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 considerablement la difficulté, á 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 possibiilty 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—alternates 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^{-5} (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

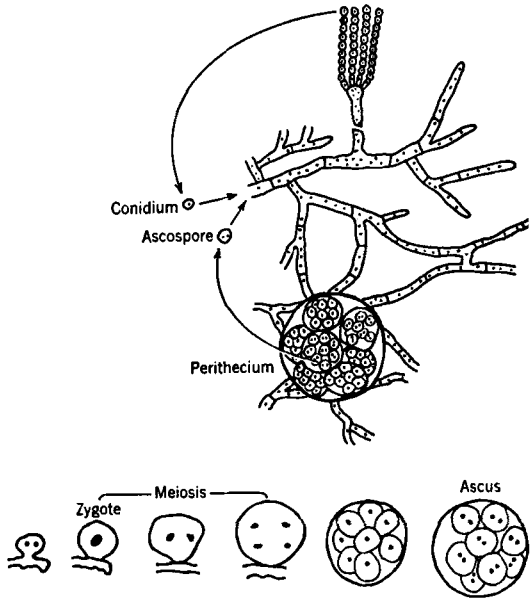


Fig. 1. Life cycle of *A. nidulans*.

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

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).

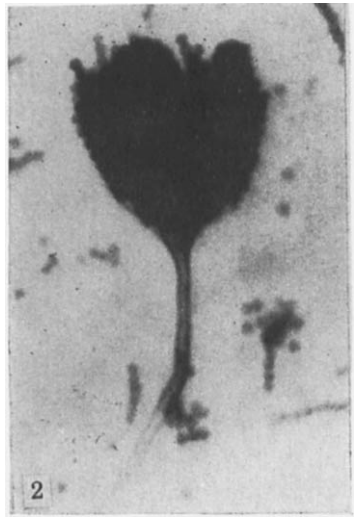


FIG. 2. Conidial head of *A. nidulans*.

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.

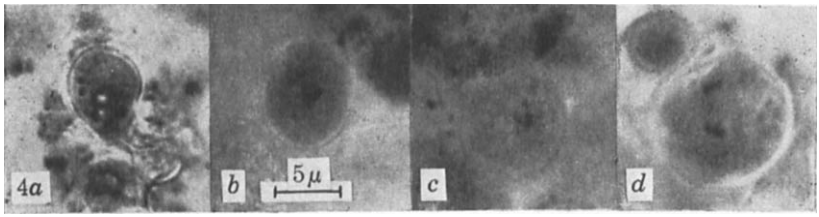


FIG. 4. Four stages in the ascus primordium: (*a*) two nuclei presumably about to fuse; (*b*) the nucleus of the zygote; (*c*) first meiotic metaphase—four structures, one out of focus; (*d*) first meiotic anaphase.

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 *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. 4*a, 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 perithecium. 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 (w_a) 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 S_δ. This strain again differs by a single known allele (s_d) 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 ($7H_2O$), 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* NaOH; 2 g. nucleic acid in 15 ml. *N* 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 (first peptic, then tryptic). 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* 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

Radius of Colonies of *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.

	Hours after inoculation									
	48	96	120	144	168	192	216	240	264	288
Complete Medium										
Dish 1 (mm.):	12	24	30	36	43	49	56	63	70	75
Dish 2 (mm.):	12	25	32	38	45	52	58	65	72	78
Minimal Medium										
Dish 1 (mm.):	7	19	24	30	36	42	47	53	60	66
Dish 2 (mm.):	8	19	25	30	36	41	47	53	59	66

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

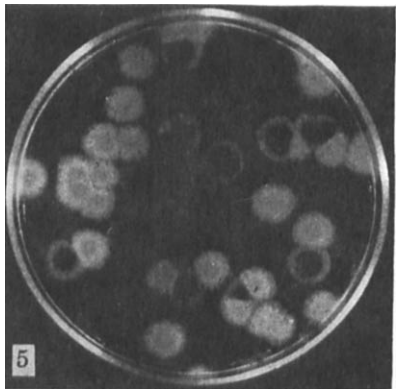


FIG. 5. Colonies from a cross segregating for yellow and green conidia; 48 hours after plating the ascospores.

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

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 Hülle 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 perithecium are needed, the perithecium is carefully cleaned of mycelium, Hülle 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 perithecium 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_a) from wild type, and a parathiotrophic mutant also differing in one gene (s_d) 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 *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).

$w\ ad_1\ y$: white conidia; incompletely cell-localized; adenine-requiring; X-ray induced from y; simultaneous occurrence of the two mutant alleles w and ad_1 (1947).

$w_n\ paba_1\ bi_1$: white conidia; P.A.B.A.- and biotin-requiring; spontaneous from $paba_1\ bi_1$ (1951).

$bi_1\ pr_1$: reduced extracellular proteolytic activity (Fig. 6); biotin-requiring; ultraviolet induced from bi_1 (1951).

$paba_1\ y\ co$: compact colony; yellow conidia; P.A.B.A.-requiring; origin of co unknown; detected 1951 in strain $paba,\ y$.

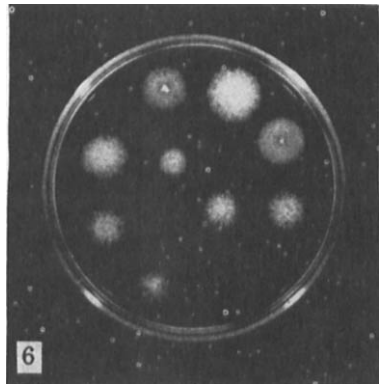


FIG. 6. Plate-tests on milk medium of segregants for the "visible" characters: colors of conidia and proteolysis; the latter revealed by presence or absence of clear ring around the colonies.

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_n 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).

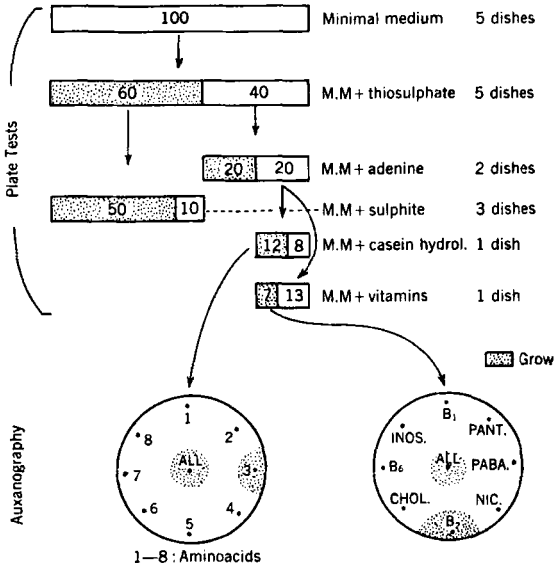


FIG. 7. Diagrammatic sequence in the routine for characterization of auxotrophs: plate-tests followed by auxanography.

TABLE 2

Mutants Obtained by "Total Isolation"

Series	*Strain treated*	*Isolates* (no.)	Total (no.)	Total (%)	*Auxotrophs* * S	N	A.A.	N.A.	Vit.	Untested
X-rays, 50,000 r.										
19-11-46	*y*	201	7	3.5	0	1	0	2	1	3
28-1-47	w_a	338	7	2.1	1	1	1	2	0	2
28-2-47	+	262	8	3.1	0	2	0	0	2	4
15-3-48	*y*	364	8	2.2	1	4	0	0	3	0
J.A.R.-1	*y thi*	1372	21	1.5	3	2	2	2	7	5
J.A.R.-2	bi_1	671	26	3.9	1	3	7	3	2	10
S-1, S-2	bi_1	230	4	1.7	0	0	1	0	3	0
	Total X-rays	3438	81	2.4	6	13	11	9	18	24
U.V. (in brackets survival rates)										
S-3, S-4 (5%)	bi_1	400	5	1.3	1	0	1	2	1	0
S-5 (25%)	bi_1	500	2	0.4	0	0	0	0	2	0
S-6 (12.5%)	bi_1	1070	5	0.5	0	4	0	0	1	0
	Total U.V.	1970	12	0.6	1	4	1	2	4	0

* 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

(1946) "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).

