival under starvation of the conidia of double-auxotrophic mutants was really due to their being more heterotrophic. This was probed in three ways: (1) by trying whether mutants of specific types would be eliminated in a starvation experiment when the starvation medium was supplemented with the corresponding growth factors; (2) by comparing the survival rates under starvation of conidia of strains with different
TABLE 6

Comparison of the Types of Auxotrophs Obtained from Total Isolation and Starvation (96 hr. or more)

<table>
<thead>
<tr>
<th>Type of auxotroph</th>
<th>Total isolation</th>
<th>Starvation (96 hr.)</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Not fully tested</td>
<td>24</td>
<td>7</td>
<td>31</td>
</tr>
<tr>
<td>Parathiotrophic</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sulphite</td>
<td>5</td>
<td>284</td>
<td>289</td>
</tr>
<tr>
<td>Thiosulphate</td>
<td>2</td>
<td>14</td>
<td>16</td>
</tr>
<tr>
<td>Paraazotrophic</td>
<td>17</td>
<td>3</td>
<td>20</td>
</tr>
<tr>
<td>Nitrite</td>
<td>13</td>
<td>0</td>
<td>13</td>
</tr>
<tr>
<td>Ammonium</td>
<td>4</td>
<td>3</td>
<td>7</td>
</tr>
<tr>
<td>Amino Acids</td>
<td>12</td>
<td>28</td>
<td>40</td>
</tr>
<tr>
<td>Arginine</td>
<td>1</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>Arginine/ornithine</td>
<td>5</td>
<td>0</td>
<td>5</td>
</tr>
<tr>
<td>Arginine/ornithine/proline</td>
<td>1</td>
<td>14</td>
<td>15</td>
</tr>
<tr>
<td>Lysine</td>
<td>3</td>
<td>7</td>
<td>10</td>
</tr>
<tr>
<td>Proline</td>
<td>0</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>Ornithine</td>
<td>0</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Methionine</td>
<td>1</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>Methionine/cystine</td>
<td>1</td>
<td>2</td>
<td>3</td>
</tr>
<tr>
<td>Phenylalanine</td>
<td>0</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Isoleucine</td>
<td>0</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Tryptophan</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Nucleic Acid components</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Adenosine</td>
<td>10</td>
<td>134</td>
<td>144</td>
</tr>
<tr>
<td>Cytidine/Uridine</td>
<td>1</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>Vitamins</td>
<td>22</td>
<td>15</td>
<td>37</td>
</tr>
<tr>
<td>Biotin *</td>
<td>4</td>
<td>0</td>
<td>4</td>
</tr>
<tr>
<td>P.A.B.A.</td>
<td>2</td>
<td>3</td>
<td>5</td>
</tr>
<tr>
<td>Pantothenate</td>
<td>1</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>Nicotinic/anthranilic acid</td>
<td>4</td>
<td>1</td>
<td>5</td>
</tr>
<tr>
<td>Nicotinic/tryptophan/anthranilic acid</td>
<td>3</td>
<td>1</td>
<td>4</td>
</tr>
<tr>
<td>Riboflavin</td>
<td>1</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>Pyridoxin</td>
<td>1</td>
<td>7</td>
<td>8</td>
</tr>
<tr>
<td>Choline</td>
<td>1</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>Aneurin *</td>
<td>3</td>
<td>2</td>
<td>5</td>
</tr>
<tr>
<td>Multiple</td>
<td>2</td>
<td>0</td>
<td>2</td>
</tr>
<tr>
<td><strong>Totals</strong></td>
<td><strong>93</strong></td>
<td><strong>485</strong></td>
<td><strong>578</strong></td>
</tr>
<tr>
<td><strong>From Isolates</strong></td>
<td><strong>5408</strong></td>
<td><strong>1825</strong></td>
<td><strong>7233</strong></td>
</tr>
</tbody>
</table>

* Quantitative data not homogeneous, because in some series the strain used was either biotin- or aneurin-requiring.
single nutritional requirements; and (3) by comparing the survival rates of the conidia of strains with double requirements when starved of one, the other, or both required growth factors.

a. Isolation of New Types of Auxotrophs. The results of two experiments for the selective isolation of new types of mutants are shown in Table 7. The technique was the usual one except that, instead of

<table>
<thead>
<tr>
<th>TABLE 7</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Search for Previously Unobtained Tryptophan, Inositol, and Guanosine Auxotrophs by Adding, after Starvation, Minimal Medium Plus These Growth Factors and Biotin Instead of Complete Medium</strong></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Hours of Starvation</th>
<th>Conidia plated</th>
<th>Colonies</th>
<th>Iso-</th>
<th>New Types</th>
</tr>
</thead>
<tbody>
<tr>
<td>S-6 (U.V., 12.5% survival)</td>
<td>124</td>
<td>450,000</td>
<td>133</td>
<td>112</td>
<td>12</td>
</tr>
<tr>
<td></td>
<td>168</td>
<td>1,300,000</td>
<td>422</td>
<td>234</td>
<td>12</td>
</tr>
<tr>
<td>S-7 (U.V., 4% survival)</td>
<td>112</td>
<td>315,000</td>
<td>3,404</td>
<td>1,000</td>
<td>5</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>1,346</td>
</tr>
</tbody>
</table>

The two new auxotrophs required tryptophan and guanosine, respectively. Of the other 27 auxotrophs, 26 were parathiotrophic and 1 adenine-requiring. These 27 evidently grew enough to be isolated even in the absence of the required growth factor.

complete medium, the top layer added after starvation was of minimal medium plus biotin (5 mg./l.), inositol (14 mg./l.), guanosine (60 mg./l.), and DL-tryptophan (10 ml. of 0.05 M solution per liter). The 1346 isolates yielded 29 auxotrophs; of these, 27 were of types already obtained (26 parathiotrophic, 1 adenine-requiring) and 2 were of the desired new types, i.e., 1 tryptophane-requiring and 1 guanosine-requiring. The experiment was therefore successful in providing two out of the three types, not previously obtained, which could have come up under the conditions used. Though the numbers are not sufficient for concluding that the previous failure was significant, the following evidence makes it very probable. Figure 10 shows that the two mutants barely grow on complete medium but that they grow well on minimal medium supplemented with the required growth factors.

b. Elimination of Specific Types of Auxotrophs. The longer survival of double auxotrophs (i.e., requiring biotin plus an additional growth factor) under starvation was investigated first as follows: if it were due to starvation for the additional growth factor, in the presence of this growth factor the difference should be annulled. Thus, for ex-
Fig. 10. Two mutants (TRYPT, requiring tryptophan; and GUAS, requiring guanosine), of types not obtained before the use of a selective technique, grow well on minimal medium plus tryptophan, adenine, and guanosine (left) but not on complete medium (right). Two other mutants (NIC, requiring nicotinic acid or tryptophan; AD, requiring adenine) grow well on both.

ample, parathiotrophic mutants might not be recovered preferentially from starvation experiments in which thiosulphate was added to the starvation medium. This expectation was fully borne out (Table 8). After irradiation with ultraviolet (survival 5%) the conidia of the biotin strain were embedded as usual in two sets of dishes: one of minimal medium, the other of minimal medium plus 1g./l. Na$_2$S$_2$O$_3$·5H$_2$O. After incubation for 139 hours, complete medium was added, and of the colonies which developed, 400 from each set of dishes were isolated and characterized. Of the 35 auxotrophs recovered from the minimal medium dishes, 24 were parathiotrophic, and 11 were of other types; of the 17 recovered from minimal medium + thiosulphate dishes, 1 was parathiotrophic and 16 were of other types. Clearly, when not starved of thio-

<table>
<thead>
<tr>
<th>Types of mutants</th>
<th>Total number of nutritional isolates</th>
<th>Unable to utilize sulphate</th>
<th>Requiring amino acids</th>
<th>Requiring purines</th>
<th>Requiring vitamins</th>
</tr>
</thead>
<tbody>
<tr>
<td>Minimal</td>
<td>400</td>
<td>35</td>
<td>24</td>
<td>1</td>
<td>8</td>
</tr>
<tr>
<td>Minimal supplemented with thiosulphate</td>
<td>400</td>
<td>17</td>
<td>1</td>
<td>3</td>
<td>8</td>
</tr>
</tbody>
</table>

Ultraviolet treatment (5% survival) followed by 139 hours of starvation on minimal medium or minimal medium + thiosulphate (Na$_2$S$_2$O$_3$·5H$_2$O, 1g./l.).
sulphate the double auxotrophs, biotin-thiosulphate-requiring, do no longer survive preferentially; the other types of auxotrophs, however, are found in the expected proportions in both sets of plates.

A practical outcome of this finding is that the elimination of unwanted types of auxotrophs in order to save labor can be achieved simply by using as a starvation medium one which contains the corresponding growth factor(s).

c. Survival Rates of Different Auxotrophs under Starvation. A comparison was made of the rates of survival in minimal medium of four mono-auxotrophic strains:

\( bi \), green conidia, biotin-requiring;
\( y bi \), yellow conidia, biotin-requiring derived by crossing from the former;
\( wa lys \), white conidia, lysine-requiring;
\( ad \), green conidia, adenine-requiring.

The results, based on addition of complete medium at 0, 48, 72, 96, and 120 hours (approximately) and counting of survivors are shown

![Graph showing survival rates of different auxotrophs](image-url)

**Fig. 11.** Rate of dying off under starvation of conidia of strains with different single nutritional requirements: adenine (\( ad \)), lysine (\( lys \)), and biotin (\( bi \) and \( y bi \)).
graphically in Fig. 11. It is clear that there are enormous differences in the rates of dying-off between the different auxotrophs: e.g., after 120 hours in minimal medium, 30% of the conidia of the \( ad_t \) strain are still viable, compared with 12% of those of the \( w_a \ lys \) strain, 1.2% of those of the \( y bi_t \) strain, and 0.2% of those of the \( bi_t \) strain.

The next step was that of determining the rate of dying off of a double auxotroph (\( w ad_t bi_t \): white conidia, adenine- and biotin-requiring) in minimal medium, in minimal medium plus biotin, and in minimal medium plus adenine, respectively. In the first, starvation is for both adenine and biotin, in the second for adenine alone, and in the third for biotin alone. Determination of survivors was done at the same intervals as in the previous tests. Furthermore, a proportion of conidia of both strains \( y bi_t \) and \( ad_t \) was added to the inoculum in minimal medium, a proportion of conidia of \( y bi_t \) to the inoculum in minimal medium plus adenine, and a proportion of conidia of \( ad_t \) to the inoculum in minimal medium plus biotin. The results are summarized graphically in Fig. 12.

---

**Fig. 12.** Rate of dying off under starvation of a strain requiring biotin and adenine. \( x \): starved of adenine alone; \( \Delta \): starved of biotin alone; \( o \): starved of both. For comparison, broken lines show rates of dying off of two strains having either requirement only.
The rates of dying-off of $y \textit{bi}_1$ and of $ad_1$ were the same in minimal medium or in minimal medium plus the irrelevant growth factor. The double auxotrophs, however, died off at a rate almost identical to that of $ad_1$ when starved of adenine only, at a rate approaching that of $y \textit{bi}_1$ when starved of biotin only, and at a rate very nearly equal to that of $ad_1$ when starved of both. It could be concluded, therefore, that at least in respect to these two requirements, the rate of dying-off in minimal medium was largely determined by the requirement for which starvation led to the slowest rate of dying-off. Clearly, the degree of heterotrophy had no appreciable role in the results.

The next step was that of taking three double auxotrophs (requiring biotin plus another growth factor) from those obtained by total isolation and five from those obtained by starvation and comparing their survivals after 112 hours incubation at $41^\circ C$, in minimal medium, in minimal plus biotin, and in minimal plus the other growth factor, respectively. The growth factors involved, besides biotin, were thiosulphate, lysine, arginine, P.A.B.A., and pyridoxin.

**TABLE 9**

Survival after 112 hours in Starvation Media of the Conidia of Eight Auxotrophs Requiring Biotin and Another Growth Factor, Three of Which Were Obtained by Total Isolation and Five by Starvation

<table>
<thead>
<tr>
<th>Strain</th>
<th>Origin</th>
<th>Requirements</th>
<th>% Survival after 112 hours of starvation*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>For biotin †</td>
</tr>
<tr>
<td>S3-A2</td>
<td>Total isolation,</td>
<td>Biotin and SO$_3^-$</td>
<td>0.04</td>
</tr>
<tr>
<td>S5-F35</td>
<td>Starvation</td>
<td>Biotin and SO$_3^-$</td>
<td>0.2</td>
</tr>
<tr>
<td>$bi_1 lys_1$</td>
<td>Total isolation,</td>
<td>Biotin and lysine</td>
<td>0.6</td>
</tr>
<tr>
<td>S5-F2</td>
<td>Starvation</td>
<td>Biotin and lysine</td>
<td>1.7</td>
</tr>
<tr>
<td>$bi_1 orn_1$</td>
<td>Total isolation,</td>
<td>Biotin and arginine or ornithine</td>
<td>0.02</td>
</tr>
<tr>
<td>S4-C96</td>
<td>Starvation</td>
<td>Biotin and arginine or ornithine or proline</td>
<td>1.3</td>
</tr>
<tr>
<td>S2-D1</td>
<td>Starvation</td>
<td>Biotin and P.A.B.A.</td>
<td>0.02</td>
</tr>
<tr>
<td>S5-E1</td>
<td>Starvation</td>
<td>Biotin and pyridoxin</td>
<td>0.2</td>
</tr>
</tbody>
</table>

* Taking as 100 the viable counts without starvation.
† On minimal medium plus the other growth factor.
‡ On minimal medium plus biotin.
§ On minimal medium.
The results, summarized in Table 9, are rather disconcerting. They certainly confirm the tremendous differences in the rates of dying-off between different auxotrophs. They do not suggest any simple relationship between degree of heterotrophy and survival rate. It is remarkable that starvation for biotin alone increases, in all cases, the rate of dying-off and brings it close to that of the \( \text{bi}_{1} \) strain.

Clearly the rate of dying-off under conditions of specific starvation could become a very powerful tool in the study of metabolic interrelationships. The investigation of which kinds of substances affect this rate could become as valuable as that carried out by the Hinshelwood school on what is, essentially, the opposite process: the lag phase in growing cultures.

As a tentative interpretation of our results we may suggest that, in our case, the efficiency of the starvation technique depended almost entirely on the happy choice, as the starting point, of the \( \text{bi}_{1} \) strain which worked out to have an exceedingly high rate of dying-off under specific starvation. Any additional nutritional requirement induced by irradiation is therefore likely to induce a lower rate, hence the enrichment by starvation.

As a working hypothesis it may be suggested that growth-factor requirements, starvation for which has a generalized blocking effect on metabolism (e.g., adenine, thiosulphate), are likely to slow down the rate of dying-off relative to those which distort specifically processes with no immediate general effect.

3. Characterization of Auxotrophs

Any auxotroph obtained by means of one of the techniques described in the preceding two sections is identified as such because of its inability to grow on the simplest medium adequate for the parent strain and of its ability to grow on complete medium. The simplest medium adequate for the growth of the parent will be referred to, for short, as "minimal medium," though of course it is actually minimal medium in the case of auxotrophs derived from the wild type, \( w_{4} \) or \( y \), but minimal medium + biotin in the case of auxotrophs derived from \( bi_{1} \), and minimal + aneurin in the case of auxotrophs derived from \( y \; thi \).

The identification of the additional growth factor, or factors, required by any auxotroph presents no theoretical difficulty, it is a matter of trying a number of individual supplements, or mixtures of supplements, with the minimal medium until the effective ones are found.

It is a fact that practically all newly arisen auxotrophs have a single growth-factor requirement: out of 612 so far subjected to systematic tests, we have failed to identify the additional requirement of only 33.
These may be cases of multiple requirements (due to multiple mutation, or to metabolic intricacies) or of requirements for substances not included in our routine tests. Thus, any procedure based on the test of individual growth factors would be successful in characterizing at least 95% of the auxotrophs. The practical problem is therefore one of efficiency.

In the first place, the procedure must restrict progressively the field of search. It would be absurd to try on each auxotroph one by one all available vitamins, amino acids, nucleosides, etc.; we must first identify the group of substances into which the requirement is likely to fall. This has been done by using inexpensive mixtures obtained from natural products (casein hydrolyzate, nucleic acid hydrolysates).

Secondly, if we know (as we do now) which types of auxotrophs constitute a high proportion of the total, it is economical to identify these first and carry out the further search only with the remainder.

Thirdly, there are three ways in which growth-response tests are more easily carried out: (1) spot-inoculating a number of strains on a series of differently supplemented plates (plate test); (2) spot-inoculating a number of substances on a series of plates, each inoculated all over with a particular auxotroph (auxanography), and (3) inoculating each strain in a series of test tubes with liquid minimal medium variously supplemented. We have found that the plate test is more efficient when a small number of substances (not more than five) have to be tested on a large number of strains (say twenty or more); that auxanography is more efficient in the converse case and when interactions between growth factors (inhibitions, sparing effects, etc.) are suspected; and that the tests in liquid medium are useful only as a final check.

A routine procedure of high efficiency, achieved only recently, is shown diagrammatically in Fig. 7; it was possible only after a precise knowledge of the proportion of different auxotrophs in the yield of starvation experiments became known. Before that the routine was as follows:

1. Simultaneous plate tests (20 auxotrophs per plate) on minimal medium, minimal medium plus casein-hydrolyzate (0.1 ml. of standard solution per plate), minimal medium plus nucleic acid hydrolyzate (0.06 ml. of standard solution per plate), minimal medium plus vitamins (0.02 ml. of standard solution per plate). Growth of any auxotroph on only one of the supplemented plates suggested a further search for individual amino acids; for individual purines, pyrimidines, and nucleosides; or for individual vitamins. Growth on both casein hydrolyzate and nucleic acid hydrolyzate indicated a requirement for amino groups, ammonium salts, or nitrite.

2. Auxanographic tests with all available amino acids, nucleosides, or vitamins of the strains which grew on the corresponding plates (Pontecorvo, 1949b).
The more efficient routine mentioned above and illustrated in Fig. 7 as applied to a hypothetical group of 100 auxotrophs is as follows:

1. Plate tests are made of the 100 strains on 5 dishes of minimal medium and 5 of minimal medium plus sodium thiosulphate (1 g./l.); none grows on the former, and about 60 strains grow on the latter. These are plate tested (3 dishes) on minimal medium plus sodium sulphite (1 g./l.) and about 50 grow. We have therefore classified as parathiotrophic 60 strains out of 100 by using 13 dishes. Of the 60 parathiotrophic, 50 grow on either sulphite or thiosulphate, and 10 grow only on the latter.

2. Plate test of the remaining 40 strains is done on 2 dishes of minimal medium plus adenine hydrochloride (80 mg./l.); 20 grow and are further analyzed auxanographically with the available related compounds (other purines, pyrimidines, nucleosides, and presumed precursors).

3. The 20 which did not grow on adenine are plate tested simultaneously on 1 dish each of minimal medium plus casein hydrolyzate and minimal medium plus vitamin solution; 12 strains grow on casein hydrolyzate, 7 on vitamins. The former are tested auxanographically with all available amino acids, the latter with all available B-vitamins. The negative strain is tested auxanographically for multiple requirements (see below).

a. Classification of Requirements of the Products of Crosses. The tests for the characterization of auxotrophs isolated from mutation experiments are also used for the classification of the products of crosses in which nutritional requirements are segregating (section V—3, 4). There are, however, some notable differences in the situation: (1) among the products of crosses, the kinds of requirements for which each isolate has to be classified are few and already known; and (2) a high proportion of isolates may show multiple requirements.

Since rarely more than three requirements are segregating in a cross, there are usually very many isolates to be tested against only few growth factors, and therefore the plate test is used extensively. If only one requirement segregates, the isolates fall into two classes; the isolates are tested simultaneously on dishes with and without the growth factor. If two requirements segregate, the isolates fall into four classes (requiring one, requiring the other, requiring both, requiring neither). They are plate tested simultaneously on two series of dishes, one with one growth factor and the other with the other: growth on both kinds of dishes indicates no requirement; growth on one indicates that requirement; growth on neither, both requirements (Fig. 13). The last are checked for growth on a medium with both growth factors.

If three requirements segregate A, B, C, the isolates fall into eight classes; they are plate tested on three series of dishes, each supplemented with two (AB; AC; BC) growth factors. Growth on all three indicates no requirement; growth on none indicates three requirements; growth on one indicates two requirements; growth on two indicates one
requirement. The isolates classified as having three requirements can be tested auxanographically. Figure 14 shows the kind of auxanogram given by isolates with one, two, and three requirements, respectively.

Fig. 13. Plate tests. Twenty-five segregants for P.A.B.A. and biotin requirements tested simultaneously on two plates of minimal medium, one supplemented with biotin (left) and one with P.A.B.A. (right). Growth on either, both, or neither classifies the segregants. Above, 25 strains photographed 24 hours after inoculation; below, 25 other strains photographed 48 hours after inoculation, when the biotin-requireers began to be "breast-fed."

Fig. 14. Three kinds of auxanograms given by segregants of a cross in which three growth-factor requirements segregate: ad, adenine; bi, biotin; arg, arginine. Top right, single requirement. Top left, double requirement. Bottom, triple requirement.
4. Selection of Prototrophs

by J. A. Roper

A problem discussed in sections IV-1 and IV-2 is the selection of a small proportion of auxotrophs from a mixture of auxotrophs and prototrophs. In certain cases the reverse selection, of rare prototrophs from auxotrophs, is required.

Giles and Lederberg (1948) and Kolmark and Westergaard (1949) using *Neurospora crassa* have developed a technique for the selection of prototrophic back-mutant conidia. This technique has also been used as a tool for detecting mutagenic activities (see Westergaard, 1952). The selection of prototrophs from auxotrophs is also crucial in the isolation of heterozygous diploids (Roper, 1952), and in the section on "Pseudoallelism" (VI-3) it will be shown in operation for selecting rare prototroph recombinants.

A preliminary investigation has been made of the suitability of *A. nidulans* as material for back-mutation studies. The technique is essentially like that of Westergaard (1952); two* strains of *A. nidulans* were used: bi$_i$ lys$_z$ and bi$_i$ arg$_i$. Double mutants were used in the tests to minimize dangers of undetected contamination and to facilitate crossing of back-mutants. Each strain was repeatedly purified by single conidium isolation. Conidial suspensions from 5-day-old cultures on minimal medium with biotin and lysine or biotin and arginine, respectively, were plated at high density (up to $3 \times 10^7$ per petri dish) on minimal medium with biotin alone. An aliquot of the suspension was diluted and plated on complete medium for estimation of the percentage of viable conidia. On minimal medium plus biotin, only the conidia independent of lysine or arginine could grow. Although the genotype of such reverted types was not tested, they will be called back-mutants. Plates were incubated for 7 days, during which time back-mutants were scored and isolated as they became visible. Results of these preliminary experiments are given in Table 10. No arginine-independent back-mutants were obtained. Lysine-independent back-mutants were recovered at a frequency of about $1 \times 10^{-6}$.

In the application of the technique to quantitative work a number of potential sources of error have been anticipated (Roper, 1950b); some of these have been the object of later independent work by Grigg (1952). In the first place, it is necessary both to know the initial number of nuclei under test and to ensure no increase in this number through germination of the auxotrophic conidia on the selective medium. Such germination may also introduce errors due to the possible effects on mutation of nuclear division under restricted metabolic conditions.
TABLE 10
Selection of Back-Mutants of *A. nidulans*

<table>
<thead>
<tr>
<th>Strain</th>
<th>Number of conidia tested</th>
<th>Viability on complete medium</th>
<th>Biotinless back-mutants</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Total</td>
<td>Per dish</td>
<td></td>
</tr>
<tr>
<td><em>bi, lys</em></td>
<td>$3.6 \times 10^8$</td>
<td>$3 \times 10^7$</td>
<td>$&gt;80%$</td>
</tr>
<tr>
<td><em>bi, arg</em></td>
<td>$2.1 \times 10^8$</td>
<td>$3 \times 10^7$</td>
<td>About 100%</td>
</tr>
</tbody>
</table>

Since the conidia of *A. nidulans* are uninucleate and, in the tested strains, do not germinate during the test, quantitative errors from this source are avoided. This is not so for the adenineless strain of *Neurospora* used by Kölmark and Westergaard (1949) which has multinucleate conidia germinating on minimal medium.

A second suggested source of quantitative error lies in the possible loss of prototrophs when they are selected from a large mass of auxotrophs. Especially where conidia of the test strain germinate on minimal medium or are "breast-fed" (section V-3) by growing back-mutants, recovery of rare prototrophs from a mesh of germination tubes may not be complete. In the tested strains of *A. nidulans*, no germination of auxotrophic conidia occurred after 7 days of incubation and there was no "breast-feeding" of auxotrophs even among the hyphae of a back-mutant colony (Fig. 15). However, there was still indirect evidence of

**Fig. 15.** Conidia of strain *bi, lys* (requiring biotin and lysine) on minimal medium plus biotin 100 hours after plating. Even near colonies of lysine-independent "back-mutants," the conidia do not germinate, though they may swell up. The hyphae are from one such back-mutant colony.
the inhibitory effects of high concentrations of auxotrophic conidia. During the first 72 hours of incubation back-mutant colonies were found only towards the edge of the inoculum, where the conidial density is lowest. Colonies appearing nearer the center of the plate were very delayed and normally became visible only after 72 to 120 hours of incubation. Since there is a considerable fall in viable count during the incubation period, some prototrophs may die before they can give rise to visible colonies. The extent of this inhibitory effect has not yet been investigated by means of reconstruction experiments.

For several strains of Neurospora, Grigg (1952) has found inhibition of prototrophs by auxotrophs on a sorbose minimal medium. On this medium, the efficiency of selection of prototrophs depends on the density of viable auxotrophic conidia surrounding them. Grigg has not investigated the degree to which this inhibition may depend on germination of the auxotrophic conidia; a matter which is probably of decisive importance. Kölmars and Westergaard (1952) have confirmed the inhibitory effect found by Grigg, but only for the sorbose medium. For the same conidial density range, Westergaard has shown that there is no inhibition on the glucose medium which is normally used in the Neurospora back-mutation technique. The inhibition may therefore be attributed mainly to competition for nutrients or energy.

A further source of error suggested by Grigg is intraconidial inhibitions involved in the use of multinucleate conidia of Neurospora. Even if correct for Neurospora, this cannot apply where uninucleate conidia, such as those of A. nidulans, are used.

Provided it is possible to overcome any possible inhibitory effects of auxotrophs in the selection of rare prototrophs, it seems likely that the conidia of some strains of A. nidulans should provide more suitable material for quantitative mutation studies than the macroconidia of Neurospora.

V. Methods of Genetic Analysis

1. Heterokaryosis

Heterokaryotic hyphae are formed when two strains are grown together on solid medium from mixed point-inoculum of conidia, ascospores, or mycelium. Usually, however, the heterokaryotic hyphae form only a small portion of the developing mycelium because, unless they grow much faster than the homokaryotic hyphae, the rate at which they are formed is balanced by that of the opposite process (Pontecorvo and Gemmell, 1944; Pontecorvo, 1947). When the growth rate of the heterokaryon is higher than that of the two homokaryons, it may be so under
any conditions or only under special conditions; these may or may not be controllable. In either case, the heterokaryon is “balanced”; i.e., once it constitutes an appreciable proportion of the mycelium, it is usually perpetuated through successive mass transfers of hyphal tips. The simplest examples of balanced heterokaryons are: (1) a heterokaryon, with growth habit approaching that of the wild type, formed between two mutant strains with stunted growth on any medium; and (2) a heterokaryon, able to grow on minimal medium, formed between two strains requiring different growth factors and therefore neither able to grow on that medium. In this second case it is the absence of the two growth factors which operates as a selective agent against the homokaryotic hyphae.

Three-component heterokaryons can be produced. We have obtained without difficulty heterokaryotic heads with chains of three different colors, white, yellow, and green (see further), by using a balanced heterokaryon between two strains with yellow and white conidia, respectively, and adding a third strain with green conidia. A balanced three-component heterokaryon undoubtedly could also be produced. It could be synthesized by using three strains in which three growth-factor requirements were shared two-by-two (A b c; a b C; A b C).

Even when the heterokaryon has a growth rate much greater than those of the two homokaryons, it does not easily become established automatically, though once established it perpetuates itself automatically on transfers of mycelium. To establish a balanced heterokaryon, two conditions are necessary. One is that primary heterokaryotic hyphae be formed in appreciable numbers from hyphal anastomosis. The other is that these primary heterokaryotic hyphae be enabled to multiply and escape from the meshes of parental mycelium in order that their higher growth rate may begin to tell. These two conditions are somewhat contradictory because to obtain many hyphal fusions a thick growth helps, but to enable the heterokaryotic hyphae to “escape” a thin growth helps.

Two techniques have been used to achieve the compromise, one or the other more appropriate to different combinations of strains. With one, conidia of the two strains, mixed in equal proportions in a suspension as dense as possible, are streaked over or stabbed into the agar medium. When the heterokaryon is to be formed between two strains with different nutritional requirements, this medium must be selective, i.e., minimal or lacking at least one growth factor for each strain. When the heterokaryon is to be formed between strains with stunted growth under any conditions, of course, no selective medium is used. In the first case, just enough supplemented medium must be carried over when inoculating to ensure a little initial growth of the two strains. Inocula-
tion is made by drawing parallel streaks about 2 cm. apart and 2 mm. wide, or a circle, or by making a series of stabs 1 cm. from the edge of the petri dish. After 5 to 10 days small patches of heterokaryotic mycelium may arise from the few points where one of the heterokaryotic hyphae formed succeeds in finding its way through the parental mycelium (Fig. 16).

![Fig. 16. Balanced heterokaryons arising from point inocula in minimal medium of two strains requiring adenine and aneurin, respectively. Note that the heterokaryon succeeds in "escaping" only at a few points.](image)

With the other technique, the mixed inoculum is grown for 24 hours in liquid complete medium, and then the mycelium, centrifuged and washed once, is spread out on to the agar surface of the medium, teasing out the meshes. This agar medium will again be selective in the case of strains differing in nutritional requirements.

With both techniques, as soon as patches of heterokaryotic mycelium appear, as revealed by their growth habit and, if color markers were used, by the presence of heads with mixed colors, small portions of agar with hyphal tips are transferred on to fresh medium. Usually one transfer is sufficient to establish the heterokaryon.

The use of differences in color of the conidia between the component strains is most convenient in watching the progress of a heterokaryon, in guarding against its loss, and in detecting changes in the balance between the two kinds of nuclei. As markers we have extensively used (section VI-1) the genes \( W/w \) (colored versus white conidia) and \( Y/y \) (green versus yellow conidia). Both these genes have cell-localized action (Pontecorvo, 1947), though not completely so in the case of \( W/w \). This means that in a combination of strains differing in color, a heterokaryotic head bears conidial chains some of one color and some of the other (Fig. 3) (Gossop, Yuill, and Yuill, 1940).

A well-balanced heterokaryon, say between a yellow and a white
strain, shows a thorough mixture of apparently pure yellow heads, apparently pure white heads, and mixed heads. In the last, the proportion of yellow to white chains varies all the way in different heads from almost all white to almost all yellow. The visibly mixed heads never constitute the major proportion, even in cases in which the ratio of yellow to white heads is not unduly lopsided (Table 11). This perhaps indicates that in

\begin{table}
\centering
\begin{tabular}{|l|c|c|c|}
\hline
\textbf{Heterokaryon} & \textbf{Heads} & \\
\hline
\hline
\textit{w} \textit{Y} \textit{AD}, \textit{lys} & & \\
\textit{W} \textit{y AD}, \textit{LYS} & 45 & 130 & 46 & 221 \\
\hline
\textit{w} \textit{Y} \textit{BI} \textit{lys orn}, & & \\
\textit{W} \textit{y BI} \textit{LYS ORN} & 81 & 179 & 25 & 285 \\
\hline
\end{tabular}
\caption{Distribution of Homo- and Heterokaryotic Heads in Balanced Heterokaryons}
\end{table}

* Certainly underestimated, because those with only very few conidial chains of the other color are likely to be misclassified as wholly yellow or wholly white.

the mycelium the nuclei of the two kinds tend to be clustered according to kind, and that the number of nuclei entering a conidiophore is small. In support of this second deduction stands the fact that in mixed heads, chains of conidia of the same color tend to be clustered. If the nuclei multiply abundantly within the vesicle, by reason of common descent those of one kind are more likely to be next to one another and therefore to enter groups of neighboring sterigmata.

Balanced heterokaryons are easily lost for a number of reasons. The most common are: back-mutation, non-genetic adaptation, recombination through sexual reproduction, formation of heterozygous diploid nuclei (section VII-1), and accumulation in the medium of metabolites enabling either or both component strains to grow independently. In any one of the first four instances, the heterokaryon is lost because of changes in the nuclei or the cells; as a consequence of these changes homokaryotic hyphae of a new type arise against which there is no selection. In the last instance, it is the medium itself which is no longer selective.

Heterokaryons, one of the components of which requires a vitamin, are those which more easily lose the balance as growth proceeds in a petri dish; presumably the heterokaryon produces the vitamin in excess
and releases it into the medium, enabling the vitamin-requiring component to grow independently. When the two component strains differ in the color of their conidia, the gradual increase in the proportion of homokaryotic hyphae of the vitamin-requiring one reveals itself in an increasing proportion of heads of the corresponding color. This process can be imitated artificially in a more extreme form by adding to the agar medium sufficient amounts of the growth factor (or factors) required by one strain after the mycelium has covered, say, one-half of the petri dish. The subsequent growth shows exclusively, or prevalently, heads of the corresponding color (Fig. 17). Whether or not the increase

in the proportion of homokaryotic heads implies a change in the balance between nuclei of the two kinds within the heterokaryotic hyphae will be discussed presently in connection with the general problem of "nuclear ratios" in heterokaryons.

Since the conidia in *A. nidulans* are uninucleate, a sample of conidia is a sample of nuclei. If in a heterokaryon the ratio of the two kinds of nuclei in the conidia were identical to, or highly correlated with, the same ratio in the mycelium, sampling of the conidia would be equivalent to sampling of the nuclei of the hyphae. Unfortunately, we have been unable to obtain any crucial evidence for or against this possibility.

An obvious way to test it was to compare the allele ratios in a sample of conidia with the same ratios in a sample of ascospores. Out of three cases tested involving balance between adenine requirement and biotin requirement, the conidia agree with the ascospores in one, but not in the other two (Table 12). In view of relative heterothallism (section V-4) (which implies non-random karyogamy or striking differential survival within one perithecium of different products of karyogamy), it is now

![Fig. 17. Balanced heterokaryon between a yellow aneurin-requiring and a green adenine-requiring strain. After some growth, aneurin was added to the medium; the yellow component sectored out.](image)
The genetics of Aspergillus nidulans

Table 12

Comparison between the Allele Ratios in the Conidia and Those in the Ascospores of Three Heterokaryons Balanced between ad, (Adenine Requirement) and bi (Biotin Requirement)

<table>
<thead>
<tr>
<th>Heterokaryon</th>
<th>Allele Ratios</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>w</td>
</tr>
<tr>
<td>1. w ad, y BI + W AD, Y bi</td>
<td>Conidia</td>
</tr>
<tr>
<td></td>
<td>Ascospores</td>
</tr>
<tr>
<td></td>
<td>Total</td>
</tr>
<tr>
<td>2. ad, y BI + AD, y bi</td>
<td>Conidia</td>
</tr>
<tr>
<td></td>
<td>Ascospores *</td>
</tr>
<tr>
<td></td>
<td>Total</td>
</tr>
<tr>
<td>3. ad, Y BI + AD, y bi</td>
<td>Conidia</td>
</tr>
<tr>
<td></td>
<td>Ascospores *</td>
</tr>
<tr>
<td></td>
<td>Total</td>
</tr>
</tbody>
</table>

* Only those not requiring adenine were classified. This might reduce the proportion of y in 2 and of Y in 3, thus tending to blur rather than emphasize the difference between ascospores and conidia.

The allele ratios in the conidia are significantly different from those in the ascospores in heterokaryons 2 and 3 but not in 1. Though the balance is between the same requirements in all three heterokaryons, the ratio of adenine-requiring to biotin-requiring nuclei (calculated from conidia) are 1:9; 1:17, and 1:3.5, respectively.

It is still an open question whether there is such correspondence in the case of the nuclear ratios in the hyphae.

Even though we have not carried out quantitative work by means of single hyphal-tip isolations, there is overwhelming qualitative evidence that, at least in A. nidulans, a balanced heterokaryon consists of a mixed population of hyphae, some heterokaryotic and some homokaryotic of either type.

As the colony grows, there are at least two types of interplaying dynamic equilibria: (1) between the two types of nuclei within the heterokaryotic hyphae, and (2) between the three types of hyphae. The latter is a form of syntrophism, though of a subtle kind: the heterokaryotic hyphae, which do not benefit as much as the homokaryotic hyphae from it, continuously break up to form the latter, but are also...
continuously re-formed from the latter, and the whole process is not independent of syntrophism itself. As to (1), there is no clue in *A. nidulans* of what goes on. In the work with *Neurospora* (e.g., Ryan and Lederberg, 1946; Emerson, 1948) changes in the nuclear ratios of a heterokaryotic colony have been attributed to (1). Clearly, they could have been attributed equally well to (2) or to both. There is, in fact, no evidence of whether the nuclear ratios within the heterokaryotic hyphae are adjustable at all. If they were not, the selective adjustment of the nuclear ratios in a growing colony as a whole—about which there is little doubt—equally involve the distribution of the total population of nuclei as between heterokaryotic and homokaryotic hyphae. The problem boils down to whether there is a differential rate of mitosis of the nuclei within a hypha, or a differential rate of multiplication of the hyphae according to the nuclear ratios which they happen to have.*

In many cases, balanced heterokaryons, instead of showing uniform growth of mycelium and, if color markers are present, uniform and thorough mixture of heads of the three kinds, show a patchy growth, with patches of heterokaryotic hyphae alternating with others of homokaryotic hyphae, often growing thinly. The complex dynamic equilibrium between and within hyphae mentioned above, is unstable, and growth proceeds in a see-saw way. In one case, a partial explanation of the phenomenon may be offered. Heterokaryons between an arginine-requiring strain and strains requiring some other growth factor (especially lysine) give this alternation of good and poor growth, and color markers show the areas of good growth to be mainly heterokaryotic and the others mainly homokaryotic. It is known that arginine intake or utilization is inhibited competitively by exogenous lysine and vice versa (section VI-4) (Pontecorvo, 1950, 1952c). A strain which requires arginine, and presumably synthesizes lysine, whenever growing on limiting amounts of arginine will accumulate lysine and therefore interfere with its own further utilization of arginine. In a balanced heterokaryon, whenever growth happens to slow down in any part of the heterokaryotic colony because of the available amount of arginine, it will stop altogether because of the accumulation of lysine. The other component of the heterokaryon, however, will grow and accumulate more arginine, which will, in its turn, overcome the endogenous inhibition. If the balanced heterokaryon is one between a lysine-requiring and an arginine-

*In two interesting papers, Jinks (1952, *Heredity* 6, 77-87; *Proc. roy. Soc.*, B, 140, 83-106) produces new very precise quantitative evidence of selective adjustment in the nuclear ratios of wild heterokaryons of *Penicillium*. This work, however, still does not provide the necessary crucial evidence as to the problem of how the nuclear ratios are adjusted.
requiring strain, the pendulum equilibrium is even more marked, because for each of the two components there is no compromise between optimal growth and self-poisoning.

Whether these unstable balanced heterokaryons oscillating between the two homokaryotic conditions behave in this way by adjusting the nuclear ratios within or between hyphae is, again, unknown.

The study of heterokaryons has revealed complexities not suspected at the start (Pontecorvo, 1947).

2. Crossing

*Aspergillus nidulans* being homothallic, when we grow two strains in mixed culture, the ascospores of any one ascus may derive their nuclei from a selfed zygote of one strain, from a selfed zygote of the other strain, or from a crossed zygote. For genetic analysis, we are interested only in the asci of crossed origin, and therefore we must aim at the maximum proportion of these.

Before the recent discovery of "relative heterothallism" in *A. nidulans* (Hemmons, Pontecorvo, and Bufton, 1952; see also section V-4), a reasonable working hypothesis was that to obtain the maximum proportion of crossed asci it was necessary to secure in the mycelium as thorough a mixture as possible of nuclei derived from the two strains to be crossed. With random distribution of the two kinds of nuclei in the mycelium and random karyogamy between any two of them, a mycelium in which the nuclei of the two kinds were in equal proportions would yield 50% asci of crossed origin.

Starting from these considerations (which we know now to be wrong) two techniques of crossing were developed. One made use of the fact that in a balanced heterokaryon nuclei of the two strains are present and multiply side by side. Another made use of the fact that hyphal anastomoses occur readily soon after germination of the conidia and, therefore, packing together in equal proportions conidia of two strains in non-selective agar medium ensures a high number of inter-strain anastomoses. If the inoculation is at such a high density that very little growth can take place before the formation of the perithecia, a high proportion of the heterokaryotic hyphae formed will remain heterokaryotic up to the time of the formation of the perithecia. A proportion of perithecia will thus start with nuclei of both kinds.

The first technique ("heterokaryon cross") simply consists in forming a balanced heterokaryon, as mentioned in the previous section, and waiting for at least 10 days, when mature asci begin to be present. The heterokaryon can be kept for months in a cupboard, the agar can be left to dry off, and the ascospores can be used at any later time, because
they are not ejected from the perithecia. The heterokaryon cross is applicable only to pairs of strains between which a balanced heterokaryon can be formed.

The second technique ("mixed inoculum cross") consists in mixing together two suspensions of conidia, one from each of the strains to be crossed, containing equal numbers of viable conidia of each kind. The mixed suspension is then plated, aiming at not less than 5 million conidia per petri dish. A bottom layer of complete medium is first poured; the mixed suspension is spread onto the surface of this layer, and then a second layer of not more than 5 ml. of complete medium is poured on top. The mixed inoculum cross has the advantage of being applicable to any combination of strains, irrespective of whether either, both, or neither requires any growth factor or has a reduced growth rate.

Which one of the two techniques is to be preferred in any one case it is not easy to decide. On the one hand, with complete homothallism, the heterokaryon technique could give as optimal results not more than 50% asci of crossed origin, and this only in the ideal case of a balanced heterokaryon in which all the mycelium were heterokaryotic and the ratio of the nuclei of two kinds were selectively adjusted to 1:1. This is certainly an uncommon occurrence (Beadle and Conrartd, 1944; Pontecorvo, 1947) and therefore one could have expected the heterokaryon technique to yield usually well below the maximum theoretical 50% crossed asci. The discovery of relative heterothallism, however, makes this inference groundless, because a self-fertile strain may yield almost 100% crossed asci when in combination with a second one, and only a few per cent when in combination with a third one. Clearly, the nuclear ratios in the heterokaryon cannot account for proportions of crossed asci in excess of 50%.

The mixed inoculum technique, on the other hand, still gives up to 100% hybrid asci in certain combinations of strains, and in others it gives proportions of hybrid asci which may approximate or fall short of the theoretical maximum for complete homothallism.

Whichever the technique used to obtain asci of crossed origin, once these are available they must be used for genetic analysis. Three techniques of genetic analysis have been developed: (1) recombinant selection from random samples of ascospores, (2) perithecium analysis, and (3) ascus analysis. The first two have been used extensively in our work; ascus analysis has not.

Ascus analysis in *A. nidulans* does not differ in principle from ascus analysis in yeast or in any other species in which the spores are not linearly arranged. In practice, some technical modifications were neces-
sary to adapt it to *A. nidulans*; they have been developed by Miss Hemmons, and they will be published fully at a later date. The data from ascus analysis in *A. nidulans* require the treatment developed by Lindgren (1949) and Whitehouse (1950, and personal communication for linked loci) involving the use of three loci, two by two.

Another quite novel technique of genetic analysis has been developed; it is based on mitotic recombination in artificially produced heterozygous diploids. Since it does not make use of sexual reproduction, it will be dealt with separately in sections VII-1 and VII-2.

3. **Recombinant Selection from Random Samples of Ascospores**

This was the first technique used for genetic analysis in *A. nidulans*, and indeed, in any homothallic fungus (Pontecorvo 1949a). It is still widely used in our work, especially for detecting and estimating close linkage.

In a cross of two strains of a heterothallic organism, a random sample of ascospores is a sample of the products of meiosis of a large number of zygotes, all of which were of crossed origin. In a homothallic organism, it is a sample of the products of meiosis of zygotes, some of which were of crossed origin and some of selfed origin of either type. Plating of such a sample can yield genetic information only if we have some means of distinguishing between the ascospores derived from selfed asci and those derived from crossed asci. This can be done by using two pairs of genetic markers for identifying and selecting recombinants; and of course, recombinants can only result from crossed karyogamy. Segregations at other loci are then studied only among the selected recombinants. The principle underlying this technique is the same as that underlying Lederberg’s (1947) technique for crossing bacteria.

**Notations**

*Italic capitals:* wild type or dominant alleles.

*Italic lower case:* mutant or recessive alleles.

*Loci determining nutritional requirements:* the symbols use the first two or three letters of the growth factor required; e.g., *LYS/lys*, locus at which a mutant allele determines lysine requirement; *Bi/bi*, locus at which a mutant allele determines biotin requirement. Different loci at which mutant alleles determine apparently the same requirement (“mimics”) are distinguished by a subscript; e.g. *AD₁/ad₂*, *AD₂/AD₁*, etc., different loci at which mutant alleles determine a requirement for adenine.

*Crosses:* the two genotypes of the strains crossed are separated by the symbol //.

*Zygotes:* the genotype of the heterozygotes from a cross is indicated by separating the symbols of the alleles by a fraction sign, loci known to be linked having a common fraction sign. All the alleles contributed by one strain are above the signs,
those contributed by the other strain below. Thus, the heterozygotes from a cross
\[ \text{lys} \ Y\ Bl//\text{LYS} \ y\ bi \]
will be represented by \[ \text{LYS} \ y\ hi \]
being known to be linked.

A full list of identified loci is on p. 202, Tab. 17.

The way in which recombinants for the two pairs of desired markers are selected in any one case may be (1) visual, when differences classifiable by inspection are used, e.g., colors of conidia; (2) automatic, when, for example, differences in nutritional requirements are used and plating is on selective media which permit the growth of only certain types of recombinants; (3) a combination of both; or (4) based on testing individual isolates, and using for Mendelian analysis any one or more recombinant types.

For example, a mutant allele at the \( Y/y \) locus gives light yellow conidia instead of green as in the wild type. Three mutant alleles at the \( W/w \) locus give colorless (white) conidia and are epistatic to \( Y/y \). From a cross between two strains, one with yellow (\( WY \)) and one with white conidia (\( wY \)), recombinant ascospores (\( WY \)) give origin to colonies with green conidia. We can select green colonies and classify them for other segregating genes. Alternatively, we may use, say, a mutant allele at the \( \text{LYS}/\text{lys} \) locus, which determines a requirement for lysine, and one at the \( Bl/bi \) locus, which determines a requirement for biotin. From a cross between two strains, one lysine-requiring (\( \text{lys} BI \)) and the other biotin-requiring (\( \text{LYS} bi \)), recombinant ascospores (\( \text{LYS} BI \)) are the only ones capable of giving origin to colonies on a medium lacking both growth factors. We can isolate these and classify them for other segregating genes.

For a cross between two strains differing at all of the four loci just mentioned (e.g. \( \text{lys} w_a Y\ BI//\text{LYS} \ W \ y\ bi \)), we can select in three ways: color alone, requirements alone, and one color and one requirement. In the first case we plate a random sample of ascospores on medium containing both lysine and biotin; we identify visually the \( WY \) recombinant colonies because of their green color; we isolate them and classify them (section IV-3) as to their requirements: \( \text{lys} BI; \text{LYS} bi; \text{LYS} BI; \text{or lys} bi \). In the second case we plate on medium devoid of lysine and biotin, and we classify as to their colors the colonies which came up (all necessarily \( \text{LYS} BI \) : \( w_a Y \) (white); \( WY \) (green)); \( w_a y \) (white) (\( w_a \) is epistatic to \( Y/y \)). In the third case, we plate either on medium containing biotin but not lysine or on medium containing lysine but not biotin. On these partially selective media one of the two parental types is capable of growing (the \( \text{lys} w_a Y\ BI \) type on lysine medium, and the \( \text{LYS} W \ y\ bi \) type on biotin medium). We
select, therefore, the recombinants between colors and requirements. Thus, on biotin medium, we discard the yellow, because they will include a proportion of parental combinations \((LYS \ W \ y \ bi)\) derived both from selfed and from crossed asci, but we isolate and classify all the white and, of course, the green. On the other hand, on lysine medium, we discard the white but isolate and classify the yellow and the green.

In the examples of completely or partially selective plating given above, at least one of the criteria for selection is based on the automatic sieve of the medium. When, however, the proportion of crossed asci in a cross is high, the automatic sieve of the medium is not essential. All the colonies from a non-selective medium are isolated and typed, and those which are recombinant in a desired way are retained for further classification as to other segregating loci. Thus, for example, we may plate on medium containing biotin and lysine, isolate all the yellow, and use for classification as to \(BI\) or \(bi\) all those which are lysine requiring, and therefore certainly of crossed origin, or we may use for classification as to colors all the double requirers \(lys \ bi\), etc.

![Fig. 18. "Breast feeding." When the prototrophs from a cross segregating for biotin requirement have formed large colonies, they release biotin in the medium in quantity sufficient for growth of biotin requirers (small colonies).](image)

Clearly, in each cross a number of selections are possible. The choice of the most efficient ones is a matter of experimental design, but also of experience. For instance, the requirement for a growth factor is sometimes satisfied by syntrophism; thus, biotin-requiring colonies do grow on biotinless medium if a number of non-requiring colonies are growing in the same petri dish and presumably secreting biotin ("breast feeding") (Fig. 18). Furthermore, certain genotypes determine a considerable delay in germination. This means that, in a plating where
colonies of these genotypes should come up, this delay may lead to the loss of a proportion of them because of smothering by others. These two examples of the kinds of practical difficulties encountered show where experience helps.

We must now discuss the question of how to identify and measure linkage. In the cross given above, as an example, if the four loci segregated independently, no matter which two were used for selection, barring differential viability, the other two should give 1:1:1:1 ratios or the modified 1:2:1 ratio due to epistasis of \( w_a \). On the other hand, if there were linkage and/or differential viability, the ratios would be distorted in certain characteristic directions.

In this cross, the workable types of selection of recombinants at two loci permitting the classification of segregants at both the other two loci, are the following:

<table>
<thead>
<tr>
<th>Method of selection</th>
<th>Selected recombinants</th>
<th>Classifiable combinations as to the other two loci</th>
</tr>
</thead>
<tbody>
<tr>
<td>Visual</td>
<td>( W Y )</td>
<td>lys ( BI ) LYS ( bi ) lys ( bi ) LYS ( BI )</td>
</tr>
<tr>
<td>Nutritional, automatic</td>
<td>LYS ( BI )</td>
<td>( W y ) ( w_a ) ( Y ) ( w_{a} ) ( y ) ( W Y ) indistinguishable</td>
</tr>
<tr>
<td>Nutritional, requiring complete testing</td>
<td>lys ( bi )</td>
<td>( W y ) ( w_a ) ( Y ) ( w_{a} ) ( y ) ( W Y ) indistinguishable</td>
</tr>
</tbody>
</table>

The number of useful selections is restricted by the epistasis of \( w_a \) over \( Y/y \).

