ISOLATION OF NUTRITIONAL MUTANTS OF ASPERGILLUS NIDULANS.

being a thesis presented by

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General Introduction.

The demonstration by Garrod that a human abnormality, inherited as a simple Mendelian recessive, consisted of lack of a specific enzyme activity, probably represents one of the earliest integrations of genetics and biochemistry. (see Garrod 1923). It was shown that alcaptonurics, who are homozygous recessives, lack the enzyme activity responsible for the oxidation of homogentisic acid. This acid is secreted in the urine which blackens on exposure to air to give the visible expression of the disorder.

The study of the inheritance of biochemical properties in organisms came to be known as biochemical genetics and notable developments in the subject have included studies on the inheritance of flower colour pigments (Onslow 1925, see also Scott Moncrieff 1937 and Lawrence 1950) and similar work on the pigments of mammalian coat colour (Wright 1942) and of eye colour pigments in *Drosophila melanogaster* (see Ephrussi 1942). Work has continued in the field pioneered by Garrod, the investigation of biochemically anomalous conditions in man inherited in

Mendelian fashion (see Haldane 1942 and Penrose 1950). In recent years also, microorganisms have become objects of genetic study and in the development of the biochemical genetics of microorganisms, the work of Beadle and Tatum (1941) on the heterothallic ascomycete, <u>Neurospora crassa</u> has played a very important part. These workers evolved a technique for the production and isolation of nutritional mutants in <u>Neurospora</u> which has proved to be of great help to the microbial geneticist.

The minimum requirements for growth of the wild type strain of <u>Neurospora crassa</u> are met by a simple medium ("Minimal medium") consisting of the usual inorganic salts, a carbon source (e.g. glucose) and the vitamin, biotin. Nutritional mutant strains differ from the wild type in being unable to grow on minimal medium unless one or more growth factors are added to it. These growth factors are believed to be essential metabolites any one of which the wild type strain is able to synthesise but a nutritional mutant is not. Knight (1936) and Lwoff (1943) have discussed the evolutionary implications of differences in the growth factor requirements of various organisms and developed the hypothesis that a requirement for a substance as a growth facter often (but by no

means always) implies inability to synthesise it. The importance of Beadle and Tatum's work is that in providing mutants of a single organism, which have different growth factor requirements, the way has been opened for the genetic analysis of syntheses of essential metabolites and with the application of their method in other microorganisms a wider approach has been possible to the study of heredity and variation in these microorganisms.

Spontaneous mutations occur at a low rate in all organisms which have been investigated. This rate, however, can be greatly increased by means of various mutagenic agents. These include Xrays (Muller 1927), ultra violet light (Altenburg 1931; for microorganisms see Hollaender and Emmons 1941 and Kolmark and Westergaard 1949) and a wide range of chemicals (for reviews see Auerbach 1949). As far as is known, the action of any one mutagenic agent is non-specific as to the spectrum of genes affected; there is thus a random induction of mutations. (see Muller 1940, 1947 and Auerbach 1949).

In the production of nutritional mutants in microorganisms with a life cycle like that of *Aspergillus nidulans* (see Part I. Material and Methods),

wild type cells are treated with a mutagenic agent and subsequently single uninucleate haploid cells are plated on to a so called 'complete' medium containing in addition to the minimal requirements of the wild type strain, mixtures of amino acids, vitamins, purines and pyrimidines and other known and unknown substances. These nutritional mutant cells which can utilise the diffusible metabolites they require from the complete medium will grow up along with the unchanged wild type cells. Since there is no way of morphologically distinguishing wild type from nutritional mutant, a random sample of the clones developing from single cells has then to be isolated and tested for the ability of individual clones to grow on the minimal medium sufficient for growth of the wild type strain. Nutritional mutants will not grow on this medium and their specific additional growth requirements are later identified. The percentage of nutritional mutants found among isolates tested, varies with the dosage of mutagenic agent, etc., but under optimum conditions is usually between 1 and 5% when the technique of isolation which has just been described is used. This is an adaptation (Pontecorvo 1947), to organisms having uninucleate haploid cells, of the technique originally devised by

Beadle and Tatum (1945) for Neurospora and is known as the Total Isolation Method.

It can be seen that the main problem in the isolation of nutritional mutants is that they are rare; a relatively large number of colonies have to be tested for a throughput of a small percentage of nutritional mutants. Their isolation can be contrasted with the isolation of bacterial mutants resistant to bacteriophage or drugs (Luria and Delbruck 1943, Demerec 1945, and Lederberg 1950) and of back mutants among nutritional mutants of Neurospora, for example, where the synthesis of a specific metabolite again becomes possible and is no longer required in the medium (Giles and Lederberg 1948 and Kolmark and Westergaard 1949). Resistant mutants and nutritional back mutants occur infrequently but the methods of isolation ensure that in each case only the desired mutants will grow up, even among a large population, either by plating sensitive cells on to a medium containing the drug or phage in the former case or by plating cells requiring a specific metabolite on to a medium lacking this metabolite in the latter case. The isolation of these mutant types is therefore automatic as distinct from the isolation of nutritional mutants.

To lessen the labour involved it was natural that

attempts should be made to improve on Beadle and Tatum's method and various modifications which have been made in the original technique, for the isolation of nutritional mutants in Neurospora and other microorganisms are described in the introduction to Part II. This thesis deals with a selective method of isolation of nutritional mutants which has been developed using the homothallic ascomycete, Aspergillus nidulans, and which is much more efficient than the Total Isolation method.

Fries (1948a, 1948b) made the discovery that when the uninucleate haploid conidia of different strains of the heterothallic ascomycete, Ophiostoma multiannulatum are suspended in a liquid medium containing all the ingredients necessary for growth of the wild type strain, apart from aneurin and pyridoxin, then the rate at which conidia from any one strain die off in this starvation medium is apparently dependent on the number of growth factor requirements the strain has. The results suggested that the length of survival was greater, the greater the degree of heterotrophy, or in other words, conidia from a nutritional mutant with, for example, one growth factor requirement additional to those of the wild type, survived starvation in the liquid medium

longer than conidia from the wild type strain. The implication of Fries' work will be discussed in Part I of this thesis. In that part of the thesis it is shown that in Aspergillus nidulans the survival of the uninucleate conidia of certain nutritional mutants on minimal medium is related to their degree of heterotrophy in the same way as in Ophiostoma multiannulatum.

As a result of his work, Fries developed a technique for the isolation of nutritional mutants in Ophiostoma multiannulatum. Briefly, the method was to suspend conidia from, for example, the wild type strain, in the liquid starvation medium mentioned above, for a requisite period, and then plate the conidia on to a complete medium. Under suitably selected starvation periods the percentage of nutritional mutants among the colonies which developed from surviving conidia approached that obtained after various mutagenic treatments. Among the conidia of the mother strain suspended in liquid medium would be a small percentage of spontaneous mutants which had one additional growth factor requirement. In effect, under the conditions of starvation, conidia from spontaneous mutants were enriched because of their greater degree of survival.

In Part II of this thesis, the development of a selective technique for the isolation of nutritional mutants in <u>Aspergillus nidulans</u> based on the same principle of differential survival under starvation is described. Fries' methods have been modified substantially notably by subjecting the conidia of the mother strain to mutagenic treatment prior to starvation. In this way the percentage of nutritional mutants found after starvation can be increased to over 50% of the isolates made.

Certain quantitative and qualitative differences between the types of nutritional mutants found after starvation and by Total Isolation in <u>Aspergillus nidulans</u> suggested that the conidia of certain nutritional mutants survived starvation better than others. Fries' suggestion that the length of survival under starvation was an expression of the number of growth factor requirements a strain had, was apparently an oversimplification and in Part III where certain experiments are described which were carried out in an investigation of the mechanism of differential survival under starvation, it was shown that the type of growth factor requirements rather than the number of growth factor requirements determines the survival of conidia from different nutritional mutants of <u>Aspergillus nidulans</u>.

In Part IV a description is given of the adaptation of the starvation technique in the selective isolation of special types of nutritional mutants - some of which were never found by other isolation methods.

The conclusions drawn from the relevant experiments are discussed at the end of each of the four parts of the thesis and some major topics are further considered in the final discussion at the end of the thesis.

PART I. Preliminary Reconstruction Experiments.

1. Introduction.

In this part of the thesis the results of some experiments are described which were carried out using certain nutritional mutant strains of *Aspergillus nidulans* to ascertain whether the findings of Fries (1948a, 1948b) on starvation cultures in *Ophiostoma multiannulatum* were applicable in *Aspergillus nidulans*. His results are reviewed at some length in this introduction.

Nutritional mutants can arise spontaneously during the growth of a wild type microorganism and multiply side by side with wild type. If the microorganism is grown on a medium which only supports the growth of wild type cells (a minimal medium) then the nutritional mutants may be sustained syntrophically, but a medium which supplies the growth factors required by nutritional mutants will, of course, more readily support their multiplication. In *Ophiostoma multiannulatum* Fries (1948a) measured the proportion of nutritional mutant conidia among a wild type population grown on a complete medium. The method was to plate out random samples of conidia on to a complete medium and

characterise the growth factor requirements of the individual colonies developing from single conidia. He gave a figure of 0.03% nutritional mutants. It is clear therefore that in a wild type culture containing many million conidia, many hundreds of these will be nutritional mutants.

The minimal requirements for growth of the wild type strain of *Ophiostoma multiannulatum* are met by the usual inorganic salts, ammonium tartrate, a carbon source (e.g. glucose) and the vitamins B1 (aneurin) and B6 (pyridoxin). Fries (1948a, 1948b) discovered that if conidia of this wild type strain grown on a complete medium, were suspended in a liquid medium containing all the required growth substances apart from aneurin and pyridexin then, among the surviving conidia, the percentage of nutritional mutants increased with the length of the period in this starvation medium. In one experiment, for example, when the total living conidia had decreased to below 1% of the original, after 100 days at 25°C in the starvation medium, he found 0.8% nutritional mutants among survivors (Fries 1948b).

It was important to distinguish whether the increase in the percentage of mutants among survivors after starvation was due to a selective or a mutagenic

action of starvation or both. Two methods were employed by Fries (1948a, 1948b) for this purpose: fluctuation tests and reconstruction experiments.

1. Fluctuation Tests.

When a large population of cells from a strain of the bacterium Escherichia coli known to be sensitive to a specific bacteriophage are exposed to this phage then it is found that a very small number of the cells are in fact resistant and are able to grow in the presence of the phage. Luria and Delbruck (1943) described a method to distinguish between two possibilities: the induction of resistance in a small number of cells by the phage or the growth of resistant cells which were present in the population as the result of rare spontaneous mutation prior to the application of the phage.

If the phage induced resistance, presumably at random, then in samples from replicate cultures the number of resistant strains should tend to occur in the same proportions.

On the other hand, if there was a selection of pre-existing mutants then a feature would be the chance grouping of such mutants, each clone of resistant cells arising from one independent mutation. During growth the proportion of mutants should therefore increase. However this is an erratic feature

when a low mutation rate is being considered since a rate mutation, by chance, might occur early or late in the growth of a population.

If the growth rates of phage sensitive and phage resistant cells are assumed to be equal and if the inoculation of replicate cultures is made by a number of cells small enough to render unlikely the chance of resistant mutants being present initially, then, after growth, a wide fluctuation in the proportion of resistant mutants present in samples from replicate cultures distinguished the selection of rare spontaneous mutations from the induction of mutations where the same proportion of mutants would be expected in similar samples. Stemming from Luria and Delbruck's original work Newcombe (1949) has described a modified technique for distinguishing the spontaneous or inductive origin of mutants resistant to phage or drugs (see also Ryan 1952 and Lederberg and Lederberg 1952).

Fries (1948a, 1948b), applying analogous reasoning to that of Luria and Delbruck, considered that the large qualitative and the quantitative differences in the types of nutritional mutants found after starvation in replicate experiments, suggested that starvation was acting by favouring the survival of mutants of spontaneous origin rather than by increasing the mutation rate.

2. Reconstruction Experiments.

In a typical reconstruction experiment, conidia of the wild type strain of *Ophiostoma multiannulatum* and conidia from a nutritional mutant having a growth factor requirement for hypoxanthine were mix-inoculated in known ratios in liquid medium of the usual inorganic salts, ammonium tartrate and glucose. After varying periods in this liquid starvation medium the percentage survivors of each strain and thus the proportion of wild type to mutant type conidia, surviving, was estimated. Initially the ratio of wild type to mutant type was 974:26, but when the total surviving conidia after starvation had dropped to 2% of the original after 40 days at 25°C then this ratio was 8:992. From this and other reconstruction experiments which gave similar results, Fries (1948a, 1948b) concluded that starvation was a selective process, conidia from the less heterotrophic strains being less able to survive the particular starvation conditions which he employed. If starvation had exerted a mutagenic effect in the reconstruction experiment which has been described then the assumption was that the ratio of wild type to mutant type would remain the same during the period of starvation, since there would then be no selection favouring either, but a mutagenic action of starvation presumably affecting both in a similar manner.

Reconstruction experiments are in effect, very much simplified reconstructions of the conditions prevailing when conidia from a wild type strain are suspended in starvation medium. In the reconstruction experiments normally only one type of mutant is considered whereas in starvation cultures of wild type, for example, there are many different nutritional mutant types present as the result of spontaneous mutation. Also the ratio of mutant type to wild type is very much greater in reconstruction experiments. It has been emphasised that any conclusions drawn from such reconstruction experiments should be tentative (see Witkin 1947 and Lederberg 1948) since they are at the best only rough approximations of the conditions which actually prevail. However from the evidence available from fluctuation tests and reconstruction experiments it did appear to Fries (1948a, 1948b) that the starvation method be employed was a selective process and that under the starvation conditions, the viability of conidia was directly proportional to their degree of heterotrophy.

The wild type strain of <u>Aspergillus nidulans</u> is able to grow on a medium consisting of the usual inorganic salts, nitrogen in the form of nitrate, and

a carbon source such as glucose. Since it had already been shown by Pontecorvo (unpublished) that conidia of a biotin requiring mutant of this fungus, obtained after X ray irradiation, died off relatively quickly on the minimal medium supporting the growth of the wild type strain, it was decided to carry out preliminary experiments to make an accurate measurement of the dying off rate of the conidia of this mutant under starvation, i.e. on a medium containing all the ingredients necessary for growth of the wild type but lacking biotin, which is specifically required by this mutant. After this, reconstruction experiments were carried out using the biotin requiring strain and more heterotrophic strains derived from it, each of which, in addition to requiring biotin, needed one other growth factor.

The behaviour of these various strains under starvation conditions is described in this part of the thesis.

2. Material and Methods.

Material:

a) Aspergillus nidulans is a homothallic ascomycete, order Plectascales, family Aspergillaceae (see Thom and Raper 1945 for full mycological details).

The life cycle of this fungus from the growth of an asexual spore (a conidium) proceeds with the development of the branching hyphae consisting of multinucleate cells. During the growth of this coenocytic mycelium conidiophore heads are produced. Asexual conidia arise in chains from the sterigmata borne on these conidiophores. The details of the sexual process are unknown but the resulting perithecia (or fruiting bodies) contain a great number of spherically shaped asci within which are borne the ascospores. Eight of these spores, not arranged in a linear order, are found in each ascus (see Plate 1).

A technique for the genetical analysis of homothallic species (Pontecorvo 1949b) has made it possible to investigate the genetics of this fungus by a number of workers at the Department of Genetics in Glasgow (Pontecorvo 1952).

The wild type strain of Aspergillus nidulans has

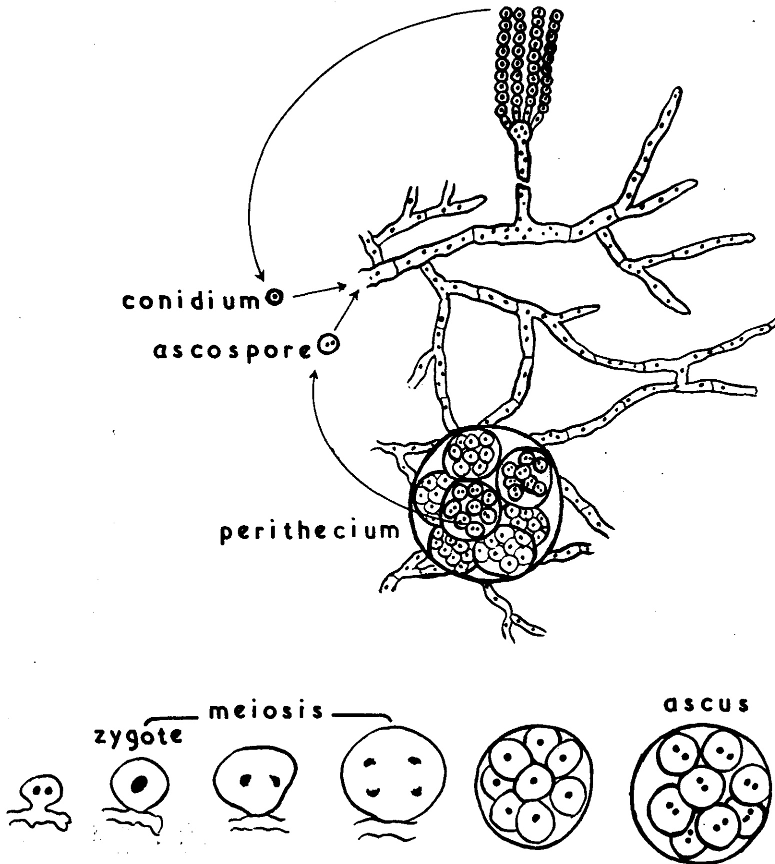


Plate 1. Diagrammatic representation of the life cycle of Aspergillus nidulans.

green conidia. Mutant strains can arise spontaneously, or by mutagenic treatment, which have either yellow or white conidia. The original wild type strain and a spontaneously occuring white 'alba' mutant were supplied by Mr. E. Yuill. These strains are referred to as + and Wa. Colour mutants (yellow and white) have been isolated in the laboratory by Dr. Pontecorvo and others from the wild type strain. Nutritional mutants have also been isolated from the wild type strain and colour mutants following treatment with X rays and ultra violet light. Other strains having combinations of mutant spore colour and nutritional deficiencies have been isolated by workers in the Department of Genetics as the result of recombination following crosses, using the routine technique for homothallic species mentioned above.

The strains used in the preliminary reconstruction experiments were:-

1. A green spored strain (symbol bi) requiring biotin for growth and obtained by X ray treatment of the wild type strain in 1948. This strain, incidentally, requires relatively enormous quantities of biotin for growth (see later).

2. A yellow spored strain (symbol y bi thi) requiring

biotin and the "thiazole" moiety of aneurin. This strain was obtained as a recombinant from a cross between a yellow spored biotin requiring mutant and a thiazole requiring mutant.

3. A white spored strain (symbol bi w ad) requiring biotin and adenine for growth and isolated as a recombinant from a cross involving a white spored adenine requiring strain and the biotin requiring strain.

The difference between biotin requirement in these three strains and the biotin independent wild type is determined by a difference in a single allele the same in all three mutants.

b) Media.

These are media which are used routinely in the Department of Genetics at Glasgow.

All the chemicals used in the preparation of media were of Analytical Reagent standard, unless otherwise stated.

1. Minimal Medium:

Ingredients:

15 gms. $NaNO_3$	1.3 gms. $MgSO_4 . 7H_2O$
1.3 gms KCl	3.8 gms KH_2PO_4
1 crystal $FeSO_4$	1 minute crystal $ZnSO_4$
25 gms Glucose	25 gms Powdered Agar (Davis' Brand)

Distilled water was added to the ingredients (withholding glucose and agar) to make the volume up to 2½ litres. This was boiled and the agar was added. The pH was then adjusted to 6.5 with NaOH or HCl and the medium steamed in a Koch apparatus for one to one and a half hours until all the agar had melted. The glucose was then added and the pH adjusted to 6.5 if necessary. The medium was then filtered through paper pulp in a Buchner funnel (the pulp and funnel having been previously thoroughly washed with boiling distilled water). After being made up into suitable aliquots in 100 ml and 250 ml conical flasks, the medium was autoclaved at 10 lbs pressure for 10 minutes.

2. Routine Complete Medium (1 litre quantity).

Ingredients:

2 gms Peptone (Oxoid Bacteriological)
1 gm Yeast Extract ('Yestrel')
6 gms $NaNO_3$
0.52 gms $MgSO_4$
0.52 gms KCl
1.52 gms KH_2PO_4
5 ml Casein Hydrolysate (see below)
3 ml Hydrolysed Yeast Nucleic acid (see below)
1 ml vitamin solution (see below)
10 gms Glucose
15 gms agar.

Made up to 1 litre with tap water.

The procedure followed in making up the medium was that for mimimal medium apart from any specially clean precautions taken. The casein hydrolysate and

hydrolysed yeast nucleic acid were added at the beginning with the other ingredients but the vitamin solution was not added until after filtration. The pH of the medium was 6.0 ± 0.2. The medium was autoclaved in suitable aliquots for 10 minutes at 10 lbs pressure.

Preparation of Vitamin Solution:

5 mg Aneurin HCl.	5 mg Calcium Pantothenate.
5 mg para aminobenzoic acid.	15 mg Nicotinamide.
50 microgms Biotin.	

The constituents were dissolved in 50 ml of double distilled water in an acid cleaned conical flask which was then plugged with cotton wool and autoclaved for 10 minutes at 10 lbs pressure.

Preparation of Casein Hydrolysate:

200 gms of B.D.H. light white soluble casein was mixed rapidly in a 2 litre conical flask with a mixture of 170 ml concentrated HCl and 80 ml of 15% $TiCl_3$ and then heated on a water bath till the mixture was uniform, autoclaved for $\frac{3}{4}$ hour at 120°C, cooled and brought to pH 6.2 with 40% NaOH (circa 230 ml). A sample in a test tube was boiled, filtered and tested by addition of NaOH and HCl for further precipitate. The bulk, after further retests is adjusted to condition for optimum precipitation, and brought carefully to the boil, boiled for ten minutes, stood for two hours and

filtered through paper pulp on a Buchner funnel.

The filtrate is adjusted to pH 6 - 6.2 if necessary, diluted to one litre and stored over chloroform in a refrigerator at 4°C. The storage time should not exceed three months. The final solution is 20% with respect to casein.

Preparation of Hydrolysed Yeast Nucleic Acid:

2 gms of Yeast Nucleic acid + 15 ml of Normal HCl
2 gms of Yeast Nucleic acid + 15 ml of Normal NaOH

Each mixture is raised slowly to the boil and boiled gently for ten minutes. Both give clear pale brown solutions. The two hyrdolysates are then mixed and brought to pH6, filtered hot and the volume made up to 40 ml. The solution is stored over chloroform at 4°C. A precipitate forms on cooling the solution; this should be taken along with the clear solution when used in making medium. The solution is equivalent to 100 mg of yeast nucleic acid per ml. Storage time should not be longer than six months.

Normal Saline:

0.87% NaCl in distilled water was made up into suitable aliquots and sterilised for 10 minutes at 10 lbs pressure.

Calzolene Solution:

1 part by volume calzolene oil to 1000 parts

distilled water, aliquoted and sterilised for 10 minutes at 10 lbs pressure.

c) <u>Fine Chemical Solutions</u>:

These were made up in double distilled water. They were sterilised by autoclaving for 10 minutes at 10 lbs pressure.

Biotin Solution:

A stock solution containing 25 microgms/ml was held. 5 microgms of biotin per litre of minimal medium assures good growth of the biotinless strain. This is enormously more than is usually needed by organisms unable to synthesise biotin.

Aneurin Solution:

A stock solution containing 20 microgms of aneurin hydrochloride per ml was made up and autoclaved when it splits into the "thiazole" and pyrimidine constituants of aneurin. 5 ml of this solution per litre of minimal medium is sufficient for good growth of the thiazoleless strain.

Adenine Solution.

A stock solution was used containing 1.25 mg of Adenine Hydrochloride per ml. 45 ml of this solution were needed per litre of minimal medium to give good growth of the adenineless strain.

d) Glass Ware

The Petri dishes used in these experiments were washed dishes further cleaned by filling with distilled water, autoclaving and then rinsing several times in distilled water.

Methods:

a) Temperature. The incubation temperature was 37°C.

b) Cultures. Master cultures were established for each strain of *Aspergillus nidulans* which was used by making single ascospore isolations from stock culture. The isolations were made through the courtesy of Miss L.H. Hemmons, who used a de Fonbrune Micromanipulator in doing them. New master cultures were established for each strain every four months from single ascospore isolations of the preceding masters. In every new establishment the culture was checked as to its nutritional requirements. The master cultures were incubated for two or three weeks (to allow of ascospore formation) and then stored at room temperature. In all the experiments conducted, six day old serial sub cultures from mass transfer of conidia were used; these cultures were grown at 37°C.

The master cultures were grown on slopes of a

modified minimal medium, favouring ascospore formation, supplemented with the required growth factor or growth factors, the sub cultures on slopes of minimal medium, supplemented with the required growth factor or growth factors.

c) Conidial Suspensions.

Conidia were removed from slopes by touching the surface with a wetted platinum loop and then immersing it in a sterile solution containing 4 parts of normal saline to 1 part of calzolene solution. (Normally about 3 ml of this made up solution was used in a boiling tube). The conidial chains were then thoroughly broken up by pipetting up and down with a Pasteur pipette until the great majority of conidia had separated out singly.

d) Spore Count and Dilution Methods.

The spore density of a suspension similar to that mentioned above was measured using a haemocytometer.

Normal saline was made up in 20 ml Universal Containers in 9 ml quantities so that suitable serial dilutions could be made for platings after the density had been estimated. Normally not less than 0.05 ml and not more than 0.2 ml of final dilution was plated out per petri dish. This amount was spread evenly over the medium surface with a glass spreader.

e) Plating Technique

A thin layer of minimal medium was poured into each Petri dish, sterilised with filter paper under the top to prevent excessive condensation. After the agar had set, suitable numbers of conidia were spread on top and allowed $\frac{1}{2}$ hour before another thin layer of minimal medium cooled at 50°C was poured on top to submerge the spores in each dish. This second layer of minimal medium prevented the displacement of conidia during subsequent manipulations. The plates were then incubated for the required starvation period after which they were removed from the incubator, enriched with a third layer of complete medium, or supplemented minimal medium (cooled at 50°C before addition) and then returned to incubate (see Fig.1.)

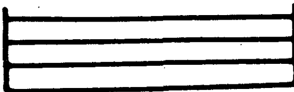
After a further 36 hours, the surviving conidia had given origin to visible colonies and after 48 hours, sporulation had begun.

f) Special Methods.

In the group of preliminary reconstruction experiments the strain with a single requirement, biotin (symbol bi) had green conidia, the strains with double requirements had in the one case yellow conidia - the yellow spored biotinless, thiazoleless strain -(y bi thi), and in the other, white conidia - the white spored

1. Spores spread on first layer of Minimal Medium (Starvation Medium)

2. Spores submerged by adding a second layer of Minimal Medium and then incubated at 37°C



3. Complete Medium added as a third layer after starvation period. Colonies develop on subsequent incubation at 37°C

FIG. 1. Plating method in the Starvation Technique.

biotinless, adenineless strain - (bi w ad). The advantage of using such different colour mutants are obvious. The viability of the conidia of each strain, during a starvation experiment was followed merely by counting the colonies of the respective colour coming up after delayed enrichment. There was no need to test the growth factor requirements of the seperate colonies, which, of course, saved a considerable amount of time.

3. Dying off rates of conidia of strains with different nutritional requirements, under starvation conditions.

1. The dying off rate of conidia of the biotinless strain (bi).

A pilot experiment was carried out to establish the dying off rate of conidia of the biotinless strain on minimal medium.

A thin layer of agar minimal medium was poured into each of 27 Petri Dishes. A conidial suspension from a 6 day old sub culture, was made up. A Haemocytometer count was done and by arranging suitable dilutions, a spore suspension containing 800 spores per ml was prepared; 0.1 ml of this suspension was spread on to each of the plates giving an estimated count of 80 conidia per dish. Half an hour was allowed for the spores to settle into the medium and then another thin layer of agar minimal medium cooled at 50°C was poured on to each plate. The plates were divided into nine series (A to I), three plates in each series. On solidification of the second layer of medium, the A series had a third layer of minimal medium, supplemented with biotin, added immediately. This was the control series, i.e. with no starvation. All the plates were then incubated at 37°C. Colonies were visible after 36

hours on the control plates; these were marked, colony counts were made after 48 hours incubation when sporulation had begun. The remaining series (B to I) had minimal supplemented with biotin added after periods of incubation ranging from 42 hours to 123 hours and colony counts were made as in the control series.

The experimental results (Table 1) showed that after 96 hours starvation, the percentage of survivors was about 1% of the control.

The next experiment which was conducted in a similar fashion, confirmed the dying off rate of the conidia from the biotin requiring mutant (see Table 2). In this experiment and in all subsequent experiments, complete medium was added as a 3rd layer instead of supplemented minimal medium.

One further experiment was carried out under acid clean conditions. The minimal medium was prepared as has been described except that shredded agar, washed for several hours in tap water and then thoroughly rinsed in distilled water, was used and the glucose was recrystallised before use (see Materials Part III). The results of the experiment are shown in Table 3.

It appears from the results of these three

TABLE 1. Dying off rate of the conidia of the biotinless strain kept for periods of θ to 123 hours on minimal medium (starved of biotin).

