INDISPENSABLE GENE FUNCTIONS IN NEUROSPORA*

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The point was raised by Delbrück[1] in 1946 that the apparent absence of multifunctional genes in Neurospora might be an artifact arising from the screening methods employed in securing biochemical mutants. Qualitatively, this argument may be summarized as follows: Certain gene functions are indispensable; that is to say, they cannot be compensated by the use of complex media. If the frequency of indispensable functions is high, there will be a high probability that a multifunctional gene has at least one indispensable function. Mutation of such multifunctional genes would be lethal on complete medium and would pass unnoticed. The detectable mutations would involve a preponderance of unifunctional genes, and an erroneous impression would be created that mutations usually affect single biochemical functions. Such a possibility was potentially a serious objection to any generalization about gene action, such as the one gene-one enzyme hypothesis, based solely on the properties of biochemical mutants.

In order to evaluate this suggestion Horowitz[2, 3] has proposed an experimental estimate of the relative frequency of mutations involving indispensable functions. The experiment consists in obtaining mutants which differ from wild type in not growing on minimal medium at certain temperatures, usually in the higher portion of the normal temperature range. These mutants can be maintained on any medium at a favorable temperature, irrespective of the nature of the phenotype at unfavorable temperatures. They are transferred to complete medium at an unfavorable temperature, and those which do not grow have lost functions which are, by definition, indispensable. Such mutants are irreparable in the sense that the particular complete medium used in the experiment does not restore growth at the unfavorable temperature. This procedure can be considered adequate to find the relative frequency of indispensable functions only if it is assumed that "genes controlling indispensable functions are just as likely to yield temperature alleles as those controlling dispensable functions."[4] The proportion of irreparable temperature mutants has been taken as a direct representation of the proportion of indispensable functions and has been reported to be 46% in *Neurospora crassa* and 23% in *Escherichia coli*.[4, 5] If the inherent assumption is accepted, it would appear from these experiments that the greater part of the genome in these organisms is accessible to the methods of biochemical genetics. If this were true, it would lend some credence to generalizations arising from the study of biochemical mutants, and indeed it is partly on this basis that the one gene-one enzyme hypothesis has been supported.

Another method for obtaining irreparable Neurospora mutants has been described.[6] Mutants obtained by this method may be referred to as heterokaryon mutants because the mutant nuclei are propagated indefinitely under conditions of heterokaryotic symbiosis with nuclei bearing normal genes capable of performing the indispensable functions. They may also be described as recessive lethal mutations, by analogy with similar mutations maintained as heterozygotes in diploid organisms. The heterokaryotic cultures produce both heterokaryotic and homokaryotic conidia, and the latter are easily tested for their ability to grow on various media. The irreparable mutants can grow only as heterokaryons with genetically complementary nuclei, but the reparable mutants can also grow as homokaryons if complete medium is provided. This method has the advantage that the reparable mutants obtained thereby are absolutely the equivalent of biochemical mutants obtained by other general methods. It is likely, but not entirely certain, that all of the possible irreparable mutants can also be detected by this method. The question of whether any are undetectable need not seriously concern us, however, because our results differ from those of Horowitz in the direction which cannot be explained on this basis. When 190 of the heterokaryon mutants obtained after ultra-violet irradiation were tested on Neurospora complete medium, only seven showed any homokaryotic growth. Over 96% of these mutants had lost indispensable functions, in strong disagreement with the results obtained with temperature mutants.

We have been reluctant to base any conclusions on the discrepancy between induced heterokaryon and temperature mutant data because a certain proportion, not precisely known, of the induced heterokaryon mutants are chromosomal deficiencies, or otherwise genetically complex. One would expect most of the multiple losses to be irreparable even if the frequency of indispensable functions is low. The selection of temperature alleles certainly excludes genetic complexity, and therefore a comparison of data obtained by the different methods has seemed unwarranted. This difficulty has now been overcome by the use of spontaneous heterokaryon mutants. It is our basic assumption that spontaneously occurring mutations rarely involve more than one locus at a time and should therefore yield stocks which are comparable with temperature mutants, at least with respect to the relative frequency with which they are reparable. It will be seen that there is in fact a highly significant difference between the reparable proportions of heterokaryon and temperature mutants.