The following data (kindly supplied by Miss L. Hemmons) can illustrate the kind of results obtained:

Cross: \( \text{lys } w_a Y BI//LYS W y bi \)

<table>
<thead>
<tr>
<th>Selected recombinants</th>
<th>Segregations at other loci</th>
<th>Parental</th>
<th>Recombinant</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>lys ( BI ) LYS ( bi )</td>
<td>lys ( bi ) LYS ( BI )</td>
</tr>
<tr>
<td>( W Y )</td>
<td></td>
<td>64</td>
<td>8</td>
</tr>
<tr>
<td>( W y )</td>
<td></td>
<td>5</td>
<td>110</td>
</tr>
<tr>
<td>( w_{a} ) ( Y )</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>( w_{a} ) ( y )</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>( W Y )</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>( LYS BI )</td>
<td></td>
<td>2</td>
<td>39</td>
</tr>
<tr>
<td>( lys ) ( bi )</td>
<td></td>
<td>22</td>
<td>28</td>
</tr>
</tbody>
</table>

132
<table>
<thead>
<tr>
<th>Cross</th>
<th>Selection</th>
<th>Segregations at other loci</th>
<th>Recombination fraction</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Unusable classes</td>
<td>Cross overs</td>
</tr>
</tbody>
</table>

1. **lys w Y BL Y THI**
   - LYS BL, THI
   - W Y
   - LYS bi, lys bi, LYS BI, lys BI

2. **lys w Y BL, Y THI**
   - LYS BL, THI
   - W Y

3. **Y BL, thi**
   - BL, THI

4. **ad, Y BL, Y bi**
   - AD, BI

Recombination fraction: 0.052 ± 0.005

Homogeneity (pooling 1b, 1s: 2a, 2b; and 2c, 3, 4b): χ² = 3.12; P ≈ 0.65
TABLE 14
Crosses Involving the $Y/y$ and $BI/bi$ Loci in Repulsion: $Y\ bi/y\ BI$

<table>
<thead>
<tr>
<th>Cross</th>
<th>Selection</th>
<th>Segregations at other loci</th>
<th>Unusable classes</th>
<th>Crossovers</th>
<th>Non-crossovers</th>
<th>Recombination fraction</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>(\frac{AD_1}{ad_1}\ \frac{Y\ bi}{y\ BI})</td>
<td>(\frac{AD_1}{ad_1}\ BI)</td>
<td>(Y) (y)</td>
<td>8(\ Y)</td>
<td>8(\ y)</td>
<td>(\frac{161}{153})</td>
</tr>
<tr>
<td></td>
<td>(\frac{AD_1}{ad_1}\ y)</td>
<td>(\frac{AD_1}{ad_1}\ y)</td>
<td>(bi) (BI)</td>
<td>8(\ bi)</td>
<td>8(\ BI)</td>
<td>(\frac{150}{142})</td>
</tr>
<tr>
<td>2.</td>
<td>(\frac{ad_1}{AD_1}\ \frac{w}{y\ bi\ PYRO})</td>
<td>(\frac{AD_1}{AD_1}\ BI\ PYRO)</td>
<td>(w\ y) (w\ Y)</td>
<td>38(\ w\ y)</td>
<td>1(\ w\ Y)</td>
<td>1(\ W\ y)</td>
</tr>
<tr>
<td></td>
<td>(\frac{ad_1}{AD_1}\ y)</td>
<td>(\frac{AD_1}{AD_1}\ y)</td>
<td>(w\ y) (w\ Y)</td>
<td>3(\ w\ y)</td>
<td>46(\ w\ Y)</td>
<td>46(\ W\ y)</td>
</tr>
<tr>
<td>3.</td>
<td>(\frac{S_a}{S_a}\ \frac{AD_1}{ad_1}\ \frac{Y\ bi}{y\ BI})</td>
<td>(\frac{S_a}{S_a}\ AD_1\ y)</td>
<td>(\frac{S_a}{S_a}\ \frac{S_a}{S_a})</td>
<td>3(\ S_a)</td>
<td>72(\ S_a)</td>
<td>72(\ S_a)</td>
</tr>
<tr>
<td></td>
<td>(\frac{S_a}{S_a}\ y)</td>
<td>(\frac{S_a}{S_a}\ y)</td>
<td>(\frac{S_a}{S_a}\ \frac{S_a}{S_a})</td>
<td>4(\ S_a)</td>
<td>44(\ S_a)</td>
<td>47(\ S_a)</td>
</tr>
<tr>
<td>4.</td>
<td>(\frac{AD_1}{ad_1}\ \frac{w}{Y\ bi})</td>
<td>(\frac{AD_1}{ad_1}\ BI)</td>
<td>(w\ y) (w\ Y)</td>
<td>59(\ w\ y)</td>
<td>3(\ w\ Y)</td>
<td>3(\ W\ y)</td>
</tr>
<tr>
<td></td>
<td>(\frac{ad_1}{ad_1}\ W)</td>
<td>(\frac{ad_1}{ad_1}\ W)</td>
<td>(\frac{Y\ BI}{y\ bi\ Y\ bi\ y\ BI})</td>
<td>0(\ Y\ BI)</td>
<td>6(\ y\ bi)</td>
<td>4(\ y\ BI)</td>
</tr>
</tbody>
</table>

Recombination fraction: 0.049 ± 0.008
Homogeneity (pooling 2a, 2b, 3a, 4a, 4b): \(\chi^2 = 0.68; P = 0.85\).

* Not classified as to PYRO/pyro.
Clearly, neither are the four combinations at the *lys* and *bi* loci equally frequent when we select the color recombinants, nor are the color combinations in 1:2:1 ratios when we select the prototrophs or the double requirers. Two departures are obvious: first, among the *W Y*, the *lys* are less frequent than the *LYS* (69 to 118); this could be due to lower viability or to linkage in coupling of *lys* and *wa*. Linkage, however, must be excluded because the other two selections show no shortage of *wa* when we select the *LYS*, nor of *W* when we select the *lys*. Second, selection for *Y* brings about a shortage of *bi* (13 *bi*: 174 *BI*), selection for *BI* brings about a shortage of *y* (2 *W y*: 40 *W Y*), and selection for *bi* brings about a shortage of *Y* (3 *W Y*: 22 *W y*). The parental combinations being complementary to those which are short (*Y* with *BI*, and *y* with *bi*), this shortage can be taken as an indication of linkage between the *Y/y* and *BI/bi* loci.

Data are available from three more crosses with the loci *Y/y* and *BI/bi* again in coupling. They are collected in Table 13 together with those already given. Not all the workable selections have been carried out in each cross. The results all support linkage. A test of homogeneity over all the sets of results in coupling gives *P* ≈ 0.65, and the pooled recombination fraction is 95/1814 = 0.052 ± 0.005.

**Table 15**

Detection, by Means of Double Crossovers, of Linkage Between Loci 50 or More Units Apart

<table>
<thead>
<tr>
<th>Cross</th>
<th>Selection</th>
<th>Double crossovers</th>
<th>Single crossovers</th>
<th>Recombination fraction *</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td><em>wa d, AD, Y</em></td>
<td><em>THI</em></td>
<td><em>AD, AD, THI</em></td>
<td><em>w</em></td>
</tr>
<tr>
<td></td>
<td><em>W AD, ad, y</em></td>
<td><em>THI</em></td>
<td><em>AD, AD, THI</em></td>
<td><em>y</em></td>
</tr>
<tr>
<td>2.</td>
<td><em>W AD, y BI</em></td>
<td><em>BI</em></td>
<td><em>w AD, BI</em></td>
<td><em>bi</em></td>
</tr>
<tr>
<td>3.</td>
<td><em>wa d, y</em></td>
<td><em>BI</em></td>
<td><em>w AD</em></td>
<td><em>bi</em></td>
</tr>
</tbody>
</table>

* Double crossovers/doubles + singles.  
  * *wa*, *ad*, and *y* are known to be closely linked.  
  Crosses 2 and 3 suggest linkage in coupling between *ad* and *y* and *ad* and *wa*, respectively (results of cross 3 non-significant). Cross 1 suggests linkage in coupling between *wa* and *ad*.  

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* *wa*, *ad*, and *y* are known to be closely linked.  
  Crosses 2 and 3 suggest linkage in coupling between *ad* and *y* and *ad* and *wa*, respectively (results of cross 3 non-significant). Cross 1 suggests linkage in coupling between *wa* and *ad*.  

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135
The next step is that of testing the $Y/y$ and $Bl/bi$ loci in repulsion. Clearly, it would be best to compare the results from crosses in which the same other genes are segregating in the same parental combinations as in the coupling crosses. These results, however, are not available except in one case (cross 4 in coupling and cross 1 in repulsion). The data from the four available crosses in repulsion are collected in Table 14. A test of homogeneity on these sets gives $P \approx 0.85$. The recombination fraction from these crosses in repulsion is $32/650 = 0.049 \pm 0.008$.

A test of homogeneity over the coupling and repulsion data combined gives $\chi^2 = 4.5$ and $P \approx 0.80$. The results can therefore be pooled and the recombination fraction is $127/2464 = 0.0515 \pm 0.0045$.

In the present case, the conclusion is unequivocal, since qualitatively consistent and quantitatively homogeneous results have been obtained despite the fact that the linkage of $Y/y$ and $Bl/bi$ has been tested in crosses involving six other loci. As will be shown (Tables 15 and 18), there is some evidence that two of these other loci—$W/w$ and $AD_1/ad_1$—are on the same chromosome as $Y/y$ and $Bl/bi$ but more than 50 c. Morgan away. This would require the special mathematical treatment (Bailey, 1951) for the case of selection based on two loci including the segment over which recombination is measured. If the evidence as to these further linkages were confirmed, a situation like this would occur in cross 4, selection a in coupling; and in crosses 1, selection a; 2, selections a and b; and 4, selection a; all in repulsion (Tables 13 and 14).

Whenever viability effects occur, the treatment of the data from any one or more types of selection could be carried out following Mather (1951, Chapter VIII, 21) provided data were available from both coupling and repulsion crosses. The problem, of course, has to be explored by the statistician for some of the new aspects which selection of recombinants introduces. When the loci used for selection are not linked with those the linkage of which has to be estimated, the situation is simple. But it is not simple when there is linkage between the two groups of loci. Bailey (1951) has started to deal with the ease of selection for two loci including the segment over which an estimate of linkage is wanted. There are, however, other cases for which the help of the statistician would be most welcome. Some will be considered presently.

In any series of crosses segregating at least at three loci—$A/a$, $B/b$, $C/c$—there are three possible ways of changing the procedure. The first is that of changing the distribution of alleles between the strains to be crossed: e.g., $AB C//a b c$, $a B C//A b c$, $a b C//A B c$, or $A b C//a B c$ ("phase" permutations). The second is that of using for selection different pairs of loci: e.g., $A$ and $B$, $A$ and $C$, or $B$ and $C$.
(‘marker’ permutations). The third is that of using for selection in any one cross one or the other or both of the two reciprocal recombinations of alleles at the two chosen loci: e.g., in cross A B C//a b C when selecting on loci A and C, we may use either or both recombinant classes A C and a c (‘allele’ permutations).

If we carried out on a sufficiently large scale all possible crosses differing in ‘phase,’ and in each cross we used all two-by-two ‘marker’ permutations for selection, and in each of these we selected both ‘allele’ permutations, we should have complete information for detecting and estimating any linkage and any viability.

Clearly it is necessary to decide how far we can reduce the necessary number of permutations. Bailey’s (1951) analysis has dealt only with a special case, that of a single linkage group, using two ‘phase’ permutations, one ‘marker’ combination, and one ‘allele’ combination. Bailey has not considered the information to be gained by the use of both ‘allele’ permutations at any pair of loci, and/or more than one ‘marker’ permutations. For the spade-work in Aspergillus, where a large number of loci are ready for analysis, and in many other microorganisms, a generalized mathematical treatment of this kind is badly needed.

There are, in addition, innumerable special problems. For instance, linkage between three loci showing 50% recombination two-by-two may be detected by making use of interference in double crossovers as in the examples of Table 15. In this case again, the problem is one of valid detection of linkage, as distinct from viability effects, and of its estimation.

Another problem, which has been considered by Roper (section VI-3), is that of estimating linkage between loci determining nutritional requirements by comparing the proportion of spores giving origin to colonies on a medium which selects for two given loci with the proportion on a medium which selects for two others. In cases of extremely close linkage, this is a very convenient way of estimating it, though the precise statistical treatment has to be developed.

Pending the development of correct methods of treatment for crosses based on recombinant selection, we shall have to use treatments undoubtedly questionable, but sufficient for the immediate purpose of building tentative chromosome maps.
4. Perithecium Analysis and Relative Heterothallism
by L. M. Hemmons, G. Pontecorvo, and A. W. J. Bufton

Homothallism may be defined as the ability of a strain whose nuclei are derived from a single haploid nucleus to go through a complete sexual cycle. Heterothallism can then be defined as the inability to complete the sexual cycle without karyogamy between nuclei of different origin. On the basis of these definitions, the three strains of *Aspergillus nidulans* (section III-1) from which all the others were derived in our work are unquestionably homothallic.

The evidence is as follows:

1. Fully fertile strains can be derived by isolation of single ascospores from each of these three strains and from a yellow spore mutant \((y)\), obtained by irradiation of the wild type, from which most of our yellow spore strains have been derived.

2. The eight spores of one ascus from each of these four strains (wild type, \(w_a, s_d,\) and \(y\)) were dissected and single-ascospore cultures established. Each of the eight spores from one ascus gave origin to fertile strains.

3. The eight spores of an ascus from one of the single-ascospore strains of (2) were again dissected, and single-ascospore cultures established (seven out of eight in the case of the wild type). All cultures were fertile.

4. The majority of the mutant strains derived from the four above-mentioned are still self-fertile and remain so after single-ascospore isolation. The majority of recombinant strains obtained from crossing are also self-fertile after single-ascospore or single-conidium isolation.

Nobody has investigated hitherto what happens in a homothallic species when nuclei of different origins are confronted within the hyphae. Clearly, this could not be done before the development of the technique for genetic analysis in homothallic species (section V-3). The investigation of this problem has revealed the existence of what we call "relative heterothallism," namely, the formation of crossed asci in excess of 50% in certain combinations of strains (Hemmons, Pontecorvo, and Bufton, 1952). The technique which has led to the accidental discovery of relative heterothallism ("perithecium analysis") is valuable *per se* as an additional means for genetic analysis in *A. nidulans* and undoubtedly in other homothallic species.

The first point investigated was: What kind or kinds of asci are to be found in individual perithecia in a cross made by mixed inoculum technique? By using color markers \((y/Y)\) it is not necessary to dissect the eight spores of each ascus to see whether the ascus was derived from
a selfed or from a crossed zygote. It is sufficient to crush the whole ascus on a small square of cellophane placed over the surface of agar medium. The colony resulting from each crushed ascus will be either uniformly of one color (yellow or green), if the ascus was of selfed origin, or show sectors of both colors, if the ascus was of crossed origin (Fig. 19). Even if the viability of the ascospores were as low as 50%.

![Fig. 19. Each colony originated from a whole ascus from a cross y bi, //++, the asci in each petri dish coming from one perithecium. Top left, all asci selfed green; top right, all asci selfed yellow; below, all asci of crossed origin.](image)

provided it were not strongly differential, misclassification of an ascus due to the failure of all the spores of one type to develop would be unimportant.

To ensure high viability of ascospores, the perithecia must be fully ripe. Asci from such perithecia are liable to burst when the perithecium is teased open with needles in order to liberate the asci. Hence, rarely can more than 10 unbroken ascis be extracted from each perithecium.

From a cross (mixed inoculum technique) of wild type and yellow (Y//y) 52 perithecia yielded 7 or, more unbroken asci, and a test of viability of the ascospores gave viable counts of more than 80%. Each ascus, when crushed as mentioned above, gave a colony either uniformly green or uniformly yellow or sectored yellow-green, revealing the selfed (Y//Y or y//y) or crossed (Y//y) origin of the ascus. The seven or more asci from each perithecium were crushed on separate dishes (Fig. 19), and each perithecium was then classified according to the types of colonies which its ascus yielded. The results were as follows:

18 perithecia gave asci all alike, producing only yellow colonies
14 perithecia gave asci all alike, producing only green colonies
13 perithecia gave asci all alike, producing only sectored colonies
7 perithecia gave asci of more than one kind

Thus, each of 18 perithecia yielded 7 or more asci all of Y/y origin, each of 14 perithecia yielded asci all of Y/Y origin, each of the 13 perithecia yielded asci all of Y/y origin, and each of the remaining 7 perithecia yielded asci of more than one origin. In detail:

4 yielded asci some of y/y and some of Y/Y origin
2 yielded asci some of Y/Y and some of Y/y origin
1 yielded asci some of Y/Y and some of y/y origin
0 yielded asci of all three origins

A random sample of 1793 ascospores from this cross gave 55% Y and 45% y. Taking these proportions to represent the proportions of the two types of nuclei in the mycelium, we may calculate the distributions of asci of the three possible origins to be expected on each of the three following hypotheses.

First, we may make the hypothesis that the nuclei are distributed at random in the mycelium and that karyogamy takes place between any two nuclei at random. In this case, the selfed yellow and the crossed and selfed green asci should be in proportions p^2:2pq:q^2, where p = 1 — q = 0.55 represents the proportion of y nuclei in the mycelium. The actual distribution of the asci from the above 52 perithecia was: 189 selfed yellow, 136 crossed, 133 selfed green. There is thus a shortage of crossed asci, and the first hypothesis is untenable.

Second, we may make the hypothesis that the nuclei of the two kinds are not distributed at random in the mycelium (say, somewhat clustered according to kind), but within one perithecium karyogamy is at random. In this case, any one perithecium which yielded crossed asci at all should yield the three kinds of ascus in binomial distribution, with p and q having different values for each of these perithecia. This is not the case: out of 19 perithecia which yielded crossed asci, 13 yielded only crossed asci, 4 yielded crossed asci and selfed y/y but no selfed Y/Y, and 2 yielded crossed asci and selfed Y/Y but no selfed y/y. The second hypothesis must also be rejected.

We are therefore left with the hypothesis discussed in section II-2 that very few, usually two, nuclei give origin to all nuclei of the ascus primordia in one perithecium, presumably by some system of conjugate divisions.

A further analysis of the yield of individual perithecia from the cross already mentioned was carried out by sampling 200 or more ascospores from each perithecium. Individual perithecia were stripped of
mycelium, Hülle cells, and conidia by rolling them repeatedly with a needle over the surface of agar medium under the dissecting microscope. Each perithecium cleaned in this way was then crushed in 0.1 to 0.2 ml. of saline, and the resulting suspension, diluted as necessary, was plated on three to four dishes to give 200 to 300 colonies. The hard integument of the perithecium of *A. nidulans* makes it withstand the rolling well. The results were as follows:

<table>
<thead>
<tr>
<th>Types of colonies produced by each perithecium</th>
<th>Only yellow</th>
<th>Only green</th>
<th>Yellow and green</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>In ratio *</td>
<td></td>
<td>In ratios *</td>
</tr>
<tr>
<td></td>
<td>of 1:1</td>
<td></td>
<td>different from 1:1</td>
</tr>
<tr>
<td>No. of perithecia</td>
<td>43</td>
<td>30</td>
<td>18</td>
</tr>
<tr>
<td></td>
<td>14</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* Significant level taken as 0.05.

These results agree with the previous ones, based on isolation of whole asci, in showing that: (1) there is a tendency for individual perithecia to produce asci all of one kind, i.e., either all selfed yellow, or all selfed green, or all crossed, and (2) less than 15% of the perithecia in this cross contain asci of more than one kind. A part of this 15% is accounted for by the occasional development of two perithecia, one inside the other or one fused to the other ('‘twins’'), as can be verified microscopically.

The finding that a high proportion of perithecia which contain crossed asci contains only, or almost only, asci of this kind, opened the way to a new method of genetic analysis. If perithecia of this kind (for short: ‘‘crossed’’ perithecia) could be identified, random samples of ascospores from them could be used without selection for Mendelian analysis. A sample of ascospores from one or more ‘‘crossed’’ perithecia is, in fact, equivalent to a sample of gametes from a cross in higher organisms.

Clearly, one cannot identify a ‘‘crossed’’ perithecium without sampling the ascospores to find whether they give 1:1 allele ratios for certain markers. In a cross like the one exemplified above, these perithecia constituted about one-sixth of the total. It was necessary, therefore, to find some way of avoiding extensive platings from the five-sixths of the wrong kind. This was done by making small assay platings of aliquots of the spore suspension from each of a number of perithecia and preserving the major part of each suspension in the refrigerator. After 2 days, one or more of the suspensions, which in the assay plating
had given the correct allele ratios, were further plated on non-selective medium on a scale sufficient for the complete analysis of all segregating genes. Later, the discovery that in certain crosses the proportion of "crossed" perithecia could approach 100% made assay platings unnecessary in these cases.

This technique—which we call "perithecium analysis"—was further refined by a quick method of estimating the upper proportion of "crossed" perithecia in any cross involving visible markers. A number of perithecia were cleaned as mentioned above, crushed on the surface of a non-selective agar medium, and the ascospores streaked out. Up to 10 perithecia could be streaked on a petri dish. After growth, if the cross involved one pair of color alleles (e.g., Y//y), each perithecium would produce one of three kinds of streak: yellow, green, or bicolor. If two pairs of color alleles were segregating (e.g., wY//Wy), the streaks would be yellow, white, or tricolor (yellow, white, green) (Fig. 20). The proportion of bicolor (or tricolor) streaks indicates the highest possible proportion of perithecia suitable for perithecium analysis.

An example of perithecium analysis is given in Table 16. The cross involved three linked loci, and the samples of ascospores were taken from two perithecia, each giving for the color markers (Y/y) allele ratios not significantly different from 1:1. Full classification for the other two segregating genes gave good allele ratios also for these. The results from the two perithecia were statistically homogeneous and when pooled could be treated as those from a three-point backcross in higher organisms.

The limitations and the usefulness of perithecium analysis as compared with the method of recombinant selection (section V-3) are apparent from the example given. As for limitations, first, perithecium
analysis is inefficient for accurate estimates of linkage when linkage is close. In fact, 721 colonies had to be isolated and fully tested in order to obtain 54 recombinants between \( y \) and \( bi; \) with recombinant selection (see, e.g., Table 14, cross 4), classification by inspection would have given the same results. When it comes to obtaining recombinants and estimating recombination fractions as low as 1/1000, let alone the extremely low ones characteristic of pseudo-alleles (section VI-3), perithecium analysis is out of the question.

### TABLE 16

**Perithecium Analysis in the Three-Point Cross:** \( paba, y BI,//PABA, Y bi, \)

<table>
<thead>
<tr>
<th>Perithecium I</th>
<th>( paba, Bi, )</th>
<th>( PABA, bi, )</th>
<th>( paba, bi, )</th>
<th>( PABA, BI, )</th>
<th>Totals</th>
</tr>
</thead>
<tbody>
<tr>
<td>( y )</td>
<td>144</td>
<td>2</td>
<td>11</td>
<td>39</td>
<td>196</td>
</tr>
<tr>
<td>( Y )</td>
<td>0</td>
<td>156</td>
<td>42</td>
<td>10</td>
<td>208</td>
</tr>
<tr>
<td>Totals</td>
<td>144</td>
<td>158</td>
<td>53</td>
<td>49</td>
<td>404</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Perithecium II</th>
<th>( paba, Bi, )</th>
<th>( PABA, bi, )</th>
<th>( paba, bi, )</th>
<th>( PABA, BI, )</th>
<th>Totals</th>
</tr>
</thead>
<tbody>
<tr>
<td>( y )</td>
<td>117</td>
<td>3</td>
<td>5</td>
<td>24</td>
<td>149</td>
</tr>
<tr>
<td>( Y )</td>
<td>1</td>
<td>125</td>
<td>20</td>
<td>22</td>
<td>168</td>
</tr>
<tr>
<td>Totals</td>
<td>118</td>
<td>128</td>
<td>25</td>
<td>46</td>
<td>317</td>
</tr>
</tbody>
</table>

**Allele ratios:**

\( Y/y \) 376/345 \( (P = 0.25) \); \( PABA,/paba, \) 381/340 \( (P = 0.15) \); 
\( BI,/bi, \) 357/364 \( (P = 0.75) \)

**Crossover values:**

\( paba, y \) 0.18 ± 0.02
\( y - bi \) 0.075 ± 0.009

**Combinations:**

- **Parental**
  - \( paba, y BI \)
  - \( PABA, Y bi \)
  - Total: 542

- **Crossovers in I**
  - \( paba, Y bi \)
  - \( PABA, y BI \)
  - Total: 125

- **Crossovers in II**
  - \( paba, y bi \)
  - \( PABA, Y BI \)
  - Total: 48

- **Doubles**
  - \( paba, Y BI \)
  - \( PABA, y bi \)
  - Total: 5

Secondly, the validity of perithecium analysis rests on the choice of perithecia which yield only, or practically only, crossed asci. The
preliminary identification of such perithecia is based on their giving 1:1 allele ratios at the loci used as markers. These ratios may not be obtained when there is close linkage between an allele lowering viability and one of the marker loci. To avoid this difficulty, it is helpful to use more than one locus as marker; the two color loci $W/w$ and $Y/y$ are obviously convenient.

As for the advantages, perithecium analysis utilizes all products of meiosis, and therefore all the usual treatments developed in classical genetics for genetic ratios can be applied to it unmodified. There is no need to wait for the development of special statistical treatments, as mentioned in section V-3. Furthermore, the fact thatplatings are made on non-selective media avoids the common complication that the viability of certain types of segregants is different on different media.

One of us (A.W.J.B.), while carrying out perithecium analysis with a certain cross, found that all the perithecia tested (30) contained crossed asci. This was quite unexpected, and a quick search was started, by means of the streaked perithecium technique, among all other available crosses. For this search, at least 8 perithecia from each cross were streaked. In some of the crosses the plates available had been prepared as mixed inocula, in other crosses as balanced heterokaryons, and in others as both. It worked out that the method of crossing was not crucial. The results showed that a proportion of "crossed" perithecia in excess of 50%, or even up to 100%, was by no means uncommon; in the 27 crosses examined to date, 16 were of this kind. The 27 crosses included 21 different strains and 17 identified loci.

Although the analysis of the mechanism of relative heterothallism is still far from complete, the following tentative conclusions can be drawn so far:

1. Relative heterothallism has arisen in the laboratory, because it occurs between certain pairs of strains derived exclusively by successive mutations from one and the same original fully self-fertile strain (wild type).

2. It cannot be clearly associated with any one identified locus, because from a cross giving 100% crossed asci one can recover pairs of recombinants differing from each other at precisely the same loci and yet yielding less than 50% crossed asci.

3. It is found in crosses in which either, both, or neither parent is fully self-fertile; unlike absolute heterothallism, it is not associated with self-sterility.

4. It is not obviously connected with the ease with which two strains form balanced heterokaryons.

Apart from being technically convenient for genetic analysis, rela-
tive heterothallism is certainly a phenomenon deserving full investigation. Its occurrence in natural populations of homothallic species is likely, to say the least, and it may have to be considered together with heterokaryosis as one of the factors in the genetic systems of such species.

VI. Formal Genetics

1. Identification of Loci

Of the 600 mutant strains available, only very few have been used so far in genetic tests. The 27 used are listed in Table 17; they are all due to independent mutation and there are several groups of "mimics," i.e., phenotypically indistinguishable in the relevant respect. The mimics include: 3 independent cases of mutation to colorless conidia; 4 to adenine/hypoxanthine requirement (out of more than 100 found); 4 to biotin/desthiobiotin requirement; 2 to P.A.B.A. requirement (out of 6 found); 4 to arginine/ornithine requirement (out of 5 found). There are other groups of "mimics" as yet untapped, among which are the overwhelming one of about 300 independent mutations to thiosulphate/sulphite requirement and about 20 to thiosulphate requirement. The only groups at present under systematic investigation are those of the adenine, P.A.B.A., and pyridoxine requirements.

The genetic tests carried out included the three methods described in section V, i.e., recombinant selection, perithecium analysis, and balanced heterokaryosis. The last is dependable only in very clear-cut cases, since in the others failure to obtain a balanced heterokaryon is not significant and success may be simulated by adaptation of one or both strains.

The detailed results of the crosses made cannot be given here. Those for the study of the bi and the paba pseudo-alleles are given in section VI-3. Those which have led to the detection and estimation of linkage between BI1/bi1 and Y/y were given in section V-3. In section VI-2 more data will be given on the 11 linked loci (or 7, if each series of pseudo-alleles is considered as one locus) belonging to the bi linkage group, as we call it. The only other probable example of linkage so far includes the two loci pr1 and co; but the work on these, in collaboration with Mr. A. W. J. Bufton, is still incomplete.

Of the loci not belonging to either of these linkage groups, LYS/lys and THI/thi have been used most extensively. The lys allele has a viability of about 60%. The other mutants have been tested in various combinations, and for each there is at least prima facie evidence that
### Table 17

**Mutant Alleles Used in Crosses up to April, 1952**

<table>
<thead>
<tr>
<th>Symbol</th>
<th>Designation</th>
<th>Phenotype</th>
<th>Strain and mode of origin</th>
<th>Linkage and allelism *</th>
</tr>
</thead>
<tbody>
<tr>
<td>$w,_{n}$</td>
<td>White alba</td>
<td>Colorless conidia</td>
<td>$+$: spontaneous, 1936 †</td>
<td>All alleles of one another and of $W$, and epistatic to $Y/y$. &quot;'bi group'&quot; (†)</td>
</tr>
<tr>
<td>$w_{n}$</td>
<td>White new</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>$y$</td>
<td>Yellow</td>
<td>Yellow conidia</td>
<td>$y$: X-rays, 1946</td>
<td>&quot;'bi group'&quot;</td>
</tr>
<tr>
<td>$co$</td>
<td>Compact</td>
<td>Compact colony</td>
<td>$paba, bi$: spontaneous, 1951</td>
<td></td>
</tr>
<tr>
<td>$pr_{t}$</td>
<td>Poorly proteolytic-1</td>
<td>Reduced proteolytic activity</td>
<td>$bi_{t}$: U.V., 1951</td>
<td>See $co$</td>
</tr>
<tr>
<td>$ad_{t}$</td>
<td>Adenine-1</td>
<td>Adenine/hypoxanthine</td>
<td>$y$: X-rays, 1946</td>
<td>&quot;'bi group'&quot;; all not allelic</td>
</tr>
<tr>
<td>$ad_{t}$</td>
<td>Adenine-2</td>
<td></td>
<td>$y$: X-rays, 1946</td>
<td></td>
</tr>
<tr>
<td>$ad_{t}$</td>
<td>Adenine-3</td>
<td></td>
<td>$y$: X-rays, 1946</td>
<td></td>
</tr>
<tr>
<td>$ad_{t}$</td>
<td>Adenine-4</td>
<td></td>
<td>$y$: X-rays, 1946</td>
<td></td>
</tr>
<tr>
<td>$bi_{t}$</td>
<td>Biotin-1</td>
<td></td>
<td>$+$: X-rays, 1947</td>
<td>&quot;'bi group'&quot;; $bi_{t}$, $bi_{s}$, and $bi_{t}$, pseudo-alleles; $bi_{t}$, incompletely tested</td>
</tr>
<tr>
<td>$bi_{s}$</td>
<td>Biotin-2</td>
<td>Biotin/desthiobiotin</td>
<td></td>
<td></td>
</tr>
<tr>
<td>$bi_{s}$</td>
<td>Biotin-3</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>$bi_{t}$</td>
<td>Biotin-4</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Allele</td>
<td>Gene Symbol</td>
<td>Phenotypic Effect</td>
<td>Mutational Effect</td>
<td>Notes</td>
</tr>
<tr>
<td>--------</td>
<td>-------------</td>
<td>-------------------</td>
<td>-------------------</td>
<td>-------</td>
</tr>
<tr>
<td>paba1</td>
<td>P.A.B.A.1</td>
<td>p-Aminobenzoic acid</td>
<td>bi: X-rays, starvation, 1951</td>
<td>&quot;bi group,&quot; pseudo-alleles</td>
</tr>
<tr>
<td>paba2</td>
<td>P.A.B.A.6</td>
<td>p-Aminobenzoic acid</td>
<td>bi: U.V., starvation, 1951</td>
<td></td>
</tr>
<tr>
<td>pyro1</td>
<td>Pyridoxine-4</td>
<td>Pyridoxine</td>
<td>bi: U.V., starvation, 1951</td>
<td>Not in &quot;bi group&quot;</td>
</tr>
<tr>
<td>s2</td>
<td>S-delta</td>
<td>Sulphite/thiosulphate</td>
<td>+: N-mustard, 1948 †</td>
<td>Not in &quot;bi group&quot;</td>
</tr>
<tr>
<td>thi</td>
<td>Thiazole</td>
<td>Aneurin/thiazole</td>
<td>y: X-rays, 1946</td>
<td>No linkage with lys or with &quot;bi group&quot;</td>
</tr>
<tr>
<td>nic1</td>
<td>Nicotinic-2</td>
<td>Anthranilic/nicotinic acid</td>
<td>+: X-rays, 1947</td>
<td>Not allelic; no linkage with &quot;bi group&quot; or thi</td>
</tr>
<tr>
<td>nic2</td>
<td>Nicotinic-3</td>
<td>Anthranilic/tryptophan/nicotinic acid</td>
<td>y thi: X-rays, 1948</td>
<td></td>
</tr>
<tr>
<td>orn1</td>
<td>Ornithine-1</td>
<td>Arginine/ornithine</td>
<td>y thi: X-rays, 1949</td>
<td>At least two different loci; no linkage with &quot;bi group&quot;</td>
</tr>
<tr>
<td>orn2</td>
<td>Ornithine-2</td>
<td>Arginine/ornithine</td>
<td>bi: X-rays, 1949</td>
<td></td>
</tr>
<tr>
<td>orn3</td>
<td>Ornithine-3</td>
<td>Arginine/ornithine</td>
<td>y thi: X-rays, 1948</td>
<td></td>
</tr>
<tr>
<td>orn4</td>
<td>Ornithine-4</td>
<td>Arginine/ornithine</td>
<td>bi: U.V. starvation, 1950</td>
<td></td>
</tr>
<tr>
<td>lys</td>
<td>Lysine</td>
<td>Lysine</td>
<td>w: X-rays, 1947</td>
<td>No linkage with &quot;bi group,&quot; thi, orn, or panto</td>
</tr>
<tr>
<td>panto</td>
<td>Pantothenate</td>
<td>Pantothenate</td>
<td>y thi: X-rays, 1948</td>
<td>No linkage with &quot;bi group,&quot; lys, thi, or orn</td>
</tr>
</tbody>
</table>

* The linkage group from w to ad1 will be referred to as the "bi group."
† From Mr. E. Yuill.
‡ From Dr. Hockenhull.
Table 18
Summary of Tests for Detecting Linkage in the bi Linkage Group

<table>
<thead>
<tr>
<th>Region</th>
<th>Cross *</th>
<th>Selection</th>
<th>Segregations</th>
<th>Tentative recombination fractions †</th>
</tr>
</thead>
<tbody>
<tr>
<td>y–bi,</td>
<td></td>
<td></td>
<td>y</td>
<td>Y</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>50</td>
<td>42</td>
</tr>
<tr>
<td>y–ad,</td>
<td>adą y BI,</td>
<td>AD, BI,</td>
<td>y</td>
<td>Y</td>
</tr>
<tr>
<td></td>
<td>AD, Y bi,</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>84</td>
<td>56</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>130</td>
<td>11</td>
</tr>
<tr>
<td>paba–y</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>PABA, y bi,</td>
<td>PABA, BI,</td>
<td>y</td>
<td>BIi</td>
</tr>
<tr>
<td></td>
<td>paba, Y BI,</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>W ad, PABA, y BI,</td>
<td>W AD, PABA,</td>
<td>y</td>
<td>bi,</td>
</tr>
<tr>
<td></td>
<td>w, AD, paba, Y bi,</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>82</td>
<td>20</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>23</td>
<td></td>
</tr>
<tr>
<td>paba–bi,</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>W ad, PABA, y BI,</td>
<td>w, PABA,</td>
<td>y</td>
<td>PABA,</td>
</tr>
<tr>
<td></td>
<td>w, AD, paba, Y bi,</td>
<td></td>
<td></td>
<td>paba,</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>12</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>y–ad,</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>adą y BI, AD, thi</td>
<td>AD, AD, THI</td>
<td>y</td>
<td>Y</td>
</tr>
<tr>
<td></td>
<td>AD, Y bi, ad, THI</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

See Tables 13 and 14 pooled results of crosses in coupling and repulsion.
| $bi_{-}ad_{1}$ | $ad_{1} y BI_{1} AD_{1}$ | $AD_{1} AD_{1}$ | $\frac{y}{BI_{1}}$ | $\frac{BI_{1}}{bi_{1}}$ | $2 \times 0.05 = 0.007$ |
| $w-ad_{1}$ | $\frac{AD_{1} PABA_{1}}{w_{n} AD_{1} paba_{1} Y bi_{1}}$ | $w_{Y}$ | $W_{Y}$ | $wY$ and $wY$ | 102 / 241 = 0.42 |
| $w-ad_{1}$ | $\frac{AD_{1} BI_{1}}{w_{n} AD_{1} paba_{1} Y bi_{1}}$ | 113 | 14 | 148 | 275 |
| $ad_{1-y}$ | $\frac{AD_{1} PABA_{1} BI_{1}}{w_{n} AD_{1} paba_{1} Y bi_{1}}$ | 102 | 1 | 120 | 223 |
| $ad_{1-y}$ | $\frac{AD_{1} AD_{1} THI}{w_{n} AD_{1} paba_{1} Y bi_{1}}$ | 14 | 35 | 49 | 14 / 55 = 0.26 |
| $ad_{1-y}$ | $\frac{AD_{1} AD_{2} THI}{w_{n} AD_{1} paba_{1} Y bi_{1}}$ | 20 | 55 | 75 | 34 / 124 = 0.28 |
| $ad_{1-y}$ | Peritheciun analysis | 1 | 703 | 704 | $2 \times \frac{1}{704} = 0.003$ |

* Data of crosses 2, 3, 4, and 5 from Dr. J. A. Roper. Part of the data of cross 9 from Mr. R. H. Pritchard.
† Some of the recombination fractions have been calculated from the comparison of recombination in a given region with that of the $y-bi$ region taken as 0.05.
‡ Cross 3 (see Table 23) was also segregating for $BI_{1}, bi_{1}/bi_{1}, BI_{1}$, which are irrelevant here.
§ The recombination fraction was also estimated to be about 0.003 from the ratio of $AD_{1}, AD_{1}$ among the viable ascospores, this cross yielding almost exclusively crossed asci.
the mutant phenotype is due to a single locus not belonging to the bi linkage group just mentioned and not linked with other tested loci.

Clearly, to advance quickly in the mapping of the chromosomes, we need to rationalize the procedure for testing against one another the enormous number of mutants available.

2. The bi Linkage Group

The first linkage detected was that between the $Y/y$ and the $BI_1/bi_1$ loci; the data are summarized in Tables 13 and 14. The pooled recombination fraction from coupling and repulsion is $0.0515 \pm 0.004$. The second linkage found was between $AD_2/ad_2$ and the two just mentioned. It was detected as follows (Table 18). Plating on minimal medium a random sample of ascospores from the cross $ad_2 y BI_1/AD_1 Y bi_1$, 50 yellow and 42 green colonies were obtained. Without linkage of $ad_2$, such a plating should have yielded about 87 yellow and 5 green. The conclusion was that $Y/y$ was located about midway between $AD_2/ad_2$ and $BI_1/bi_1$. Further crosses confirmed this conclusion. The available information on crosses involving $ad_2$ and $y$ in coupling and repulsion gives a recombination fraction of between 0.06 and 0.08.

The third linkage—between $paba_1$ and $y$—was detected by Mr. A. W. J. Bufton. The recombination fractions (data by Dr. J. A. Roper) are 0.148 and 0.198, respectively, in two crosses in repulsion. The $paba_1/bi_1$ recombination fraction (repulsion) in one of these crosses is 0.158 (Table 18).

The fourth linkage detected was between $ad_4$ and $bi_1$. Crosses 6 and 7 in Table 18 indicate the kind of evidence. In cross 6, if $ad_4$ segregated independently, selection of $AD_4 AD_4$ recombinants should yield a small excess of $Y$ because of the loose linkage between $ad_3$ and $y$ (see cross 9). Instead of this, the $Y$ constitute only 0.10 of the total. This can only be due to close linkage. The $AD_4/ad_4$ locus could be either beyond $bi_1$ or between $y$ and $bi_1$. The next cross shows that it is beyond $bi_1$. Thus the calculation of the recombination fraction requires Bailey’s (1951) treatment, since the segment over which it has to be calculated lies between the two loci used for selection. Unfortunately, our data are not suitable for this treatment. But cross 7 permits a crude indirect estimate of recombination between $bi_1$ and $ad_4$. The recombination fraction between $bi_1$ and $y$ has been estimated independently with good precision as 0.05. In cross 7 there are 15 crossovers between $bi_1$ and $y$ and 2 between $bi_1$ and $ad_4$; thus $2/x = 15/0.05$ gives 0.007 as the desired estimate. This admittedly questionable method has been used repeatedly in Table 18.

Finally we come to three loci—$W/w$, $AD_1/ad_1$, and $AD_2/ad_2$—
which are better considered together. In the first place, \( ad_1 \) and \( ad_3 \) are very closely linked. This has been shown in two ways; firstly, with perithecial analysis, only one \( AD_1 \ AD_3 \) crossover was obtained among 703 colonies from a cross in repulsion; the recombination fraction is thus \( 2 \times 1/703 = 0.003 \). Secondly, with recombinant selection, the proportion of \( AD_1 \ AD_3 \) recombinants among viable ascospores was found to be again about 0.003. Since cross 9 gave almost exclusively crossed asci, this proportion can be taken to be as practically identical to the recombination fraction. Recent work by Mr. R. H. Pritchard with heterozygous diploids shows that \( ad_1 \) and \( ad_3 \) are not pseudo-alleles.

That \( ad_1 \) and \( ad_3 \) as well as \( w \) belonged probably to the \( bi \) linkage group was found by making use of interference in multiple crossovers as mentioned on p. 193 and shown in Table 15. We can select crossovers in the \( bi \) group and see how other markers in the same group segregate. Crosses 5, 8, and 9 show the results: when we select a crossover between \( ad_1 \) and \( ad_3 \), both \( w \) (cross 8) and \( y \) (cross 9) show linkage with the \( ad_1 \)–\( ad_3 \) region; the recombination fractions so obtained (0.26 and 0.28) are, as expected in the case of linkage, smaller than when calculated on single crossovers. Many other crosses in which only single crossovers between the \( ad_1 \)–\( ad_3 \) region and either \( w \) or \( y \) were measured showed free recombination. Slightly more stringent conditions—such as making one crossover compulsory over a long region (cross 5)—yield recombination fractions (about 0.46) smaller than 0.50 but considerably greater than those obtained when the compulsory crossover is in the \( ad_1 \)–\( ad_3 \) region.

We can now construct a tentative linkage map of the \( bi \) chromosome. The recombination fractions, crude and questionably derived as they are, leave very little doubt as to the sequence of loci and give a rough idea of the distances:

\[
\begin{array}{ccccccc}
\text{w} & 50 & ad_1 & 0.3 & \text{paba}_{1,18} & ad_2 & 6-8 & y & 5 & bi_{1,11} & 0.7 & ad_4 \\
26-46 & \text{(doubles)} & & & 15-20 & 16 & & & & & & 28 & \text{(doubles)}
\end{array}
\]

In the course of the work on the \( bi \) linkage group we have come across cases of disturbed segregations which can be attributed only to chromosomal rearrangements. One case in Table 12 determines spurious close linkage between \( ad_1 \) and \( y \) in a particular strain. Another determines absence of single crossovers in the region \( paba_{1,1} \)–\( ad_2 \) when a strain \( ad_1 \ y \) is used.
3. Pseudo-Allelism

by J. A. Roper

A number of cases have been reported of closely linked loci mutant alleles at which determine similar phenotypes. For example "lozenge" (Green and Green, 1949), "star-asteroid," "bithorax" (Lewis, 1945, 1950), "singed" (Ives and Noyes, 1951) and "white" (MacKendrick and Pontecorvo, 1952) in Drosophila; "brachyury" (Dunn and Caspari, 1943) in the mouse; perhaps Rh in man (Fisher, 1946); inositol (Giles, 1952) and nicotinic acid requirement in Neurospora (Bonner, 1950); biotin requirement in Aspergillus (Roper, 1950a); adenine requirement in Aspergillus (Pontecorvo, 1952c).

In some of these cases, whether or not the close linkage is a matter of chance is unknown. In other instances it has been shown that the investigated alleles constitute a pseudo-allelic series. Pseudo-alleles are, for all general purposes, alleles of one gene at one locus; closer investigation, however, reveals crossing-over between different pseudo-alleles. Further, such alleles show an effect which has been described as a position effect in that the genotypes $m_1m_2/++$ and $m_1+/+m_2$ give different phenotypes. The former is wild type or nearer to wild type than the latter, which is mutant, or more extreme.

There have been several approaches to the study of pseudo-alleles. Probably the most completely investigated pseudo-allelic series is the case of "lozenge" (Green and Green, 1949). The study of "lozenge" followed the observation (Oliver, 1940; Oliver and Green, 1944) of wild-type progeny from females of genotype $lz^+/lz^+$ Lewis (see 1950) has found cases of pseudo-allelism associated with repeats and deduces a connection between repeats and pseudo-alleles. In the present instances of pseudo-allelism found in A. nidulans, the working hypothesis which prompted the investigation has already been published (Pontecorvo, 1950) and can briefly be stated as follows. Close linkage might be expected between some of the genes acting on any one series of biochemical reactions where the intermediates are non-diffusible, labile, or present in very low concentration. It seems more likely, however, that the cases of close linkage which have so far resulted from the research prompted by this working hypothesis are concerned with intra-genic rather than inter-genic organization (Pontecorvo, 1952b; 1952c).

For the initial investigation three biotin-requiring strains of A. nidulans, independently obtained by X-ray treatment, were used. In their responses to known and possible intermediates in biotin synthesis the three strains were identical (Table 19).

Despite the failure to distinguish the strains biochemically, crosses
TABLE 19
Responses of Three Biotin-Requiring Strains of *A. nidulans* to Intermediates
in Biotin Synthesis

<table>
<thead>
<tr>
<th></th>
<th>(bi_1)</th>
<th>(bi_2)</th>
<th>(bi_3)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pimelic acid</td>
<td></td>
<td></td>
<td>+</td>
</tr>
<tr>
<td>7,8-Diamino pelargonic acid *</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Desthiobiotin</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Biotin</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

* Kindly supplied by Professor B.C.J.G. Knight.

of the strains, two by two, gave in every case, though very infrequently, biotin-independent types. If these biotin-independent types arose as a result of recombination, and not, say, mutation, the requirements would be conditioned by alleles at three different closely linked loci. The steps taken to establish that this was the case were as follows:

Pontecorvo, Forbes, and Adam (1949) had already shown that a locus for biotin requirement in one strain (\(bi_1\)) was linked with and approximately 5 c. Morgan distant from the \(y\) locus (See section V-2 and V-3 for meaning of symbols). The mutant alleles determining the biotin requirements in the other two strains were provisionally designated \(bi_2\) and \(bi_3\). The locus of each of these was tested for linkage with the \(y\) locus. Only one cross, involving the \(bi_2\) locus, is exemplified (Table 20).

This cross showed that mutation in a single gene determines the biotin requirement and that the locus of this gene is linked some 4 to 5 c.

TABLE 20
Cross to Locate the \(bi_2\) Locus with Respect to the \(y\) Locus:

\[
\begin{array}{ccc}
\text{ad, Y BI, THI} & \text{ad, y bi,} & \text{THI} \\
\text{AD, y bi} & \text{THI} & \text{THI} \\
\end{array}
\]

<table>
<thead>
<tr>
<th>Spores</th>
<th>Recombinants</th>
<th>Colonies</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>plotted on selected by medium</td>
<td>Yellow</td>
</tr>
<tr>
<td>Minimal medium with biotin</td>
<td>(THI\ AD_1)</td>
<td>182</td>
</tr>
<tr>
<td>Minimal medium with aneurin</td>
<td>(BI_2\ AD_1)</td>
<td>10</td>
</tr>
</tbody>
</table>

(77 tested: 74\(bi_2\), 3\(BI_1\))

(60 tested: 57\(BI_1\), 3\(bi_2\))

(32 tested: 18\(THI\), 14\(thi\))
Morgan from the \( y \) locus. A similar cross was made to test the \( bi_5 \) locus. Again, it was shown that mutation in a single gene determines the biotin requirement and that the locus \( bi_5 \) was some 4 to 5 c. Morgan from the \( y \) locus.

A series of crosses, details of three of which are given in Table 21,

**TABLE 21**

CROSSES TO DETERMINE THE ORDER OF THE LOCI \( bi_5, bi_5, \) AND \( bi_5 \) WITH RESPECT TO ONE ANOTHER AND THE \( y \) LOCUS:

<table>
<thead>
<tr>
<th>Cross 1:</th>
<th>( bi_5 BI_7 )</th>
<th>( BI_7 bi_5 )</th>
<th>( BI_7 BI_7 )</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Recombinants selected from platings on minimal medium</strong></td>
<td>Green</td>
<td>Yellow</td>
<td></td>
</tr>
<tr>
<td>Cross 1:</td>
<td>( BI_7 BI_7 )</td>
<td>0</td>
<td>9</td>
</tr>
<tr>
<td>Cross 2:</td>
<td>( BI_7 BI_7 )</td>
<td>27</td>
<td>0</td>
</tr>
<tr>
<td>Cross 3:</td>
<td>( BI_7 BI_7 )</td>
<td>48</td>
<td>3</td>
</tr>
</tbody>
</table>

showed two facts: (1) that all three biotin loci were on the same side of the \( y \) locus, and (2) that if the biotin-independent types were the result of recombination, and not of some other process, then the order of the loci with respect to one another and the \( y \) locus was \( y bi_5 bi_5 bi_5 \).

The results so far did not unambiguously prove recombination as an explanation of the biotin-independent types. Further, if recombination did in fact occur, it was also necessary to obtain an estimate of the recombination frequencies between the biotin loci.

To test for recombination as an explanation of these results, a series of crosses was made of which one example is given in Table 22. The crosses were designed in such a way that: (a) if the biotin independent types arose as a result of recombination, the recombinants would show the expected segregations for linked and freely recombining markers; (b) more than half of the recombinants would differ from either parent by at least two alleles; and (c) freely recombining nutritional markers used in addition to the biotin markers would allow, as shown below, an easy estimation of the recombination frequencies between the closely linked loci. Both \( a \) and \( b \) would give unambiguous proof of crossing-over as opposed to mutation.

The results of these crosses showed, for the reasons given above, that recombination between the biotin loci did take place. A crude calculation of the recombination frequencies was made as follows: colonies obtained on minimal medium with biotin (Table 22) were from ascospores
TABLE 22
Cross to Detect and Estimate the Frequency of Recombination Between Two bi Loci:

\[ W AD, y bi, BI, thi/w ad, Y BI, bi, THI \]

<table>
<thead>
<tr>
<th>Total number of ascospores plated on</th>
<th>Recombinants selected</th>
<th>Colonies</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>White</td>
</tr>
<tr>
<td>Minimal medium with biotin</td>
<td>(62.7 \times 10^8)</td>
<td>(AD, THI)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>133 tested,</td>
</tr>
<tr>
<td></td>
<td></td>
<td>all bi</td>
</tr>
<tr>
<td>Minimal medium with aneurin and adenine</td>
<td>(3.48 \times 10^6)</td>
<td>(BI, BI)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>21 (ad, THI)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>16 (ad, thi)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2 (AD, thi)</td>
</tr>
</tbody>
</table>

The results show that strain \(w ad, Y BI, bi, THI\) has a chromosome rearrangement such that the locus \(ad\), instead of segregating independently of the \(y\) locus, is linked about 2 c. Morgan from it.

necessarily recombinant for the freely recombing alleles \(AD\) and \(THI\). On this medium, \(62.7 \times 10^8\) ascospores gave 636 colonies. On minimal medium with aneurin and adenine the colonies were from ascospores recombinant for the alleles \(BI\) and \(BI\). If the biotin alleles recombined freely, then \(62.7 \times 10^8\) ascospores should have given 636 colonies, which was not the case. In fact, \(3.48 \times 10^6\) ascospores gave only 76 colonies. These figures allow estimation of the recombination frequency by the following formula:

\[
\text{Recombination fraction (\%)} = \frac{p}{q} \times \frac{50}{1}
\]

where \(p\) and \(q\) are, respectively, the number of recombinant colonies obtained for the closely linked loci and the number expected for free recombination. For the \(bi\) and \(bi\) loci, the estimated recombination frequency was approximately 0.1\%. A similar cross using the loci \(bi\) and \(bi\) gave a recombination frequency of 0.04\%. 

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The accuracy of this method of estimation may be open to criticism mainly for the reasons discussed in section IV-4, where it is suggested that high concentrations of auxotrophs may inhibit growth of rare prototrophs. However, in the first place the density of ascospores used in this work was not usually higher than $10^6$ per plate. In the second place, in one cross investigated for this specific purpose no inhibitory effect was found over a range of densities from $10^2$ to $10^6$ per plate (Table 23). Further, estimations were made of one recombination frequency with ascospores from heterokaryons having widely different percentages of hybrids and, therefore, different proportions of prototrophs. No serious variation in the estimated values was found. It seems likely that any error from this source is less than errors due to variations in viability on different media and experimental errors involved in serial dilutions and platings. For these reasons it is impossible, at present, to give standard errors for these very low recombination frequencies. It can only be said that estimations of any one recombination frequency by a series of different crosses gave values in which the extremes differed by less than 50% of the mean of all the estimations.

Since a chromosome rearrangement was detected in crosses of the type shown in Table 22, it was thought necessary to repeat part of the work with strains having no known chromosomal abnormalities. Two such crosses are shown in Table 23. In one of these the critical arrangement of two markers, one on each side of the $bI$ loci, was used. These crosses entirely confirmed the previous results.

Finally, one cross was made between two strains having the same biotin allele, $bi_I$. This cross (Table 24) gave no results in any way resembling recombination of the $bi_I$ alleles. This was so, although the high number of ascospores plated on the biotinless medium would have detected a "recombination" frequency much smaller than those measured when different biotin alleles were used. This again confirms that the three biotin alleles $bi_1$, $bi_2$, and $bi_3$ differ and that their loci do recombine.