TABLE 3

Comparison of the Efficiency of Total Isolation, Delayed Enrichment, and Starvation for the Isolation of Auxothrophic Mutants

	X-rays (50,000 r.)			*U.V.* (5% survival)	
	Total isolation	Delayed enrichment	Starvation (≥ 96 hr.)	Total isolation	Starvation (≥ 96 hr.)
Isolates	3438	71	344	400	777
Mutants	81	4	94	5	308
Per cent	2.4	5.6	27	1.3	39

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_1) 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_1 and $y\ bi_1$) were mixed in known proportions with conidia (white) of a biotin-adenine requirer ($w\ ad_1\ bi_1$) or with conidia (yellow) of a biotin-aneurin requirer ($y\ bi_1\ thi$). 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_1 (biotin-requiring, green) or of the $y\ bi_1$ (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

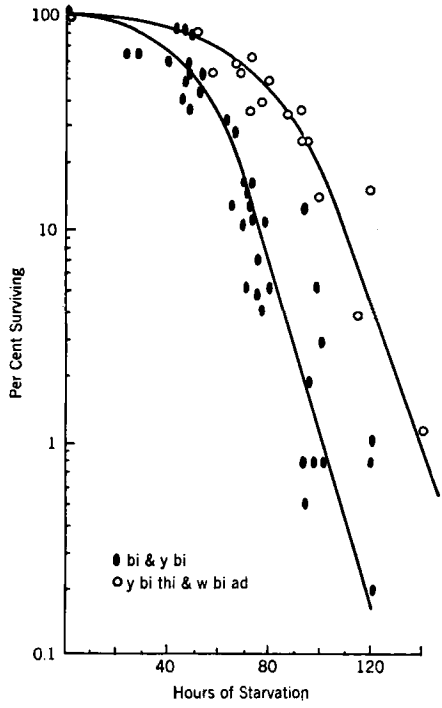


FIG. 8. Reconstruction experiments: under starvation the conidia of strains requiring biotin and aneurin or adenine (*y bi$_1$ thi* and *w ad$_1$ bi$_1$*) die off at a slower rate than conidia of strains requiring biotin only (*bi$_1$* and *y bi$_1$*).

the colonies developed thereafter was 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

TABLE 4

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. Series S-5: U.V., 25% survival.

Hours of starvation	*Conidia plated* *	*Colonies*	*Isolates*	*Auxotrophs among isolates*		*Auxotrophs per 10^6 spores plated*
	(no.)	(no.)	(no.)	(no.)	(%)	
X-rays, 50,000 r.						
0	63,000	335	230	4	1.7	92
46	45,000	184	115	2	1.7	71
74	45,000	129	88	3	3.4	97
96	630,000	165	119	18	15.6	39
111	1,010,000	130	93	31	33.3	42
117	560,000	44	40	12	30	24
123	1,120,000	42	38	11	28.9	11
147	2,240,000	57	54	22	40.8	10
U.V. (5% survival)						
0	23,100	1010	400	5	1.25	545
114	2,640,000	889	300	56	18.7	63
137	1,980,000	191	137	89	65	62
143	2,640,000	362	200	91	45.5	66
161	2,640,000	254	140	72	51	49
U.V. (25% survival)						
0	3,000	734	500	2	0.4	975
96	56,700	348	160	2	1.25	77
112	112,800	383	175	12	6.9	234
123	56,700	147	123	10	8.1	212
136	112,800	104	86	24	27.9	260
159	225,000	185	160	35	21.9	180

* 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 ("paraazo-

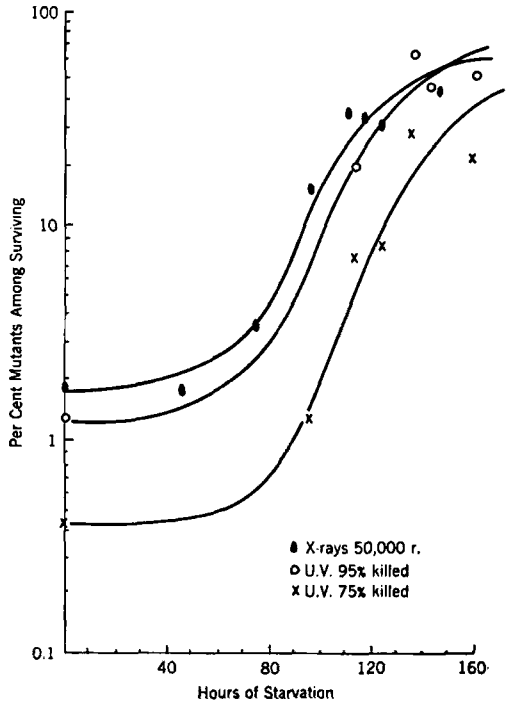


FIG. 9. Rise in the proportion of double auxotrophs (requiring biotin plus another growth factor) among survivors with increasing length of "starvation" of irradiated conidia.

trophic"). 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

Effect of Time of Starvation on the Proportions of the Different Types of Auxotrophs Recovered

Hours of Starvation *	*Isolates* (no.)	Total (no.)	Not fully tested (no.)	*Auxotrophs* †					
				Fully tested					
				Total	S.	N.	A.A.	N.A.	Vit.
0	5408	93	24	69 *(100)*	7 *(10)*	17 *(25)*	12 *(17)*	11 *(16)*	22 *(32)*
96–117	887	131	3	128 *(100)*	71 *(55)*	0 *(0)*	9 *(7)*	37 *(29)*	11 *(9)*
118–139	384	134	0	134 *(100)*	81 *(60)*	2 *(2)*	6 *(4)*	42 *(31)*	3 *(2)*
140–161	554	220	4	214 *(100)*	146 *(68)*	1 *(.5)*	13 *(6)*	55 *(25)*	1 *(.5)*

* 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 Houlahan, 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)

Type of auxotroph	*Total isolation*		*Starvation (96 hr.)*		*Total*	
Not fully tested	24		7		31	
Parathiotrophic	7		298		305	
Sulphite		5		284		289
Thiosulphate		2		14		16
Paraazotrophic	17		3		20	
Nitrite		13		0		13
Ammonium		4		3		7
Amino Acids	12		28		40	
Arginine		1		0		1
Arginine/ornithine		5		0		5
Arginine/ornithine/proline		1		14		15
Lysine		3		7		10
Proline		0		2		2
Ornithine		0		1		1
Methionine		1		0		1
Methionine/cystine		1		2		3
Phenylalanine		0		1		1
Isoleucine		0		1		1
Tryptophan		0		0		0
Nucleic Acid components	11		134		145	
Adenosine		10		134		144
Cytidine/Uridine		1		0		1
Vitamins	22		15		37	
Biotin *		4		0		4
P.A.B.A.		2		3		5
Pantothenate		1		0		1
Nicotinic/anthranilic acid		4		1		5
Nicotinic/tryptophan/anthranilic acid		3		1		4
Riboflavin		1		1		2
Pyridoxin		1		7		8
Choline		1		0		1
Aneurin *		3		2		5
Multiple		2		0		2
TOTALS	93		485		578	
FROM ISOLATES	5408		1825		7233	

* 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 7

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

Experiment	*Hours of starvation*	*Conidia plated*	*Colonies*	*Isolates*	*Auxotrophs* Total	*Auxotrophs* New types
S-6 (U.V., 12.5% survival)	124	450,000	133	112	12	1
	168	1,300,000	422	234	12	0
S-7 (U.V., 4% survival)	112	315,000	3,404	1,000	5	1
				1,346	29	2

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-

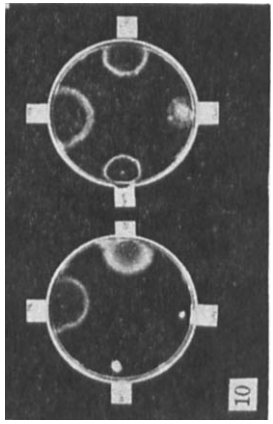


FIG. 10. Two mutants (TRYPT, requiring tryptophan; and GUAS, requiring guanosine), of types not obtained before the use of a selective technique, grow well on minimal medium plus tryptophan, adenine, and guanosine (*left*) but not on complete medium (*right*). Two other mutants (NIC, requiring nicotinic 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./1. $Na_2S_2O_3 \cdot 5H_2O$. 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 8

Elimination of Auxotrophs of an Unwanted Type by Supplementing the Starvation Medium with the Corresponding Growth Factor

Starvation medium	*Total number of isolates*	*Total nutritional mutants*	*Types of mutants*			
			Unable to utilize sulphate	Requiring amino acids	Requiring purines	Requiring vitamins
Minimal	400	35	24	1	8	2
Minimal supplemented with thiosulphate	400	17	1	3	8	5