Plate Series.	Plate Nos.	Period of Starvn. in Hours.	Haem. Estim. per Plate.	Colony Count per Plate.	Total Colony Count.	%age Viability taking control as 100%.
A (control)	1 2 3	0	80	41 45 46	132	100%
B	1 2 3	42	80	37 38 42	117	88.6%
C	1 2 3	47	80	10 17 24	51	35.9%
D	1 2 3	66	80	15 13 11	39	29.5%
E	1 2 3	71	80	3 4 8	15	11.4%
F	1 2 3	77	80	8 3 2	13	10.6%
G	1 2 3	90	80	2 3 1	6	4.5%
H	1 2 3	96	80	0 1 0	1	0.8%
I	1 2 3	123	80	0 0 0	0	0%

TABLE 2. Dying off rate of conidia of the biotinless strain kept for periods of from 0 to 92 hours on minimal medium.

Plate Series	Plate Nos.	Period of Starvn. in Hrs.	Haem. Estim. per Plate.	Colony Count per Plate.	Total Colony Count.	‡ Correction Factor.	Comparative Colony Counts.	%age Viability taking control as 100%.
A (control)	1	0	97	56	192	1	192	100%
	2			47				
	3			42				
	4			47				
B	1	23	97	28	126	1	126	65.6%
	2			35				
	3			31				
	4			32				
C	1	28	97	31	128	1	128	66.7%
	2			35				
	3			27				
	4			35				
D	1	40	97	24	118	1	118	61.5%
	2			32				
	3			26				
	4			36				
E	1	52	97	19	84	1	84	43.8%
	2			25				
	3			28				
	4			12				
F	1	64	194	16	49	2	24.5	12.5%
	2			9				
	3			10				
	4			14				
G	1	76	970	25	86	10	8.6	4.5%
	2			18				
	3			23				
	4			20				
H	1	92	970	0	0	10	0	0%
	2			0				
	3			0				
	4			0				

‡ The correction factor is found by dividing the haemocytometer estimate by the control haemocytometer estimate. Comparative colony counts are then obtained by dividing the total colony count by the correction factor.

TABLE 3. Dying off rate of conidia of the biotinless strain kept for periods of from 0 to 100 hours on minimal medium. (Experiments conducted under acid clean conditions).

Plate Series	Plate Nos.	Period of Starv^{n}. in Hrs.	Haem. Estim. per Plate.	Colony Count per Plate.	Total Colony Count.	Correction Factor.	Comparative Colony Counts.	%age Viability taking control as 100%.
A (control)	1 2 3 4	0	105	59 62 58 67	246	1	246	100%
B	1 2 3 4	40	105	61 53 66 58	238	1	238	96.8%
C	1 2 3 4	46	105	50 43 54 65	212	1	212	86.2%
D	1 2 3 4	53	105	45 41 29 51	166	1	166	67.5%
E	1 2 3 4	64	210	46 31 29 50	156	2	78	31.7%
F	1 2 3 4	70	1050	92 86 97 105	380	10	38	15.5%
G	1 2 3 4	76	1050	42 37 28 14	121	10	12.1	4.9%
H	1 2 3 4	87	1050	23 27 19 29	98	10	9.8	4.0%
I	1 2 3 4	94	1050	1 7 3 8	19	10	1.9	0.8%
J	1 2 3 4	100	1050	5 2 10 4	21	10	2.1	0.8%

experiments that the proportion of conidia of the biotin requiring strain which survive starvation is the same whether biotin or complete medium is added at the end of the starvation period and further that when starvation is conducted under acid clean conditions, the dying off rate of the conidia is not affected.

It is to be expected that trace amounts of biotin would be present in minimal medium unless conditions of strict acid cleanliness are observed and since biotin is normally required in relatively minute quantities by organisms which do not synthesise it, it would of course be important to observe such acid clean conditions if biotin was to be excluded from minimal medium. The implications here are that the amount of biotin required by the biotin requiring mutant is relatively so enormous that the metabolic processes which lead to the dying off of the conidia under starvation are unaffected whether starvation is conducted under normally clean or acid clean conditions.

The data obtained in the three experiments are plotted on a graph (see Fig.2), where, plotting the logarithm of percentage survivors against the period of starvation, there is apparently an initial period of up to 40 hours during which no appreciable death of conidia occurs; the conidia then begin to die off rapidly in a logarithmic manner.

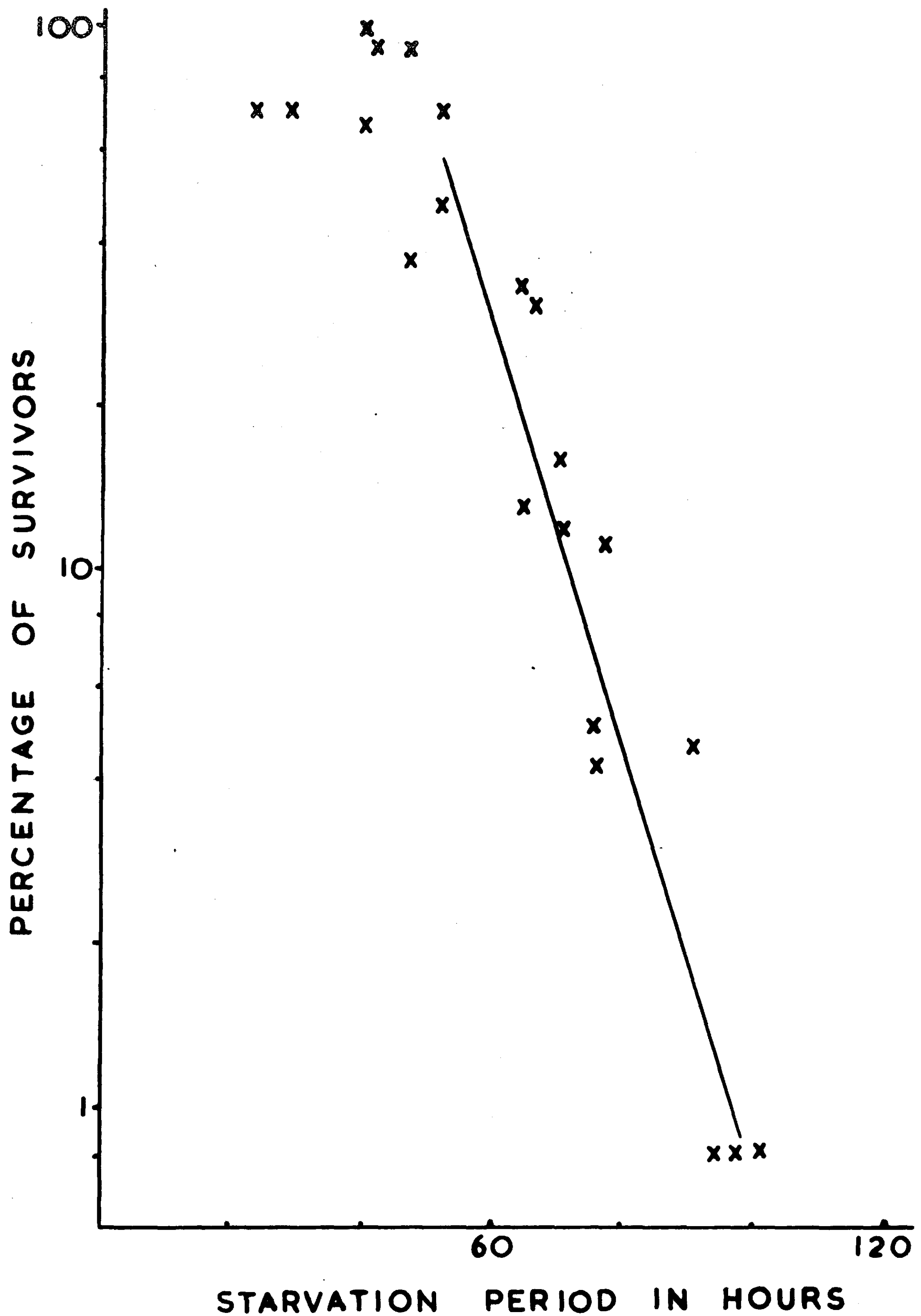


FIG. 2. The dying off rate of conidia from the biotin requiring strain is shown with progressive starvation on minimal medium. The results are from the three experiments described in the text.

2. <u>The dying off rate of conidia of the biotinless thiazoleless strain (y bi thi).</u>

The results of a similar starvation experiment to that described, and involving the yellow spored biotinless, thiazoleless strain (y bi thi) are shown in Table 4. Fig.3 illustrates that again there appears to be a period before the conidia begin to die off logarithmically. The logarithmic slope is less steep than that in Fig. 2 so that, on minimal medium, the conidia of this strain survive longer than those of the biotinless strain.

3. <u>Reconstruction experiment involving the biotinless and the biotinless, thiazoleless strains.</u>

After two separately broken up suspensions of bi and y bi thi strains had been prepared and haemocytometer counts done, the suspensions were diluted and mixed so that the final suspension contained an estimated 1400 bi conidia and 400 y bi thi conidia. 24 plates of minimal medium were arranged in 6 series of 4 plates each. 0.1 ml of the thoroughly mixed suspension was spread on the control series and 0.2 ml on the remaining plate series. After a 2nd layer of minimal medium had been added to all the plates and a 3rd layer of complete medium to the control plates,

TABLE 4. Dying off rate of conidia of the biotinless, thiazoless strain kept for periods from 0 to 139 hours on minimal medium.

Plate Series.	Plate Nos.	Period of Starv^{n}. in Hours.	Haem. Estim. per Plate.	Colony Count per Plate.	Total Colony Count.	%age Viability taking control as 100%.
A (control.)	1	0	86	42	128	100%
	2			45		
	3			41		
B	1	54	86	23	74	57.8%
	2			28		
	3			23		
C	1	66	86	27	77	59.9%
	2			28		
	3			22		
D	1	71	86	25	71	55.2%
	2			19		
	3			27		
E	1	77	86	17	51	39.8%
	2			14		
	3			20		
F	1	87	86	14	43	33.6%
	2			11		
	3			18		
G	1	93	86	9	31	24.2%
	2			12		
	3			10		
H	1	115	86	1	5	3.9%
	2			1		
	3			3		
I	1	139	86	2	2	1.1%
	2			0		
	3			0		

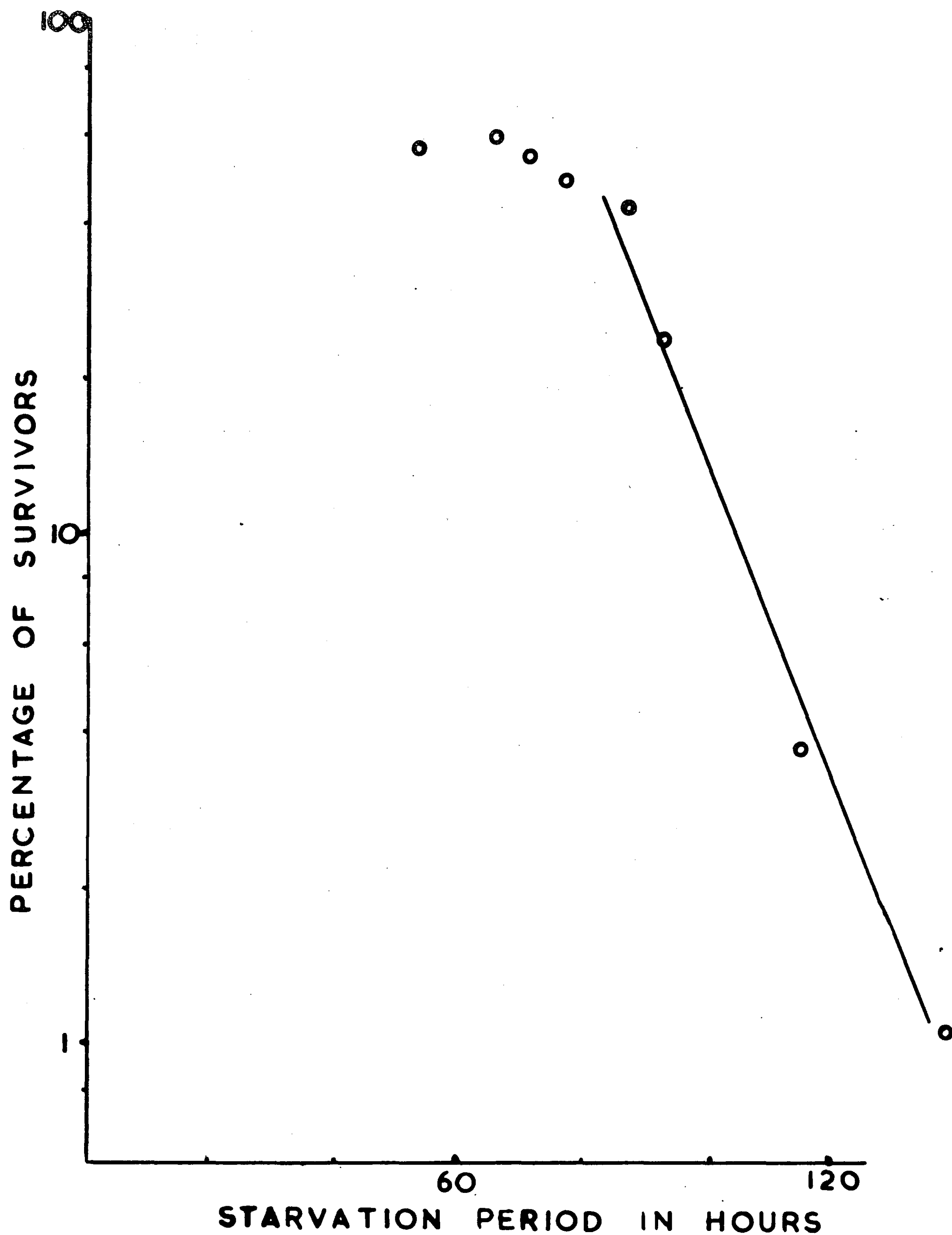


FIG. 3. The dying off rate of conidia from the yellow spored, biotinless, thiazoleless strain when starved on minimal medium.

all the plates were then incubated at 37°C. After 36 hours the non-sporulating colonies which grew up on the control plates were marked. These plates were removed from the incubater after 48 hours, when sporulation had begun and the number of colonies growing on each plate was rechecked.

The colour of the conidia was not distinguishable on some of the smaller colonies but it was found that if the plates were left at room temperature for a further 48 hours, conidial colour developed on all colonies, although the colonies themselves remained compact. Further incubation at 37°C would of course have also developed the conidial colour, but the colonies would also have merged, making it difficult to distinguish them separately. The green and yellow colonies were then counted on each control plate and checked with the total colony counts made after 48 hours incubation. A similar procedure was followed in the other plate series which were starved for different lengths of time. The experimental results (see Table 5) were plotted, as before, in Fig. 4, which illustrates the greater viability of the yellow biotinless, thiazoleless conidia under starvation conditions.

4. <u>Reconstruction experiment involving the biotinless and the white spored biotinless, adenineless strains.</u>

TABLE 5. Reconstruction Experiment.
Dying off rates of conidia of the biotinless (bi) and conidia of the biotinless, thiazoleless (y bithi) strains on minimal medium.

Plate Series	Plate Nos.	Period of Starv^{n}. in Hrs.	Haem. Estim. per Plate		Colony Count per Plate		Total Colony Count		Correction Factor		Comparative Colony Counts.		%age Viability taking control as 100%	
			bi	ybithi	bi	ybithi	bi	ybithi	bi	ybithi	bi	ybithi	bi	ybithi
A (control)	1	0	140	40	73	39	264	140	1	1	264	140	100%	100%
	2				49	41								
	3				77	26								
	4				65	34								
B	1	69	280	80	18	37	87	144	2	2	43.5	72	16.4%	51.4%
	2				27	22								
	3				11	45								
	4				31	40								
C	1	74	280	80	11	29	37	175	2	2	18.5	87.5	7.1%	62.6%
	2				13	44								
	3				5	55								
	4				8	47								
D	1	78	280	80	8	38	27	139	2	2	13.5	69.5	5%	49.5%
	2				9	21								
	3				3	31								
	4				7	49								
E	1	92	280	80	0	21	0	70	2	2	0	35	0%	25.2%
	2				0	12								
	3				0	28								
	4				0	9								
F	1	98	280	80	0	16	0	40	2	2	0	20	0%	14.0%
	2				0	8								
	3				0	7								
	4				0	9								

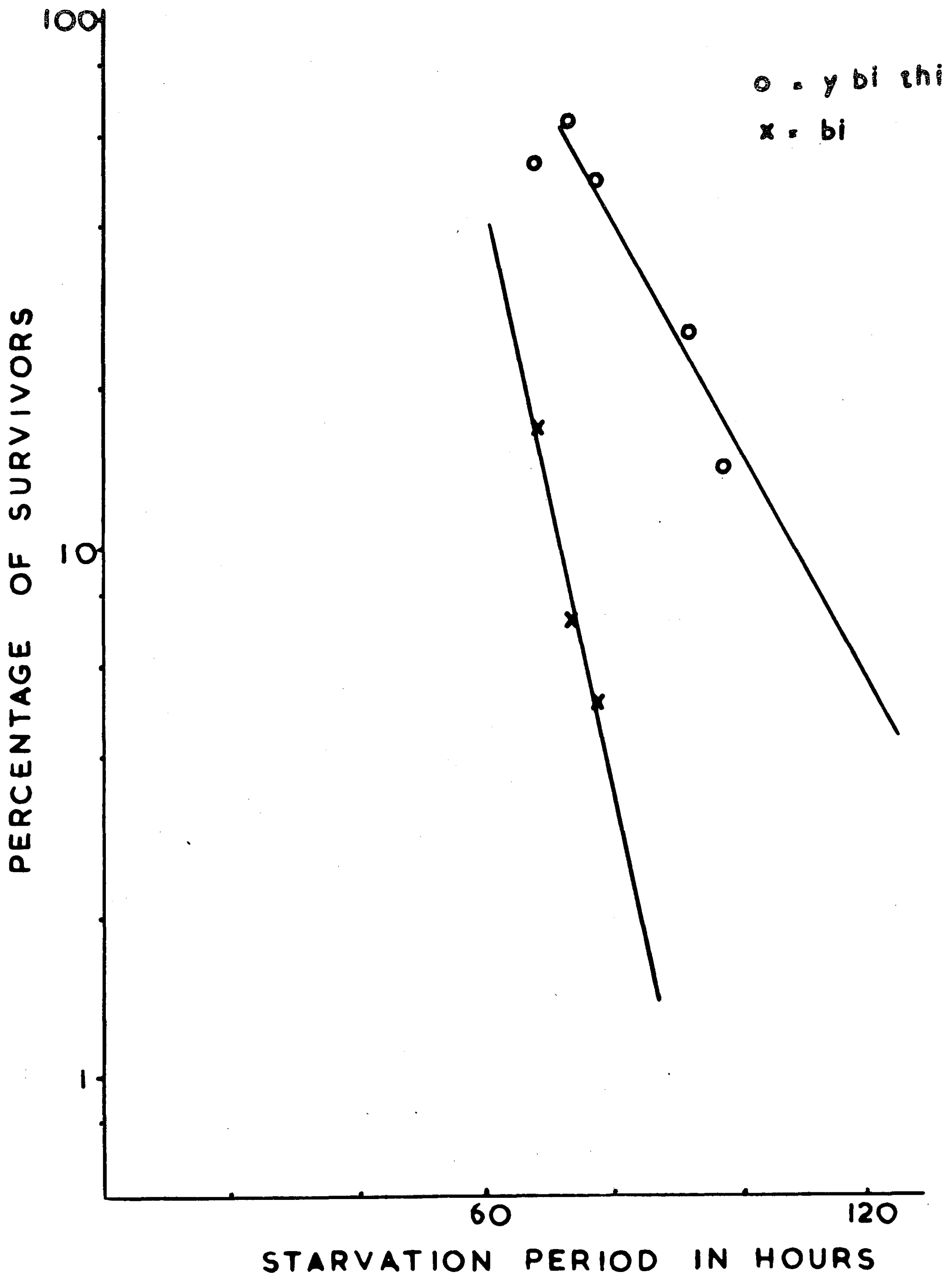


FIG. 4. Reconstruction Experiment: Conidia from the strain with a double requirement (y bi thi = biotin and "thiazole" requiring) die off less quickly than conidia from the strain with a single requirement (bi = biotin requiring) when both types are starved on minimal medium

This experiment was essentially the same as the one described above and the results are shown in Table 6. Again the strain with two requirements survives the longer under conditions of starvation (see Fig. 5).

Plate 2 shows the results of a reconstruction experiment involving the green spored biotinless strain and the white spored biotinless, adenineless strain. The ratio of doubly deficient colonies to singly deficient colonies increases with the length of starvation in minimal medium.

TABLE 6. Reconstruction Experiment.
Dying off rates of conidia of the biotinless (bi) and conidia of the biotinless, adenineless (biwad) strains on minimal medium.

Plate Series	Plate Nos.	Period of Starv^{n}. in Hrs.	Haem. Estim. per Plate.		Colony Count per Plate		Total Colony Count		Correction Factor		Comparative Colony Counts.		%age Viability taking control as 100%	
			bi	biwad	bi	biwad	bi	biwad	bi	biwad	bi	biwad	bi	biwad
A (control)	1	0	120	44	67	31	215	99	1	1	215	99	100%	100%
	2				59	26								
	3				41	22								
	4				48	20								
B	1	48	240	88	37	42	146	168	2	2	73	84	34%	84.8%
	2				29	41								
	3				41	36								
	4				39	49								
C	1	92	240	88	9	18	44	102	2	2	22	51	10.2%	51.5%
	2				15	23								
	3				8	30								
	4				12	31								
D	1	96	240	88	3	18	4	69	2	2	2	34.5	0.9%	34.3%
	2				1	15								
	3				0	16								
	4				0	20								
E	1	120	240	88	0	9	0	37	2	2	0	18.5	0	18.7%
	2				0	6								
	3				0	15								
	4				0	7								

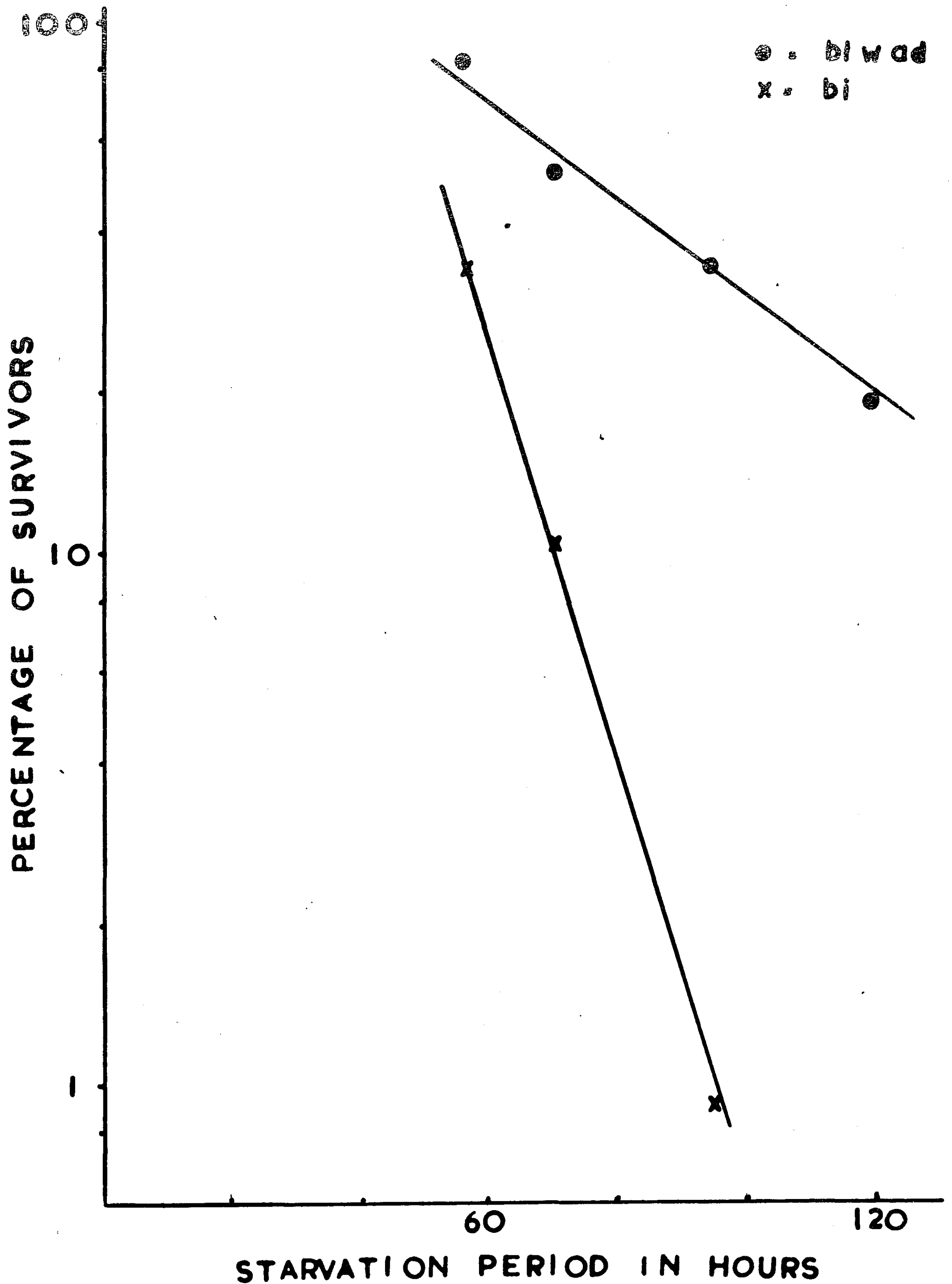


FIG. 5. Reconstruction Experiment: Conidia from the mutant with two requirements (bi w ad = biotin and adenine requiring) survive longer on minimal medium than conidia from the mutant with a single requirement (bi = biotin requiring)

Starvation Period on Minimal Medium.

0 Hrs. 72 Hrs. 96 Hrs.

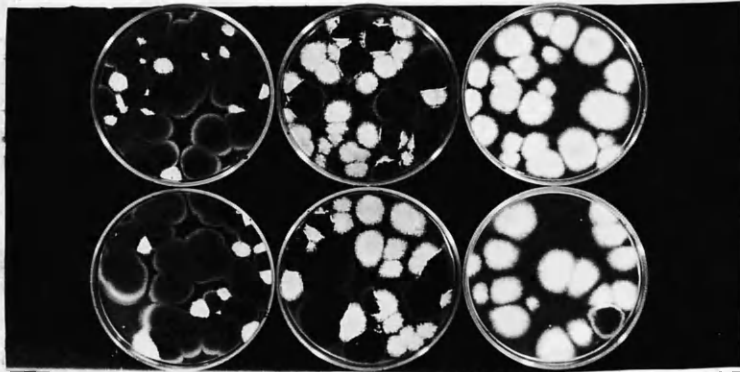


Plate 2. The dark coloured (green) colonies have developed from conidia of the biotin requiring mutant (bi) and the white colonies from conidia of the biotin and adenine requiring mutant (bi w ad). The proportion of spores plated for each strain was the same in each case so that, as starvation progresses, the greater degree of survival of the double nutritional mutants can be seen.

4. Discussion and Conclusions.

It is apparent that what Fries discovered concerning the behaviour of strains of *Ophiostoma multiannulatum* under starvation (Fries 1948a, 1948b) has been confirmed in the nutritional mutants of *Aspergillus nidulans* so far investigated. In the latter fungus, conidia of a biotin requiring strain have been shown to die off more rapidly in minimal medium than do the conidia of another two different strains each having one other nutritional requirement (adenine and "thiazole" respectively) in addition to that of biotin.

A composite plot of all the results obtained has been made in Fig. 6 which shows clearly the slower dying off rates conidia of the two double nutritional mutants have, relative to conidia of the biotin requiring mutant.