Further tests were then made in an attempt to differentiate, biochemically, the phenotypes determined by each of the three mutant biotin alleles. All tests, including cross-feeding experiments, failed to show biochemical differences in the effects of the three mutant alleles. Finally, for some combinations of biotin alleles, diploid strains were prepared (see section VII-1). The phenotypes of these diploids and of some heterokaryons are listed in Table 29, section VII-1. A number of critical genotypes, particularly heterozygotes with two mutant biotin alleles in coupling (e.g., $bi_2$ $bi_I/BI_2$ $BI_I$) have not yet been tested because of the difficulty of obtaining strains with two mutant biotin alleles in coupling.
### TABLE 23

**Crosses To Estimate the Recombination Frequency between the bi, and bi, Loci Using Linked Markers on One Side or on Both Sides of These Loci**

<table>
<thead>
<tr>
<th>Crosses</th>
<th>Recombinants selected</th>
<th>Number of ascospores plated per dish</th>
<th>Total number of ascospores plated</th>
<th>Colonies</th>
<th>Yellow</th>
<th>Green</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Cross:</strong> $AD_{y} bi, BI, thi$</td>
<td>$ad_{y} Y BI, bi, THI$</td>
<td>$18 \times 10^{3}$</td>
<td>$180$</td>
<td>$11$</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Minimal medium with biotin</td>
<td>$AD_{y} THI$</td>
<td>$18 \times 10^{3}$</td>
<td>$130$</td>
<td>$11$</td>
<td>(all bi)</td>
<td></td>
</tr>
<tr>
<td>Minimal medium with aneurin and adenine</td>
<td>$BI, BI_{y}$</td>
<td>$1.8 \times 10^{6}$</td>
<td>$2$</td>
<td>$31$</td>
<td>(both $AD_{y}, THI$)</td>
<td></td>
</tr>
<tr>
<td>Cross: $y bi_{y} BI, AD_{y}, thi$</td>
<td>$Y BI_{y} bi_{y} ad_{y}, THI$</td>
<td>$4 \times 10^{6}$</td>
<td>$16 \times 10^{1}$</td>
<td>$41$</td>
<td>$6$</td>
<td></td>
</tr>
<tr>
<td>Minimal medium with biotin</td>
<td>$AD_{y} THI$</td>
<td>$3.5 \times 10^{3}$</td>
<td>$7 \times 10^{3}$</td>
<td>$2$</td>
<td>$0$</td>
<td></td>
</tr>
<tr>
<td>Minimal medium with aneurin and adenine</td>
<td>$BI_{y}, BI_{y}$</td>
<td>$6.4 \times 10^{6}$</td>
<td>$18.2 \times 10^{6}$</td>
<td>$0$</td>
<td>$11$</td>
<td></td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td></td>
<td></td>
<td>$4.88 \times 10^{5}$</td>
<td>$138$</td>
<td>$15$</td>
<td>(all bi)</td>
</tr>
<tr>
<td>Minimal medium with aneurin</td>
<td>$3.5 \times 10^{3}$</td>
<td>$7 \times 10^{3}$</td>
<td>$0$</td>
<td>$0$</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Minimal medium with aneurin and adenine</td>
<td>$6.4 \times 10^{6}$</td>
<td>$18.2 \times 10^{6}$</td>
<td>$0$</td>
<td>$11$</td>
<td>(6 $AD_{y}, thi$)</td>
<td></td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td></td>
<td></td>
<td>$3.06 \times 10^{7}$</td>
<td>$0$</td>
<td>$15$</td>
<td>(9 $AD_{y}, THI$)</td>
</tr>
</tbody>
</table>

By the calculation used on p. 211 the recombination frequency between the $bi_{y}$ and $bi_{y}$ loci on the basis of the above data is 1.2% in the first cross and 0.08% in the second.
TABLE 24
Cross Involving Identical Biotin Alleles in the Two Strains:

\[ AD_1 \ y \ bi \_ thi \]
\[ ad_2 \ Y \ bi \_ THI \]

<table>
<thead>
<tr>
<th>Ascospores plated on</th>
<th>Number of ascospores plated</th>
<th>Recombinants selected</th>
<th>Colonies</th>
</tr>
</thead>
<tbody>
<tr>
<td>Minimal medium</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>with biotin</td>
<td>( 9 \times 10^8 )</td>
<td>( AD_2 THI )</td>
<td>175</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>14</td>
</tr>
<tr>
<td>Minimal medium</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>with aneurin and adenine</td>
<td>( 54 \times 10^6 )</td>
<td>( BI )</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>0</td>
</tr>
</tbody>
</table>

By analogy with the lozenge loci, it is expected that a genotype such as that above would give a wild-type phenotype.

Preliminary investigations have also been made on two p-aminobenzoic acid-requiring strains of \( A. \ nidulans \). The results so far indicate that in each strain a single allele determines the difference from wild type and that the loci of the two mutant alleles recombine with a frequency of about 0.002%. It is not yet known whether the wild-type alleles at the two \( paba \) loci determine different biochemical reactions.

Any hypothesis to explain the results obtained for the \( bi \) loci must account for two facts: (1) the extremely close linkage of three loci, mutant alleles at which determine similar and probably identical phenotypes; such close linkage for three loci is hardly likely to be a matter of chance; and (2) the fact that the phenotype of the diploid, \( bi_2 BI_1/BI_2 bi_1 \), and the heterokaryon, \( bi_2 BI_1 + BI_2 bi_1 \), are mutant and not wild type as expected from the fact that each mutant \( bi \) allele tested separately is recessive.

Two explanations at present seem possible. The first, and less likely, is that the results are convalidating the working hypothesis which prompted the investigation. The wild-type alleles at the three \( bi \) loci would then control different biochemical reactions, the differences not being detected by the tests used. The wild-type alleles would function normally only when they were all three on one and the same chromosome. This would be the case if the intermediates in the reactions controlled by the wild-type alleles were labile, or non-diffusible, or present in very low concentration. Thus the unexpected phenotypes would be explained.
The essentials of the second and more plausible hypothesis have been considered by Muller (1947; see also Raffell and Muller, 1940), Goldschmidt (1944, 1946, 1950) and Pontecorvo (1952b, 1952c). That is, that the gene as a working unit in physiological action is based on a chromosome segment larger than either the unit of mutation or recombination. Mutation at different sites in the bi gene gives at least three, and possibly many, mutant alleles, any one of which inactivates the gene. In some cases recombination between alleles will be possible. The phenotype of the double heterozygote in repulsion is then as expected, since, in this diploid, both biotin genes are inactivated by mutations at different sites. Assembly of all the normal parts of the gene on a single chromosome is necessary for the normal functioning of the gene. Bonner (1950) has suggested that a similar situation may apply to the Q-locus in Neurospora. Stephens (1951) has made a critical analysis of the possible association between repeats and pseudo-alleles and finds no case in which the association is satisfactorily proved. As Goldschmidt (1950) has pointed out, an hypothesis such as the second outlined above requires no such association. However, a variety of cases have been labeled as pseudo-allelomorphism, and it may be that they do not constitute a homogeneous group.

4. Physiological Genetics

The non-systematic observations reported in the present section are by-products of the main genetic work. We have deliberately not investigated sequences of biochemical reactions except where this work was necessary for the approach to a genetic problem, as, for example, the study of pseudo-alleles.

These observations fall under two headings: types of nutritional mutants, and novel properties of some, i.e., properties not found or not described in other microorganisms.

The detailed classification of 578 auxotrophs was given in Table 6. This table did not include: (a) 5 mutants (3 requiring sulphite, 1 adenine, and 1 unknown factor) out of 203 isolates obtained after X-rays and starvation for less than 96 hours; (b) 29 mutants (1 requiring guanosine, 1 tryptophan, 1 adenine, and 26 sulphite or thiosulphate) obtained out of 1346 isolates in the selective experiment summarized in Table 7; and (c) 52 mutants (25 parathiotrophic, 5 requiring amino acids, 16 nucleic-acid components, and 7 vitamins, but not further classified) obtained out of 800 isolates in the selective experiment summarized in Table 8. Adding on to Table 6 the auxotrophs under a and b, but not those under c because they are incompletely classified, we have a grand total of 612 auxotrophs. For only 33 of these have we failed to identify a
single growth factor capable of satisfying the requirements of each strain; but for 24 the tests have not been complete or decisive. Even if all 33 were cases of genuine multiple requirements, this would constitute only about 5% of the total.

A glance at the list of auxotrophs in Table 6 and a comparison with corresponding lists for *Penicillium notatum-chrysogenum* (Bonner, 1946), *Ophiostoma* (Fries, 1945), and *Neurospora* (Tatum, Barratt, Fries, and Bonner, 1950) shows certain similarities and certain striking differences. In the first place, the high proportions of arginine, lysine, and adenine requirers is common in all four species. The high proportion of parathiotrophic mutants found in *Aspergillus* and *Ophiostoma*, however, does not seem to be paralleled in *Neurospora* or *Penicillium*, where there is a correspondingly high proportion of methionine/cystine requirers. Whether this is a real difference or simply due to the array of compounds used in the tests, is not clear from the published accounts.

In *Aspergillus*, inositol and histidine auxotrophs have not been obtained, not even among 1346 isolates in a selective technique experiment (Table 7) which yielded the two previously unobtained types requiring guanosine and tryptophan. In view of the considerable proportion of inositol requirers in the three other species, the failure in *Aspergillus* seems to be of some interest.

Two groups of auxotrophs in *Aspergillus* show novel properties: the arginine requirers and the nicotinic acid requirers. Twenty-four mutants responding to arginine or to related compounds have been obtained (Table 25). Citrulline is ineffective in all strains, whether they

---

TABLE 25

<table>
<thead>
<tr>
<th>No.</th>
<th>Arginine</th>
<th>Ornithine</th>
<th>Proline</th>
<th>Glutamic Acid</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>5</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>14</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>1</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>2</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Tot. 24</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

None responds to citrulline. Lysine inhibits competitively the utilization of arginine or ornithine but has a sparing effect on proline.

DL-α-Amino-δ-OH-valeric acid (kindly supplied by Dr. J.R.S. Fincham) has shown no growth-promoting activity when tested on one proline-requiring strain, on two arginine/ornithine/proline-requiring strains, on three arginine/ornithine-requiring strains, and on the one arginine-requiring strain. It has an inhibitory effect, not competitive, on the utilization of proline by the proline-requiring strain.
are able to utilize ornithine or proline or not. Other sources of guanido and ureido groups are also ineffective (e.g., guanidino acetic acid, creatine, etc.) alone or in combination with aspartic acid. Most of the strains have been tested for interactions with lysine; lysine inhibits competitively growth on exogeneous arginine or ornithine, with complete inhibition at molar ratios of about 2:1. It has, however, a striking sparing effect (Fig. 21) on exogeneous proline, also for a strain responding only to proline. α-Amino-δ-OH-valeric acid is ineffective.

Eight of the ten lysine-requiring strains have been tested for response to α-amino adipic acid, kindly supplied by Dr. Neuberger, and to α, ε-diaminopimelic acid, kindly supplied by Mrs. Work: neither is effective. The same strains are competitively inhibited by arginine and by ornithine similarly to what Doermann (1944) found in Neurospora.

By crossing, a recombinant has been obtained which requires both arginine/ornithine and lysine; it grows only within very finely balanced limits of concentrations of arginine and lysine.

Another interesting group of nine mutants obtained in Aspergillus, and apparently not yet described in other molds, responds to anthranilic acid, or nicotinic acid. Their growth responses are summarized in Table 26. They are essentially of two kinds: those which respond to anthranilic acid and 3-OH-anthranilic acid, but not to compounds in the tryptophan pathway; and those which, like the Neurospora mutants, respond to the whole series. It is significant that all these mutants are "adaptable"; i.e., they show a lag phase of several days but will eventually
TABLE 26
Growth Responses of 9 Mutants Requiring Nicotinic Acid or Related Substances

<table>
<thead>
<tr>
<th>Mutants</th>
<th>nic&lt;sub&gt;i&lt;/sub&gt;, nic&lt;sub&gt;j&lt;/sub&gt;, nic&lt;sub&gt;k&lt;/sub&gt;, nic&lt;sub&gt;l&lt;/sub&gt;</th>
<th>nic&lt;sub&gt;m&lt;/sub&gt;, nic&lt;sub&gt;n&lt;/sub&gt;, nic&lt;sub,o&lt;/sub&gt;, nic&lt;sub&gt;p&lt;/sub&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anthranilic acid</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Indole</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>DL-Tryptophan</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>3-OH-Tryptophan</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>L-Kynurenine sulphate</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>3-OH-kynurenine</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>3-OH-anthranilic acid</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Quinolinic acid</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Nicotinic acid</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

All the strains grow without supplement after a lag of 3 to 4 days: DL-phenylalanine delays this adaptation, which is not transmitted through the conidia. The efficiency of DL-tryptophan for the four strains responding to it is about 1/200 of that of nicotinic acid.

Dr. Neuberger, Dr. Weidel and Dr. Thorpe have kindly supplied the above 3-OH analogues.

These results suggest (Pontecorvo, 1950) that in Aspergillus, besides the route of biosynthesis of nicotinic acid via tryptophan, there must be an alternative short-cut from anthranilic acid to 3-OH-anthranilic acid.

VII. Diploids and Mitotic Recombination
by G. Pontecorvo and J. A. Roper

1. Isolation and Properties of Heterozygous Diploids

Attempts at obtaining diploid nuclei in the vegetative cells of filamentous fungi have been made repeatedly (review Beneke and Wilson, 1950). Most were based on the use of polyploidogenic agents and aimed at doubling the chromosome number of individual nuclei. In no case, however, was crucial genetic or cytological evidence of polyploidy obtained, though Sansome with Penicillium (1949) and with Neurospora (1950) went farthest in this direction. In the light of what will be discussed here, it seems also possible that certain results of Quintanilha (1938) and Papazian (1950), i.e., the occasional occurrence of recombinant genotypes in heterokaryons, may well be due to formation of diploid nuclei followed by mitotic recombination (section VII-2).
The technique with which diploids were obtained in *A. nidulans* (Roper, 1952) was based on a different approach: instead of looking for diploid nuclei originated from individual haploid nuclei the chromosome complement of which had been doubled, the attempt was made to obtain them as a consequence of fusions between haploid nuclei. The two haploids could then be different in genetic markers and the resulting diploid heterozygous. Thus heterozygosis itself could be used for detecting and isolating the diploid strains, at the same time making sure that their nuclei were actually diploid.

The technique based on this approach has been fully successful in *A. nidulans* (Roper, 1952) and has been applied to *A. niger* (Pontecorvo, 1952a), where sexual reproduction does not occur. It is now being applied to other imperfect fungi. An unexpected consequence of diploidy in the vegetative cells of both these species worked out to be the high frequency with which segregation and recombination occur at mitosis, a fact of far-reaching theoretical and practical interest (section VII-2).

Roper's technique in its present routine version is as follows. The conidia of a balanced heterokaryon, when plated on non-supplemented medium, give origin to no colonies at all or to rare delayed heterokaryons arising from new anastomoses. If, however, by fusion between nuclei, *one of each parental kind*, heterozygous diploid nuclei have arisen, the conidia carrying these will give origin to colonies usually able to grow on non-supplemented medium. The use of color markers makes the isolation of diploids even easier: e.g., if the balanced heterokaryon is between a white and yellow strain, the diploid will be green, besides being able to grow on a medium which is inadequate for the parent strains. When using color markers, it is not even necessary to plate the conidia; occasionally a green sector, or small patch of mycelium, arises in a growing heterokaryon and a green (diploid) strain can be established from it. A proportion of our diploids has been isolated in this last way (Table 27).

To obtain the diploids, Roper (1952) treated with d-camphor vapor the balanced heterokaryon, on the reasonable assumption that it might stimulate either the coalescence of pairs of resting, or prophase nuclei next to one another within a heterokaryotic hypha, or the coalescence of pairs of spindles in metaphase or anaphase. However, we know now that very rarely heterozygous diploid nuclei do arise in the hyphae even without camphor treatment (Table 28). It remains to be seen whether camphor increases the frequency of fusions, or selects the diploids, or simply helps them to become established by a dilution effect, which might help the rare diploid hyphae to "escape" from the meshes of the heterokaryon. In the same way (section V-1), dilution by teas-
TABLE 27
Heterozygous Diploids Obtained in *A. nidulans* and in *A. niger*

<table>
<thead>
<tr>
<th>How obtained</th>
<th>How isolated</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>A. nidulans</strong></td>
<td></td>
</tr>
<tr>
<td>w ad, Y BI, bi, Thl</td>
<td>Camphor</td>
</tr>
<tr>
<td>W AD, y bi, BI, thi</td>
<td>Sector</td>
</tr>
<tr>
<td>w, Y BI, lys orn,</td>
<td>Camphor</td>
</tr>
<tr>
<td>W y, bi, LYS ORN,</td>
<td>Sector</td>
</tr>
<tr>
<td>w ad, Y</td>
<td>Camphor</td>
</tr>
<tr>
<td>W ad, y LYS</td>
<td>Plating</td>
</tr>
<tr>
<td>w ad, Y BI, lys</td>
<td>Spontaneous</td>
</tr>
<tr>
<td>W ad, y bi, LYS</td>
<td>Sector</td>
</tr>
<tr>
<td>w paba, AD, y bi,</td>
<td>Spontaneous</td>
</tr>
<tr>
<td>W PABA, ad, y BI,</td>
<td>Sector</td>
</tr>
<tr>
<td>y bi, BI, AD, thi</td>
<td>Camphor</td>
</tr>
<tr>
<td>Y BI, bi, ad, THI</td>
<td>Plating</td>
</tr>
<tr>
<td>w ad, y LYS</td>
<td>Spontaneous</td>
</tr>
<tr>
<td>w ad, Y lys</td>
<td>Plating</td>
</tr>
</tbody>
</table>

| **A. niger** | |
| (A1) Fawn, aneurin requiring | Spontaneous |
| (F92) Olive, histidine requiring | Sector |
| (A33) Fawn, arginine requiring | Spontaneous |
| (F104) Olive, casein digest requiring | Sector |
| (A35) Fawn, guanosine requiring | Spontaneous |
| (F92) Olive, histidine requiring | Sector |

* The code numbers of the two strains from which the diploid is derived are in brackets above and below the fraction sign.

Ing out the mycelium is known to help rare heterokaryotic hyphae to escape from the parental mycelium when an attempt is made to form balanced heterokaryons. That the dilution is drastic is shown by the fact that, after camphor treatment of a colony, growth restarts from isolated points along the hyphal tips and in the body of the colony; only a small proportion of the mycelium seems to survive the treatment.
TABLE 28
Frequency of Heterozygous Diploid Conidia in Balanced Heterokaryons

<table>
<thead>
<tr>
<th>Conidia plated on selective medium (no.)</th>
<th>Diploid colonies (per 10^6 conidia)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Conidia plated on selective medium (no.)</td>
<td>Diploid colonies (per 10^6 conidia)</td>
</tr>
<tr>
<td>Camphor 4,200,000</td>
<td>1141</td>
</tr>
<tr>
<td>Control 487,000,000</td>
<td>122</td>
</tr>
<tr>
<td>Camphor 12,000,000</td>
<td>41</td>
</tr>
<tr>
<td>A. niger A35 + F92</td>
<td></td>
</tr>
<tr>
<td>Camphor 1,760,000</td>
<td>8</td>
</tr>
<tr>
<td>Control 8,700,000</td>
<td>3</td>
</tr>
</tbody>
</table>

The ease with which the technique yields diploids is shown in Table 28. So far, with the eight balanced heterokaryons of *A. nidulans* to which the technique has been applied, the diploid has been obtained in every case at the first attempt. The same success has been obtained in *A. niger* in all three combinations attempted.

The question of how, as a rare accident, two nuclei happen to fuse in these filamentous fungi might have a general interest. It is known that binucleate cells occur often in animals, including mammals, especially in the male gonads. Usually the two nuclei divide synchronously but form separate spindles; one case, however, (Pontecorvo, 1943, p. 34), has been recorded of almost certain formation of a fused spindle. If this were the mechanism operating in the formation of our diploids, it would not be so interesting. But if the fusion of the nuclei took place otherwise than by coalescence of two spindles, then we would have a valuable tool for investigating substances and conditions which affect the fusion of two nuclei. We may well have a new way of approaching the problem of regulated nuclear fusion as it occurs at fertilization in all organisms with a sexual cycle.

The crucial point in Roper’s technique is the ability to select the diploid conidia which, even after camphor treatment, form an exceedingly small proportion of the total (Table 28), and to recognize the diploid strains by their phenotype. The selection is based on the assumption that most nutritional requirements which behave as recessives
in heterokaryons would behave as recessives in diploids as well, and therefore a diploid heterozygous for two requirements would show neither. This has proved to be the case for all nine requirements tested so far in *A. nidulans* (Table 29) and for all five tested in *A. niger*. There

<table>
<thead>
<tr>
<th>Phenotypes</th>
<th>Heterokaryon</th>
<th>Heterozygote</th>
</tr>
</thead>
<tbody>
<tr>
<td>$y$</td>
<td>Yellow or green</td>
<td>Green</td>
</tr>
<tr>
<td>$\bar{y}$</td>
<td>green</td>
<td></td>
</tr>
<tr>
<td>$w$</td>
<td>White or colored</td>
<td>Colored</td>
</tr>
<tr>
<td>$\bar{w}$</td>
<td>colored</td>
<td></td>
</tr>
<tr>
<td>$w$</td>
<td>White</td>
<td>White</td>
</tr>
<tr>
<td>$w_a$</td>
<td>Non-requirer</td>
<td>Non-requirer</td>
</tr>
<tr>
<td>$L_{ys}$</td>
<td>Non-requirer</td>
<td>Non-requirer</td>
</tr>
<tr>
<td>$L_{YS}$</td>
<td>Non-requirer</td>
<td>Non-requirer</td>
</tr>
<tr>
<td>$O_{RN_1}$</td>
<td>Non-requirer</td>
<td>Non-requirer</td>
</tr>
<tr>
<td>$a_{D_1}$</td>
<td>Non-requirer</td>
<td>Non-requirer</td>
</tr>
<tr>
<td>$a_{D}$</td>
<td>Non-requirer</td>
<td>Non-requirer</td>
</tr>
<tr>
<td>$a_{D_1}$</td>
<td>Non-requirer</td>
<td>Non-requirer</td>
</tr>
<tr>
<td>$a_{D}$</td>
<td>Non-requirer</td>
<td>Non-requirer</td>
</tr>
<tr>
<td>$panto$</td>
<td>Non-requirer</td>
<td>Non-requirer</td>
</tr>
</tbody>
</table>

TABLE 29

Dominance in Heterokaryons and in Heterozygotes of *A. nidulans*

<table>
<thead>
<tr>
<th>Phenotypes</th>
<th>Heterokaryon</th>
<th>Heterozygote</th>
</tr>
</thead>
<tbody>
<tr>
<td>$b_i$</td>
<td>Non-requirer</td>
<td>Non-requirer</td>
</tr>
<tr>
<td>$B_l$</td>
<td>Non-requirer</td>
<td>Non-requirer</td>
</tr>
<tr>
<td>$b_i$</td>
<td>Non-requirer</td>
<td>Non-requirer</td>
</tr>
<tr>
<td>$B_l$</td>
<td>Non-requirer</td>
<td>Non-requirer</td>
</tr>
</tbody>
</table>

is an exception which confirms the rule. In the case of the pseudo-alleles $b_i$ and $B_l$ (section VI-3), the phenotype of the diploid doubly heterozygous *in repulsion* is biotin-requiring, like that of the corresponding heterokaryon.

The use of conidial differences as additional markers to identify diploids was based again on the assumption that the mutant alleles would be recessive. There was, in this case, no information to go by from the heterokaryons because both color markers used in *A. nidulans* ($y$ and $w$) are autonomous in action; the color of the conidia formed by heterokaryons is determined by the type of nucleus segregated in each chain. However, the guess that the two mutant alleles would be recessive in the double heterozygote was correct; the conidia of heterozygotes of consti-
tion \( W/w \ Y/y \) are green. On the other hand, the color markers used in \( A. \ niger \) (fawn and olive, the wild type being black), are not autonomous; i.e., each heterokaryotic head has chains uniform in color and this color varies from one head to another, ranging from fawn to olive through black, presumably according to the nuclear ratios; the diploids have uniformly black heads.

Apart from being phenotypically as expected, the heterozygous diploids of \( A. \ nidulans \) are unequivocably identifiable also on the basis of other properties: diameter of conidia (Table 30); meiosis (section II-3); low viability of the ascospores; degeneration of the majority of asci after meiosis; presence of 16-spore asci; segregation in the asci; mitotic segregation and recombination.

### Table 30

Diameter of Conidia of Haploid and Diploid Strains of \( A. \ nidulans \) and \( A. \ niger \)

<table>
<thead>
<tr>
<th>( A. \ nidulans )</th>
<th>Conidia measured (no.)</th>
<th>Mean diameter (( \mu ))</th>
</tr>
</thead>
<tbody>
<tr>
<td>Diploids ( w \ adj \ Y \ BI, bi, ) ( THI ) ( W AD, y bi, BI, ) ( thi )</td>
<td>55</td>
<td>4.3</td>
</tr>
<tr>
<td>( w_a AD, Y BI \ lys ) ( W ad, y bi \ lys ) ( LYS ) ( W ad, y LYS ) (yellow conidia)</td>
<td>82</td>
<td>3.8</td>
</tr>
<tr>
<td>Wild type</td>
<td>85</td>
<td>3.1</td>
</tr>
<tr>
<td>( y ) (yellow conidia)</td>
<td>89</td>
<td>3.2</td>
</tr>
<tr>
<td>( A. \ niger ) Diploids</td>
<td></td>
<td></td>
</tr>
<tr>
<td>A1/F92 (black conidia)</td>
<td>53</td>
<td>5.4</td>
</tr>
<tr>
<td>A35/F92 (black conidia)</td>
<td>58</td>
<td>5.3</td>
</tr>
<tr>
<td>II/33d (olive conidia) ( \dagger )</td>
<td>62</td>
<td>5.5</td>
</tr>
<tr>
<td>Haploids</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Wild type (black conidia)</td>
<td>52</td>
<td>4.3</td>
</tr>
<tr>
<td>680F (olive conidia)</td>
<td>64</td>
<td>4.7</td>
</tr>
</tbody>
</table>

* Mitotic recombinant from \( w_a AD, Y lys \) \( W ad, y LYS \)

† Mitotic recombinant from A1/F92.

Measurements with eyepiece micrometer of pieces of chains of conidia in suspension.
As to the diameter of the conidia, the data of Table 30 need no comment. About meiosis, the little which can be said is that it is certainly tetraploid (section II-3). As to the low viability of the ascospores (less than 1 in 50) and the degeneration of the majority of the asci after meiosis, they were expected, of course. However, they are far too severe to be accounted for entirely by the formation of unbalanced products as a consequence of tetraploid meiosis. These disturbances may be in part an additional manifestation of whatever causes the formation of 16-spore asci. Crosses between haploids and diploids will probably tell whether or not a "maternal effect" of the diploid mycelium plays a part.

As to the 16-spore asci (Fig. 22), they came, of course, as a surprise; so far they have never been found in haploid strains and they have been found in every diploid strain. In the diploid strains, individual perithecia with 16-spore asci carry these almost exclusively with an occasional 8-spore ascus and rare asci with any number of spores from a single gigantic one to 16. In certain diploids, however, one finds side by side typical perithecia, as those just described, and perithecia packed with asci, all 8-spored, as in haploid strains. The analysis of these perithecia has not started. If the asci arise parthenogenetically in a diploid heterozygous for known markers, these asci should show ordinary diplohaploid segregation; if they arise from patches of mycelium which have reverted to the haploid condition, usually they should not segregate at all. It will have to be seen whether or not the formation of perithecia with 8-spore asci in certain diploids is in any way connected with relative heterothallism (section V-4).

As to segregation in the 16-spore asci, the low viability of the ascospores is a serious obstacle. Ascus dissection is clearly out of the question, but random sampling of ascospores is possible. We have so far
carried out only qualitative analysis, mainly to ascertain whether a
diploid was segregating for the markers for which it was supposed to
be heterozygous. This has usually proved to be the case, with two ex¬
ceptions: a diploid originally heterozygous for *lys* lost this mutant allele
within the course of a few sub-cultures, and a diploid supposed to be
heterozygous for *panto* did not segregate for it. Evidently *lys*, and
presumably *panto*, reduce the fitness in heterozygous condition and are
therefore supplanted by their normal alleles as soon as mitotic recombina¬
tion (see below) produces homozygotes for these.

Of the ascospores produced by diploids, some are haploid and some
diploid. To ascertain whether triploid ascospores also are produced, as
expected, would require a special type of genetic analysis or substantial
improvements in the cytological examination of meiosis; neither has been
attempted.

As to mitotic segregation and recombination, they occur in every
diploid strain of *A. nidulans* and *A. niger* so-far examined, i.e., in the
eleven strains listed in Table 27 and in a large number of strains de¬
derived from the above.

Promoted by the classical work of Stern (1936) on somatic crossing-
over in *Drosophila* we deliberately looked for the occurrence of mitotic segre¬
gation and recombination as soon as diploids were obtained: we did
not expect however, to find this process occurring as regularly and fre¬
quently as it does.

2. Mitotic Recombination and Its Use in Genetic Analysis

Mitotic segregation manifests itself in heterozygous diploids of *A.
nidulans* in the following way. Colonies of a green-spore diploid strain
(heterozygous for *w Y/W y* and purified by single conidium micromanip¬
ulation) started from point inoculum on agar medium show a number
of single heads, or spots with a few heads or small sectors bearing white
or yellow conidia (Fig. 23). Alternatively, plating of green conidia
from such a strain yields green colonies, most of which show a few single
heads, small patches of heads, or sectors with white or yellow conidia.

Isolation and purification, by single conidium micromanipulation,
of yellow segregants ("first order" segregants) gives strains most of
which again produce spots with white heads ("second order" segre¬
gants). The converse, of course, cannot be tested because of the epistasis
of *w*. The segregants, both of first and second order, are usually still
diploid, as shown by their producing 16-spore asci and segregating via
ascospores for other markers for which the parent strain was heterozyg¬
gous. In some cases the diameter of the conidia of the segregants has
been measured and found to fall within the diploid range (Table 30).
Fig. 23. Colonies of a diploid heterozygous $w^Y\over W^y$ (green) showing yellow or white spots and one white sector, due to mitotic recombination.

Besides segregation, the process may involve also recombination. For instance, a diploid derived from haploids $W^a d^2 y^2$ $LYS$ and $w^a D^2 \over y^2$ $LYS$, yielding yellow segregants, some of which were lysine-requiring, and white segregants, some of which adenine-requiring (Table 31), besides other types of recombinants. Mitotic recombination of this kind has been obtained consistently from all diploids analyzed both in $A.\nidulans$ and $niger$. The problems raised by the occurrence of mitotic segregation and recombination in the vegetative cells, and the possibilities opened by it, are somewhat overwhelming. We shall try to visualize some and illustrate the kind of preliminary attack opened on a few.

In the first place, there is the question of the rate of occurrence and of the regularity of the process. The difficulty here is the same as in the measurement of mutation rates; i.e., the clonal distribution of the segregant nuclei. If we sample the conidia of a heterozygous diploid, the proportion among them of homozygotes for any one marker would permit an estimation of the segregation rate only if it were known that the segregant nuclei did not multiply differentially: in fact, it is certain that in most cases they do. In one sample of plated conidia from each of four diploids, all heterozygous at $w/W$ locus, the following proportions of homozygotes for the recessive were obtained:

From diploid: $w^a AD^2 y^2 BI^2$ $\over W^2 ad^2 y^2 bi^2$ $lys\over LYS$ : 3 out of 763

From diploid: $w^a AD^2 y^2 BI^2$ $\over W^2 ad^2 y^2 bi^2$ $lys\over LYS$ : 0 out of 70

From diploid: $w^a AD^2 y^2 BI^2$ $\over W^2 ad^2 y^2 bi^2$ $lys\over LYS$ : 0 out of 236

From diploid: $w^a paba AD^2 y^2 BI^2$ $\over W^2 paba ad^2 y^2 bi^2$ $lys\over LYS$ : 3 out of 531
### TABLE 31

An Example of Mitotic Recombination

Diploid, green conidia prototroph, purified by single conidium micromanipulation:

\[
\begin{align*}
\frac{w^{a} A D_{1} Y}{W a d_{1} y} & \quad lys \\
\rightarrow & \quad 16\text{-spore asci, segregating for yellow, white, and green and for the requirements.}
\end{align*}
\]

"First order" mitotic segregants and recombinants isolated by inspection

<table>
<thead>
<tr>
<th>White, requiring:</th>
<th>Yellow, requiring:</th>
</tr>
</thead>
<tbody>
<tr>
<td>Adenine and lysine</td>
<td>Adenine and lysine</td>
</tr>
<tr>
<td>Adenine</td>
<td>Adenine</td>
</tr>
<tr>
<td>lysine</td>
<td>lysine</td>
</tr>
<tr>
<td>Neither</td>
<td>Neither</td>
</tr>
<tr>
<td>Lysine</td>
<td>Lysine</td>
</tr>
<tr>
<td>2</td>
<td>6</td>
</tr>
<tr>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>7</td>
<td>1</td>
</tr>
<tr>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

Purified by single conidium micromanipulation

- 5 out of 7 give "second-order" white mitotic recombinants
- 16-spore asci segregating for lysine and adenine requirements, for yellow and white

"Second order" mitotic segregation for lysine and adenine requirements, for yellow and white
Taken at their face value, these results indicate a proportion of homozygotes of about 1 in 300. Clearly, mitotic segregation is not a very rare event. Furthermore, 20 colonies from plating of conidia of the first of the above strains were carefully scanned when about 1 cm. in diameter: a total of 21 white spots and 17 yellow spots were identified in them. This gives roughly an average of one white or yellow visible segregant spot per 35 sq. mm. of colony surface. But to be visible, a segregant spot must include at least one head; for each such spot identified there must be many hundreds carrying only one or few white or yellow conidia which escape detection.

A second problem is that of the mechanism of mitotic segregation and recombination. Any interpretation of the results must account for the fact that the majority of segregants are still diploid and heterozygous for some of the markers present in the parent strain. Almost certainly, however, somatic reduction to the haploid condition, with or without recombination between non-homologous chromosomes, also occurs. For instance, a proportion of biotin-independent mitotic recombinants from diploid $y bi^6 BI_t AD_4 thi/Y BI_t bi_t ad_4 THI$ (see section VI-3) were probably haploid because: (1) they failed to segregate further, (2) they abundantly produced 8-spore asci only; and (3) they had conidia of haploid size. If confirmed and extended, results of this kind will provide excellent material for testing Huskins' ideas on reduction in somatic tissues. Haploidization, however, could not possibly account for more than a minor fraction of the segregants and recombinants obtained, which, it must be emphasized again, are mainly diploid.

As an interpretation of the results, we are therefore left with the theory of somatic crossing-over as developed by Stern (1936) for Drosophila and applied by Demerec (1936) to the investigation of cell-localized lethal gene action. The theory is that somatic crossing-over occurs in a small proportion of diploid nuclei in mitosis, that it takes place at the four-strand stage and that, barring non-disjunction and multiple crossovers, it must inevitably lead to segregation at heterozygous loci distal to any point of exchange.

In trying to test the theory of somatic crossing-over in A. nidulans, we have one marked advantage and one minor limitation. The former is that we can isolate segregant cells and analyze their genotypes, whereas the work in Drosophila is limited almost exclusively to the identification of the phenotypes of these cells. True, nuclei originated from mitotic crossing-over have been recovered in certain cases in the pollen in higher plants and in the sperm in Drosophila, but this is not as satisfactory as the possibility of recovery from cells only a few mitotic divisions removed from the one in which the process took place. The minor limi-
tion is that in *A. nidulans* we do not have a pair of closely linked markers, both identifiable by inspection, such as the classical *y* used by Stern and by Demerec in *Drosophila*.

The results to date with *A. nidulans* (and with *A. niger* within the limits imposed by the absence of cross-checking via sexual reproduction) seem to substantiate fully Stern's theory of somatic crossing-over. However, we have not yet recovered in one and the same diploid nucleus the two complementary products of somatic crossing-over. Until this is done, we feel that only tentatively can mitotic recombination be taken to result from a crossing-over-like process.

As markers, we have used more extensively the two color loci *W/wa* and *Y/y*, two "nutritional" loci on the same chromosome (*ADg/ad* and *BI*/*bi*), and the *LYS/lys* locus, which segregates independently of all

### TABLE 32

Mitotic Recombinants from Diploid Strains of *A. nidulans*

<table>
<thead>
<tr>
<th>Diploids Green</th>
<th>Recombinants</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>White</td>
<td>Yellow</td>
</tr>
<tr>
<td></td>
<td><em>bi</em></td>
<td><em>BI</em></td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>6</td>
</tr>
<tr>
<td></td>
<td><em>bi</em></td>
<td><em>BI</em></td>
</tr>
<tr>
<td></td>
<td>20</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1. <em>w</em> Y <em>BI</em></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>W</em> <em>y</em> <em>bi</em></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2. <em>w</em> <em>AD</em> <em>Y</em></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>lys</em> <em>Lys</em></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>W</em> <em>ad</em> <em>y</em></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>LYS</em></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td><em>ad</em></td>
<td><em>AD</em></td>
</tr>
<tr>
<td></td>
<td>lys LYS</td>
<td>lys LYS</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>13</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>47</td>
</tr>
<tr>
<td></td>
<td></td>
<td>52</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3. <em>w</em> <em>AD</em> <em>Y</em></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>lys</em> <em>Lys</em></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>W</em> <em>ad</em> <em>y</em></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>LYS</em></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td><em>ad</em></td>
<td><em>BI</em></td>
</tr>
<tr>
<td></td>
<td>lys LYS</td>
<td>lys LYS</td>
</tr>
<tr>
<td></td>
<td>7</td>
<td>13</td>
</tr>
<tr>
<td></td>
<td></td>
<td>20</td>
</tr>
</tbody>
</table>

* The symbols of the alleles are used to indicate the phenotypes of the recombinants.

† This strain, originally heterozygous for *LYS/lys*, *ORN/orn*, and *PANTO/panto*, was mixed in these respects when analyzed. The sample of white and yellow is not a random sample in this case.
the others. Experiments with seven more markers (five of which are in
the linkage group just mentioned) have not yet gone far enough to be
reported here.

Since mitotic segregants constitute only a small proportion of a
growing colony, they have to be selected out. The selection is visual as
to the conidial color markers, but enrichment is necessary as to the nu-
tritional markers. A technique to this end is now available (Forbes,
1952); it is analogous to the penicillin technique for isolating bacterial
mutants since it is based on the preferential killing by SO$_2$ of proto-
trophs pregerminated in minimal medium, where auxotrophs do not
germinate.

With three strains the results of visual selection of mitotic segre-
gants differing from the green parent, heterozygous diploid ($W/w_a$
$Y/y$), in being white or yellow, are shown in Table 32. For strains 1
and 2 the selection of color segregants was carried out exclusively in large
colonies grown from point inoculum of conidia by picking out of each
segregant spot a single head (white or yellow). For strain 3 (see
also Table 33), some of the color segregants were also picked from small
colonies obtained by plating conidia. In this case, picking not more than
one segregant per colony ensures that the same segregant clone is not
isolated more than once. In view of the methods of selection, the pro-
portions of yellow to white segregants tested are not representative of
the actual proportions in the colonies.

Keeping in mind (section V-1, 2) that the loci $AD_g/ad_g$, $Y/y$,
and $B_l/b_l$ are closely linked and in this order, that the $W/w_a$ locus is prob-
ably on the same chromosome, but more than 50 units beyond $AD_g/ad_g$
(Table 18), and that the locus $LYS/lys$ segregates independently of
these four, the following points of interest arise from Tables 32 and 33.

1. Simultaneous segregation at more than one locus does not occur
at random; the alleles in coupling at closely linked loci tend to segregate
together. Thus all the yellow from diploid 1 are also homozygous for
$b_l$; all but one of the yellow from diploid 2 are also homozygous for
$ad_g$; and all the yellow from diploid 3 are also homozygous for $ad_g$
and $b_l$. Homozygosis at the freely segregating locus $LYS/lys$ occurs in 2
out of 52 white and 2 out of 16 yellow in diploid 2, and in 14 out of 31
white and 7 out of 20 yellow in diploid 3. Simultaneous homozygosis
for $w_a$ and the recessive alleles in the $y$ region, which if on the same
chromosome is more than 50 units away, occurs in 5 out of 11 white in
1, in 5 out of 47 in 2 and in 10 out of 31 in 3. It is to be noted that $w_a$
is in repulsion relative to these other recessive alleles in all three cases.

2. Simultaneous segregation at two non-linked loci occurs far in ex-
cess of what would be expected from the frequency of segregation at each
### TABLE 33 *

Mitotic Recombinants from Diploid:

\[
\frac{w_AD_YBI}{W_ad_ybi} \quad \frac{\text{lys}}{\text{LYS}}
\]

<table>
<thead>
<tr>
<th></th>
<th>(AD_YBI)</th>
<th>(ad_ybi)</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Green</strong></td>
<td>lys</td>
<td>lys</td>
<td></td>
</tr>
<tr>
<td></td>
<td>0</td>
<td>0</td>
<td>240</td>
</tr>
<tr>
<td><strong>White</strong></td>
<td>lys</td>
<td>lys</td>
<td></td>
</tr>
<tr>
<td></td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td><strong>Yellow</strong></td>
<td>lys</td>
<td>lys</td>
<td></td>
</tr>
<tr>
<td></td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td>lys</td>
<td>lys</td>
<td></td>
</tr>
<tr>
<td></td>
<td>0</td>
<td>0</td>
<td>240</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th></th>
<th>(ly S)</th>
<th>(LY S)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Green</strong></td>
<td>lys</td>
<td>lys</td>
</tr>
<tr>
<td></td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td><strong>White</strong></td>
<td>lys</td>
<td>lys</td>
</tr>
<tr>
<td></td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td><strong>Yellow</strong></td>
<td>lys</td>
<td>lys</td>
</tr>
<tr>
<td></td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td>lys</td>
<td>lys</td>
</tr>
<tr>
<td></td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th></th>
<th>(AD_YBI)</th>
<th>(ad_ybi)</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Green</strong></td>
<td>lys</td>
<td>lys</td>
<td>197</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>195</td>
<td></td>
</tr>
<tr>
<td><strong>White</strong></td>
<td>lys</td>
<td>lys</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>0</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td><strong>Yellow</strong></td>
<td>lys</td>
<td>lys</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>0</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td>lys</td>
<td>lys</td>
<td>200</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>196</td>
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</table>

<table>
<thead>
<tr>
<th></th>
<th>(AD_YBI)</th>
<th>(ad_ybi)</th>
<th>Total</th>
</tr>
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<tbody>
<tr>
<td><strong>Green</strong></td>
<td>lys</td>
<td>lys</td>
<td>6</td>
</tr>
<tr>
<td></td>
<td>2</td>
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<td></td>
</tr>
<tr>
<td><strong>White</strong></td>
<td>lys</td>
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<td>15</td>
</tr>
<tr>
<td></td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td><strong>Yellow</strong></td>
<td>lys</td>
<td>lys</td>
<td>18</td>
</tr>
<tr>
<td></td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td>lys</td>
<td>lys</td>
<td>21</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>0</td>
<td></td>
</tr>
</tbody>
</table>

* The symbols of the alleles are used to indicate the phenotypes of the recombinants.

** Random sample of 240 colonies out of 763 (of which 3 white, no yellow) from plated conidia.

† Some by isolation of heads from mosaic spots; some from white colonies obtained in 1.

‡ Random sample of 200 colonies out of 1716 (of which 7 were white and 17 yellow) from pregerminated conidia treated with \(SO_2\).

|| The white and the yellow out of the 1516 colonies not included in the random sample in 3.

locus. For instance, simultaneous segregation for \(lys\) occurs in 21 out of 119 yellow or white in diploids 2 and 3. We know (p. 226, and Table 33) that homozygosis for \(y\) or \(lys\) did not occur once among 236 conidia of diploid 2 and among 763 conidia of diploid 3. Among the latter, homozygosis for \(wa\) occurred three times. Yet we find now that about 1 in 6 of the color segregants are also homozygous for \(lys\). Even though the data are very limited, the converse is also true: Table 33 shows that in selecting for \(lys\), 1 out of the 3 \(lys\) obtained was yellow. Clearly (Pontecorvo, 1952a), somatic segregation occurs in a small pro-
portion of nuclei, but in those in which it occurs it tends to involve more than one chromosome and (if \( W/w \) were really on the same chromosome as \( Y/y \)) more than one region of a chromosome.

3. If the tentative location of \( W/w_a \) on the same chromosome as \( AD_2/ad_2, Y/y, \) and \( BI_1/bi_1 \) were confirmed, the results of Table 33 would leave no doubt as to recombination of linked genes. All the cases in which homozygosis for \( w_a \) and for one or more of the recessive alleles in the \( ad_2-bi_1 \) region occurred would imply multiple exchanges in one chromosome. Precisely, if the centromere were either beyond \( W/w_a \) or beyond \( BI_1/bi_1 \), three exchanges involving chromatids 1,3 and 4,2; 2,4 would be necessary. If the centromere were between \( W/w_a \) and \( AD_2/ad_2 \), two disparate exchanges would be required, involving one arm each. Barring these as examples of crossing-over, there is only one other case in Table 33 of recombination between unquestionably linked loci, i.e., the yellow adenine-independent recombinant from diploid 2. This recombinant was fully tested (Table 31) and its genotype is as expected. However, a diploid of this genotype could also have arisen by \( Y \rightarrow y \) mutation in the parent strain.*

As mentioned before, a technique by Forbes (1952) makes it possible to select the auxotrophic segregants from the mass of parental prototrophic conidia. This technique is still being improved, but the results of Table 33 (obtained in collaboration with Mr. E. C. Forbes) show that it is no longer impossible to isolate these segregants: a yield of 4 auxotrophs out of 200 tested colonies was obtained in a sample enriched by this technique as compared with 0 out of 240, in the untreated control. The number of auxotrophs obtained is unfortunately too small to expect any recombinant for the linked markers among them. We hope to get these as soon as the technique will be perfected.

Other examples of what can be only either mitotic crossing-over between closely linked markers or mutation have been obtained: e.g., biotin-requiring green from diploids of constitution \( Y \ BI_1, \) and biotin and adenine-independent green from diploids of constitution \( y \ bi_2 \ BI_1, AD_1. \)

\* Unquestionable evidence of mitotic recombination between loci which are certainly linked has now been obtained. From a diploid with \( paba_i \) and \( y \) in coupling, out of 51 yellow recombinants visually selected, 39 were \( paba_i \) and 12 \( PABA_i. \) The two loci show 15-20% recombination at meiosis. These results suggest that the \( PABA_i/paba_i \) locus is proximal and that, in terms of mitotic recombination, the 'distance' between the centromere and this locus is about three times that between this locus and \( Y/y. \)
Though every detail of our results is in agreement with the theory of mitotic crossing-over, we shall not take it as proven until we can recover the complementary products of one exchange. Work to this end is in progress. It may be mentioned, for instance, that in *A. niger* the diploid doubly heterozygous for fawn and olive has conidia considerably lighter than the wild type. Often in a growing colony near to spots segregant for fawn, spots darker than the diploid are found; when isolated and purified, these give origin to strains segregating for olive but not for fawn. We deduce that the darker types are "twin" products, homozygous for the wild-type allele of fawn. Though we have no such convenient situation in *A. nidulans*, this finding suggests that in the vicinity of a recessive segregant spot detectable by inspection we should find the corresponding dominant homozygote not distinguishable by inspection from the heterozygous parent.

The fact that at mitosis the alleles of closely linked loci tend to segregate together and to recombine as a group with other non-linked loci makes it possible to carry out genetic analysis via mitotic recombination. In *A. nidulans*, for instance, out of a total of 101 segregants tested which could have shown recombination between two of the three closely linked loci in the *ad*/bii region, only one showed it. On the other hand, between 7 and 50% of those which could have shown recombination between non-linked loci did show it. In a species without sexual reproduction, results of this kind can be used to detect linkage groups.

The analysis, however, may go further. If mitotic crossing-over will be proved to be the cause of segregation (and all seems to point this way), then we shall be able to locate the centromere in a sequence of linked genes as the point at which the direction of linked segregation is inverted. If we have the sequence a-b-c-centromere-d-e-f, homozygosis for d will usually carry with it homozygosis for e and f, but not so often for a, b or c. On the other hand, homozygosis for c will carry with it homozygosis for b and a, but not so often for d, c, or f. On the basis of this reasoning and of certain results with mitotic recombination in *A. nidulans*, we have tentatively located the centromere in the bii linkage group between *adi* and *pabai*. Unfortunately this is a region where an independent check by ascus analysis is not easy.

The production of heterozygous diploids and the use of mitotic recombination has opened the way to genetic analysis and "breeding" in asexual species of filamentous fungi. The first results in applying our technique to *A. niger* show that this way is relatively easy and extremely promising both in fundamental research and practical applications.
VIII. Summary and Conclusions

The work reported in this paper is obviously spadework in extension rather than in depth. In the process of this spadework, results of general implication have been obtained, and some have been followed up even if outside our main line of interest. A summary of the salient points will make the general picture clearer.

1. The genetic analysis of a homothallic fungus has been carried out for the first time and shown to be perfectly manageable.

2. The formal genetics of *A. nidulans* has gone as far as the identification of at least 18 loci, 5 of which, and probably 7, are in one linkage group.

3. Two of the regions investigated for pseudo-allelism (the *hi* and the *paba* loci) show it. A third investigated region (*ad*$_1$ and *ad*$_2$) is not of this kind. Taken together with results in organisms ranging from *Drosophila* to maize, where pseudo-allelism has been found almost invariably whenever looked for, this may suggest that recombination between some of the members of one allelic series is the rule rather than the exception.

4. The detailed investigation of the asci of individual perithecia has led to the discovery of relative heterothallism, i.e., the fact that a self-fertile strain may take part preferentially in outcrossing, if given the opportunity.

5. The biochemical genetics of *A. nidulans*, though similar to that of *Neurospora* and *Ophiostoma*, has revealed certain interesting differences in detail: e.g., the inability of citrulline to replace ornithine for strains responding to ornithine or arginine; the inability of tryptophan to replace anthranilic acid for certain strains responding to anthranilic acid or nicotinic acid; the competitive inhibition by lysine of exogenous arginine or ornithine and its sparing effect on exogenous proline; etc.

6. The production of strains carrying in their vegetative cells diploid nuclei heterozygous for known markers has opened the way to a more thorough study of somatic segregation and recombination than could be possible in higher animals or plants. Genetic recombination can now be obtained outside the sexual cycle, and this has already been done with the asexual species *A. niger*.

7. The comparative physiological genetics of heterozygotes and heterokaryons in one and the same species is now possible. This was one of the missing links in the study of the relations between spatial distribution and action of genes which prompted the present work (Ponte-corvo, 1947, 1950, 1952b, 1952c).

It is a great pleasure to acknowledge the valuable technical
assistance of Mr. E. C. Forbes. During the course of this work financial support in the form of grants for research and fellowships has been received from The Department of Scientific and Industrial Research, the Medical Research Council, and Messrs. Distillers Company Ltd. Mr. K. D. Macdonald carried out the work reported in section IV-2 while holding a Research Scholarship from the Carnegie Trust for the Universities of Scotland.

IX. References

Davis, B. D., 1948, Isolation of biochemically deficient mutants of bacteria by penicillin. J. Amer. chem. Soc. 70, 4267.
1948b, Viability and resistance of spontaneous mutations in Ophiostoma representing different degrees of heterotrophy. Physiol. Planter 1, 330-341.


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Raffell, D., and Muller, H. J., 1940, Position effect and gene divisibility considered in connection with three strikingly similar acute mutations. *Genetics* 25, 541-583.


Parasexual Recombination in *Penicillium chrysogenum*

**By G. Pontecorvo and G. Sermonti**

*Department of Genetics, University of Glasgow*

SUMMARY: Roper's (1952) technique for the isolation in filamentous fungi of strains carrying in their hyphae diploid nuclei heterozygous for known markers has been applied with minor modifications to a third species: *Penicillium chrysogenum*. One of these modifications is the use of 'dwarf' (stunted growth) mutants for growing balanced heterokaryons. Heterozygous diploid strains of *P. chrysogenum* behave like those of the other two species in yielding, vegetatively, new strains with recombined properties. This behaviour can be marshalled for the deliberate breeding of improved industrial strains.

In two species of filamentous fungi, *Aspergillus nidulans* (Roper, 1952; Pontecorvo & Roper, 1952, 1953; Pontecorvo, Tarl Gloor & Forbes, 1954) and *Aspergillus niger* (Pontecorvo, 1952; Pontecorvo, Roper & Forbes, 1953), recombination between properties of different strains may take place outside, or in the absence of, a sexual cycle. Processes leading to gene recombination otherwise than via sexual reproduction are called 'parasexual' (Pontecorvo, 1953). The present paper reports the discovery of a parasexual cycle in a third species, *Penicillium chrysogenum*, the mould used for the industrial production of penicillin. This discovery was the outcome of the application, with minor modifications, to this species of the techniques developed for the other two species.

The differences in properties used for the present work (colours of conidia, growth rates, colony morphology and nutritional requirements) were chosen exclusively for their convenience as experimental markers. The discovery that recombination occurs in respect of these properties makes it certain that it also occurs in respect of inherited properties of other kinds. Clearly, this knowledge makes possible the deliberate breeding of improved strains for penicillin production. A preliminary report of the work in this paper has been published (Pontecorvo & Sermonti, 1953).

The production of marked strains

Unless explicitly stated, the techniques used in the present work are the same as those used as a routine for *Aspergillus nidulans* (Pontecorvo, 1953) and applied to *A. niger* (Pontecorvo et al. 1953).

**Media.** Minimal medium (MM): a modified Czapek-Dox. Complete Medium (CM): a complex medium containing yeast extract, casein hydrolysate, etc. Incubation was at 26°C.

**Organisms.** Two starting strains of *Penicillium chrysogenum* were used; one with white conidia, turning very pale green after long incubation at room temperature, and one with yellow-brown conidia. The former, kindly supplied by Dr J. C. Calam (Imperial Chemical Industries, Manchester) was a mutant obtained after nitrogen mustard treatment. It is designated here with the
symbol \( w \). The yellow strain (symbol \( y \), code no. 1086) was obtained previously by one of us (G.S.) after ultraviolet irradiation of strain \( Q176.47.1564 \). Both were prototrophs, i.e. able to grow well on medium MM, like the green-spored strain \( Q176 \), to which both ultimately traced back.