Ultraviolet treatment (5% survival) followed by 139 hours of starvation on minimal medium or minimal medium + thiosulphate ($Na_2S_2O_3 \cdot 5H_2O$, 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$_1$, green conidia, biotin-requiring;
y bi$_1$, yellow conidia, biotin-requiring derived by crossing from the former;
w$_a$ *lys*, white conidia, lysine-requiring;
ad$_1$, 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

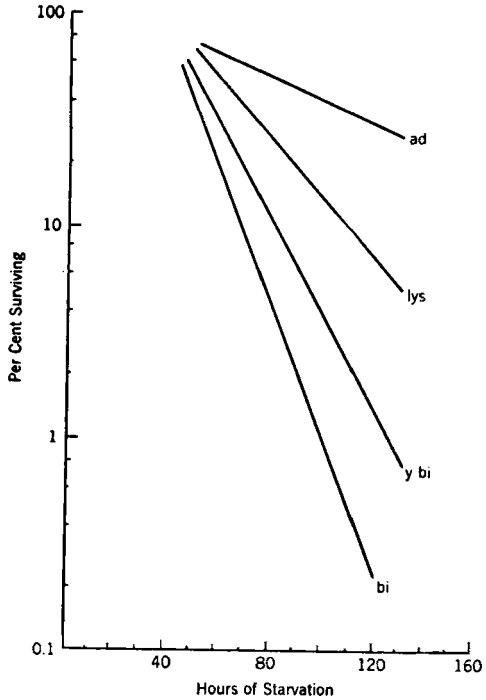


FIG. 11. Rate of dying off under starvation of conidia of strains with different single nutritional requirements: adenine (*ad*), lysine (*lys*), and biotin (*bi* and *y bi*).

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*$_1$ strain are still viable, compared with 12% of those of the *w*$_a$ *lys* strain, 1.2% of those of the *y bi*$_1$ strain, and 0.2% of those of the *bi*$_1$ strain.

The next step was that of determining the rate of dying off of a double auxotroph (*w ad*$_1$ *bi*$_1$: 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*$_1$ and *ad*$_1$ was added to the inoculum in minimal medium, a proportion of conidia of *y bi*$_1$ to the inoculum in minimal medium plus adenine, and a proportion of conidia of *ad*$_1$ to the inoculum in minimal medium plus biotin. The results are summarized graphically in Fig. 12.

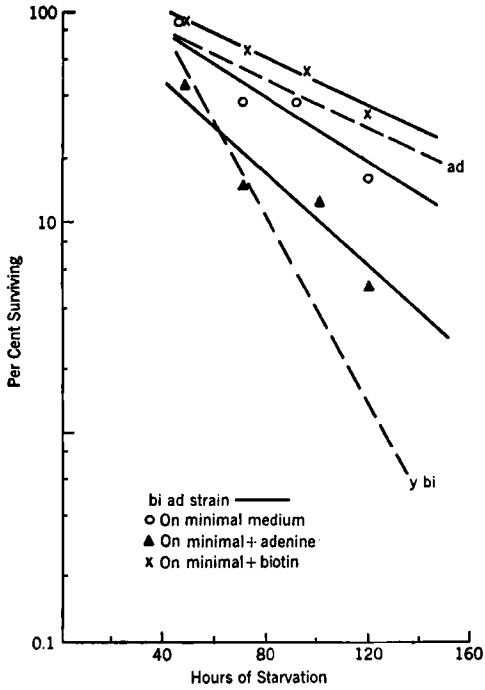


FIG. 12. Rate of dying off under starvation of a strain requiring biotin and adenine. x: starved of adenine alone; ▲: starved of biotin alone; o: starved of both. For comparison, broken lines show rates of dying off of two strains having either requirement only.

The rates of dying-off of *y 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 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°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

			*% Survival after 112 hours of starvation**		
Strain	*Origin*	*Requirements*	For biotin †	For the other growth factor ‡	For both §
S3–A2	Total isolation,	Biotin and SO_3^-	0.04	39.0	36.0
S5–F35	Starvation	Biotin and SO_3^-	0.2	51.0	44.0
bi, lys$_s$	Total isolation,	Biotin and lysine	0.6	2.8	1.2
S5–F2	Starvation	Biotin and lysine	1.7	0.9	1.5
bi, orn$_s$	Total isolation,	Biotin and arginine or ornithine	0.02	0.04	0.001
S4–C96	Starvation	Biotin and arginine or ornithine or proline	1.3	13.3	4.5
S2–D1	Starvation	Biotin and P.A.B.A.	0.02	0.08	0.003
S5–E1	Starvation	Biotin and pyridoxin	0.2	3.0	5.3

* 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 *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 *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_a 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 (A B; A C; B C) 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

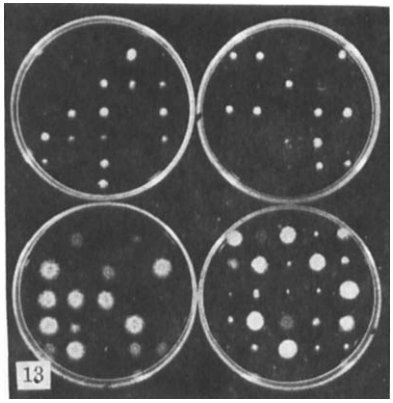


FIG. 13. Plate tests. Twenty-five segregants for P.A.B.A. and biotin requirements tested simultaneously on two plates of minimal medium, one supplemented with biotin (*left*) and one with P.A.B.A. (*right*). Growth on either, both, or neither classifies the segregants. *Above*, 25 strains photographed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

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.

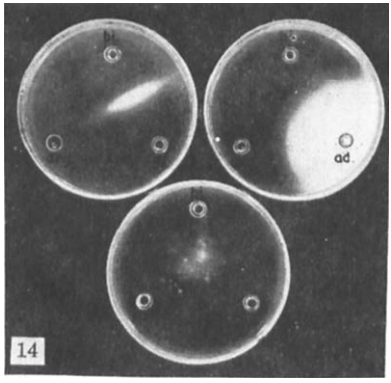


FIG. 14. Three kinds of auxanograms given by segregants of a cross in which three growth-factor requirements segregate: *ad*, adenine; *bi*, biotin; *arg*, arginine. *Top right*, single requirement. *Top left*, double requirement. *Bottom*, triple requirement.

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 Kölmark 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: $bi_1\ lys_2$ and $bi_1\ arg_1$. 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 3×10^7 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 in 1.4×10^8.

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*

Strain	*Number of conidia tested*		*Viability on complete medium*	*Biotinless back-mutants*
	Total	Per dish		
bi, lys$_2$	3.6×10^9	3×10^7	> 80%	25
bi, arg$_1$	2.1×10^9	3×10^7	About 100%	0

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

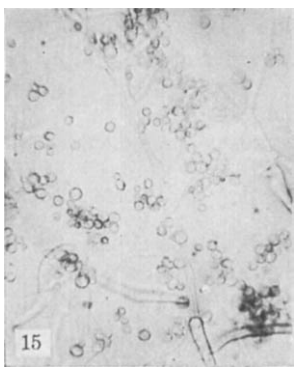


FIG. 15. Conidia of strain *bi, lys$_2$* (requiring biotin and lysine) on minimal medium plus biotin 100 hours after plating. Even near colonies of lysine-independent "back-mutants," the conidia do not germinate, though they may swell up. The hyphae are from one such back-mutant colony.

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. Kölmark 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).

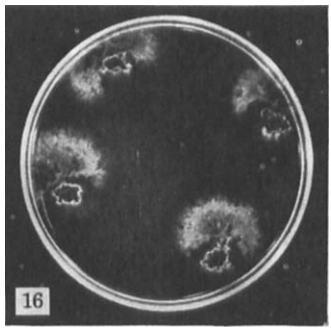


FIG. 16. Balanced heterokaryons arising from point inocula in minimal medium of two strains requiring adenine and aneurin, respectively. Note that the heterokaryon succeeds in "escaping" only at a few points.