Methods and Results.—Spontaneous recessive lethal mutations were detected and maintained in the methionine-amycelial nuclear component of a heterokaryon between ornithine (29997)A and methionine-amycelial (4894-422)A in the following manner: The macroconidia from week-old cultures of the heterokaryon are plated in minimal sorbose agar and incu-

bated four days at 33°C. Since no supplements are added to the plates, all colonies originate from heterokaryotic cells. The colonies are isolated in tubes of minimal glycerol agar, where they conidiate profusely within a few days. The conidia of each isolate are suspended in water and surface-streaked on sorbose agar with added methionine. The methionine permits the growth of methionine-amycelial homokaryons. When the streaks are examined after 48 hours of incubation the normal heterokaryotic colonies are, in most cases, heavily interspersed with morphologically distinct amycelial homokaryons. Occasional streaks show none of these homokaryons, and these streaks represent cases where a lethal mutation was carried by the amycelial nuclei in the single conidium from which the culture was grown. Such mutations are detectable if they originate during the growth of the preceding (initial) culture, or even in the heterokaryotic conidia themselves, since a large proportion of the latter contain but a single amycelial nucleus in addition to one or more of the ornithine nuclei. The heterokaryons bearing the desired mutant nuclei were reisolated from the streaks. In this manner a total of 58 mutants was obtained from 2764 isolates.

The cultures from which the mutants were selected were interrelated through a lineage of mass transfers and it is possible for any number of the mutants to have shared a common mutational origin. All repetitions of the same mutant must be discarded. The mutants were therefore tested for homology by the following method.[7] A conidial suspension in water is prepared from each stock. The suspensions are mixed in all the possible pairwise combinations, of which a total of $n(n-1)/2$ can be formed from n mutants. Each mixture is centrifuged to a firm pellet and a portion of each pellet is transferred to a sorbose agar surface and incubated for 12 hours, by which time the conidia have coalesced. The germinating conidia establish cytoplasmic connections at innumerable points, and nuclei which were previously in separate cells now find themselves together in a common cytoplasm. The coalescent masses are then isolated in glycerol agar tubes where they produce a profusion of aerial hyphae and conidia. The purpose of the foregoing procedure is to mix as thoroughly as possible the nuclei from the contributing stocks, so that the conidia of the mixed coenocyte will have the best chance of containing new combinations of nuclei. Some of the conidia formed by the culture which results from such a mixture may be expected to contain at least one amycelial nucleus from each of the contributing heterokaryons, and no other type of nucleus. Cells with this nuclear constitution are homokaryotic for amycelial and are therefore potentially able to initiate amycelial colonies. However, they are viable only if the lethal mutations are not homologous. Thus the conidia of the mixture may produce some amycelial colonies whereas the original components produce none. The presence of such amycelial colonies shows that

the two mutations are different, and also very satisfactorily confirms the most important basic postulate of the system; namely, that the nuclei which carry lethal mutations are cryptically propagated in the heterokaryons.

The data from such an experiment can be conveniently summarized in the form of figure 1. The mutants are arranged in order along adjacent