The marked strains required for the present work were strains differing from the wild type in three pairs of alternative characters and from each other in six pairs. Two pairs (white versus green conidia, yellow versus green conidia) were already available in the two starting strains mentioned above. The other four pairs were obtained by successive ultraviolet irradiations and they consisted either in differences in nutritional requirements or differences in growth rates (e.g. dwarf versus normal colony size).

Table 1. Results of ultraviolet irradiation (4 min. at 30 cm.) of dishes of CM agar plated with about 1000 conidia each

<table>
<thead>
<tr>
<th>Strain irradiated</th>
<th>Viable colonies isolated (%)</th>
<th>Nutritional requirements</th>
<th>Dwarf</th>
</tr>
</thead>
<tbody>
<tr>
<td>( w )</td>
<td>White, no requirements</td>
<td>2</td>
<td>238</td>
</tr>
<tr>
<td>( y )</td>
<td>Yellow, no requirements</td>
<td>5</td>
<td>160</td>
</tr>
<tr>
<td>( 22y )</td>
<td>Yellow, hypoxanthine</td>
<td>17</td>
<td>325</td>
</tr>
<tr>
<td>( 7w )</td>
<td>White, thiosulphate</td>
<td>6</td>
<td>150</td>
</tr>
<tr>
<td>( 38y )</td>
<td>Yellow, ( NO_2^- )</td>
<td>0·2</td>
<td>125</td>
</tr>
</tbody>
</table>

Suspensions of \( 10^4 \) conidia/ml. were spread in volumes of 0·1 ml./plate (1000 conidia) over the agar surface of a series of Petri dishes containing CM agar. The dishes were irradiated by exposing them to an Hanovia XI low-pressure mercury lamp for 4 min. at 30 cm. distance. Colonies which grew up (random sample) after such treatment (Table 1) were isolated and screened for mutants with additional growth factor requirements (Pontecorvo, 1953b). Furthermore, four dwarf mutants were picked by inspection from some of the irradiation series. The mutants were purified by micromanipulation of single conidia or by plating followed by single colony isolation.

Synthesis of balanced heterokaryons

Previous attempts by one of us (G.P.) to form balanced heterokaryons between pairs of strains each differing from the wild type in one additional nutritional requirement had been unsuccessful. A similar failure is mentioned
by Bonner (1946) in respect of pairs of strains of *Penicillium notatum*; yet heterokaryosis in this species (Pontecorvo & Gemmell, 1944; Sansome, 1947) was known to occur. The technique used in our attempts was the one which has invariably proved effective with other species of moulds (Pontecorvo, 1947, 1953b). In its most recent version it involves the following steps: (1) inoculation into liquid CM of a thick suspension of about equal numbers of conidia of each of two strains requiring different growth factors; (2) incubation for a time sufficient to obtain a thin mesh of mycelium; (3) removal of this mesh, which is washed by dipping in MM, and thoroughly broken up by teasing out over the surface of plates of MM agar; (4) incubation of these until growth starts from a few points; (5) isolation of growing hyphal tips from these points on to MM agar. The length of incubation (under 4) required for the ‘escape’ of the heterokaryotic growth varies with the species and with the particular pair of strains used (4 days for most combinations in *Aspergillus nidulans* and in *A. niger*).

The growth rate on agar medium of *Penicillium chrysogenum* is about one-fifth of that of *Aspergillus nidulans*. It was therefore expected that the time of incubation necessary for the escape on MM agar of heterokaryotic growth would be much longer. In fact, in the unsuccessful attempts mentioned above, after 2 or 3 weeks the MM plates would begin to show widespread growth, but this proved to be syntrophic, not heterokaryotic. The conditions making it possible for balanced heterokaryotic mycelium to become established to the exclusion of, or in equilibrium with, homokaryotic mycelium have been discussed by Pontecorvo (1953b). It appeared likely that the failure just mentioned might be due to the very low growth rate of *P. chrysogenum*. A growth rate small compared to the rate of diffusion of metabolites may not confer on the heterokaryon an advantage over the two syntrophically growing homokaryons sufficient for the heterokaryon to become established.

This kind of reasoning suggested two ways out. First, the use of pairs of strains with a very much decreased growth rate not capable of being restored to normal by the supply of growth factors; the possibility of syntrophism would thus be excluded. Strains of this type constitute an appreciable proportion of those obtained after irradiation and are identified by the tiny colonies they form even on CM agar. They will be referred to as ‘dwarf’ strains. Balanced heterokaryons with normal growth rate, formed between pairs of dwarf strains, have been described in other species (Dodge, 1942; Pontecorvo, 1947). Dodge’s work gave, in fact, the first example of balanced heterokaryosis.

Secondly, the use of pairs of strains with more than one growth factor requirement, in order to make their metabolic interdependence in the absence of the growth factors more interlocked, and therefore presumably favouring intracellular (heterokaryotic) rather than intercellular (syntrophic) co-operation. This metabolic interlocking might be particularly effective when the growth factor requirements of the two strains are metabolically related in pairs, as in the case of different blocks on the same pathway of synthesis; e.g. requirement of adenine or hypoxanthine versus adenine only; for methio-
Parasexual recombination

nine or cystine versus methionine only. Both types of strain combinations were used and both led to the successful isolation of balanced heterokaryons, as presently described.

_Balanced heterokaryons between dwarf strains._ The first combination attempted between two dwarf strains was that of 22y dw 1 and 7w dw 4, i.e. of one dwarf strain with yellow conidia and requiring adenine or hypoxanthine, and another dwarf strain with white conidia and requiring cystine or thiosulphate. On MM agar, strain 7w dw 4 does not grow at all; strain 22y dw 1 grows to a barely visible size (Pl. 1, fig. 1). On CM agar 7w dw 4 forms very tiny dome-shaped colonies and 22y dw 1 irregularly shaped colonies, highly convoluted and seldom growing beyond 1 cm. diameter (Pl. 1, fig. 1).

The balanced heterokaryon was obtained by incubating for 5 days in a test-tube in liquid CM a thick mixed suspension of conidia of the two strains, removing the mycelium formed, teasing this out on the surface of MM agar plates and incubating for a further 9 days. After this time a little growth had occurred at various points, and out of some of these there grew small tufts of more vigorous mycelium with white heads. Isolation of growing tips from these tufts on to MM agar plates gave uniformly good growth with macroscopically white sporing surface (Pl. 1, fig. 1). Isolation on to CM agar gave mainly white growth of the kind just mentioned, alternating with patches obviously of 22y dw 1 type.

That most of the mycelium on MM agar, and most of the whitish regions of the mycelium on CM agar, were heterokaryotic and balanced was suggested by their growth rate and non-dwarf habit, and was proved by the following tests: (1) successive transfers both on MM agar and on CM agar, of hyphal tips from heterokaryons grown on MM or on CM, gave growth with the characters just described; (2) a proportion of single hyphae isolated from heterokaryous gave origin again to heterokaryotic growth; (3) platings of conidia from colonies derived from single hyphae gave the two types of parental strains: 22y dw 1 and 7w dw 4 (Table 2).

_A second combination of dwarf strains was prepared from 7w dw 5 and 38y dw 6. The former has white conidia and requires cystine or thiosulphate; the latter has yellow conidia and requires NH$_4^+$ or NO$_3^-$. In this case, the heterokaryons were established by isolating small bits of more vigorous mycelium which arose at the region of contact between colonies of the two types grown on CM agar. These more vigorous tufts were quite evident after_

Table 2. _Plating on supplemented medium of conidia from balanced heterokaryons in order to recover the two component types_

<table>
<thead>
<tr>
<th>Balanced heterokaryon</th>
<th>Medium</th>
<th>Yellow No.</th>
<th>Yellow %</th>
<th>White No.</th>
<th>White %</th>
</tr>
</thead>
<tbody>
<tr>
<td>22y dw 1 + 7w dw 4</td>
<td>CM</td>
<td>222</td>
<td>78.2</td>
<td>62</td>
<td>21.8</td>
</tr>
<tr>
<td>22y 13 + 7w 16</td>
<td>MM + adenine + methionine</td>
<td>16</td>
<td>8.5</td>
<td>171</td>
<td>91.5</td>
</tr>
</tbody>
</table>
12 days from the inoculation of the plates. Evidently, even on CM agar, the heterokaryon between the two dwarf strains used had a sufficient selective advantage over either dwarf component to be able to escape from the homokaryotic mycelia.

Mass hyphal transfers on to MM agar from these more vigorous tufts grew at a rate approaching that of the wild type and developed a macroscopically yellowish sporing surface. No further work was carried out with this combination.

Following the notations used in previous papers (e.g. Pontecorvo, 1953b), heterokaryons are designated by the code numbers of the components, joined by the sign +. Thus the two heterokaryons mentioned so far will be designated: $22y \, dw1 + 7w \, dw4$ and $7w \, dw5 + 38y \, dw6$, respectively.

**Balanced heterokaryons between strains each having two nutritional requirements.** Balanced heterokaryons were synthesized between strains $22y \, 13$ (yellow, requiring adenine or hypoxanthine, and methionine; inhibited by cystine) and $7w \, 16$ (white, requiring methionine or cystine or thiosulphate, and adenine; inhibited by hypoxanthine). Neither of these two strains shows any growth on MM agar and their conidia do not even germinate on it. They both grow well on MM agar supplemented with the respective pairs of growth factors; $22y \, 13$ grows well and $7w \, 16$ grows poorly on CM agar.

The balanced heterokaryons $22y \, 13 + 7w \, 16$ were obtained in the same way as in the case of $7w \, dw5 + 38y \, dw6$, i.e. by isolation on to MM agar of tufts of vigorous mycelium arising from the region of contact between colonies of the two strains growing on CM agar. The selective advantage of the heterokaryon on CM agar may perhaps depend, in this case, on overcoming the inhibitions mentioned above.

The heterokaryon grows well on MM agar; macroscopically its sporulating surface is yellowish. The same three tests mentioned before gave proof of the heterokaryotic condition: (1) perpetuation of the heterokaryon by mass transfers of hyphae on MM agar; (2) perpetuation of the heterokaryon by single hypha isolation; (3) recovery of both component strains by plating conidia of colonies grown from single hyphae (Table 2). An additional visual test was that on addition of the appropriate growth factors to MM agar, the white, the yellow or both components would sector out.

**Properties of heterokaryons.** The balanced heterokaryons of *Penicillium chrysogenum*, in contrast with those of other species (*Aspergillus nidulans*, Pontecorvo, 1953b; *A. niger*, Pontecorvo et al. 1953) can be maintained by massive transfers from the sporing surface. As in those other species, the conidia of *P. chrysogenum* are uninucleate (Tonolo & Urbani, 1952). Thus in a heterokaryon/nuclei of each kind should segregate when the conidia are formed. That this is substantially so is shown by the results of platings of conidia at density of the order of 100 conidia/plate (Table 3). However, when platting massive numbers of conidia, rare heterokaryotic colonies arise within 48 hr., at the rate of a few per $10^8$ plated conidia (Table 3; Pl. 1, figs. 2, 3). It is probable that these colonies originate from bits of heterokaryotic mycelium, accidentally present in the inoculum.
Parasexual recombination

As mentioned before, one of the three heterokaryons formed \((22y\ dw1 + 7w\ dw4)\) has macroscopically a white sporing surface; the other two have yellowish surfaces. The structure of the penicillus in \(Penicillium\ chrysogenum\), with loose and divergent conidial chains, makes it impossible to tell whether chains of different colour arise from the same conidiophore. Furthermore, the two colours (white and yellow) are not sharply distinguishable under the microscope. Nevertheless, the fact remains that all heterokaryons have, in different proportions, both white and yellow penicilli but no green ones, while diploids (see below) have green penicilli. The colour of individual conidia seems thus to depend upon the kind of nucleus segregated into each of them. In this respect the colour markers used in \(P.\ chrysogenum\) behave more like those used in \(Aspergillus\ nidulans\) (Pontecorvo, 1953b) than like those used in \(A.\ niger\) (Pontecorvo et al. 1953).

### Table 3. Heterozygous diploids obtained from conidia of balanced heterokaryons plated in minimal medium

<table>
<thead>
<tr>
<th>Balanced heterokaryons</th>
<th>Plated conidia</th>
<th>New hetero-karyotic colonies arising*</th>
<th>Whole colonies</th>
<th>Sectors from heterokaryotic colonies</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>No. per dish</td>
<td>Per 10^6 plated conidia</td>
<td>Per 10^7 plated conidia</td>
</tr>
<tr>
<td>22y\ dw1 + 7w\ dw4</td>
<td>77 x 10^6</td>
<td>2.6 x 10^6</td>
<td>769</td>
<td>10</td>
</tr>
<tr>
<td>22y 13 + 7w 16</td>
<td>8.4 x 10^6</td>
<td>0.6 x 10^6</td>
<td>1083</td>
<td>138</td>
</tr>
</tbody>
</table>

* When plating conidia of balanced heterokaryons at densities of the order of 10^6 per plate a few tens of colonies arise within 48 hr. on MM. They turn out to be balanced heterokaryons, presumably arising from bits of mycelium or from rare bi- or multinucleate conidia carrying nuclei of the two kinds. Macroscopically their sporing surface is white in the case of heterokaryon (1), or yellowish in the case of heterokaryon (2), and their morphology normal (Pl. 1, figs. 2, 3).

† Diploids are identified either as green spored colonies of normal morphology and growth rate or as green sectors out of the newly arising heterokaryons (Pl. 1, figs. 2, 3).

**Isolation of heterozygous diploids**

**Diploid from heterokaryons \(22y\ dw1 + 7w\ dw4\).** From heterokaryons grown for \(2-3\) weeks on MM agar, conidia (washed twice in water) were plated on MM agar at the rate of \(2.6 \times 10^6\) conidia/plate. After \(48\) hr. a mean of about twenty-eight colonies/plate were visible. After a few days' further incubation all but two of these colonies developed a macroscopically visible whitish sporing surface; tests on about thirty of them showed that they were heterokaryons, as mentioned before (Table 3). Two, however, grew better than the others and developed green penicilli. Isolation, purification by single conidium micromanipulation, and further tests (see later) showed these two colonies to be the desired heterozygous diploids. Diploids are designated by interposing a fraction sign between the symbols of the strains associated in the heterokaryon which gave them origin:

\[
\frac{22y\ dw1}{7w\ dw4}, \quad \text{or} \quad \frac{22y\ dw1}{7w\ dw4}.
\]
On keeping the plates for 2 weeks or more, about 1% of the heterokaryotic colonies produced one macroscopically visible green sector (Table 4; Pl. 1, fig. 2). Isolation and tests of strains from such sectors shows them also to be diploids. Incidentally, after such prolonged incubation the plates show a background growth of minute colonies (Pl. 1, fig. 2) of type 22y dwI, presumably adapted; these minute colonies represent about 5% of the conidia of this type present in the plated suspension (Table 4).

Table 4. Properties of heterozygous diploid 22y dwI/7w dw4 compared with those of the heterokaryon, of the two marked haploid strains, and of the original haploid wild type Q176 (see also Pl. 1, fig. 1)

<table>
<thead>
<tr>
<th>Strain</th>
<th>Growth habit on CM</th>
<th>Individual conidia</th>
<th>Single conidia</th>
<th>Hyphae</th>
<th>Segregation through conidia and sectors</th>
</tr>
</thead>
<tbody>
<tr>
<td>Q176</td>
<td>Normal</td>
<td>Green</td>
<td>None</td>
<td>None</td>
<td>Parental types, except for very rare diploids (10^-7)</td>
</tr>
<tr>
<td>22y dwI</td>
<td>Dwarf</td>
<td>Yellow</td>
<td>Hypox.</td>
<td>Hypox.</td>
<td>Small proportion (10^-3) of parental and new types</td>
</tr>
<tr>
<td>7w dw4</td>
<td>Dwarf</td>
<td>White</td>
<td>Cystine</td>
<td>Cystine</td>
<td></td>
</tr>
<tr>
<td>22y dwI + 7w dw4 (heterokaryon)</td>
<td>Normal or white</td>
<td>Hypox. or cystine</td>
<td>None</td>
<td></td>
<td></td>
</tr>
<tr>
<td>22y dwI/7w dw4 (diploid)</td>
<td>Normal</td>
<td>Green</td>
<td>Giant</td>
<td>None</td>
<td></td>
</tr>
</tbody>
</table>

**Diploid from heterokaryon 22y 13 + 7w 16.** From heterokaryons grown for about 2 weeks on MM agar, conidia (washed twice in water) were plated on MM agar at the rate of 0.6 x 10^6 conidia/plate. The results were similar to those of the previous example with the difference that, (a) the newly arisen heterokaryotic colonies had, macroscopically yellowish sporing surface; (b) relative to the number of conidia plated, the proportions of newly arisen heterokaryotic colonies and of the green diploid colonies was about ten times higher (Table 4); (c) the green sectors from the heterokaryotic colonies were about twice as frequent; (d) there was no delayed background growth of parental types (Pl. 1, fig. 3). Isolation from one of the green colonies, purification by single conidium micromanipulation and further tests led to the establishment of diploid 22y 13/7w 16.

**Properties of diploids.** The diploids differ from the heterokaryons, from the haploids which formed the heterokaryons and from the haploid wild type in a number of ways. These have been tabulated (Table 5) for the case of diploid 22y dwI/7w dw4 and some of them are evident on Pl. 1, fig. 1. These differences also apply, mutatis mutandis, to the case of diploid 22y 13/7w 16. The size of the conidia is not easily measurable because of considerable variations in shape. However, a visual comparison (Pl. 1, fig. 4) leaves no doubt that diploids have larger conidia, a fact suggesting that Sansome (1949) was right when she deduced that the ‘gigas’ strains isolated by her in the closely related species Penicillium notatum were (homozygous) diploid. As diploids heterozygous for nutritional...
requirements, colours of conidia and habit of growth, show features approaching those of the wild type (no requirement, green conidia and normal growth) all the mutant properties used as markers in this work are recessive. In the diploids segregation and recombination take place, as shown in detail in the next section, i.e. a small proportion of individual conidia or hyphae of the diploids give rise to strains different from the diploid from which they arose, in that they show one or more properties of the original marked strains associated either in the original way or in new ways.

Table 5. First-order segregants from diploid:

<table>
<thead>
<tr>
<th></th>
<th>Requiring Prototrophs</th>
<th>Requiring hypoxanthine</th>
<th>Requiring cystine</th>
<th>Requiring both</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>A. Obtained as whole colonies*</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Green</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dwarf</td>
<td>5</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>5</td>
</tr>
<tr>
<td>Normal</td>
<td>964</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>965</td>
</tr>
<tr>
<td>Yellow</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dwarf</td>
<td>2</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>3</td>
</tr>
<tr>
<td>Normal</td>
<td>2</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>2</td>
</tr>
<tr>
<td>White</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dwarf</td>
<td>6</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>6</td>
</tr>
<tr>
<td>Normal</td>
<td>12</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>12</td>
</tr>
<tr>
<td>Non-sporing</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Normal</td>
<td>17</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>17</td>
</tr>
<tr>
<td></td>
<td>1008</td>
<td>2</td>
<td>0</td>
<td>0</td>
<td>1010</td>
</tr>
</tbody>
</table>

| B. Obtained as sectors† |                       |                        |                   |               |       |
| Green              |                       |                        |                   |               |       |
| Dwarf              | 0                     | 0                      | 0                 | 0             | 0     |
| Normal             | 0                     | 0                      | 1†                | 0             | 1     |
| Dwarf              | 1                     | 0                      | 0                 | 0             | 1     |
| Yellow             |                       |                        |                   |               |       |
| Normal             | 5                     | 0                      | 0                 | 0             | 5     |
| White              |                       |                        |                   |               |       |
| Dwarf              | 0                     | 0                      | 0                 | 0             | 0     |
| Normal             | 14                    | 0                      | 0                 | 0             | 14    |
|                    | 20                    | 0                      | 1                 | 0             | 21    |

* Conidia of 22y dw 1/7w dw 4 were plated on CM, and 1010 colonies were classified as to colour, morphology and requirements.
† Sectors, differing in colour or growth habit from the mother colonies, were isolated from a proportion of the 964 green, normal, prototroph colonies, and classified as to colour, morphology and requirements.
‡ Though this sector had green conidia and non-dwarf habit, it was isolated because of its rather thinner growth than the rest of the mother colony: it turned out to be cystine-requiring.

Segregation and recombination in diploids

In Aspergillus nidulans and A. niger (Pontecorvo & Roper, 1953; Pontecorvo et al. 1953; Pontecorvo et al. 1954) mitotic segregation and recombination occurs once about every 100 divisions in heterozygous diploids. Its detectable result is the production of diploid nuclei homozygous for one or more of the recessive
markers or of haploid nuclei with one or more of the recessive markers. A patch of mycelium homokaryotic for one of these nuclei (for short, ‘segregant’ nuclei) will have the recessive marker property or properties. When these properties are detectable by inspection (e.g. colour of conidia) the patch of mycelium may be identified visually and a strain isolated from it. In the two other species mentioned above this visual identification is easy even in the case of a single head in a background of thousands with the wild type colour. In *Penicillium chrysogenum* individual heads differing in colour from the background are difficult to recognize, and only patches of homokaryotic segregant mycelium with a substantial number of segregant heads can be identified visually and isolated.

Thus one way of detecting mitotic segregation and recombination of colour markers is to examine a number of diploid colonies (preferably originated from single conidia) for the presence of sectors with penicilli of one of the recessive colours; isolation from these patches will permit the classification of each segregant in respect of other properties also (e.g. nutritional requirements, growth habit, etc.). Another way in which segregation, not only of colour markers but of any markers, can be recognized is by plating conidia of the diploid and classifying individually the resulting colonies. This classification can be visual as to colour of conidia and growth habit, but requires the testing of individual colonies for nutritional requirements. Segregation and recombination was analysed extensively in the case of diploid *22y dw1/7w dw4* (see later). As to diploid *22y13/7w16*, we only went so far as to observe that it did segregate for colour markers. An extensive analysis of this and other diploids was carried out later at the Istituto Superiore di Sanità, Rome, by one of us (G.S.), and it will be the subject of a separate publication.

**First-order segregants.** First-order segregants from diploid *22y dw1/7w dw4* were obtained by plating conidia of a subculture (purified by single conidium micromanipulation) on fully supplemented medium; 1010 colonies (Table 5) from this plating were transferred to plates of minimal agar. After 2 days those colonies which showed no further growth were picked out and tested for nutritional requirements and colour. The remaining colonies were transferred back on to supplemented medium, kept until the colour appeared on the sporing surface, and then classified for colour and morphology.

A proportion of the 964 green prototroph normal colonies (out of the total of 1010) showed sectors; some were yellow, some white and some non-sporulating or with a growth habit different from that of the mother colony. Twenty-one of these sectors were isolated, each from a different colony, and further tested. Table 5 shows the classification of the forty-six segregants obtained as whole colonies after plating, and of the twenty-one segregants detected as sectors in some of the colonies arising from platings. The proportion of segregants among the conidia of the diploid is about 5% (46/1010). No recombination of parental recessive characters occurred among the sixty-seven first-order segregants isolated either as colonies or as sectors (Table 5).

**Second-order segregants.** The first-order segregant with green conidia, normal growth and hypoxanthine requirement was tested for further segregation after
Parasexual recombination

purification by single conidium isolation. One yellow and two white sectors from colonies of this strain gave yellow or white, hypoxanthine-requiring strains, with normal growth. A white hypoxanthine-requiring strain is an example of recombination between recessive properties of the starting strains.

CONCLUSIONS

The experiments reported show the occurrence in *Penicillium chrysogenum* of the parasexual processes already found in two other species of filamentous fungi. In *Penicillium* the analysis has gone only so far as to identify three out of the four steps which were studied in detail in the two other species, namely: (1) formation of heterokaryons; (2) formation of heterozygous diploid nuclei within heterokaryons; (3) recombination in the diploid nuclei. We have not yet looked for and identified the formation of haploid nuclei from diploid nuclei (Pontecorvo *et al.* 1953a; Pontecorvo, 1954). As, however, this is a consequence of accidents of mitosis of a type known to occur in all higher and lower organisms in which it has been looked for, it is a reasonable assumption that it will be found also in *Penicillium*.

*Penicillium* thus may have, like the two other species, a complete parasexual cycle (Pontecorvo, 1953a). This involves alternation of haploid and diploid stages, the possibility of storing gene variation under the cloak of dominance in both heterokaryons and heterozygotes and the possibility of gene recombination.

Because of its low growth rate and certain details of its morphology, *Penicillium chrysogenum* is a species much less suitable than *Aspergillus nidulans* or *A. niger* for the detailed analysis of the parasexual processes themselves. It is, however, a species of great economic importance. The discovery of the parasexual processes in it is relevant in two respects. First, this third species of filamentous fungus again shows the occurrence of these parasexual processes; thus they would appear to be widespread at least. Secondly, the parasexual processes in *P. chrysogenum* can be used for practical purposes: (a) deliberate 'cross-breeding' for the production of improved industrial strains; (b) the identification of different genetic blocks in the analysis of the biosynthesis of penicillin.

One of us (G. S.) is indebted to the Istituto Superiore di Sanità, Rome, for a grant enabling him to take part in this work at the Department of Genetics, University of Glasgow. This work is part of a general programme supported by the Nuffield Foundation.

REFERENCES


G. Pontecorvo and G. Sermonti


**EXPLANATION OF PLATE**

Fig. 1. +, the haploid green wild type; 1, the haploid dwarf, yellow, hypoxanthine-requiring 22y dw1; 4, the haploid dwarf, white, cystine-requiring dwe4; HK, the heterokaryon between 22y dw1 and 7we dwe4; D, the diploid derived from the heterokaryon. Left: on complete medium. Right: on minimal medium. White circles indicate the positions of inoculum on MM of the two dwarf strains.

Fig. 2. Plating on MM of conidia of heterokaryon 22y dw1 + 7we dwe4 (2·6 x 10⁶ dish): there are about twenty heterokaryotic colonies (large) per dish, and a background of minute colonies of type 22y dw1. About 1% of the heterokaryotic colonies show, like the one in the centre, a green diploid sector.

Fig. 3. Plating on MM of conidia of heterokaryon 22y 13+7we 16: results similar to those of Fig. 2, but no background growth. Two heterokaryotic colonies show green diploid sector.

Fig. 4. Conidia of heterokaryon 22y dw1 + 7we dwe4 (left) and of the diploid derived from it (right) about x 400.

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THE PARASEXUAL CYCLE IN FUNGI

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INTRODUCTION

The present article is an essay rather than a review. The discovery in 1952 by Pontecorvo & Roper (1) of parasexual recombination in Aspergillus nidulans, i.e., recombination of hereditary determinants outside the sexual cycle, has been followed by research on two questions. First, which processes underlie what we now know to be a cyclical series of events, the parasexual cycle? Second, how widespread is the parasexual cycle among filamentous fungi, and what part does it play in their genetic systems, particularly in the case of species in which the sexual cycle is not known to occur? There is a corollary to the second question, relevant to the applied fields of phytopathology and industrial fermentations: what is the bearing of the parasexual cycle on variation in nature and in the factory? In nature because it might, and indeed Buxton's work (2) suggests that it does play a part in the variation of host-pathogen relationships; and in the factory because it might, and the work of Caglioti & Sermonti (3) shows that it can be harnessed to the “breeding” of more productive strains.

With the exception of work on heterokaryosis, which we now know to be one of the steps in the cycle, practically all the published work on the parasexual cycle has been done either in our Laboratory or by workers previously associated with it, at the Istituto Superiore di Sanità, Rome, and at the Plant Pathology Department, Rothamsted Experimental Station.

Research on the first question has led to an understanding of the sequence of steps in the cycle, and to a working knowledge of some of these steps (4, 5). This working knowledge suggests new ways of investigation of the phenomenon observed by Buller (6), Quintanilha (7), Papazian (8), and Raper (9) in the Basidiomycetes, i.e., the formation of nuclei of new types in dikaryons. Furthermore, this knowledge is already sound enough for use in the mapping of chromosomes. In A. nidulans, for instance (5), certain operations for mapping are more conclusive and less laborious if the analysis is through the parasexual cycle than if it is carried out in the usual ways through the sexual cycle.

Research on the second question has ascertained that the parasexual cycle occurs in the asexual species Aspergillus niger (10, 11), Penicillium chrysogenum (3, 12) and Fusarium oxysporum (2), besides the homothallic A. nidulans in which it was first found. Recombination also occurs in Streptomyces coelicolor (13), but here it is not yet known whether or not the underlying process is the same as in the four species of fungi mentioned above. In short, a search for the parasexual cycle in four species of filamentous fungi.

1 The survey of literature pertaining to this review was completed in January, 1956.
has found it in all of them: it is legitimate to conclude that it cannot be a rare oddity.

As to the part which the paraseuxal cycle plays in nature in the systems of heredity and variation of the species in which it occurs, there is only the remarkable work on *F. oxysporum* to quote (2). Variation in natural populations is a huge field of research in which the mycologist, including the industrial mycologist, and the phytopathologist should have a more direct interest than the geneticist. Yet at a discussion at the New York Academy of Sciences as recently as 1954, of fourteen papers on the topic "Speciation and Variation in asexual fungi" (14), only one realised what a change in outlook is imposed by the existence of the paraseuxal cycle.

**The Elements of the Paraseuxal Cycle**

The paraseuxal cycle is known in great detail in *A. nidulans* (15), where the existence of a sexual cycle permits a thorough control of each step by means of standard genetic techniques. In the other species mentioned before, the complete cycle has been identified in *P. chrysogenum* (3, 12, 15, 16, 17).

In *A. niger* and *F. oxysporum* some of the steps are not yet quite established.

The steps of the cycle are the following: (a) Fusion of two unlike haploid nuclei in a heterokaryon. (b) Multiplication of the resulting diploid heterozygous nucleus side by side with the parent haploid nuclei in a heterokaryotic condition. (c) Eventual sorting out of a homokaryotic diploid mycelium which may become established as a strain. (d) Mitotic crossing over occurring during multiplication of the diploid nuclei. (e) Vegetative haploidisation of the diploid nuclei.

Events (a), (d) and (e) occur at rather low rates. For the sake of orienting the reader, let us say: 1 in $10^6$ or $10^7$ for fusion of two unlike haploid nuclei in a heterokaryon, 1 in 500 for the incidence of crossing over per diploid nucleus and 1 in 1000 for the haploidisation of diploid nuclei. The actual variation in these figures from one combination of strains to another and from one species to another is very large. However, they reveal one of the reasons why diploid strains have not been identified in nature and why they are unlikely to be identified without a deliberate selective search: in the absence of selection against haploids these should outnumber the diploids as the ratio of the rate of haploidisation versus that of fusion, i.e., 1000 to 1.

**Fusion of unlike nuclei.**—This process is inferred from the fact that, in a heterokaryon, diploid nuclei arise which are heterozygous for all the markers in which the two types of haploid nuclei present in the heterokaryon differed. In fact the original technique by Roper (18) for the synthesis, identification, and isolation of diploids was precisely based on the expectation, on general grounds, that a mycelium heterozygous for known markers should show certain properties. These properties are expected to be different, in predictable ways, from those of the two mycelia carrying either of the two kinds of haploid nucleus or from those of the herokaryon itself. In the laboratory it is only a matter of choosing, for the formation of a heterokaryon, a suitable
THE PARASEXUAL CYCLE IN FUNGI

combination of strains differing in known nuclear markers. For instance, if the two strains, by virtue of two different mutant presumably recessive alleles, require two different growth factors, the heterokaryon and the diploid heterozygote derived from it will require neither, but the heterokaryon and the heterozygote will be distinguishable in one important respect. The heterokaryon, carrying nuclei of two kinds, will give origin to a proportion of hyphae homokaryotic of either kind. There is more: in a species with uninucleate conidia the two kinds of nuclei will be invariably sorted out in the formation of conidia. In the heterozygote, on the other hand, neither type of segregation will occur, except for the rare mitotic segregation and recombination to be described later. The conidia from the heterokaryon will not grow on nonsupplemented medium, but if among them there is an odd one carrying a diploid heterozygous nucleus, this one will be able to form a colony. Under such selective conditions, the diploid may also become established as a sector out of a heterokaryotic colony: this is of common occurrence with \textit{A. niger} (11) and \textit{P. chrysogenum} (12).

The heterozygous diploids, besides having the phenotypic properties expected on general grounds and segregating mitotically, as described below, have a volume and deoxyribonucleic acid content of uninucleate conidia double that of haploid conidia (4, 18, 19).

Nothing is known of the way in which fused nuclei arise. We only know that they do arise and crude measurements of the rate at which they arise are available in some cases (15). It would be interesting to know something about this process, which may well be only a rare accident, but this knowledge is not essential to an understanding of the parasexual cycle, just as a knowledge of the details of fertilisation is not essential for understanding the biological consequences of sexual reproduction.

Recently Elliott (20) has synthesised triploid strains of \textit{A. nidulans} by applying Roper's technique to heterokaryons between haploids and diploids. These diploids must, of course, be homozygous for one or more recessive markers, suitable for selection.

\textit{Mitotic crossing over.}—During multiplication, heterozygous diploid nuclei give origin occasionally to nuclei still diploid but homozygous for one or more of the markers previously heterozygous. Early in the work (1, 10, 11, 18, 21) it became clear that similarly to what Stern (22) found in \textit{Drosophila}, mitotic crossing over could account for this segregation. This became certain after investigating diploids of \textit{A. nidulans} heterozygous for linked markers, the locations of which along one chromosome were known (4). Since then work with a large number of different diploids has amply confirmed this conclusion. The matter has also been clinched by the recovery of the two complementary products of mitotic crossing over in single nuclei (23, 24).

Mitotic crossing over produces from, say, one out of 500 heterozygous nuclei A/a, two daughter nuclei A/A and a/a. If the nuclei are heterozygous for a number of linked markers, segregation in any one nucleus occurs only for the markers distal to the position of exchange in that nucleus, e.g.:
The proximal markers (i.e., those between the centromere and the position of exchange) remain unaffected, and so do those on the other arm of the same chromosome pair and those on different chromosome pairs.

The incidence of exchanges is sufficiently rare to permit us to disregard the occurrence of more than one exchange in any one nucleus. This means that the order of genes along the chromosomes can be determined easily by means of mitotic crossing over, and even more unequivocally than by means of meiotic crossing over (5).

The positions of the centromeres can also be determined. In the example just given, if we assume that the centromere is between the loci c/C and d/D, then homozygosis at the d/D locus would always go with homozygosis at e/E and f/F but not at a/A, b/B or c/C, and homozygosis at c/C would always go with homozygosis at b/B and a/A but not at d/D, e/E or f/F.

In *A. nidulans* mapping of two chromosomes by means of mitotic crossing over has already led to the building of "mitotic" maps with a total of 12 loci and two centromeres (5). These maps confirm absolutely the linear order of loci and centromeres determined independently by means of meiotic analysis via the sexual cycle.

In view of the relative rarity of mitotic crossing over, an essential point for mitotic mapping is to have available a number of markers suitable for selection. For example these can be "visible" markers, such as differences in colour of the conidia detectable even in a small number of heads. Or they may be markers conferring to the mycelium homokaryotic for one allele properties which lend themselves to selection: for instance, homozygotes for an allele conferring resistance to Acriflavine (25) are much more resistant than heterozygotes; hence the homozygotes arising by mitotic crossing over from heterozygotes can be selected as vigorous sectors out of heterozygous colonies grown in the presence of Acriflavine.

Another type of useful markers are "suppressors," i.e., recessive alleles restoring the ability to grow in the absence of a particular growth factor to strains homozygous for mutants determining requirement for that growth factor. In this case a diploid heterozygous for the "suppressor" and homozygous for the allele determining the requirement requires the growth factor, but a homozygote for the suppressor, produced by mitotic crossing over does not, and can therefore be selected on a medium lacking the growth factor (5, 24).

In the genetics of phytopathogenic fungi, genes affecting the host range can conceivably be used as selective markers.
The absolute incidence per nucleus of mitotic crossing over is 500 to 1000 times lower than that of meiotic crossing over. The question then arises of whether or not the relative distribution along any one chromosome is the same for mitotic and meiotic crossing over. A first answer to this question is available (5): the relative distribution along two chromosomes of *A. nidulans* with a total of nine intervals studied is definitely different at meiosis as compared with mitosis. The work so far does not reveal any general feature in this difference. In two arms there is a relative excess of mitotic crossing over in an interval of about 20 units next to the centromere but in a third arm a gross excess is in an interval of 10 units starting 20 units away from the centromere.

It has been suggested (5, 26) that analysis via mitotic crossing over in tissue cultures might be used for mapping the chromosomes of slow breeding higher organisms, including man.

All the detailed work on the process of mitotic crossing over has been done with *A. nidulans* because in this species it is possible to check any deduction by means of ordinary genetic analysis. In species in which a sexual cycle is unknown this cross check is impossible. For this reason the preliminary work with *A. niger* (11) and *P. chrysogenum* (12) did not even attempt to use mitotic crossing over for identifying linkage groups and constructing chromosome maps.

The knowledge gained in *A. nidulans* by the analysis in parallel via mitotic and via meiotic crossing over has now made it possible to start this kind of work with completely asexual species. The first linkage group in an asexual species, *P. chrysogenum*, has been identified by Sermonti (27).

Haploidisation.—Segregation of the markers for which a diploid is heterozygous occurs during vegetative multiplication not only as a consequence of mitotic crossing over. It is also the consequence of a process of haploidisation in which whole chromosomes, but not chromosome parts, re-assort at random (4).

The details of the process of haploidisation are almost completely unknown. In *A. nidulans* diploid nuclei produce haploids at a rate lower than that at which they undergo mitotic crossing over. For instance, diploids homozygous for the most distal marker in one chromosome arise anything from 1.5 to 10 times as frequently as haploids carrying the marked chromosome.

A working hypothesis used for planning further work was that haploidisation is the consequence of accidental breakdown at mitosis in the separation of the chromatids to the two poles: *in a proportion of cases* one daughter nucleus arises with a single set of chromosomes (4). Breakdowns of this kind are well known in higher organisms.

Two of the testable consequences of this hypothesis are that the same process which produces haploid nuclei should also: (a) produce diploid nuclei homozygous for whole chromosomes, and (b) produce aneuploid nuclei. Both predictions have been verified in unpublished work of Pontecorvo &
Käfer on *A. nidulans*. The quantitative aspects, however, are such that these results do not constitute as good evidence as they would seem in support of the working hypothesis. Briefly, it is found that diploids homozygous for a whole chromosome, well marked on both arms, are homozygous for other chromosomes less frequently than expected from random distribution.

On the other hand the use of diploids marked on several chromosomes has shown that the majority of haploids originate as aneuploids, i.e., they are “monosomic” for some chromosomes but still “disomic” for others. This imbalance puts a strong premium on nuclei which eliminate the extra chromosomes, so that the aneuploid repeatedly throws out more vigorous sectors which remain constant thereafter. Analysis of these sectors shows that in a proportion of cases the disomic chromosome pair was still heterozygous. The situation is in most respects the same as that of the *Neurospora* disomics (28).

It is clear that a considerable proportion of the products of haploidisation must be imbalanced and nonfunctional and therefore be eliminated. The process of haploidisation is at present under study, both by means of diploid strains marked on both members of each chromosome pair and by means of triploids.

**Significance of the Parasexual Cycle**

The use of markers has permitted to follow through heterokaryosis, diploid heterozygosis, and haploidisation the fate of various parts of the genotype. The result of the parasexual cycle is that starting from a culture containing a mixture of genetically different haploid strains, one would end up ultimately with a much more varied mixture. The latter would include: (a) haploid strains like the starting ones; (b) haploid strains which have recombined in all possible ways the chromosomes and chromosome parts of the starting strains, and (c) a small proportion of diploid strains homozygous and heterozygous for all possible associations and recombinations of markers of (a) and (b). It is not to be excluded that also a small proportion of triploids, and higher polyploids, would be present. The actual proportions of the various strains would be determined in any one case by the external conditions which would keep the less fit combinations at low levels.

Clearly a genetic system based on the parasexual cycle has all the elements which are found in a genetic system based on sexual reproduction, and some novel ones. Within a population, it provides for the storage, both in heterokaryotic and heterozygotic condition, of a large amount of gene diversities originated by mutation or by any other mechanism which may produce such diversities. It also provides for the re-assortment of all these diversities both in haploid and in diploid condition, ready for the sieve of natural (or artificial) selection.

The main novel features of the parasexual cycle are two. One is that though it is made up of steps which, considered together, are more or less like those of the sexual cycle, the precise sequence of these steps is not fixed as in
the sexual cycle. Moreover, haploidisation is achieved rather wastefully, though it occurs only in a very minor proportion of all diploid nuclei. The second feature is the occurrence, even in one mycelium, of both heterokaryosis and heterozygosis. Heterokaryosis may involve nuclei of the same ploidy or of different ploidies, and even more than two types of nucleus in one mycelium. This joint play of heterozygosis and heterokaryosis permits a latitude in the storing of gene variation vastly greater than in other organisms. For instance, in most higher organisms gene variation can only be stored in diploid heterozygotes, and in the Basidiomycetes, as far as it is known, only in heterokaryons or dikaryons. On the whole, the parasexual cycle seems to be less perfect but more flexible than the sexual cycle.

There is no need to make a case for harnessing such a system in the artificial selection of industrially valuable moulds. Obviously in an asexual species the parasexual cycle can be used for “breeding” improved strains just as the sexual cycle is used for producing better varieties of flowering plants, and, for example, better breeds of dogs and sheep. Technically the difficulties are far smaller in industrial microbiology than in horticulture or animal breeding.

It is legitimate, however, to ask whether the parasexual cycle plays more than an irrelevant part in natural populations. The answer is that we do not know yet, but it would be surprising if a system with potentialities as great as those of sexual reproduction were merely a laboratory curiosity.

Admittedly, variation in the characters used for the laboratory work (growth factor requirements, color of conidii, etc.) has not been so far of the kind which makes up most of the differences between populations in nature. Also admittedly, the very little evidence available as to heterokaryosis in nature shows that while a high proportion of isolates from nature are heterokaryotic, monokaryotic strains collected in different localities more often than not refuse to form heterokaryons with one another (29).

Fortunately variation of a type with the greatest adaptive significance has now been added to the list by the work of Buxton (2). Two races of the pathogen Fusarium oxysporum pisi differ in host range: one is pathogenic for the pea variety “Onward” but not for the variety “Alaska,” and the other for both. Neither race is pathogenic for “Delwiche Commando.” A diploid synthesised by means of Roper’s technique from the two races had the broader host range (both “Onward” and “Alaska”) and of the recombinants from this diploid three were also pathogenic for “Delwiche Commando”: more precisely, two were pathogenic for “Onward” and “Delwiche” though they differed in nutritional requirements, and one for all three, “Onward,” “Alaska,” and “Delwiche.”

The fact that parasexual recombination can determine variation in the host range of pathogens opens a wide field of research both fundamental and applied mainly to phytopathology, i.e., the study of what part recombination plays in the origin of “new” pathogenic races. This question, asked rather academically in 1947 (30), becomes now quite practical.
27. Sermonti, G. (In press)
Proceedings of the Royal Physical Society

Mapping the Chromosomes by Means of Mitotic Recombination. By G. Pontecorvo and Etta Kafer, Department of Genetics, University of Glasgow

(Read at the meeting of 23rd January 1956)

Previous work with heterozygous diploids of Aspergillus nidulans showed that segregant nuclei, and consequently strains, arise vegetatively by means of two distinct and independent processes: one is mitotic crossing over, the other is haploidisation (Pontecorvo, Tarr Gloor and Forbes, 1954). In the formation of haploid nuclei the chromosomes segregate and recombine as units, i.e. with no crossing over between linked genes. The aim of the work reported here was to find out how practicable it is to use mitotic crossing over and haploidisation for mapping the chromosomes (including the location of centromeres) and what differences, if any, there are between the distribution of crossing over along the chromosomes at meiosis and that at mitosis.

1. Locating Centromeres.—Multiple exchanges being rare, mitotic crossing over produces, from a heterozygous nucleus, two daughter nuclei each homozygous for one of the two homologous chromosome segments distal to a position of exchange. In respect of the proximal segment or of the other arm of the chromosome in question the two daughter nuclei remain usually unchanged. Thus if we have four linked markers A, B, C, D, in this order, and we find that homozygosis for C always goes with homozygosis for D but not for A and B, and homozygosis for B always goes with homozygosis for A but not for C and D, we deduce that the centromere is between B and C.

To locate the centromere in the BI chromosome of Aspergillus we have synthesised (Roper, 1952) a diploid of the following constitution:

\[ \text{wm} + \text{pro1} + + \text{bi1} \]
\[ + \text{ad14} + \text{paba1} y \text{bi1} \]

(see further on for symbols).

It was known that the loci pro1, paba1, y and bi1 were all on the same arm, with pro1 nearest to the centromere. The question was whether ad14 was also on that arm and therefore between pro1 and the centromere, or on the other arm, i.e. with the centromere between it and pro1.

As the diploid was homozygous for bi1, it was possible to select segregants homozygous for ad14 by means of the "starvation" technique (Macdonald and Pontecorvo, 1953) applied to conidia of this diploid. Three such segregants were obtained and they were all still heterozygous +/y. Should ad14 have been on the same arm as y, they would all have been homozygous y/y.

Localisation of this centromere was confirmed later by a more usual method. By means of tetrad analysis Mr N. Strickland—who permits us to quote his results—estimated the recombination fraction between the centromere and ad14 as 0.20 ± 0.04, and that between the centromere and pro1 as 0.17 ± 0.02. The recombination fraction between ad14 and pro1 estimated by single strand analysis is known to be about 0.30: hence meiotic analysis has confirmed that the centromere is between the two loci in question.

2. Mapping Mitotically.—Preliminary work (Pontecorvo and Kafer, 1954) with three diploids heterozygous for the same five markers on the BI chromosome in different coupling arrangements gave remarkably consistent results. This suggested that mapping on a larger scale by means of mitotic crossing over was quite feasible and the work was therefore extended to include both arms of the BI chromosome and one arm of another
Mapping the Chromosomes by Means of Mitotic Recombination 17

chromosome (the W chromosome). Furthermore, two new methods of selection of recombinants had become available; we were no longer limited to select recombinants for the “visible” markers only: y (yellow conidia) and w (white conidia), or to use the cumbersome starvation technique. The new selections possible were: (a) of homozygotes for a recessive suppressor (su) of ad20 (Pritchard, 1955): a diploid homozygous ad20/ad20 and heterozygous su/+ is adenine-requirer; segregants from it of su/su constitution are adenine-independent and can therefore be selected on adenine-less medium; (b) of homozygotes for an incompletely dominant (ACR) conferring resistance to acriflavine (Roper, unpublished): a diploid ACR/+ is partially resistant; segregants from it of ACR/ACR constitution are much more resistant and can therefore be selected on acriflavine containing medium.

Two diploids, marked on four chromosomes, were synthesised for this work:

**DIPLOID Y**

\[
\begin{array}{cccccc}
pyrO & chol & ACR & w & + & su \\
+ & + & + & + & + & ad20 \\
\end{array}
\]

**DIPLOID Z**

\[
\begin{array}{cccccc}
pyrO & chol & ACR & + & + & + \\
+ & + & + & + & + & w \\
\end{array}
\]

\[
\begin{array}{cccccc}
+ & + & + & + & + \\
+ & + & + & + & + \\
\end{array}
\]

Symbols of Mutant Alleles.—(a) Requirements: \textit{pyrO} = pyridoxine; \textit{chol} = choline; \textit{ad1} = adenine (absolute requirement); \textit{ad14} = adenine (partial requirement); \textit{ribo} = riboflavine; \textit{an} = aneurine; \textit{pro} = proline; \textit{paba} = p-aminobenzoate; \textit{bi} = biotin; (b) Conidial colours: \textit{y} = yellow; \textit{w} or \textit{w+} = colourless (white), epistatic; \textit{w+y+} = green, wild type.

Because of the dominance relationships, the diploids were wild type (green) in colour and non-requirers of the growth factors, except adenine because of homozygosis for ad20, and showed the partial resistance to acriflavine characterising heterozygotes.

Three selections of segregants were applied to the two diploids, using simple precautions to avoid isolating the same clone twice. These selections were: (1) Selection for recessive colours: by isolation from yellow or white spots identified under the stereoscopic microscope; (2) Selection for “suppressed” ad20: by isolation from vigorous sectors produced by colonies grown on adenineless medium; (3) Selection for full acriflavine resistance: by isolation from vigorous sectors produced by colonies grown on acriflavine-containing medium. The segregants selected in any one of these ways were divided into haploids and diploids, classified as to phenotypes, and the diploids were classified as to genotype by means of analysis through ascospores or through further mitotic segregation.

As to the diploids, selection (1) for yellow segregants identifies crossing over in regions V, VI or VII (Table II) of the “right” arm of the BI chromosome; selection (2) for “suppressed” ad20 segregants identifies crossing over in regions I, II, III or IV of the “left” arm of the BI chromosome; and selection (3) for full acriflavine resistance.
identifies crossing over in regions $a$ or $b$ of the $W$ chromosome. In diploid $Y$, in which the proximal markers are all in coupling with each of the three markers used for selection, the position of crossing over in each arm is deduced directly from the phenotype of each diploid segregant. In diploid $Z$, with all unselected markers in repulsion, it is necessary to ascertain the complete genotype of the relevant arm of each segregant in order to deduce the position of crossing over.

The types and proportions of haploids confirmed the conclusions of Pontecorvo, Tarr, Gloor and Forbes (1954) that in the processes of haploidisation whole chromosomes are reassorted at random. A new observation was that the great majority of haploids do not arise as such but as hyperploids for one or more chromosomes and become soon balanced through elimination of the extra chromosome/s. This matter is being investigated further.

Another new observation concerns the diploids. A proportion of them (about 8 per cent.) are homozygous for one whole chromosome while still heterozygous for the others. Double crossovers across the centromere are unlikely to account for the origin of most of these diploids. It is possible that they are products of the same kind of process which yields the aneuploids-haploids.
Mapping the Chromosomes by Means of Mitotic Recombination

Out of a total of about 1400 diploid segregants analysed, 12 were the result of two crossovers, one in each of two chromosomes. By testing the mycelium which had given origin to 3 out of these 12 segregants, it was possible to ascertain for one that the two crossovers had occurred in two successive steps: it seems likely that this "second order" segregation accounts for most of these double crossovers. Another 2 diploids were the result of two crossovers, one in each arm of the BI chromosome. Finally 1

### TABLE II

**Comparison of Maps of the W and BI Chromosomes of Aspergillus nidulans**

*Based on Meiotic and on Mitotic Crossing over*

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<thead>
<tr>
<th>Units</th>
<th>W chromosome</th>
<th>BI chromosome</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>24 18 50</td>
<td>38 19 7 20 17 8 0 3 5</td>
</tr>
<tr>
<td>Relevant regions</td>
<td>† a b</td>
<td>† I II III IV V VI VII †</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Crossovers per cent. of total crossovers in each arm</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>W chromosome</strong></td>
</tr>
<tr>
<td>(42 units)</td>
</tr>
<tr>
<td>Region</td>
</tr>
<tr>
<td>† a b</td>
</tr>
<tr>
<td>18 82 (139)</td>
</tr>
<tr>
<td>Mean</td>
</tr>
<tr>
<td>Mean</td>
</tr>
</tbody>
</table>

(In brackets: total number of segregants on which per cent. were based)

<table>
<thead>
<tr>
<th>Diploid Y</th>
<th><strong>ACR w</strong></th>
<th>su ribo an</th>
<th>+</th>
<th>+</th>
<th>+</th>
<th>+</th>
<th>+</th>
<th>+</th>
<th>ad20 bi</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>+ + + + + + + + + + +</td>
<td>pro paba y ad20 +</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Diploid Z</td>
<td><strong>ACR +</strong></td>
<td>+ ribo an ad14 +</td>
<td>+</td>
<td>+</td>
<td>y ad20 +</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>+ w + su + + + + pro paba + ad20 bi</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Diploid was the result of two crossovers in the same arm of the BI chromosome or of mutation at the y locus.

Table I gives the numbers of diploid segregants obtained from the two diploids with each of the three selections: they are classified according to the region, in which crossing over occurred. The diploid segregants visually selected for w are omitted because they do not add any information. All were of the type expected from each diploid on the basis that the locus of w is proximal to that of ACR, i.e. the 75 isolated from diploid Y were all ACR/ACR and the 54 isolated from diploid Z were all +/+. From Table I we have omitted 51 diploids homozygous for all the markers of either member of the BI chromosome pair: as said before, these segregants (8 per cent. of all diploid segregants) arise through some process unlikely to be mitotic crossing over.

Table II gives the mitotic crossovers in any one region as the percentage proportion
of the total crossovers in the arm of which that region is a part. It will be noted that there is surprisingly high homogeneity between the results with diploid Y—in which all the markers in each arm were in coupling with the most distal marker used for selection (ACR, su or y)—and those with diploid Z—in which the markers were in repulsion. This shows that differential viability does not affect the results.

At the bottom of Table II, the meiotic map length of each region is also calculated as a percentage of the total meiotic map length of the arm. This makes the data of the meiotic maps comparable to those of the mitotic maps. Clearly the relative distribution of crossing over along the chromosomes is different at meiosis and mitosis. There does not seem to be any systematic trend in this difference. Two out of three of the regions proximal to the centromeres—all about 20 units long—account for relatively more (about twice) crossing over at mitosis than at meiosis, but the third accounts for much less (about 1/7th).

Clearly a much wider survey is required to discover any consistent trend.