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

TABLE 11

Distribution of Homo- and Heterokaryotic Heads in Balanced Heterokaryons

Heterokaryon	*Heads*			
	Yellow	White	Mixed *	Total
w_a Y AD, lys + *W y ad, LYS*	45	130	46	221
w_a Y BI lys orn, + *W y bi LYS ORN,*	81	179	25	285

* 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

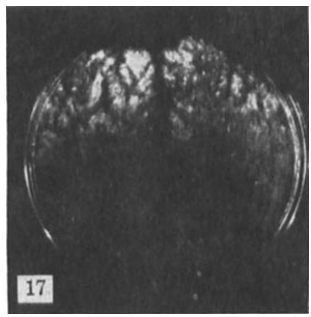


FIG. 17. Balanced heterokaryon between a yellow aneurin-requiring and a green adenine-requiring strain. After some growth, aneurin was added to the medium; the yellow component sectored out.

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

TABLE 12

Comparison between the Allele Ratios in the Conidia and Those in the Ascospores of Three Heterokaryons Balanced between ad_1 (Adenine Requirement) and *bi* (Biotin Requirement)

	Heterokaryon					Allele ratios
			w	*W*		
1.	*w* ad_1 *y BI*	Conidia	44	395	439	1:9
	+					
	W AD_1 *Y bi*	Ascospores	12	70	82	1:6
			56	465	520	
			Y	*y*		
2.	ad_1 *y BI*	Conidia	280	16	296	17:1
	+					
	AD_1 *Y bi*	Ascospores *	489	175	664	3:1
			769	191	960	
			Y	*y*		
3.	ad_1 *Y BI*	Conidia	286	1019	1305	1:3.5
	+					
	AD_1 *y bi*	Ascospores *	1191	2333	3524	1:2
			1477	3352	4829	

* 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.

clear that there is no necessary correspondence between the allele ratios in the conidia and those *in the ascospores.* 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—could 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*

A. 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 Coonradt, 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 Lindegren (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. AD_1/ad_1, AD_2/ad_2, 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 *lys Y BI//LYS y bi* will be represented by $\frac{lys}{LYS}\ \frac{Y\ BI}{y\ bi}$, the two loci *Y/y* and *BI/bi* 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 (*W y*) and one with white conidia (*w Y*), recombinant ascospores (*W Y*) 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 *LYS/lys* locus, which determines a requirement for lysine, and one at the *BI/bi* locus, which determines a requirement for biotin. From a cross between two strains, one lysine-requiring (*lys BI*) and the other biotin-requiring (*LYS bi*), recombinant ascospores (*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.

From a cross between two strains differing at all of the four loci just mentioned (e.g. *lys* w_a *Y BI//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 *W Y* recombinant colonies because of their green color; we isolate them and classify them (section IV-3) as to their requirements: *lys BI; LYS bi; LYS BI;* 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 *LYS BI*): w_a *Y* (white); *W y* (yellow); *W Y* (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 *lys* w_a *Y BI* type on lysine medium, and the *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.

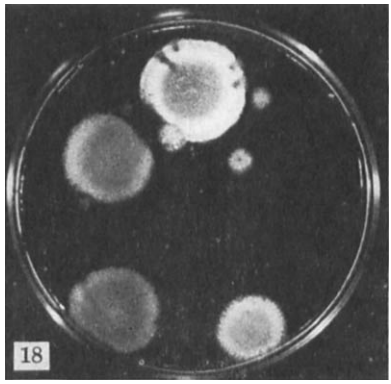


FIG. 18. "Breast feeding." When the prototrophs from a cross segregating for biotin requirement have formed large colonies, they release biotin in the medium in quantity sufficient for growth of biotin requirers (small colonies).

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:

Method of selection	*Selected recombinants*	*Classifiable combinations as to the other two loci*			
Visual	*W Y*	*lys BI*	*LYS bi*	*lys bi*	*LYS BI*
Nutritional, automatic	*LYS BI*	*W y*	w_a *Y*	w_a *y*	*W Y*
			indistinguishable		
Nutritional, requiring complete testing	*lys bi*	*W y*	w_a *Y*	w_a *y*	*W Y*
			indistinguishable		

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: *lys* w_a *Y BI//LYS W y bi*

Selected recombinants	*Segregations at other loci*			
	Parental		Recombinant	
	lys BI	*LYS bi*	*lys bi*	*LYS BI*
W Y	64	8	5	110
	W y	w_a *Y*	w_a *y*	*W Y*
LYS BI	2	39		40
lys bi	22	28		3

TABLE 13

Crosses involving the *Y/y* and *BI/bi* Loci in Coupling: *y bi/Y BI*

Cross	Selection	Segregations at other loci			
		Unusable classes	Cross-overs	Non-cross-overs	Recombination fraction
1. $\frac{lys\ \ w_a\ \ Y\ BI_1\ \ THI}{LYS\ \ W\ \ y\ bi_1\ \ thi}$	a. *LYS BI*$_1$ *THI*	$w_a y$ and $w_a Y$ 177	*W y* 14	*W Y* 194	$\frac{14}{208}$
	b. *LYS W y THI*	.....	*BI* 4	*bi* 37	$\frac{4}{41}$
	c. *LYS W Y THI*	.....	*bi* 0	*BI* 46	$\frac{0}{46}$
2. $\frac{lys\ \ w_a\ \ Y\ BI_1}{LYS\ \ W\ \ y\ bi_1}$	a. *LYS BI*$_1$	$w_a y$ and $w_a Y$ 39	*W y* 2	*W Y* 40	$\frac{2}{42}$
	b. *lys bi*$_1$	$w_a y$ and $w_a Y$ 28	*W y* 22	*W Y* 3	$\frac{3}{25}$
	c. *lys W y*	.....	*BI* 4	*bi* 46	$\frac{4}{50}$
	d. *W Y*	.....	*LYS bi*$_1$ *lys bi*$_1$ 8 5	*LYS BI*$_1$ *lys BI*$_1$ 110 64	$\frac{13}{187}$
3. $\frac{Y\ BI_1\ \ thi}{y\ \ bi_1\ \ THI}$	*BI*$_1$ *THI*	.....	*y* 3	*Y* 82	$\frac{3}{85}$
4. $\frac{ad_1\ \ Y\ BI_1}{AD_1\ \ y\ \ bi_1}$	a. *AD*$_1$ *BI*$_1$	.....	*y* 49	*Y* 974	$\frac{49}{1023}$
	b. *AD*$_1$ *Y*	.....	*bi*$_1$ 3	*BI*$_1$ 104	$\frac{3}{107}$
				Total	$\frac{95}{1814}$

Recombination fraction: 0.052 ± 0.005
Homogeneity (pooling 1b, 1c; 2a, 2b; and 2c, 3, 4b): $\chi^2_4 = 3.12$; P ≈ 0.65

TABLE 14

Crosses Involving the *Y/y* and *BI/bi* Loci in Repulsion: *Y bi/y BI*

Cross	Selection	Segregations at other loci			
		Unusable classes	Cross-overs	Non-crossovers	Recombination fraction
1. $\frac{AD_1}{ad_1}\ \frac{Y\ bi}{y\ BI}$	a. $AD_1\ BI$	.....	$\frac{Y}{8}$	$\frac{y}{153}$	$\frac{8}{161}$
	b. $AD_1\ y$	.....	$\frac{bi}{8}$	$\frac{BI}{142}$	$\frac{8}{150}$
2. $\frac{ad_1}{AD_1}\ \frac{W}{w}\ \frac{Y\ bi}{y\ BI}\ \frac{pyro}{PYRO}$	a. $AD_1\ BI\ PYRO$	$w\ y$ and $w\ Y$ 38	$W\ Y$ 1	$W\ y$ 35	$\frac{1}{36}$
	b. $AD_1\ BI$ *	$w\ y$ and $w\ Y$ 46	$W\ Y$ 3	$W\ y$ 46	$\frac{3}{49}$
3. $\frac{S_d}{s_d}\ \frac{AD_1}{ad_1}\ \frac{Y\ bi}{y\ BI}$	a. $S_d\ AD_1\ y$	.....	$\frac{bi}{3}$	$\frac{BI}{72}$	$\frac{3}{75}$
	b. $AD_1\ y$	.....	$\frac{bi}{s_d\ \ S_d}$ 4 2	$\frac{BI}{s_d\ \ S_d}$ 44 47	$\frac{6}{97}$
4. $\frac{AD_1}{ad_1}\ \frac{W}{w}\ \frac{y\ BI}{Y\ bi}$	a. $AD_1\ BI$	$w\ y$ and $w\ Y$ 59	$W\ Y$ 3	$W\ y$ 69	$\frac{3}{72}$
	b. $ad_1\ W$	.....	$Y\ BI$ 0 $\quad y\ bi$ 0	$Y\ bi$ 6 $\quad y\ BI$ 4	$\frac{0}{10}$
				Total	$\frac{32}{650}$

Recombination fraction: 0.049 ± 0.008

Homogeneity (pooling 2a, 2b, 3a, 4a, 4b): $\chi^2_3 = 0.68$; $P \approx 0.85$.