Fries suggested that the reason for the longer survival of more heterotrophic conidia might be that they were in a deeper state of dormancy so that under starvation conditions their metabolism proceeded at a lower rate (Fries 1948a, 1948b). This may also be the case in those strains of *Aspergillus nidulans* whose behaviour under starvation has been examined. Presumably the metabolic rate of conidia from double nutri-

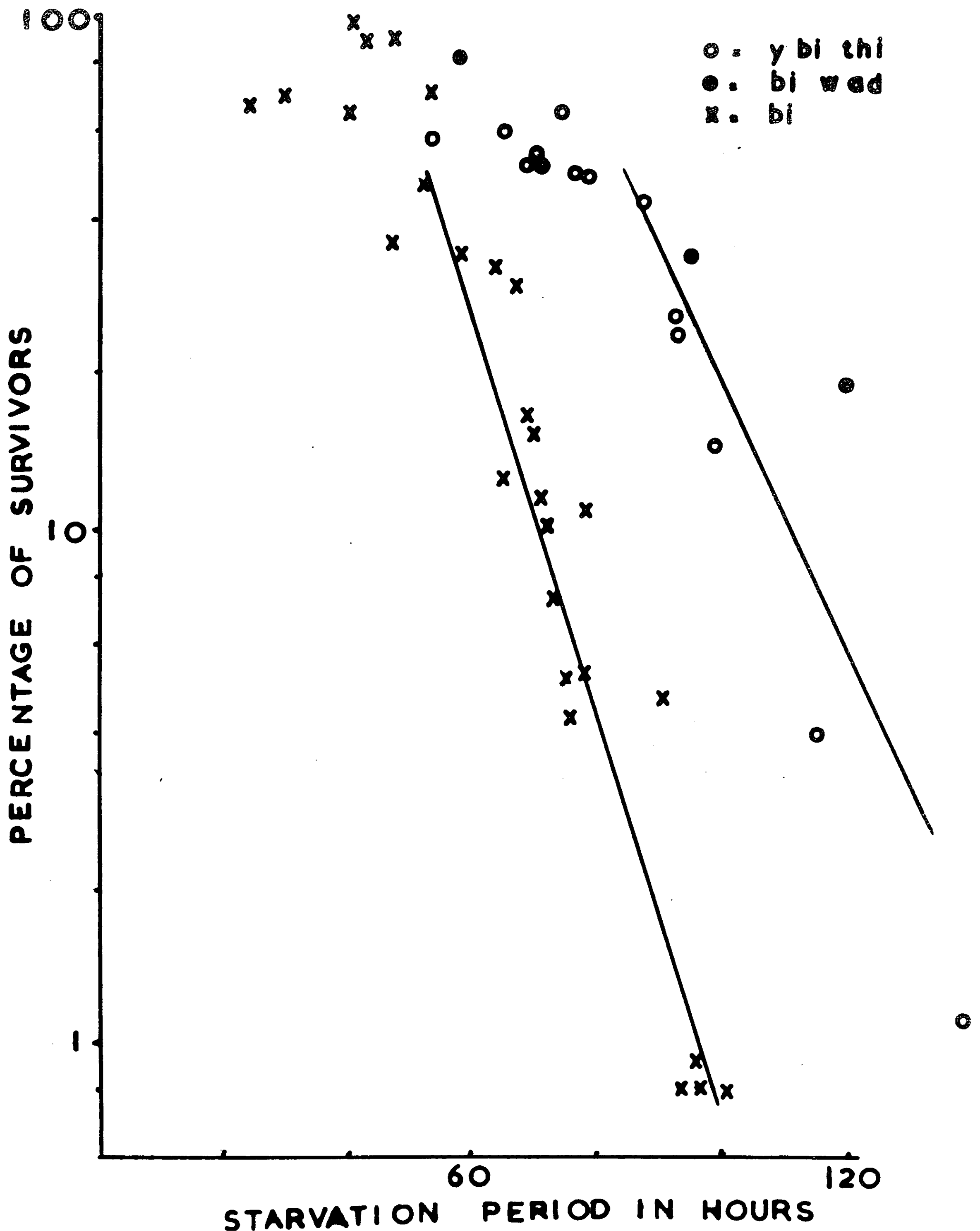


FIG. 6. Combined results of all the experiments conducted in the first part of this thesis. Conidia from the two mutants with double requirements (y bi thi = biotin and "thiazole" requiring; bi w ad = biotin and adenine rquiring) clearly survive longer than conidia of the mutant with a single requirement (bi = biotin requiring) when starvation is conducted on minimal medium.

tional mutants is lower on minimal medium than of conidia from the biotin requiring mutant; death resulting from the non-satisfaction of growth requirements will therefore be reached after a longer time in the former case.

In view of the encouraging results with the reconstruction experiments, it was decided to try to develop a technique for the isolation of nutritional mutants in Aspergillus nidulans making particular use of the biotinless strain.

Summary:

1. The dying off rate of conidia of a biotinless strain of Aspergillus nidulans, on minimal medium, without biotin, was measured and found to be logarithmic after an initial phase of no dying off. The proportion of survivors is reduced to 0.8% after 96 hours at 37°C.

2. Reconstruction experiments with this strain and two other strains requiring "thiazole" and adenine, respectively, in addition to biotin, showed that conidia of the latter strains survived starvation in non-supplemented minimal medium for a longer period.

PART II. Development of a technique for the isolation of Nutritional Mutants in *Aspergillus nidulans*, based on starvation.

1. Introduction.

Lederberg (1948) has evaluated the conditions which have to be satisfied before mutagenic activity can be assigned to a physical or chemical agent. It is not enough to show that the proportion of mutants increases among the survivors of any particular treatment since these mutants might be selected by the treatment from those present originally, as the result of spontaneous mutation. Either it has to be shown that there is an absolute increase in the number of mutants or that the agent used is non-selective.

In microorganisms, improved methods of detecting mutants have been used to determine whether or not specific agents are mutagenic. Mutations to drug or phage resistance in bacteria (Luria and Delbruck 1943, Demerec 1945, Witkin 1947, Lederberg 1950) and back mutation among nutritional mutants of *Neurospora* (Ryan 1946, Giles and Lederberg 1948, Kolmarkand Westergaard 1949) are easily distinguishable, in either case, from among large populations of non-mutant types,

since it can be arranged that only those mutant types will grow. It then becomes possible to test vast numbers of cells so that even low mutagenic activity can be detected. However, in the case of bacteria, until the nature of hereditory factors is more conclusively established and until genetical analysis becomes possible in the strains under test, caution must be exercised in ascribing mutagenic activity to specific agents, in the normal sense (Auerbach 1949).

Using the back mutation method, Kolmark and Westergaard (1949), Jensen, Kolmark and Westergaard (1949), and Giles and Lederberg (1948) have demonstrated that X rays, ultra violet light and certain chemical agents are mutagenic in _Neurospora_. Certain criticisms of the validity of the _Neurospora_ back mutation method made by Grigg (1952) have apparently been satisfactorily answered by Kolmark and Westergaard (1952) (see also Jinks 1952).

Apart from demonstrating an absolute increase in mutants, reconstruction experiments in which artificial mixtures of mutant and non mutant cells are subjected to a supposedly mutagenic agent, afford a tentative way of distinguishing between selection and mutagenesis. In the later case, the killing rates of mutant and non mutant cells are similar (see Witkin 1947).

In the studies to be described in this part of the thesis, mutations were induced both by X rays and ultra violet light. These agents which have been used extensively to produce mutations in microorganisms, appear to be non specific in their action (Muller 1940, Muller 1947). In comparing their effects it has been shown that X rays are more drastic in that they produce a greater number of chromosone breaks, whereas the effect of ultra violet light is closer to the type of nuclear changes which arise spontaneously (Stadler 1941). The physico-chemical actions of these radiations have been widely investigated in a desire to elucidate the manner in which they induce mutations (Hollaender and Emmons, 1941, 1946, Lea 1946, Catcheside 1948, Muller 1950).

The mode of action of ionising radiations has been explained by the 'target' theory. This hypothesis supposes that a single ionisation produced within a certain volume has a certain probability of inducing one mutation. When X ray dosage is measured in roentgen units (a measure of the number of ionisations per unit volume) then there is a linear relationship between dosage and mutations produced among survivors, a fact which supports the target theory.

Recent work has shown that radiochemical reactions

might be important in the mutagenic action of short wave radiations. For example, a reduction in oxygen tension or the addition of certain substances which can act as reducing agents, has the effect of increasing the survival of cells of Escherichia coli on X ray irradiation. Presumably molecular oxygen is required in some radiochemical reactions. (Thoday and Read 1947, and Stapleton, Billen, and Hollaender 1952). The present indications appear to be that although ionisations might not act directly, the target theory gives the best account of observable genetic changes. (Horowitz and Mitchel 1951).

Ultra violet light in contrast to X rays, has a very low penetrating power and is effective in a different manner. The ultra violet wave lengths which are most strongly mutagenic approximate to those which are most absorbed by nucleic acids. These acids are of primary importance in chromosomal make-up and it would seem that ultra violet absorption by them is the activating agency of mutagenesis (Hollaender and Emmans 1941). Certain recent work has illustrated that peroxide and organic peroxides play some rôle in ultra violet mutagenesis (Haas, Clarke, Wyss and Stone 1950). Irradiated broths retain mutagenic activity, probably by the formation of organic peroxides (produced through the intermediacy of peroxide formed during

irradiation). These organic peroxides have been shown to be potent mutagens in Neurospora (Dickey, Cleland and Lotz 1949). However, since pre-irradiation of media is only effective at wavelengths below 2000Å, the results obtained cannot account fully for the effects of direct ultra violet irradiation where the peak of mutagenic activity is at 2,600Å (Hollaender and Emmons 1941). The dosage/effect relationship in ultra violet irradiation is obscure. It is known that there is a fall off in the percentage of mutants among survivors after a certain dosage is reached. In Neurospora, for example, a killing rate above 99% produces a fall off in mutation rate (Hollaender and Emmons 1941, Hollaender Sansome, Zimmer and Demerec 1945). The explanation has been offered that induced mutants may be more sensitive to the killing effects of radiations (Catcheside 1948).

It was discovered recently that visible light reverses the lethal and mutagenic effects of ultra violet. This phenomenon of photoreactivation was first noticed in Streptomyces griseus (Kelner 1949a), and has since been described and more fully interpreted in other microorganisms (Dulbeggo 1949, Kelner 1949b, Novic and Slizard 1949, Brown 1951, and Newcombe and Whitehead 1951). Certain photosensitive products of u/v irratiation are apparently responsible for the

reversible effect of light (Newcombe and Whitehead 1951). In investigations on heat reactivation of a similar nature, an effect has been demonstrated only in certain Escherichia coli strains (Stein and Meutzer 1950, Anderson 1951).

The killing and mutagenic effects of X rays do not appear to be susceptible to either type of reactivation (Dulbecco 1950, Anderson 1951).

Following the induction of mutation, methods essentially similar to those employed in Neurospora (Beadle and Tatum 1945) have been used in the isolation of nutritional mutants from many other microorganisms. Other modified and improved techniques have also been developed which will be discussed shortly.

Among the fungi, and apart from Neurospora, nutritional mutants have been isolated in Absidia glauca (Giles 1947), Allomyces spcs. (Yaw 1950), Aspergillus nidulans and Aspergillus oryzae, (Pontecorvo 1946), Aspergillus terreus (Rapor, Coghill and Hollaender 1945), Coprinus fimetarius (Fries, L. 1948), Glomerella cingulata (Market 1950), Ophiostoma multiannulatum (Fries 1945), Penicillium notatum (Pontecorvo and Gemmel 1944, Pontecorvo 1947, Bonner 1946) and Ustilago maydis (Perkin 1949). Nutritional mutants have also been isolated in Actinomyces flaveolus (Kelner

1947) and among the following bacteria, Acetobacter melanogenum (Gray and Tatum 1944), Achromobacter fischeri (Farghally, Miller and McElroy 1948), Azotobacter agilis (Karlsson and Barker 1948), Bacterium subtilis (Burkholder and Giles 1947), Bacterium aerogenes (Devi, Pontecorvo and Higginbottom 1947), Escherichia coli (Gray and Tatum 1944), and Salmonella typhimurium (Plough, Young and Grimm 1950). Similar mutants have been found in bacteriophage (Delbruck 1948), yeasts (Lindegren 1949) and the unicellular algae Chlamydomonas moewusii (Lewin 1952) and Chlorella spps. (Granick 1949).

Beadle and Tatum (1945) have detailed the methods which they employed in isolating nutritional mutants from Neurospora. Macroconidia, asexual multinucleate haploid spores, are treated with a mutagenic agent and then applied to protoperithecia of the opposite mating type. The ascospores which are produced after sexual fusion and meiosis are haploid; these are placed on to complete medium and the colonies which grow up are isolated and tested for their growth factor requirements. Those which do not grow on Neurospora minimal medium are classified as to their specific growth factor requirements after further tests.

The ascospores require to be heat treated for

germination to occur but it has been shown that chemical activation is equally successful (Emerson 1948).

The Total Isolation method developed by Pontecorvo (1947) for organisms with uninucleate haploid cells is of course much less laborious than Beadle and Tatum's original method since cells are plated out directly following mutagenic treatment without the laborious methods of spermatising protoperithecia, etc., entailed in Neurospora. However, in addition to bearing macroconidia Neurospora crassa also produces small asexual spores known as microconidia. These spores, which are haploid and uninucleate can be plated out directly following mutagenic treatment (Tatum, Barrat, Fries and Bonner 1950) so that Pontecorvo's method can in fact be used in Neurospora, although certain difficulties have to be overcome because of the spreading colonial growth and the readiness with which heterocaryons are formed (Beadle and Coonradt 1944). However this can be overcome by limiting the growth of the colony. A restricted colony growth can be obtained either genetically as has been done by Lindegren and Lindegren (1941), who used a strain with a single gene difference, or by adding substances to the medium which restrict growth (Tatum, Barrat, and Cutler 1948, 1949). These substances include a synthetic detergent known as Tergitol and

sorbose. The limited growth of inositolless mutants of Neurospora (Beadle 1944) and Ophiostoma multiannulatum (Fries 1949a) on certain concentrations of inositol are examples of other paramorphic effects. Tatum et al (1950) used an inositolless mutant in their experiments in isolating nutritional mutants from mutagenically treated microconidia.

Improved methods of mutant isolation have been described in other microorganisms. Ledeberg and Tatum (1946) introduced a delayed enrichment technique using Escherichia coli; an agar minimal plate was poured in three layers to the middlelayer of which had been added the mutagenically treated cells, while it was still in the liquid state. This stratification keeps the cells in a colony from contaminating the entire plate in the next step. After a short period of incubation, visible colonies are marked; a fourth layer of complete medium was added and the plates reincubated. Any further colonies which developed were isolated and tested as possible mutants. The principle of the method was that non-mutants would grow up after the first incubation and nutritional mutants after further incubation with added complete medium. However, delayed growth of non-mutant cells, following the mutagenic treatment, prevented the technique from being wholly successful,

although there was an increase in the percentage of mutants isolated. In *Aspergillus nidulans* (Pontecorvo 1952) a similar method increased the percentage of mutants by a factor of two or three over the normal Total isolation technique. Meyersburg, Pomper, and Cutter (1949) have described a quick method for marking the colonies which grow up after preliminary incubation. A photograph is taken on high speed/high contrast paper, and after the second incubation, the plates are superimposed on the photographic paper. Colonies not shown on the paper are isolated. It is claimed that this method saves a considerable amount of time when a large throughput is desired.

A limited enrichment method has been used in *Escherichia coli* (Davis 1949). The treated cells are plated on to a medium containing, for example, a limited amount of casein hydrolysate. After a period of incubation, any tiny colonies are observed under a microscope and marked. Persistently tiny colonies (following further incubation) are picked up as possible mutants. It was found that over 50% of the isolates were in fact mutants. The limited amount of casein hydrolysate will only allow amino acid requiring mutants to carry out microscopic growth. A similar technique has been employed in *Ophiostama multiannulation* (Fries 1950).

Another technique developed for bacteria makes use of the fact that the antibiotic, penicillin, is only active against growing bacteria. (Davis 1948, Lederberg and Zinder 1948). Cells are incubated in liquid minimal medium containing penicillin (but see below). The wild type cells commence growth and are killed. Davis found over 80% of the surviving colonies were nutritional mutants. Certain precautions are necessary for the workings of this method. Over-crowding leads to syntrophic effects and also a direct plating on to minimal medium, following mutagenic treatment, yields no mutants. Apparently there is a phenotypic lag in the adjustment of mutated cells to their new genotype, so that an intermediate cultivation is necessary (Davis 1950, see also Adelberg and Myers 1952). A technique based on these same principles and using SO_2 as a killing agent has been applied by Forbes (1952) in *Aspergillus nidulans*.

A filtration method has been described in *Ophiostoma multiannulatum* (Fries 1947). Treated conidia are suspended in liquid minimal medium for a time and then filtered through a suitable filter. On suspension in minimal medium, unmutated wild type conidia germinate so that on filtration, the non-germinated mutant conidia pass more easily through the filter. 12 to 15% mutants

were isolated by this technique as against 1 - 2% mutants by Total Isolation methods. This technique has been used in Aspergillus nidulans (Hockenhull 1948).

It has been shown that if treated cells of Achromobacter fischeri are incubated in the cold (8°C) then induced nutritional mutants have a selective advantage over wild type cells (Farghally, Millar and McElroy 1948).

In the starvation technique which has been developed for the isolation of nutritional mutants in Aspergillus nidulans, it will be seen that use is made both of the technique devised by Lederberg and Tatum (1946)(see above) and of the observations of Fries (1948a, 1948b) on starvation cultures (Macdonald and Pontecorvo 1950). The layer plating methods are very similar to those employed by the former workers only the delay period prior to enrichment with complete medium is very much extended to allow the development of any differential survival effect.

Conidia of the biotin requiring mutant of Aspergillus nidulans are treated with a mutagenic agent and layer plated on to agar minimal medium. After suitable periods in this starvation medium, complete medium is added and a random sample of

isolates made from the colonies which develop. A substantial number of these isolates turn out to be nutritional mutants. The success of the technique depends on the fact that mutant conidia which are induced by the treatment and have one other growth factor requirement in addition to biotin, survive starvation for a longer period and are thus enriched. Experiments are described in this part of the thesis in which nutritional mutants were isolated by the starvation technique, following X ray and u/v irradiation.

A section is devoted to the methods which were used to classify the large number of nutritional mutants which were isolated. The methods employed by most workers to characterise the growth factor requirements of nutritional mutants appear to be those of Beadle and Tatum (1945). The colonies which grow up on complete medium, following mutagenic treatment are isolated on to complete medium slopes. These are tested for growth on minimal medium, those which grow being discarded as non mutants. The remainder are then tested on various components of the complete medium and later, depending on the response to certain components, tested on substances known to be contained in the various fractions.

Fries (1948b), however, has described a method in

Ophiostoma multiannulatum which avoids the wasteful cultivation of non mutants on complete medium slopes. Conidia from each colony growing after mutation has been induced, are spotted in marked positions on Petri dishes containing agar minimal medium (about 20 spots per dish). Those sub cultures showing no growth after incubation are then rescued on to complete medium slopes, and later tested for their specific requirements.

The Auxanographic method is of considerable use in mutant characterisation (Pontecorvo 1949a). A Petri dish is poured with seeded agar minimal medium. Various substances can then be spotted in marked positions and growth round the spot where a particular nutrient is placed indicates a requirement for that substance. This technique is also extremely useful in showing up antagonistic and other effects between different growth factors.

Both auxanography and the methods of Fries have been used in the characterisation of nutritional mutants in the work to be reported.

Finally, the efficiency of the starvation method is discussed and some comparisons are made between the type of mutants isolated by this method and by other techniques.

2. Material and Methods.

Material.

a) Strain used:

The biotin requiring mutant of *Aspergillus nidulans* which was used in the experiments described in Part I was established as the parental culture on minimal medium supplemented with biotin. Sub cultures were made by mass transfer of conidia on to minimal medium with added biotin. In all the experiments 6 day old sub cultures were employed which had been grown up at 37°C.

b) Media:

Only media not hitherto described are listed. They are routinely used in the Department of Genetics at Glasgow.

Ultra complete medium:

The constituents were the same as in routine complete medium with the following new constituents added per litre of medium.

Hydrolysed Thymus Nucleic Acid (see below) : 3 ml

Casein Digest (see below) : 5 ml

Hydrolysed Blood Corpuscles (see below) : 3 ml

Hydrolysed Blood Plasma (see below) : 3 ml

Additional Vitamin Solution (see below) : 1 ml

This medium was made up in the same way as routine complete medium. All the extra constituents were added with the other constituents, apart from the vitamin solution which was added after filtration.

The preparation of the extra constituents:

Casein digest: Combine peptic and tryptic digest of casein and equivalent to 125 mg of Casein per ml. pH c.6. Stored over chloroform at 4°C in dark, preferably for not more than three months.

Hydrolysed Thymus Nucleic Acid: This was prepared in exactly the same way as hydrolysed yeast nucleic acid which has already been described (Part I. Material and Methods).

Hydralysates of Blood: 25 ml of Burroughs Welcome oxalated horse blood was centrifuged and plasma and corpuscles were separated.

Corpuscles: Packed corpuscles were mixed with 25 ml 2N HCl and autoclaved for 10 minutes at 15 lbs pressure. The mixture was then cooled and brought to pH 10 with NaOH, and filtered. The solution was then diluted to 75 ml with H_2O, keeping the pH at 10 and stored over chloroform at 4°C in dark, preferably for not longer than 2 months. Each 3 ml of this solution was equivalent to corpuscles in 1 ml of horse blood.

Plasma: The supernatant plasma (following

centrifugation) was mixed with 25 ml of 2N HCl and autoclaved for 10 minutes at 15 lbs pressure, cooled, the pH adjusted to 7 with NaOH and filtered. The solution was then diluted to 50 ml at pH7 and stored as for corpuscles hydrolysate. 1 ml of this solution was equivalent to plasma from 0.5 ml blood.

Additional Vitamin Solution:

Inositol	0.5 gms.	Desthiobiotin	50 microgms.
Folic acid	5 mg.	Riboflavin	3 mg.
Pyridoxin	5 mg.		

This was prepared in the same way as the vitamin solution used in routine complete medium.

Supplemented Media.

In the plate test methods used in the characterisation of nutritional mutants (and to be described later), agar minimal medium, with added desthiobiotin (5 microgms/litre), was supplemented with certain fractions of ultra complete medium including

Casein Hydrolysate) 5 ml of the standard solution was required per 100 ml of medium for good growth of amino acid requiring strains.

Vitamins) 0.2ml of normal and 0.2 ml of additional vitamin solutions were used per 100 ml of medium.

Adenine) 5 ml of the 1.25 mg/ml solution was used per 100 ml of medium.

Sodium Thiosulphate) 0.2 ml of a 50gm/100ml solution

was used per 100 ml of medium. This solution was sterilised over a period of three days for ½ hour each day at 95°C.

Sodium Sulphite) as for sodium thiosulphate.

c) Mutagenic Agents.

X rays.

The X ray source was a Watson 90/30 Trolley set Type C (Power Mobilix - Austerity Model). The tube was an air cooled Aeromax 12. The wavelength was 0.5Å, the filtration being equivalent to 0.5 mm Aluminium. Irradiation was with a tungsten target of 85KV and 4mA and over ½ hour, the time used, the dosage was approximately 50,000 roentgen.

Ultra violet rays.

A Hanovia XI, low pressure mercury lamp was employed. The model had a 12 inch filter-jacketed tube. The consumption was 30 watts and the maximum operating intensity was reached within half an hour of switching on, 87% of the output being in the 2537Å region, which comes close to the peak line for mutagenic activity.

In the experiments to be described, irradiation was done at distances of 40 cms and 45 cms from the tube. In each experiment the u/v dosage is expressed as a killing rate calculated from viable counts after

irradiation relative to conidia plated (estimated by haemocytometer).

Methods.

a) X ray irradiation: This method is routine in the Department of Genetics at Glasgow.

A square of agar covered with a thick felt of conidia was cut out from a six day old sub of the biotin requiring mutant and placed in a sterile Petri dish whose lid was replaced by a cellophane cover. The dish was then placed in position on the X ray unit. After the required dosage of 50,000 roentgen had been given, the agar square was removed and placed on the inside of a boiling tube containing a few ml of saline and calzolene solutions. The conidia on the agar were washed down into the liquid with the aid of a Pasteur pipette and the agar was then discarded. The conidial chains were then broken up and the spore density estimated by haemocytometer count.

b) Ultra violet irradiation:

A dense suspension of conidia was made in saline and calzolene solution and from this a final 10 ml suspension was prepared containing a million conidia per ml. This was poured into a flat Petri dish. The depth of the liquid was 1.5m.m. From previous data

available it was shown that, with the lamp at a distance of 45 cm from the inside bottom of the dish (measured from the centre of the tube), an irradiation of 8 minutes gave a 95% kill, and at 40 cm an irradiation of 5 minutes gave a 75% kill. The Petri dish lid was removed during irradiation and the plate was rocked for 5 seconds every 15 seconds to below the point when splashing would occur. The conditions observed in all experiments, with regard to the unavoidable exposure of the conidia to light, were standardised as much as possible to equalise photoreactivation effects.

3. X ray Irradiation.

A direct plating of irradiated conidia on to a starvation medium would only be effective if there was no delay in phenotypic expressions necessary for differential survival to operate. The obvious way to decide was to irradiate conidia, plate out on starvation medium and determine the proportion of mutants among survivors as starvation progressed.

In the first experiment, conidia of the biotin requiring mutant of Aspergillus nidulans were X ray irradiated (50,000 roentgen dosage) and plate embedded in known numbers on the starvation medium, in this case minimal medium. The techniques involved have already been described (see Fig.1). The control plates were enriched with a top layer of ultra complete medium and all the plates were then incubated at $37^{\circ}C$, the starvation plates being enriched over increasing periods of time. The complex ultra complete medium was used in the hope of isolating a very varied selection of nutritional mutants.

The isolation methods and the characterisation of nutritional mutants are described in a later section.

The results of this experiment showed that the percentage of nutritional mutants among survivors does

increase very markedly as starvation progressed (Table 7). Another experiment carried out in a similar manner confirmed these results (Table 8).

Details from both experiments are summarised in Table 9.

Apparently the increase in mutant percentage does not continue beyond a certain period of starvation (Plate 3). Presumably delayed germination in a proportion of the biotinless conidia may account for this. Such a delay, produced by irradiation, is probably non genetical (see Davis 1949). If a proportion of conidia of the mother strain has germination delayed, then it would be expected that this proportion would survive starvation longer than the remaining conidia of the mother strain. The general effect would be to increase the survival of conidia of the mother strain on minimal medium relative to nutritional mutant conidia. The ratio of mutant type to mother type will increase as long as the mutants have a greater degree of survival under starvation. From the experimental results, it appears that a point may be reached where any further enrichment of mutant conidia is masked by long surviving mother strain conidia (presumably having long delayed germination).

Naturally the nutritional mutants themselves die

off as starvation progresses, as can be seen in Fig. 7, where the logarithm of the number of nutritional mutants per one million plated conidia is plotted against the period of starvation.

TABLE 7. Conidia of the biotinless strain (bi) X ray irradiated (50,000r) and kept for periods of from 0 to 111 hours on minimal medium before the addition of ultra complete medium.

Plate Series	Plate Nos.	Period of Starvn in Hours.	Haem. Estim. per Plate.	Colony Counts per Plate.	Total Colony Count.	Correction Factor.	Comparative Count	%age viability taking control as 100%	No. of isolates made per plate	Total No. of Isolates	Among Isolates.		
											No. of non-mutants	No. of nutritional mutants	%age of nutritional mutants
A (control)	1 2 3	0	9,000	49 54 47	150	1	150	100%	38 37 37	112	111	1	0.9%
B	1 2 3	46	15,000	65 67 52	184	5/3	110.4	73.6%	29 49 37	115	113	2	1.7%
C	1 2 3	74	15,000	49 43 37	129	5/3	75.4	50.3%	33 26 29	88	85	3	3.4%
D	1 2 3	96	9,000	49 39 37	125	10	12.5	8.3%	32 25 23	80	70	10	12.5%
E	1 2 3	111	150,000	46 23 22	91	50/3	5.5	3.7%	27 15 14	56	39	17	30.1%

TABLE 8. Conidia of the biotinless strain X ray irradiated (50,000r) and kept for periods of from 0 to 147 hours on minimal medium before the addition of ultra complete medium.

Plate Series	Plate Nos.	Period of Starv^n in Hours.	Haem. Estim. per Plate.	Colony Counts per Plate.	Total Colony Count.	Correction Factor.	Comparative Count	%age viability taking control as 100%	No. of isolates made per plate	Total No. of Isolates	Among Isolates		
											No. of non-mutants	No. of nutritional mutants	%age of nutritional mutants
A (control)	1	0	9,000	35	185	1	185	100%	30	118	115	3	2.6%
	2			54					28				
	3			52					28				
	4			44					32				
B	1	96	90,000	10	40	10	4	2.2%	10	39	31	8	20.5%
	2			6					6				
	3			11					11				
	4			13					12				
C	1	111	140,000	10	39	$\frac{140}{9}$	2.5	1.4%	8	37	23	14	37.8%
	2			15					15				
	3			4					4				
	4			10					10				
D	1	117	140,000	14	44	$\frac{140}{9}$	2.7	1.5%	13	40	28	12	30.0%
	2			11					11				
	3			10					8				
	4			9					8				
E	1	123	280,000	7	42	$\frac{280}{9}$	1.4	0.8%	6	38	27	11	28.9%
	2			15					14				
	3			9					8				
	4			11					10				
F	1	147	560,000	11	57	$\frac{560}{9}$	0.9	0.5%	11	54	32	22	40.8%
	2			13					12				
	3			15					14				
	4			18					17				

TABLE 9. Combined Data from tables 7 and 8.
Conidia of biotinless strain x ray irradiated 50,000r.