Conclusions.——The analysis of mitotic crossing over in two chromosomes involving three arms and nine relevant loci has shown that:

(a) the linear order of loci and centromeres can be determined by this kind of analysis. Mitotic mapping is feasible and in some favourable cases less laborious than meiotic mapping

(b) the relative distribution of mitotic crossing over along the chromosomes differs from that of meiotic crossing over but no systematic feature is so far apparent in this difference.

Mitotic crossing over and the “parasexual cycle” can be used, and have already been used, for genetic analysis and deliberate breeding in asexual fungi (Pontecorvo, 1954). From the present work it is clear, furthermore, that mitotic crossing over may make possible the formal genetics of higher organisms, including man, in tissue cultures.

References


CHAPTER I

GENETIC ANALYSIS AND ITS RESOLVING POWER

"ANALYSIS," in the Oxford dictionary, is defined as "resolution into simple elements." In genetic analysis we must be clear about what we resolve and into what simpler elements.

Classical genetic analysis is based on the results of breeding and by means of them resolves the genome into linkage groups, and each linkage group into loci. By also making use of cytological techniques and combining them with breeding techniques it goes further: it establishes on which chromosome each linkage group has its structural basis and to which small section of the chromosome each locus corresponds.

Mainly as a consequence of the development of microbial genetics, genetic analysis has increased enormously its resolving power in recent years, so much so that it now goes beyond that of physical or chemical techniques applied to biological organisation. I hope to substantiate this contention and make it more precise than was possible in 1952 when it was first put forward.

The essential process on which genetic analysis is based is recombination. Consider the analogy with microscopy, which is based instead on diffraction. The resolving power attained in microscopy depends on the quality of the microscope and on other technical details, but we know that it has a theoretical limit set by the wavelength of the light used. So far, in genetic analysis the resolving power has been limited only by the refinement of techniques. What
the ultimate limit is we do not know, nor can we deduce from theory. Recent advances make it possible to venture a few guesses.

Recombination can be defined as any process which gives origin to cells or individuals associating in new ways two or more hereditary determinants in which their ancestors differed: for instance, cells with determinants $Ab$ or $aB$ descending from other cells with $AB$ or $ab$.

Until less than fifteen years ago, only two processes of recombination were known: sexual reproduction and infection. Now we know that there are more. For instance, transformation by means of desoxyribonucleic acid (Avery, MacLeod, and McCarthy, 1944) and virus-mediated transduction (Zinder and Lederberg, 1952) in bacteria, the parasexual cycle (Pontecorvo, 1954) in fungi, etc.

We recognise recombination by observing in a line of descent certain cells or individuals—recombinants—which show new associations of properties. Recombination of properties, however, is only the detectable secondary effect of reassociation of subcellular structures determining differences in such properties.

In the type of recombination on which classical genetic analysis is based, i.e., recombination in sexual reproduction, these structures are the chromosomes and their linearly arranged elements. The latter are recognised as genes as a consequence of their specific activities in metabolism and development.

In sexual reproduction recombination of chromosomes and their elements takes place at meiosis and it is the result of the independent segregation of nonhomologous chromosome pairs and of crossing over between members of a chromosome pair, respectively.

Crossing over (whatever its precise mechanism, see Chapter IV) can be formally described as the reciprocal exchange of linear bonds at corresponding positions along pairs of homologous chromosomes. These exchanges are microscopically observable in suitable material. In a population of cells going through meiosis, the incidence of exchanges between any two given points in one chromosome pair is highly correlated with the physical distance between these two
RESOLVING POWER

points. This incidence can vary from an average of over five exchanges per cell per chromosome pair to none.

In the analysis of the results of breeding experiments we recognise recombination not by microscopic examination of cells in meiosis but by the proportion of recombinant individuals in later generations of a cross. From the fraction of recombinant individuals one can calculate the fraction of recombinant gametes from which those individuals originated. The two coincide, of course, in the case of haploid organisms.

If the proportion of the recombinant gametes formed—e.g., $Ab$ and $aB$—is smaller than that of gametes with the ancestral associations—$AB$ and $ab$ ("parental")—we say that there is linkage between the two genes $A/a$ and $B/b$. This is almost the same as saying that along a chromosome pair between the position of the difference $A$ versus $a$ and that of the difference $B$ versus $b$ there is an incidence of less than one exchange as the average of a large number of cells in meiosis.

The measure of linkage is based on the fraction of recombinant gametes out of the total tested. If the two positions just mentioned are either on different pairs of chromosomes or so far apart on one pair that there are on the average one or more exchanges between them, the recombinant gametes are formed in equal proportions with the "parental" gametes. In this case we say that there is "free recombination" between the two genes $A/a$ and $B/b$.

Three or more genes closely linked two by two (i.e., showing much less than 50% recombination) reveal the additiveness of recombination fractions. If the recombination fraction between $A/a$ and $B/b$ is, say, .05 and that between $B/b$ and $C/c$ is .04, that between $A/a$ and $C/c$ will be approximately either the sum of (.09) or the difference between (.01) the other two. This additiveness makes it possible to represent the recombination fractions graphically as segments in a linear sequence—$A/a$, $B/b$, $C/c$ in the first case, or $A/a$, $C/c$, $B/b$ in the second case—with the length of the intervals proportional in suitable scale to the recombination fractions.

Overlooking certain refinements required by the occurrence of
multiple exchanges, interference, etc., which lead us to correct the recombination fractions and turn them into "crossover values," a linkage map consists essentially of this. The "position" of a gene on the linkage map is called its locus.

Thus, by means of breeding experiments, we measure the incidence of recombination between a group of linked loci and we resolve a linkage group into crossover values between loci. This gives a valid but rather abstract picture. We do not make it much more concrete by representing the crossover values graphically as a linkage map. In the same way we do not make more concrete the tabulation of volumes versus pressures of a gas if we turn it into a graph, though for certain people like me it is easier to grasp the meaning of a graph than that of a table or an equation.

Because of the approximate additiveness of recombination fractions, linkage maps are quite meaningful but are still only graphic expressions of certain numerical relationships. They are more meaningful than the analogy of pressures and volumes because the material structure—the chromosome—which underlies the relations expressed in the map actually has a linear arrangement at least at first approximation.

The concepts and the methods which have led to the construction of linkage maps in organisms with a standard sexual cycle have been of tremendous value in extending genetic analysis to systems in which recombination is not based on meiotic crossing over. The results of these new adventures are evident in the successful mapping of bacteriophages and bacteria, and in the use of mitotic analysis in fungi (Chapter V). Furthermore, in bacteria, mapping has made use of a refreshing variety of natural and artificial processes: transformation, transduction, conjugation, and finally mechanical or radiation-induced fragmentation of the "chromosome."

The combined work of cytology and genetics in higher organisms over the last fifty years has led to a substantial understanding of the relations between linkage map and chromosome, and in particular between recombination fractions and incidence of crossing over at meiosis. It has also taught us to be aware of the limitations of
genetic maps as pictures of the actual spacing of loci on the chromosomes. One has only to keep in mind how different the results are if we compare, for example, the loci in the X chromosome of *Drosophila melanogaster* mapped: 1) cytologically in the mitotic chromosomes by means of X-ray breakage; 2) cytologically in the salivary gland chromosomes; 3) genetically by means of meiotic crossing over, and 4) genetically by means of mitotic crossing over. This difference becomes embarrassing if we compare the meiotic maps of the male, in which crossing over does not occur, with those of the female.

In spite of these obvious limitations we must attempt to give a concrete meaning to the numerical relationships expressed in linkage maps. This means that we must aim at a description in chemical and physical terms of the chromosomes and of the processes taking place in them. Among these processes the most prominent are replication, crossing over, and the part played by the chromosomes in the metabolism of the cell. We are, of course, very far from this ambitious end. There are eminent geneticists who think that this is no concern of the geneticist but of the biochemist, the biophysicist, and the physiologist.

**RESOLUTION AT THE INTER-GENIC LEVEL**

As mentioned above, genetic analysis resolves pairs of loci not too far apart by detecting recombination between them and measuring its amount. The closer the two loci, the smaller the amount of recombination. Consequently the detection and measurement of recombination between two loci very close to each other requires the classification of a large number of products of meiosis: as long as we are below the ultimate limit—wherever it may be—the resolving power is determined only by the size of the sample that we are able, and are prepared, to classify.

Remarkable increases in resolving power have resulted in recent years precisely from increases in the size of the analysable samples. This is mainly the consequence of the introduction of microorganisms in genetical research and the development of selective tech-
niques which pick out automatically rare recombinants from a mass of nonrecombinant cells. The result is that recombination fractions of one in a million have been measured. But larger samples can still be handled. There should be no insuperable technical difficulty in actually reaching the ultimate limit. Perhaps it has already been reached in the analysis of recombination in bacteriophage (Benzer, 1957) and Salmonella (Demerec et al., 1956).

Let us first consider the resolving power of genetic analysis using exclusively results from breeding experiments; as if we did not know of the existence and significance of chromosomes and as if we had no clue as to their chemistry. Table 1 gives examples taken from the literature of the closest linkages recorded between different genes in five organisms, ranging from a mammal to fungi. I am using here the

<table>
<thead>
<tr>
<th>Organism</th>
<th>Total map units</th>
<th>Pairs of genes</th>
<th>Recombination as fraction $\times 10^4$</th>
</tr>
</thead>
<tbody>
<tr>
<td>DROSOPHILA</td>
<td>280</td>
<td>$w$ and $rst$ (I)</td>
<td>% of total map</td>
</tr>
<tr>
<td></td>
<td></td>
<td>$sp$ and $bl$ (II)</td>
<td>0.2</td>
</tr>
<tr>
<td></td>
<td></td>
<td>$ey$ and $ci$ (IV)</td>
<td>0.3</td>
</tr>
<tr>
<td>MAIZE</td>
<td>904</td>
<td>$a_1$ and $sh_2$</td>
<td>0.25</td>
</tr>
<tr>
<td></td>
<td>1,350*</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>1,620</td>
<td>$se$ and $d$</td>
<td>0.16</td>
</tr>
<tr>
<td></td>
<td>1,954*</td>
<td></td>
<td></td>
</tr>
<tr>
<td>MOUSE</td>
<td>660</td>
<td>$y$ and $ad16$</td>
<td>0.05</td>
</tr>
<tr>
<td></td>
<td></td>
<td>$ad15$ and $paba1$</td>
<td>0.5</td>
</tr>
<tr>
<td>ASPERGILLUS</td>
<td>380*</td>
<td>$co4$ and $arg2$</td>
<td>0.4</td>
</tr>
<tr>
<td></td>
<td>800*</td>
<td>$q$ and $lys$</td>
<td>0.5</td>
</tr>
</tbody>
</table>

* Indicates total maps calculated by multiplying the chiasma frequency by 50. Other total maps calculated by adding the lengths of all linkage groups. For those organisms for which both estimates are available, the larger has been used, as more likely to be nearer the true value. In the case of close linkages the recombination fractions, expressed as $\%$, are equivalent to map units. (References: Mouse—Carter, 1955; Grünberg, 1952; Slizynski, 1955; Aspergillus—Pritchard, 1955; Calef, 1957; Käfer, 1958; Neurospora—Singleton, 1953; Barratt, Newmeyer, Perkins, and Garnjobst, 1954; Maize—Darlington, 1934; Rhoades, 1950.)
term "gene" in the vague classical meaning based on the tacit and wrong assumption that the ultimate units of transmission and of difference in heredity are one and the same thing. This matter will come up for closer scrutiny in Chapter II.

In the examples given in Table 1, the two members of each pair of closely linked genes considered have quite different effects on the phenotype. It is reasonable to suppose that members of each pair are relatively independent in the part they play in metabolism or development. Their recombination fractions vary from 5 in $10^4$ to 5 in $10^8$. These recombination fractions represent from about 1 to 10 ten-thousandths of the total map for each organism.

We could follow Muller (1916, 1926) and use these ratios to make a minimal estimate of the total number of genes in each organism. It would be minimal, of course, because on the one hand there is no reason why the closest linkage found so far in any one organism should be just the closest occurring in that organism. On the other hand, as the detailed genetic analysis of an organism proceeds, the total identified map increases. In Drosophila, however, this analysis is so advanced that the map has not increased in the last thirty years or more. This means that the outermost genes of each linkage group already identified are really very near the ends.

RESOLUTION AT THE INTRA-GENIC LEVEL

When Muller made his estimates the following facts were not known. A gene, defined as a unit of physiological action (the matter is discussed later in this chapter and more fully in Chapter II) may have its chromosomal basis on a section of chromosome of considerable length and containing a number of "sites" of mutation (Pontecorvo, 1952), each separable from the others by recombination.

The work by Green, E. B. Lewis and Mackendrick with Drosophila, that by Roper, Pritchard, Forbes, Calef and others with Aspergillus, that by Giles with Neurospora and, foremost, the remarkable work by Benzer with phage T4 and by Demerec and co-
workers with Salmonella, all point the same way. Recombination (be it by means of reciprocal exchanges as in crossing over or by other processes) resolves not only genes but also mutational sites of one and the same gene.

We have at present no evidence that the processes which recombine two sites within a gene are necessarily different from those which recombine two sites, one in each of two adjacent genes (see Chapter IV). In one case (Pritchard, 1955) there is an example of the recombination fraction between two sites in one gene being greater than that between one of these sites and another in a different gene nearby. The fact that we do not have evidence to the contrary does not exclude, of course, that there may actually be a difference between inter- and intra-genic recombination; a problem well worthy of a considerable research effort at the present moment (see Chapter IV).

In the majority of the cases analysed in Aspergillus and in Drosophila recombination between alleles of one gene does occur as a rare but regular event, not as an exception. The two complementary expected types of recombinants do arise and in some cases these complementary types have been recovered from one cell, i.e., they originate as a consequence of one recombinational event. It seems legitimate, therefore, to consider these cases as examples of crossing over between alleles. However, there are features of this intra-genic crossing over, and in general of crossing over within small intervals, which are not yet clear (see Chapter IV).

The examples of unidirectional transfer in heterozygotes described by Lindegren (1953)—who called them "conversion"—and Roman (1956) in yeast, by M. Mitchell (1955) and Case and Giles (1957) in Neurospora, by Strickland (1958) in Aspergillus, by Demerec (1928) in *Drosophila virilis*, and perhaps some of those described by Laughnan (1955) in maize, are very interesting though still completely obscure. They are unquestionably a source of confusion in certain cases of analysis of intra-genic crossing over. However, they are examples of something different from the intra-genic crossing
over analysed in at least six allelic series in Aspergillus, and at least four in Drosophila. They are also different from the intra-genic recombination analysed by Benzer (1955) and Streisinger and Franklin (1956) in phage, by Morse, Lederberg and Lederberg (1956) in *Escherichia coli*, and by Demerec and co-workers (1956) in Salmonella. The difference is clear even on a purely formal analysis (Chapter IV). It would become even clearer if the results by H. K. Mitchell (1957) of differential effects on crossing over and conversion by temperature shocks were confirmed and extended.

We have now to consider the resolving power of recombination when it takes place between mutational sites within one gene (i.e., between allelic mutants) rather than between mutational sites of different genes (i.e., between non-allelic mutants).

When we test for recombination two mutants originated by distinct mutational events and allelic with each other, we are of course trying to resolve very closely linked sites, so much so that up to a few years ago crossing over between alleles was not known to occur. In some cases presumably the linkage may even be complete (a point hard to prove): mutation will have actually recurred at the “same” site, or there may be a minute structural rearrangement which prevents recombination.

It is not surprising that the examples of closest linkage so far detected come from tests between alleles. Table 2 gives examples of recombination fractions measured between alleles in various organisms. The smallest recombination fractions so far measured between alleles are of the order of $10^{-6}$ in Aspergillus and of $10^{-5}$ in Drosophila.

We can now attempt a minimal estimate of the total number of mutational sites in one organism from the ratio between the total map length and that of the smallest recombination fraction measured, following in this the argument used by Muller (1916, 1926) for estimating the total number of genes. There is here at least one assumption which must be made explicit. It is that the incidence of recombination between two adjacent mutational sites is the same along the whole map. The well-known fact that the distribution of
TABLE 2
EXAMPLES FROM FOUR ORGANISMS OF THE RATES OF RECOMBINATION BETWEEN MUTATIONAL SITES OF ONE GENE (CISTRON)

<table>
<thead>
<tr>
<th>Organism, gene and reference</th>
<th>Mutational sites so far identified No.</th>
<th>Sum of recombination fractions between the two outermost sites (a)</th>
<th>Recombination fraction between the two closest sites (b)</th>
<th>Inferred minimal number of sites per cistron Ratios a/b a/b*</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>DROSOPHILA</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1. bx</td>
<td>5</td>
<td>$3 \times 10^{-4}$</td>
<td>$3 \times 10^{-5}$</td>
<td>10</td>
</tr>
<tr>
<td>2. lz</td>
<td>3</td>
<td>$1.4 \times 10^{-3}$</td>
<td>$6 \times 10^{-4}$</td>
<td>2.5</td>
</tr>
<tr>
<td>3. w</td>
<td>4</td>
<td>$5.6 \times 10^{-4}$</td>
<td>$8 \times 10^{-6}$</td>
<td>70</td>
</tr>
<tr>
<td><strong>ASPERGILLUS</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1. bi</td>
<td>3</td>
<td>$1 \times 10^{-3}$</td>
<td>$4 \times 10^{-4}$</td>
<td>2.5</td>
</tr>
<tr>
<td>2. ad8</td>
<td>6</td>
<td>$1.8 \times 10^{-3}$</td>
<td>$15 \times 10^{-4}$</td>
<td>360</td>
</tr>
<tr>
<td>3. paba</td>
<td>2</td>
<td></td>
<td>$1 \times 10^{-6*}$</td>
<td></td>
</tr>
<tr>
<td>4. pro3</td>
<td>2</td>
<td></td>
<td>$1 \times 10^{-6*}$</td>
<td></td>
</tr>
<tr>
<td><strong>PHAGE T4</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1. r(II)A</td>
<td>39</td>
<td>$4.3 \times 10^{-2}$</td>
<td>$1.3 \times 10^{-4}$</td>
<td>330</td>
</tr>
<tr>
<td>2. r(II)B</td>
<td>18</td>
<td>$3.5 \times 10^{-2}$</td>
<td>$1 \times 10^{-3}$</td>
<td>35</td>
</tr>
<tr>
<td>3. k</td>
<td>6</td>
<td>$2.0 \times 10^{-2}$</td>
<td>$2 \times 10^{-4}$</td>
<td>100</td>
</tr>
<tr>
<td><strong>SCHIZOSACCHAROMYCES POMBE</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1. ad2</td>
<td>3</td>
<td>$4 \times 10^{-4}$</td>
<td>$1.5 \times 10^{-4}$</td>
<td>2.5</td>
</tr>
<tr>
<td>2. ad7</td>
<td>9</td>
<td>$1.5 \times 10^{-3}$</td>
<td>$6 \times 10^{-6*}$</td>
<td>251</td>
</tr>
</tbody>
</table>

* Indicates the smallest recombination fraction so far measured in each organism.


The data of Demerec and co-workers on Salmonella do not lend themselves to the treatment used in this table. They are very relevant, however, for the numbers of sites already identified as separable by recombination in transduction, e.g., 11 sites in the $hiA$ gene (Hartmann, 1957); 12 sites in the $cysB$ gene (Clowes, quoted by Demerec, 1956). The same can be said of the data of Roman (1956) with yeast, who has identified from 4 to 26 alleles as different in each of seven loci; out of a total of 83 independently arisen mutants at these loci, 73 were different as shown by the tests of complementary repair (see Chapter IV).

crossing over per unit of physical length of the chromosome is demonstrably not uniform does not necessarily make nonsense of this assumption. The chromosome may be, in fact, it probably is (Ris, 1957), differently packed along its length, so that the lengths meas-
ured under the microscope (e.g., Rhoades, 1950; Gall, 1956) may not have a uniform relation to lengths of the ultimate fibre.

The argument is as follows: on the assumption just made, the minimum recombination fraction so far measured in an organism can only be equal to, or an integer multiple of, the ultimate fraction, i.e., that occurring between two adjacent mutational sites. Clearly this estimate of the total number of sites—if the method is valid at all—can only be in error by defect: the smallest fraction so far measured is not necessarily the smallest occurring, and the map so far measured can only be smaller than or equal to the real total map. The results for three organisms are given in Table 3.

TABLE 3
MINIMAL ESTIMATES OF THE TOTAL NUMBER OF MUTATIONAL SITES IN THREE ORGANISMS

<table>
<thead>
<tr>
<th>Total map units</th>
<th>Smallest recombination fraction measured</th>
<th>Total number of sites</th>
</tr>
</thead>
<tbody>
<tr>
<td>(a) DROSOPHILA</td>
<td>$w^A_w^*$</td>
<td>$8 \times 10^{-4}$</td>
</tr>
<tr>
<td>(b) ASPERGILLUS</td>
<td>$paba1-paba6$</td>
<td>$1 \times 10^{-4}$</td>
</tr>
<tr>
<td>(c) PHAGE T4</td>
<td>$rII55-rII247$</td>
<td>$1.3 \times 10^{-2}$</td>
</tr>
</tbody>
</table>

References: Drosophila—Mackendrick, 1953 and unpublished; Aspergillus—Roper, unpublished; Phage T4—Benzer, 1957, p. 91. The total map in phage is an extrapolation of the estimates from detailed mapping of a small region to the whole genome.

The conclusion is that the total number of mutational sites may well be of the order of ten million in organisms like Aspergillus and Drosophila and of ten thousand in organisms like bacteriophage (Table 3).

By the same sort of reasoning we can now go further and estimate the number of sites making up a gene. But before doing this we shall have to introduce some terminology, anticipating its full discussion in Chapter II.

In place of the term "gene," and to avoid its vague meaning, in previous publications (Pontecorvo, 1955; Pontecorvo and Roper, 1956; Pontecorvo, 1956) the terms "region," "section of allelism,"
or “set” have been used. They were meant for a portion of the linkage map, and, by extrapolation, of the chromosome, containing the mutational sites at which recessive mutants are allelic with one another. In its turn “allelism” was considered to be a purely functional relation, the operation for identifying which is perfectly clear (Pontecorvo, 1952, 1955): two recessive mutants \( m_1 \) and \( m_2 \) are allelic to one another when they are not complementary, i.e., when the heterozygote \( m_1/m_2 \) has a mutant phenotype. Non-complementarity (which denotes unity of function) usually goes with the \textit{cis-trans} (or Lewis) effect, i.e., the double heterozygote in \textit{trans} is mutant but that in \textit{cis} is not.

Benzer (1957) has adopted these two functional criteria and has proposed the term “cistron” for the map segment underlying a unitary function, as shown by non-complementarity and \textit{cis-trans} effect of a set of recessive mutants. This very acceptable term will be used here instead of the term gene in every case in which it is less equivocal. It should be stated emphatically, however, that the criteria of allelism (and therefore those for defining a “cistron”) as given above are not absolute. There are relationships between recessive mutants which are intermediate between allelism and complementarity. In addition, of course, those criteria cannot operate in the case of dominants (see Chapters II and III).

The number of sites in a gene or cistron can be estimated the same way as the total number of sites in the genome, i.e., dividing the total “length” of a cistron by the smallest interval measured, the assumption being again that the amount of recombination in any measured interval is either equal to or an integer multiple of an ultimate unit of recombination which, in its turn, is that between two adjacent mutational sites.

In cistrons in which more than two mutational sites have already been identified and located we can obtain this estimate by dividing the measured “length” of the cistron by the smallest element in that cistron. This would give an even greater underestimate of the total number of sites in that cistron than if we divided that “length” by the smallest element measured in the whole genome (marked with
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an asterisk in Table 2). Even this very conservative estimate gives figures of tens or hundreds of mutational sites per cistron. The less conservative estimate (last column in Table 2) gives figures reaching even into the thousands. In both these ways of estimating the number of sites per cistron, both the identified part of the cistron and the smallest segment within it are measured in a way which can only produce an error by defect: we are testing for recombination a small sample of mutants of independent origin of each cistron, and the probability is small that we have picked up in this sample both the two outermost sites of that cistron and two which are the closest together possible. For this reason the estimates are interesting only in the cases of high values: 360 sites in the *ad8* cistron of *Aspergillus*, 70 in the *w* cistron of *Drosophila*, 330 in the *rIIA* cistron of phage T4, and 251 in the *ad7* cistron in *Schizosaccharomyces pombe*.

How representative are these estimates for all the cistrons (genes) in the genome? Apart from the small number of different cistrons on which our conclusions are based, there is one important fact which suggests caution. The cistrons analysed for recombination between alleles of independent mutational origin are, obviously, cistrons of which a number of mutants were available. It is conceivable, to say the least, that cistrons with many mutational sites are precisely those more likely to yield mutants: our sample could very well be strongly biased in favour of cistrons with an unusually high number of sites. As I suggested some years ago (Pontecorvo, 1952) we must keep an open mind and be prepared to find genes with all degrees of complexity, from those based on thousands of mutational sites to those based on few.

For this reason it does not seem very profitable at present to go one step further with speculation and calculate, from the total number of sites and the mean number in the two or three cistrons so far measured in three organisms, the total number of cistrons in an organism. If we wished to indulge in this, the results would be: 3,500 in *Drosophila*; 5,000 in *Aspergillus*, and 100 in phage.

But the important conclusion that at least some genes may have many mutational sites is supported from another angle, i.e., the pro-
portion of independently arisen mutants which represent recurrence of mutation at the same site of a cistron, or in other words, the proportion of allelic mutants which, with a given resolving power, have not been found to be distinguishable from one another by means of recombination. Here the conclusion is wholly operational. In Drosophila, in which the classification of $10^4$ gametes is already hard work, Green (1955a) and Green and Green (1956) for the $l_z$ and $f$ cistrons, and Mackendrick (1953) for the $w$ cistron, have failed to obtain recombination between quite a number of pairs of alleles. So has Dunn (1956) in the case of the $t$ system in the mouse, and so have all the workers on human and animal antigens. But in Aspergillus experiments with a resolving power of $10^{-6}$ have so far never failed to yield recombinants between any two allelic mutants of independent origin. This statement is based on 23 mutants belonging to 7 cistrons:

<table>
<thead>
<tr>
<th>Cistron</th>
<th>Alleles tested No.</th>
</tr>
</thead>
<tbody>
<tr>
<td>$ad8$</td>
<td>6</td>
</tr>
<tr>
<td>$bi$</td>
<td>3</td>
</tr>
<tr>
<td>$ad9$</td>
<td>6</td>
</tr>
<tr>
<td>$pro1$</td>
<td>2</td>
</tr>
<tr>
<td>$pro3$</td>
<td>2</td>
</tr>
<tr>
<td>$paba$</td>
<td>2</td>
</tr>
<tr>
<td>$Acr$</td>
<td>2</td>
</tr>
<tr>
<td>Total</td>
<td>23</td>
</tr>
</tbody>
</table>

Dr. H. Levene has kindly calculated for me that the probability of obtaining by chance a distribution like this would be less than one in a million if there were no more sites in each cistron than those already identified; it would become about one in fifty for seven sites per cistron, and about one in four for fifteen sites per cistron, on the basis of equal probability of mutation per site and an equal number of sites per cistron.

If the mutability varied greatly from one site to another (as it certainly does) the results obtained would indicate an even greater
number of sites. In fact, the finding, in Aspergillus, that out of nine intervals measured, three are extremely small (Table 2) shows that there are micro-regions of very high mutability: the "hot spots" of Benzer (1957). The work of Benzer (1957) and Streisinger and Franklin (1956) with phage, and of Z. Hartman (1956) and P. E. Hartman (1956) with Salmonella, and of Giles (1956) with Neurospora suggests that different sites of one cistron do differ grossly in mutation rates and that sites with high mutability may be clustered.

If the number of sites varied greatly from one cistron to another, again the results obtained would underestimate the number of sites. As to the extent of this variation in number of sites between cistrons, we have no clue.

Another way of estimating the number of sites per cistron could be that of comparing the average "distance" between two sites taken at random in a cistron with the average "distance" of two sites taken at random one in each of two adjacent cistrons. The greater the number of sites within a cistron, the greater the ratio of the average distance between two sites in one cistron to the average distance of two sites one in each of two adjacent cistrons. Unfortunately the extreme values of these ratios (for the calculation of which I am again indebted to Dr. H. Levene) are 2 in the case of only two sites per cistron and 3 in the case of an infinite number, the latter value being already attained for about twenty sites. This means that an analysis of this kind is not very sensitive and could give some information only if we had many more examples of analysis of several sites in a cistron and for many more adjacent cistrons.

Going back to Table 1, we have seen that most of the recombination fractions between neighbouring cistrons so far measured are of the order of $10^{-3}$; though that between $m$ and $d_y$ in Drosophila (Table 8) is as low as $10^{-5}$ (Slatis and Willermet, 1954). With one exception in Aspergillus ($y$ to $ad16$: 0.0005; $ad16$ to $ad8$: 0.0014, Pritchard, 1955, see map on Table 10) these recombination fractions are larger than those measured between sites of one cistron, and this
by a factor of the order of 10. The argument just developed suggests that the maximum ratio between average inter-cistron recombination and average intra-cistron recombination should be 3, for the case of very many sites per cistron, and provided that recombination between the two contiguous sites one of each of two adjacent cistrons were of the same kind and of the same frequency as that between any two contiguous sites of one cistron. The discrepancy between the expected factor of 3 and the found factor of 10 is obviously not significant for a number of reasons not only statistical, but it will be wise to keep it in mind.

The conclusions to be drawn so far are that genetic analysis has already resolved the linkage maps down to fractions of the order of a ten-millionth of the map. The results of measurements of this kind and of the location of mutants of independent origin suggest that the total number of mutational sites separable by recombination within a section of chromosome in which recessive mutants are allelic to each other (a cistron or gene, for short) may well be in the order of hundreds or thousands. Let us see whether we can give structural, or even better, chemical meaning to these conclusions.

RESOLUTION AT THE MOLECULAR LEVEL

This discussion so far has been based exclusively on the results of breeding experiments: the resolving power has been expressed exclusively in terms of recombination fractions as fractions of the total map, also based on recombination. Six years ago, repeating the pioneer attempt by Muller (1935), I tried to translate the meagre measurements of minute recombination fractions available then into lengths of chromonema. This required using recombination fractions from one organism (Aspergillus) and length of the presumably fully stretched chromonema from another one (Drosophila, salivary gland chromosomes). We can do better now that Benzer’s (1955, 1957) brilliant analysis of the $rIIA$ cistron in bacteriophage T4 has shown what can be done. For a few organisms besides phage, we know the DNA content of the haploid complement or particle, and we can express the smallest recombination fractions measured
as fractions of this DNA content (Pontecorvo and Roper, 1956).

The underlying assumptions are disputable even in the case of phage. They are rash in the other cases. We attempt these calculations only because they may be useful in bringing to light what the problems are, and in thinking out experiments. The assumptions are (Benzer, 1955; Pontecorvo and Roper, 1956): 1) that DNA constitutes the structural backbone of the linkage map and that all the DNA is in this backbone; 2) that recombination involves exchanges of nucleotide linkages either by a process of breakage and reunion or by one of change in copy-choice (Lederberg, 1955); 3) that each nucleotide linkage, or small multiple of nucleotide linkages, has the same probability of taking part in recombination as any other one; and 4) that mutational sites are separated from one another, along the whole length of the chromosome, by one nucleotide linkage (better, a pair in the Watson-Crick model) or by a fixed small multiple of pairs of nucleotide linkages.

In the case of bacteriophage T4, on which Benzer’s analysis was based, there is at least some evidence that the first assumption is approximately true, though Levinthal’s (1956) experiments suggest that not all the DNA qualifies. If the genetic information in phage is contained in the DNA, it is also possible that nucleotide linkages are actually those involved in recombination.

The results of Table 4 are for five organisms, for which measurements of total linkage map, of total DNA per genome and of very small recombination fractions are available. They confirm Benzer’s suggestion for phage by extending it to other organisms on the assumptions made above. The smallest recombination fractions measured to date would, for at least some organisms, represent only a few nucleotide pairs. This appears to be the case not only in phage, but also in Escherichia coli and Aspergillus nidulans and perhaps Drosophila, and it is conceivable that it could be so also for the mouse and maize if only the resolving power of recombination were increased sufficiently.

The next step in this speculation, still following Benzer, is that the ultimate unit of crossing over is a single nucleotide bond (3.4 A.)
or perhaps a fixed multiple of nucleotides, say ten, i.e., one complete turn in the Watson-Crick double helix or 34 A. A further speculation is that a mutational site is the segment of duplex DNA helix between two nucleotide linkages relevant for crossing over, i.e., one nucleotide pair or a small number of nucleotide pairs, say ten. A cistron or gene would then work out to be based on a length of helix, say, 1,000 nucleotide pairs long. Perhaps this is what is needed to determine the specific arrangement of aminoacids in a protein (cf. Ingram, 1957; Crick, 1958). In terms of only one Watson-Crick duplex helix as the basis of the linkage map, the length of a cistron would work out as some 3,400 A. and correspondingly less if the basis of the linkage map were a multi-stranded structure made up of several Watson-Crick helices.

The trouble in all this—apart from the unwarranted assumptions on which it is based—is that our analysis measures the internodes between mutational sites and we are now identifying the separators with the separated. Even in phage, where it looks as if nothing but
the DNA carried the genetic information, this is a rather hazardous step. In higher organisms it is completely gratuitous. Let us examine some of the difficulties to which it leads.

In closely related groups, like the Anura and the Urodeles, the DNA content of the nucleus differs by a factor of 20 times: does this mean that there are 20 times more genes, or that genes are 20 times more complex, or that the bulk of the DNA has nothing to do with linkages involved in crossing over, or that the multiplicity of DNA helices of the chromonema can vary as much as from one to twenty between two groups of organisms even closely related taxonomically?

Another point: Table 4 shows that the amount of DNA per unit of recombination increases by a factor of 10,000 times when we consider a series of organisms starting with phage and ending with the mouse or maize. This matter will come up again in Chapter IV. It certainly shows that the relations between recombination and DNA are not direct and simple.

Clearly the attempt to give chemical content to the resolving power of genetic analysis is useful in the case of bacteriophage but perhaps not yet in the case of higher organisms. We do not know, in chemical terms, what we are trying to resolve and into which elements. Yet, in purely genetical terms we can express the resolving power of genetic analysis quite validly: this resolving power is at present of the order of one ten-millionth of the linkage map. Even if the whole material of the nucleus went up to make nothing but the linear bonds which we resolve, this resolving power would still be astonishing.

Genetic techniques turn out to be more sensitive than biochemical ones, as I contended at the beginning of this chapter. They make profitable use of the enormous amplifying system of the organism. Biochemistry, however, is beginning to catch up and attempt to give structure, i.e., spatial organization, to chemical reactions within the cell. Dr. Hotchkiss, my predecessor in the Jesup Lectures, has shown that genetic analysis of transforming principles of Pneumococcus, which are pure DNA, detects by means of recombination differences
RESOLVING POWER

between two parts of a macro-molecule. Biochemistry cannot yet do so when, as in the case of transforming principles and genes, that macro-molecule is one out of thousands of not very different ones, or better, is a part of an enormously greater aperiodic complex (Schrödinger, 1943). Surely there is a case for joining forces in the field of the fine structure of the genetic materials.

The present chapter contains a number of statements of faith and day-dreams rather than documented inductions or deductions. The documentation is reserved for later chapters, but some of the statements of faith will remain such. Science would not be able to advance as it does if it had to rely exclusively on the scientific method.
CONCLUSIONS

THE six chapters of this book discuss in detail the present trends in certain basic fields of genetics. It may be useful now to summarise. Some mistaken ideas are on the way out; others—not necessarily correct—have appeared either as replacements or as novelties.

THE GENETIC CODE

The most obvious wrong idea on its way out is that of the particulate gene, i.e., of the genetic material as beads on a string in which each bead is an ultimate unit of crossing over, of mutation and of specific activity. This picture was not merely crude: it was wrong because it implied an unnecessary, and almost certainly non-existent, structural differentiation between the beads and the string. Against it Goldschmidt has fought for years and Muller stressed long ago that it was not called for either on theoretical or on experimental grounds. What has replaced it is the picture of a nonrepetitive linear sequence of building blocks of only a few different kinds, the unique groupings of which determine unique functions. Each of these functions we now call a “cistron.”

The analogy of the genetic material with a written message is a useful commonplace. The important change is that we now think of the message as being in handwritten English rather than in Chinese. The words are no longer units of structure, of function, and of copying, like the ideographic Chinese characters, but only units of function emerging from characteristic groupings of linearly arranged letters. Miscopying has now become misspelling: a mistake in letters or in their order, not usually a mistake in words. In this analogy,
letters correspond to mutational sites exchangeable by crossing over, words correspond to cistrons, and misspellings to mutations. When the confusion of the present transitional period will be over and when knowledge about the primary functions of the genetic material will be sounder, we may be able to use again the term gene without danger, both for the group of mutational sites, the function of which is to determine the amino-acid sequence of a protein, and for the function itself. This is what I shall do in the following paragraphs.

It will have to be seen whether or not—continuing with the analogy—the words usually follow one another without overlaps. If so they may join by links not different from those between letters in cursive, or they may follow one another with the interposition of meaningless short sequences. They might also be linked by bonds of a different nature from those between letters. But in this case either all crossing over would have to be intra-genic or there would be two different kinds of crossing over—inter- and intra-genic—a possibility for which there is no evidence, to say the least.

The picture of the genetic material as a continuous nonrepetitive sequence is the result of the refinement of genetic analysis. I gave it shape some years ago though most of its experimental basis came later, mainly from work with Aspergillus, bacteriophage, and Salmonella.

While this picture was emerging from genetic analysis, equally promising became the search for what, in terms of physical chemistry, the letters and words of the genetic message are. First, from work with the transforming principles of Pneumococcus, then from bacteriophage, and finally from tobacco mosaic virus, it became clear that genetic information could be coded—and actually is coded in these organisms—in either desoxyribo- or ribo-nucleic acids. It will have to be seen if in respect of DNA this is the case in all organisms or only in what Stanier calls the “lower protista,” which have DNA not conjugated with histones.

Parallel with the realisation that genetic information could reside in nucleic acids came the brilliant contribution of Watson and Crick. The complementary duplex structure which they proposed for
DNA, and which seems to be essentially correct, offers just what is required for the genetic material: 1) it is an aperiodic structure with infinite possibilities for coding information; 2) it suggests a new model for replication, i.e., complement formation by each of the two parts of a duplex structure; 3) it is capable of carrying the kind of information which is necessary for specifying the 20 aminoacids found in proteins.

The convergence of the structural analysis of DNA with the biological analysis of the genetic material is one of the most exciting events in biology. It is only fair to remember, however, that Astbury was the first to suggest, twenty years ago, some of the biological implications of the structure of DNA, which was then just beginning to be formulated.

Even if the Watson-Crick ideas turned out to be wrong in respect of the mechanism of replication, they would have had a most fruitful effect: they have led to formulate more clearly what are the problems of replication of the genetic material, and of determination of phenotypic differences. They have also led to a series of most elegant experiments and we shall soon know if it is possible to homologise the map of the mutational sites of a gene (perhaps in terms of nucleotide pairs) with the sequence of aminoacid residues of the protein, the specificity of which is determined by that gene.

GENE ACTION

These advances, both in ideas and experiments, have made the one gene-one enzyme hypothesis of Beadle more plausible now, in some modified form, than it was ten years ago.

While it seems likely that part of the genetic message consists of specifying individual proteins, a question for the future will be whether this is the only way in which the genetic material works. This is the same as asking whether the only information in the genetic message is in the individual words and none emerges from the arrangement of words and groups of words.

The existence of position effects shows, of course, that supragenic arrangement is relevant. This evidence, however, does not
help to discriminate between relevance at the code level or relevance at levels removed from it. In other words, the question is whether irrespective of their location two genes determine the same two proteins (which may work differently according to where they happen to be synthesized) or the two genes, if differently located, determine different proteins.

Another very big question, related to the preceding one, is that of the amount and nature of the genetic information required for morphogenesis.

It would be useful in this respect to estimate the number of different proteins in one or more organisms with little or no morphogenesis, e.g., bacteria and moulds. If this number were of the same order as the estimated number of cistrons there would be a strong suggestion that in these organisms the bulk of the genetic material has but one function: one gene-one protein. Then the basic question in the genetics of morphogenesis would become that of whether or not the genes operating on morphogenetic processes also act by nothing more than determining the specificity of proteins. In the affirmative, morphogenesis would result from the epigenetic evocation of protein-forming systems, with gene-determined information for each protein. In the opposite case, we would have to search the genetic material for information of a nature other than that of specifying aminoacid sequences.

It would not be surprising if a part of the genetic control of morphogenesis were of the first kind: But it would be surprising if it were all, and the “higher fields” mentioned in Chapter III had no reality.

It may be advisable to start looking at the problems of morphogenesis from the angle of information theory: it could well be as rewarding as it has been in the case of the genetic determination of protein specificity.

REPLICATION AND RECOMBINATION

The genetic material has to be replicated every time a cell divides, and has to recombine, typically at meiosis. While it is clear now of
what replication consists, recombination is still as baffling a problem as it ever was.

In replication, an existing structure, made up of a unique linear sequence of a large number of building blocks of a few different kinds, determines the selection of an identical sequence out of an almost infinite number possible for the arrangement of building blocks from a random pool.

Whether or not the existing sequence is a duplex polynucleotide replicating by complement formation or, as Stent suggests, by transfer of information to an RNA-protein intermediate, or is something as yet not described, is a matter of detail: the essentials of replication can hardly be different from those indicated above.

The study of recombination suffers from the same disease as Italian literature: that of having reached the highest peak too early in its life. The result is subsequent scholasticism. The *Divina Commedia* of recombination was Darlington’s *Recent Advances in Cytology*.

After thirty years of scholastic development, recombination has to be approached anew with a completely fresh mind. What is needed is to start with information other than that from microscopic observation, which is too coarse.

The facts that have to be kept in mind are those from genetics, especially tetrad analysis and analysis of minute regions, those from electronmicroscopy, and those from the correlation of meiotic stages with the time of replication of the chromosomal materials.

There are three main questions: 1) What are the basic molecular processes underlying recombination and what relations do they bear to those of replication? 2) Are these molecular processes fundamentally the same from phage to man? 3) If they are the same, what accounts for the great differences in their end results between various organisms?

Among the important new facts to be kept in mind are mitotic crossing over and the clustering of exchanges. They force us to reconsider the dogma that cytologically visible pairing, as observed at zygotene and later, is a condition for crossing over. Discontinuous
contacts extending over minute homologous regions, and possibly occurring well before zygotene, seem to be a better model.

This model reduces the enormous differences in incidence of crossing over between organisms, or between sexes of one organism, or between meiosis and mitosis, merely to differences in the incidence of these contacts. Furthermore, this model is compatible both with the occurrence of crossing over well before meiotic prophase and with its coincidence with the time of duplication of the chromosomal material, known to be completed before zygotene. Cytologically visible pairing along the whole length of homologous chromosomes—a mechanical device essential for segregation—may well turn out to have nothing to do with crossing over.

The last three years have witnessed a promising beginning in the electronmicroscopy of the chromosomes, and in the use of autoradiography to follow the fate of their atoms in replication.

One of the results of electronmicroscopy I should like to single out: what Ris calls the elementary chromosome fibril appears to be about 100 Å in diameter and made up of two fibres of nucleoprotein about 50 Å in diameter lying side by side. When deproteinised each of these fibres is about 20 Å in diameter.

If confirmed, this structure would have two interesting features: one is its symmetry across the longitudinal axis; the other is that it would offer the means of satisfying, within one chromosome, the requirement for mating of identical structures which Stent suggests to be necessary for replication.

Clearly, decisive advances in the electronmicroscopy of chromosomes are badly needed to fill the appalling gap between molecular structure and microscopic structure.

There is a widespread expectation that crossing over will turn out to be based not on mechanical breaks and reunions but on some sort of switch in copying-choice as part of the replication process. The use of autoradiography with meiotic chromosomes, so successfully used by Mazia and Plaut and Taylor with mitotic chromosomes, may show how well grounded is this expectation.

New techniques but, above all, an unprejudiced mind, are nec-
necessary for a start towards an understanding of the most basic of all genetic phenomena: recombination.

THE VERSATILITY OF RECOMBINATION

An unexpected development of the last fifteen years is the discovery that sexual reproduction is not the only process of recombination: transduction, transformation, cytoplasmic infection, mitotic recombination, autogamy, are all recent additions. “Merozygotic” processes, i.e., processes of transfer of only part of the genome, are now taken for granted. This means, incidentally, that the narrow view that genetics is nothing but the study of the modes of formation of the gametes can no longer be entertained even formally. Its holders could still cling to it, however, by stretching the term gamete to any vehicle of genetic information from one cell to another.

In respect of evolution theory, the discovery of the versatility of recombination has made nonsense of the specious arguments which used to be produced for reconciling with neo-Darwinism the widespread occurrence of asexual microorganisms. We realise now that if in an organism there is no obvious sexual cycle, we had better find out which other process of recombination is operating. But perhaps the most promising outcome of these advances is the fact that they have opened up the field of the genetics of somatic cells.

Two obvious approaches come immediately to mind. One is the study of differentiation as a process involving transformation-like or transduction-like transfer of genetic information from cell to cell.

The other, for which I am responsible, is the analysis of the genotype of a donor by means of mitotic segregation in cultures of its cells. This kind of analysis, which bypasses sexual reproduction, may soon well make the knowledge of the genetics of man and of other slow-breeding organisms more extensive than that of Drosophila.
Pontecorvo: I am sure that some of you must have felt like me, in the last three days, a growing admiration for the success of the chemist and a sense of frustration for the genetical approach in human genetics. It seems to me that it is a really sad moment when the chemist has to tell the geneticist e.g. that haemoglobin G must be determined by a mutation in “cistron” α rather than in “cistron” β, on purely chemical grounds. The geneticist can do nothing, or very little, about it in the case of Man, while in the case of experimental organisms, the geneticist was many years ahead of the chemist in showing what sort of ultimate structure and function the genetical material must have. We must find short cuts to genetical
analysis in Man. The present methods are quite inadequate, especially when we come to problems like those which we are debating: fine genetic structure and gene action. For these problems we need extremely high genetic resolution if we are to get anywhere.

About five years ago, when I was just beginning to become senile (in Prof. Penrose’s definition), I thought that some work which we had done in the previous ten years on somatic segregation in lower organisms might give a clue as to how to overcome the impossibility of experimental breeding and the difficulty of breeding in large numbers in the case of Man. How and why could somatic segregation and recombination be used in genetical analysis in Man? First of all, there are two ways of using it. One is to study somatic segregation in the soma and the other is to study it in cultures derived from the soma.

The work with *Aspergillus* and other lower organisms has given quite clear ideas both of the techniques that can be used, and of the processes at work. (Incidentally, in lower organisms which have no true soma we have to use the term “mitotic” rather than “somatic” segregation.) These techniques for handling mitotic segregation in the analysis of the genotypes of diploid cells multiplying clonally are so easy, and have such advantages over analysis based on sexual reproduction, that we use them now as a routine in a number of species of lower organisms. The location of the centromeres, the determination of the order of loci, and the assignation of an unlocated mutant to a linkage group are quite simple.

Now, what the *Aspergillus* work has led us to realize is that there are at least three processes of somatic segregation and recombination. In the case of one of these, i.e. somatic crossing over, we do not know whether or not it occurs in mammals, including Man. We know that it occurs in certain insects. We know that it possibly occurs in certain plants, but we do not know about its occurrence in mammals. The other two processes are “non-disjunction” and what I have called “haploidization”. The former could, in fact, be only a different result of the same process which underlies haploidization.

Consider a cell heterozygous for two linked genes: $\frac{AB}{ab}$. The cell divides mitotically and starts a clone of cells. At a certain point—this is a rare event—one of the cells of this clone gives origin not to two daughter cells with a genotype like its own but to two daughter cells of one of the following types. One is homozygous $AB$, the other is homozygous $ab$, or alternatively one is still heterozygous $A/a$, but homozygous $B$, and the other is still heterozygous $A/a$ but homozygous $b$. This is what somatic crossing over produces. The details of it are now perfectly clear. It was discovered by Stern in *Drosophila*.
in 1936, and the amount of information we have now added makes Stern’s interpretation certain. Somatic crossing over permits the identification of the location of the centromeres and of the sequence of loci in one arm of any one chromosome. As I said, we have no clue as yet as to whether or not somatic crossing over occurs in vertebrates and in particular in Man.

A second process of somatic segregation is haploidization. Consider a cell heterozygous for a number of markers on a number of chromosomes. Such a cell, by an accident of mitosis, gives origin to a cell which has lost one of its chromosomes. This puts a premium on further losses, so that in the clonal line derived from that cell, eventually either one or the other member of each of the other pairs of chromosomes is lost until finally a completely haploid set is attained. This haploid set may of course be made up of any association between the members of the various chromosome pairs. There is, therefore, recombination between whole chromosomes, and different recombinant sublines arise in the clone.

**Brenner:** Why do you say there is a premium on further losses?

**Pontecorvo:** This is an experimental fact in *Aspergillus nidulans* (which has eight chromosomes) and I believe it to be likely in other cases. If you isolate segregant cells only a few nuclear generations after segregation for one marker has occurred, you can follow the occurrence of further losses until the complete haploid set is attained. On the other hand, another compensatory process can occur, namely non-disjunction, about which I shall talk in a moment. When a loss of one member of a chromosome pair takes place there is a premium put on duplication of the now haploid (“monosomic”) chromosome. This produces homozygosis for one whole chromosome, but all the rest remains heterozygous.

**Luria:** Would you think that there is a premium on further losses, or that there may be in such lines a mechanism that continues to favour losses?

**Brenner:** Could they be clonal?

**Pontecorvo:** They could be both, i.e. accidental losses selected and further losses favoured by the same cause which produced the first loss. My guess is that the most common situation is simply one of selection, and not that of some permanent damage in the cell which is transmitted in the cell lineage and goes on producing chromosome losses. A third possibility would be that the imbalance, resulting from one accidental loss, favours further losses.

**Lederberg:** It should be stressed that you are culturing an organism in a nearly normal habitat, corresponding to the one in which its chromosome complement has evolved. The same consideration does not necessarily apply equally to tissue culture, or to transplanted
tumours whose new environment may well select for, as well as induce, karyotypic innovations.

Pontecorvo: Closing this parenthesis, the third process of somatic segregation is “non-disjunction”. The first step is the same as in haploidization, namely one member of one chromosome pair is accidentally lost, and the compensatory process is simply that of non-disjunction of the monosomic chromosome. Non-disjunction is known to occur in every organism which has been investigated cytologically and/or genetically. I repeat that all three processes of somatic segregation are rare, of the order of once in a few hundred or thousand cells.

There is no doubt that while we do not know whether or not somatic crossing over occurs in Man—and it is possible that it does not—these other two processes must occur: they have been seen in careful cytological work with organisms of all kinds, including tissue cultures of Man.

Even if somatic crossing over did not occur in Man, these other two processes of accidental—or perhaps non-accidental—loss of chromosomes, and compensatory processes like non-disjunction would be quite enough to carry out genetical analysis via somatic segregation to a very considerable extent. They permit individual genes to be assigned to individual linkage groups. They do not permit, as mitotic crossing over does, the establishment of the linear order of the genes on one chromosome.

Somatic segregation, therefore, could be used for genetical analysis in Man even if limited to these two processes. It could be used in the soma itself, or in tissue cultures. In the soma, somatic segregation would lead to small or large clones of tissues differing in genotype from the rest as a consequence of one of the processes mentioned above. That this expected consequence of somatic segregation could be tested relatively simply, occurred three years ago to Dr. Goudie in Glasgow. He searched for it in the red cells of AB individuals. The matter was then taken up independently by Atwood who developed a far more refined technique, using isotopic chromic acid, and confirmed one of Goudie’s conclusions. There is no doubt that the erythrocytes of individuals heterozygous for blood group antigens always seem to carry a small proportion of cells which have only one of two possible alleles. There is, of course, no proof at all that this is due to somatic segregation. But it simply means that somatic segregation is not excluded at least as a possible source of some of the unexpected cells.

The limitations of the study of somatic segregation in the soma are very great. We can see some of them already in the classical example of the study of somatic segregation: Stern’s work with Drosophila.
In Drosophila there were only 4 or 5 markers which lent themselves to the study of somatic segregation in the soma, because the markers have to be of a very special kind. First, they have to be detectable in small patches, representing only a small proportion of the total body. This proportion could be small when dealing with fluid tissues like blood but in the case, e.g. of mosaic patches in the skin one needs patches of quite a substantial size to distinguish them from the background. An exception is the case of the bristles in Drosophila in which individual bristles of recessive type in a background of dominants can be detected. A second and more serious limitation is that any recessive allele which has non-autonomous action would not be detectable in a segregant patch because the diffused substances from the surrounding tissues (heterozygous and phenotypically dominant) would mask the mutant genotype. So it seems to me that the scope of the study of somatic segregation in the soma is rather limited. However, it has some interesting possibilities, especially with the blood. I do not know whether Prof. Ceppellini has carried out the plan which he was considering in 1958, to study the formation of doubly homozygous red cells in individuals heterozygous at two linked loci both determining antigenic properties. These are very interesting possibilities and I think it would be very good if Ceppellini and others with the same kind of skill will try them out.

**Ceppellini:** No, unfortunately I cannot. I was thinking of the MN, Ss, Hunter, Henshow, Mi and Vw chromosomes (cf. Race and Sanger, 1959; Blood Groups in Man. Oxford: Blackwell). Probably some of these factors are not too closely linked; in any case it would be interesting to follow the behaviour of other related specificities in cell populations selected for a change in one of them (for instance M cells separated from MN blood). However, disappearance of an antigen up to now has only been observed for the ABO groups; of course it is possible that the change only corresponds to a developmental disturbance of the phenotype.