* 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 w_a. Linkage, however, must be excluded because the other two selections show no shortage of w_a 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 *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 $P \approx 0.65$, and the pooled recombination fraction is $95/1814 = 0.052 \pm 0.005$.

TABLE 15

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

	Cross	*Selection*	*Double crossovers*	*Single crossovers*	*Recombination fraction* *
1.	$\frac{w\ ad_1\ AD_3\ y}{W\ AD_1\ ad_3\ y}\ \frac{THI}{thi}$	$AD_1\ AD_3\ THI$	w 9	W 29	$\frac{9}{38}$
2.	$\frac{W\ AD_1\ ad_3\ y}{W\ ad_1\ AD_3\ Y}\ \frac{thi}{THI}$	$AD_1\ AD_3\ THI$	y 14	Y 35	$\frac{14}{49}$
3.	$\frac{w\ ad_1\ y\ BI}{W\ AD_1\ Y\ bi}$	$W\ ad_1$	Y and $Y\ bi$ 21	y and $y\ bi$ 28	$\frac{47}{107}$
		$w\ AD_1$	BI 26	bi 32	

* Double crossovers/doubles + singles.

ad_1 and ad_3 are known to be closely linked.

Crosses 2 and 3 suggest linkage in coupling between ad_3 and *y*, and ad_1 and *y*, respectively (results of cross 3 non-significant). Cross 1 suggests linkage in coupling between *w* and ad_1.

The next step is that of testing the *Y/y* and *BI/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 $\chi^2_8 = 4.5$ 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 *BI/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 *BI/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 case 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., A B 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%,

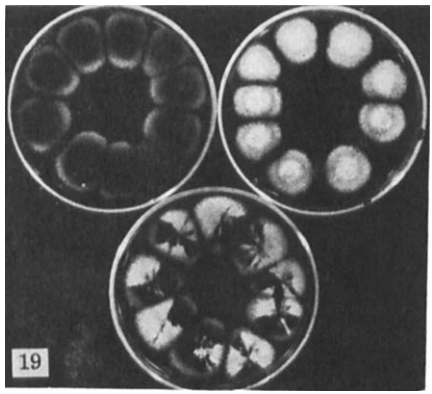


FIG. 19. Each colony originated from a whole ascus from a cross *y bi*, //+, the asci in each petri dish coming from one perithecium. *Top left*, all asci selfed green; *top right*, all asci selfed yellow; *below*, all asci of crossed origin.

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 asci 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 asci 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
52

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:

Plating of Random Sample of More Than 200 Ascospores from Each of 105 Perithecia. Cross: *Y//y*

Types of colonies produced by each perithecium

	Only yellow	Only green	Yellow and green	
			In ratio * of 1:1	In ratios * different from 1:1
No. of perithecia	43	30	18	14

* 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., *w Y//W y*), the streaks would be yellow, white, or tricolor (yellow, white, green) (Fig. 20). The proportion of bicolor (or tricolor) streaks indicates the highest

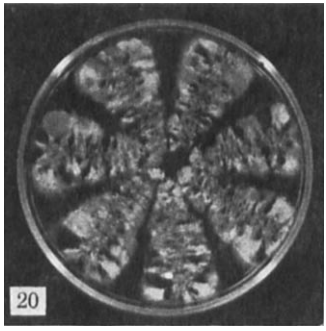


FIG. 20. Each streak from the ascospores of one perithecium from a cross segregating for *Wy* and *wY*; all perithecia contain exclusively, or mainly, crossed asci.

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$_1$ y BI$_1$//PABA$_1$ Y bi$_1$*

		paba$_1$ Bi$_1$	*PABA$_1$ bi$_1$*	*paba$_1$ bi$_1$*	*PABA$_1$ BI$_1$*	Totals
Perithecium I						
	y	144	2	11	39	196
	Y	0	156	42	10	208
	Totals	144	158	53	49	404
Perithecium II						
	y	117	3	5	24	149
	Y	1	125	20	22	168
	Totals	118	128	25	46	317

Allele ratios: *Y/y* 376/345 ($P = 0.25$); *PABA$_1$/paba$_1$* 381/340 ($P = 0.15$); *BI$_1$/bi$_1$* 357/364 ($P = 0.75$)

Crossover values: *paba$_1$ — y* 0.18 ± 0.02
y — bi 0.075 ± 0.009

Combinations:

Parental	*paba$_1$ y BI*	261	542
	PABA$_1$ Y bi	281	
Crossovers in I	*paba$_1$ Y bi*	62	125
	PABA$_1$ y BI	63	
Crossovers in II	*paba$_1$ y bi*	16	48
	PABA$_1$ Y BI	32	
Doubles	*paba$_1$ Y BI*	1	6
	PABA$_1$ y bi	5	
			721

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 that platings 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 BI_1/bi_1 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 pr_1 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

Alleles			*Strain and mode of origin*	*Linkage and allelism* •
Symbol	Designation	Phenotype		
	Visible			
w_a	White alba	Colorless conidia	+: spontaneous, 1936 †	All alleles of one another and of *W*, and epistatic to *Y/y*. "*bi* group" (?)
w	White		*y*: X-rays, 1946	
w_n	White new		*paba*$_1$ *bi*$_1$: spontaneous, 1951	
y	Yellow	Yellow conidia	+: X-rays, 1946	"*bi* group"
co	Compact	Compact colony	*paba*$_1$ *y*: unknown, 1951	Not in "*bi* group," probably linked with *pr*$_1$
pr$_1$	Poorly proteolytic-1	Reduced proteolytic activity	*bi*$_1$: U.V., 1951	See *co*
	Nutritional			
ad$_1$	Adenine-1	Adenine/hypoxanthine	*y*: X-rays, 1946	"*bi group*"; all not allelic
ad$_2$	Adenine-2		*y*: X-rays, 1946	
ad$_3$	Adenine-3		*y thi*: X-rays, 1948	
ad$_4$	Adenine-4		*bi*$_1$: X-rays, 1948	
bi$_1$	Biotin-1	Biotin/desthiobiotin	+: X-rays, 1947	"*bi* group"; *bi*$_1$, *bi*$_2$, and *bi*$_3$, pseudo-alleles; *bi*$_4$, incompletely tested
bi$_2$	Biotin-2		*y thi*: X-rays, 1948	
bi$_3$	Biotin-3		*y thi*: X-rays, 1948	
bi$_4$	Biotin-4		*y*: X-rays, 1948	

paba$_1$	P.A.B.A.-1	*p*-Aminobenzoic acid	*bi*$_1$: X-rays, starvation, 1951	"*bi* group," pseudo-alleles
paba$_6$	P.A.B.A.-6	*p*-Aminobenzoic acid	*bi*$_1$: U.V., starvation, 1951	
pyro$_4$	Pyridoxine-4	Pyridoxine	*bi*$_1$: U.V., starvation, 1951	Not in "*bi* group"
s$_d$	S-delta	Sulphite/thiosulphate	+: N-mustard, 1948 ‡	Not in "*bi* group"
thi	Thiazole	Aneurin/thiazole	*y*: X-rays, 1946	No linkage with *lys* or with "*bi* group"
nic$_2$	Nicotinic-2	Anthranilic/nicotinic acid	+: X-rays, 1947	Not allelic; no linkage with "*bi* group" or *thi*
nic$_3$	Nicotinic-3	Anthranilic/tryptophan/ nicotinic acid	*y thi*: X-rays, 1948	
orn$_1$	Ornithine-1	Arginine/ornithine	*y thi*: X-rays, 1949	At least two different loci; no linkage with "*bi* group"
orn$_2$	Ornithine-2	Arginine/ornithine	*bi*$_1$: X-rays, 1949	
orn$_3$	Ornithine-3	Arginine/ornithine	*y thi*: X-rays, 1948	
orn$_4$	Ornithine-4	Arginine/ornithine	*bi*$_1$: U.V. starvation, 1950	
lys	Lysine	Lysine	*w*$_a$: X-rays, 1947	No linkage with "*bi* group," *thi*, *orn*$_1$, or *panto*
panto	Pantothenate	Pantothenate	*y thi*: X-rays, 1948	No linkage with "*bi* group," *lys*, *thi*, or *orn*$_1$