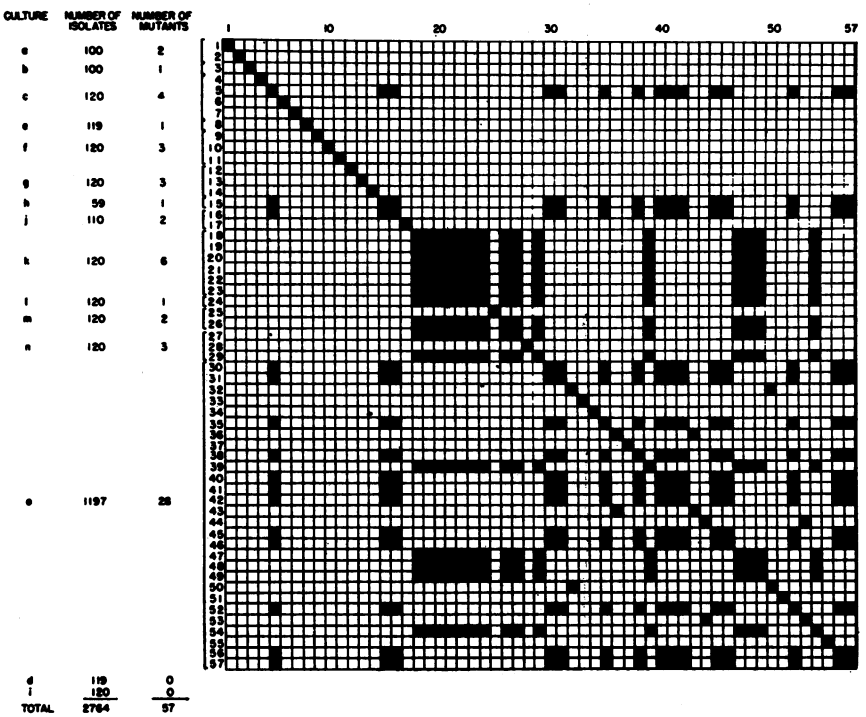

FIGURE 1

Homology tests of 57 spontaneous heterokaryon mutants. The mutants are numbered in chronological order of isolation. The solid squares represent homologous tests; that is, mixtures which produced no amycelial colonies. The open squares are nonhomologous pairs: all produced numerous amycelial colonies. The interpretation of the figure is fully described in the text.

sides of the square to form a coordinate system in which the result of each test may be represented in duplicate, once on each side of the diagonal. This arrangement facilitates recognition of three possible conditions which may pertain to a mutant. First, it may be homologous with no other members of the series; this is true of mutant number one, for example. Second, it may belong to a group of identical mutants, as does mutant number five. The identity of the members of the group is reflected in an exact coincidence

of their patterns of homology. Third, it may participate in an inclusive complex, as when one mutant is homologous with two others and these in turn are not homologous with each other. We have found many examples of inclusive complexes among mutants isolated from radiation experiments, and it seems likely that these represent chromosomal deficiencies. It is noteworthy that no such complexes have been found among the spontaneous mutants, but the series is too small to be entirely certain that they do not occur.

Conidia formed by each mixture were streaked on the surface of sorbose agar with methionine and in 1383 of the 1596 tests shown in figure 1 numerous amycelial colonies were found. One mutant (an irreparable mutant from culture *j*) was omitted from figure 1 because, although all of its combinations yielded amycelial colonies, the number of these colonies was in most cases extremely small, and it was felt that the status of the mutant was somewhat equivocal. A sharp all-or-none criterion of homology was applicable to every combination of the remaining 57 mutants. It can be seen from the distribution of those tests which showed no amycelial colonies that five sets of identities are revealed, comprising two groups of 15 each and three groups of two each. The 36 mutants in these five groups may have been derived from only five mutations. Alternatively, some or all of them may represent unusually mutable loci. For the purpose of the experiment each group is counted as a single mutant, and these five, together with the 21 mutants which showed no homology, make a total of 26 non-allelic mutants. If the basic assumption of a predominantly unitary nature of these spontaneous mutations is to be valid, it is important that cases of non-disjunction or any type of chromosomal instability leading to multiple gene losses be detected and excluded from the experiment. There are not enough mutants in the series to insure the detection of small or medium-sized deficiencies as inclusive complexes. Here we rely on the established rarity of such deficiencies as spontaneous events. Spontaneous loss of a whole chromosome may be sufficiently frequent to occur in the series. It is not yet certain whether losses of this magnitude would be viable, even in a heterokaryon with normal nuclei. Assuming that they are viable in a heterokaryon, it is unlikely that they would have escaped detection as inclusive complexes if they account for a significant number of the mutants.

The 26 non-allelic mutants occurred among a total of 2764 isolates, an incidence unquestionably much higher than the incidence of spontaneous biochemical mutants. Biochemical mutants are identical, as a class, with reparable heterokaryon mutants. Therefore we expect only a small proportion of the heterokaryon mutants to be reparable. This expectation is confirmed by experiment. The frequency of reparable mutants was found by streaking conidia of each mutant stock on Neurospora complete medium. Elaborate precautions were taken to make this medium comparable to that

of Horowitz. In two cases (mutants 1 and 14) amycelial colonies appeared. Two out of the 26 are reparable, whereas the corresponding data for temperature mutants are 14 out of 26. The difference is highly significant, $P < 0.001$.