**Pontecorvo:** Yes, I only say that the evidence so far is not against somatic segregation as the cause of some of the aberrant red cells identified.

**Neel:** Dr. Atwood a few weeks ago said that he has now observed a striking increase in the proportion of these exceptional cells in individuals who have received $^{32}$P for therapeutic reasons. I believe he feels that the increase is more likely to be of a mutational origin than from somatic crossing-over with segregation.

**Pontecorvo:** I do not think that holds at all, because radiation and many mutagens affect at the same time abnormal segregation, crossing-over, mutation, and development of the phenotype.
**Harris:** What do you think may be the difference in relative frequency between mutation and somatic segregation?

**Pontecorvo:** I do not know. It depends on the organism, on the locus, on external conditions and on the genotype.

**Harris:** In the Atwood situation, for instance, is the general idea that segregation of this sort occurs, say, ten times more frequently than mutation?

**Pontecorvo:** I do not think you can use this sort of argument; in *Aspergillus*, for distal genes somatic crossing-over is of the order of $10^{-4}$, haploidization is of the order of $10^{-5}$, and non-disjunction is rarer than either of them, because it requires coincidence of two events.

**Kalckar:** Can you in this case distinguish between a loss of a gene and the loss of the expression of a gene? I presume that experiments on $^{32}$P incorporation are meant as a tool for distinguishing between these two possibilities.

**Neel:** I do not know; but one would assume that the action of $^{32}$P is on nucleated cells.

**Kalckar:** Differentiation often brings about a loss of expression of a gene.

**Pontecorvo:** You cannot distinguish loss from mutation when you are dealing with a single marker. You can distinguish loss from mutation when you are dealing with a number of markers, some of which, at least, are linked.

Well, this is enough as to somatic segregation in the soma. It is clear that one can think of doing the same sort of analysis in tissue culture. One could establish cultures from suitable tissues of healthy donors. These donors differ from one another in genotypes. The formation of segregant clones in tissue culture would be a means of detecting heterozygosis—any particular marker which can be detected.

The problem can be approached in two ways. One is to start from individuals who differ from one another in genetically determined features and try to see whether these differences can be identified at the cell level. The other way is the opposite one, i.e. to establish cultures from a large number of individuals and submit them to all possible screening tests in an attempt to identify differences between cultures, then go back to the individuals and see whether the effect of the genetic difference—discovered at the cellular level—is identifiable in the individual as a whole either as a difference in morphological or physiological characters or in any other kind of feature.

As some of you may know, in my laboratory we are attempting both approaches. In respect of the second, we are testing for differences between cultures from individual donors in reactions to a
spectrum of viruses, we are testing for differences in antigens, enzymes and other proteins, and we propose to test for differences in reaction to drugs. The obvious thing is to begin with a few clearly identifiable markers. After a difficult start I am quite confident that things will become feasible. But I want to give you an idea of my estimate of the pace of work, because I think there is too much expectation for what the approach via tissue cultures will do. I would be extremely happy if within the next ten years we could begin to get somewhere.

*Luria*: I do not know if this is appropriate, but may I ask Prof. Penrose for the definition of senility in a geneticist?

*Penrose*: This is Pontecorvo’s interpretation of an aside which was made for my own amusement.

*Harris*: Could we have Pontecorvo’s interpretation?

*Pontecorvo*: I thought that the very reasonable idea was that when a general geneticist becomes senile he turns to human genetics.
METHODS OF MICROBIAL GENETICS
IN AN APPROACH TO HUMAN GENETICS

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1. Principles of Genetic Analysis

Genetic analysis—whether in unicellular or in multicellular organisms—is the description of the genetic constitution ("genotype") of a cell, a colony or an individual. It requires, (i) the existence of differences in properties ("phenotypes") between cells, colonies or individuals; (ii) the demonstration that a difference in phenotype between two lines of descent stems from a difference in structural cell elements which can be brought together into one line and later again sorted out ("segregated"); and (iii) the observation that, when more than one difference exists, segregation may lead to re-sortment ("recombination") between elements contributed by two lines of descent, each determining one of the differences.

Classical genetic analysis is based on the segregation and recombination that result from sexual reproduction. Here the structures involved are the chromosomes, and what segregates and recombines in any one case may be a pair of differences in only a few atoms at two sites on the structure, many hundred-million-atoms long, of a chromosome set. In sexual reproduction, the process which brings together in one cell chromosomes differing at one or more sites from two lines of descent is "karyogamy", i.e., the fusing of two haploid nuclei which in higher animals and plants takes place at fertilization. The process which brings about segregation and recombination of these differences is "meiosis", which in the higher animals and plants takes place just before gametogenesis. The process which brings about the segregation and recombination of these differences is "meiosis", which in higher animals and plants takes place just before gametogenesis.

Before the developments of microbial genetics in the last 15 years, the only way of carrying out genetic analysis was via the results of sexual reproduction, and mainly by making use of appropriately designed experimental matings. This meant that in organisms in which sexual reproduction does not occur, or—as in man—is not open to experimental manipulation, or the length of time between generations is long, or the number of progeny per pair small, genetic analysis was impossible or very limited in scope.

Microbial genetics has changed all this. One of its main contributions is the realization that sexual reproduction is not the only process for bringing together and recombining genetic structures from different lines of descent. A corollary is that we are no longer limited to the use of sexual reproduction as the only tool for genetic analysis.

2. The "Parasexual Cycle" in Filamentous Fungi

This process—the "parasexual cycle" (Pontecorvo, 1954)—consists of a series of rare events which take place during the vegetative growth of some of these filamentous fungi. In the species—Aspergillus nidulans—in which the parasexual cycle was first discovered (Pontecorvo & Roper, 1952), and then investigated to the fullest extent and harnessed for genetic analysis (Pontecorvo, Tarr Gloor & Forbes, 1954; Pontecorvo, Tarr Gloor & Forbes, 1954; Pontecorvo & Käfer, 1956, 1958; Käfer, 1958; Forbes, 1959; Roberts, 1959), a normal sexual cycle also occurs. The formal genetics based on the latter was sufficiently advanced to make it possible to compare the results obtained via the sexual cycle with those via the parasexual cycle. The location of genes in their linkage groups and the linear order of the genes within one linkage group are the same when deduced by means of either of the two methods.

Later, the formal genetic analysis of a number of species in which the sexual cycle is not known to occur has been worked out to greater or lesser extent by various authors using exclusively the parasexual cycle (see, for example, Pontecorvo & Sermoni, 1954, for Penicillium chrysogenum). The most extensive work of this kind to date is that of Lhoas (1961, and unpublished) who has identified in the asexual Aspergillus niger 32 genes and located them in their linear order in six linkage groups.

Naturally, the extensive use of the parasexual cycle in formal genetic analysis, both of species with and without a sexual cycle, has led to improvements in techniques. Certain steps in this analysis—e.g., the identification of linkage groups (Forbes, 1959)—are more conveniently carried out by means of the parasexual cycle than by means of classical procedures. The parasexual cycle in filamentous fungi, typically in Aspergillus and Penicillium, consists of the following elements:

- i. formation of diploid nuclei by rare, and probably accidental, nuclear fusion in a multinucleate mycelium in which the nuclei are usually haploid (Roper, 1952). If the mycelium is "heterokaryotic"—i.e., carries haploid nuclei genetically of two or more kinds—fusion may occur between two nuclei of different kinds, and the diploid resulting will be heterozygous;
i. Mitotic crossing-over

The results of the work with *A. nidulans* have revealed that the modalities of mitotic crossing-over are precisely as postulated by Stern (1936) to explain the patterns of somatic mosaicism which he studied in the fruit fly *Drosophila*.

Mitotic crossing-over, a diploid nucleus heterozygous at a number of loci, some on the same arm of one chromosome and some on different arms or on different chromosomes, gives origin, in a proportion of cases, to two daughter diploid nuclei ("segregant") which are homozygous at all the loci distal to the position of crossing-over; the other loci, i.e., those proximal on the same arm, those on the other arm of the same chromosome and those on other chromosomes, remain unaffected. The two segregant daughter nuclei are complementary, in the sense that, in the relevant section of a chromosome, one is homozygous for the alleles derived from one parent and the other homozygous for the alleles from the other parent. For instance, nuclei of homozygous constitution

\[ a \quad b \quad c \quad d \quad e \quad f \quad A \quad B \quad C \quad D \quad E \quad F \]

in which crossing-over has occurred between *d* and *e* produce, in a proportion of cases, daughter diploid nuclei of constitution

\[ a \quad b \quad c \quad d \quad e \quad f \quad A \quad B \quad C \quad D \quad E \quad F \]

and

\[ a \quad b \quad c \quad d \quad e \quad f \quad A \quad B \quad C \quad D \quad E \quad F \]

(The two fractions indicate two chromosome pairs, the dot indicates the centromere, and the capital versus small letters indicate alleles.)

Nuclei in which mitotic crossing-over has occurred between the centromere and *d* will give, in a proportion of cases, daughter nuclei homozygous at all three marked loci in that arm, i.e.,

\[ a \quad b \quad c \quad d \quad e \quad f \quad A \quad B \quad C \quad D \quad E \quad F \]

and

\[ a \quad b \quad c \quad d \quad e \quad f \quad A \quad B \quad C \quad D \quad E \quad F \]

And nuclei in which it has occurred between *e* and *f* will give:

\[ a \quad b \quad c \quad d \quad e \quad f \quad A \quad B \quad C \quad D \quad E \quad F \]

and

\[ a \quad b \quad c \quad d \quad e \quad f \quad A \quad B \quad C \quad D \quad E \quad F \]

Because of the rarity of multiple exchanges, nuclear segregant for *f* (i.e., *ff* or *FF*) will be of only three types: either homozygous at the *f* locus only, or homozygous at both the *f* and the *e* loci, or homozygous at all three loci in the "left" arm. The fact that segregants homozygous at *e* are homozygous at *f* but not necessarily at *d*, and that segregants homozygous at *d* are homozygous at both *e* and *f* loci, reveals that these three loci are linked, that they are on the same arm of one chromosome, and that the order is centromere-*d*-*e*-*f*.

Thus, mitotic crossing-over permits the identification of "half" linkage groups, i.e., from the centromere to the end of one chromosome arm, and the determination of the linear order of loci within each "half" linkage group.

Besides the fly *Drosophila*, for which Stern (1936) formulated the model of somatic crossing-over to explain somatic mosaicism, and the fungus *A. nidulans* (see review: Pontecorvo & Kafer, 1958) in which the proposed model was shown to be unquestionably correct, the occurrence of mitotic crossing-over has been verified in a number of other species of filamentous fungi.

ii. Mitotic non-disjunction

The genetic consequences of this process are that, from diploid nuclei heterozygous at a number of loci on both arms of one chromosome pair, other diploid nuclei arise which are homozygous at all of these loci.

Thus, a nucleus

\[ a \quad b \quad c \quad d \quad e \quad f \quad A \quad B \quad C \quad D \quad E \quad F \]

as a consequence of mitotic non-disjunction of the multiply marked chromosome, will give origin to nuclei

\[ a \quad b \quad c \quad d \quad e \quad f \quad A \quad B \quad C \quad D \quad E \quad F \]

or

\[ a \quad b \quad c \quad d \quad e \quad f \quad A \quad B \quad C \quad D \quad E \quad F \]

Mitotic non-disjunction thus identifies linkage groups or, better, identifies which loci are in one chromosome, irrespective of arm, by the fact that the alleles derived from one parent segregate en bloc, i.e., as completely linked.

The process underlying mitotic non-disjunction is still not fully clear. All the evidence so far suggests (see, for example, Kafer, 1960) that it is one of the alternatives to a monosomic nuclear lineage, i.e., nuclear lineage in which one of the two members of a chromosome pair has been lost; the other alternative is complete haploidization, as explained in the next section. If the other member fails to segregate normally at a mitotic division, so that both daughter chromosomes are included in the same daughter nucleus, this nucleus is fully diploid again and completely homozygous in respect of that chromosome.

Mitotic non-disjunction thus identifies the loci of one chromosome pair, though not their linear order along the chromosome.

iii. Haploidization

Haploidization consists in the origin, from diploid nuclei, of haploid nuclei in which the different chromosome pairs have segregated at random with no intra-chromosome recombination (Pontecorvo *et al.*, 1954). The haploids produced by a diploid have therefore two striking features: there is free recombination (50%) between genes located on different chromosome pairs, and no recombination (i.e., complete linkage) between those located in the same chromosome pair.
For instance, a diploid of constitution
\[ a \ b \ c \ d \ e \ f \]
\[ A \ B \ C \ D \ E \ F \]
will give origin to the following types of haploids in about equal proportions:
\[ ab \ cd \ ef \ ab \ CD \ ef \ ab \ cd \ EF \ ab \ CD \ EF \]
\[ AB \ CD \ EF \ AB \ cd \ EF \ AB \ CD \ ef \ AB \ cd \ ef \]

No recombinant types in respect of loci on the same chromosome pair (e.g.: \( ab \ cd; ef \)) are produced.

Haploidization seems to be a step-wise process: accidental loss of one chromosome is followed by successive losses, through a number of nuclear divisions, of one member of each of the other pairs of chromosomes until the fully haploid condition is attained (Käfer, 1958; Pontecorvo & Käfer, 1958).

Thus haploidization, like mitotic non-disjunction, identifies the loci of one chromosome pair, though not their linear order on the chromosome.

The use of haploidization in formal genetic analysis is exceptionally convenient because it permits the immediate and unmistakable determination of linkage groups. The criterion is: production of haploid recombinants in respect of two markers means no linkage; and absence of such recombinants means linkage. Since recombination can only be at the rate of 50% or not at all, a small sample of haploids is sufficient to distinguish between the two.

3. Problems of Genetic Analysis via Mitotic Segregation in Man

It is clear that, in principle, the methods of genetic analysis via mitotic segregation developed for artificially synthesized diploids in filamentous fungi could be applied to the naturally occurring diploid somatic cells of higher organisms, including man. The essential conditions for this are that mitotic segregation should occur in somatic cells, and that a proportion of the genetically determined differences in phenotype between individuals should be recognizable at the cellular level.

The study of mitotic segregation in fungi required first a method for obtaining and recognizing cells with heterozygous diploid nuclei (Roper, 1952). In the case of man, every individual is heterozygous at many loci. Thus, the availability of heterozygous cells is not a problem.

As to somatic segregation, the evidence for its occurrence in man and other mammals is suggestive. In man, Goudie (1957) and Atwood & Scheinberg (1958) have found that individuals of AB blood group have a small proportion of their red cells which show only one of the two antigens. This of course would be precisely the consequence of somatic segregation by any one of the three ways described in fungi, but at least three other processes are obvious candidates: mutation, loss of a chromosome segment and phenotypic failure.

In the mouse, Klein & Klein (1959) have shown that tumours from strains heterozygous at the histocompatibility locus give origin regularly—if sufficient selection is applied by means of implantation into an incompatible host—to strains homozygous in respect of one of the alleles.

The evidence as it may, there certainly is nothing in it against the occurrence of somatic segregation, to say the least. However, with the large amounts of heterozygosis characteristic of man and other mammals, we would expect somatic mosaicism—the consequence of segregation—to be very common. Yet it is very rare. This suggests that in the soma there are conditions which either keep low the incidence of somatic segregation or prevent the multiplication of the segregants or prevent the expression by the segregants of their phenotypic difference from the rest of the soma.

Among the systems which could prevent the multiplication of the segregants, a high amount of heterozygosis for deleterious recessives is an obvious possibility. Segregation of a particular marker—by means of any one of the three processes illustrated in fungi—would automatically bring with it homozygosis or hemizygosis of the whole of the chromosome carrying the marker or of the sections distal to the marker: a deleterious recessive linked with the marker could therefore become homozygous or hemizygous and expose the segregant lineage to elimination or reduced multiplication.

Among the systems which could prevent the expression, and therefore the recognition, of segregants, any type of gene action mediated by diffusible or circulating substances ("non-autonomous") could be responsible.

Leaving open the question of whether or not somatic segregation in one form or another occurs in the soma—it may be rare and the segregant cells liable to be at a disadvantage or to go undetected—there can be little doubt that it occurs when somatic cells are grown in artificial culture media in vitro. Loss of individual chromosomes, polysomy of others, etc., are events so common in cell cultures that they are considered a routine nuisance. However, like mitotic non-disjunction and step-wise haploidization in fungi, these accidents of mitosis can become useful when the aim is that of obtaining cells homozygous or hemizygous in respect of particular chromosomes.

Furthermore, some chemical treatments inducing and/or selecting monosomes, haploids and non-disjunctional diploids, are already routine in work with fungi (Lhoas, 1961, and unpublished; Morpurgo, 1961). My own attempts at inducing chromosome losses in cultured human fibroblasts suggest that this is not going to be a serious problem.

In conclusion, while we have no clues as to the occurrence of somatic crossing-over in man, other processes of somatic segregation certainly occur, at least in cultured somatic cells, and at least as accidents of mitosis. These other processes are adequate for genetic analysis to the extent of assigning genes to their linkage groups. They are not adequate, however, for determining the linear order of the genes within one linkage group. But this is a refinement which can wait.

Cellular "markers"

The real problem is not that of somatic segregation—spontaneous or induced, in the soma or in cultured cells—but that of "markers", i.e., of genetically determined differences between individual persons which are recognizable at the cellular level, particularly in culture.

Screening programmes for the identification of such cellular markers have been in progress for a few years in various laboratories, including my own. The scarcity of published reports speaks for itself. Galactosaeemia and acatalasia are the only two well-established examples of genetically determined differences between individuals which are recognizable in fresh explants and persist in long-term
cultures of their cells. Other persistent differences—e.g., in the induction of alkaline phosphatase by a substrate—are known between cultures established from different donors, but it is not yet certain whether or not these differences stem from the genetic constitution of the donors.

The question then arises as to why the search for cellular markers has been, so far, not rewarding. The unfortunate fact that negative results are not usually published makes it impossible to know, more than by hearsay, how many of the more obvious genetically determined differences in antigens, enzymes, other proteins, etc., have been looked for and not found between cultures of cells established from donors known to differ in any one of these respects. The kind of difficulty that may be encountered is illustrated by a finding of my colleague, Dr G. Le Bouvier, of our laboratory, who has kindly permitted me to mention it here. The A and B antigens, demonstrable by mixed agglutination in cell suspensions from skin biopsies, are no longer detectable in established cultures derived from such suspensions.

The scarcity of reports of a difference in antigens or proteins—detectable in biopsies of somatic tissues of different donors—which persists in the cultures established from those biopsies, suggests the need for a new approach in the search for cellular markers. It is clear that in highly differentiated organisms like mammals a proportion of the genetic information—perhaps a large proportion—determines the conditions for the synthesis of a protein rather than the structure of it. The new approach should be based on this obvious consideration. For instance, about 20 genetically determined variants of haemoglobin are known, yet its synthesis in the soma is limited to the erythrocytes: somatic cell cultures from individuals differing in their haemoglobins are all alike in producing no haemoglobin. Yet it is conceivable that we may find ways of persuading, e.g., a culture of skin fibroblasts, to produce haemoglobin. In this hypothetical case it would be surprising if the haemoglobin were not of the particular genetic type which the donor had in his red cells.

Recent research points the way. Cox & MacLeod (1961) found that different strains of cells differ persistently in respect of their alkaline phosphatase activity: some have a high "constitutive" activity, some develop activity after growth in the presence of a corticosteroid hormone, and some are unable to respond to the hormone.

Cox & Pontecorvo (1961) have found that permanent skin fibroblastic cultures from individual donors differ in their development of the activity in response to phenylphosphate, which is a substrate of the enzyme. Most cultures have no detectable constitutive activity. The majority, however, develop very high activity when grown in the presence of the substrate; some do not. None is inducible by the corticosteroid hormone. If we had not found a system of induction, most of these cultures would have been negative and therefore indistinguishable from one another.

This is, of course, an obvious conclusion in microbial genetics, where a considerable amount of work has been on variation in inducible (or "de-repressible") enzyme systems. In genetic analysis via somatic cells we have the difficulty that a protein, or other substance, normally present in the cells of a certain tissue, and showing quantitative or qualitative genetic variation between individuals, may not be produced in cultures of that or of other tissues. If we wish to detect variation in respect of that substance between cultures from different donors, we have to determine first the conditions for its induction.

On the other hand, the inducibility, mentioned above, of alkaline phosphatase by a corticosteroid hormone in certain strains, and by substrate in others, shows the sort of variation likely to occur in the systems of induction. This means that there may well be plenty of cellular markers waiting to be discovered in the systems of induction of individual proteins. It is profitable at the present stage to put more emphasis on a search in this direction than in that of genetic variation of the proteins themselves.

The techniques of microbial genetics can be applied to cell cultures because technical advances (Puck, 1958) permit us to handle them very much like micro-organisms. Genetic analysis of a culture of somatic cells is now feasible in much the same way as that of a culture of, say, protozoa. A much more exciting possibility is, however, the use of a culture of somatic cells for the genetic analysis of the donor of those cells, thus by-passing the stumbling block of sexual reproduction. The main immediate challenge is that of cellular markers.

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The Leeuwenhoek Lecture
Microbial genetics: retrospect and prospect

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Introduction

I wonder whether Anthony van Leeuwenhoek would have considered me as an appropriate choice for this lecture. For the past six years my interests have been in the rather unmanageable field of the genetics of cultured human somatic cells. These cells are animalcules only because we make them so. I can therefore look at the genetics of micro-organisms as an outsider, admittedly not wholly dispassionate. This is a pleasant task, because if there is a field of biology which has made great, unexpected and illuminating advances since 1940, this is it.

1. Classical Genetics

Genetic analysis up to that time consisted in deducing the genetic constitution ('genotype') of an individual, which underlies its relevant somatic characters ('phenotype'), from the distribution of these characters among its ascendants and its descendants. It therefore required the analysis of the results of breeding—experimental or not—and was limited to organisms with sexual reproduction. It was particularly illuminating in those multicellular organisms in which there is a clear distinction between germ cells ('gametes') and soma. Here, short of a preformistic process, it is clear that we can distinguish between determinants of hereditary characters and the characters themselves.

By about 1920 the hereditary differences between individuals in sexually reproducing multicellular organisms were known to stem from highly localized differences along the chromosomes contributed to the fertilized ovum in two sets, one from each parent. The active foci of the chromosomes, at which such differences were detectable by their effects, were called the genes. The rare process by which different forms of a gene ('alleles') arose was called mutation. The re-assortment between the maternal and the paternal contributions of genes in the formation of each gamete, which occurs at meiosis, was called gene recombination.

Sexual reproduction, stripped of all its paraphernalia, is essentially an alternation, in the life cycle of an organism, of karyogamy—i.e. nuclear fusion—with meiosis. The first brings together two chromosome sets, one from each parent, and the second sorts out again ('segregates') and re-assorts ('recombines') among different gametes the two parental contributions.

The proportion of gametes in which the paternal and maternal contributions in respect of two genes are recombined is correlated with their distances apart along...
one chromosome pair. Genes are thus linearly arranged along the chromosomes, and the frequency with which they recombine permits us to identify their linear order and estimate their distances. If two genes are so close together that they recombine on the average only in one out of 10,000 gametes, the work required to resolve the two genes is too great in higher organisms. This clearly sets a practical limit to resolution by recombination analysis in higher organisms where the classification of a few thousand gametes—based on the characters of the individuals arising from them—is the limit of what is feasible.

By 1940 classical genetics had led to the following important generalizations:

(i) The existence of a germ plasm or, as we say now, ‘genetic material’, as distinct from other cell elements, is validly inferred. The genetic material is a structure located in the chromosomes in the form of a linear array of active sections, the genes.

(ii) The genetic material is replicated by a template mechanism, in other words by a system in which structure, above and probably at the molecular level, is preserved at every step. It is subject to rare mutation at a number of points in its submicroscopic structure, and the mutated structures are capable of replication in the mutated form.

(iii) The activity of individual genes is in some way expressed in, or mediated by, the synthesis or the activity of individual kinds of macromolecules, particularly enzymes and other proteins. The process by which a gene is replicated is similar to that by which the gene produces its effects (de Vries 1899).

(iv) While the activity of the genetic material is open to regulatory control at every level of cellular and somatic organization, and indirectly to control by the environment, the structure of the genetic material is not open to regulation, i.e. mutation is functionally random.

By 1940 no precise idea had emerged as to the ultimate chemical structure of the genetic material. Chromosomes were known to be mainly made up of deoxyribonucleo-proteins and DNA was known to be found almost exclusively in the nucleus. The tacit expectation was that the protein carried the specificity.

2. The beginning of microbial genetics

It is not surprising that the development of genetic analysis in micro-organisms should have lagged for almost 40 years, or indeed 75 years, if we take as the birth of genetics Mendel’s paper of 1865. The historical accident was that genetics started with higher organisms. It therefore developed ideas and methods difficult to adapt to micro-organisms. The result was a sort of tacit understanding that geneticists should steer clear of micro-organisms and microbiologists of genetics. The reasons were simple.

First, in large groups of micro-organisms, particularly bacteria and many fungi, sexual reproduction was unknown to occur; in others, like the homothallic fungi, there was no method of utilizing it in experimental crosses. Genetical analysis without the use of sexual reproduction was almost a contradiction in terms up to as late as 1945.
Secondly, in micro-organisms, particularly unicellular ones, there is no clear distinction between soma and gametes. The difficult gap to bridge was between the methodology developed for inheritance via gametes from one generation to the next of multicellular individuals, and inheritance from cell to cell.

The way out took shape gradually between 1936 and 1946 with the realization that the stumbling blocks were either unreal or could be by-passed. The basis for this changed attitude, and for the subsequent spectacular advances, came from a number of directions. One is that sexual reproduction—or something very similar—turned out to occur among groups of micro-organisms in which it was unknown before. The other is that it turned out to be not the only process of gene recombination.

Winge (1935) working with yeast, Moewus (1936) with Chlamydomonas, Sonneborn (1939) with Paramecium, and Lederberg & Tatum (1946) with the bacterium Escherichia coli, found that conjugation of cells in pairs followed by segregation and recombination of genes does occur in unicellular organisms. This disposed of the idea that genetic analysis was impossible in such organisms because of the absence of sexual reproduction. The occurrence of sexual reproduction in a proportion of filamentous fungi had already been known for a long time. By now we can add the Mycetozoa (Dee 1962), and the Actinomycetes (Sermonti & Spadon-Sermonti 1955; Hopwood & Sermonti 1962) to this series. Even in bacteriophages Delbrück & Bailey (1946) found that processes of segregation and recombination occur, and in other viruses other workers later found similar effects. Altogether sexual, or similar processes, are now known to occur in every major group of micro-organisms in which a deliberate search has been conducted.

The widespread occurrence of sexual reproduction among micro-organisms came as a surprise, but a bigger surprise was the discovery that there are other processes which bring together and recombine, in one line of descent, genes from different lines of descent.

The first novel process of gene recombination discovered was transformation in Pneumococcus. The transfer of hereditary properties from dead to living bacteria, described by Griffith (1928), was shown by Avery, MacLeod & McCarty (1944) to be due to the transfer of DNA. The antigenic type of the cells from which the DNA was extracted would develop in a proportion of cells of a strain of different antigenic type treated with that DNA. The transformed cells would then transmit the acquired character indefinitely and synthesize DNA of the previously foreign type. This was important, not only as the first example of a new process of recombination—i.e. not based on conjugation and meiosis—but because it called the attention to DNA as the possible chemical basis of gene specificity.

It was not until 1952 that a further process of necrophilous transfer of hereditary determinants was discovered by Hayes (1952) in Escherichia coli. At present DNA-mediated transformation is known in a number of bacteria, and has been claimed (Szybalski & Joannes 1962) for human cells in tissue culture.

The second novel process of recombination to be discovered was ‘transduction’ in bacteria (Zinder & Lederberg 1952): here, a bacterial virus liberated by lysis from cells of one strain of bacteria carries genes of that strain to cells of another

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strain, where they recombine with the new host genes. The virus need not persist in the new cells: it may be considered merely as a rather complex means of transport. Transduction is now known to be possible by the mediation of structures other than viruses, that is genetic elements which are normal cell components. They have been called ‘episomes’ by Jacob & Wollman (1958). Previous Leeuwenhoek lecturers have dealt with the question of whether viruses are episomes which have gone astray, or episomes are viruses which have become well behaved. Particularly Lwoff’s (1953) imaginative unravelling of lysogeny has shown how meaningless questions of this kind can be, a matter that Pirie (1937) had characteristically put into a nutshell quite a long time ago.

The third novel process of recombination to come to light was the parasexual cycle in filamentous fungi (Pontecorvo & Roper 1952). This process, which occurs in species both with sexual reproduction and without it, is made up of all the individual components of karyogamy and meiosis, but they are not organized exactly. Segregation, recombination between whole chromosomes and between sections of a chromosome pair, and an alternation of haploidy and diploidy, all occur in isolation as rare accidents of nuclear division in vegetative cells rather than in a precise sequence, as in karyogamy and meiosis. Genetic analysis by means of the parasexual cycle in one species (Aspergillus nidulans) in which ordinary analysis via sexual reproduction was already well advanced, gave qualitatively identical results. In other words, the distribution of genes between chromosomes and their sequences along each chromosome are the same when established with either of the two methods. On the basis of this finding, the parasexual cycle has been used for mapping genes on the chromosomes in a number of species without sexual reproduction (e.g. Lhoas 1961).

The twin discoveries that sexual reproduction is widespread and that it is not the only system of recombination in micro-organisms, and the advances in the use of both, would have been very difficult without a number of powerful techniques. Some, like the possibility of selecting the odd cell with an unusual property out of huge numbers with different properties, were routine in microbiology. Others were new, such as the technique of Beadle & Tatum (1941) for the isolation of nutritional mutants—with the tremendous impetus it gave to biochemical genetics—and Beadle’s (1945) ‘one gene-one enzyme’ generalization which followed it.

Also exceptionally fruitful was the theory and methodology of Luria & Delbrück (1943) for the study of mutation in microbial populations. This clarified the old controversy of training versus selection in the origin of mutants, such as those resistant to drugs, in the presence of the drug:

3. Molecular genetics

By the early fifties, the formal genetics of several types of micro-organisms was in full development. Recombination analysis, based on old and new processes of recombination, and making use of the wealth of genetic markers and techniques, then reached a decisive stage. It was no longer—as in the previous 50 years—a matter of building chromosome maps for micro-organisms like those of Drosophila or maize. This soon became unexciting routine, so much so that it was somewhat
neglected and we are still suffering from that. The real challenge was to use the much higher resolving power of recombination analysis in micro-organisms to push its refinement to its physical limits, rather than to its practicable limits.

The first attempt was by Roper (1950), using *Aspergillus nidulans*. On the basis of Roper's results, and of others from *Drosophila* (Lewis 1945), I proposed a model of the gene which has since been confirmed (Pontecorvo 1952a). The model was that of a linear array of many sites of mutation exchangeable by recombination. The function of this structure was unitary, as shown by the fact that in a diploid nucleus carrying two alleles of one gene, each defective at a site different from that of the other (in *trans* arrangement; Haldane 1941; Pontecorvo 1950, 1952b), the function would fail, while in a diploid nucleus carrying on one chromosome the normal allele and on the homologous chromosome an allele defective at both sites (cis arrangement) the function would not fail. We must remember that in 1951, when this model was put forward, we had as yet no idea of the chemical structure underlying the genetic material.

Other attempts (with various micro-organisms) followed that of Roper in the analysis of the fine structure of individual genes, until in 1955 Benzer produced his now classical, masterly analysis of the *rIIA* and *rIIB* genes in bacteriophage T₅. Benzer resolved the structure of each of these genes into large numbers of linearly arranged sites of mutation.

By that time the Watson & Crick (1953) model of *DNA* was generally accepted. It had also become clear that the structure of *DNA* would fit very well the model of the gene just mentioned, and that *DNA*—at least in bacteria and bacteriophages—was the most obvious candidate as the carrier of the specificity of the genetic material.

Benzer (1955) was therefore able to equate the structure of the *RII* genes with a length of *DNA* a few hundred nucleotide pairs long, and the ultimate unit of recombination and of mutation with a single nucleotide pair, or a small number of pairs, a conclusion which has now been brilliantly confirmed by Henning & Yanofsky (1962) in *Escherichia coli*.

Simultaneously with these advances in the recombinational analysis of the genetic material, the theoretical and experimental unravelling of the structure of nucleic acids and proteins and of the processes of their synthesis was proceeding at a rate (on the average: one Nobel Laureate per annum) comparable to that of nuclear physics following the discovery of artificial disintegration. I will very briefly summarize the exciting picture which has arisen from the fruitful combination of genetic analysis with the biochemical investigation of nucleic acid and protein structure and synthesis:

1. **Coding.** The sequence of amino acids in a polypeptide which a cell may be able to synthesize is encoded in the sequence of nucleotide pairs in a segment of *DNA*—a gene—a few hundred nucleotides long (Crick 1958). The coding ratio is probably three nucleotides for one amino acid.

2. **Transcription.** The transcription of the sequence of nucleotides of a gene into the sequence of amino acids in a protein is by a two-step template process: a ‘messenger’ *RNA* (Jacob & Monod 1961; Brenner, Jacob & Meselson 1961)
complementary to the DNA is assembled on the DNA and then moves out to a ribosome, where in its turn it acts as template for the assembly of the amino acids. The messenger may be short-lived in some cases in bacteria, but probably not generally so.

(3) Replication. The replication of DNA is by a template process. Each of the two complementary strands of DNA acts as a template so that two new pairs of complementary strands are formed from one pre-existing pair, and each of these two is made up of one new and one old strand (Watson & Crick 1953; Meselson & Stahl 1958).

Remarkably, the old expectation of classical genetics, that replication and activity of the genes would turn out to involve related template-like processes (e.g. Muller 1947, p. 24) has been fully vindicated. On the other hand, I am unable to recollect, in classical genetics, any suggestion that the gene template itself should be a duplex complementary structure, replicating by double-complement formation, or that the ultimate linearity of the genetic material reflects the linearity of the structure of the polypeptides which it determines. For both these completely original ideas—the only two utterly novel in the whole of molecular genetics—we are indebted to Watson and Crick.

Among the enthusiasts of molecular biology, there is at present the feeling that the phase of decisive usefulness of recombination analysis is finished. If this is so, its swan song was last year's work of Crick, Barnett, Brenner & Watts-Tobin (1961). They were able to provide the strongest argument, up to then, for a trinitarian nature of the genetic code as a result of a series of brilliant, perfectly self-consistent and purely genetical experiments which required no assumption as to the chemical nature of the genetic material.

Even if recombination analysis were dead, and the formal genetics of the future should consist in matching in test tubes DNAs and messenger RNAs (Hall & Spiegelman 1961) from vegetative (or somatic) cells, the fact remains that we still do not have the faintest idea of what the molecular processes underlying recombination are. Yet recombination is a basic biological process.

4. The stability of the genetic material

The present picture of the replication of DNA and of the relations between DNA and proteins makes sense of one of the paradoxes of biology. Classical genetics discovered and accepted this paradox, and expressed it—perhaps unfortunately—in the statement that mutation is random. Many otherwise brilliant minds outside genetics, especially among physiologists, embryologists and microbiologists, found it absolutely unpalatable. For 62 years they kept genetics in the doghouse as unsound and obscurantist.

The paradox is this. Already in the sixth century B.C., as Menenius Agrippa’s apologue shows, it was quite clear that the components of a living system interact reciprocally in the most various and unexpected ways. The ability to ‘regulate’ and ‘repair’ is one of the distinctive features of life, and only since the development of electronics and cybernetics do we accept them as a consequence of complexity and not of a miracle. Yet, the genetic material, when it comes to its
replication, seems to violate such universal rule: metabolic processes are incapable of changing the DNA template in any specifically adaptive way. They can change it at random, i.e. with a negligible probability for any one change to have a feedback effect related to the stimulus which induced it.

In view of what we now think about the structure of the genetic material, its template-like mode of replication, and the way in which it determines—via proteins—the metabolic potentialities of the organism, all this is no longer surprising. The experimental discovery by classical genetics of the randomness of mutation, and the understanding by molecular genetics of why mutation is random, are among the great modern contributions to general biology.

5. Regulation of gene activity

Thus, mutation in the sequence of DNA bases (including loss or rearrangement of sections of DNA) is a non-adaptive process, or, in other words, the structure of the genetic material is not subject to regulatory change. It is even clearer, however, that the expression of the genetic material, both in micro-organisms and in higher animals and plants, is subject to regulation—qualitative and quantitative—at all levels of organization: the chromosome, the nucleus, the cell, the tissue, and even the whole organism in its relation to its environment.

A most important consequence of regulation of gene expression is that cells with identical genotype, i.e. with identical DNA, can have different properties as a consequence of different previous experience. This difference in properties may even be maintained for an indefinite number of cell generations, though the difference in experience which caused it has been transitory.

Beale (1954) gave a striking demonstration of some such occurrence. A clone of Paramecium is divided in two halves; one is grown for a short time at 15 °C and the other at 25 °C: the two halves develop two different antigens. After both are transferred to 20 °C they go on for an indefinite number of cell generations maintaining this difference. It can be shown, however, that neither half has changed its relevant genotype.

As pointed out by Delbrück (1949), an appropriate interlocking of two metabolic pathways—e.g. when a product of one pathway inhibits the other and vice versa—leads to an unstable state, stabilized only when either pathway has excluded the other. I gave a slightly more complicated example from Aspergillus (Pontecorvo 1952b) of a case of oscillation between two pathways.

The various levels at which interlocking occurs have been enumerated very clearly by Waddington (1962, p. 7). With the additional complexities introduced by feedback inhibitions, ‘allosteric’ reactions (Monod & Jacob 1961), etc., the interlocking of pathways is a well-known feature of the activities of enzyme systems, i.e. it is well known at the level of reactants which are themselves distant products of gene activity. This level need not concern us here.

For the present discussion a more interesting type of interlocking is that operating not at the level of intermediary metabolism but at the level of primary gene action: i.e. on the process of transcription of the gene. That gene interaction
of this kind should be widespread has been a creed of classical genetics for long, but it has never been formulated precisely. A precise model with exceptionally heuristic value has now been proposed by Jacob & Monod (1959). Because of its interest I shall summarize it here as a basis for discussion, in the latest version known to me, with apologies for errors and omissions (Jacob & Monod 1959, 1961, 1963; Monod & Jacob 1961).

The Jacob–Monod model is as follows:

(i) There are two kinds of gene: structural and regulatory. A structural gene acts by supplying the information for the aminoacid sequence of a polypeptide. It does this by transcribing its DNA base sequence into the base sequence of messenger-RNA, which 'programmes' the ribosomes for the assembly of the amino acids of the polypeptide.

(ii) A regulatory gene acts by blocking specifically the transcription of one or more structural genes. It does this by producing a specific substance, a repressor (a 'genotropic' substance in Waddington's (1962) apt terminology), which combines with a section of the structural gene, the operator. For simplicity Jacob & Monod (1963) assume that the operator is also the section from which the transcription of the structural gene starts. In some cases, at least in bacteria, it is known that this transcription involves a stretch of DNA including more than one gene. It will be remembered that Crick et al.'s (1961) work suggests that transcription is polarized.

(iii) The blocking of the transcription of a stretch of DNA by a repressor is in certain cases activated (e.g. in the case of repression of enzyme synthesis) and in other inactivated (e.g. in the case of induction of enzyme synthesis) specifically by compounds of small molecular weight of metabolic or external origin: the effectors. A repressor must therefore be able to recognize both an operator, i.e. a sequence of DNA nucleotides, and a compound of small molecular weight, the effector. For this reason, Jacob & Monod (1963) argue that repressors are proteins.

An important feature of the Jacob–Monod model is that of 'allosteric' relations, i.e. there is no necessary steric relation between an effector and the active site of an enzyme the synthesis of which is induced or antagonized by the effector via its action on the repressor. Effectors can thus interconnect metabolic processes which would otherwise have no necessary chemical connexion.

In bacteria at least two types of relation between regulator and structural genes are already known. One occurs in the case in which two or more structural genes, determining the aminoacid sequences of the two or more enzymes of a metabolic pathway, are located in sequence to one another on the chromosome. (This, incidentally, is a widespread feature of the formal genetics of bacteria (Demerec 1956), but it is almost unknown in other micro-organisms and in higher organisms.) In this case, the whole series of functionally related and serially linked genes may have a single operator on which the repressor produced by a regulator gene acts and thereby blocks the transcription of the whole series.

For a group of genes co-ordinated by linkage in this way, so that their transcription, and its repression, occur en bloc, Jacob, Perrin, Sanchez & Monod (1960) coined the word operon. In more recent versions of their model, Jacob & Monod
(1963) have realized that the linkage of genes related functionally is a special case of the model which they overstressed initially. They now define an operon as an operator plus one or more structural genes not necessarily related in function. It is possible, of course, that the usual size of the unit of transcription is a segment of DNA longer than one gene.

In another type of relation between regulatory and structural genes, known in bacteria, either there is no close linkage between a number of functionally related structural genes blocked by a common regulator, or there is linkage between some but not all. Here each structural gene, or each group, should have an operator identical to that of the others and recognized by one repressor (Gorini, Gunderson & Burger 1961).

A variant of this assumption is relevant to heteropolymeric proteins (i.e. made up of more than one kind of polypeptide (Pontecorvo 1960)) in the particular case in which the same polypeptide is a component of two or more different proteins. This is the well-known case of the three human haemoglobins, all three of which have the $\alpha$-polypeptide; in addition, each has another distinctive polypeptide: $\beta$, in the case of adult haemoglobin, $\gamma$ in the case of foetal haemoglobin, and $\delta$ in the case of the $A 2$ haemoglobin. In a case like this, the single structural gene for the common polypeptide could be that on which a repressor acts, and the co-ordinated repression of the two or more proteins would be mediated by regulation of the synthesis of the common polypeptide.

The important point is that the Gorini and other examples show that, even in bacteria, linkage is not the only way to achieve co-ordinated repression. In other words, the rate of synthesis of a number of enzymes, or other proteins, sequentially or otherwise related in function, can be co-ordinately regulated at the level of primary gene action without close linkage of the corresponding structural genes.

In micro-organisms more complex than bacteria, with their genetic material enclosed within a nuclear membrane, relations between regulator and structural genes are beginning to come to light (e.g. tyrosinase in Neurospora, Horowitz, Fling, Macleod & Watanabe (1961); $\beta$-glucosidase in yeast, Halvorson (1961); alkaline and acid phosphatases in Aspergillus, Dorn (1962)).

In these organisms close linkage between structural genes for enzymes acting in sequence in a pathway is very exceptional. For co-ordinated repression of a number of unlinked structural genes, coding for a number of functionally related enzymes, we have to think of systems which act otherwise than via an operon including the relevant genes.

From what has already been discussed, a single regulator could act either by repressing a single structural gene, coding for a polypeptide common to all the enzymes—each of which would have to be heteropolymeric—or by repressing all of the relevant structural genes—each of which would have to carry an identical operator. Both of these systems are likely to be widespread in nucleated micro-organisms and in higher animals and plants where, incidentally, heteropolymeric proteins seem to be rather common. The reasons for these, and other, suggestions will be given in the following sections.
6. Cascade regulation

Unlike bacteria, but like higher animals and plants, fungi have a nuclear membrane and do not show the sequential linkage, characteristic of bacteria, between structural genes determining enzymes of a pathway. Consideration of certain features—admittedly still very crudely known—of fungal genetics, suggests two modifications of the Jacob–Monod model which are likely to be important in these and in higher organisms and which may well apply in a rudimentary form also to bacteria.

One of these modifications is the genotropic regulation of regulatory genes: 'cascade regulation'. The other is the effective localization of repressors, to be discussed in § 7. The former makes the model more pliable; the latter more precise.

While Jacob & Monod do not explicitly exclude genotropic regulation of regulators (see, e.g. figure 5, p. 399, Monod & Jacob 1961), their emphasis throughout is on genotropic regulation of structural genes or, concisely, the one-regulator model. Waddington (1962, p. 24) explicitly mentions regulation of regulators as a possibility, and coins the apt term 'cascade repression' for it. Perhaps an even more appropriate term would be 'cascade regulation', which will be used here.

'Cascade regulation' may be defined as regulation of the primary action (transcription) of a regulator gene by a repressor, a primary product of another regulator gene, which may or may not require activation (see § 8). By specifying primary product of a gene I exclude, for instance, the products of the activity of an enzyme for which a gene codes.

At the present stage we can limit ourselves to considering the interesting consequences of cascade regulation in its simplest model, concisely, the two-regulator model. More complex systems, involving more than two regulators, cyclical series of regulators, refinements of the distribution of regulators and structural genes into operons, etc., are all conceivable, and indeed likely to occur.

The two-regulator model of cascade regulation is as follows: one regulator gene \( R_2 \) produces a repressor \( \rho_2 \) which acts on another regulator gene \( R_1 \) producing a repressor \( \rho_1 \) which acts on a structural gene \( S \) producing, via messenger RNA, polypeptide \( \sigma \).

\[
\begin{align*}
\text{Regulator gene} & \quad \text{Regulator gene} & \quad \text{Structural gene} \\
\begin{array}{c}
R_2 \\
\rho_2 \quad \text{repressor} \\
\end{array} & \quad \begin{array}{c}
R_1 \\
\rho_1 \quad \text{repressor} \\
\end{array} & \quad \begin{array}{c}
S \\
\sigma \quad \text{polypeptide} \\
\end{array}
\end{align*}
\]

Effectors \( e_1 \) and \( e_2 \), of metabolic or external origin, have a role to play in the two-regulator model, just as they have in the Jacob–Monod model. In fact, the former is compatible with a simplification in respect of the effectors: they need have only a negative action, i.e. that of interfering with the genotropic action of the repressors.
In the scheme of cascade regulation given above we can distinguish four typical cases, depending on whether either, both or neither effector $e_1$ and $e_2$ play a part in a given system. Furthermore, in the cases in which one or two effectors could play a part, they may be present or absent:

Case I. There are no effectors; $R_2$ is active to the extent of producing the second-order repressor $p_2$ at a rate adequate for the full repression of $R_1$; no first-order repressor $p_1$ is produced, and the structural gene $S$ is transcribed at maximal rate. The synthesis of polypeptide $\sigma$ is ‘constitutive’.

Case II. The action of $p_2$ on $R_1$ can be antagonized by effector $e_2$. When $e_2$ is present $p_1$ is produced and $S$ is not transcribed. The synthesis of polypeptide $\sigma$ is ‘repressible’.

Case III. $R_2$ is not sufficiently active to repress completely $R_1$. The action of $p_1$ on $S$ can be antagonized by effector $e_1$. When $e_1$ is present $S$ is transcribed. The synthesis of polypeptide $\sigma$ is ‘inducible’.

Case IV. The action of $p_1$ on $S$ can be antagonized by effector $e_1$, and that of $p_2$ on $R_1$ by effector $e_2$. There are four possibilities according to whether $e_1$ only, $e_2$ only, both or neither are present. The results are superficially similar to, but potentially distinguishable from those in one or other of the three previous cases. Specifically, when both effectors are present, the results are superficially as in Case I; when $e_2$ only is present they are as in Case II; when $e_1$ only is present they are as in Case III; when both are absent the results are again as in Case I.

In a system in which the effective intracellular concentrations of both effectors were open to experimental control, certain specific predictions could be made. For instance, in the absence of $e_1$ the otherwise apparently ‘constitutive’ synthesis of polypeptide $\sigma$ becomes ‘repressible’ by $e_2$, and in the presence of $e_2$ it becomes ‘inducible’ by $e_1$.

Thus, with cascade regulation, a difference between ‘inducible’ and ‘repressible’ polypeptide synthesis could be that an effector inactivates a first-order repressor in the first case and a second-order repressor in the second case. Constitutive synthesis could be one in which effectors do not play a part, or in which other relevant parts of the system are absent or cancel each other out (see §7). It seems likely that there should be many cases in which the synthesis of a polypeptide is both inducible and repressible, i.e. under the control of two effectors, one antagonizing the primary repressor and the other the secondary repressor. If this is so, a difference between inducible, repressible and constitutive polypeptide synthesis might only be a matter of balance between the inactivation, by effectors, of the first order and of the second-order repressor.

We can now consider the effects, in the case of cascade regulation, of the types of mutation so elegantly identified and incorporated in the Jacob–Monod model:

(a) mutation of a regulator gene to the production of a less active or inactive repressor;

(b) mutation of a regulator gene to the production of an active repressor less sensitive to antagonism by an effector;

(c) mutation in the operator of a regulator gene ($R_2$) to lesser sensitivity to a repressor ($p_2$) produced by another regulator gene ($R_2$);
(d) mutation in the operator of a structural gene \((S)\) to lesser sensitivity to a repressor \((p)\), with or without relevant consequences on the properties of the polypeptide for which \(S\) codes;

(e) mutation in the structural gene \((S)\) determining failure of synthesis of the polypeptide, or synthesis of an altered polypeptide.

The only type not described by Jacob & Monod is type (c), the existence of which is assumed here on the basis that such type of operator mutation is already known in the case of structural genes.

With cascade regulation, the various types of mutation described above and their combinations give again all the results incorporated in the Jacob–Monod one-regulator model. But the two-regulator model predicts specifically in addition certain effects which can therefore be used to test it. A few examples will suffice:

(A) In Case I ('constitutive' p. 11) a mutation of type (a) in \(R_t\) produces a recessive loss of the ability to synthesize the polypeptide, which can be compensated by a type (a) mutation in \(R_j\). This is the case referred to in genetic jargon as a 'recessive extra-cistron suppressor'. The distinctive feature is that both mutations map at loci other than the structural locus.

(B) In Case I ('constitutive') or II ('repressible') a type (a) mutation in \(R_j^t\), producing a recessive failure of synthesis of the polypeptide, can be restored by a type (d) mutation in \(S\) which will behave as dominant in genetic jargon a 'dominant extra-cistron suppressor'.

(C) In Case II ('repressible') a type (b) mutation in \(R_j^t\) produces a 'constitutive' (i.e. non-repressible) mutant dominant in the heterozygote but not in the heterokaryon (see §7) which does not map, as the type (d) mutants in the Jacob–Monod model, at the \(S\) locus, but maps at the \(R_j^t\) locus.

(D) In Case I ('constitutive') and partially in Case III ('inducible') a type (c) mutation in the \(R_j\) operator causes a failure of synthesis of the polypeptide, dominant in the heterozygote but not in the heterokaryon (see §7). Further mutation, this time of type (a), will restore the synthesis but only when the second mutant is in cis arrangement with the first mutant or homozygous. In most cases, type (c) mutations should be far less frequent than type (a).

In genetics of bacteria, fungi and higher organisms there are, of course, many examples formally fulfilling the requirements under (A) or (B). Their precise analysis—which demands as a first step the rigorous identification of the \(S\) gene or genes as distinct from the regulators—has not even begun. Yet, the occurrence of intranuclear localization, described in the next section, supports an interpretation based on cascade regulation as the simplest for some of these examples.

Mutations producing effects (C) and (D) are not yet known. A system for isolating selectively those under (D) in diploid Aspergillus nidulans is now available (Apirion 1962). The properties predicted for mutants of this type are so unusual and restrictive that if it occurs at all it will lend very strong support to the model.

There have been recent reports both about bacteria (Garen & Echols 1962) and about fungi (Horowitz et al. 1961; Halvorson 1961) of systems for the interpretation of which more than one regulator gene is postulated. For instance, Garen & Echols (1962) have found that constitutive mutants for the (repressible) synthesis
of alkaline phosphatase in *Escherichia coli* map at two loci other than structural. Mutants at one locus are of the usual constitutive type, i.e. they show maximal rate of synthesis irrespective of the presence of the effector (orthophosphate). Mutants at the other locus are essentially of two types. Those of one type are unable to synthesize the enzyme, like most mutants at the structural locus. Those of the other type are able to synthesize it but at a low rate, and this rate is insensitive to the effector.

Garen & Echols (1962) refer to the two loci as 'regulators' and interpret the features of this system by assuming that one regulator gene produces an inducer which an enzyme, produced by the other regulator gene, transforms into a repressor in the presence of orthophosphate. This interpretation is of course plausible, except semantically. If we start using the term 'regulator gene' for a gene producing an enzyme which catalyzes the formation of a substance which represses a structural gene, then the whole idea of *genotropic* regulation becomes confused with all sorts of interactions at the level of intermediary metabolism which feed back on the genes. This is precisely a familiar type of confusion in classical genetics which the Jacob–Monod model has done so much to remove, and which Waddington (1962, p. 7, figure 1) has clarified. Apart from this criticism on the use of terms, an interpretation based on cascade regulation, with the effector inactivating the second-order repressor (Case II, p. 11), might be equally plausible in this case.

In closing this section I do not wish to suggest that cascade regulation is universal in nucleated organisms. The two-regulator system is its simplest form and we may expect increasingly diverse and more elaborate forms in organisms with increasing complexity. Nevertheless, the single regulator system of Jacob & Monod, which is certainly widespread in bacteria, need not have been totally supplanted in nucleated organisms, nor need rudimentary cascade regulation be absent from bacteria.

In addition, we should not expect *all* structural genes in higher organisms to be under regulator gene control. Even in organisms as complex as man there are certain proteins—for instance, catalase—which are synthesized in all types of cells and their synthesis seems to be *indifferent to* internal or external conditions. The corresponding structural genes could be examples of systems with no genotropic regulation, unless they are examples of such a perfect and complex genotropic regulation that their activity is completely buffered against all sorts of variation in intra- and extracellular conditions.