A	B	C	D	E	F	G
Period of Starv^{n} in Hours.	Haem. Estim. Total No. of Condia Plated	Total No. of Colonies which grew.	Total No. of Colonies which were isolated	Total No. of Nutritional Mutants.	%age of Nutritional Mutants among isolates. ($\frac{E}{D}$ x 100)	No. of Nutritional Mutants per 10^6 plated Spores ($\frac{ExC}{BxD}$ x 10^6)
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

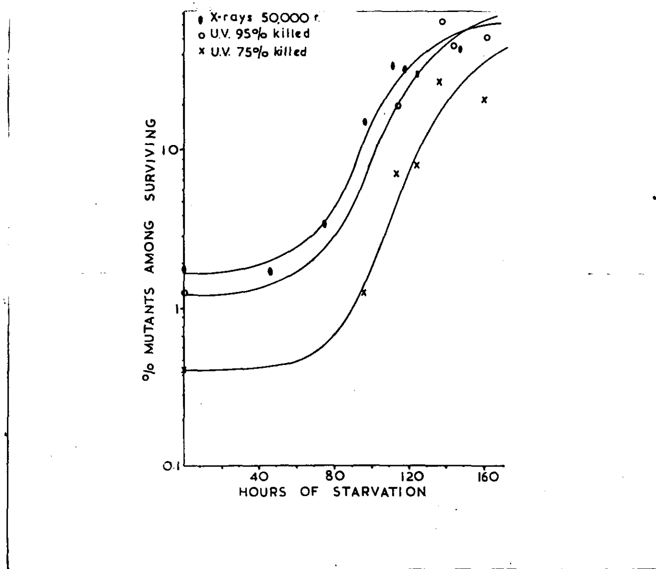


Plate 3. After X ray and u/v irradiation of conidia of the biotin requiring mutant (bi) the proportion of nutritional mutants among survivors increases to between 40 - 60% after 140 - 160 hours starvation from about 1 - 2% with no starvation.

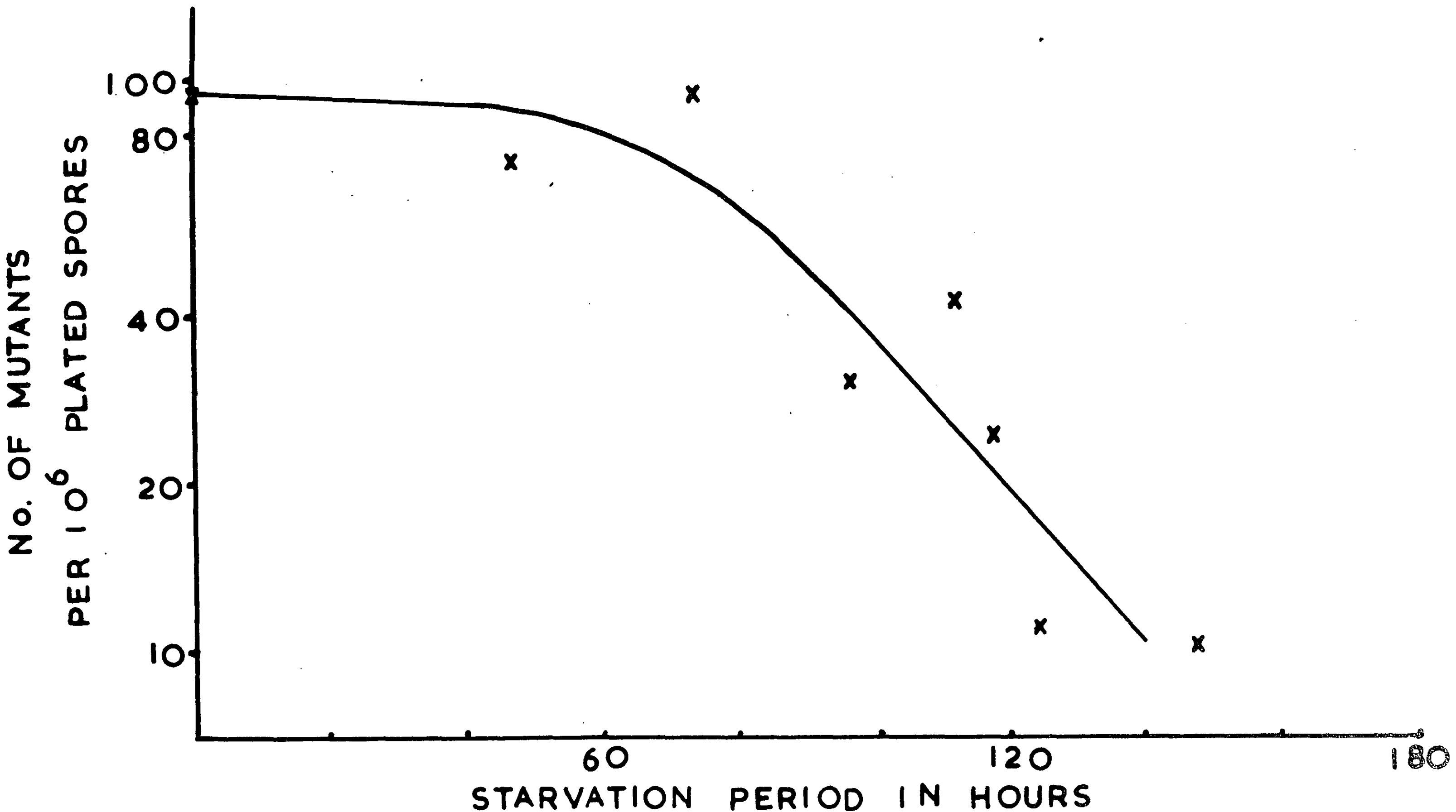


FIG. 7. Data from Table 8: Combined results of X ray experiments. Nutritional mutant conidia die off as starvation progresses on minimal medium.

4. Ultra violet irradiation.

In two starvation experiments conducted on u/v irradiated conidia, the dosage given produced in the region of a 95% kill. In one other experiment a 75% killing rate was produced.

The methods of u/v irradiation, plating, starvation, etc., have already been described. As in the X ray experiments, the plates were enriched with ultra complete medium after starvation.

The experimental results confirm those already obtained for X ray treated conidia. (Tables 10 - 13. Fig. 8. Plate 3).

A 75% kill was used in the hope of minimising as far as possible non-genetic delayed germination effects, concomitant with a reasonable initial number of mutants. It appears that even at this dosage the percentage of mutants did not increase beyond a certain point (Plate 3).

A comparison was made between reconstruction and irradiation dying off rates (Plate 4). The reconstruction results are from various experiments described before and later, and the irradiation results from the experiments described in this section.

Irradiated biotinless conidia obviously survive longer on a starvation medium than do non-irradiated

biotin conidia, a function of the slower dying off rates of the induced mutants present in any irradiated sample, and probably also of the delayed germination of a proportion of the non-mutant conidia.

TABLE 10. Conidia of the biotinless strain u/v irradiated (95% kill) and kept for periods of from 0 to 161 hours on minimal medium before addition of ultra complete medium.

Plate Series	Plate Nos.	Period of Starv^{n} in Hours.	Haem. Estim. per Plate.	Colony Counts per Plate.	Total Colony Counts.	Correction Factor.	Comparative Count	%age viability taking control as 100%	No. of isolates made per Plate	Total No. of Isolates	Among Isolates		
											No. of non-mutants	No. of nutritional mutants	%age of nutritional mutants
A (control)	1	0	2,200	97	612	1	612	100%	40	200	198	2	1%
	2			98					32				
	3			119					32				
	4			105					33				
	5			111					31				
	6			82					32				
B	1	114	110,000	25	120	50	2.4	0.4%	23	100	69	31	31%
	2			10					9				
	3			15					12				
	4			39					32				
	5			10					7				
	6			21					17				
C	1	137	330,000	23	191	150	1.3	0.2%	19	138	49	89	65%
	2			40					26				
	3			29					22				
	4			29					20				
	5			32					23				
	6			38					28				
D	1	161	440,000	23	254	200	1.3	0.2%	15	140	68	72	51%
	2			47					27				
	3			30					18				
	4			48					33				
	5			45					26				
	6			61					21				

TABLE 11. Conidia of the biotinless strain u/v irradiated (95% kill) and kept for periods of from 0 to 143 hours on minimal medium before addition of ultra complete medium.

Plate Series	Plate Nos.	Period of Starv^{n} in Hours.	Haem. Estim. per Plate.	Colony Counts per Plate	Total Colony Counts	Correction Factor.	Comparative Count	%age viability taking control as 100%	No. of isolates made per Plate	Total No. of Isolates	Among Isolates		
											No. of non-mutants	No. of nutritional mutants	%age of nutritional mutants
A (control)	1	0	1,650	61	398	1	398	100%	33	200	197	3	1.5%
	2			72					33				
	3			62					33				
	4			63					33				
	5			64					33				
	6			76					35				
B	1	114	330,000	112	769	200	3.8	1%	33	200	175	25	12.5%
	2			92					33				
	3			96					33				
	4			139					33				
	5			150					33				
	6			180					35				
C	1	143	440,000	66	362	$\frac{800}{3}$	1.6	0.4%	40	200	109	91	45.5%
	2			42					30				
	3			42					32				
	4			78					37				
	5			83					29				
	6			51					32				

TABLE 12. Combined data from tables 10 and 11.
Conidia of the biotinless strain u/v irradiated (95% kill).

A	B	C	D	E	F	G
Period of Starv^{n} in Hours.	Haem. Estim. Total No. of Conidia Plated	Total No. of Colonies which grew.	Total No. of Colonies which were isolated	Total No. of Nutritional Mutants.	%age of Nutritional Mutants among isolates. ($\frac{E}{D}$ x 100)	No. of Nutritional Mutants per 10^6 plated Spores. ($\frac{ExC}{BxD}$ x 10^6)
0	23,100	1010	400	5	1.25%	545
114	2,640,000	889	300	56	18.7%	63
137	1,980,000	191	138	89	65%	62
143	2,640,000	362	200	91	45.5%	66
161	2,640,000	254	140	72	51%	49

B	1	96	6300	35	348	75/4	18.6	2.5%	28	160	158	2	1.25%
	2			64					32				
	3			63					38				
	4		12600	40					16				
	5			68					26				
	6			78					20				
C	1	112	12600	21	383	75/2	10.2	1.4%	19	175	163	12	6.9%
	2			22					17				
	3			42					23				
	4		25000	85					35				
	5			96					31				
	6			117					50				
D	1	123	6300	32	147	75/4	7.8	1.0%	29	123	113	10	8.1%
	2			16					11				
	3			51					40				
	4		12600	17					15				
	5			15					12				
	6			16					16				
E	1	136	12600	15	104	75/2	2.8	0.4%	11	86	62	24	27.9%
	2			15					11				
	3			20					19				
	4		25000	9					8				
	5			33					28				
	6			12					9				
F	1	159	25000	19	185	75	2.4	0.3%	19	160	125	35	21.9%
	2			18					17				
	3			39					32				
	4		50000	32					30				
	5			38					30				
	6			39					32				

TABLE 13. Conidia of the biotinless strain u/v irradiated (75% kill) and kept for periods of from 0 to 159 hours on minimal before the addition of ultra complete medium.

Plate Series	Plate Nos.	Period of Starv^{n} in Hours.	Haem. Estim. per Plate.	Colony Counts per Plate.	Total Colony Counts.	Correction Factor.	Comparative Count	%age viability taking control as 100%	No. of isolates made per Plate	Total No. of Isolates	Among Isolates: No. of non-mutants	Among Isolates: No. of nutritional mutants	Among Isolates: %age of nutritional mutants
A (control)	1	0	126	41	734	1	734	100%	32	500	498	2	0.4%
	2			37					28				
	3			40					20				
	4			35					20				
	5			45					25				
	6			33					32				
	7			44					38				
	8			22					21				
	9			22					11				
	10			40					33				
	11			35					32				
	12			34					28				
	13			31					15				
	14			29					15				
	15			22					15				
	16			35					15				
	17			22					15				
	18			23					15				
	19			20					15				
	20			24					15				
	21			25					15				
	22			27					15				
	23			28					15				
	24			20					15				

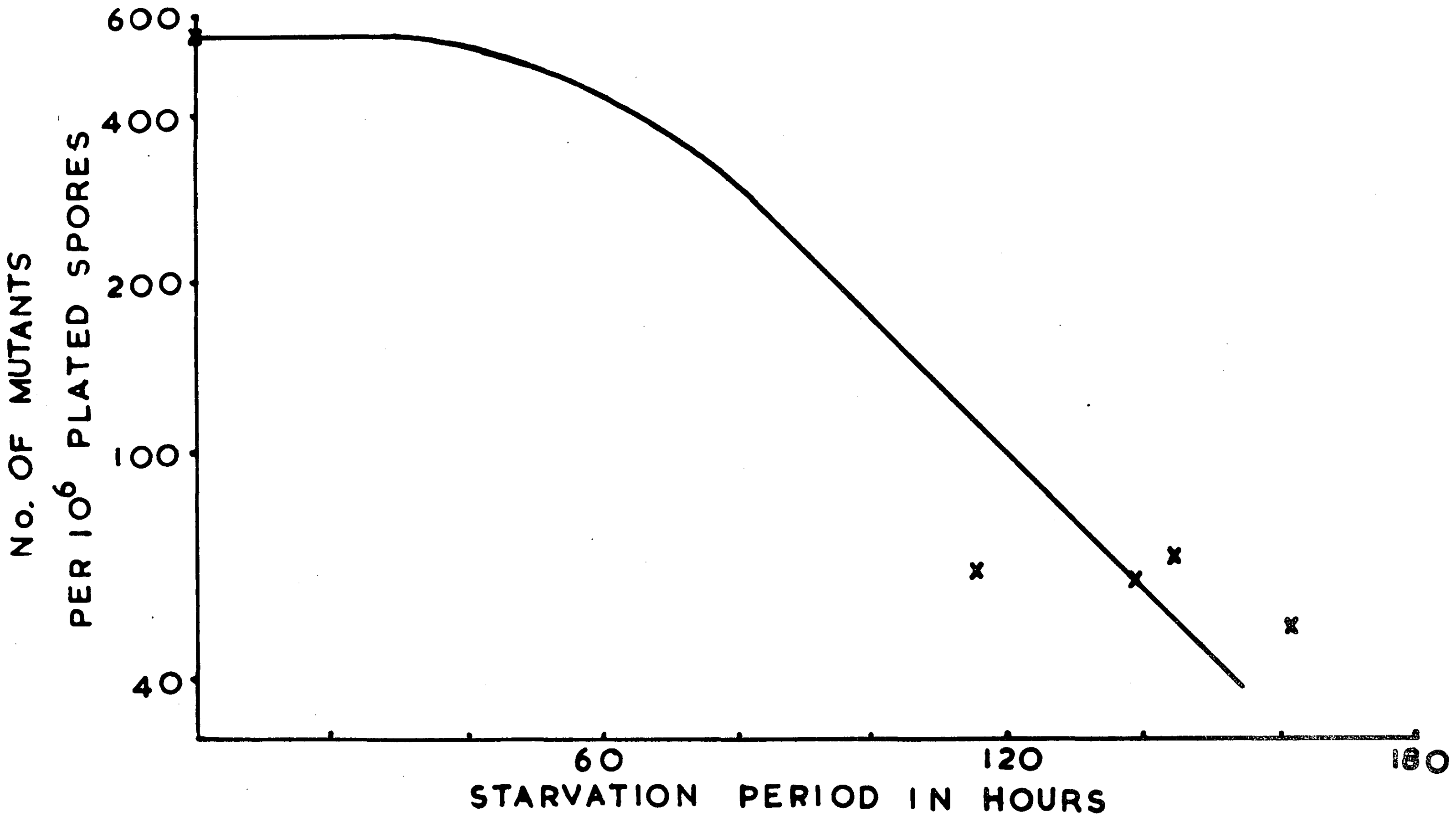


FIG. 8. Data from Table 11: Combined results of u/v 95% kill. As in fig. 7, nutritional mutant conidia die off as starvation progresses on minimal medium.

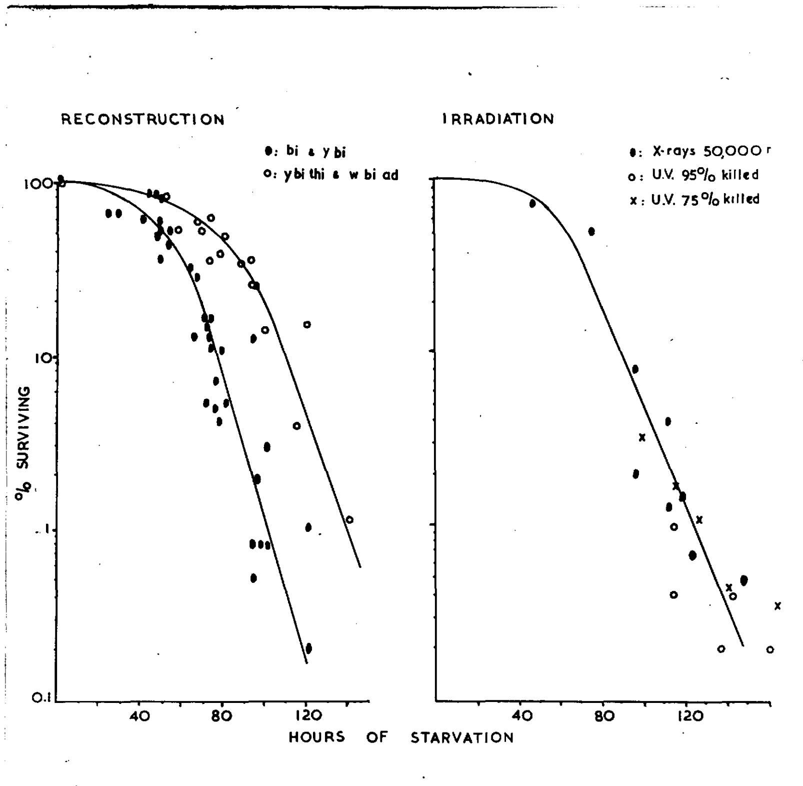


Plate 4. In the graph on the left, the conidia of strains bi and ybi, requiring biotin, are shown to die much faster on minimal medium than conidia of strains derived from them (y bi thi and bi w ad) and requiring "thiazole" and adenine in addition to biotin. A comparison of both graphs shows that conidia of the biotin requiring mutant survive on minimal medium longer after mutagenic treatment than with no treatment, due to the increased proportion of mutants and probably also to delayed germination effects.

5. Routine identification of growth factor requirements of nutritional mutants.

After nutritional mutants have been established as such, two general methods of characterisation were used.

a) The Plate Test Method: This involves the testing of mutant strains for ability or otherwise to grow on the minimal medium sufficient for the parent strain, supplemented with fractions of complete medium. The agar medium was poured into Petri dishes and twenty strains could be tested in marked positions on each dish. In the starvation experiments the parental minimal medium was minimal medium with added desthio-biotin. This substance was used in place of biotin since the parent strain will utilise both equally well and it was hoped that a mutant might be isolated with a metabolic block between desthiobiotin and biotin. (No such mutant was in fact found).

b) Auxanography: Conidia of a nutritional mutant were seeded into agar medium (minimal for the parent strain) while it was still molten (but below 55°C). The medium was then poured into one or more Petri dishes and on solidification was incubated for about 8 hours. Growth factor substances were then spotted in different positions on the dish or dishes and, after further incu-

bation, an area of growth round a particular nutriant indicated a requirement for that substance.

The rule is that when a large number of strains have to be tested on a few substances, plate test methods are used. In the opposite case, when a few strains have to be tested on many different substances, auxanography is used.

<u>The isolation and characterisation of nutritional mutants</u>.

After the conidia surviving irradiation and starvation had been grown on ultra complete medium for 36 hours, the plates were removed from incubation and colony counts made. At that time, the colonies had not begun to spore so that mycelial isolates could safely be made without fear of intercolony spore contamination. These isolates were done with the aid of a fork shaped needle (made by twisting two strands of michrome wire together) and were placed in marked positions on plates of minimal medium supplemented with desthiobiotin, twenty isolates per dish. When the colonies were rather dense, a random sample was taken of those which were discrete. No attempt was made, at any time, to select colonies either by their size or morphological characteristics. The starvation experiment plates were then re-incubated and any further

colonies which developed were counted and those which could be isolated were picked up as before, care being taken to avoid intercolony spore contamination at this point.

The test plates were then incubated for 48 hours and the non-growing colonies were rescued on to ultra complete medium slopes. These colonies were able to survive on the tiny piece of complete medium which came over with each isolate. The slopes were then incubated for 5 to 6 days, when the majority had spored. Any further plate tests were made by conidial inoculations (apart, of course, from the non-sporing colonies, where mycelia inoculations were used); these were done from below with the plates inverted to prevent spore scatter. Normally at this point, duplicate plate tests were made on parental minimal medium and ultra complete medium so that morphological mutants, characterised by poor growth on both media, could be discarded.

Experience regulated further plate testing. Since most of the mutants isolated from starvation experiments were unable to utilise sulphate, requiring sulphur in a more reduced form, the next series of plate tests were done on the parental minimal medium supplemented with thiosulphate, those mutants which responded were then tested on a sulphite medium. The

next commonest type of requirement was for adenine, so that those strains showing no growth on the thiosulphate plates were tested on parental minimal medium with adenine added. Those strains remaining were then tested on casein hydrolysate and vitamin supplemented plates. Strains responding to either of the latter two supplements were finally characterised by auxanography.

A diagrammatic representation of mutant characterisation is shown in Plate 5 while Plates 6 - 8 show actual plate tests.

Nutritional mutants were listed as unclassified only after they had been shown not to respond to the substances or groups of substances listed below. Each substance was tested singly, apart from when otherwise mentioned, on auxanographic plates.

a) Amino acids and related compounds.

L Arginine HCl	L Lysine HCl	L Ornithine 2 HCl
L Proline	DL Methionine	L Cystine
DL Phenylalanine	Leucines (Natural)	DL Leucine
L Tyrosine	L Histidine HCl	DL Tryptophane
L Glutamine	L Asparagine	L Cystein
Glycine	L Glutamic acid	L Aspartic acid
DL Serine	DL Threonine	L Alanine
Anthranilic acid	L Citrulline	Kynurenine
L Isoleucine	L Valine	

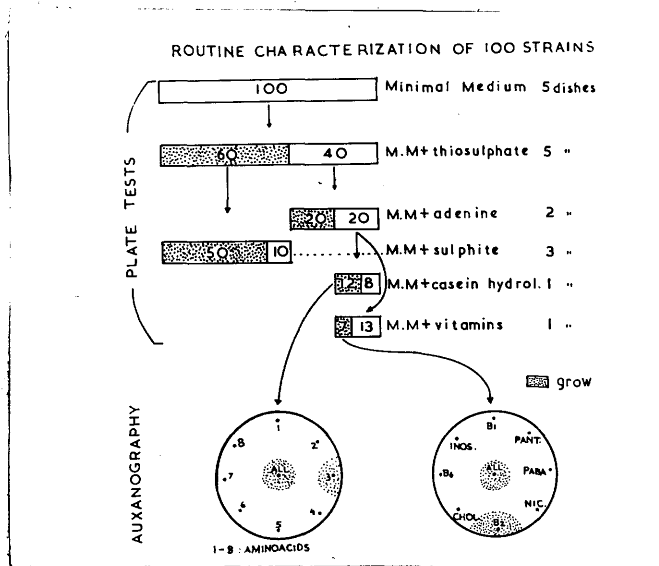


Plate 5. Diagrammatic representation of the methods used in characterising the growth factor requirements of nutritional mutants isolated by the starvation method.

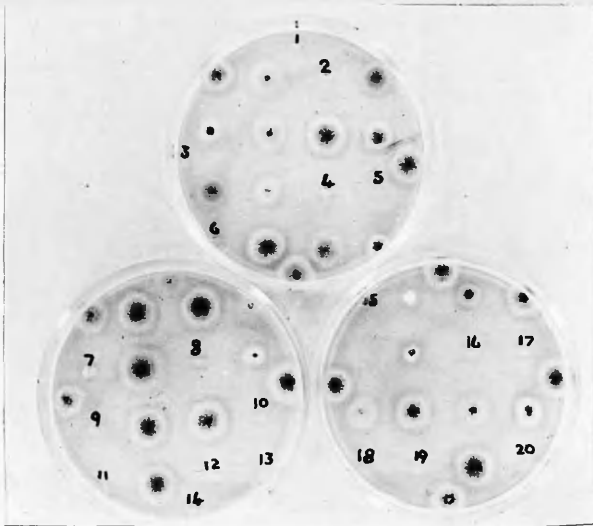


Plate 6. Isolates made from 60 colonies, arising on complete medium from conidia surviving mutagenic and starvation treatment, on to 3 plates of parental minimal medium. 20 isolates (marked 1-20) failed to grow and were rescued on to complete medium.

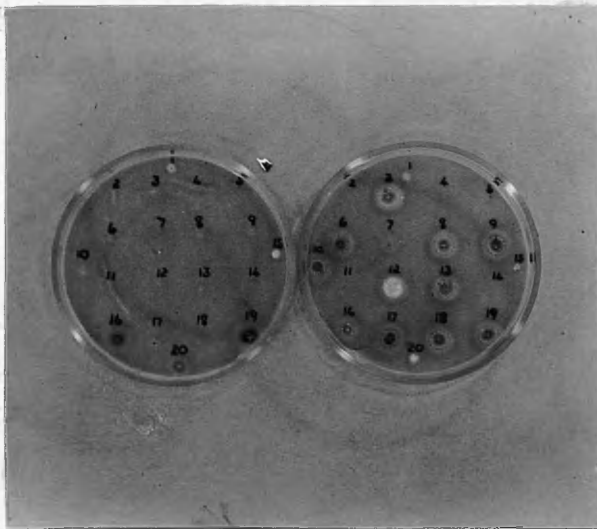


Plate 7. These 20 isolates were tested on parental minimal medium (left) and on this medium with thiosulphate (right). Isolates 16, 19 and 20 discarded as non-mutants. Isolates 3, 6, 8, 9, 10, 12, 13, 17 and 18 classified as thiosulphateless and the remainder tested as below.

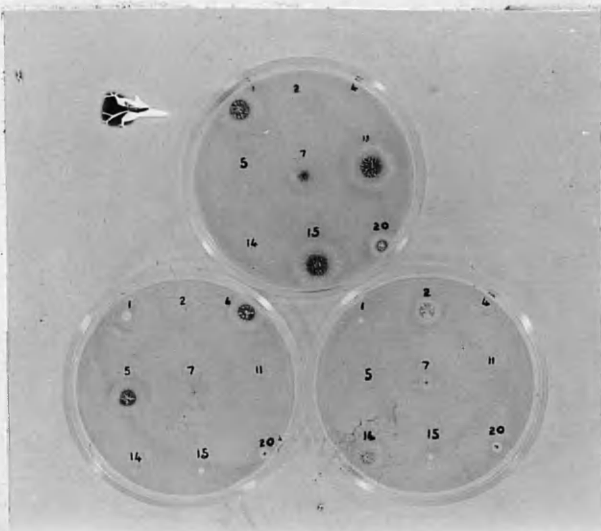


Plate 8. Remaining isolates tested on parental minimal medium with adenine (top), vitamins (left) and with casein hydrolysate (right). Further characterisation was carried out by auxanography.

b) Vitamins.

Ca Pantothenate	Riboflavin	Pyridoxin
Inositol	Nicotinic acid	Nicotinamide
Biotin	Desthiobiotin	Aneurin
Paraminobenzoic acid	Folic acid	Choline HCl

c) Purines, Pyrimidines, and their nucleosides.

Adenine HCl	Adenosine	Guanosine
Guanine	Uridine	Cytidine

Phenylalanine, p-aminobenzoic acid, tyrosine, and anthranilic acid were made up in a saturated solution and tested together

Leucine, isoleucine and valine were spotted in triangular fashion to facilitate the detection of interactions.

Out of 501 nutritional mutants tested, only eight remained unclassified; of these, seven respond to casein hydrolysate and probably require two seperate amino acids. There is some evidence that the remaining strain requires components from yeast nucleic acid hydrolysate and from casein hydrolysate.

Table's 14 - 16 show the classification of the nutritional mutants from starvation experiments.

Nutritional Mutants obtained in Starvation Experiments.

TABLE 14. X ray dosage 50,000r

1st Experiment

Series	Period of Starv^n in Hrs	No. of Isolates made	Number of mutants responding to:-						Unclassified Mutants	Total
			Adenine	SO_3^{--}	$S_2O_3^{--}$	NO_2^-	Amino Acids	Vitamins		
A	0	112	0	0	0	0	1 Arg/orn	0	0	1
B	46	115	0	2	0	0	0	0	0	2
C	74	88	1	1	0	0	0	0	1	3
D	96	80	2	5	2	0	0	1 pyridoxin	0	10
E	111	56	2	10	0	0	2 Arg/orn/prol.	1 aneurin 1 Nicotin-amide.	1	17

2nd Experiment

A	0	118	0	0	0	0	0	1 nicotinamide 1 aneurin 1 P.A.B.A.	0	3
B	96	39	0	5	1	0	1 cystine	1 Riboflavin	0	8
C	111	37	1	11	0	0	1 Arg/orn/prol 1 lysine	0	0	14
D	117	40	4	2	1	0	1 lysine	2 pyridoxin 1 P.A.B.A.	1	12
E	123	38	1	4	2	2 NH_4^+	1 proline	1 P.A.B.A.	0	11
F	147	54	0	12	3	1 NH_4^+	1 lysine 1 cystine 1 Arg/orn/prol. 1 Phenylalanine.	0	2	22

Nutritional Mutants obtained in Starvation Experiments.

TABLE 15. u/v dosage 95% kill.

Series	Period of Starv^n in Hrs.	No. of Isolates made	Number of mutants responding to:-						Unclassified Mutants	Total
			Adenine	SO_3^{--}	$S_2O_3^{--}$	NO_2^-	Amino Acids	Vitamins		
1st Experiment.										
A	0	200	1	1	0	0	0	0	0	2
B	114	100	22	5	1	0	0	2 pyridoxin	1	31
C	137	138	32	50	2	0	2 lysine 1 proline 2 Arg/orn/prol	0	0	89
D	161	140*	14	53	1	0	2 Arg/orn/prol. 1 lysine.	0	1	72
2nd Experiment.										
A	0	200	1	0	0	0	1 Arg/orn/prol.	1 P.A.B.A.	0	3
B	114	200	3	19	0	0	2 Arg/orn/prol.	1 P.A.B.A.	0	25
C	143	200	34	52	1	0	3 Arg/orn/prol. 1 Ornithine	0	0	91

Nutritional Mutants obtained in Starvation Experiments.