Discussion.—Evidently the relative frequency of mutations involving indispensable functions is higher for heterokaryon mutants than for temperature mutants. This knowledge does not clarify the question of multifunctional genes. Quantitative relationships between the frequencies of multifunctional genes and of irreparable mutants can at present be founded only on completely artificial assumptions. When a mutation involves an indispensable function one does not know whether more than one function at the genic level has been lost, but the notion is certainly a gratuitous one.

Of greater interest is the possibility that the disagreement can be explained. First, it will be worth while to outline the reasons for believing that the heterokaryon method offers the more reliable approach to the true proportion of indispensable functions. The growth of heterokaryons, and the compatibility of their components, are influenced by many genetic and environmental factors. We know of no case, however, of a biochemical mutant which will not participate in a heterokaryon, provided that independently determined conditions for compatibility are present. Indeed, it would seem unreasonable that a mutant would accept a material provided in the medium, and yet not accept the equivalent material provided through the action of complementary nuclei. Heterokaryon mutants arise within a pre-existing heterokaryon, where the components are already known to be compatible. It is therefore reasonable to believe that there is little or no restriction on the variety of reparable mutants which are accessible by the heterokaryon method. It is a conservative assumption, then, that the two methods detect the reparable mutants with equal relative efficiencies; so the very fact that the heterokaryon method yields relatively more irreparable mutants implies that this method gives a more nearly correct result. We arrive at the surprising conclusion that there must be a class of irreparable mutants undetectable by the temperature method. In other words, the potentiality of mutating to a temperature allele is relatively less frequent among genes having indispensable functions than among those having dispensable functions.

Two investigations into the causes of temperature-sensitive phenotypes offer a key to this anomaly. Maas and Davis[8] have found that a mutant of *Escherichia coli*, requiring pantothenate above 30°C., possesses an enzyme (coupling β-alanine with pantoic acid) which is rapidly and irreversibly inactivated in the unfavorable temperature range where the corresponding enzyme of the wild type is stable. Horowitz and Fling[9] have shown that the thermostability of tyrosinase in *Neurospora crassa* is governed by a pair of alleles, and have found evidence that the difference in thermostability is

caused by a structural difference between the enzymes. If we were to risk generalizing on these cases, we would surmise that many temperature alleles are characterized by the production of abnormally thermolabile proteins. Temperature alleles of this type would necessarily be restricted to loci at which the normal gene product is of sufficient complexity, and otherwise so constituted, as to permit the existence of differentially thermolabile structural analogues. Heterokaryon mutants are subject to no such restriction. Among biochemical mutants the necessary condition for such temperature alleles may be general, for it is only a moderate extrapolation on present knowledge to suppose that all the dispensable functions are performed by enzymes. Indeed, Horowitz and Leupold[4] have presented evidence of the random occurrence of temperature alleles among biochemical mutants. Perhaps the temperature mutant data more nearly represent the ratio of the dispensable to the indispensable among enzyme controlled reactions than among gene functions.

This explanation implies a broad subdivision of the genome into two categories. First, there is a class of genes whose functions may be surmised in a general way from studies on biochemical mutants. Each member of this class determines the structure of a product, such as an enzyme, which is structurally capable of existing as a thermolabile analogue, and is rather likely to be dispensable. An enzyme, for example, is dispensable if it catalyzes some step in the synthesis of a low molecular weight compound which can be supplied to the mutant. Second, there is a larger class of genes which perform entirely unknown functions. If these functions are synthetic, then the hypothetical products formed under control of these genes are structurally incapable of existing as thermolabile analogues and are seldom, if ever, dispensable. These meager clues are of little help in characterizing the indispensable functions explicitly, but they are enough to make it clear that these genes are, in a sense, a natural class. As Horowitz and Leupold[4] have assiduously pointed out, reparable and irreparable temperature mutants are fortuitous classes, being determined by the availability of factors in the medium rather than by a fundamental difference in the genes. The genes which are inaccessible to the temperature method, on the other hand, share in common this rather fundamental property of being unable to mutate to temperature alleles.