7. Intra-nuclear localization of repressors

Unlike bacteria and higher organisms, certain fungi—among those analyzed genetically the ascomycete *Aspergillus nidulans* (review, Pontecorvo 1958) and the basidiomyecete *Coprinus lagopus* (Lewis 1961 and unpublished)—have vegetative bi- or multinucleate cells which may carry either haploid or diploid nuclei. In another fungus, *Neurospora crassa*, also with multinucleate cells, true diploid nuclei do not seem to occur, but the next best are nuclei disomic for a particular chromosome. Systems of this kind offer the valuable possibility of comparing the effects of the same combination of mutant and normal alleles within one nucleus.
(heterozygote) and between two kinds of nucleus in a bi- or multinucleate cell (heterokaryon). An additional dimension (Pontecorvo 1950, 1952b) for the expression of a difference between cis and trans arrangement of alleles is therefore available.

It is a common experience that in the majority of cases there is no obvious difference between the heterozygote and the corresponding heterokaryon. On general grounds, however, it was expected that cases of such a difference would come to light (Pontecorvo 1950), and the first example was in fact found (Pontecorvo 1952b, pp. 228–229) soon after Roper (1952) made it possible to obtain heterozygous diploids in *Aspergillus*. Formally the situation can be represented as follows:

$$\frac{m_1 + m_2}{m_1}$$

compared with $$\frac{(m_1 +)}{(m_1 +)} \frac{(m_2 +)}{(m_2 +)}$$

(formalism: mutant alleles; +: normal alleles; brackets identify individual nuclei; fraction signs separate alleles between homologous chromosomes.) The critical comparison is between A, in which the two normal alleles are within one nucleus (cis) and B, in which they are in separate nuclei (trans). Situation B' can be ignored for the time being.

In the last few years a number of further instances of differences in phenotype between heterozygote and heterokaryon have come to light—in *Aspergillus*: Roberts (1962), Apirion (unpublished); in *Coprinus*: Lewis (1961, and unpublished); in *Neurospora* disomics: Case & Giles (1960). All these cases indicate effective localization within the nucleus of the reactants in the processes, determined by the + alleles, which lead to the normal phenotype. Of the following four examples at least the first three can be interpreted easily in terms of the two-regulator model, with the additional unexpected deduction that the reactants effectively localized within the nucleus are repressors.

Example 1. Each of two recessive unlinked mutants (*f3* and *f101*) of *Aspergillus nidulans* differ from the wild type in being unable to utilize acetate as only carbon source (Apirion 1962, and unpublished). The heterokaryon (*f3* +) (+ *f101*) between the two mutants—kept balanced by means of other nutritional requirements—has the mutant phenotype. The diploid double heterozygote $\frac{f^3}{f^3 +}$ has the normal phenotype. Formally, the two mutant alleles—each of which is recessive to its normal allele in the heterozygote and in the heterokaryon—are recessive in the double heterozygote and dominant in the corresponding heterokaryon. Thus, both normal alleles have to be within one nucleus for the production of the normal phenotype: their functions are interdependent and effectively localized within one nucleus. This example, however, may turn out to be more complex (three loci?).

Example 2. A recessive mutant of *A. nidulans* (*meth2*) differs from the wild type in requiring methionine for growth. Another unlinked mutant (*su8*), with no known effect by itself, restores the ability of *meth2* to grow without methionine. The diploid $\frac{meth2}{meth2 +}$ requires methionine, while the heterokaryon $(meth2 +) (meth2 su8)$. 

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does not (Luig, unpublished). Formally \( su8 \) is recessive in the heterozygote and dominant in the heterokaryon; in other words, the function mediated by the normal allele—\( su8 \)—which \( su8 \) cannot perform, is effectively localized within one nucleus.

**Example 3.** Another similar example in *Aspergillus*, but in this case with close linkage, is that of \( paba22 \) (recessive requirement for \( p \)-aminobenzoate) and \( su1 \), which in combination with \( paba22 \) in the haploid restores the ability to grow without \( p \)-aminobenzoate. Luig (1962) found that the diploid \( \frac{paba22}{
\begin{array}{c}
\text{Heterokaryon} \\
\text{Genotype} \\
\text{Products} \\
\end{array}
\end{array}
\) 

\[
\frac{f3^+}{f101^+} = S^+ 
\]

(f3 +) (+ f101)

\[
\begin{array}{c}
\text{Heterokaryon} \\
\text{Genotype} \\
\text{Products} \\
\end{array}
\end{array}
\]

\[
\frac{f3^+}{f101^+} = S^+ 
\]

(f3 +) (+ f101)

\[
\begin{array}{c}
\text{Heterokaryon} \\
\text{Genotype} \\
\text{Products} \\
\end{array}
\end{array}
\]

\[
\frac{f3^+}{f101^+} = S^+ 
\]

(f3 +) (+ f101)

\[
\begin{array}{c}
\text{Heterokaryon} \\
\text{Genotype} \\
\text{Products} \\
\end{array}
\end{array}
\]

\[
\frac{f3^+}{f101^+} = S^+ 
\]

(f3 +) (+ f101)

\[
\begin{array}{c}
\text{Heterokaryon} \\
\text{Genotype} \\
\text{Products} \\
\end{array}
\end{array}
\]

\[
\frac{f3^+}{f101^+} = S^+ 
\]

(f3 +) (+ f101)

\[
\begin{array}{c}
\text{Heterokaryon} \\
\text{Genotype} \\
\text{Products} \\
\end{array}
\end{array}
\]

\[
\frac{f3^+}{f101^+} = S^+ 
\]

(f3 +) (+ f101)

\[
\begin{array}{c}
\text{Heterokaryon} \\
\text{Genotype} \\
\text{Products} \\
\end{array}
\end{array}
\]

\[
\frac{f3^+}{f101^+} = S^+ 
\]

(f3 +) (+ f101)
Interpretation of Example 2
\[ \text{meth}^{2+} = R_t^+ \quad \text{and} \quad \text{su}^{8+} = R_t^+ \]
meth2 is a mutant type \( a \) failing to produce \( \rho_2 \), therefore \( \sigma \); su8 is a mutant type \( a \) failing to produce \( \rho_1 \)

\[
\text{Heterokaryon} \quad \text{Heterozygote} \\
(meth2 +) \quad (meth2 \ su8) \\
(R_t^- \ R_t^+ \ S^+) \quad (R_t^- \ R_t^+ \ S^+ \ S^+) \\
\sigma \text{ produced, methionine not required} \quad \sigma \text{ not produced, methionine required}
\]

Interpretation of Example 3
\[ paba^{22+} = R_t^+ \quad \text{and} \quad su^{1+} = R_t^+ \]
both paba22 and su1 are mutants type \( a \)

\[
\text{Heterokaryon} \quad \text{Heterozygote} \\
(paba^{22} +) \quad (paba^{22} \ su1) \\
(R_t^- \ R_t^+ \ S^+) \quad (R_t^- \ R_t^+ \ S^+ \ S^+) \\
\sigma \text{ produced, pABA not required} \quad \sigma \text{ not produced, pABA required}
\]

Interpretation of Example 4
\[ sup^{-3+} = R_t^+ \quad \text{and} \quad sup^{-5+} = S^+ \]
(or vice versa)
sup-3 is a mutant type \( a \) failing to produce \( \rho_2 \), therefore \( \sigma \); sup-5 is a mutant type \( e \) failing to produce \( \sigma \), an enzyme determining methionine requirement in the presence of me-1.

\[
\text{Heterokaryon} \quad \text{Heterozygote} \\
(sup^{-3} +) \quad (+ sup^{-5}) \\
(R_t^- \ R_t^+ \ S^+) \quad (R_t^- \ R_t^+ \ S^+ \ S^+) \\
\sigma \text{ not produced, methionine not required} \quad \sigma \text{ produced, methionine required}
\]
The examples given and interpreted above are, of course, no more than straws in the wind. They show without question that certain products of gene activity are effectively localized within the nucleus. That these products are repressors, i.e. products of the primary activity of regulator genes, is so far a pure assumption. It should not be difficult, however, to test this assumption in a few cases.

The effective localization could have several causes, for instance: a substance locally produced at extremely low rate and/or captured on the spot, and/or labile, and/or destroyed or diluted below effectiveness when outside the nucleus, or physically unable to escape from the nucleus etc.

One point should be made clear: the examples given of failure by the normal allele in one nucleus to compensate for a mutant allele in another nucleus in a heterokaryon, are not of the spurious type which abounds in the Neurospora literature (see de Serres 1962), a rich spawning ground for red herring. In Aspergillus, heterokaryons are forced by growth on a medium lacking at least two growth factors, other than those under test, each required by one of the component strains; the heterokaryotic condition is then verified by successive transfers, by using autonomous colour markers, and occasionally by plating for diploids. In Coprinus the dikaryotic condition can be verified unequivocally by inspection for the presence of clamp connexions.

Another point to be stressed is that should the hypothesis of the intra-nuclear localization of repressors turn out to be correct, we should not expect all cases of a difference between heterozygotes and heterokaryons to involve repressors. An instance is intra-cistron complementation, i.e. the production of an almost normal enzyme by a heterozygote or heterokaryon carrying two different alleles of a structural gene each producing an inactive form of the enzyme.

A current hypothesis for intra-cistron complementation is that it results from ‘hybrid’ polymerization between the two differently defective kinds of polypeptide (Fincham 1959). If polymerization occurs as the polypeptides are released from the ribosomes where they have been assembled, we might expect very marked localization effects in a heterokaryon; especially in cases of low rate of enzyme synthesis. In a heterokaryon the two mutants are in different nuclei; each of the two kinds of messenger-RNA is liberated from one nucleus and it is likely that it may programme preferentially the ribosomes in the vicinity of that nucleus. The two kinds of defective monomers would then be concentrated in different parts of the cell, and the proportion of ‘hybrid’ polymerization would be low. In a heterozygote both kinds of messenger-RNA are liberated from each nucleus and therefore the ribosomes programmed by one or the other RNA would be thoroughly interspersed. The proportion of ‘hybrid’ polymerization could approach that expected on random association.

Perhaps this sort of localization underlies the difference between heterokaryon and heterozygote found by Roberts (1962) in the case of certain heteroallelic Aspergillus mutants defective in an enzyme of sorbitol metabolism. It would also be interesting to know whether the reduced efficiency of intra-cistron complementation in heterokaryons between Neurospora leucineless mutants, found by Gross

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In closing, work in Luria's laboratory (1961, p. 210), on β-galactosidase synthesis in *Escherichia coli* transduced by phage P1 dl, is relevant. In the transduced cells \( R_f^+ \) and \( S^- \) (symbols of my model) are carried on the bacterial chromosome. The phage carries either \( R_f^+ S^+ \) or \( R_f^+ S^- \). The repression by \( \rho_f \) of \( S^+ \) carried by the phage is much more effective when \( R_f^+ \) is on the phage than when it is only on the bacterial chromosome. Luria favours an explanation based on local concentration of repressor. This could be a primitive form of the intranuclear localization which, as suggested in this section, seems to be characteristic of nucleated organisms.

8. A MODEL FOR INTRANUCLEAR REPRESSION

So far there is no direct evidence on the chemical nature of repressors even in bacteria, let alone in nucleated organisms. For bacteria, Jacob & Monod (1963) argue that repressors must be proteins since a repressor must recognize on the one hand the operator, i.e. a stretch of DNA, and on the other hand an effector, i.e. a metabolic or external compound of low molecular weight.

For the case of nucleated organisms and with cascade regulation this property of dual recognition needs reconsideration. In principle, not all repressors—defined as primary products of regulator genes—need have it. For instance, in the case of repressible polypeptide synthesis (Case II, p. 11), the two-regulator model requires a recognition of an effector by \( \rho_2 \) but not, or not always, by \( \rho_1 \). The opposite applies to the case of inducible polypeptide synthesis (Case III, p. 11). The recognition ability which must be assumed for all repressors is for the base sequence of a specific operator, presumably by complementary base pairing, and for this an RNA transcript of part or all of the regulator gene is adequate.

A second point is that histones are characteristic intranuclear proteins in search of employment. Stedman & Stedman (1951) found them a hypothetical job long ago: that of specific inhibitors of the genes. This hypothesis is coming back into favour (Leslie 1961; Huang & Bonner 1962; Allfrey & Mirsky 1962). Could repressors be histones for which regulator genes code? This seems to be unlikely for the following reasons.

In higher organisms the number of recognizably different histones seems to be not large, and variation between histones from different sources not great (Neelin & Butler 1961). Thus it looks as if histones may not occur in the enormous range of specificities required of repressors in higher organisms. We have to think in terms of thousands of structural genes, most of them regulated by one or more regulators, and each regulator producing a repressor specific for one operator. Even if usually more than one structural gene were repressed by the same repressor—by one of the mechanisms discussed before—the number of specific repressors to be thought of is at least several hundreds but more likely several thousands.

Yet, Jacob & Monod's argument that a protein is needed for the dual recognition of a nucleic acid and an effector is unassailable. The point which I wish to make is that the range of specificities required for the recognition of operators is likely to be one or two orders of magnitude greater than that required for the
recognition of effectors. In other words, we need to think of not more than several tens of different effectors, while we have to think in terms of several hundreds, possibly thousands, of different operators.

If this estimate is correct then the histones can still come into the picture with two functions as a middleman between repressors and effectors. One function is the recognition of small molecular weight effectors, with each molecular species of histone specific for one effector; the other is the recognition of repressors. Each regulator gene comes into the picture by producing an RNA transcript (the repressor) which recognizes: (a) the DNA base sequence of an operator, and (b) one out of a few tens of different histones. The repressor may share this second recognition with other repressors produced by other regulator genes. Coding for histones would require only a few tens of structural genes: the actual synthesis of histones might well be intranuclear.

On this model the repressor, an RNA transcript of the regulator gene, has a base sequence a part of which is specific for the base sequence of one or a few out of several thousand operators (DNA), and another part specific for one out of several tens of histones. The histone combines with the repressor RNA, or with a major part of it, and activates it. The activated repressor selects the relevant operator and blocks it. If an effector for which the histone is specific is present, the combination of the repressor with the histone is obstructed, or the fit of the active repressor-histone onto the operator is affected.

Clearly, the purely hypothetical mechanism proposed here has been inspired by the now well established mechanism for the adaptors in protein synthesis, which was no less imaginary when first suggested by Crick (1958). Instead of the twenty or so sRNAs, the twenty or so activating enzymes and the twenty amino acids, we have several thousand repressor RNAs, and several tens of histones and effectors. This hypothetical mechanism has the attractive feature that the simultaneous repression and de-repression of the synthesis of a number of proteins can be achieved in a further way, besides the three already discussed: a single effector, of metabolic or external origin, can affect simultaneously the rate of synthesis of a number of proteins because of the specificity for it of a single histone, activator of a number of repressors.

We come to the conclusion that at least four genotropic mechanisms for coordinate regulation are already known or conceivable:

1. The Jacob–Monod operon, with linkage in sequence of a number of structural genes. This determines not only qualitative co-ordination but also strictly quantitative co-ordination (Ames & Garry 1960; Beckwith, Pardee, Austrian & Jacob 1963).

2. The Gorini identical operator in a number of unlinked structural genes.

3. The operator of a structural gene coding for a polypeptide common to a number of heteropolymeric proteins.

4. The histone activating the first- or second-order repressors of a number of unlinked structural genes.

Mutually exclusive co-ordination is also conceivable. When the first-order repressor of a structural gene is the second-order repressor of another structural gene, the activities of the two structural genes are alternative.
If we add to the models for multiple *genotropic* regulation of protein synthesis the possible ways in which two or more genotropic systems can be interlocked via cytoplasmic metabolites, as suggested by Monod & Jacob (1961), and the interlocking at the level of enzyme activities suggested long ago by Delbrück, it seems that there is now a quite adequate theoretical framework for an initial experimental attack on cell differentiation.

9. The meaning of the nucleus

There is a discontinuity between the genetics of bacteria and the genetics of other more complex organisms which sets bacteria apart even from the micro-fungi and the green unicellular algae. This discontinuity consists of the absence of a distinct nucleus. Besides not having a nuclear membrane to keep their genetic material separate from the endoplasmic reticulum and other cytoplasmic structures, the bacteria have a number of distinctive features. They have much less DNA per genome, they show linkage in sequence of genes coding for enzymes acting in sequence and they do not show even the rudiments of morphogenesis. On two further distinctions—the inability to synthesize steroids and possibly histones—the evidence is not conclusive.

All these features become significant if one considers the development of cascade regulation and perhaps of other *genotropic* control mechanisms up the ladder of complexity to the gigantic level which we can guess for higher animals.

An organism like yeast, or the slightly more complex *Aspergillus nidulans*, is not likely to have many more structural genes than *Escherichia coli*. If it did, it should have a correspondingly greater number of proteins. Unless the difference in DNA—by a factor of 2 and 4, respectively—is made up mainly by genetically inert material, regulator genes of known and unknown types and not structural genes must account for it. Let us attempt an estimate.

Taking some 1000 nucleotides as the average DNA length of a structural gene (i.e. 300 amino acids as the average length of a polypeptide) how long should a repressor RNA for such a gene be? The repressor has to identify one operator out of several thousand and one histone out of several tens. Unambiguous individuation of the operator alone is likely to require a sequence of 100 deoxyribonucleotides at the very least. Correspondingly, the regulator gene, of which the repressor is assumed to be an RNA transcript, should have a length of these 100 nucleotides and perhaps a length for the recognition of the histone. We come to the conclusion that while on the whole regulator genes can be shorter than structural genes they are not likely to be as short as one-tenth.

The difference by a factor of about 2 in the DNA per genome between yeast and *Aspergillus* suggests, therefore, that even in an organism barely more complex than yeast there are at the very minimum two regulator genes per structural gene.

This is quite a surprising conclusion; a job for part at least of the enormous amount of DNA in, say, mammals seems now to be available (Pontecorvo 1958). We are also a long way ahead of the one gene-one enzyme, or even the one gene-one polypeptide generalization when we are compelled to realize that the proportion
of genes coding for polypeptides becomes smaller and smaller as the total number of genes increases up the scale of organic complexity.

With an increasing proportion of regulator genes the seclusion of the genetic material in the nucleus makes sense. In electron micrographs the unencumbered, almost structureless appearance of the nuclear sap contrasts with the fantastic structural organization of the cytoplasm. Economy in the synthesis of repressors and histones is achieved by confining regulator, operator and histone within the nucleus. The reciprocal search of repressors for histones and of activated repressors for operators is facilitated by the absence of structural barriers.

The techniques for the identification of different 'informational' RNAs, so ably designed and used by the group led by Spiegelman, should now make it easy to identify, in nucleated organisms, the repressor RNAs (rRNA) as distinct from messenger RNAs (mRNA), soluble RNAs (sRNA) and the informational RNA, or RNAs, ultimately built into ribosomes. The point is that repressor RNAs should represent the major proportion of the molecular species of RNA found exclusively in the nucleus and possibly conjugated with histones.

Another of the peculiarities of the genetics of bacteria—the operon including functionally related structural genes: the 'functional operon' for short—is also a feature discarded by organisms with a higher development of more complex genotrophic regulatory mechanisms.

The 'functional operon' has an understandable appeal for embryologists who are desperately searching for systems of co-ordinated switching on, or off, of a number of functions in differentiation. That appeal is deceptive, for two reasons. One, not cogent, is that at least one other genotrophic mechanism, and possibly three, for switching on and off simultaneously a number of genes are already known to exist, or are plausibly inferred (see p. 19). The other is that the very feature of quantitative co-ordination which makes the functional operon so effective, and therefore so widespread, in the utterly extrovert world of the bacteria, makes it too rigid both functionally and evolutionarily in more complex organisms in which the relations of cells with the environment are so much more indirect.

Evolutionarily, the 'functional operon' is a hindrance because, just as an inversion of a chromosome, it makes difficult the evolutionary change of arrangement of genes along the chromosomes, a change which certainly occurs but the significance of which is still not clear. Functionally it is too crude because it does not allow for finesse of modulation of the activities of the individual genes in an operon.

Consider, for instance, the finesse of co-ordinated regulation possible in a simple system of cascade regulation with \( \rho_2 \) repressing \( R^A, R^B \) and \( R^C \), which, in their turn, repress the unlinked structural genes \( A, B \) and \( C \). With \( \rho_2 \) not produced or inactive, \( A, B \) and \( C \) are all fully repressed. With partial \( \rho_2 \) activity \( A, B \) and \( C \) are all active but their activities can be individually adjusted, for instance, by three effectors. This would not be possible if \( A, B \) and \( C \) were in an operon, even with cascade regulation.

Besides the above arguments suggesting that the 'functional operon' should not be a widespread feature of the genetics of organisms more complex than bacteria,
there is one much more powerful: in spite of plenty of opportunities for showing its existence, the functional operon is conspicuous for its almost complete absence outside the bacteria and no wishful thinking will create it. A cursory look at the classical literature—i.e. the literature up to 1959—is quite conclusive. In fungi the ‘functional operon’ for enzymes acting in a pathway is, to say the least, extremely rare. In higher organisms sequential linkage between genes acting sequentially on a morphogenetic process is at least equally rare.

In this lecture I have tried to convey the idea that genetics has been the tool for some of the most exciting advances in biology of this century. The latest, barely explored but potentially very important, is the precise formulation by Jacob and Monod of the problems of genotropic regulation. Evidence already available from organisms with a nuclear membrane suggests certain expansions and modifications of that formulation which was based exclusively on evidence from bacteria.

I have omitted in this lecture the consideration of the usefulness that the knowledge gained from microbial genetics has for the problems posed by the formal genetics of higher organisms, including man. Of particular importance is the possibility of by-passing the stumbling block of sexual reproduction by genetic analysis of somatic cells. This may require either the use of some of the novel processes of gene recombination found in micro-organisms (Pontecorvo 1962) or the chemical identification and analysis of transcripts of the genes (Hall & Spiegelman 1961).

On second thoughts, I am sure that Leeuwenhoek would have welcomed the possibility that the study of animalcules could help in the understanding of the genetics of larger animals. After all, in a letter of 16 July 1683 (trans.: Sirks 1959) he described accurately dominance in the inheritance of coat colour in the rabbit.

REFERENCES

Avery, O. T., MacLeod, C. & McCarthy, M. 1944 J. Exp. Med. 79, 137–158.
Beale, G. H. 1954 The genetics of Paramecium aurelia. Cambridge University Press.
My purpose today is to state in a somewhat unusual form the essentials of the current views on the nature of heredity. I shall be satisfied if at the end the impression will be that I have produced nothing more than obvious platitudes.

Mendel did not distinguish explicitly between factors and characters in his papers and perhaps he did not even have this distinction clear in his own mind; yet we have no alternative but to readmit in his paper, which would make no sense without it.

This distinction, later expressed as one between genotype and phenotype, underlies the idea of 'genetic material', an idea which has been exceptionally fruitful for over 50 years but which is no longer needed. The present clearer understanding of the part which the various cell structures and processes play in heredity makes it superfluous. In fact the expression 'genetic material' offers unlimited opportunities to those who don’t mind falling into the trap of circular argument.

The most illuminating contribution of Mendelian genetics, and of its progeny—biochemical genetics and molecular biology—is that we can now distinguish quite clearly, right down to the molecular level, two utterly different types of cellular process: template and stepwise.

A template process is one in which there is no necessary chemical restriction to the order in which building blocks of a small number of kinds—four nucleotides, twenty amino acids and so on—can be polymerized into macromolecular structures, but the actual arrangement selected in any one case is uniquely determined by a pre-existing one, identical, complementary or otherwise related.

The distinctive feature of a template process of synthesis is that the uniqueness of the product is wholly predetermined in the uniqueness of the template. With trivial provisos (e.g. the inability of viral RNA-primed polymerase to polymerize RNA other than that of the virus), the enzymes taking part in a template process of synthesis have no relevance to the uniqueness of the product. They catalyse the formation of simple bonds—a peptide bond; a pentose 3,5-phosphate bond, an α-glucoside bond, etc.—with indifference to the sequence of the building blocks that they bind together.

This feature of template processes underlies the very special way in which templates can change without thereby being destroyed: i.e. only by mutation or recombination, i.e. by change of one kind of building block into another, or by exchange for another or by deletion, addition or change of sequence of building blocks. Mutational or recombinational changes are accompanied by another outstanding feature: the changed template replicates as of the changed type, is
transcribed as of the changed type and is translated as of the changed type. We
realize why Luria could state, recently, that specific mutagens, i.e. mutagens
selective for one gene, can only be substances with information not much smaller
than that of the gene. Mutation and replication are such basic features of living
systems that there are those who would equate them to life itself.

Stepwise processes are all those metabolic processes in which it is the specificity
of an enzyme which determines the specificity of a product, usually with no
possible alternatives. Processes of this kind occur mostly as a series of steps:
substance $A$ acted upon by enzyme $\alpha$ gives substance $B$; substance $B$ acted upon
by enzyme $\beta$ gives substance $C$, etc. Since the specificity of an enzyme is itself the
result of a series of template processes, we are back to the postulate which Crick
propounded eight years ago, i.e. that the flow of information is essentially one way:
stated in the present terms, from template processes to stepwise processes. This
does not mean that there is no feedback of stepwise processes (and of the external
environment) on template processes. It does mean that this feedback can only
affect the rates of template processes but not the specificities of their products.

Characteristic of stepwise processes is that the sequence of steps from $A$ to $Z$
via, say, $M, N, P$, can be replaced by alternative ones, say, via $\beta, \gamma, \omega$. The
replacement in evolution of a pathway of synthesis or degradation by another one,
without changing an end product (say an amino acid or $CO_2$), is well known in
comparative biochemistry.

An important question that should be explored now is if the thermodynamics
of template systems and that of stepwise systems differ in some basic feature.

It is clear that the phenomena which we put under the vague umbrella of
‘heredity’ (I should not like to attempt a definition) are based essentially on
template processes. Cases of heredity based on self-perpetuating alternative
steady states—i.e. on special interactions of stepwise processes—are becoming more
and more elusive. Nevertheless, we should not conclude that they cannot exist.

We should ask ourselves, furthermore, how many different types of template
process, besides the few already identified, exist and operate not only in heredity
but in the related fields of differentiation and morphogenesis.

The best-known series of template processes are those involving the chromosomal
DNA, its transcription into a series of RNAs (transcrf., or $sRNA$, ribosomal $RNA$
and messenger $RNA$) and the translation of $RNA$ into polypeptides. The cuckoo
part that $DNA$ viruses and $RNA$ viruses play in this game is also fairly clear. Yet
the current picture, satisfactory for bacteria, is not too good for nucleated cells.
where the existence of messenger $RNAs$ migrating out of the nucleus to the ribo¬
somes, or with the ribosomes, is doubtful.

Molecular biologists have made only weak attempts to search for different
classes of ribosomes. There was an anticlimax of interest when, barely four years
ago, the individual ribosome turned out not to be the template for translation.
However, it could be that there are as many species of ribosomes, or, say, of their
$32S$ components, as there are different codons, $sRNAs$ and activating enzymes.
Could it be, furthermore, that the function of messenger $RNA$ in nucleated cells
is almost wholly intranuclear? It could then consist in selecting codon-specific
ribosomes according to the sequence of codons in the messenger and fixing them in this sequence, on some surface, e.g. the nascent endoplasmic reticulum, which would then carry them into the cytoplasm. It would make a pleasing evolutionary picture if it were found that even in bacteria the messenger function were that of ordering in their correct sequence codon-specific ribosomes rather than acting directly as the template for the assembly of the amino acids in a polypeptide. It will be apparent, of course, that there are difficulties, but not insuperable ones, due to the size of the ribosomes relative to the spacing of codons on the messenger RNA.

If there are still plenty of question marks in the most thoroughly investigated series of template processes, that from chromosome to protein, the picture is completely blank in other spheres.

It is now quite clear that mitochondria, plastids and kinetosomes carry DNA. Does it act as a template, and for what? The DNA of the ciliate macronucleus replicates autonomously and, presumably, codes for proteins but is scrapped now and then and replaced by micronuclear DNA. Could the DNA of mitochondria, plastids and kinetosomes in other kinds of cell bear a similar relation to nuclear DNA? What are the relations of non-chromosomal inheritance to organelle DNA? We shall hear, no doubt, on this point from Drs Beale and Sager.

Even more obscure are the problems raised by template systems based on macromolecules other than nucleic acids. In the case of polysaccharides we do not even know whether the priming function known for glycogen synthesis in vitro has any significance in vivo. In other words, there is no clear evidence that polysaccharide synthesis in vivo involves a template process.

When, finally, we come to supramolecular structures such as mitochondria and plastids, membranes and, for example, the pattern of kinetosomes in Paramecium, in the formation of all of which template processes are suspected, the picture is only one of exciting challenge. I am glad that the even more formidable problems of pattern at the multicellular level will be the job of other speakers.

We can pay no greater tribute to Mendel than to recognize how well aware he was of his Pandora's box.
The chromosomes of one or other of the "parents" of a hybrid cell are usually gradually lost over successive generations. X-irradiation, y-irradiation or BUDR labelling of one "parent" before fusion can be used to predetermine which of the two sets of chromosomes will be preferentially lost. The fusion of somatic cells of different species produces hybrids which, in most cases, preferentially lose some or all of the chromosomes of one of the parental species on prolonged subculture. So far, however, there has been no general way of predetermining which of the parental sets will be preferentially reduced. I report here a technique to this end in the belief that it will constitute a useful tool in the use of somatic cells for formal genetic analysis.

The idea stems from work carried out more than 30 years ago. The problem was how to obtain individuals recombinant with respect to whole chromosomes from two species of Drosophila the hybrids of which were sterile so that the recombinants desired could not be produced by means of meiosis and fertilization. The solution was to X-irradiate the haploid sperms of one species and then to use these to fertilize eggs from triploid females of the second species. Triploid females produce a high proportion of eggs with one or more chromosomes in duplicate ("disomic"). Irradiation induces potential breaks in the sperm chromosomes and their subsequent elimination in the cleavage divisions of the zygote. An egg, disomic for a chromosome homologous to one broken in the spermatozoon which fertilized that egg, would give rise to a diploid zygote carrying exclusively the two maternal chromosomes in question. The same procedure was applied successfully at the intraspecific level by using triploid females with multiple recessive genetic markers and diploid males of the same species with the corresponding dominant alleles.

The problem of inducing a preferential elimination of chromosomes from interspecific or intraspecific mammalian somatic cell hybrids is similar. In both cases there is redundancy in the chromosome sets and loss of chromosomes is not necessarily lethal. A partial solution for interspecific cell hybrids came in 1967 when Weiss and Green discovered that hybrids between somatic cells of mouse and man would gradually lose the human chromosomes on continued culture. Cell lines would therefore eventually arise which had retained only one or a few human chromosomes. By this means human genes can be assigned to individual human chromosomes and linkages recognized or excluded. A similar elimination occurs in Chinese hamster-man hybrids. This directional elimination of chromosomes from interspecific hybrids is very useful for locating genes specifying those human proteins which can be distinguished from their mouse, or Chinese hamster, counterparts. It is of little use, however, for locating human genes known only because of the effects of recessive alleles.

Irradiation of Parent Cells

The technique described here allows the experimenter to choose which of the parental chromosome contributions shall be preferentially eliminated in hybrid somatic cells. It involves X or y-irradiation of the cells of one "parent" before they are fused with those of the other. An alternative to X-irradiation is to label the chromosomes of one "parent" with bromodeoxyuridine (BUDR) and thus sensitize them to visible light.

With the first assay system I used, the loss from hybrid clones of the chromosomes of the treated "parent" can be detected unmistakably. All but one of the chromosomes of...
one parental cell—Chinese hamster \(3T3\) IMP—were morphologically quite distinct from those of the other—mouse \(3T3\) TK-. Furthermore, this combination of strains yields hybrid cells which, on prolonged culture, lose "spontaneously" only very few chromosomes and this loss is not markedly preferential for either species (but see ref. 13). Thus the loss of chromosomes of either species can be detected by morphological classification and count of the chromosomes in the cells of individual hybrid clones each originated from an independent fusion (Figs. 1 and 2).

\[\text{Table 1: Chromosome Counts on Hamster–Mouse Hybrid Cells with One Irradiated Parent}\]

<table>
<thead>
<tr>
<th>Hybrid clone</th>
<th>Irradiated hamster (22 chromosomes)</th>
<th>Irradiated mouse (mode 66 chromosomes)</th>
<th>Hybrid Clone</th>
</tr>
</thead>
<tbody>
<tr>
<td>3T3/wg-a</td>
<td>1.7 ± 0.1</td>
<td>3.7 ± 0.2</td>
<td>3T3/wg-a</td>
</tr>
<tr>
<td>3T3/wg-b</td>
<td>2.8 ± 0.6</td>
<td>4.5 ± 0.5</td>
<td>3T3/wg-b</td>
</tr>
<tr>
<td>3T3/wg-c</td>
<td>3.1 ± 0.4</td>
<td>5.0 ± 0.2</td>
<td>3T3/wg-c</td>
</tr>
<tr>
<td>3T3/wg-d</td>
<td>4.2 ± 0.7</td>
<td>6.0 ± 0.3</td>
<td>3T3/wg-d</td>
</tr>
<tr>
<td>3T3/wg-e</td>
<td>5.3 ± 0.9</td>
<td>7.0 ± 0.4</td>
<td>3T3/wg-e</td>
</tr>
<tr>
<td>3T3/wg-f</td>
<td>6.4 ± 1.0</td>
<td>8.0 ± 0.5</td>
<td>3T3/wg-f</td>
</tr>
<tr>
<td>3T3/wg-g</td>
<td>7.5 ± 1.1</td>
<td>9.0 ± 0.6</td>
<td>3T3/wg-g</td>
</tr>
<tr>
<td>3T3/wg-h</td>
<td>8.6 ± 1.2</td>
<td>10.0 ± 0.7</td>
<td>3T3/wg-h</td>
</tr>
<tr>
<td>3T3/wg-i</td>
<td>9.7 ± 1.3</td>
<td>11.0 ± 0.8</td>
<td>3T3/wg-i</td>
</tr>
<tr>
<td>3T3/wg-j</td>
<td>10.8 ± 1.4</td>
<td>12.0 ± 0.9</td>
<td>3T3/wg-j</td>
</tr>
</tbody>
</table>

* Irradiated "parent".
* One clone, out of fourteen, in which the loss was in the opposite direction to that expected.

Survival of Unirradiated Chromosomes

Irradiation with 600 r, of the hamster cells induced subsequent elimination from individual hybrid clones of up to six hamster chromosomes in addition to those lost even without irradiation (about two). Irradiation of the mouse cells induced elimination of up to nine mouse chromosomes. Hybrid clones from non-irradiated cells of these two strains retain about twenty hamster and sixty-five mouse chromosomes after 2 months in HAT medium. In the selective system used, the retention by a hybrid clone of at least one mouse chromosome and one hamster chromosome is a condition for survival. The

![Fig. 1 A typical karyogram of Chinese hamster strain \(3 \text{ IMP}\): twenty-two chromosomes, all but one (bottom right) distinguishable from those of mouse strain \(3T3 \text{ TK}\) (bottom three rows of Fig. 2).](image)

![Fig. 2 Karyogram of a hybrid clone from irradiated (600 r) hamster cells. The hamster chromosomes are arranged in the top two rows in the same order as in Fig. 1; eight, indicated by \(x\), are missing. The sixty-six mouse chromosomes in the lower three rows correspond to the modal complement of mouse strain \(3T3 \text{ TK} \).](image)
hybrid clones which become established in this system represent therefore a selected sample of karyotypes with fewer chromosome losses than if selection were not applied.

Larger doses of irradiation produce more chromosome losses, but entail several disadvantages. A proportion of hybrid clones gradually dies and chromosome aberrations are more frequent. Furthermore, among surviving clones there are many with multiple sets of chromosomes, chiefly from the non-irradiated parent. It is not clear what proportion of these surviving clones with multiple sets of chromosomes of one parent originated from multiple fusions (for example, two mouse cells and one hamster), initial or in succession, or from fusions involving a polyploid parental cell. As polyploid cells constitute at most 10°, in the two strains used, the proportion of hybrids with a polyploid contribution from one parent, obtained when high doses of irradiation are used, is much higher than expected from the proportion of pre-existing polyploids.

For example, in an experiment in which either parent was irradiated with 1,000 r. of thirteen established clones from irradiated mouse cells, five had a tetraploid hamster complement and a a variable reduced mouse complement (minimum in one clone: forty-two). Conversely, of eight established clones from irradiated hamster cells, five had a mouse complement of 120 or more and a reduced hamster complement (minimum in one clone: eleven), two had about normal mouse complements and reduced hamster complements, and one had a normal mouse complement and a less than tetraploid hamster complement. This last hybrid probably originated from fusion of a hamster cell with forty-four chromosomes, many of which were later lost, with a normal (about sixty-six chromosomes) mouse cell.

In an experiment with the highest dose of γ-rays used so far—1,600 r.—no hybrids became established from irradiated hamster cells, and only three clones from irradiated mouse cells. They all carried an almost complete tetraploid hamster complement and only twenty to thirty mouse chromosomes. All this suggests that when—as after heavy irradiation—a chromosome complement becomes very imbalanced, hybrids with a multiple contribution from the unirradiated parent survive preferentially. The imbalance in this case is relatively large.

BUDR Labelling

As an alternative to X or γ-irradiation, the cells of one parent can be grown in the presence of BUDR for one or two replication cycles and then exposed to visible light, which induces breaks in chromosomes which have incorporated BUDR. Clearly, both the X or γ-rays and the BUDR techniques can be applied to intraspecific (for example, man-man or mouse-mouse, and so on) cell hybrids between pairs of genetically marked primary or secondary explants.

I thank Dr C. Basilico for strain 3 T3 TK−; Dr A. Westerfield for strain 3 T3 $IMP^{−}$; Miss S. Henderson for help with the cultures, Dr D. Roscoe for the supply of Sendai virus and Dr D. Roscoe, Dr M. Fried and Dr J. Renwick for stimulating discussions.

Received February 4, 1971.

3 Pontecorvo, G., J. Genet., 45, 51 (1943).
13 Scalletta, J. L., Rusforth, N. B., and Ephrussi, B., Genetics, 57, 107 (1967).
Chapter VIII  Cell genetics

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Opening remarks

The contributions to this Symposium deal with somatic cell hybridization. They are strictly limited to the uses of it for two purposes: cytological identification of which human chromosome carries a given gene and detection or exclusion of linkage between two or more genes.

The principles so far applied are the same as those developed and routinely used by my colleagues and myself in the early 1950's for formal genetic analysis in fungi via vegetative cells (Pontecorvo, 1969). It gives me great satisfaction to quote from my plea at the Ciba Symposium of 1959 for applying similar principles to human somatic cells in culture. I then said that: '... accidental - or perhaps non-accidental - loss of chromosomes and non-disjunction would be quite enough to carry out genetic analysis via somatic segregation to a very considerable extent. They permit individual genes to be assigned to individual linkage groups.' And later on: '... I would be extremely happy if within the next ten years we could begin to get somewhere' (Pontecorvo, 1959).

We have got somewhere in just over ten years, though, as often happens, by an unexpected technical roundabout. In 1959 I was thinking of, and making attempts about, chromosome elimination or non-disjunction in diploid cultures from heterozygous individuals. The results - e.g. by the use of p-fluorophenylalanine, which acts as magic with diploid fungi - have been unconvincing, to put it mildly.

The solution came from two advances. Ephrussi and his collaborators (1965) showed that hybrids capable of indefinite multiplication could be obtained between cultured cells of different species. Weiss and Green (1967) then made the most useful and unexpected discovery that man x mouse hybrids eliminate successively human chromosomes. Recently Puck's group (Kao and Puck, 1971) found that the same occurs, even more rapidly, in man x Chinese hamster hybrids.

The work which is the subject of this Symposium stems from these two basic advances joined with substantial improvements in the cytological identification of individual human chromosomes.

There are already some remarkable successes: the detection of at least one autosomal linkage, the exclusion of linkage between about 15 autosomal other loci and the assignation of at least two genes to their chromosomes. In formal genetics the first steps are notoriously the hardest. We are now probably not far from the phase of exponential growth.

Professor Siniscalco is going to report in another Symposium on another promising advance in the use of somatic cell hybrids: the identification of the linear order of some linked genes based on hybrid clones which have lost segments of a relevant chromosome (the X in his first attempt). This is in principle similar to the use of somatic crossing-over in Drosophila or fungi.
Hybrids of human and mouse (or Chinese hamster) cells will continue to be useful for some time for at least two reasons. First, they spontaneously lose human chromosomes. Second, as Ephrussi pointed out long ago, the occurrence of a number of interspecific differences in enzymes makes it possible to use as genetic cell markers human enzymes for which detectable variation between individuals is rare or unknown.

In the former respect, however, there are now new possibilities opened by the technique for directing chromosome elimination (Pontecorvo, 1971). This technique induces elimination of the chromosomes of one ‘parent’ by irradiating the cells of that ‘parent’ just before fusion (alternatively, the chromosomes of one ‘parent’ are labelled by BUdR before fusion and the hybrids exposed to blue light). This means, among others, that we can now use man × man somatic cell hybrids for the detection of linkages. It also means that we can exhumate my old hope of using human diploid heterozygous explants and induce in them segregation, either in the form of monosomy or of non-disjunctional homozygosis.

REFERENCES


I have been continuing to investigate the elimination of chromosomes from hybrids produced by crossing cells irradiated (X- or gamma rays) immediately prior to fusion with nonirradiated cells (1). The system chosen is one in which the chromosomes of the 2 parent species could be distinguished and in which no substantial loss of the chromosomes of either parent from the hybrids occurs without irradiation. One of the parental lines was a TK-deficient mouse 3T3 line, with a mode of 66 acrocentric chromosomes and no biarmed chromosomes (kindly supplied by Dr. Basilico). The other parental line was an HGPRT-deficient Chinese hamster line, with 22 chromosomes, all distinguishable from the mouse chromosomes (kindly supplied by Dr. Westerveld). Hybrid clones between these two lines have almost exactly the sum of the chromosome complements of the parental cells (see Fig. 1). Chromosome loss from these hybrids is minimal over at least the first two months of subculture in HAT.

In a series of experiments either the Chinese hamster cells or the mouse cells were irradiated just prior to fusion with unirradiated cells of the other species, and the hybrids were isolated as clones within 10 to 20 days and maintained in HAT. The hybrid clones showed extensive loss of the chromosomes of the irradiated parent. A karyotype of one such hybrid is shown in Fig. 2. In this case there are only 11 hamster chromosomes left, a loss of 11 chromosomes. The present stage of the work is summarized in Table 1, for experiments with irradiation doses up to 1500 rads. The loss of chromosomes was found to be dependent upon the irradiation dose. For example, when the mouse cells were irradiated at 600 rads, there was a mean loss of 3.2 mouse chromosomes from the hybrids. At 1000 rads the average loss of mouse chromosomes was 11.3. At 1500 rads the average loss was 25.7. Similar results were obtained when the hamster cells were irradiated prior to fusion. At 600 rads, an average of 2.7 hamster chromosomes were lost from the hybrids. at 1000 rads 5.2 chromosomes, and at 1500 rads 10.9 chromosomes. Control hybrids in which neither parent cell was irradiated showed no substantial chromosome loss. It is clear that irradiation before fusion can cause the loss of many chromosomes of the irradiated parent from the hybrids.

Not surprisingly, after irradiation of one of the parental cells, fewer hy-
FIG. 1. Top left: karyogram of the mouse line. Top right: karyogram of the hamster line. Bottom: typical karyogram of a clone of hybrids between nonirradiated cells.

FIG. 2. Karyogram of a hybrid clone between irradiated hamster cells and nonirradiated mouse cells.
TABLE 1. Loss of chromosomes of the irradiated parent

<table>
<thead>
<tr>
<th>Dose rads</th>
<th>Mouse cells irradiated</th>
<th>Hamster cells irradiated</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Clones no.</td>
<td>Mouse chromosomes lost</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Mean</td>
</tr>
<tr>
<td>0</td>
<td>21&quot;</td>
<td>2.7</td>
</tr>
<tr>
<td>600</td>
<td>6</td>
<td>3.2</td>
</tr>
<tr>
<td>1000</td>
<td>13</td>
<td>11.3</td>
</tr>
<tr>
<td>1500&quot;</td>
<td>13</td>
<td>25.7</td>
</tr>
</tbody>
</table>

* Provisional data, analysis in progress.

logenome is eliminated at the irradiated parent level. However, if the rate of hybrid formation is compared to the survival rate of the irradiated parental cells, it is apparent that there has been an enormous amount of rescue. Cells that are rendered inviable by irradiation are rescued by fusion with nonirradiated cells. This, of course, is the predicted result. For example, at 1500 rads the surviving fraction of irradiated hamster cells is reduced to 0.08%. The number of hybrid clones obtained when these irradiated cells are hybridized with nonirradiated mouse cells is about 2% of the number obtained when nonirradiated hamster cells are hybridized with mouse cells. Since the decrease in viability (over 1000-fold) is so much greater than the decrease in hybridization frequency (50-fold) it is clear that there has been significant rescue. Similar results have been obtained by Dr. Morgan Harris in hamster-hamster hybrids.

I have also obtained some information on the number of chromosome complements in these hybrids. Some of the hybrids (with either parent irradiated) contain a 2 S chromosome complement of the nonirradiated parent. Hybrids with a 2 S complement of one or the other parent are also observed occasionally in control crosses. Significantly, in the hybrids with one irradiated parent, the 2 S contribution was always from the nonirradiated parent. This could be the consequence of fusion with tetraploid cells, which are always present in the populations in a small proportion, or of multiple fusion. However, the conditions of fusion were such as to minimize the possibility of multiple fusion. Furthermore, to account for the frequency of hybrids with a 2 S complement by fusion with tetraploid cells the pre-existing tetraploid cells would have had to fuse at a much higher rate than the diploid cells. Finally, the 2 S complement is either from the mouse or the hamster cell, depending on which parental cell was not irradiated. It is well known that following irradiation, there is a delay in chromosome replication in irradiated nuclei. My hypothesis is that this delay is carried over in the heterokaryons. In heterokaryons with one irradiated and one nonirradiated nucleus, in some cases, the nonirradiated nucleus has time to go through an additional division before fusion with the other nucleus.
It is interesting that when mouse cells are given high doses of irradiation the number of mouse chromosomes in the hybrids can be greatly reduced. as a maximum, in the range of irradiation used, from 66 chromosomes down to about 10. The same results are not obtained with heavily irradiated hamster cells. The hamster cells have 10 large metacentric chromosomes. These large chromosomes are less frequently found to be lost in the surviving hybrid clones. This is an unexpected result considering the mode of action of irradiation: in rough approximation, the larger a chromosome the greater its chance of undergoing breakage and successive elimination. Out of 26 clones from irradiated hamster cells, not one has fewer than five large metacentric hamster chromosomes. It would appear that there is very strong selective pressure to retain some of the large hamster chromosomes in the hybrids, even though the hybrids have the complete mouse genetic information. The results suggest that, even in mouse-hamster hybrids in which there is no substantial spontaneous preferential chromosome loss, there is something that acts to conserve part of the chromosomes of one species more than those of the other. Clearly, this is unlikely to be a simple matter of a full set of genetic information. It may be important to look at the interactions between two or more chromosomes and between centromeres and the spindle, for example.

Finally, I have tested this technique on man-mouse hybrids. If the mouse cells are irradiated before fusion with human diploid cells, the number of hybrids formed is greatly reduced. However, those hybrids that do grow segregate human chromosomes, as do hybrids with nonirradiated cells.

REFERENCES


DISCUSSION

Tashjian: The rescue experiments described by Pontecorvo are intriguing to me because of the observations we have made concerning the extraordinary degree of resistance to X-irradiation in hybrid cells [Little, J. B., Richardson, U. I., Tashjian, A. (1972): Proc. Natl. Acad. Sci. 69:1363]. We have seen this phenomenon with rat-mouse hybrid strains. We are far from understanding the mechanism; nevertheless, it is interesting that after hybridization you see rescue of cells that would have died unless they had been hybridized.

Thompson: Dr. Pontecorvo, how long after fusion did you characterize the hybrids?

Pontecorvo: Between 1 and 3 months after fusion.
Gerald: How much chromosome damage appears as a result of irradiation?

Pontecorvo: The results are very informative. If you look at the hybrid clones as soon as you can (2 to 3 weeks after fusion), you find many cells with chromosome aberrations as expected. However, if you look later, the retained chromosomes appear normal. In fact, there is very little of what I expected would make the chromosomal analysis difficult. There is little joining of two mouse chromosomes to form new metacentrics or cleavage of metacentric hamster chromosomes to form telocentrics. It seems that chromosomes are still being eliminated by some cells of a clone 20 days after irradiation, while the other cells have already “cleansed” themselves, no doubt by the usual breakage-reunion-breakage process.

Watkins: Have you ever tried irradiating both parents to see if there is some kind of mutual rescue?

Pontecorvo: I have wanted to do that but have not done it yet.

Siniscalco: Did you try to irradiate human cells and then fuse them with mouse cells?

Pontecorvo: Yes. It does not help very much.
THE BATESON LECTURE 1974

"Alternatives to sex": genetics by means of somatic cells.

by

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William Bateson, whose memory we are here to honour, put Mendelian genetics on the map in this country and contributed decisively to its early developments. Among other things, he coined the word "Genetics" to designate the study of the "physiology of descent", and he discovered linkage.

By a fortunate coincidence, the title of my lecture honours the memory of another great John Innes man: J.B.S. Haldane (1955). He used the expression "alternatives to sex" to include the approach developed in Glasgow during the previous few years. These two John Innes men stood at the dawn of two eras of genetics: that of genetics via sexual reproduction and that of genetics via "alternatives to sex".

The field, now fashionably called "somatic cell genetics", includes two distinct, though complementary and overlapping approaches. One is the use of somatic cells in culture, very much like the use of cultures of microorganisms, for the study of general problems: mutation, replication, differentiation, metabolism of macromolecules, selection, social behaviour etc. (reviews: Ephrussi, 1972; Harris, 1970; Puck, 1972; Stoker, 1972). The other is the use of somatic cells, mainly in culture, as an alternative to germ cells for the genetic analysis of the individual from which the somatic cells were taken. In this talk I shall limit myself exclusively to this second field. Let me try first to summarize how it all started.

THE INITIAL WORK WITH ASPERGILLUS NIDULANS.

The initial approach, about 1952, in Glasgow made use of the mould Aspergillus nidulans. The formal genetics of this homothallic Ascomycete had been worked out in considerable detail in the previous 5 years by standard techniques based on sexual reproduction. Then my colleague J.A. Roper (1952) developed a simple, way of synthesizing strains carrying in their vegetative cells (I shall call them "somatic") diploid nuclei heterozygous for any desired markers. The wild-type strains have haploid nuclei. It became therefore possible to ask the question: does some form of segregation and recombination occur in these diploid...
"somatic" cells? The idea came to me from the work of 15 years earlier by Curt Stern (1936) on somatic crossing over in Drosophila.

After finding that indeed somatic segregation and recombination did occur, though at a low frequency, it took three years to identify the two processes at work and to forge them into efficient tools for routine genetic analysis. Anticipating, it turned out that one of them, somatic crossing over à la Stern, had very limited use as a tool. The other process - involving chromosome loss - turned out to be the basis for a substantial advance in the methods of formal genetics (Pontecorvo et al., 1954; Pontecorvo, 1958; Forbes, 1959; McCully and Forbes, 1965). Since 1968 methods identical in principle to those developed for Aspergillus are in routine use for human genetic analysis by means of somatic cell hybrids (review: Ruddle, 1972).

The possibility of cross-checking the results of analysis based on what came to be called "parasexuality" with the previous ones obtained via sexual reproduction was, of course, the great asset of Aspergillus. This is why the work was so conclusive. There are now at least 20 times as many workers using Aspergillus as there were in those early days: the only part of the work shown to be wrong is a minor hasty cytological conclusion of my own.

The techniques developed then were soon applied to other moulds, including some producing antibiotics. In some of these species sexual reproduction was not known to occur and the "alternatives to sex" were the only way of doing genetic analysis. The first of these asexual species to be analysed was Aspergillus niger in 1954.

More important, I became convinced that these alternatives should be usable in somatic cell cultures of higher organisms. In some of these, for example: man, the use of the results of sexual reproduction for genetic analysis is hopelessly restricted by ethical and practical reasons. Clearly a break-through in human genetics could only come from methods by-passing sexual reproduction.

When I first proposed this in the mid-50's and more boldly and precisely at the Ciba Symposium of 1958 (Pontecorvo, 1959), with few exceptions professional human geneticists were not enthusiastic. I had no choice but to try on my own. This was 1956. The U.S. National Institutes of Health and later the M.R.C. gave a generous hand. Unfortunately my initial line of attack was a failure, mainly because some of the ancillary techniques were not ripe then. They are now, and as you will hear, I have taken up again that initial approach.
Much later, about 1968, the general approach worked out for *Aspergillus nidulans* in the 50's became routine, *mutatis mutandis*, for human genetics. This was the consequence of an utterly unexpected development: the discovery by Weiss and Green (1967) that mouse/man hybrid somatic cells eliminate the human chromosomes on prolonged culturing. The results are spectacular. In the last 6 years - in fact mainly in the last 3 years - about 9 times as many human genes have been assigned to chromosomes or linkage groups as in the whole previous work in human genetics. Unfortunately, quantity is beginning to depress quality.

Table 1

<table>
<thead>
<tr>
<th>Family studies*</th>
<th>Somatic Cell hybridization</th>
<th>In situ annealing</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>25</td>
<td>61</td>
<td>6</td>
<td>92</td>
</tr>
</tbody>
</table>

* Mainly based on linkage to one or more other loci assigned by means of somatic cell hybridization.