TABLE 16. u/v dosage 75% kill.

Series	Period of Starv^n in Hrs	No. of Isolates made	Number of mutants responding to:-						Unclassified Mutants	Total
			Adenine	SO_3^{--}	$S_2O_3^{--}$	NO_2^-	Amino Acids	Vitamins		
A	0	500	0	0	0	0	0	1 choline 1 riboflavin	0	2
B	96	160	0	1	0	0	0	1 pyridoxin	0	2
C	112	175	3	8	0	0	1 arg/orn/prol.	0	0	12
D	123	123	3	7	0	0	0	0	0	10
E	136	86	6	16	0	0	0	1 pyridoxin 1 nicotin-amide.	0	24
F	159	160	7	24	0	0	1 lysine 1 isoleucine	1 aneurin	1	35

6. The Efficiency of the Starvation Method and a comparison of mutants obtained with other techniques.

For comparative purposes, a list of nutritional mutants obtained from *Aspergillus nidulans* both by Total Isolation and Starvation Methods is shown in Table 17 and 17A. Most of the Total Isolation data has been very kindly made available by Dr. G. Pontecorvo and Dr. J.A. Roper (see Pontecorvo 1952).

In an initial comparison between Total Isolation, Delayed Enrichment (Pontecorvo 1952, c.f. Lederberg and Tatum 1946) and Starvation Techniques, the over all efficiency of the starvation method can be clearly seen (Plate 9). A more particular comparison between Total Isolation and Starvation Methods shows large quantitative differences, when the mutants are distributed into certain main groups (Plate 10). The most striking quantitative differences are shown by sulphiteless and adenineless mutants. From Total Isolation data available, these two mutant groups are in fact less numerous than mutants requiring vitamins or amino acids (Fig 10). The vast quantitative distortion in the spectrum of mutants after starvation by both sulphite and adenine requiring strains can only be reasonably explained by their being very much

A Summary of nutritional mutants obtained in *Aspergillus nidulans*.

TABLE 17: by Total Isolation Methods. TABLE 17A: by the Starvation Method.

	17: A	17: B	17: C	17: D	17: E	17A: A	17A: B	17A: C	17A: D	17A: E
Parental Strains.	bi, y, y_2, y thi, Wa, and +	bi	bi	bi	bi, y, y_2, y thi, Wa, and +.	bi	bi	bi	bi	bi
Mutagenic agent and dosage and starv^n period where applicable.	X rays 50,000r.	u/v 95% kill	u/v 75% kill	Total u/v (B+C)	Grand Total (A+D)	X rays 50,000r 46 Hrs. and 74 Hrs.	X rays 50,000r 96 Hrs. or more	u/v 95% kill 96 Hrs. or more	u/v 75% kill 96 Hrs. or more	Total 96 Hrs or more (B+C+D)
Parathiotrophic										
Sulphiteless	4	1	0	1	5	3	49	179	56	284
Thiosulphateless	2	0	0	0	2	0	9	5	0	14
Parazotrophic										
Nitriteless	11	0	0	0	11	0	0	0	0	0
NH_4-less	2	0	0	0	2	0	3	0	0	3
Amino Acids.										
Arginine	1	0	0	0	1	0	0	0	0	0
Arginine/Ornithine	4	1	0	1	5	0	0	0	0	0
Arg./Orn./Prol.	1	0	0	0	1	0	4	9	1	14
Lysine	3	0	0	0	3	0	3	3	1	7
Proline	0	0	0	0	0	0	1	1	0	2

Ornithine	0	0	0	0	0	0	0	1	0	1
Methionine	1	0	0	0	1	0	0	0	0	0
Methionine/Cystine	1	0	0	0	1	0	2	0	0	2
Phenylalanine	0	0	0	0	0	0	1	0	0	1
Isoleucine	0	0	0	0	0	0	0	0	1	1
Casein Hydrolysate (not single Acid)	0	0	0	0	0	0	4	2	1	7
Nucleic Acid Comp.										
Adenosine	8	2	0	2	10	1	10	105	19	134
Uridine/Cytidine	1	0	0	0	1	0	0	0	0	0
Vitamins										
Biotin (Data not comparable).	4	0	0	0	4	0	0	0	0	0
P-aminobenzoic acid.	1	1	0	1	2	0	2	1	0	3
Pantothenate	1	0	0	0	1	0	0	0	0	0
Nicotinic/Anthranilic Acids	4	0	0	0	4	0	1	0	0	1
Nicotinic/Anthranilic/Tryptophane	3	0	0	0	3	0	0	0	1	1
Riboflavin	0	0	1	1	1	0	1	0	0	1
Pyridoxin	0	0	0	0	0	0	3	2	2	7
Choline	0	0	1	1	1	0	0	0	0	0
Aneurin (Data not comparable).	3	0	0	0	3	0	1	0	1	2
Unknown.	2	0	0	0	2	0	0	0	0	0
Unknown Factors or Multiple.	0	0	0	0	0	1	0	0	0	0
Mutants:-										
fully tested	57	5	2	7	64	5	94	308	83	485
not fully tested.	24	0	0	0	24	0	0	0	0	0
Total Mutants	81	5	2	7	88	5	94	308	83	485
Total Isolates	3438	400	500	900	4338	203	344	777	704	1825
%age of Mutants.	2.35%	1.25%	0.4%	0.78%	2.03%	2.35%	27%	39.6%	11.9%	26.6%

EFFICIENCY OF DIFFERENT TECHNIQUES FOR THE ISOLATION OF MUTANTS IN ASPERGILLUS NIDULANS.

	X-RAYS 50.000 r.			U.V. 95% killed
	TOTAL ISOLAT.	DELAYED ENRICH.	STARV. ≥96 h.	STARV. ≥96 h.
ISOLATES	3438	71	344	777
MUTANTS	81	4	94	309
Percent	2.4%	5.6%	27%	39%

Plate 9. The efficiency of the starvation technique is very much greater than that of other techniques for the isolation of nutritional mutants.

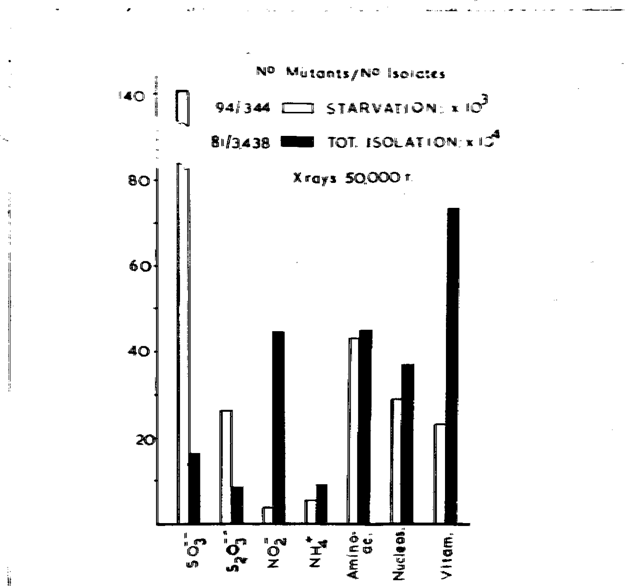


Plate 10. The spectrum of mutants obtained with the starvation technique covers the same qualitative range as Total Isolation. There are, however, large quantitative differences and some qualitative differences in detail.

better able to survive starvation treatment than any other group of mutants.

A more careful analysis of specific requirements illustrates that there are also qualitative differences present (see Tables 17 and 17A). For example, when the mutants which grow on arginine were examined for their responses to ornithine and proline, it was shown that all the arginine requiring mutants isolated by the starvation method would also grow both on ornithine and proline; whereas, among those picked up by Total Isolation methods, a requirement for arginine or ornithine but not proline was most common, although the other two types were also present (i.e. those which would only grow on arginine and those which grew equally well on arginine, ornithine and proline). Mutants unable to utilise nitrate but which grow on nitrite or a more reduced form of nitrogen, are also absent from the starvation isolates.

Another comparison which can be made is that of the relative efficiency of the starvation method over different starvation periods (Table 18, Figs. 9-12).

It is clear that the period of starvation is of considerable importance with regards to the efficiency of the technique for the isolation of any one group of mutants. From the experimental results, those mutants

requiring vitamins are most abundant among isolates after a period of about 4½ days starvation but after 6 to 7 days starvation, however, less are isolated than would be by normal Total Isolation. On the other hand, for example, the technique becomes more efficient for the isolation of amino acid requiring mutants as starvation progresses up to the maximum period which was done.

Nutritional Mutants classified as to their occurence in different starvation periods.

TABLE 18.

Period of Starv^{n} in Hrs.	No. of Isolates	Mutants responding to:-						Unclassi-fied Mutants	Total Mutants.
		Purines and Pyrimid-ines	SO_3^{--}	$S_2O_3^{--}$	NO_2^-	NH_4^+	Amino Acids	Vitamins		
0 ‡ (control)	4338	11	5	2	11	2	12	21	24	88
∴ per	1000	2.54	1.15	0.46	2.54	0.46	2.76	4.84	5.53	20.28
96-117	887	37	66	5	0	0	9	11	3	131
∴ per	1000	41.71	74.41	5.64	0	0	10.15	12.40	3.38	147.68
118-139	384	42	77	4	0	2	6	3	0	134
∴ per	1000	109.38	200.51	10.41	0	5.21	15.63	7.81	0	348.96
140-161	454	55	141	5	0	1	13	1	4	220
∴ per	1000	121.15	310.57	11.01	0	2.23	28.63	2.23	8.81	484.58

‡ Includes all Total Isolation data from Table 17.

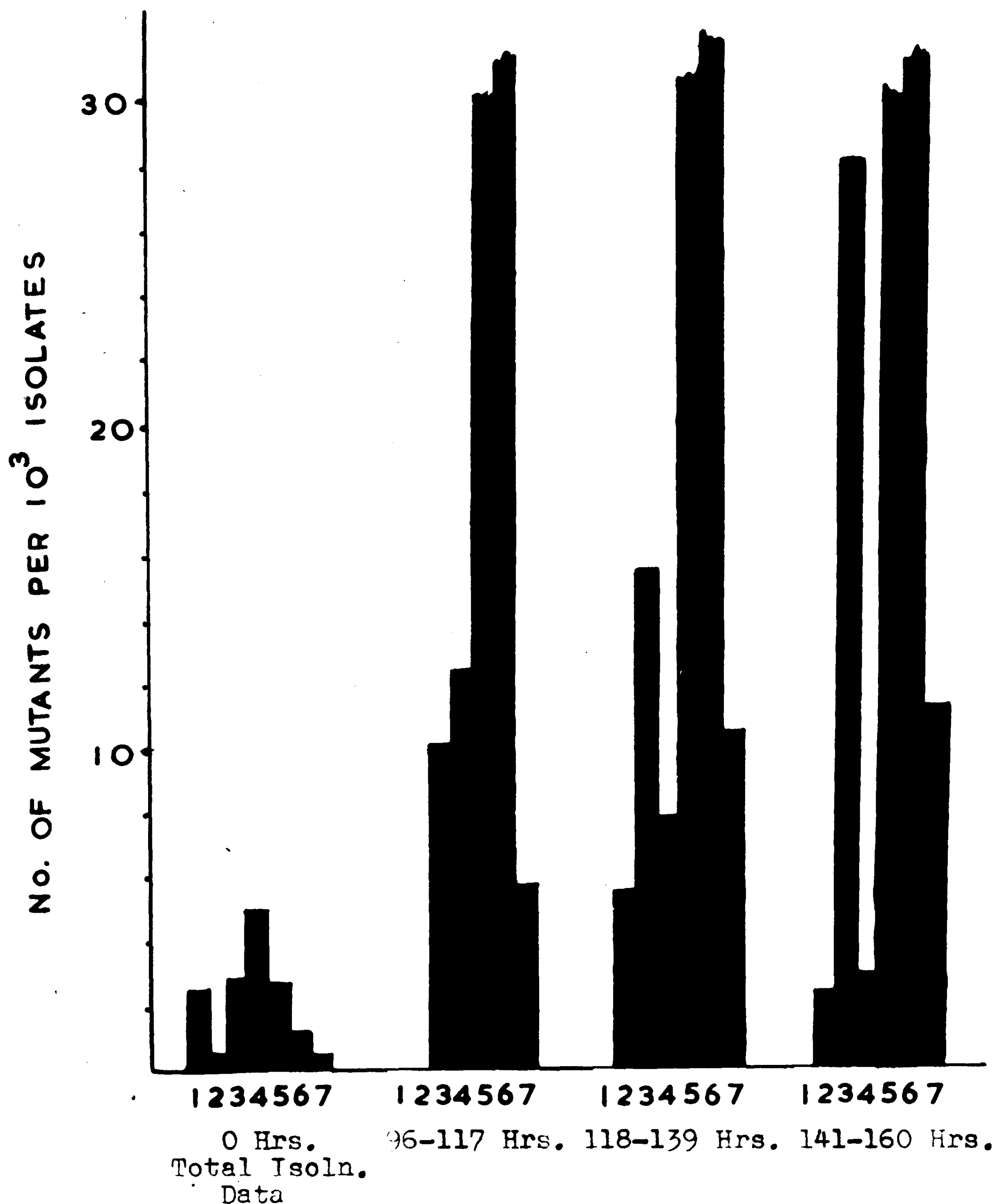


FIG. 10. This is similar to fig. 9, but the ordinate scale has been adjusted to show the quantitative variation more clearly in groups of mutants other than those requiring sulphite, or purines or pyrimidines.
The abcissa notations are the same as in fig. 9.

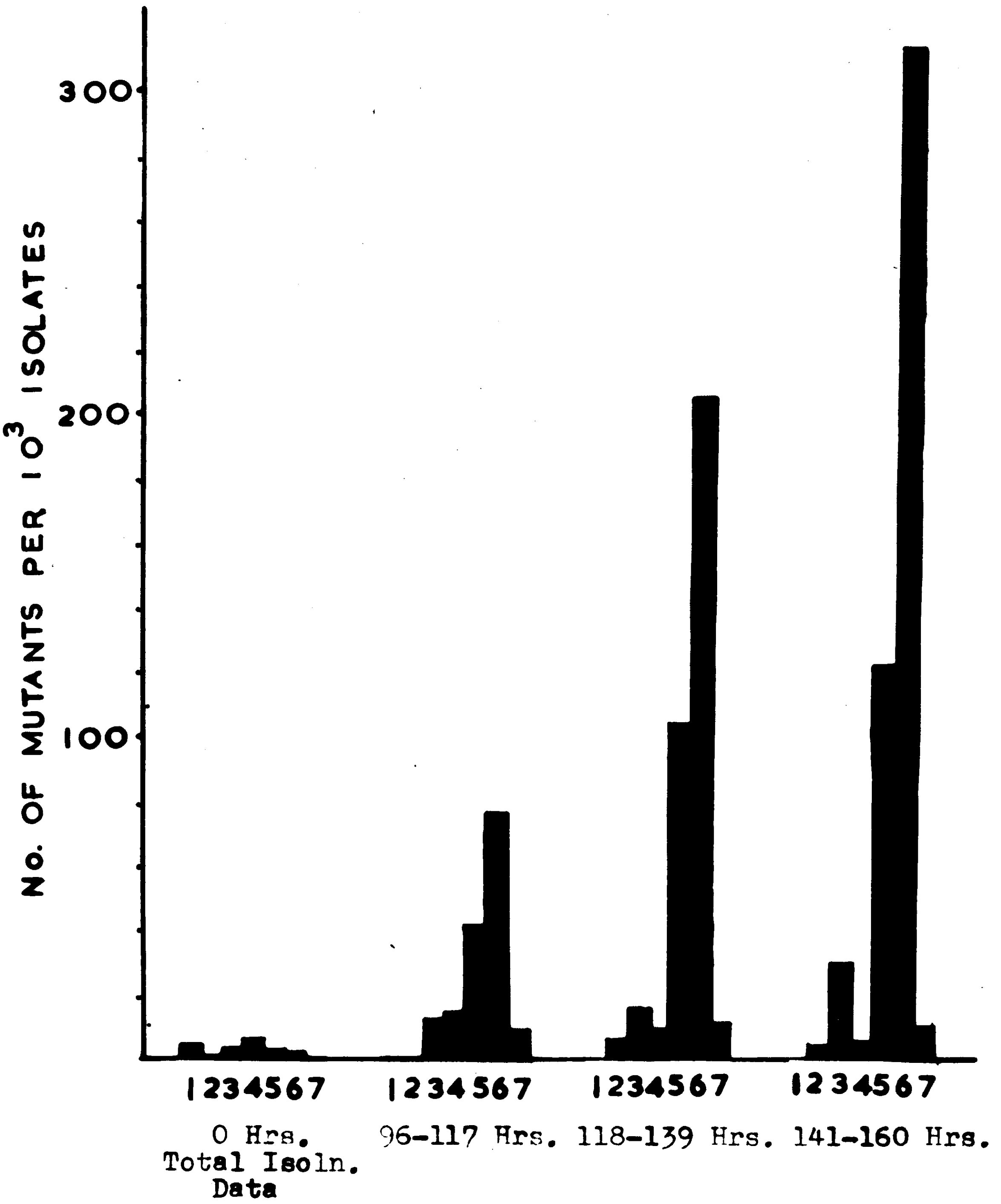


FIG. 9. The quantitative spectra of groups of mutants are shown over different starvation periods. Large quantitative differences are apparent.

Abcissa No.	1	2	3	4	5	6	7
Mutant group requiring:-	NO_2^-	NH_4^+	Amino Acids	Vits	Pur & Pyr	SO_3^{--}	$S_2O_3^{--}$

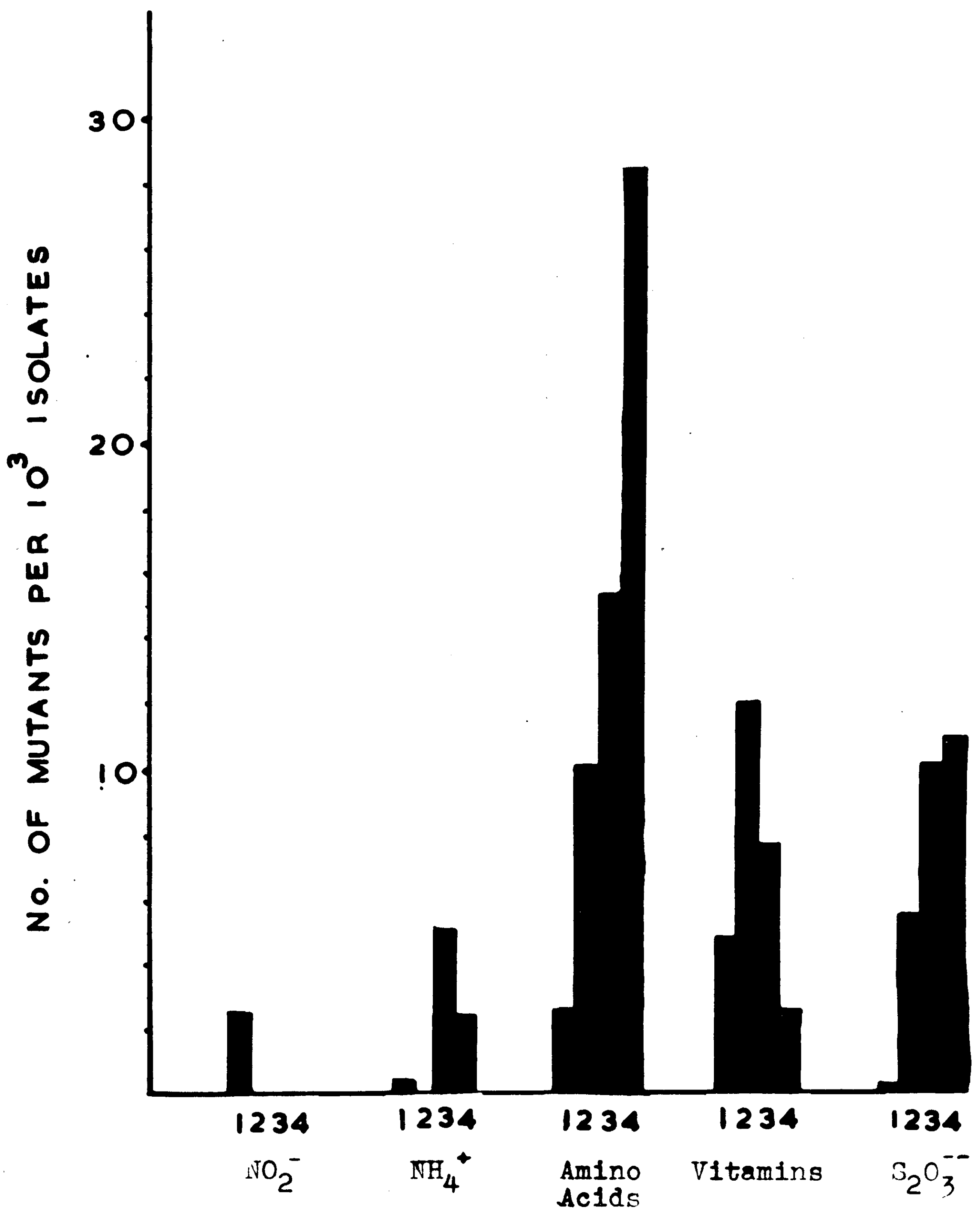


FIG. 12. The quantitative variation in each group of mutants is shown over different periods of starvation. The abcissa notations are as in fig. 11.

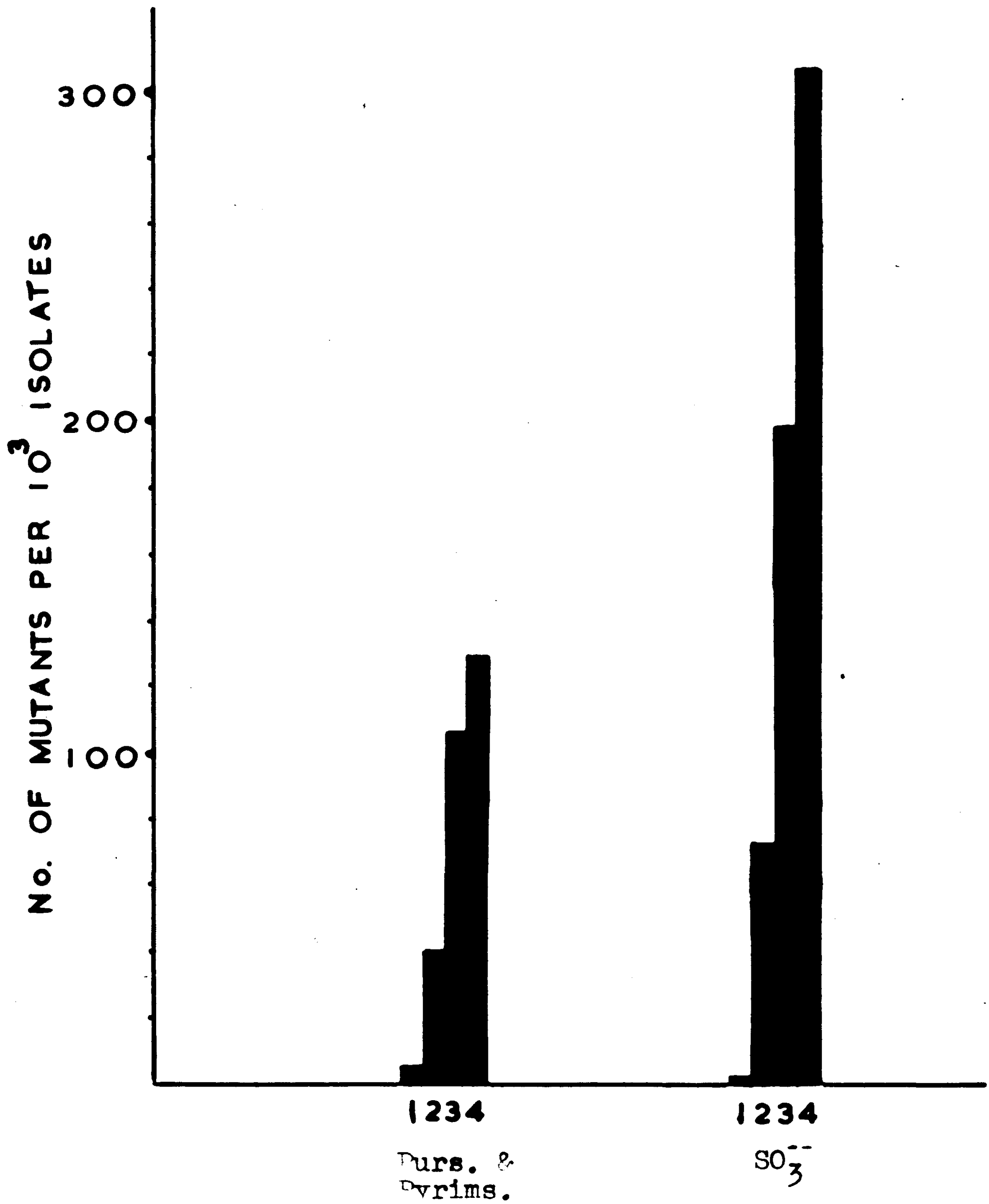


FIG. 11. The quantitative distribution of two groups of mutants is shown over different starvation periods.

Abcissa No.	1	2	3	4
Period of Starvation in Hours	0 (Total Isoln.)	96-117	118-139	140-161

7. Discussion and Conclusions.

In his starvation experiments, Fries suspended untreated conidia from individual strains of Ophiostoma multiannulatum in a liquid starvation medium and after periods ranging from about 30 to 50 days at 25°C, plated out suspensions on to complete medium, isolating and testing those single colonies which developed from surviving conidia. On the average close on 1% of the isolates proved to be nutritional mutants (Fries 1948a, 1948b).

In the starvation method which has been developed for the isolation of nutritional mutants in Aspergillus nidulans and which has been described in this part of the thesis, Fries' methods have been telescoped somewhat and the experimental manipulations rendered more simple. The efficiency of the starvation method has also been greatly increased notably by pre-treatment of conidia of the mother strain with u/v or X rays prior to starvation. The method in Aspergillus nidulans is to layer plate the treated conidia of the biotin requiring mutant on to minimal medium and after 4 to 6 days at 37°C, add a top layer of complete medium. Over 40% of the isolates made from the individual colonies which develop are nutritional mutants.

Delayed phenotypic expressions are not uncommon among certain bacterial mutants (Demerec and Latarjet 1946, Davis 1948, and Newcombe and Scott 1949) and as has been mentioned, it was conceivable that on subjecting conidia with newly induced nutritional deficiencies to the effects of immediate starvation, a phenotypic lag might occur in their reaction to the environment and continue long enough to allow those conidia to die off as quickly as the parental biotinless strain. The fact that, in general at least, no such effect was found might be an expression of the lack of capacity of conidia to store growth factors which, after mutation, they are no longer able to synthesise; since if sufficient of such growth factors were present for normal germination to proceed then presumably there would in fact be a phenotypic lag.

Out of 501 nutritional mutants which were isolated by the starvation method, all but 8 were completely characterised and were shown to require one other growth factor in addition to the biotin requirement common to all. These results are of interest in connection with Horowitz's approach to the one gene/one enzyme hypothesis (Horowitz 1950). The origin of this hypothesis is not clear, but the idea is implied in the works of Garrod, Onslow, Haldane and others, and has

been more recently formulated by Beadle (1946). Since then, he has reclarified the conception of the hypothesis which he now calls the one gene/one function hypothesis, to take into account the fact that the specifity of nucleic acids, non enzymatic proteins and perhaps other compounds are gene controlled (Beadle 1951). The suggestion is that a given gene is concerned in a primary way with but one single enzyme. Whether a gene may act as a template in the production of a specific enzyme or not can only be conjectured but the hypothesis implies that an enzyme cannot arise de novo in the absence of the corresponding gene. The hypothesis is then that all the genes in an organism are each concerned in a primary way with individual enzymes or functions.

In _Neurospora_ many mutants with single growth factor deficiencies have been shown by genetical analysis to differ by single genes from wild type. Out of 484 independently occuring nutritional mutants, which have been obtained in this organism, 405 or 84% require one single growth factor and it can be reasonably assumed that in these 405 mutants one gene is concerned with but one function (Horowitz 1950). The requirements of the remaining 16% have not been worked out but these will probably include mutants having requirements for single growth factors not so far tested and mutants with two

seperate gene mutations.

The question arises whether all the genes in _Neurospora_ are unifunctional? One appreach is to make a search for multifunctional genes.