* The linkage group from *w* to *ad*$_4$ 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

Region	*Cross* *	*Selection*	*Segregations*			*Tentative recombination fractions* †
y–bi_1	See Tables 13 and 14 pooled results of crosses in coupling and repulsion					$\frac{127}{2464} = 0.0515 \pm 0.0045$
			y	Y	Total	
y–ad_2	1. $\frac{ad_2\ y\ BI_1}{AD_2\ Y\ bi_1}$	$AD_2\ BI_1$	50	42	92	$\frac{50 \times 0.05}{42} = 0.06$
	2. $\frac{PABA_1\ ad_2\ y\ BI_1}{paba_1\ AD_2\ Y\ bi_1}$	$AD_2\ BI_1$	84	56	140	$\frac{84 \times 0.05}{56} = 0.075$
	3.‡ $\frac{ad_2\ Y}{AD_2\ y}\ \frac{THI}{thi}$	$AD_2\ THI$	130	11	141	$\frac{11}{141} = 0.078$
$paba_1$–y	4. $\frac{PABA_1\ y\ bi_1}{paba_1\ Y\ BI_1}$	$PABA_1\ BI_1$	67	198	265	$\frac{198 \times 0.05}{67} = 0.148$
	5. $\frac{W\ ad_1\ PABA_1\ y\ BI_1}{w_n\ AD_1\ paba_1\ Y\ bi_1}$	$W\ AD_1\ PABA_1$	82	20	102	$\frac{20}{102} = 0.196$
$paba_1$–bi_1	5. $\frac{W\ ad_1\ PABA_1\ y\ BI_1}{w_n\ AD_1\ paba_1\ Y\ bi_1}$	$w_n\ PABA_1$	BI_1 20	bi_1 3	23	$\frac{6}{38} = 0.158$
		$w_n\ BI_1$	$PABA_1$ 12	$paba_1$ 3	15	
y–ad_4	6. $\frac{ad_3\ y\ BI_1\ AD_4}{AD_3\ Y\ bi_1\ ad_4}\ \frac{thi}{THI}$	$AD_3\ AD_4\ THI$	y 21	Y 2	23	$\frac{5}{48} = 0.104$
		$AD_3\ AD_4$	22	3	25	

bi_1–ad_4				y		Y			
				BI_1	bi_1	BI_1	bi_1		
	7. $\frac{ad_2\ y\ BI_1\ AD_4}{AD_2\ Y\ bi_1\ ad_4}$		$AD_2\ AD_4$	11	1	15	2	29	$\frac{2 \times 0.05}{15} = 0.007$
w–ad_1				Wy		WY	wy and wY		
	5. $\frac{W\ ad_1\ PABA_1\ y\ BI_1}{w_n\ AD_1\ paba_1\ Y\ bi_1}$		$AD_1\ PABA_1$	82		20	139	241	$\frac{102}{241} = 0.42$
			$AD_1\ BI_1$	113		14	148	275	$\frac{127}{275} = 0.46$
			$AD_1\ PABA_1\ BI_1$	102		1	120	223	$\frac{103}{223} = 0.46$
				W			w		
	8. $\frac{w\ ad_1\ AD_3\ y}{W\ AD_1\ ad_3\ Y}\ \frac{THI}{thi}$		$AD_1\ AD_3\ THI$	41			14	55	$\frac{14}{55} = 0.26$
ad_3–y				y			Y		
	9. $\frac{ad_1\ AD_3\ Y}{AD_1\ ad_3\ y}\ \frac{THI}{thi}$		$AD_1\ AD_3\ THI$	14			35	49	$\left.\begin{matrix}\\ \\ \end{matrix}\right\}\ \frac{34}{124} = 0.28$
			$AD_1\ AD_3$	20			55	75	
ad_1–ad_3						ad_1 or ad_3			
	9. $\frac{ad_1\ AD_3\ Y}{AD_1\ ad_3\ y}\ \frac{THI}{thi}$		Perithecium analysis	$AD_1\ AD_3$		or $ad_1\ ad_3$			$2 \times \frac{1}{704} = 0.003$ §
				1		703		704	

* 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 $BI_2\ bi_1/bi_2\ BI_1$, which are irrelevant here.

§ The recombination fraction was also estimated to be about 0.003 from the ratio of $AD_1\ AD_2$ 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 ± 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 $ad_2\ y\ BI_1//AD_2\ Y\ bi_1$, 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).

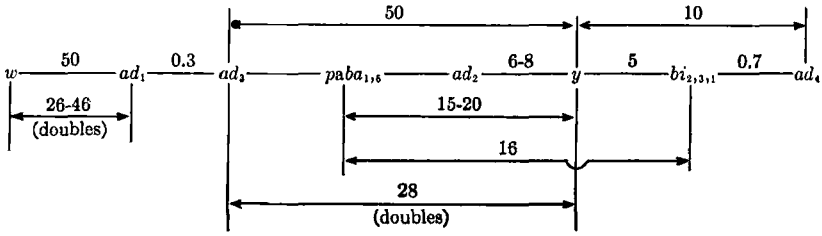
The fourth linkage detected was between ad_4 and bi_1. Crosses 6 and 7 in Table 18 indicate the kind of evidence. In cross 6, if ad_4 segregated independently, selection of $AD_3\ AD_4$ recombinants should yield a small excess of Y because of the loose linkage between ad_3 and y (see cross 9). Instead 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_3/ad_3—

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 perithecium 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:



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 $paba_1$–ad_2 when a strain ad_2 *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-asteriod," "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 $m_1\, m_2/+\ +$ 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^g/lz^s. 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

	bi_1	bi_2	bi_3
Pimelic acid	—	—	—
7,8-Diamino pelargonic acid *	+	+	+
Desthiobiotin	+	+	+
Biotin	+	+	+

* 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:

$$\frac{ad_1\ Y\ BI_2}{AD_1\ y\ bi_2} \quad \frac{THI}{thi}$$

Ascospores plated on	*Recombinants selected by medium*	*Colonies*	
		Yellow	Green
Minimal medium with biotin	*THI AD*$_1$	182 (77 tested: 74bi_2, 3BI_2)	192 (60 tested: 57BI_2, 3bi_2)
Minimal medium with aneurin	$BI_2\ AD_1$	10	227 (32 tested: 18*THI*, 14*thi*)

Morgan from the *y* locus. A similar cross was made to test the bi_3 locus. Again, it was shown that mutation in a single gene determines the biotin requirement and that the locus bi_3 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_1, bi_2, and bi_3 with Respect to One Another and the *y* Locus:

1. $\frac{y \quad BI_2\, bi_1}{Y \quad bi_2\, BI_1}$ 2. $\frac{y \quad bi_2\, BI_1}{Y \quad BI_2\, bi_1}$ 3. $\frac{y \quad bi_2\, BI_2}{Y \quad BI_2\, bi_2}$

Recombinants selected from platings on minimal medium	*Colonies*	
	Green	Yellow
Cross 1: $BI_2\, BI_1$	0	9
Cross 2: $BI_2\, BI_1$	27	0
Cross 3: $BI_2\, BI_2$	48	3

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_2 bi_3 bi_1.

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$_1$ y bi$_2$ BI$_1$ thi/w ad$_1$ Y BI$_2$ bi$_1$ THI

Ascospores plated on	*Total number of ascospores plated*	*Recombinants selected*	*Colonies*		
			White	Yellow	Green
Minimal medium with biotin	62.7×10^3	*AD$_1$ THI*	312 133 tested, all *bi*	318 140 tested, all *bi*	6 5 tested, all *bi*
Minimal medium with aneurin and adenine	3.48×10^6	*BI$_2$ BI$_1$*	39 21 *ad$_1$ THI* 16 *ad$_1$ thi* 2 *AD$_1$ thi*	4 1 *AD$_1$ thi* 3 *AD$_1$ THI*	33 18 *ad$_1$ THI* 15 *ad$_1$ thi*

The results show that strain *w ad$_1$ Y BI$_2$ bi$_1$ THI* has a chromosome rearrangement such that the locus *ad$_1$*, instead of segregating independently of the *y* locus, is linked about 2 c. Morgan from it.

necessarily recombinant for the freely recombining alleles *AD* and *THI*. On this medium, 62.7×10^3 ascospores gave 636 colonies. On minimal medium with aneurin and adenine the colonies were from ascospores recombinant for the alleles *BI$_2$* and *BI$_1$*. If the biotin alleles recombined freely, then 62.7×10^3 ascospores should have given 636 colonies, which was not the case. In fact, 3.48×10^6 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$_2$* and *bi$_1$* loci, the estimated recombination frequency was approximately 0.1%. A similar cross using the loci *bi$_3$* and *bi$_1$* gave a recombination frequency of 0.04%.