The problem of why this property should be so closely correlated with indispensability is likely to remain unsolved until the functions are characterized. It would be fruitless now, if not impossible, to exhaustively list the possibilities, but there is one which is of sufficient interest to warrant discussion; namely, the possibility of gene-controlled complex intermediates in protein synthesis. A most attractive feature of the one gene-one enzyme hypothesis is its agreement with the experimental fact that a large number of biosynthetic reactions appear, when studied genetically, to

be mutually independent. This result would be expected if, as has been suggested,[4] each enzyme which uniquely catalyzes such a biosynthetic reaction were totally synthesized from its simple residues under control of a single gene. However, if specific complex intermediates are shared in common by a fair number of different enzymes or other large molecules, the same result would be produced. A mutation affecting a single complex intermediate would affect the synthesis of a number of different enzymes, and therefore almost invariably lead to loss of an indispensable function. The detectable mutations would be confined practically to genes controlling the final assembly of the complex fragments into macromolecules. The experimental evidence of genetic independence of biosynthetic reactions, then, suggests only that there are many examples of a one gene-one enzyme relationship at this final level, but does not imply that the total synthesis of an enzyme involves but a single gene. It may equally well reflect the near impossibility of compensating for the loss or the alteration of a complex intermediate. Because of its size, its specificity, and its ephemeral existence in the organism, such an intermediate would be extremely difficult to obtain, or to effectively supply to a mutant. At the same time, its structure would be such as to preclude its existence in two differentially thermolabile forms. Polypeptides of intermediate size have precisely the properties required of the hypothetical gene products. Thus, it is not implausible to assume that many genes indirectly participate in the synthesis of a protein, although only one gene can be inferred from the usual genetic evidence.

The one gene-one enzyme hypothesis has seemed to perish under the weight of exceptions, and our findings will inevitably be interpreted as one of these. It should be understood, however, that the core of the hypothesis is the coincidence of gene and product; not the contention that the product must always be an enzyme. Whereas the various lines of evidence which have been discussed here favor the conclusion that in the great majority of cases enzyme production is not the immediate function of the gene, it must be noted carefully that the data cannot in any sense be interpreted as evidence against a basically unitary hypothesis of gene action. This concept, so appealing in its simplicity, is still tenable. It will remain so unless it is proved that the exceptions are not secondary consequences of changes in single products. Heuristically, we are justified in generalizing the one gene-one enzyme hypothesis into a one gene-one action hypothesis, where the action, in most cases, awaits description.

Summary.—The proportion of Neurospora mutants which have lost indispensable functions is higher among mutants obtained by the heterokaryon method than among those obtained as temperature alleles. This finding is explained by postulating a class of genes which are, because of the nature of their actions or gene products, exempt from mutating to tempera-

ture alleles. Practically all members of this class have indispensable functions. These genes may have no role in enzyme synthesis, or they may be remotely involved, through controlling the formation of complex intermediates. A majority of the genes in *N. crassa* are in this class.

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*IMMUNOGENETIC STUDIES OF PSEUDOALLELISM IN DROSOPHILA MELANOGASTER. I. ANTIGENIC EFFECTS OF THE LOZENGE PSEUDOALLELES**

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It has been proposed[1, 2] that the phenotypic similarities and position effects of pseudoalleles may be explained by the hypothesis that such loci are concerned with successive steps in a chain of reactions which occurs at the site of the genes in the chromosomes:

$$S \xrightarrow{a^+} A \xrightarrow{b^+} P.$$

Localization could be the result of limited amount or diffusibility of the intermediate A. The sequence of reactions would then proceed to completion only if the wild alleles of the adjacent loci are on the same chromosome, but not if they are on the different members of a pair of homologs.

This hypothesis seems not to be contradicted by position pseudoalleles with morphological effects in Drosophila, and indeed has led to the discovery of pseudoallelism among certain classes of biochemical mutants in Aspergillus.[1, 3, 4] It is a particular form of the "kinetic" hypothesis of