Most of the young, able and enthusiastic workers in this field use the *Aspergillus* methodology without being aware of where they got it from. This methodology is now beginning to move back to the world of plants where it originated.

**GENETIC ANALYSIS BY MEANS OF SOMATIC CELLS.**

Genetic analysis means the resolution of the genetic material into its component elements. The techniques for genetic analysis vary tremendously in resolving power, especially between one organism and another. In viruses we consider poor any analysis short of the recognition of nucleotide sequences. In man we are happy if we can identify a gene by its specific effect on the phenotype and assign it to a chromosome or to a linkage group. We are even happier if we can determine the chromosome arm, and exceedingly happy if we can place that gene in its correct order relative to other genes or identify the chromosome band where it lies.

In man resolution almost down to nucleotide sequences has been achieved only in four very special cases all based on *in situ* annealing techniques (review: Hirschhorn and Boyer, 1974). Three involve...
repetitive sequences: the satellite-DNAs, the 5S ribosomal RNAs and the ribosomal RNAs. One involves the haemoglobin a and b messengers, which are almost the only messengers that reticulocytes produce. The beautiful work of Hirschhorn and his colleagues based on in situ annealing of labelled messenger, has led to the location of the two relevant haemoglobin genes on chromosomes 2 and 5 (or 6). Hirschhorn’s results are disputed on the grounds that they are physically impossible. H.J. Muller once told me that the physicists had to correct their calibration of an X-ray tube when his mutation results disagreed with their previous calibration. The carbon dating technique is another recent example.

Granted that the ultimate aim in genetic analysis is the resolution of nucleotide sequences and the identification of the active macromolecular products encoded in them, for the present in higher organisms we must be contented with much coarser successive steps. These are: (1) THE IDENTIFICATION OF A GENE by its phenotypic effect and its segregation; (2) THE RECOMBINATION OF THAT GENE with others, syntenic (Renwick, 1971), i.e. carried in the same chromosome pair, or not. (3) THE LOCATION OF THAT GENE within the linear order of other syntenic genes, and (4) THE IDENTIFICATION OF THE CHROMOSOME ARM AND BAND where that gene is physically located.

In classical genetics all of these steps required the use of sexual reproduction. Segregation at meiosis and complementation in heterozygotes permitted to implicate a gene as the cause of a difference in phenotype. Recombination at meiosis was the basis of the two other steps. Cytogenetical techniques, using appropriate structural heterozygotes, were the basis for the physical location.

The work with heterozygous diploid strains of Aspergillus showed that all of these operations could be done with "somatic" cells, taking advantage of the two rare processes already mentioned: somatic crossing-over and chromosome loss.

The consequence of somatic crossing-over in a chromosome pair is to produce, from one heterozygous nucleus, two diploid daughter nuclei homozygous in certain precise respects. One is homozygous for all the alleles of one member of that chromosome pair at the loci distal to the position of crossing-over, the other is homozygous at the same loci for the alleles of the other member of that chromosome pair. The linear order of loci can be established by classifying, and an estimate of "distances" made by the relative frequencies of a sample of segregants. Since multiple exchanges are rare enough to be disregarded,
the positions of three loci - centromere - A - B - C, in this order -
can be established by the fact that homozygosis at A always goes with
homozygosis at B and C and homozygosis at B always goes with homozygo-
sis at C.

Differently from Drosophila, in Aspergillus it was possible to
isolate and grow the segregants produced by somatic crossing-over and
analyze them by crosses. The results entirely confirmed Stern's ded-
uctions of 15 years earlier on the modalities of the process. Unfortu-
nately, for a number of technical reasons, somatic crossing over
turned out to be of limited use in formal genetics by means of somatic
cells.

The other rare process discovered in Aspergillus is quite a differ-
ent matter. Even in that species it made the assignment of a gene to
a linkage group and the detection of synteny and of chromosome re-
arrangements (e.g. translocations) much simpler than by traditional
techniques. But its real value was that it suggested a way of assign-
ing a gene to its chromosome and detecting synteny between two or more
genes in higher organisms by means of somatic cells.

This process was misnamed "haploidization" because one, its most
obvious, consequence is to produce haploid from diploid somatic cells.
It consists in a maldistribution of chromosomes at mitosis, usually
affecting one or a few chromosomes at a time. Each maldistribution
produces mono-or trisomy. When repeatedly occurring, with strong sele-
cution against the imbalanced nuclei, the ultimate result is a popu-
lation of mainly haploid nuclei reassorting the members of different
chromosome pairs in all possible ways.

Since this is a process which involves whole chromosomes, not
chromosome segments, in any one haploid clone the alleles at different
loci on the same member of a chromosome pair are all present or all
absent. In other words in respect of syntenic loci only the parental
associations are present among the haploids. The alleles at two non
syntenic loci, on the other hand, appear at random in any one of the
four possible associations, two parental and two recombinant, in indivi-
dual haploid clones. A recent example of the application of this
rationale to man-Chinese hamster hybrids is given by Van Someren
Their results are in Table 2. The numbers in the 2 x 2 tables are
those of the primary clones showing the presence of either one (+-),
or the other (-+), or both (++) or neither (--) of the three enzymes

taken two by two at a time.

Table 2

<table>
<thead>
<tr>
<th></th>
<th>PGM&lt;sub&gt;3&lt;/sub&gt;</th>
<th>IPO-B</th>
<th>PGM&lt;sub&gt;3&lt;/sub&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>+ -</td>
<td></td>
<td>+ -</td>
<td></td>
</tr>
<tr>
<td>11 0</td>
<td></td>
<td>18 0</td>
<td>12 1</td>
</tr>
<tr>
<td>ME&lt;sub&gt;1&lt;/sub&gt;</td>
<td>-</td>
<td>- 16</td>
<td>- 0 16</td>
</tr>
<tr>
<td></td>
<td>ME&lt;sub&gt;1&lt;/sub&gt;</td>
<td></td>
<td>IPO-B</td>
</tr>
</tbody>
</table>

The assignment of a locus to a particular linkage group could be done in *Aspergillus* on the same reasoning as the detection of synteny. But it required the use of genetically multiply marked strains because in this species the individual chromosomes are too small to be identifiable. A diploid, synthesized between a multiply marked strain and a strain carrying a non-assigned gene, produces a number of haploid clones carrying the markers and the non-assigned gene in an array of associations. The non-assigned gene will appear only in the parental associations with the marker (or markers) of the chromosome pair on which it is located.

In a nutshell, chromosome loss provides a very efficient tool because two syntenic loci give zero recombination, while two non-syntenic loci give 50% recombination.

The technique of chromosome loss for detecting synteny and assigning loci became even more efficient after two further technical advances. One was Morpurgo’s (1961) discovery that parafluorophenylalanine enormously increases the rate of segregation; followed by Lhoas (1961) demonstration that it acts on haploidization. The other was the routine construction and use of ”tester” strains with at least one marker per identified linkage group (Forbes, 1959; McCully and Forbes, 1965).

The rationale of this chromosome loss technique, applied to man/mouse somatic cell hybrids since Weiss and Green’s discovery of 1967 has changed the pace of human formal genetics (Table 1). Instead of using parafluorophenylalanine on heterozygous diploids, one uses human somatic cells hybridised to mouse or Chinese hamster cells. The hybrid system acts on human chromosomes like parafluorophenylalanine acts on *Aspergillus* chromosomes: the human chromosomes are lost one or a few
at a time and the rodent chromosomes retained. Two human loci are syntenic if they are either lost or retained together in a series of hybrid clones.

The assignment of a gene to its chromosome does not necessarily require in man the use of genetic markers and tester strains. Human chromosomes, differently from *Aspergillus*, can be identified microscopically by their morphology and banding patterns. Thus in man/rodent hybrid cells one can assign a gene to its chromosome by finding, in a series of hybrid clones, which human chromosome has to be retained in order that the hybrid cells may express the phenotype determined by the human unassigned gene.

**INITIAL ATTEMPTS WITH HETEROZYGOUS HUMAN CELLS.**

In 1956 I took up the challenge of trying to do with heterozygous diploid human somatic cells what my colleagues and I had done with *Aspergillus* heterozygous diploid "somatic" cells. The design was to grow human skin fibroblasts from individuals heterozygous at a number of loci expressed in cultured cells. If chromosome loss or non-disjunction occurred, it was hoped that clones should arise either fully haploid, or hemi- or homo-zygous at one or more of the loci.

No human marker usable for selection was available at that time for the isolation of the expected segregants. Thus it was imperative to find some way of inducing a manageable rate of chromosome loss. The various treatments tried - obviously, parafluorophenylalanine was the first - were ineffective. It also became apparent that aneuploid human clones were unlikely to survive for long enough until the attainment of a new balance.

This old approach I have now taken up again. The time seems to be ripe for three reasons. First, there is now an agent which induces human chromosome loss. Second some of the human inborn errors of metabolism are now known to be usable as selective markers. Third, the use of inactivated Sendai virus helps in the fusion of human cells. These may be from two individuals differing in genetic characters expressed in cell cultures, or from an individual known to be heterozygous for suitable characters.

As to the first reason, De Carli and his colleagues (Rossi *et al.*, 1971; De Carli, 1972) in Pavia showed three years ago that a short treatment of human cultured cells with the antymycotic griseofulvin causes a tremendous scatter of chromosome counts from over tetraploid to subdiploid. A couple of weeks after the treatment mostly tetra-
ploid or diploid counts are found in the cultures. My colleague Dr. Riddle and I have followed with time-lapse microcinematography what happens to human skin fibroblasts after griseofulvin treatment. There is violent elimination of packets of chromosomes from cells in mitosis and utter disruption of the spindle: hence the formation of cells with such a scatter of chromosome counts. Clearly, griseofulvin seems to be what we need.

As to selective markers, there are at least three errors of metabolism in man - Lesch-Nyan syndrome, galactosaemia and xeroderma pigmentosum - which lend themselves to selection. Finally the possibility of producing tetraploids, by fusion of cells from either the same or different donors, may be of valuable help in reducing the imbalance of aneuploid clones on their way to become homozygous. Martin and Sprague, by using cells heterozygous for a chromosomal marker, have shown a few years ago that indeed human tetraploid cultured cells are suitable for segregation analysis.

Why bother about pure human somatic cells, when the man/rodent hybrids have been and are likely to go on being such a wonderful tool? The answer is simply that the estimated 1000 or so human genes suitable for mapping by means of the rodent/man hybrids (or indeed other hybrids which may be synthesized in the future) are prevalently of a special class. They are mainly "inbuilt markers", in Ephrussi's expression, i.e. genes determining interspecific differences, such as, typically, a species difference in the electrophoretic form of an enzyme. In human genetics we are interested in intraspecific differences, those that make each individual unique. Only about 1/3 of all these "in-built markers" will be polymorphic in man. Anyway, most of the genes in man which have more immediate interest are known because of the pathological effects of their recessive alleles, and recessive alleles do not lend themselves to mapping by the rodent/man hybrid cell technique.

Don't misunderstand me. I do not wish to belittle what we can still get from the rodent/man hybrid technique. One thousand genes on the human map, even of a kind that has little direct interest for bread-and-butter human genetics, will be of enormous value as a frame for all the other, in my view, more interesting genes. It will tremendously speed up the mapping of these other, even indirectly by helping the analysis by means of family studies. Finally, it may reveal features of the gene maps and of gene interactions which we can-
not even guess.

Nevertheless, I am firmly convinced that an important complementary pathway in the use of somatic cells for human genetics will be that of using explants from heterozygotes, or synthesizing tetraploid heterozygotes by fusion between cells from homozygotes.

And now that I have dealt with the prehistory, and guessed at the rosy future, of genetics via somatic cells, let me deal with a few salient points of what is going on now.

CURRENT HIGHLIGHTS.

After the initial discovery in 1967 by Weiss and Green of the loss of human chromosomes in man/mouse somatic cell hybrids, three most useful technical advances have followed. One, from Bodmer's group (Nabholz et al., 1969), was the use of human blood white cells as the human component in man/mouse, and later man/Chinese hamster hybrids. This made it possible to make hybrids with the somatic cells of any donor, without the need of selective markers in them.

The second advance was the use for hybridisation of cells from individuals carrying translocations or deletions. This made it possible to go further than merely assigning genes to chromosomes: they may be assigned to chromosome arms and bands within one arm.

The third was, of course, the spectacular advance in cytological techniques, which are improving every month. It is now possible to identify each of the 23 human chromosomes by its banding pattern.

There has also been a promising - though by no means complete - joining of forces between the "hybridizers" and the "family mappers". Any one gene recognizable both in cultured cells and the whole individual, assigned to a chromosome or to a linkage group by means of one method, can be used to determine synteny in respect of any other gene mapped by the other method. The "somatic cell" and "family" maps are gradually coalescing. For example, on human chromosome 1, of the 18 loci assigned to it, 7 came from somatic cell mapping, 6 from "family" mapping, 4 from both and one from in situ annealing and autoradiography of cDNA.

One result of somatic cell hybridization of considerable interest also for medical genetics is the ascertainment of whether two or more indistinguishable recessive conditions, occurring in different individuals or families, are determined by the same or different genes. These tests of complementation can be carried out by fusing cells from
two affected individuals and examining heterokaryotic or hybrid cells for their phenotype. Tests of this kind have added among others xeroderma pigmentosum (De Weerd-Kastelein et al., 1972) to the list of genetical disorders in which the same clinical phenotype may result from mutation at different loci.

This leads me to a point that supports the well known, but not endearing, belief of geneticists that the spade work for many biochemical problems is often much more easily done with simple genetical techniques than with rigorous biochemical ones. For example, it is of considerable general interest to know the proportions of monomeric, dimeric etc. enzymes in a number of species. A chemical answer requires the complete purification of each enzyme and its analysis: a Sisyphaean task. Electrophoresis of crude cell extracts gives a first approximate estimate. Cells from heterozygotes for two electrophoretically different forms of an enzyme, or hybrid somatic cells between two species differing in the electrophoretic mobility of an enzyme show in all cases so far both forms of the enzyme. In addition, in a majority of cases they show one or more new electrophoretic bands. These are inferred to be - and in a few cases they have been shown to be - molecular hybrid forms originated by association of polypeptide chains of the two kinds. Association occurs in the cases in which the active enzyme is a polymer. Thus an enzyme can be provisionally classified as monomeric, dimeric etc. on the basis of the number of bands, in addition to the original ones, which are shown in heterozygotes or in interspecific hybrids. Professor Harry Harris has permitted me to show his data based on a sample of 50 human enzymes, some from human heterozygotes and some from man/rodent hybrids. This spade work already tells us that about 1 in 5 human enzymes are monomeric, a matter of considerable general interest. One question to which there should be already an answer is whether or not enzyme polymorphism in human populations is more common in respect of monomeric or of polymeric enzymes. The population genetics of the two kinds of enzymes is likely to be different.

I mentioned that in Aspergillus nidulans the use of somatic crossing-over did not play as important a role as was initially hoped in sequencing genes by means of somatic cells. In the first place it required the use of a selective marker placed at the distal end of each chromosome arm. These were hard to come by. In the second place, in that species, sequencing of genes can be done very easily by means of sexual reproduction.
Table 3
Number of polypeptide chains, inferred from heterozygotes or interspecific somatic hybrids, in 50 human enzymes

<table>
<thead>
<tr>
<th>MONOMERIC</th>
<th>DIMERIC</th>
<th>HOMOPOLYMERIC</th>
</tr>
</thead>
<tbody>
<tr>
<td>13</td>
<td>26</td>
<td>1</td>
</tr>
<tr>
<td>10</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

(courtesy of Professor H. Harris, Galton Laboratory)

In man, sequencing of genes by family studies is an extremely slow process. Any help from other quarters would be most welcome. Unfortunately somatic crossing is not known to occur in human somatic cells of normal individuals. The beautiful cytological work of German and his collaborators (German and Pugliatti Crippa, 1966) on cells of individuals affected by Bloom's syndrome, the consequence of a very rare recessive mutant, shows a tremendous amount of sister chromatid exchanges and what may be interpreted as exchanges at homologous positions between homologues. The technique used is a very ingenious one based on the fact that bromodeoxyuridine incorporation in the chromosomes reduces their stainability with Giemsa or fluorescent dyes. A pulse of bromodeoxyuridine followed by staining after one or two chromosome replications shows semiconservative replication much more simply than autoradiography. This technique applied by German (personal communication) to normal cells shows, after two replications, one chromatid of each chromosome intensely stained and one much less so. Applied to Bloom's disease cells it shows the two chromatids as two complementary sequences of intensely stained and poorly stained segments. The challenge is now either to devise conditions or treatments (mitomycin C has been suggested by German and La Rock (1969) ) which will induce something similar to Bloom's disease in normal cultured cells, or to introduce Bloom's allele into them. In somatic hybrids between Bloom cells and rodent cells the effect of Bloom's gene is, as expected, recessive.

There are so many areas of somatic cell genetics I do not know what else to choose.

I shall finish with two minor contributions of mine. One is the possibility of predetermining which of the two parental chromosome complements should be eliminated in hybrid cells. This can be done by irradiation, or by sensitization with bromodeoxyuridine, of the
cells of one "parent" just before fusion. It can be applied both to inter- and intra-specific hybrids (Poncetcorvy, 1974).

The other is the use of the repeated backcross. The backcross (A crossed to B and the F1 back to A, or B) has long been one of the most powerful tools of genetics via sexual reproduction. It may become equally useful in genetics via somatic cells, for instance for introducing in cultured somatic cells a gene required for a particular purpose, such as Bloom's syndrome gene.

Since this is a gathering of people interested in plant cells I could not finish without mentioning the momentous experiment of Carlson (1972) and his colleagues who produced whole hybrid plants from the fusion of protoplasts of *Nicotiana glauca* and *N. langsdorffi*. I know that there are doubts about the nature of the results. But such doubts cannot belittle the fact that this was just the kind of experiment which had to be done. The additional examples to be reported at this Symposium by Melchers and Gamborg respectively, confirm that the breakthrough has taken place. While in the case of animals somatic cell fusion is, fortunately, only an analytical tool, in the case of plants - with their totipotentiality of somatic cells - it is also a synthetic tool with fantastic possibilities. What I regret is that most of the workers in this much younger field are unwilling to borrow as much as possible from the much wider experience of their colleagues in the animal field. Since at different times I have had a foot in both and learned from both, I hope this encouragement will not be taken amiss.

The excitement and bubbling activity of the then new Mendelian genetics at the time of Bateson are now repeated with the "alternatives to sex". Bateson and Haldane would have enjoyed the present time tremendously.

REFERENCES


De Carli, L. (1972). Personal communication.


Ephrussi, B. Hybridization of somatic cells. Princeton University Press. (1972)


INTRODUCTION

Inactivated Sendai and other viruses (1, 2) increase the rates at which mammalian somatic cell hybrids arise. However, virus treatment seems to be ineffective with some kinds of cells, production of the virus is laborious, the activity of various batches is variable and liable to decay, and the possibility of introducing fragments of viral information into the treated cells is not to be ignored. For all these reasons the search for chemical “fusogens” effective on mammalian cells has gone on for some years (see, for instance, 3–6). The results to date have not warranted dispensing with Sendai virus.

In the novel field of higher plant protoplast fusion (7) polyethylene glycol (PEG) has recently become established as a powerful fusogen (8–10). So far the published reports do not claim more than a few divisions for the products of plant protoplast fusion. Nevertheless, it seemed worthwhile to try this botanical device on mammalian cells where the techniques for determining success or failure are a matter of established routine. The results, summarized here, were quite positive. In a test system well tried before (11, 12), uninucleate mouse × Chinese hamster hybrid cells capable of indefinite multiplication arose after PEG treatment at rates at least as high as those obtained with Sendai virus. Furthermore, PEG treatment has yielded
hybrids even in notoriously recalcitrant combinations of cells, such as early passage human skin fibroblasts and human lymphocytes.

MATERIALS AND METHODS

In preliminary tests a number of variations of the PEG treatment have been tried, such as treating both parental cells in suspension, both in mixed monolayer or one as monolayer and the other overlaid on it. The details given below are for the treatment of mixed monolayers of equal numbers of Chinese hamster hypoxanthine guanine phosphoribosyltransferase-deficient (HGPRT) cells (strain wg, clone 1) and mouse thymidine kinase-deficient (TK) cells (strain 3T3) (for details see reference 11).

Cells of each strain in equal numbers ($10^5$-$10^6$) are inoculated as a mixed culture in a 25-cm$^2$ flask in Dulbecco's medium with 15% fetal-calf serum (D15) and incubated overnight. 10 g of polyethylene glycol 6000 (either "pure" Koch-Light; or 6000-7500 mol. wt., BDH), sterilized by autoclaving, are dissolved in 10 ml of Dulbecco's medium without serum (D). The mixed culture, washed twice with 5 ml of phosphate buffer saline (with Ca and Mg), is overlaid for 5-15 min at 37°C with 1.5-3 ml of PEG solution. At the end of this time the cells have shrunk somewhat. The film of PEG solution, after draining off as much as possible, is very gradually diluted with D, for instance in five steps at intervals of 5 min, adding 0.3, 0.6, 1.2, 2.4, and 4.8 ml of D. (more rapid dilution kills the cells.) After this, the liquid is removed and replaced with 5 ml D15. At this juncture the morphology of the cells changes dramatically. After 30 minutes D15 medium is renewed. Bi- or multinucleate cells are formed following the introduction of D15, go on increasing in proportion for many hours, and then start to decline. After overnight incubation and, if necessary, trypsinization and plating, D15 supplemented with hypoxanthine, aminopterin, and thymidine (HAT) (13) replaces D15, and is renewed every 2-3 days. By the fifth day small colonies of possible hybrid cells begin to appear among the dying parental cells.

RESULTS

In the three hybridizations carried out between wg-1 and 3T3(TK$^-$) the yields of presumptive hybrid colonies per plated cell (of one parent) were $2.2-9.5 \times 10^{-4}$ in the PEG-treated series and $2.6-9 \times 10^{-4}$ in the untreated controls (see Table 1).

*Early Passage Human Skin Fibroblasts.* Mixed monolayers of early passage cells from a case of Lesch-Nyhan syndrome (LN) and a case of xeroderma pigmentosum (XP), treated as above, gave tetraploid presumed hybrids after successive enrichments by selection with HAT and UV light
Hybridization by Means of Polyethylene Glycol

Table 1. Presumptive hybrid colonies obtained from fusion of Chinese hamster and mouse cells

<table>
<thead>
<tr>
<th></th>
<th>Total cells in mixed monolayers</th>
<th>Total hybrid colonies*</th>
<th>Hybrid colonies per 10⁸ cells of one parent</th>
</tr>
</thead>
<tbody>
<tr>
<td>Controls</td>
<td>6 × 10⁸</td>
<td>23</td>
<td>7.6</td>
</tr>
<tr>
<td>PEG-6000-treated</td>
<td>7.9 × 10⁸</td>
<td>1764</td>
<td>446.6</td>
</tr>
</tbody>
</table>

* Mixed confluent monolayers of equal numbers of Chinese hamster 
  wgs-1 (HGPRT⁻) and mouse 3T3 (TK⁻) cells were treated as indicated.

** The colonies were presumed to be hybrid cell colonies on the basis of (1) chromosome
  constitution on a sample of colonies, (2) morphology, and (3) ability to grow in HAT.

(30–60 ergs/mm²). With these human fibroblasts the formation of bi- or
multinucleate cells is easily followed under phase contrast. Starting from
about 1% before treatment it reaches a maximum (up to 42% in the experi-
ments to date) around 40 hours after treatment and then declines over a few
days. My colleague, Dr. Peter Riddle, has succeeded in recording by time-
lapse microcinematography the actual fusion of cells.

Mouse or Human Fibroblasts and Human Lymphocytes. Monolayers in
Falcon flasks of either human fibroblasts LN or XP or of the 3T3 (TK⁻)
mouse fibroblast line were overlaid for 3–5 min with the PEG solution men-
tioned above. The solution was then drained and a suspension in 0.3 ml
phosphate buffer saline (with Ca and Mg) of washed column-purified
peripheral blood lymphocytes (in some cases PHA-stimulated) was overlaid
on the monolayers (10:1). Very effective agglutination occurred after a few
minutes of gentle rocking. Gradual dilution with D and finally replacement
with D15 led to the usual massive formation of multinucleate cells. One or
more lymphocyte nuclei (?) appeared to have been taken up in a proportion
of fibroblasts. Gradual enrichment by HAT or UV light, according to the
kind of fibroblast used, led to cultures resistant to the selective agent. Fur-
ther characterization is in progress.

ACKNOWLEDGMENTS

I am indebted to Professors E.C. Cocking and J.A. Lucy for valuable
suggestions and stimulating discussions and for informing me of the success
in their laboratories (14) in fusing yeast protoplasts and hen erythrocytes.
To Dr. O.L. Gamborg, whose laboratory has done most of the work on
protoplast fusion by PEG, I am indebted for reprints and preprints.

LITERATURE CITED

  124:46–47.


REMINISCENCES ON GENETICS : FROM MENDELISM TO RECOMBINANT DNA*

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It is a great honour that the Raman Research Institute Trust has done me by asking me to deliver on this anniversary day, the Gandhi memorial lecture. Mahatma Gandhi's ideals and dedication are badly needed in today's world, mad with violence and crushing individuality. I am going to talk about a field of science which, in my lifetime, has made enormous strides. Most I have witnessed myself. Like in any other field of science the powerful tools it now provides for mankind can be put to good or evil use. This is where Gandhi's ideals and wisdom would be most needed.

An awareness of the existence of heredity, that is-of a tendency for variation not to be distributed at random between individuals of a species but to be somehow related to descent, must go back a long time in human history. The fact that offspring resemble their parents more than unrelated individuals is what, no doubt, neolithic farmers first used in improving cultivated plants and domestic animals. The simple device was that of selecting the most desirable individuals as parents of the next generation.

But the history of the attempts towards an understanding of the mechanism of heredity is full of pitfalls and false ideas. From Hippocrates and Aristotle to as recently as Darwin, for instance, the idea persisted that the features of the individual were somehow directly transmitted to its progeny through the germ cells. The pure fiction of the "homunculus", the mini-man believed to be visible through the microscope in the sperm head, was still alive 200 years ago. After all we still say "Johnny has inherited his father's blue eyes" implying something like "Johnny has inherited his father's watch". Yet in the Bible, Genesis 30, we find a garbled account which can be interpreted as showing that Jacob knew how to breed black lambs out of a white flock.

To find a first comprehensive scientific approach to the mechanism of heredity we have to come to Mendel in 1866. He disposed once and for all of any mechanism implying that the characters of the individual were themselves somehow transmitted through the germ cells. He showed that heredity is mediated by factors (we now call them "genes") uninfluenced by the somatic features, and that the somatic features are the consequence of the nature (we now call it "information") of such factors. He also showed that different factors are transmitted separately from each other and distinguished clearly between the genetic constitution of an

* The Gandhi Memorial Lecture delivered at Raman Research Institute, Bangalore on 30 January 1983.
individual—its "genotype" and its characters—
its "phenotype".

Mendel's contribution was so far ahead of his time that it remained ignored until 1900 when Tschermak, DeVries and Correns rediscovered it. Even Darwin, whose theory of evolution by natural selection of small variations was so much in need of some knowledge of heredity, seems to have been unaware of Mendel's decisive paper.

Of the two main ideas of Mendel, indirect inheritance by transmission of information via the gametes and particulate nature of that information, the latter was qualified in 1905 by Bateson's discovery of linkage. The qualification is that two or more genes need not be transmitted independently, but may be transmitted together in the same gamete in a higher proportion than expected by chance. Thus genes fall into "linkage groups". It is to the credit of Morgan's school at Columbia University, particularly Sturtevant, Muller and Bridges, to have shown that the number of linkage groups in a species corres-

ponds to the number of chromosome pairs in the somatic cells of that species. Evidence of various kinds from other workers, especially that sex determination is based on the distribution of the members of a special pair of chromosomes, led Morgan's school to the generalization which goes under the title of "Chromosome theory of Mendelian inheritance".

This generalization states that the chromosomes carry the genes in a linear arrangement and the physical distance between two genes on the same chromosome pair is correlated to the frequency with which the two genes are exchanged at meiosis before gametogenesis.

By 1915 the essentials of the mechanism of hereditary transmission were clear. Two basic points were still obscure. One, how do genes exert their effects on the characters of the individuals? Two, what are the properties of the gene material?

The first decisive contribution to the problem of gene action had come earlier from Garrod, a physician in Oxford, in 1908. He had shown that
certain "inborn errors of metabolism" in man, each the result of deficiency in a particular enzyme, were inherited as single Mendelian recessives. It took almost forty years, to 1945, for Beadle's generalization "one gene one enzyme", refined and expanded in 1956 by Benzer as "one cistron-one polypeptide chain".

To the second question - what are the properties of the gene material? Muller gave a brilliant and definitive answer in a paper of 1922. These are reproduction and mutation. Replication by itself is not unique to the gene material: crystallization, for instance, is a non-biological analogue. Mutation by itself, as a stochastic process, has also many non biological analogues. But replication with mutation, that is replication persisting inspite of mutation, is indeed a unique feature of the gene material. Muller asked what extraordinary structure the gene material must have to give it this carte blanche ability to go on replicating in the changed form whenever change has occurred. The answer to this question came in 1953, thirty years later, with Watson and Crick's double helix structure for DNA.

Muller concluded: given a material with these fateful properties, evolution would automatically follow. Remember, this was at a meeting in 1922. Osborn, the distinguished palaeontologist, was in the chair. As Muller reported, Osborn commented: "I am glad you have a sense of humour". The idea of a material, other than the whole cell, with such properties, let alone forming the building blocks for evolution, was outrageous in 1922. We now take it for granted.

In the same paper Muller compared the gene material with bacteriophages and prophetically suggested that a chemical and physical attack on them could throw light on the nature of the genes. Thus Muller qualifies as the forerunner, indeed a founder, of molecular biology.

The telling title of another paper which Muller wrote in 1924 "The gene as the basis of life" - shows a powerful imagination capable of building models and generalising from the basis of hard experimental evidence. In biology, as distinct from the physical sciences, the combination of deduction and induction was still frowned upon. "Real" biology was supposed to be equal to careful gathering of facts. Darwin, of course, seventy years before had already been a victim of this sort of prejudice, to which he had to reply: how can you gather facts if you do not have a model to test? We can well consider Muller's ideas of 1922 as the second major milestone in genetics, taking Mendel's as the first.

The technology of genetic analysis, and for that of genetic synthesis, based on the chromosome theory made great strides between 1915 and 1950. It also opened the way to many useful applications in animal and plant breeding. But that technology required the experimental use of sexual reproduction and the classification of the kinds and proportions of gametes produced by informative individuals. It could not be applied effectively to organisms in which practical or ethical considerations stood in the way of using sexual reproduction. Man is of course one such organism. Consequently, its genetics was very poorly known in comparison with most other aspects of its biology. This was the situation up to 1968, when the new technology "via" somatic cell genetics came on to the scene.

Two approaches converged to produce this new technology. One was the development in the early 50s by my colleagues - especially J. A. Roper - and myself of a series of procedures for answering the question: does some sort of gene segregation and recombination occur in somatic cells, albeit as a rare event, and if it does, can it be harnessed to genetic analysis and synthesis? The answer was in the affirmative and we developed procedures first with the mould Aspergillus nidulans.

In this mould fusion of vegetative cells occurs regularly and is followed, at a low but manageable rate, by fusion of their nuclei. The nuclei resulting from fusion may undergo during multiplication two processes of segregation and recombination. One is "somatic crossing over", as masterly discovered and analysed by Stern in Drosophila in 1936. The other is a progressive loss of chromosomes. The latter turned out to be most valuable for assigning genes to their chromosomes. This prompted me to suggest, at the CIBA Symposium of 1958, that the same
procedure should be applied to human cells in culture.

The other approach was the development, mainly by Ephrussi and his school, of the techniques for isolating hybrid cells from fusion of mammalian somatic cells in culture. The breakthrough came in 1968 when Mary Weiss and Howard Green found that in hybrids between human and mouse cells the human chromosomes were rapidly eliminated. Application of the same rationale worked out for Aspergillus 18 years earlier led to a procedure for identifying which human chromosome carries a given human gene. This procedure does not require mutants of the genes which one wishes to assign to their chromosomes because the differences, particularly electrophoretic, between the human form of a protein and its mouse counterpart can be used, in Ephrussi's expression, as "built-in markers".

Application and refinements of this procedure have given spectacular results in formal human genetics. Up to 1968, except for sex-linked genes, it was not known for even a single human gene which chromosome carried it. Today there are more than 300 so assigned, and for an increasing proportion of them, the analysis goes as far as the location in a chromosome band. Combined with other techniques— in situ annealing, identification or restriction enzyme markers, D.N.A. sequencing etc.— formal human genetics, based on somatic cell fusion, is now one of the frontiers of genetics. It was its Cinderella in 1968.

Another extremely useful application of somatic cell genetics is the production of "hybridomas". These are clones of somatic cell hybrids which produce specific antibodies. They were first produced by Milstein and Kohler about 10 years ago. They opened a vast field both in research, for instance, for the identification and study of cell surface proteins, and as potential therapeutic agents, particularly for targeting anti-cancer agents.

Applied recently to higher plants, somatic cell genetics is a floodgate of possible practical and scientific applications. The possibility of growing whole plants from somatic hybrid cells has enormous potentialities not only in plant breeding but in the study of all sorts of basic biological problems: differentiation, evolutionary relationships and many others. Melchers has been one of the pioneers in this field, and produced whole hybrid plants from fusion of somatic protoplasts of potato with those of tomatoes.


15. Mary Weiss and Howard Gzeen, 1983
As I mentioned before, between 1915 and 1950 mendelian genetics made great strides in many directions, both fundamental and applied. Among these was the reconciliation of Darwinian natural selection with the new understanding of heredity and mutation. It gave birth between the 20s and 30s to population genetics. The founders of this new approach were Fisher, Haldane, Sewall Wright and Tchetverikov.

By the early 50s Mendelian genetics had produced a picture of the gene material which completely vindicated Muller’s pioneer model of 1922. The gene material was viewed as a continuous linear structure of a few kinds of building blocks each capable of mutation and of reciprocal exchange with an homologous one. Individual segments of the material, the genes, determined the ability of a cell to synthesize a particular protein, or, more precisely a particular polypeptide chain. Mutation, as a change in quantity, quality or sequence in a gene, was what determined a change, qualitative or quantitative, in the relevant polypeptide chain.

The fine genetic analysis carried out by E. B. Lewis and M. M. Green in Drosophila and by
my colleagues and myself both in Drosophila and in Aspergillus nidulans had shown that there was no discontinuity in the material of each chromosome, such as was unnecessarily implied in the old picture, of genes as beads on a string. Benzer, a few years later, with his masterly analysis of fine genetic structure in bacteriophages gave a complete and definitive picture. The term "cistron", which he coined for a segment of genetic material which codes for one polypeptide chain, has now replaced in precise language the older term "gene".

The question of what the genetic material actually was in chemical terms, rather than what it had to be as deduced from its genetic properties, was still quite open throughout the 40s, in spite of Avery's demonstration in 1940 that the "transforming principle" in Pneumococcus was DNA. Even at the 1946 Cold Spring Harbor Symposium on "The genetics of Microorganisms", memorable for Lederberg's demonstration of recombination in bacteria and Hershey and Delbruck's in bacteriophages or for Harriet Taylor's demonstration of linked transformation by pure DNA, there was still dispute as to whether the gene material was DNA, nucleoprotein to protein. Even the demonstration by Hershey and Chase a few years later that the DNA of phage, and not the protein, enters the bacterial host cell and there multiplies, did not lead to general acceptance.

The breakthrough came with the momentous papers of Watson and Crick in 1953. The double helix structure of DNA which they proposed immediately satisfied all the requirements for the gene material which the previous 50 years of work had so precisely identified. Its impact on biology is only beginning to be felt. It is at least as great as that of Darwin a hundred years earlier. No doubt, the double helix is the particularly illuminating third major milestone in genetics.

The Watson-Crick model provided a full answer to what was demanded. It also provided one new idea not at all implicit in the previous work: collinearity, i.e. the linear sequence of nucleotides in the DNA of the gene corresponds to the linear sequence of aminoacids in the polypeptide chain encoded in that gene.

The earlier general view of the relation between a gene and the corresponding polypeptide chain was not that of collinearity. Beadle, for instance, in his important 1945 paper which produced the "one gene-one enzyme" generalization, suggested that a gene imprinted the final specificity on the relevant protein. We must remember that Sanger's work showing that the primary structure of insulin was a linear sequence of aminoacids was just beginning to have an impact. Globular proteins were still viewed as very complex tridimensional structures.
Long ago I asked independently Watson and Crick how they had stumbled on the idea of colinearity. The answer was identical: no other idea had ever crossed their mind. This shows how decisive ideas in science often come to those who are not too deeply steeped in a specialized field. Pasteur is a classical example of this, and in the field of DNA, Chargaff, an outstanding and brilliant biochemist, is a control example. He had produced all the data for suggesting base pairing in DNA but it was to two outsiders, far from well versed in biochemistry, to see what they meant.

The developments in genetics since 1953 are too well-known for me to dwell on. I shall only mention the Jacob-Monod model for switching genes on and off. It is still the best that we have for a start on differentiation, especially now that DNA methylation seems to play a basic role in the switch.

We come now to the mid 70s when Arber’s work on restriction enzymes opened the flood...
gate to the latest techniques in genetic analysis and synthesis: the recombinant DNA techniques.

These varied techniques, of great versatility, are extremely powerful tools in research and applications, including the production by genetically engineered bacterial cultures of valuable substances such as human insulin and interferons, renin, growth hormones etc. In research, a striking recent result is the demonstration that the difference between a human bladder carcinoma cell and its normal counterpart stems from a single nucleotide change.

I wish only, in conclusion, to emphasize a point of great intellectual and ethical interest which seems to have been overlooked. We now understand that, in principle, segments of DNA of any organism, or even of synthetic origin, can be transferred to any other organism and there become part of the recipient's genetic make up. In nature this occurs regularly as a consequence of retrovirus infection in animals and probably of Agrobacterium infection in plants. In the laboratory, vectors carrying any desired segment of DNA can be constructed routinely and used for transferring it to recipient organisms. Even more simply, microsyringe injection of foreign DNA into cell seems to work. The foreign DNA, transferred by virus, plasmid, injection or cell fusion, may become active part and parcel of the recipient's genome.

Thus, the exchange of gene material, which until recently we thought would occur almost exclusively between individuals of a species, can now be viewed as possible, albeit with probabilities ranging from near zero to very high, between any two living organisms.

We come to the concept that the whole biosphere on Earth shares a common gene pool. A new view of the unity of life on Earth becomes imperative. Let us hope that this unifying view takes roots and helps in realizing Gandhi's ideals.
Reminiscences on Genetics
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When a scientist approaches 80 and has given up working at the Lab
Bench or in the field, the only things left are writing a book, giving
boring lectures and, much more important, ruminating on what has happened
in the last 50 years and where, if anywhere, does his own contribution fit
into the general picture. It is a pity that this last activity does not
occur more often, now and then, earlier in life. It might reduce the lava
flow of literature which engulfs and destroys everything, and increase the
pleasure one gets from one own’s research.

I have dabbled in genetics since 1926, when I was an undergraduate in
Agriculture in the ancient University of Pisa, which has produced some
physicists like Galileo and Enrico Fermi. I missed by 300 years meeting
the former, but as undergraduates with Fermi and the group of students of
physics around him, we used to go hiking and skiing. That is where I
learned that entropy decreases in living things at the expense of an
increase of it in their environment. We used to offer sacrifices to
entropy by throwing boulders down from the tops of mountains: a juvenile
crime.

At Pisa a very kind and competent plant breeder, Enrico Avanzi, taught
me the elements of the genetics of that time. He and another man,
Strumelli, were introducing short straw genes into the local long straw
varieties of wheat, to reduce the crippling incidence of lodging. They
succeeded and revolutionized wheat production in Italy. About 40 years
later, Borlaugh, in Mexico, did about the same, was acclaimed by the slogan
“Green revolution” and a Nobel Prize. Well deserved! But I am sorry for
those long gone pioneers.

This episode linked to my youth leads me to a generalization: in no
other field of biology basic ideas or generalizations have arisen so
repeatedly in advance of times as in genetics, or its offshoot molecular
biology. Mendel, Muller, Barbara McClintock even Watson and Crick.
Mendel: 35 years between his paper of 1865 and the rediscovery in 1900;
Muller: 32 years between his definition of the genetic material and its
properties in 1921 and its vindication by Watson and Crick in 1953.
Barbara McClintock: about 30 years between her discovery of transposons
in corn and their rediscovery in bacteria and Drosophila which made them
respectable. Even Watson and Crick, whose landmark paper of 1953 most
certainly cannot be said to have been ignored, proposed in those papers
co-linearity between nucleotide sequence and aminoacid sequence which took
11 years to be established by Yanofsky and by the Cambridge group.

I feel that this feature of genetics, advance by leaps and bounds with
silent or pedestrian intervals in between, is one of its great
fascinations. Few areas of genetics have had long periods of turning into
a “discipline” - in Seymour Benzer’s meaning - no longer as exciting as
when they were fresh from the brain. For example, Mendelian genetics
became a "discipline" in 1915 when Morgan's school summarized their pioneer
work in the "Mechanism of Mendelian-heredity" and crystallized the
beads-on-a-string picture. A new bout started in 1940 when Muller
discarded the beads-on-a-string picture. The development of bacterial and
phage genetics in the 40s by Luria and Debruck is perhaps another example:
it became a discipline after 1946.

But there is also, in genetics, the reverse of the medal: wrong data
and generalizations immediately and uncritically accepted by the scientific
establishment (which, incidentally, means grants) delaying progress in
thought and experiments. Best example: the "tetranucleotide" structure of
DNA. We had to accept it from chemists. It delayed the acceptance of DNA
as the genetic material from 1943 - Avery's demonstration that the
"transforming" principle was D.N.A. - 1952 when Chargaff showed how
versatile DNA really was and the Hershey-Chase blender experiment left no
doubt that in bacteriophage DNA was the genetic material.

Another example: the myth of ribosome as the coding structures. It
was taken uncritically as an implicit consequence of Brachet's
demonstration, before the war, that RNA synthesis went up massively just
before a massive increase in protein synthesis. It took up to 1961 for
Brenner, Jacob, Monod and Meselson to show that ribosomes were the machine
to be programmed by messenger.

A third example: the beads-on-a-string structure of the chromosome
material, that is: the chromosome is made up of genes (the beads) which, as
we would say, code for proteins, and intergenic material (the string) where
recombination occurs. It was Muller in 1940 who showed that there was
nothing in theory and even less in data to force the implicit conclusion
that the gene as a unit of function, mutation and recombination are one and
the same thing. Later in the 40s Ed. Lewis, M.M. Green and my own group
showed in Aspergillus and Drosophila that the gene could be split by
recombination. This led me in 1951 to propose that the ultimate structure
of the genetic material was uniform, with singularities in function of
certain segments. Benzer, of course, with his masterly fine genetic
analysis in bacteriophage, and the newly established structure of DNA,
clinched the matter once and for all.

So there are plenty of examples in genetics of wrong generalizations
clogging progress. This should make genetics even more attractive to the
young. It may provide them with the satisfaction, more than in any other
approach to biology, of relegating to the dustbin foolish or unnecessary
beliefs of an older establishment. The Royal Society has as its motto
something like: don't swear on your teacher's words (not usually followed).

How true is Eddington's paradox, which I paraphrase as: "Don't trust
data until confirmed by theory". I have a nagging feeling that
"oncogenes", a truly important recent advance, are entering a phase in
which Eddington's paradox will have plenty of scope.

Now let me go through some of the developments in ideas since the 1900
"rediscovery". What did Mendel do? He dismissed the idea of
direct inheritance of body characters, of phenotypes, and he
introduced the not quite new idea of particulate inheritance. How
infrained is even today the idea that somatic characters are inherited.
When we say "Johnny inherited his father's blue eyes" we imply, without
much thought, something like "Johnny inherited his father's watch".
should say, of course "Johnny inherited the program for his father's blue eyes".

A percursor of Mendel was Jacob (cf Genesis, 30). He knew how to breed brown sheep out of his cheating father-in-law's flock of white sheep. He obviously identified and kept heterozygous rams. But the Bible gives a garbled account of his methodology.

After the "rediscovery" of Mendel in 1900, the advances in chromosome cytology by Wilson, Boveri and Sutton, and the rediscovery and misinterpretation of linkage by Bateson and Punnett in 1905, the major second generalization in genetics was the mechanism of hereditary transmission by the Morgan's school, Sturtevant, Muller and Bridges: genes are linearly arranged on the chromosomes and the frequency of recombination at meiosis between those on one chromosome is a measure of their distance apart.

What the genes are and how they work was not the concern of that masterly summary by Morgan, Sturtevant, Muller and Bridges, the 1915 classic "The mechanism of Mendelian-heredity". Yet as far back as 1908 Garrod had shown that "inborn errors of metabolism" in man, each the consequence of some enzyme deficiency, were inherited as single mendelian characters. Under the stimulus of J.B.S. Haldane this idea was extended to flower pigments and to rodent coat colours in the 30s: one gene—one metabolic step. Extended to the biosynthesis of essential metabolites in Neurospora by Beadle and Tatum this led to Beadle's nut shell of 1945: one gene—one enzyme, which Benzer refined in 1956 as one cistron—one polypeptide chain.

The late 20s and early 30s witnessed the reconciliation of Darwinism and Mendelism: Fisher, Haldane and Sewall Wright.

I am sure most of you know Haldane's reply when in the late 40s bacterial transformation was very exciting. What would you like to add to your enzymic endowment? Cellulase, of course: when you send me a dull MS I would eat it and truly say that I enjoyed it.

The modern neo-Darwinian synthesis followed much later, after the war, with Julian Huxley, Simpson, Mayr and others. Now Kimura and others are pulling it one or two pegs down, hopefully with euristic results.

But let me deal with the central stream of genetics. In 1921 Muller came out with his most powerful idea: there is a "genetic material" defined by its "fateful" properties: replication, mutation and "heterocatalysis" (what we now encompass under transcription, processing and translation). Given such material, evolution would automatically follow. He reinforced this idea in 1925 in a paper by the telling title "The gene as the basis of life". This idea had to wait till 1953 for a full vindication by the Watson/Crick structure. When Muller proposed this momentous picture, the distinguished paleontologist Osborn thought, and said so, that Muller was joking.

Muller got his Nobel Prize in 1946, the second in genetics after Morgan in 1933. The motivation was not his "gene as the basis of life" but his production of mutations by x-rays, a minor fallout of all his previous theoretical and experimental work. Anybody could have done it with those techniques and ideas. However, the time was soon after Hiroshima. Much better give him the Nobel Prize after 1953 jointly with Watson and Crick.
to Muller for having stated what the genetic material had to be and how it had to work and to Watson and Crick for having shown what it was and how it worked.

In the late 30s and early 40s and again after the war, Warren Weaver of the Rockefeller Foundation, who I believe invented the term “Molecular Biology”, started active support for a getting together of physicists and geneticists. X-ray crystallographers (Astbury and Bernal), biologists (Muller, Timofeef, Ephrussi, Waddington, Lwoff, Darlington) and theoretical physicists (Niels Bohr, Delbruck, Max Born) started having joint informal meetings in Europe: Paris, Copenhagen, Edinburgh.

In 1938-40, Muller, a refugee from Texas and from Lysenko, Max Born, a refugee from Hitler and the Stedmans, nucleic acid chemists, held regular discussions at the Institute of Animal Genetics in Edinburgh. Lotte Auerbach and I, as very much junior members, were lucky to attend. Incidentally, Luria at that time was taking up the physicists’ outlook in Fermi’s group in Rome.

This extraordinarily fruitful cross-fertilization was very active well before Schrödinger’s book of 1944 “What is life?” The defection of physicists and crystallographers towards genetics – Delbruck, Zimmer, Bernal – was well on its way. My guess is that this aroused Schrödinger’s short lived interest in biology. The book, we are told, stimulated the later wave: Wilkins, Szirmai, Benzer, Stent, Levinthal, and many others, but strangely, not particularly in Europe.

To me it gave the hope that sooner or later biochemistry would recover from the complex enshrined in the bag-of-enzymes, liver homogenates and bioenergetics. This had to wait until about 1961 when the three approaches, genetics, biochemistry and physical chemistry were amalgamated.

The 1940s of course witnessed the tremendous contribution of bacterial and phage genetics. Though eukaryotic microorganisms like yeast and fungi were already “systems” used for genetics, prokaryotic genetics originated with Delbruck and Luria’s contribution of 1941 and was clinched by Lederberg in 1946. An amusing episode at the 1946 Cold Spring Harbor Symposium was this. Lederberg, 21 years old, reported his momentous discovery. A respected old bacteriologist asked: ‘have you isolated single recombinant cells?’ Lederberg did not yet possess the savoir-faire of a university president. He put up a fight to show what a waste of time it would be.

The tremendous intellectual discipline, honesty and no-nonsense introduced by Delbruck, in his gravitation between Nashville, CalTech and Cold Spring Harbor, complemented in its early stages by the biological imagination of Luria, is hard to overstate, it was amazing even for a complete outsider like me. The phage group which they created is now the folklore of molecular biology. The atmosphere of the phage group – great competition to answer problems rather than to advance one’s career – reminds me very much of the atmosphere in the group of physicists around Fermi in the late 30s in Rome.

The other two great centres of the advances of the 40s and 50s were, of course, Cambridge, England, where Perutz and Braqq were joined by Watson and Crick, and later Brenner and the Pasteur Institute where Lwoff managed to encourage Wollman, Jacob and Monod. Both at Cambridge and CalTech there was not much knowledge of biochemistry and, even less, respect for it as it
was then. Not so at the Pasteur. This is why I believe the Pasteur group helped more than any other to raise biochemistry out of the bag-of-enzymes complex and make it what it is today: part and parcel of serious biology. The land-mark of this change is, I believe, Jacob and Monod's 1961 paper "On the control of protein synthesis".

What happened in Cambridge is so amply reported in books, films and television that I do not need to dwell on it. The 1953 papers by Watson and Crick satisfied at once all the requirements for the properties of the genetic material so clearly stated in Muller's paper of 1921 and in later developments. We knew by 1952 that: (1) the genetic material had the three "fateful" properties; (2) that its structure was linearly uniform; (3) that proteins had a primary linear structure (Sanger's work on insulin was of about that time) and (4) that there was the one gene-one enzyme relation between the two.

Major as the Watson-Crick contribution was in answering all the previously formulated questions, there were two aspects which were not in the air: duplex complementary structure and colinearity.

For sure, Pauling in his Jesse Boot Foundation Lecture in Birmingham on 28 May 1948; i.e. five years before Watson and Crick, had stated: "If the gene or virus molecule consists of, say two parts which are complementary each can serve as the mould for the production of the replica of the other". It was a brilliant flash of insight not taken any further. Muller is another example of the same sort of event. In 1921 he suggested the use bacteriophages as "systems" to study genes. His famous sentence about grinding genes in a mortar is in all science history books. But he did not follow up the idea let alone take up the experiments, so he does not deserve so much credit.

I asked both Watson and Crick independently soon after the 1953 papers, how they had stumbled on the idea of colinearity: no other had ever crossed their mind. It took 11 years for it to be confirmed by Yanofsky and the Cambridge group.

I only want to mention one step since 1953 which has opened a floodgate: the discovery of restriction enzymes by Werner Arber. He was made a foreign member of the Natl. Acad. Sci. just 5 days ago.

There is one field of genetics which has made more practical progress in the last 25 years than any other: it is not recombinant DNA or monoclonal antibodies or oncogenes. It is the field of pedestrian formal human genetics. I have a prejudiced grandparental affection for it.

In 1945, when I was appointed to my first tenure job in Glasgow University (incidentally a one-man Department of Genetics because the botany, zoology and medicine departments did not want it in each others set-up) I went to see the Dean of Medicine to ask what he wanted me to do. The answer was shattering: is there one thing in genetics of any use to medicine?
This set me thinking about what was wrong with human genetics. Mapping to that time was either by recombination or by deletion. Mapping by duration of mating was not yet discovered and unfortunately it does not work with higher organisms. Think of the possibilities if it could! So I decided that we had to find some way of bypassing sexual reproduction: "alternatives to sex" as J.B.S. Haldane called it.

I won't bother you with details: by 1958 I was able to press the Ciba Symp. on human genetics for somebody to take up human formal genetics by using human somatic cells in culture. Nobody reacted. I had no alternative but to try myself. The chosen system did not work, for reasons we now understand. Ten years later Mary Weiss and Howard Green made the breakthrough with the techniques of hybrid somatic cells developed by Ephrussi over the years and the rationale developed by my group ten years earlier. The results were spectacular. Not a single autosomal gene was mapped in 1968; there are now over 500. Combined with all sorts of modern molecular biology techniques human genetics from being the Cinderella is now one of the frontier fields.

Well what do all the recent developments suggest to an old man? We used to believe that transfer of genes was possible only within a species. We now know that, in principle, it is possible between any two living organisms. So the whole biosphere shares a single colossal gene pool. There may well be a bacterium in Australia carrying a bit of my own or your genetic material.

This gives a glimmer of hope. When, by accident or design, the wise political and military leaders of East and West will have succeeded in annihilating all advanced forms of life on Earth, odd bacteria carrying genes from more advanced forms will survive: they are much more resistant to radiation and do not mind the nuclear freeze.

They will start evolution all over again but it will take them a billion or so years less to reach the present level of self-destructive complexity.