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 10^3 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_1. This cross (Table 24) gave no results in any way resembling recombination of the bi_1 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., $bi_2\ bi_1/BI_2\ BI_1$) 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_2 and bi_1 Loci Using Linked Markers on One Side or on Both Sides of These Loci

Ascospores plated on	Recombinants selected	Number of ascospores plated per dish	Total number of ascospores plated	Colonies	
				Yellow	*Green*
Cross: $\frac{AD_2\ y\ bi_2\ BI_1}{ad_2\ Y\ BI_2\ bi_1}\ \frac{thi}{THI}$					
Minimal medium with biotin	$AD_2\ THI$		18×10^3	130	11 (all *bi*)
Minimal medium with aneurin and adenine	$BI_2\ BI_1$		1.8×10^6	2 (both $AD^2\ THI$)	31 (17 $ad_2\ thi$, 13 $ad_2\ THI$, 1 $AD_2\ THI$)
Cross: $\frac{y\ bi_2\ BI_1\ AD_4}{Y\ BI_2\ bi_1\ ad_4}\ \frac{thi}{THI}$					
Minimal medium with biotin	$AD_4\ THI$	35	70	0	0
		350	700	0	0
		3.5×10^3	7×10^3	2	0
		4×10^4	16×10^4	41	6
		8×10^4	32×10^4	97	9
		Total	4.88×10^5	138	15 (all *bi*)
Minimal medium with aneurin and adenine	$BI_2\ BI_1$	3.5×10^3	7×10^3	0	0
		3.5×10^4	7×10^4	0	0
		5.7×10^5	17.1×10^5	0	0
		3.2×10^6	9.6×10^6	0	4
		6.4×10^6	19.2×10^6	0	11
		Total	3.06×10^7	0	15 (6 $AD_4\ thi$ 9 $AD_4\ THI$)

By the calculation used on p. 211 the recombination frequency between the bi_2 and bi_1 loci on the basis of the above data is 0.12% in the first cross and 0.08% in the second.

TABLE 24

Cross Involving Identical Biotin Alleles in the Two Strains:

$$\frac{AD_2\ \ y\ \ bi_1\ \ thi}{ad_2\ \ Y\ \ bi_2\ \ THI}$$

Ascospores plated on	*Number of ascospores plated*	*Recombinants selected*	*Colonies*	
			Yellow	Green
Minimal medium with biotin	9×10^8	*AD₂ THI*	175	14
Minimal medium with aneurin and adenine	54×10^8	*BI*	0	0

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 phenotoype 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

Growth Response of 24 Mutants Requiring Arginine or Related Substances

No.	*Arginine*	*Ornithine*	*Proline*	*Glutamic Acid*
1	+	−	−	−
5	+	+	−	−
14	+	+	+	−
1	+	+	+	+
2	−	−	+	−
1	−	+	−	−
Tot. 24				

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 guanidino 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

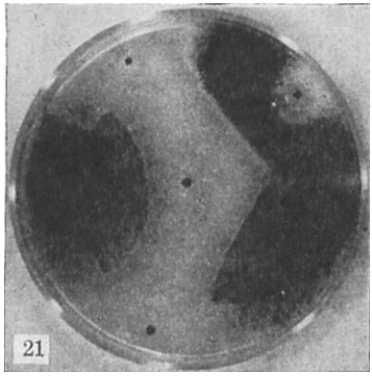


FIG. 21. Auxanography of an arginine/ornithine/proline-requiring strain. Spots clockwise from top: glutamic acid, arginine, ornithine, citrulline, proline; in the center: lysine. Note inhibition by lysine of growth on arginine or ornithine and sparing effect of lysine on proline.

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-aminoadipic 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

	Mutants	
	nic$_1$, *nic*$_3$, *nic*$_8$, *nic*$_9$	*nic*$_2$, *nic*$_4$, *nic*$_5$, *nic*$_6$, *nic*$_7$
Anthranilic acid	+	+
Indole	+	—
DL-Tryptophan	+	—
3-OH-Tryptophan	—	—
L-Kynurenine sulphate	+	—
3-OH-kynurenine	+	+
3-OH-anthranilic acid	+	+
Quinolinic acid	+	+
Nicotinic acid	+	+

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.

grow at an almost normal rate without the growth factor. This adaptation is not transmitted through the conidia.

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*

	How obtained	*How isolated*
A. nidulans		
$\frac{w\ ad_1\ Y\ BI_2\ bi_1\quad THI}{W\ AD_1\ y\ bi_2\ BI_1\quad thi}$	Camphor	Sector
$\frac{w_a Y\ BI_1\quad lys\quad orn_1}{W\ y\ bi_1\quad LYS\quad ORN_1}$	Camphor	Sector
$\frac{w_a\ AD_2\ Y\quad lys}{W\ ad_2\ y\quad LYS}$	Camphor	Plating
$\frac{w_a\ AD_2\ Y\ BI_1\quad lys}{W\ ad_2\ y\ bi_1\quad LYS}$	Spontaneous	Sector
$\frac{w_a\ Y\ BI_1\quad LYS\quad panto\quad orn_1}{W\ y\ bi_1\quad lys\quad PANTO\quad ORN_1}$	Camphor	Plating
$\frac{w_n\ paba_2\ AD_2\ Y\ bi_1}{W\ PABA_1\ ad_2\ y\ BI_1}$	Spontaneous	Sector
$\frac{y\ bi_2\ BI_1\ AD_4\quad thi}{Y\ BI_2\ bi_1\ ad_4\quad THI}$	Camphor	Plating
$\frac{w\ ad_1\ y\quad LYS}{w_a\ AD_1\ Y\quad lys}$	Spontaneous	Plating
A. niger *		
(A1) Fawn, aneurin requiring / (F92) Olive, histidine requiring	Spontaneous	Sector
(A33) Fawn, arginine requiring / (F104) Olive, casein digest requiring	Spontaneous	Sector
(A35) Fawn, guanosine requiring / (F92) Olive, histidine requiring	Spontaneous	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

		Conidia plated on selective medium (no.)	*Diploid colonies* (no.)	*Diploid colonies* (per 10^6 conidia)
A. nidulans				
y bi$_3$ BI$_1$ AD$_4$ thi + *Y BI$_3$ bi$_1$ ad$_4$ THI*	Camphor	4,200,000	1141	271
	Control	487,000,000	122	0.25
w$_6$ AD$_3$ Y lys + *W^r ad$_3$ y LYS*	Camphor	12,000,000	41	3.4
A. niger				
A35 + F92	Camphor	1,760,000	8	4.6
	Control	8,700,000	3	0.4

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 29

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

	Phenotypes			*Phenotypes*	
	Heterokaryon	Heterozygote	Heterokaryon		Heterozygote
$\frac{y}{Y}$	Yellow or green	Green	Pseudo-alleles		
$\frac{w}{W}$	White or colored	Colored	$\frac{bi_1\ BI_2}{BI_1\ BI_2}$	Non-requirer	Non-requirer
$\frac{w}{w_a}$	White	White			
$\frac{lys}{LYS}$	Non-requirer	Non-requirer	$\frac{BI_1\ bi_2}{BI_1\ BI_2}$	Non-requirer	
$\frac{orn_1}{ORN_1}$	Non-requirer	Non-requirer	$\frac{bi_1\ BI_2}{BI_1\ bi_2}$	Requirer	Requirer
$\frac{ad_1}{AD_1}$	Non-requirer	Non-requirer			
$\frac{ad_2}{AD_2}$	Non-requirer	Non-requirer			
$\frac{ad_4}{AD_4}$	Non-requirer	Non-requirer			
$\frac{panto}{PANTO}$	Non-requirer	Non-requirer			

is an exception which confirms the rule. In the case of the pseudo-alleles bi_1 and bi_2 (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*