Many gene functions must be irreparable from the environment and if it is assumed that the distribution of such functions is random between unifunctional and multifunctional genes, then the possibility of finding the latter type genes will be less than the former. Horowitz (1950) has attempted to estimate the fraction of irreparable gene functions by reference to so called 'temperature' mutants in _Neurospora_. At 25°C some mutants grow on minimal medium but at a higher temperature (35°C) they will only grow on complete medium and are found to have specific single growth factor requirements at this temperature. Other mutants which also grow normally at 25°C on minimal medium are found to be unable to grow at 35°C, even on complete medium. Presumably in the latter case, functions which are not reparable from the environment have been lost when growth is at 35°C. The proportion of the two types of mutants is 1 : 1 approximately. If the assumption is made that the likelihood of genes mutating to temperatures alleles is the same whether they have reparable or irreparable functions, then a measure of the fre-

quency of both functions is available. That is to say, of all gene functions, approximately half are irreparable under the experimental conditions involved. On this basis it becomes possible to correct data allowing for the fact that unifunctional genes are more likely to be recovered than multifunctional genes. Assuming that the unclassified 16% of nutritional mutants in <u>Neurospora</u> were mutants of bifunctional genes, Horowitz (1950) estimated the correct frequency of unifunctional genes as follows:-

$$\text{Observed frequency} = \frac{84}{84 + 16} = 0.84$$

$$\text{Corrected frequency} = \frac{84 \times 2}{84 \times 2 + 16 \times 4} = 0.73$$

i.e. about 73% of the genes in <u>Neurospora</u> are unifunctional. This estimate agrees very closely with the result obtained when the Poisson distribution is applied to account for genes with more than two functions in the unclassified 16%. It would appear unlikely that selection is the cause of such a high frequency of unifunctional genes being recovered and since the figure obtained could be a minimum one, the evidence is not necessarily inconsistent with all genes being unifunctional.

Making similar assumptions, the evidence in <u>Ophiostoma multiannulatum</u> and <u>Aspergillus nidulans</u> is

even more suggestive that all the genes in these micro-organisms are unifunctional. In Ophiostoma 435 out of 463 or 94% mutants require single growth factors (Fries 1947) and in Aspergillus 98% of the 501 mutants isolated after starvation each require one growth factor.

The use of a complex medium in the hope of isolating a great variety of mutants does not recommend itself. As has been mentioned, out of 501 mutants isolated only 8 remain uncharacterised; seven of the latter grew on casein hydrolysate supplemented minimal (and probably are double mutants having two growth factor requirements) while there is some evidence that the remaining strain requires components of both yeast nucleic acid and casein hydrolysates. Probably none of these nutritional mutants have growth factor requirements of an unusual nature and, although blood hydrolysates were incorporated in the complete medium, no mutants requiring haem derivatives were in fact isolated.

It is becoming increasingly clear that certain types of mutants can never be isolated when complete media are used, even although they can survive when the required growth factor alone is supplied exogenously. Such mutants are inhibited by other substances present in the complete media (e.f. Lein, Mitchell and Houlahan 1948). Fortunately other techniques have

been devised which will be discussed in the introduction to Part IV of this thesis, and the starvation method has also been adapted so that these types of mutants can be picked up. These selective starvation methods will be described in Part IV.

The efficiency of the starvation method over other isolation techniques has been amply demonstrated. The obvious conclusions to be drawn from the large quantitative differences in the types of mutants isolated after starvation, when compared with those from Total Isolation, is that there is a selection in progress for certain nutritional mutants by virtue of their slower dying off under starvation. It would appear that the dying off rates of strains requiring one other growth factor in addition to biotin are not due solely to their increased degree of heterotrophy as Fries (1948a, 1948b) has suggested but dependent also on the specific additional requirement. This will be discussed more fully in the next part of the thesis.

The qualitative differences are also of some interest. Presumably those mutants not found after starvation die off relatively quickly.

Certain mutants have been isolated after starvation and not by Total Isolation, and include those which

require phenylalanine, isoleucine, pyridoxin, etc. This is almost certainly due to the comparatively small number of mutants which were obtained by the latter method. It is extremely unlikely that any mutants not obtainable by Total Isolation would come up after starvation unless perhaps if delayed germination was concomitant with a growth factor requirement. There is no evidence of this among any of the mutants isolated.

It has been suggested that the qualitative and quantitative spectrum of nutritional mutants obtained in microorganisms is a measure more of gene stability than of the particular action of a mutagenic agent and furthermore that the genes most susceptible to change do not necessarily control the same biosynthetic processes as evidenced by the distribution of nutritional mutant types in various fungi and bacteria. (Tatum, Barrat, Fries and Bonner 1950).

For instance, in Neurospora crassa the commonest types of nutritional mutants are those requiring methionine adenine, arginine and lysine (Tatum et al 1950), whereas in Escherichia coli the commonest type is proline requiring (Tatum 1946) and in Saccharomyces cerevisiae mutants requiring histidine, adenine, tryptophane, lysine and methionine are the most fre-

quently found (Reaume and Tatum 1949, and Bevan, unpublished).

A reassessment is made of this problem here. It would appear that the main factors of importance in determining the qualitative and quantitative distribution of nutritional mutants found in individual microorganisms, after mutagenic treatment, are as follows:

a) The mumber of genes involved in the synthesis of specific essential metabolites,

b) The mutability of these genes,

c) The mutagenic agent employed,

d) The isolation method used.

a) The number of genes involved in the synthesis of a particular essential metabolite will obviously have a bearing on the likelihood of a mutation blocking that synthetic pathway. It would be expected that the greater the number of genes, the more chance there was of a mutation occuring. However, this conception would be modified, to some extent, in considering the question of gene mutability.

b) Stadler (1930, 1932) has shown that genes affecting endosperm character in maize have widely different mutation rates. Apart from such individual gene differences, certain genes may affect the muta-

bility of others or of the whole organism. It has been shown that in maize the presence of a certain dominant gene increases enormously the mutation rate of another recessive gene to its dominant allele form (Rhoades 1941). In _Drosophila_ the frequency of over all mutation rate as measured by the percentage of sex linked recessive lethals may be increased by a factor of ten by the presence of a specific gene in the 2nd chromosome (Demerec 1937). It is reasonable to assume that similar variations will be found in microorganisms and in _Escherichia coli_, for example, the mutation rates towards resistance to different bacteriophages varies considerably (Demerec and Fano 1945).

c) Turning to the question of mutagenesis, although X rays and u.v. light are apparently non-specific in their mutagenic activity, it cannot be presumed, with regards to the production of nutritional mutants, that they produce the same quantitative results, although there is no evidence that the results are qualitatively distinguishable. Of the large number of chemical substances now known to be mutagenic all of them have been described as being aspecific in their action, apart from a few possible exceptions (Auerbach 1949).

In barley there is a different distribution of certain albino mutants following nitrogen mustard

treatment, than after irradiation (Gustafsson and MacKey 1948). However until the genetic nature of these mutants is established, it is possible that the types of changes in chromosome structure may account for the difference. Few large chromosomal rearrangements are found after nitrogen mustard treatment as compared to the relatively large number found after irradiation (Auerbach 1949). Hadorn, Rosin, and Bertani (1949) have suggested that phenol, which is weakly mutagenic in *Drosophila*, may act specifically on certain phenol sensitive loci; certain lethal alleles turn up repeatedly in widely separated experimental series. On the other hand, Jensen, Kolmark and Westergaard (1949) failed to find any mutagenic activity for phenol using the *Neurospora* back mutation method. The serum from rabbits immunised against *Neurospora*, increased the mutation rate in this fungus and it was suggested that the antibodies against the fungus were responsible for the mutagenic action (Emerson 1944). Certain analogies between antibodies and enzymes has led to the hope, as yet unconfirmed, that more specific mutagenesis might in fact become possible (Emerson 1945, Hindle and Pontecorvo unpublished quoted in Auerbach 1949).

It remains possible, though there is no real evidence so far to support it, that a single gene varies in its susceptibilities to different mutagenic agents and that

a more careful quantitative rather than qualitative analysis in the future will demonstrate this.

d) If the mutants obtained in <u>Aspergillus nidulans</u> by Total Isolation are examined, it will be seen that vitamin requiring mutants are the most common while, among the starvation isolated mutants, sulphite-less mutants preponderate (Fig.10). Apart from these and other quantitative differences, there are biochemical differences between the types of mutants isolated by either method. For example with Total Isolation, three types of arginine requiring mutants can be picked up, differing in their ability to utilise proline or orinthine in place of arginine, only one of these types, however, occurs after starvation (Tables 17, 17a). This emphasises the misleading conclusions which can be drawn about distributions when the selective action of special techniques is ignored. Indeed, it is felt that similar isolation techniques, when used by different workers, may not be objective enough as yet for strictly valid comparisons to be made.

In conclusion, it can be said that the frequent occurence of any one type of nutritional mutant is not simply related to gene mutability, but may also be a measure of the number of genes controlling a particular chain of reactions. The frequency will also depend on

the method of isolation, if it is in any way selective and possibly on the type of mutagenic agent used.

<u>Summary</u>:

1. When conidia of a biotinless strain of <u>Aspergillus nidulans</u> are irradiated and starved on minimal medium for 96 hours or more at 37°C, then over 40% of the surviving conidia are found to be nutritional mutants.

2. 98% of the 501 mutants isolated by the starvation method were shown to have single growth factor requirements which offers support of the one gene/one function hypothesis.

3. A complex complete medium was used in the isolation of nutritional mutants but no rare or unusual mutants were found. Inhibitor substances present in complete medium may prevent the growth of certain mutants.

4. Large quantitative and a few qualitative differences are noticeable between the types of nutritional mutants found after Total Isolation and Starvation.

5. The frequent occurence of any one type of nutritional mutant in a microorganism is deemed not to be simply related to gene mutability but to be also a

measure of the number of genes involved in the synthesis of the specific metabolite, the isolation method employed, if selective, and possibly also of the mutagenic agent used.

PART III. Mechanism of Differential Survival under Starvation.

1. Introduction.

Although the starvation technique for the isolation of nutritional mutants was developed and successfully applied in *Aspergillus nidulans* (see Part II), nothing was in fact known of the mechanisms involved in differential survival. This part of the thesis describes investigations that were undertaken in an attempt to elucidate some of the problems presented.

Apart from the studies of Fries, the only observations of relevance to the behaviour of conidia under the starvation conditions employed in the work repeated here, have been those of Ryan (1948). In his microscopical investigations on the germination of macroconidia from *Neurospora crassa*, Ryan noted that whereas conidia from nutritional mutants requiring one growth factor, additional to the minimal requirements of the wild type strain, would germinate in a matter of hours on *Neurospora* minimal medium supplemented with the necessary growth factor, in the absence of the growth factor, germination although it did eventually occur, was delayed for several days.

Presumably, this delayed germination effect indicated that the metabolic rate of the macroconidia is lowered under starvation conditions. The phenomena can be compared to what occurs in conidia from strains of *Aspergillus nidulans* and *Ophiostoma multiannulatum* under similar conditions.

Fries' work has already been reviewed at length in the first part of this thesis. As the outcome of his studies with *Ophiostoma*, he suggested that, under starvation conditions, the viability of conidia would be directly proportional to their degree of heterotrophy. The results obtained in experiments using conidia from nutritional mutants of *Aspergillus nidulans* were in agreement with this (see Part I). Mutants with a requirement for biotin and one other growth factor survived starvation on minimal medium longer than a mutant with a requirement for biotin alone. However, after the starvation technique had been used for the isolation of nutritional mutants in *Aspergillus nidulans* it became apparent that Fries' interpretation was an over simplification. The vast quantitative and the qualitative differences which occured between the types of nutritional mutants found after Total Isolation and Starvation, indicated that conidia from certain nutritional mutant types were obviously better able to survive

starvation than others (see Tables 17, 17a, and Figs. 9 - 12).

Of the nutritional mutants isolated by the starvation technique, all but a few were completely characterised; these which were characterised had a requirement for biotin and one other growth factor.

The selective action of the starvation technique suggested that the survival on starvation medium of conidia from these doubly heterotrophic mutants was an expression of the particular growth factor requirement induced by mutagenic treatment, acting in conjunction with the biotin requirement and not merely one of increased degree of heterotrophy. Differential survival on starvation medium has already been shown to occur between the conidia from nutritional mutants with a different number of growth factor requirements (see Part I of this thesis and Fries 1948a, 1948b) but here, apparently, differential survival was occuring between conidia with the same number of growth factor requirements (two in this case). It might be expected that differential survival would also operate between conidia from nutritional mutants with single growth factor requirements. The only strain in _Aspergillus nidulans_ with a single requirement whose behaviour under starvation conditions was known was the strain requiring biotin. The dying

off rates of conidia from a number of other nutritional mutants with different single growth factor requirements were measured and the results are reported in this part of the thesis.

If conidia, surviving preferentially under starvation did behave in this way because of a lowered rate of metabolism it appeared conceivable that these conidia would also be better able to survive the action of sub lethal temperatures. An account is given of experiments designed to investigate the effects of starvation at sub lethal temperatures which were, however, inconclusive to say the least.

In the case of a nutritional mutant with two growth factor requirements, it was of interest to determine the relative importance of either deficiency in regulating the dying off rate of the conidia on minimal medium. One appreach which has been used and is described in this part of the thesis makes use of degrees of starvation.

For example, the conidia from a nutritional mutant requiring biotin and one other growth factor can be starved either on minimal medium, or on minimal medium supplemented with biotin, or on minimal medium supplemented with the other growth factor. Some assessment is then possible of the seperate and combined effects of these mutritional deficiences on the survival of the

of the conidia. Another method which can be used, of course, when the strains are available, is to estimate the dying off rates under starvation of conidia from the biotin requiring mutant and conidia from a mutant with the other growth factor requirement and compare these with that of conidia from the corresponding double nutritional mutant, in all cases on minimal medium. The evidence suggests that both methods give results in reasonable agreement with each other.

At the end of this part of the thesis, a general comparison, incorporating degrees of starvation, is made between the dying off rates of conidia from certain mutants obtained both by Total Isolation and Starvation methods. The primary expectation was that conidia from mutants isolated after starvation would in general be slower dying under starvation than conidia from mutants found after Total Isolation; the former technique tending to select slower dying strains.

2. Material and Methods.

Material.

a) Strains of *Aspergillus nidulans* used.

The following nutritional mutants were used in the experiments described in this part of the thesis.

The nutritional mutants described under Total Isolation were isolated either by Total Isolation or as recombinants from crosses involving strains found by Total Isolation. These were obtained by Dr. Pontecorvo and other workers in the Department of Genetics. The remaining nutritional mutants were isolated using the starvation technique.

Total Isolation Mutants.

Symbol or Code Number	Colour of Conidia.	Growth Factor Requirements.
bi	green	biotin
y bi	yellow	biotin
ad	green	adenine
wa lys	white	lysine
bi w ad	white	biotin and adenine
y bi thi	yellow	biotin and "thiazole"
bi lys_2	green	biotin and lysine
bi orn_2	green	biotin and arginine or ornithine
bi n_2	green	biotin and reduced N (NO_2)
S3A2	green	biotin and reduced S (So_3)

The strains with single deficiencies were obtained following X ray irradiation of 50,000r. Those with double deficiencies were either obtained in two stages after irradiation or as recombinants from crosses between singly deficient strains of X ray origin. An exception is S3A2 which was obtained by u/v treatment of bi (75% kill).

In all the strains with a biotin requirement, this requirement is determined by the same gene.

Starvation Treatment Mutants.

All these strains have green conidia and were derived by X ray or u/v treatment of the strain requiring biotin.

Code Number	Growth Factor requirement in addition to biotin.	Mutagenic Agent and Dosage	Period of Starv^{n}. in hours after which strain was isolated.
S5F1	"thiazole"	u/v 75% kill	159
S5F2	lysine	u/v 75% kill	159
S5F35	reduced S (sulphite)	u/v 75% kill	159
S4C96	arginine or ornithine or proline	u/v 95% kill	143
S2E11	reduced N (NH_4)	X ray 50,000r	123
S5E1	pyridoxin	u/v 75% kill	117
S2D1	p-aminobenzoic acid	X ray 50,000r	136

Master cultures of all these strains were established either from single ascospore, single conidial or single colony isolates on to slopes of minimal medium supple-

mented with the necessary growth factors. The growth factor requirements were checked. In the experiments to be described 6 day old serial sub cultures from mass transfer of conidia were used. All cultures were grown on minimal medium supplemented with required growth factors.

b) Liquid Minimal Medium.

Ingredients (2½ litre quantity)

15 gms $NaNO_3$	13gms $MgSO_4.7H_2O$
1.3 gms KCl	3.8 gms KH_2PO_4
1 crystal $FeSO_4$	1 minute crystal $ZnSO_4$

25 gms recrystallised Glucose (see below)

All the chemicals were of analytical reagent standard.

The preparation was conducted under acid clean conditions, all glass ware used being cleaned in chromic acid mixture followed by thorough rinsing in double distilled water.

The ingredients were made up to 2½ litres with double distilled water, the pH adjusted to 6.5 and the medium then autoclaved for 10 minutes at 10 lbs pressure.

Under sterile conditions the medium was then ~~aliquoted~~ poured into 100ml conical flasks in 9ml quantities. These flasks had been previously acid cleaned, dried, cotton wool plugged with and sterilised. The medium was

then stored at 4°C.

Preparation of Recrystallised Glucose:

100 gms were made up into a syrup by adding a little hot double distilled water. The syrup was heated and filtered through thoroughly washed filter paper. A volume of absolute alcohol equal to about three times the volume of the syrup was then added and the solution stored at 4°C for 24 hours. The crystals were then filtered off and dryed at 50°C.

c) Calzolene solution and Saline:

These solutions were prepared as before only in the experiments at sub lethal temperatures the preparation was carried out under acid clean conditions.

d) Fine chemical solutions:

These were all made up with double distilled water and autoclaved for 10 minutes at 10 lbs pressure.

The biotin, adenine, aneurin and thiosulphate solutions and the amount which allows good growth in mutants requiring these growth factors have been described under Material and Methods in Parts I and II.

L Lysine mono HCl:

A 50 mg/ml stock solution was held. 10 ml of this solution per litre of minimal medium assured good growth of lysine requiring mutants.

L Arginine mono HCl:

As for Lysine.

p-aminobenzoic acid:

A 100 microgm/ml stock solution was held. 1 ml of this solution per litre of minimal medium assured good growth of p-aminobenzoic acid requiring strains.

Pyridoxin:

As for p-aminobenzoic acid.

$(NH_4)_2SO_4$:

3 gms was added per litre of minimal medium for growth of strains requiring reduced nitrogen.

e) Glass Ware:

All the glass ware used in the experiments at sub lethal temperatures, prior to plating out the conidia on to complete medium, was acid cleaned.

f) Heating Unit.

In the experiments at sub lethal temperatures a Towers Heating Unit was used to heat a trough of water. The temperature control was a thermostatic mercury switch, supplied with the unit, which, when set, had a variation of not more than $\pm 0.15C^{o}$ at all the temperatures which were employed.

g) Shaker:

A mechanical shaker was devised, for use in the sub lethal temperature experiments, made mainly of Meccano parts and driven by a small electric motor. It was arranged so that four 100 ml conical flasks could be

clipped into position and shaken in the trough of water heated by the Tower's Unit.

The other materials (media, etc.) which were used have already been described in parts I and II.

Methods.

The methods used in the experiments at sub lethal temperatures are described in section 4; other methods which were employed in this part of the thesis have already been described in Parts I and II.

3. Starvation of conidia from strains with different single growth factor requirements:-

The large differences between the proportions of the various types of nutritional mutants obtained from starvation and those found after Total Isolation, suggested that the conidia from certain kinds of mutants were better able to survive starvation than others. Each mutant had two growth factor requirements so that differential survival was occuring among mutants with the same number of growth factor requirements. It was not unreasonable to assume then that different degrees of survival would also be found among conidia from mutants with single growth factor requirements.

In this section the results of three experiments are reported where the dying off rates, under starvation, of three nutritional mutants with single growth factor requirements were estimated. The experimental techniques have already been described in Part I.

In the first two experiments, conidia from the biotin requiring mutant (bi) were ~~mix~~ inoculated with conidia from a white spored lysine requiring mutant (wa lys) and starvation was carried out on minimal medium. The results are shown in Tables 19 and 20.

In the third experiment conidia from a yellow

TABLE 19. Dying off rates of conidia of the biotinless strain (bi) and conidia of the lysineless strain (wa lys) kept on minimal medium for periods of from 0 to 119 hours.

Plate Series	Plate Nos.	Period of Starv^{n}. in Hrs.	Haem. Estim. per Plate.		Colony Count per Plate		Total Colony Count		Correction Factor		Comparative Colony Counts.		%age Viability taking control as 100%	
			bi	walys	bi	walys	bi	walys	bi	walys	bi	walys	bi	walys
A (control)	1	0	30	31	18	24	85	92	1	1	85	92	100	100
	2				30	16								
	3				16	31								
	4				21	21								
B	1	47	30	31	13	24	45	94	1	1	45	94	52.9	102.2
	2				10	27								
	3				10	31								
	4				12	12								
C	1	71	60	62	4	10	21	49	2	2	10.5	28.5	12.4	30.9
	2				6	9								
	3				5	9								
	4				6	21								
D	1	95	300	310	0	54	13	135	10	10	1.3	13.5	1.9	14.8
	2				10	30								
	3				2	27								
	4				1	24								
E	1	119	300	310	0	31	2	115	10	10	0.2	11.5	0.2	12.5
	2				1	34								
	3				1	22								
	4				0	28								

TABLE 20. Dying off rates of conidia of the biotinless strain (bi) and conidia of the lysineless strain (wa lys) kept on minimal medium for periods of from 0 to 119 hours.

Plate Series	Plate Nos.	Period of Starv^{n}. in Hrs.	Haem. Estim. per Plate.		Colony Count per Plate		Total Colony Count.		Correc-tion Factor.		Compar-ative Colony Counts.		%age Viability taking control as 100%.	
			bi	walys	bi	walys	bi	walys	bi	walys	bi	walys	bi	walys
A (con-trol)	1	0	32	32	19	24	71	87	1	1	71	87	100	100
	2				19	22								
	3				15	19								
	4				18	22								
B	1	47	64	64	17	30	74	159	2	2	37	79.5	52.1	91.4
	2				22	41								
	3				23	35								
	4				12	53								
C	1	71	160	160	16	62	44	229	5	5	8.8	45.8	12.4	52.6
	2				11	49								
	3				9	57								
	4				8	61								
D	1	95	320	320	7	28	30	195	10	10	3	19.5	4.2	22.4
	2				9	51								
	3				7	48								
	4				7	68								
E	1	119	640	640	3	45	13	226	20	20	0.65	11.3	0.9	12.9
	2				3	76								
	3				5	67								
	4				2	38								

spored biotin requiring strain (y bi) were inoculated with conidia from an adenine requiring mutant with 'wild' type, green, conidia (ad) and again starved on minimal medium (Table 21).

The experimental results from these three experiments are shown in Plate 11, where the logarithm of percentage survivors is plotted against the period of starvation.

There are obviously very large variations between the dying off rates of conidia from nutritional mutants with different single growth factor requirements. The long survival of the adenine requiring mutant is of interest since, among mutants isolated by the starvation method, those requiring adenine (in addition to biotin) had relatively low dying off rates. There is, here, an indication that, in a strain with two growth factor requirements, the slow dying of its conidia during starvation may be attributed more to one of the requirements than the other; that growth factor requirement which when present singly also induces a slow dying.

The strain with yellow conidia and a biotin requirement (y bi) survives starvation for a longer period than the biotin requiring mutant with green conidia (bi), However, the difference in degree of survival is not substantial.

TABLE 21. Dying off rates of conidia from the yellow spored biotinless strain (y bi) and conidia of the adenineless strain (ad) kept on minimal medium for periods of from 0 to 120 hours.

Plate Series	Plate Nos.	Period of Starv^n in Hrs.	Haem. Estim. per Plate.		Colony Count per Plate.		Total Colony Count.		Correction Factor.		Comparative Colony Counts.		%age Viability taking control as 100%	
			ybi	ad	ybi	ad	ybi	ad	ybi	ad	ybi	ad	ybi	ad
A (control)	1	0	30	31	27	14	93	44	1	1	93	44	100	100
	2				25	11								
	3				21	9								
	4				20	10								
B	1	48	30	31	16	10	55	43	1	1	55	43	59.2	97.7
	2				13	12								
	3				12	11								
	4				14	10								
C	1	72	60	62	13	12	33	50	2	2	16.5	2.5	17.9	56.8
	2				8	11								
	3				7	17								
	4				5	10								
D	1	96	300	310	10	42	50	181	10	10	5	18.1	5.4	41.1
	2				15	33								
	3				7	52								
	4				18	54								
E	1	120	300	310	2	35	11	136	10	10	1.1	13.6	1.2	30.9
	2				5	37								
	3				3	21								
	4				1	43								

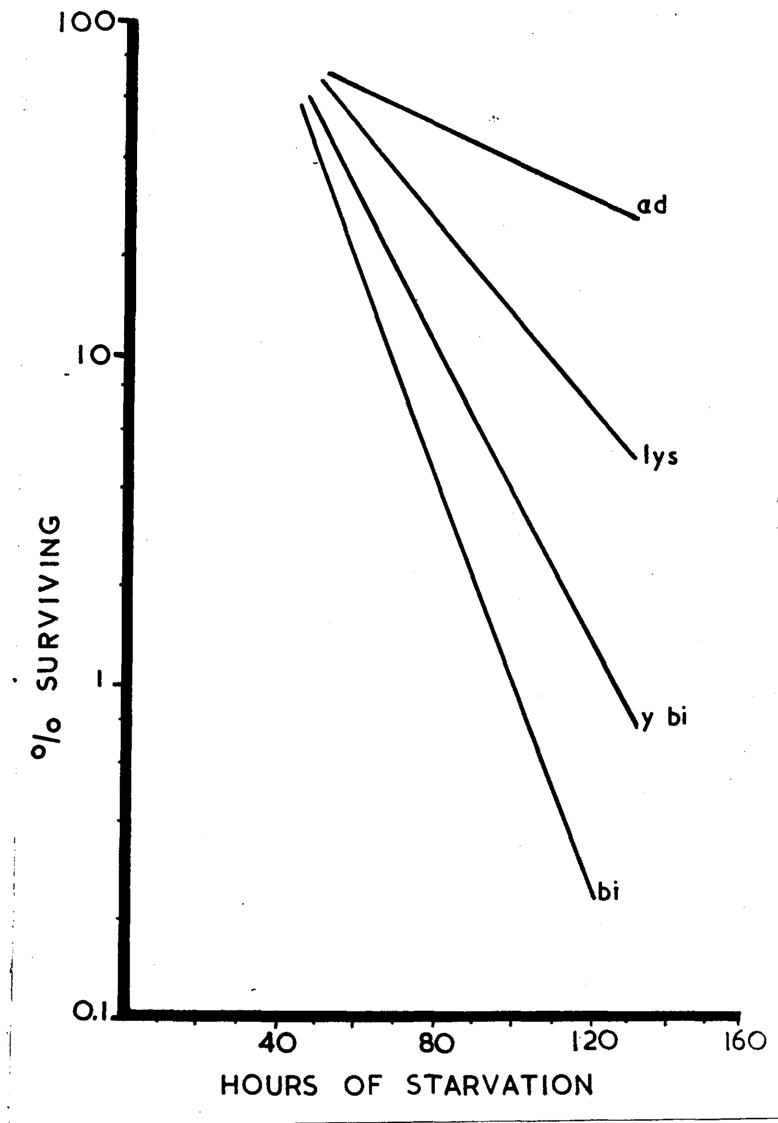


Plate 11. The conidia of four strains with different single nutritional requirements (ad = adenine requiring, lys = lysine requiring, bi and y bi = biotin requiring) die at enormously different rates on minimal medium.

The pr/imary observation from the experiments discribed in this section was the importance of the type of growth factor requirement in determing the rate of dying off of conidia under starvation. It would appear that the survival of conidia on a starvation medium is probably more specifically an expression of the type of growth factor requirements, rather than the number of growth factor requirements, or degree of heterotrophy as Fries called it.

At this stage, the provisional working hypothesis was advanced that the slow dying of conidia under conditions of starvation might be the result of a lowering of the over all metabolic rate of conidia when certain growth factor requirements remained unsatisfied, whereas quick dying might be related to the failure of specific synthetic processes.

For instance, if conidia from a strain which requires adenine have this growth factor withheld, then fundamental energy exchange processes will be blocked from the start, and the conidia should therefore die off slowly under starvation, since the general metabolism will be at a low rate.

On the other hand, conidia from a strain with, for example, a vitamin requirement (which they are deprived of on starvation) may be able to metabolise up to a point where a serious unbalance of enzyme systems leads to death.

4. Starvation at Sub Lethal Temperatures.

The experiments to be described were undertaken to investigate the process of differential survival with the idea that killing by temperature would be easier for those strains which had a low degree of survival under starvation. It was decided to proceed in the first instance on the effects of starvation at sub lethal temperatures. It was thought that, if strains which survived starvation for a long period did so because metabolism was proceeding at a low rate, then these strains might be better able to survive high temperatures. Conceivably, if conidia were metabolising at a relatively low rate, the cessation of thermo labile reactions would be immediatedly less drastic than in more actively metabolising conidia where it might be expected that such reaction blocks would lead to unbalance and death relatively quickly.

In these investigations, the practical advantage hoped for was a general speeding up of the dying off rates of conidia and hence a quicker exhibition of differential survival effects shown after starvation at 37°C. If this could be demonstrated, then an investigation of the action of sub lethal temperatures alone

(i.e. without starvation) would be carried out.