A. nidulans	*Conidia measured* (no.)	*Mean diameter* (μ)
Diploids		
$\frac{w\ ad_1\ Y\ BI_2\ bi_1}{W\ AD_1\ y\ bi_2\ BI_1}\ \frac{THI}{thi}$	55	4.3
$\frac{w_a\ AD_2\ Y\ BI}{W\ ad_2\ y\ bi}\ \frac{lys}{LYS}$	82	3.8
$\frac{w_a\ AD_2\ y}{W\ ad_2\ y}\ \frac{lys}{LYS}$ (yellow conidia)*	79	3.9
Haploids		
Wild type	85	3.1
y (yellow conidia)	89	3.2
A. niger		
Diploids		
A1/F92 (black conidia)	53	5.4
A35/F92 (black conidia)	58	5.3
II/33d (olive conidia)†	62	5.5
Haploids		
Wild type (black conidia)	52	4.3
680F (olive conidia)	64	4.7

* Mitotic recombinant from $\frac{w_a\ AD_2\ Y}{W\ ad_2\ y}\ \frac{lys}{LYS}$

† 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;

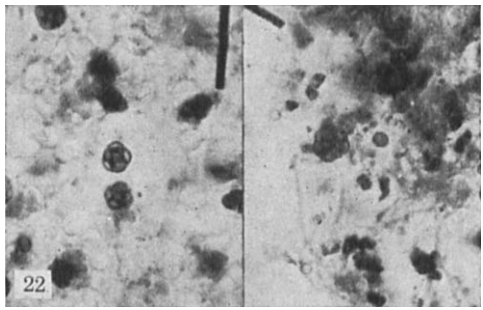


FIG. 22. *Left:* 8-spore asci of a haploid strain. *Right:* 16-spore ascus of a diploid strain, with debris of many others degenerating.

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 exceptions: 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 recombination (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 derived from the above.

Prompted by the classical work of Stern (1936) on somatic crossing-over in *Drosophila* we deliberately looked for the occurrence of mitotic segregation and recombination as soon as diploids were obtained: we did not expect however, to find this process occurring as regularly and frequently 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 micromanipulation) 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" segregants). 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 heterozygous. In some cases the diameter of the conidia of the segregants has been measured and found to fall within the diploid range (Table 30).

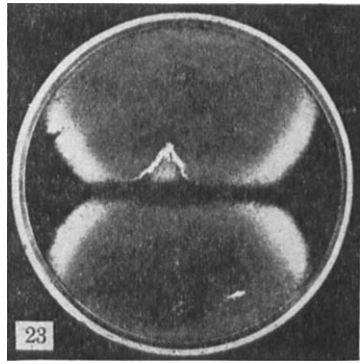


FIG. 23. Colonies of a diploid heterozygous $\frac{w\ Y}{W\ y}$ (green) showing yellow or white spots and one white sector, due to mitotic recombination.

Besides segregation, the process may involve also recombination. For instance, a diploid derived from haploids *W ad*$_2$ *y LYS* and w_a *AD*$_2$ *Y lys* yielded 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 the *w*/*W* locus, the following proportions of homozygotes for the recessive were obtained:

From diploid: $\frac{w_a\ AD_2\ Y\ BI}{W\ ad_2\ y\ bi}\ \frac{lys}{LYS}$: 3 out of 763

From diploid: $\frac{w_a\ AD_2\ y}{W\ ad_2\ y}\ \frac{lys}{LYS}$: 0 out of 70

From diploid: $\frac{w_a\ AD_2\ Y}{W\ ad_2\ y}\ \frac{lys}{LYS}$: 0 out of 236

From diploid: $\frac{w_n\ paba\ AD_2\ Y\ bi}{W\ PABA\ ad_2\ y\ BI}$: 3 out of 531

TABLE 31

An Example of Mitotic Recombination

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

$$\frac{w_s\ AD_8\ Y}{W\ ad_8\ y} \quad \frac{lys}{LYS}$$

→ 16-spore asci, segregating for yellow, white, and green and for the requirements.

↓

"First order" mitotic segregants and recombinants isolated by inspection

White, requiring:				Yellow, requiring:			
Adenine	Adenine and lysine	Neither	Lysine	Adenine	Adenine and lysine	Neither	Lysine
2	1	7	0	6	1	1	0

↓ Purified by single conidium micromanipulation

5 out of 7 give "second-order" white mitotic recombinants

↓ Purified by single conidium micromanipulation

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_2 BI_1 AD_4 *thi/Y* BI_2 bi_1 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 limita-

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/w_a and Y/y, two "nutritional" loci on the same chromosome (AD_2/ad_2 and BI_1/bi_1), and the LYS/lys locus, which segregates independently of all

TABLE 32

Mitotic Recombinants from Diploid Strains of *A. nidulans*

Diploids Green	*Recombinants* • White	Yellow	*Total*
1. † $\frac{w_a\ Y\ BI_1}{W\ y\ bi_1}$	bi_1 BI_1 : 5 6 → 11	bi_1 BI_1 : 20 0 → 20	31
2. $\frac{w_a\ AD_2\ Y}{W\ ad_2\ y}\ \frac{lys}{LYS}$	$\frac{ad_2}{lys}$ $\frac{}{LYS}$: 1 4 → 5; $\frac{AD_2}{lys}$ $\frac{}{LYS}$: 1 46 → 47; 5 47 → 52	$\frac{ad_2}{lys}$ $\frac{}{LYS}$: 2 13 → 15; $\frac{AD_2}{lys}$ $\frac{}{LYS}$: 0 1 → 1; 15 1 → 16	68
3. $\frac{w_a\ AD_2\ Y\ BI_1}{W\ ad_2\ y\ bi_1}\ \frac{lys}{LYS}$	$\frac{ad_2\ bi_1}{lys\ LYS}$: 3 7 → 10; $\frac{AD_2\ BI_1}{lys\ LYS}$: 11 10 → 21; 10 21 → 31	$\frac{ad_2\ bi_1}{lys\ LYS}$: 7 13 → 20; $\frac{AD_2\ BI_1}{lys\ LYS}$: 0 0 → 0; 20 0 → 20	51

* 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 nutritional 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 prototrophs pregerminated in minimal medium, where auxotrophs do not germinate.

With three strains the results of visual selection of mitotic segregants 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 proportions 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_2/ad_2, Y/y, and BI_1/bi_1 are closely linked and in this order, that the W/w_a locus is probably on the same chromosome, but more than 50 units beyond AD_2/ad_2 (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 bi_1; all but one of the yellow from diploid 2 are also homozygous for ad_2; and all the yellow from diploid 3 are also homozygous for ad_2 and bi_1. 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 excess of what would be expected from the frequency of segregation at each

TABLE 33 *

Mitotic Recombinants from Diploid:

$$\frac{w_a\ AD_s\ Y\ BI_1}{W\ \ ad_s\ y\ bi_1} \quad \frac{lys}{LYS}$$

	$AD_s\ BI_1$		$ad_s\ bi_1$		
1. Unselected **	*lys*	*LYS*	*lys*	*LYS*	*Total*
Green	0	240	0	0	240
White	0	0	0	0	0
Yellow	0	0	0	0	0
Total	0	240	0	0	240
2. Selected only for color †					
White	11	10	3	7	31
Yellow	0	0	7	13	20
Total	11	10	10	20	51
3. Selected only for requirements ‡					
Green	2	195	0	0	197
White	0	1	0	0	1
Yellow	0	0	1	1	2
Total	2	196	1	1	200
4. Selected for color and requirements ‖					
White	2	0	1	3	6
Yellow	0	0	0	15	15
Total	2	0	1	18	21

* 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 w_a 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 $\frac{Y\ BI_1}{y\ bi_1}$, and biotin and adenine-independent green from diploids of constitution $\frac{y\ bi_2\ BI_1\ AD_4}{Y\ BI_2\ bi_1\ ad_4}$.

* Unquestionable evidence of mitotic recombination between loci which are certainly linked has now been obtained. From a diploid with $paba_1$ and y in coupling, out of 51 yellow recombinants visually selected, 39 were $paba_1$ and 12 $PABA_1$. The two loci show 15-20% recombination at meiosis. These results suggest that the $PABA_1/paba_1$ 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_2–bi_1 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 *bi* linkage group between ad_1 and $paba_1$. 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 *bi* and the *paba* loci) show it. A third investigated region (ad_1 and ad_3) 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 (Pontecorvo, 1947, 1950, 1952b, 1952c).

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