Heat treatment was carried out by immersion of flasks containing conidial suspensions in water, which was kept at a controlled temperature by a thermostatic unit (temperature variations not more than ±0.15C°). This necessitated some changes in the techniques already in use in starvation experiments. Two main steps were now necessary, a heat treatment of conidia in liquid minimal medium (corresponding to the starvation treatment in layered minimal), followed by a plating of the conidia on to complete medium and incubation at 37°C (corresponding to the addition of complete medium at the end of the starvation period and subsequent incubation for the growth of a surviving conidia to take place).

The experimental work in the heat treatment method was carried out under strict acid clean conditions up until the conidia were plated out on complete medium in an effort to eliminate any variables which might otherwise be present.

However, to standardise this new method with the normal starvation method, the effects of two main sources of variation had to be considered, that between flasks and that between different times. If seperate flasks were to be used, as being strictly comparable, then it had to be shown that, at any one time, the number of conidia

withdrawn from each separate flask did not vary significantly, if the withdrawal volumes were kept constant. It had also to be shown that the time of withdrawal caused no significant variation intthe number of conidia, the withdrawal volume again being constant.

If no significant variations were demonstrated, then any conclusions drawn from the experimental results could be confidently attributed to the effects of heat treatment in liquid starvation medium.

In the first experiment, four 100 ml conical flasks were set up, each containing approximately 10^7 conidia of the biotin requiring strain in 10 ml of liquid minimal medium. The method was to make up a dense suspension of conidia, estimate the number of conidia by haemocytometer count, and dilute so that each ml contained approsimately 10^7 conidia. 1 ml of the diluted suspension was then pipetted in 9 ml of minimal in each of the flasks. 1 ml of conidial suspension was then withdrawn from each flask and a spore count determined for each, using four separate haemocytometers. (It had previously been demonstrated that there was no variation in spore counts between separate haemocytometers). The flasks were then placed on the shaker and heated in the water trough at a temperature of 60°C. At periods of ½ hour, 1 hour, 1½ hours, and 3 hours, 1 ml of spore suspension

was withdrawn from each flask and a spore count again determined for each. In all cases, withdrawal of conidia was made only after vigorously shaking the flasks to suspend the conidia.

An analysis of variance was done on the results and is given below.

The Haemocytameter Counts are given at each period of time for each flask.

		Time of Heat Treatment at 60°C in Hours						
		0	½	1	1½	3	Flask Totals	
Flasks	I	85	73	67	81	63	369	
	II	78	84	65	71	63	361	
	III	72	61	58	70	66	327	
	IV	74	56	67	79	70	346	
	Time Totals	309	274	257	301	262	1403	Grand Total

Analysis of Variance Table.

Main Effect	Degrees of Freedom	Sum of Squares.	Mean Square
Time	4	542.3	135.6
Flasks	3	204.95	68.32
Inter Action			
T.F.	12	547.3	45.6
Total	19	1294.55	68.1

None of these effects is significant at the 0.05 level.

It was now decided to run a range finding experiment at the arbitrarily chosen temperature of 60°C, using conidia of the biotin requiring mutant.

Two flasks were set up in the usual way, each containing 10.8×10^6 conidia in 10 ml of liquid minimal. 1 ml of spore suspension was withdrawn from each flask suitably diluted, and 0.1 ml surface plated on four plates of complete medium, in each case, so as to give an estimated 108 spores per dish. These plates, the controls, were then incubated at 37°C and colony counts made in the usual manner after 36 and 48 hours incubation (see Parts I and II). Immediatedly after the withdrawal of conidia for the control platings, the flasks were placed in the shaker and heat treated at 60°C. At intervals of $\frac{1}{4}$ hour, $\frac{1}{2}$ hour, 1 hour and $1\frac{1}{4}$ hours, 1 ml of spore suspension was withdrawn from each flask suitably diluted and plated on to complete medium as in the control series. During withdrawal of conidia, the flasks were removed from the shaker and replaced as quickly as possible. The routine was standardised to eliminate temperature variation. The results of this experiment are shown in Table 22.

Obviously very few conidia survive at 60°C after even 15 minutes at this temperature. The temperature effect was considered to be too drastic for experimental

TABLE 22. Dying off rate of conidia of the biotinless strain suspended in liquid minimal medium at 60°C for periods of from 0 to 1¼ hours.

Flask I.

Plate Series	Plate Nos.	Hrs. of Heat Treatment at 60°C	Haem. Estim. per Plate.	Colony Count per Plate	Total Colony Count.	Correction Factor.	Comparative Colony Counts.	%age Viability taking control as 100%.
A (control.)	1	0	108	105	403	1	403	100
	2			98				
	3			97				
	4			103				
B	1	¼	540	0	0	5	0	0
	2			0				
	3			0				
	4			0				
C	1	½	5400	0	6	50	0.03	0.007
	2			4				
	3			1				
	4			1				
D	1	1	10800	5	23	100	0.23	0.057
	2			4				
	3			4				
	4			10				
E	1	1¼	108000	39	174	1000	0.174	0.043
	2			42				
	3			42				
	4			51				

TABLE 22 (cont).

Flask II.

Plate Series	Plate Nos.	Hrs. of Heat Treatment at 60°C	Haem. Estim. per Plate	Colony Count per Plate	Total Colony Count.	Correction Factor	Comparative Colony Counts.	%age Viability taking control as 100%
A (control.)	1	0	108	52	220	1	220	100
	2			64				
	3			57				
	4			57				
B	1	$\frac{1}{4}$	540	1	5	5	1	0.45
	2			1				
	3			2				
	4			1				
C	1	$\frac{1}{2}$	5400	4	8	50	0.04	0.018
	2			1				
	3			2				
	4			1				
D	1	1	10800	2	9	100	0.09	0.041
	2			2				
	3			3				
	4			2				
E	1	$1\frac{1}{4}$	108000	23	80	1000	0.08	0.036
	2			20				
	3			17				
	4			20				

purposes. The results of a similar experiment using conidia from the same biotin requiring strain and carried out at the reduced temperature of 55°C are given in Table 23.

At 55°C in liquid minimal medium, a 99% kill of conidia from the biotin requiring strain is reached ~~sets~~ after about 3 hours. The conidia from this strain die off relatively quickly on minimal medium at 37°C. Conidia from a strain requiring adenine (symbol ad) have already been shown to die off relatively slowly on minimal medium at 37°C (see previous section of this part of the thesis). Conidia from this latter strain were now heat treated in minimal medium at 55°C and the experimental results are shown in Table 24.

The results indicated that conidia from the adenine requiring strain died off quicker than conidia from the biotin requiring strain, a reversal of the effect known to be the case when starvation was conducted at 37°C.

In a further experiment at 55°C, three flasks were set up, one with conidia from the biotin requiring strain, another with conidia from the adenine requiring strain, and the remaining one with conidia from strain S4096. This latter strain is a starvation isolated mutant requiring biotin and either arginine, ornithine or proline, whose conidia were known to die off relatively slowly on

TABLE 23. Dying off rate of conidia of the biotinless strain suspended in liquid minimal medium at 55°C for periods of from 0 to 3 hours.

Flask I.

Plate Series	Plate Nos.	Hrs. of Heat Treatment at 55°C	Haem. Estim. per Plate.	Colony Count per Plate	Total Colony Count.	Correction Factor.	Comparative Colony Counts.	%age Viability taking control as 100%.
A (control.)	1	0	109	66	320	1	320	100
	2			84				
	3			96				
	4			74				
B	1	1	545	93	475	5	95	29.7
	2			122				
	3			132				
	4			128				
C	1	2	1090	38	207	10	20.7	6.5
	2			53				
	3			47				
	4			69				
D	1	3	10900	69	269	100	2.69	0.9
	2			67				
	3			65				
	4			68				

TABLE 23 (cont).

Flask II.

Plate Series	Plate Nos.	Hrs. of Heat Treatment at 55°C	Haem. Estim. per Plate.	Colony Count per Plate.	Total Colony Count.	Correction Factor.	Comparative Colony Counts.	%age Viability taking control as 100%
A (control.)	1 2 3 4	0	109	40 57 68 72	237	1	237	100
B	1 2 3 4	1	545	118 123 120 107	468	5	93.6	39.9
C	1 2 3 4	2	1090	17 29 31 33	110	10	11	4.2
D	1 2 3 4	3	10900	31 39 47 50	167	100	1.67	0.7

TABLE 24 (cont).

Flask II.

Plate Series	Plate Nos.	Hrs. of Heat Treatment at 55°C	Haem. Estim. per Plate.	Colony Count per Plate.	Total Colony Count.	Correction Factor.	Comparative Colony Count	%age Viability taking control as 100%
A (control)	1	0	105	87	324	1	324	100
	2			74				
	3			89				
	4			74				
B	1	1	105	10	28	1	28	8.7
	2			9				
	3			6				
	4			3				
C	1	2	105	0	1	1	1	0.3
	2			0				
	3			1				
	4			0				
D	1	3	210	0	0	2	0	0
	2			0				
	3			0				
	4			0				

TABLE 24. Dying off rate of conidia of the adenineless strain (ad) when suspended in liquid minimal medium at 55°C for periods of from 0 to 3 hours.

Flask I.

Plate Series	Plate Nos.	Hrs. of Heat Treatment at 55°C	Haem. Estim. per Plate.	Colony Count per Plate	Total Colony Count.	Correction Factor.	Comparative Colony Counts.	%age Viability taking control as 100%
A (control)	1 2 3 4	0	105	54 49 49 48	200	1	200	100
B	1 2 3 4	1	105	8 6 6 5	25	1	25	12.5
C	1 2 3 4	2	105	0 2 1 4	7	1	7	3.5
D	1 2 3 4	3	210	0 0 0 0	0	2	0	0

minimal medium at 37°C.

The results again indicate a reversal of the degree of survival on minimal at 37°C. At 55°C conidia from the two slow dying strains when starvation is conducted at 37°C, ad and S4C96 die off quicker than the biotin requiring strain (Table 25).

The temperature was reduced to 50°C and the results of an experiment involving conidia from the biotin requiring strain and the adenine requiring strain are shown in Table 26. Conidia from the biotin requiring strain again survive the longer. A final experiment conducted at 48°C showed a similar effect (Table 27).

When starvation was conducted in liquid minimal medium and a range of temperatures from 60°C to 48°C, the effects of starvation at 37° were reversed in those strains which were investigated.

From the point of view of speeding up the dying off rates of conidia from different nutritional mutants while still preserving the differential survival effect known to be the case when starvation was conducted at 37°C these experiments were a failure. Experiments were not conducted at temperatures below 48°C since it is known that *Aspergillus nidulans* will grow at temperatures exceeding 40°C and, furthermore, if any effect could be shown at temperatures a little below 48°C whereby the

TABLE 25. Dying off rates of conidia from three strains when suspended in liquid minimal medium at 55°C for periods of from 0 to 3 hours.

biotinless strain (bi)

Plate Series	Plate Nos.	Hrs. of Heat Treatment at 55°C	Haem. Estim. per Plate.	Colony Count per Plate	Total Colony Count.	Correction Factor.	Comparative Colony Count.	%age Viability taking control as 100%
A (control)	1 2 3 4	0	96	52 57 61 64	234	1	234	100
B	1 2 3 4	1½	480	137 122 112 130	501	5	100.2	43.1
C	1 2 3 4	2	960	55 56 61 70	242	10	24.2	10.3
D	1 2 3 4	3	9600	58 66 79 60	263	100	2.63	1.1

over

TABLE 25 (cont).

biotinless, arginineless strain (S4C96).

Plate Series	Plate Nos.	Hrs. of Heat Treatment at 55°C	Haem. Estim. per Plate.	Colony Count per Plate.	Total Colony Count.	Correction Factor.	Comparative Colony Count.	%age Viability taking control as 100%
A (control)	1	0	96	72	324	1	324	100
	2			81				
	3			79				
	4			92				
B	1	1	480	8	38	5	7.6	2.4
	2			11				
	3			12				
	4			7				
C	1	2	960	3	8	10	0.8	0.3
	2			1				
	3			2				
	4			2				
D	1	3	960	0	0	10	0	0
	2			0				
	3			0				
	4			0				

TABLE 25 (cont).

adenineless strain (ad).

Plate Series	Plate Nos.	Hrs. of Heat Treatment at 55°C	Haem. Estim. per Plate.	Colony Count per Plate	Total Colony Count.	Correction Factor.	Comparative Colony Count.	%age Viability taking control as 100%.
A (control)	1 2 3 4	0	92	65 67 50 70	252	1	252	100
B	1 2 3 4	1	460	18 21 14 20	73	5	14.6	5.8
C	1 2 3 4	2	920	9 12 6 10	37	10	3.7	1.5
D	1 2 3 4	3	9200	8 9 6 9	32	100	0.32	0.1

TABLE 26 (cont).

adenineless strain (ad).

Plate Series	Plate Nos.	Hrs. of Heat Treatment at 50°C	Haem. Estim. per Plate.	Colony Count per Plate.	Total Colony Count.	Correction Factor.	Comparative Colony Counts	%age Viability taking control as 100%
A (control)	1 2 3 4	0	96	60 68 49 56	233	1	233	100
B	1 2 3 4	2	96	56 57 53 40	206	1	206	88.4
C	1 2 3 4	4	96	48 46 40 57	191	1	191	81.9
D	1 2 3 4	6	96	14 13 14 10	51	1	51	21.9
E	1 2 3 4	8	96	5 4 5 2	16	1	16	6.9
F	1 2 3 4	10	96	0 0 2 0	2	1	2	0.9

TABLE 26. Dying off rates of conidia of the biotinless and conidia of the adenineless strains when suspended in liquid minimal at 50°C for periods of from 0 to 10 hours.

biotinless strain (bi)

Plate Series	Plate Nos.	Hrs. of Heat Treatment at 50°C	Haem. Estim. per Plate.	Colony Count per Plate.	Total Colony Count.	Correction Factor.	Comparative Colony Counts.	%age Viability taking control as 100%
A (control)	1	0	92	74	314	1	314	100
	2			78				
	3			70				
	4			92				
B	1	2	92	67	306	1	306	97.5
	2			78				
	3			82				
	4			79				
C	1	4	92	32	194	1	194	61.5
	2			47				
	3			54				
	4			61				
D	1	6	92	55	177	1	177	56.4
	2			45				
	3			43				
	4			34				
E	1	8	92	7	32	1	32	10.2
	2			5				
	3			8				
	4			12				
F	1	10	92	2	6	1	6	1.9
	2			2				
	3			2				
	4			0				

TABLE 27 (cont).

adenineless strain (ad).

Plate Series	Plate Nos.	Hrs. of Heat Treatment at 48°C	Haem. Estim. per Plate	Colony Count per Plate	Total Colony Count.	Correction Factor.	Comparative Colony Counts.	%age Viability taking control as 100%
A (control)	1 2 3 4	0	94	65 63 65 58	251	1	251	100
B	1 2 3 4	2	94	57 51 63 56	227	1	227	90.5
C	1 2 3 4	4	94	28 50 57 31	166	1	166	62.2
D	1 2 3 4	6	94	52 41 41 37	171	1	171	68.5
E	1 2 3 4	8	94	27 29 34 32	122	1	122	48.6
F	1 2 3 4	9	94	24 17 13 14	68	1	68	27.1

TABLE 27. Dying off rates of conidia of the biotinless and conidia of the adenineless strains when suspended in liquid minimal at 48°C for periods of from 0 to 9 hours.

biotinless strain (bi).

Plate Series	Plate Nos.	Hrs. of Heat Treatment at 48°C	Haem. Estim. per Plate.	Colony Count per Plate.	Total Colony Count.	Correction Factor.	Comparative Colony Counts.	%age Viability taking control as 100%
A (control)	1	0	102	83	280	1	280	100
	2			71				
	3			74				
	4			52				
B	1	2	102	59	268	1	268	95.7
	2			94				
	3			65				
	4			50				
C	1	4	102	59	266	1	266	95.0
	2			77				
	3			77				
	4			63				
D	1	6	102	67	258	1	258	92.1
	2			65				
	3			66				
	4			60				
E	1	8	102	90	307	1	307	109.6
	2			85				
	3			59				
	4			73				
F	1	9	102	63	205	1	205	73.2
	2			59				
	3			53				
	4			30				

differential survival effect which occurs on starvation at 37°C, was preserved, the more tedious manipulations of the heat treatment method in liquid medium would hardly compensate any increase in dying off rates of conidia, which, at temperatures below 48°C, would not be over substantial.

However, it was decided that some experiments might be run, using the usual starvation layer plating method, where starvation was conducted in an incubator at a temperature of 41°C.

Conidia of the biotin requiring strain and of the adenine requiring strain were layer plated on minimal medium (on separate plates). Control plates had complete medium added and were incubated at 37°C. Of the remaining plates, one series was incubated at 37°C, and the other at 41°C. Starvation was conducted over increasing periods of time, After starvation, complete medium was added and incubation was at 37°C. The experimental results are shown in Table 28. The results of another experiment, in which conidia from the biotin requiring strain and the adenine requiring strain were again used are given in Table 29. Starvation was conducted at 41°C and, on addition of complete medium, the plates were incubated at 37°C. In Fig.13, data from Tables 28 and 29 are shown on a graph where the logarithm of percentage

survivors is plotted against the period of starvation.

The differential survival effect on starvation at 37°C is preserved when starvation is conducted at 41°C although the dying off rates of conidia from both strains are speeded up.

The results of the experiments in this section indicate that the effect of temperature, combined with starvation on conidia from certain nutritional mutants, bears no relation to the effects of starvation alone. Presumably, those temperatures which were employed have an effect perhaps independent of, and masking the effect shown to be the case when starvation is conducted at 37°C. Because of this, no experiments were conducted on the effects of sub lethal temperatures without starvation.

TABLE 28 (cont).

Adenineless Strain.

Starvation at 37°C.

Plate Series	Plate Nos.	Starv^n. Period in Hours.	Haem. Estim. per Plate.	Colony Count per Plate	Total Colony Count.	Correction Factor.	Comparative Colony Counts.	%age Viability taking control as 100%
A (control)	1	0	95	87	320	1	320	100
	2			77				
	3			92				
	4			64				
B	1	69	95	11	87	1	87	27.2
	2			15				
	3			31				
	4			30				
C	1	96	95	15	78	1	78	24.4
	2			22				
	3			25				
	4			16				
D	1	120	475	35	132	5	26.4	8.3
	2			29				
	3			32				
	4			36				

over

TABLE 28. Dying off rates of conidia of the biotinless and conidia of the adenineless strains on minimal medium.

Biotinless Strain.

Starvation at 37°C.

Plate Series	Plate Nos.	Starvn. Period in Hours.	Haem. Estim. per Plate.	Colony Count per Plate	Total Colony Count.	Correction Factor.	Comparative Colony Counts.	%age Viability taking control as 100%
A (control)	1	0	104	125	471	1	471	100
	2			121				
	3			111				
	4			114				
B	1	69	104	27	109	1	109	23.1
	2			24				
	3			30				
	4			28				
C	1	96	104	2	9	1	9	1.9
	2			3				
	3			2				
	4			2				
D	1	120	1040	1	13	10	1.3	0.3
	2			1				
	3			3				
	4			8				

TABLE 28 (cont).

Adenineless Strain.

Starvation at 41°C.

Plate Series	Plate Nos.	Starv^{n}. Period in Hours.	Haem. Estim. per Plate.	Colony Count per Plate.	Total Colony Count.	Correction Factor.	Comparative Colony Counts.	%age Viability taking control as 100%
A (control)	1 2 3 4	0	95	83 71 88 70	312	1	312	100
B	1 2 3 4	69	95	8 5 4 6	23	1	23	7.4
C	1 2 3 4	96	95	3 1 2 0	6	1	6	1.9
D	1 2 3 4	96	475	10 5 7 9	31	5	6.2	2.0

TABLE 28 (cont).

Biotinless Strain.

Starvation at 41°C.

Plate Series	Plate Nos.	Starvn. Period in Hours.	Haem. Estim. per Plate.	Colony Count per Plate.	Total Colony Count.	Correction Factor.	Comparative Colony Counts.	%age Viability taking control as 100%
A (control)	1	0	104	131	404	1	404	100
	2			92				
	3			84				
	4			97				
B	1	69	104	1	8	1	8	1.9
	2			1				
	3			5				
	4			1				
C	1	96	104	0	0	1	0	0
	2			0				
	3			0				
	4			0				
D	1	96	1040	0	0	10	0	0
	2			0				
	3			0				
	4			0				

TABLE 29 (cont).

adenineless strain (ad).

Plate Series	Plate Nos.	Starv^{n}. Period in Hours.	Haem. Estim. per Plate.	Colony Count per Plate.	Total Colony Count.	Correction Factor.	Comparative Colony Counts.	%age Viability taking control as 100%
A (control)	1 2 3 4	0	100	83 59 60 79	281	1	281	100
B	1 2 3 4	48	100	27 18 17 28	90	1	90	32
C	1 2 3 4	69	500	54 48 37 25	164	5	33	11.7
D	1 2 3 4	90	5000	91 86 105 90	372	50	7.4	2.6

TABLE 29. Dying off rate of conidia of the biotinless and conidia of the adenineless strain on minimal medium at 41°C.

biotinless strain (bi)

Plate Series	Plate Nos.	Starv^{n}. Period in Hours.	Haem. Estim. per Plate.	Colony Count per Plate.	Total Colony Count.	Correction Factor.	Comparative Colony Counts.	%age Viability taking control as 100%
A (control)	1 2 3 4	0	99	64 72 83 79	298	1	298	100
B	1 2 3 4	48	99	15 16 24 21	76	1	76	25.5
C	1 2 3 4	69	990	45 53 44 46	188	10	18.8	6.2
D	1 2 3 4	90	9900	8 9 13 18	48	100	0.48	0.2

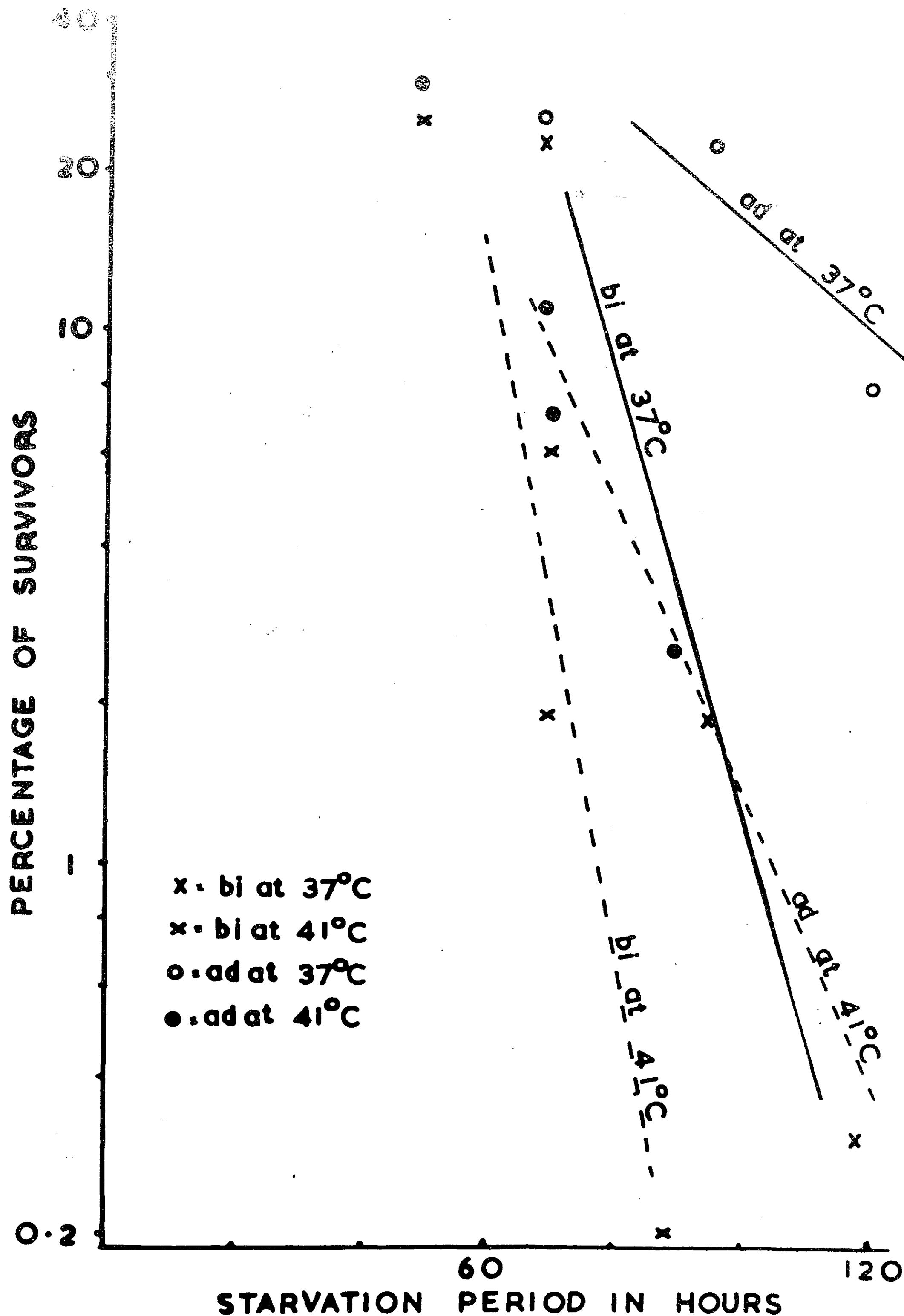


FIG. 13. The dying off rates of conidia from the biotin requiring strain (bi) and the adenine requiring strain (ad) are shown when starvation is conducted on minimal medium at temperatures of 37°C and 41°C. At 41°C, while the dying off rates are speeded up, the differential survival between strains is preserved.

5. The effects of degrees of starvation.

In an attempt to estimate the relative importance of either growth factor deficiency in determining the dying off rate under starvation of conidia from a nutritional mutant with two growth factor requirements, the following experiments were carried out. They involved starvation of the conidia not only on minimal medium (i.e. starvation for <u>both</u> factors), but also on minimal medium supplemented with either one or the other growth factor (i.e. starvation for only one, the missing, factor). The term "degree of starvation" will be used here to indicate starvation for either, as compared with starvation for both factors.

The behaviour of conidia from a white spored strain, requiring biotin and adenine for growth (symbol bi w ad) was investigated under the three possible conditions of starvation. Two other strains were employed as controls, a yellow spored biotin requiring strain (y bi) and a green spored adenine requiring strain (ad). The biotin and adenine requirements in the latter two strains are genotypically identical to those in the double nutritional mutant.

The plating methods followed the normal layer

plating technique and incubation was at 37°C.

The starvation media used were minimal medium, minimal medium, supplemented with biotin in amounts to satisfy the biotin requirement of the strains, and minimal medium with a similar sufficiency of adenine.

1. Petri dishes containing minimal medium were mix-inoculated with conidia from all three strains, the yellow spored biotin requiring strain, the green spored adenine requiring strain, and the white spored biotin and adenine requiring strain.

2. Petri dishes containing minimal medium supplemented with biotin were mix-inoculated with conidia from the green spored adenine requiring strain and the white spored biotin and adenine requiring strain.

3. Petri dishes containing minimal supplemented with adenine were mix-inoculated with conidia from the yellow spored biotin requiring strain and the double nutritional mutant.

After starvation, complete medium was added as a third layer to the plates. Conidia which survived starvation gave rise to visible colonies after reincubation, those from each strain being distinguishable by their colour, after sporulation had taken place. Colony counts were made as described in Part I. The experimental results are shown in Table 30 and in Plate 12 the

logarithm of percentage survivors is shown plotted against the period of starvation.

1. On minimal medium alone, conidia from the yellow spored biotin requiring strain die off relatively quickly, whereas conidia both from the adenine and the biotin and adenine requiring strains die off relatively slowly.

2. On minimal medium supplemented with biotin, conidia from the biotin and adenine requiring strain die off at a rate similar to conidia from the strain requiring adenine alone.

3. On minimal medium supplemented with adenine conidia from the biotin and adenine requiring strain die of at a rate approaching that of conidia from the biotin requiring mutant.

It would appear that on a biotin supplemented medium the strain requiring biotin and adenine behaves like an adenine requiring strain and on adenine supplemented medium, like a biotin requiring strain as to the rates of dying off.

It is obvious that the adenine requirement of the double nutritional mutant is the crucial one with regards to its slow dying under starvation on minimal; that requirement alone could account for the survival of conidia from this strain on minimal.

It is now apparent that the choice of the biotin

requiring strain (bi) as the mother strain in the starvation technique (see Part II) was a fortunate one. The conidia from this strain die off relatively quickly when starved on minimal medium, so that most of the induced mutant conidia with additional single growth factor requirements are likely to result in a slower dying off with consequent enrichment of these during starvation. However, if, for example, an adenine requiring strain had been used as a mother strain, most conidia with additional mutations would probably not have died off at a rate slow enough to allow their enrichment after starvation, since the mother strain had conidia which were already slow dying. The technique would then have been unsuccessful.

For the practical purposes of selecting mutants by means of the starvation technique, therefore, a mother strain is required whose conidia die off relatively quickly on minimal medium.

TABLE 30. Dying off rates of conidia from three strains under three conditions of starvation, yellow biotinless strain (y bi), adenineless strain (ad), and biotinless and adenineless strain (bi wad).

Minimal Medium (starvation for both biotin and adenine)

Plate Series	Plate Nos.	Period of Starv^n. in Hours.	Haem. Estim. Conidia per Plate.			Colony Count per Plate.			Total Colony Count.			Correc- tion Factor.	Comparative Totals.			%age Viability taking control as 100%.		
			ybi	ad	biwad	ybi	ad	biwad	ybi	ad	biwad		ybi	ad	biwad	ybi	ad	biwad
A (control)	1	0	32	29	31	24	14	12	76	53	53	1	76	53	53	100	100	100
	2					15	12	14										
	3					15	15	16										
	4					22	12	11										
B	1	48	32	29	31	8	10	8	45	44	45	1	45	44	45	59.2	83.0	84.9
	2					13	12	10										
	3					12	10	13										
	4					12	12	14										
C	1	72	64	58	62	7	21	16	24	61	38	2	12	30.5	19	15.8	57.6	35.9
	2					7	19	9										
	3					8	20	10										
	4					2	1	3										
D	1	93	160	145	155	17	24	35	48	99	95	5	9.6	19.8	19	12.6	37.4	35.9
	2					14	35	37										
	3					17	26	18										
	4					0	14	5										
E	1	120	320	290	310	5	48	32	8	163	85	10	0.8	16.3	8.5	1.1	30.8	16.0
	2					2	45	22										
	3					1	54	22										
	4					0	16	9										

over

TABLE 30 (cont).

Minimal Medium supplemented with Adenine (starvation for biotin only

Plate Series	Plate Nos.	Period of Starv^{n}. in Hours.	Haem. Estim. per Plate.		Colony Count per Plate.		Total Colony Count.		Correction Factor.	Comparative Totals.		%age Viability taking Control as 100%.	
			ybi	biwad	ybi	biwad	ybi	biwad		ybi	biwad	ybi	biwad
A (control)	1	0	35	34	16	15	89	58	1	89	58	100	100
	2				26	12							
	3				28	9							
	4				19	22							
B	1	48	70	68	17	18	73	51	2	36.5	25.5	41.0	43.9
	2				24	13							
	3				16	12							
	4				16	8							
C	1	72	350	340	26	28	90	83	10	9	8.3	10.1	14.3
	2				26	23							
	3				24	16							
	4				14	16							
D	1	93	350	340	23	24	48	70	10	4.8	7	5.4	12.1
	2				14	25							
	3				9	15							
	4				2	6							
E	1	120	700	680	5	9	14	45	20	0.7	2.25	0.8	3.9
	2				4	12							
	3				3	11							
	4				2	13							

TABLE 30 (cont).

Minimal Medium supplemented with Biotin. (starvation for adenine only)

Plate Series	Plate Nos.	Period of Starv^{n}. in Hours.	Haem. Estim. per Plate.		Colony Count per Plate.		Total Colony Count.		Correc-tion Factor.	Comparative Totals.		%age Viability taking Control as 100%.	
			ad	biwad	ad	biwad	ad	biwad		ad	biwad	ad	biwad
A (control)	1	0	30	33	13	11	61	51	1	61	51	100	100
	2				14	11							
	3				16	14							
	4				18	15							
B	1	48	60	66	17	23	67	91	2	33.5	45.5	54.9	89.2
	2				16	25							
	3				16	17							
	4				18	26							
C	1	72	60	66	18	12	58	64	2	29	32	49.2	62.7
	2				17	14							
	3				12	22							
	4				11	16							
D	1	93	150	165	35	31	114	131	5	22.8	26.2	37.4	51.4
	2				40	48							
	3				27	32							
	4				12	20							
E	1	120	300	330	39	36	169	165	10	16.9	16.5	27.7	32.3
	2				43	41							
	3				48	42							
	4				39	46							

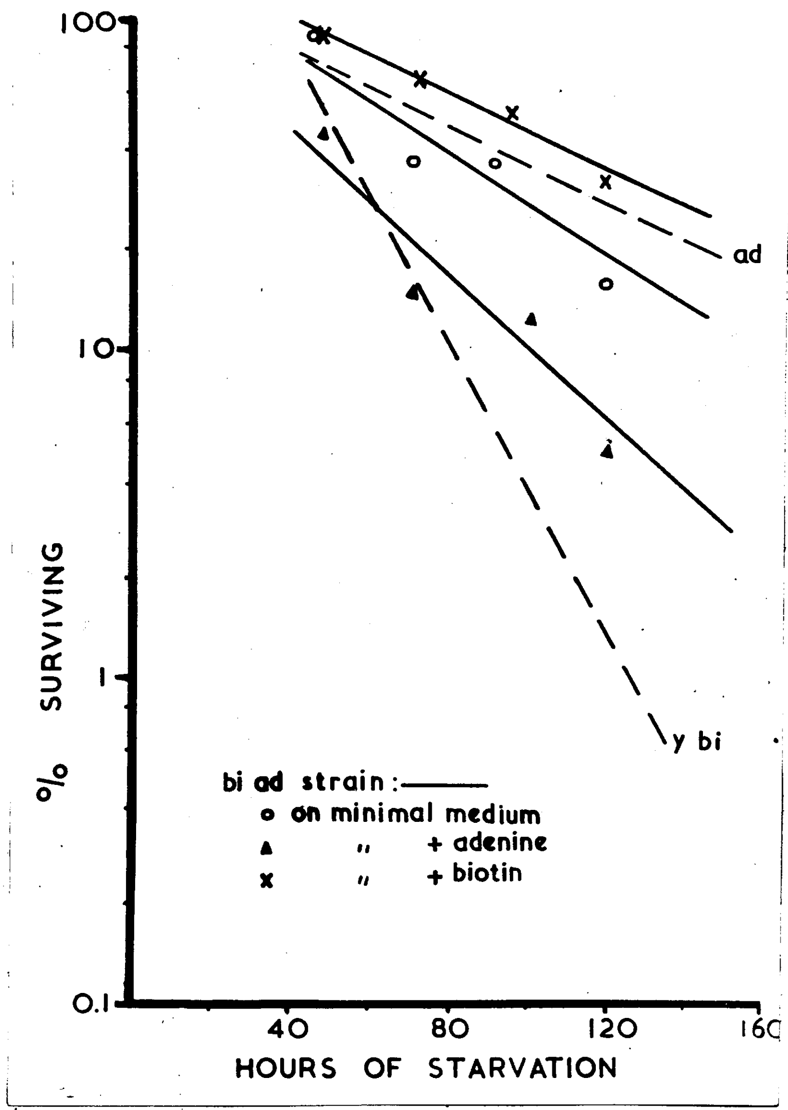


Plate 12. The dying off rates of conidia from a strain requiring biotin and adenine (bi w ad) is shown under three conditions of starvation. For comparison the dying off rates of conidia from ad (requiring adenine) and y bi (requiring biotin) are shown on minimal medium.

6. Starvation of conidia from nutritional mutants obtained by Starvation and Total Isolation: A comparison incorporating degrees of starvation.

In this section, the survival of conidia from a selection of nutritional mutants isolated both by Total Isolation and Starvation methods are compared under different degrees of starvation. All the strains used had a requirement for biotin and for one other growth factor.

Among the several strains selected, there was included one from either isolation method having one of the following requirements (in addition to biotin):- reduced sulphur (i.e. unable to utilise sulphur in the form of sulphate), reduced nitrogen (unable to utilise nitrate), arginine, lysine and "thiazole". Three types of mutants are distinguishable biochemically, among those which require arginine for growth in Aspergillus nidulans (Pontecorvo 1950b).

1. Mutants which will only grow when arginine is supplied.
2. Mutants which will grow when either arginine or ornithine is supplied.
3. Mutants which will grow when arginine, ornithine or proline is supplied.

All three types have been found after Total Isolation methods were used, with the second type predominating, but only the third type has been isolated by the Starvation method. In the comparison, a Total Isolation mutant which required arginine or ornithine (besides biotin) was compared with a starvation isolate which grew when supplied with arginine, ornithine or proline (besides biotin). Among the mutants requiring nitrogen in a form more reduced than nitrate, a strain which was found after Total Isolation and required either NO_2^- or NH_4^+ and biotin was compared with a NH_4^+ and biotin requiring starvation isolate. Both reduced nitrogen mutant types have been found after Total Isolation but only the latter after starvation.

The other comparisons were straight forward between two mutants requiring reduced sulphur in the form of sulphite, two requiring lysine, and two requiring "thiazole" (as well as biotin in each case), one of each pair from Total Isolation and the other from starvation. The nutritional requirements of these mutants in each individual comparison were biochemically indistinguishable.

Three experiments were carried out and the procedure in each was as follows. Previously, an estimation of the number of conidia to be plated on the starvation

dishes was obtained by running small scale pilot experiments.

Experimental Procedure: For each strain suitably diluted conidial suspensions were plated on to six plates of minimal, three plates of minimal supplemented with biotin, and three plates of minimal supplemented with the other growth factor required by the particular strain. Three plates of minimal medium had complete medium added as a third layer immediatedly, and were incubated at 37°C as the control. The colony counts of these plates gave the initial viability of the conidia. The remaining plates were incubated for a predetermined starvation period at 41°C, after which complete medium was added and the plates then incubated at 37°C to allow the development of visible colonies from the conidia surviving starvation. The percentage survival of conidia was then estimated under the three conditions of starvation. In each of the three experiments, conidia from the biotin requiring strain (bi) were starved on minimal medium.

As will be noticed, no attempt was made to estimate the dying off rates of the conidia from each strain, since only one period of starvation was used. However, a comparison was possible between the relative survival of conidia from each strain under different conditions

of starvation, when compared to the survival of conidia from the biotin requiring strain on minimal medium, after the same period of starvation.

The results of the three experiments are shown in Table 31 and are summarised in Table 32.

Among the limited number of nutritional mutants analysed, there does not appear to be a general correlation between the survival of conidia after starvation from different nutritional mutants and the method by which these mutants were isolated. The conidia from starvation isolated mutants do not generally survive starvation on minimal medium longer than conidia from mutants isolated after Total Isolation.

However, the results show that there is no simple relationship between the degree of heterotrophy and the survival rate; the dying off rates of conidia from different double nutritional mutants vary enormously on minimal medium. These reconstructional experiments have also confirmed some of the results obtained when using the starvation techniques for nutritional mutant isolation. For example, conidia from mutants with a requirement for sulphite (as well as biotin) obviously survive starvation on minimal medium very much longer than do conidia from any of the other mutants, which is in accordance with these mutants preponderating among

those isolated by the starvation method. Referring to the vitamin requiring mutants, conidia from strain S5E1, which requires pyridoxin as well as biotin, survive the longest on minimal medium. These are the most abundant among the vitamin requiring mutants isolated by the starvation technique (see Table 17A).

With regards to the differences in the qualitative spectrum of nutritional mutants found after starvation and Total Isolation (see Tables 17 and 17A), the behaviour of conidia, from the arginine requiring mutants, on minimal medium is of interest. Conidia from strain bi orn_2 (A Total Isolation mutant requiring biotin and arginine or ornithine) survive for a very short time, whereas conidia from strain S4C96 (a starvation isolate requiring biotin and either arginine, ornithine or proline) survive for a relatively long period.

As mentioned earlier, arginine requiring mutants in _Aspergillus nidulans_ can be classified into three types: utilising arginine only, utilising arginine or ornithine, and utilising arginine, ornithine or proline.

Only the last mentioned type occurs among starvation isolated mutants. The reconstruction experiments suggest that the absence of the second type after starvation is due to the very low degree of survival the conidia have on minimal medium. Since the first

type is also not found after starvation, it could be suggested that the slow dying of conidia from nutritional mutants of the last type is more specifically an expression of the proline requirement rather than the arginine or ornithine requirements. Proline, as well as being an intermediate in arginine biosynthesis, is apparently involved in other biosynthetic processes, since mutants with a requirement for proline alone have been obtained in Aspergillus nidulans and other micro-organisms.

TABLE 31 (cont).

Strain	Plate Nos.	Starvation on Minimal Medium + Biotin: Haem. Estim. per Plate.	Colony Count per Plate.	Total Colony Count.	Correction Factor.	Comparative Totals	%age Viability taking control as 100%	Starvation on Minimal Medium + other growth Factor.: Haem. Estim. per Plate.	Colony Count per Plate.	Total Colony Count.	Correction Factor.	Comparative Totals	%age Viability taking control as 100%	Period of Starv^{n}. in Hours.
biorn$_2$	1	580,000	142	308	2000	0.154	0.039	580,000	67	177	2000	0.089	0.021	112
	2		120						59					
	3		46						51					
S4C96	1	530	77	249	5	49.8	13.280	5,300	66	237	50	4.740	1.264	
	2		76						86					
	3		96						85					
S3A2	1	490	202	587	5	117.4	38.778	49,000	31	62	500	0.124	0.041	
	2		186						17					
	3		199						14					
S5F35	1	550	181	646	5	129.2	50.862	55,000	53	154	500	0.308	0.157	
	2		228						34					
	3		237						67					

over

TABLE 31. The results of those experiments where the survival of conidia from double nutritional mutants was measured under three possible conditions of starvation.

Strain	Plate Nos.	Control: Haem. Estim. per Plate.	Control: Colony Count per Plate.	Control: Total Colony Count.	Control: %age Viability taking control as 100%	Starvation on Minimal Medium: Haem. Estim. per Plate.	Starvation on Minimal Medium: Colony Count per Plate.	Starvation on Minimal Medium: Total Colony Count.	Starvation on Minimal Medium: Correction Factor.	Starvation on Minimal Medium: Comparative Totals.	Starvation on Minimal Medium: %age Viability taking control as 100%	Period of $Starv^{n}$. in Hours.
bi	1	106	80	234	100	53,000	42	119	500	0.238	0.102	112
	2		83				43					
	3		71				34					
biorn2	1	290	125	393	100	580,000	5	7	2000	0.0035	0.001	
	2		142				0					
	3		126				2					
S4C96	1	106	116	375	100	530	32	85	5	17.0	4.533	
	2		130				35					
	3		129				18					
S3A2	1	98	90	303	100	490	162	545	5	109.0	35.973	
	2		108				182					
	3		105				201					
S5F35	1	110	99	254	100	550	211	564	5	112.8	44.42	
	2		79				199					
	3		76				154					

TABLE 31(cont).

Strain	Plate Nos.		Starvation on Minimal Medium + other growth Factor.						Period of Starv^{n}. in Hours.
			Haem. Estim. per Plate.	Colony Count per Plate.	Total Colony Count.	Correction Factor.	Comparative Totals	%age Viability taking control as 100%	
ybithi	1	All these strains grew to some extent on Minimal Medium + Biotin.	5,400	351	989	50	19.78	8.675	108
	2			311					
	3			327					
S5F1	1		5,400	79	240	50	4.8	2.308	
	2			72					
	3			89					
bin_2	1		10,800	163	589	100	5.89	1.558	
	2			201					
	3			225					
S2E11	1		10,000	96	292	100	2.92	0.795	
	2			105					
	3			91					

over

TABLE 31 (cont).

Strain	Plate Nos.	Control: Haem. Estim. per Plate.	Control: Colony Count per Plate.	Control: Total Colony Count.	Control: %age Viability taking control as 100%	Starvation on Minimal Medium: Haem. Estim. per Plate.	Starvation on Minimal Medium: Colony Count per Plate.	Starvation on Minimal Medium: Total Colony Count.	Starvation on Minimal Medium: Correction Factor.	Starvation on Minimal Medium: Comparative Totals.	Starvation on Minimal Medium: %age Viability taking control as 100%	Period of Starv^{n}. in Hours.
bi	1	108	63	191	100	54,000	115	405	500	0.81	0.424	108
	2		69				132					
	3		59				158					
ybithi	1	108	69	228	100	2,700	25	85	25	3.4	1.404	
	2		92				34					
	3		67				26					
S5F1	1	108	84	208	100	27,000	73	219	250	0.876	0.421	
	2		63				84					
	3		61				62					
bin_2	1	108	120	378	100	10,800	221	672	100	6.72	1.774	
	2		131				252					
	3		127				199					
S2E11	1	100	116	372	100	10,000	28	139	100	1.39	0.374	
	2		133				49					
	3		123				62					

TABLE 31 (cont).

Strain	Plate Nos.	Starvation on Minimal Medium + Biotin: Haem. Estim. per Plate.	Colony Count per Plate.	Total Colony Count.	Correc-tion Factor.	Com-para-tive Totals	%age Via-bility taking control as 100%	Starvation on Minimal Medium + other growth Factor.: Haem. Estim. per Plate.	Colony Count per Plate.	Total Colony Count.	Correc-tion Factor.	Com-para-tive Totals	%age Via-bility taking control as 100%	Period of $Starv^{n}$. in Hours.
$bilys_2$	1	10,800	161	512	100	5.12	2.828	54,000	176	499	500	0.998	0.551	112
	2		202						175					
	3		149						148					
S5F2	1	10,200	65	196	100	1.96	0.908	5,100	56	188	50	3.76	1.741	
	2		59						75					
	3		72						57					
S2D1	1	27,000	10	55	250	0.220	0.083	54,000	7	26	500	0.052	0.019	
	2		14						10					
	3		31						9					
S5E1	1	2,500	59	193	25	7.72	2.992	50,000	49	208	500	0.416	0.161	
	2		61						81					
	3		73						78					

TABLE 31 (cont).

Strain	Plate Nos.	Control: Haem. Estim. per Plate.	Control: Colony Count per Plate.	Control: Total Colony Count.	Control: %age Viability taking control as 100%	Starvation on Minimal Medium: Haem. Estim. per Plate.	Starvation on Minimal Medium: Colony Count per Plate.	Starvation on Minimal Medium: Total Colony Count.	Starvation on Minimal Medium: Correction Factor.	Starvation on Minimal Medium: Comparative Totals.	Starvation on Minimal Medium: %age Viability taking control as 100%	Period of Starv^{n}. in Hours.
bi	1	100	49	178	100	50,000	17	72	500	0.144	0.081	112
	2		65				29					
	3		64				26					
bilys$_2$	1	108	51	181	100	10,800	81	226	100	2.26	1.243	
	2		67				75					
	3		63				70					
S5F2	1	102	73	216	100	10,200	121	317	100	3.17	1.468	
	2		71				107					
	3		72				89					
S2D1	1	108	82	265	100	27,000	0	2	250	0.008	0.003	
	2		94				1					
	3		89				1					
S5E1	1	100	82	258	100	2,500	81	341	25	13.64	5.287	
	2		90				59					
	3		86				201					

bi	Total Isolation	-	0.424			100		
ybithi	Total Isolation	"Thiazole"	1.404		8.675	331		2046
S5F1	Starvation	"Thiazole"	0.421		2.308	99		544
bin_2	Total Isolation	NO_2^- or NH_4^+	1.777		1.558	419		368
S2E11	Starvation	NH_4^+	0.374		0.795	88		188

bi	Total Isolation	-	0.081			100		
$bilys_2$	Total Isolation	Lysine	1.243	2.828	0.551	1535	3491	680
S5F2	Starvation	Lysine	1.468	0.908	1.741	1812	1121	2149
S2D1	Starvation	Paramino-benzoic acid.	0.003	0.083	0.019	4	102	23
S5E1	Starvation	Pyridoxin	5.287	2.992	0.161	6527	3964	199

TABLE 32. Survival of conidia from nutritional mutants under three possible conditions of starvation. Data summarised from Table 31.

Strain	Method by which strain was isolated	Growth Factor requirement of strain in addition to biotin	Percentage viability of conidia after starvation on:-			Viability taking bi starvation. Viability as 100.		
			Minimal Medium.	Minimal Medium + Biotin	Minimal Medium + other growth factor.	Minimal Medium.	Minimal Medium + Biotin	Minimal medium + other growth factor.
bi	Total Isolation	-	0.102			100		
$biorn_2$	Total Isolation	Arginine or Ornithine.	0.001	0.039	0.021	1	38	21
S4096	Starvation	Arginine, Ornithine, or Proline.	4.533	13.28	1.264	4444	13020	1239
S3A2	Total Isolation	SO_3^{--}	35.973	38.778	0.041	35268	38017	40
S5F35	Starvation	SO_3^{--}	44.442	50.862	0.157	43571	49864	154

7. Discussion and Conclusions.

Miller, Farghally and McElroy (1949) have described conditions under which nutritional mutant cells have a selective advantage over wild type cells in the luminous salt water bacterium Achromobacter fischeri. They have shown that, when cells are suspended in liquid minimal medium at 8°C, the survival of nutritional mutants is favoured. When wild type cells were treated with nitrogen mustard, suspended in minimal at 8°C for nine days, and then plated on to complete medium, the recovery rate of nutritional mutants among isolates exceeded the recovery rate with no cold treatment by about twenty times.

If untreated wild type cells were kept in minimal liquid medium at 8°C for several days and then plated on to minimal medium and incubated at 23°C, only relatively few colonies developed. However, when complete medium was added an additional large number of colonies grew up. Most of the latter colonies grew on minimal medium when subsequently tested, but about 3% required several transfers on complete medium before reverting to growth on minimal medium. The rapidity of the change in wild type cells to the apparent mutant state was inversely

proportional to the density of the suspension in minimal at 8°C.

Millar et al (1949) have postulated that, when cells are suspended in minimal at 8°C, wild type cells lose certain intermediate metabolites by diffusion more rapidly than do nutritional mutant cells. The suggestion is that certain enzyme systems may then be unstable in the absence of their specific substrates. Some of the substrates will be available from complete medium so that increased viability will be shown when cold treated wild type cells are plated on complete medium instead of minimal medium. However, the loss of substrates irreplacable from complete medium will result in the ultimate death of the organism. The stabilisation of enzymes by the action of substrates is reminiscent of the mechanism of adaptive enzyme formation and indeed these workers have suggested, like others, that there may be little fundamental difference between the synthesis and stabilisation of adaptive and constitutive enzymes (see Monod 1947).

Certain analogies can be drawn between the behaviour of cells from <u>Achromobacter fischeri</u> under cold treatment and the behaviour of conidia from <u>Aspergillus nidulans</u> under starvation.

At 8°C on a medium which only supports the growth of

wild type cells of *Achromobacter fischeri*, metabolism will be at a low rate. Nutritional mutant cells under similar conditions presumable metabolise even more slowly. These latter cells have a greater degree of survival and it has been suggested in this thesis that conidia which metabolise at a relatively low rate are those which have a selective advantage in *Aspergillus nidulans* under starvation conditions.

However, while the hypothesis of the loss of diffusible substrates more rapidly by wild type cells than nutritional mutant cells may account for the selection against the former in the luminous bacterium under cold treatment, there is no evidence to suggest that a similar mechanism can account for the selection which favours the survival of certain nutritional mutants in *Aspergillus nidulans* under starvation. Indeed, there is some evidence against it. When conidia of the biotin requiring strain (bi) of this fungus are starved on minimal medium, the addition of either biotin or complete medium at the end of the starvation period makes no apparent difference to the percentage survival of the conidia (see Part I of this thesis). There is therefore no suggestion that these conidia lose certain diffusible growth components during starvation, some of which could be replaced from complete medium.

The total number of nutritional mutants isolated by Miller et al (1949) was 51. However, their data allow no comparison to be made between nutritional mutants found by Total Isolation and after cold treatment so that there is no evidence of any selective action which might occur under cold treatment for particular types of mutants.

Referring now to the results obtained in this part of the thesis; conidia from nutritional mutants with different single growth factor requirements have been shown to die off at enormously different rates on minimal medium. It has also been found that starvation for either or both requirements of a double nutritional mutant shows that, generally, the dying off rate of conidia from such a mutant on minimal medium is mainly governed by the nutritional deficiency inductive of slower dying when acting alone.

It is clear, therefore, that under starvation conditions on minimal medium, the survival of conidia from different nutritional mutants is more an expression of the kinds than of the number of growth factor requirements as suggested by Fries (1948a, 1948b).

The behaviour of conidia under starvation from a strain requiring biotin and arginine or ornithine (symbol bi orn_2) is of interest considering the studies of

Feldott and Lardy (1951). These workers have shown that in the rat biotin has a function in the synthesis of arginine at a step prior to the conversion of ornithine to citrulline. If a similar situation exists in Aspergillus nidulans then a biotin deficient strain would not be able to synthesise ornithine unless alternative pathways existed. The fact that on minimal medium, conidia from strain bi orn_2 do not die off at a slower rate than conidia from the biotin deficient strain offers some confirmation that biotin may be connected with ornithine synthesis in Aspergillus nidulans (see Table 31); a slower dying off rate would mean the failure of a metabolic process unconnected with biotin deficiency.

The possibility that starvation might be of use in determining the relative importance of different metabolites in germination metabolism, by virtue of the dying off rates under starvation of conidia deficient in specific metabolites, remains to be investigated properly. It would be expected that the effects of various metabolites in speeding up or slowing down the dying off rate would become as informative a tool as the effect of specific metabolites in lengthening or shortening the lag phase in bacterial growth (Hinshelwood 1946).

Summary.

1. An analysis of the dying off rates under starvation on minimal medium of conidia from nutritional mutants with different single growth factor requirements showed a wide variation among them. The rate of dying off is an expression of the type of growth factor requirement.

2. The effect of starvation at sub lethal temperatures on the conidia of those nutritional mutants which were investigated bore no relation to the effects of starvation alone. The temperature effect may be independent of and mask that of starvation.

3. In general, the dying off rate under starvation on minimal medium of conidia from a nutritional mutant with two growth factor requirements can be attributed mainly to that deficiency which, when present alone, is inductive of the slower dying.

4. The working hypothesis is advanced that quick dying of conidia under starvation may be connected with the failure of specific synthetic processes and slow dying with the failure of processes, e.g. energy-yielding, which lower the general metabolic rate.

PART IV. Application of the Starvation Technique for the Isolation of Mutants of Particular Types.

1. Introduction.

The starvation technique which has been developed for the isolation of nutritional mutants in *Aspergillus nidulans* can, with slight modifications, be employed for the selective isolation of particular types of mutants. These modifications are described in this part of the thesis.

The problems are of two kinds: a) how to eliminate mutants of unwanted types, b) how to obtain mutants only of desired types.

It has been shown (Part III) that conidia from a strain requiring sulphite and biotin, normally survive for a relatively long period on minimal medium, but if they are starved on minimal supplemented with sulphite, their survival approaches that of conidia from the biotin requiring mutant on minimal medium, which is relatively short (see Table 31). If it is desired then to eliminate sulphite requiring mutants from among those nutritional mutants isolated after starvation, the procedure would be to layer plate conidia of the biotin requiring strain on minimal medium supplemented with sulphite or thio-

sulphate. Strictly speaking, the sulphite requiring mutants should not be completely eliminated, but if the dying off rates of their conidia under the degree of starvation approaches that of conidia from the mother strain, then after starvation they should be found about as frequently as they occur after Total Isolation. Other nutritional mutants should occur in the frequency they normally do after starvation.

The starvation technique can also be adapted for the isolation of nutritional mutants of specific types which have not so far been isolated by other techniques, or by the starvation technique when isolation was made from complete medium. The growth of certain bacteria ~~have~~ has long been known to be inhibited in the presence of some amino acids (see Gordon and Macleod 1926, and Gladstone 1939). After the development of the biochemical genetics of microorganisms, following the work of Beadle and Tatum (1941), certain nutritional mutants of Neurospora were shown to have their growth inhibited by antagonistic interactions. The growth of a lysine requiring mutant was inhibited in the presence of arginine (Doerman 1944), a uridine requiring mutant by adenosine (Pierce and Loring 1947), etc. In Aspergillus nidulans, competitive inhibitions were demonstrated between arginine requirement and exogenous lysine and

between guanosine and adenosine (Pontecorvo 1950a). Fries (1949b) has described similar antagonisms among nutritional mutants of Ophiostoma multiannulatum.

If the substance they require for growth is a constituent of a complete medium, it would seem apparent then that certain nutritional mutants may not grow up on such a medium, not necessarily because they are very rare, or because they will only grow under a narrow range of environmental conditions of temperature, pH, etc., but because their growth is inhibited by antagonistic substances in the complete medium.

Certain isolation techniques have been developed in which the possible effect of antagonisms is avoided. Lein, Mitchell and Houlahan (1948) have described a method in Neurospora. After mutagenic treatment, macroconidia are applied to protoperithecia of the opposite mating type and the ascospores which develop are shed on to plates of minimal medium. Among those colonies which grow up and remain discrete, the persistently small ones are isolated as putative mutants. By this method, they have isolated nutritional mutants not previously found in Neurospora, including those with requirements for histidine and tryptophane. Wikberg and Fries (1952) have developed a technique in Ophiostoma multiannulatum. Treated conidia are

suspended in liquid minimal medium for 24 hours and then filtered. Those wild type conidia which show no delayed germination effects after mutagenic treatment, will germinate in minimal and will tend to remain behind on filtration so that there will be a consequent enrichment of nutritional mutant conidia. The conidia are then plated on to a modified complete medium containing sub optimal amounts of complete medium components known to contain inhibitory substances; liver extract, sterilised by seitz filtration is also incorporated in the medium. From such a medium these workers have isolated nutritional mutants with double and triple growth factor requirements, not found by other techniques and have also found a mutant which requires for growth the tripeptide glutathione.

The adaptation of the starvation technique for the isolation of nutritional mutants in Aspergillus nidulans which have not so far been isolated from complete medium consists of allowing treated conidia from the biotinless strain to starve on minimal for a requisite period, for mutant enrichment to take place, and then adding instead of complete medium, minimal supplemented with biotin and the growth factors of the mutants which are desired. By adding supplemented minimal instead of complete medium, the effects of possible antagonistic substances present

in complete medium are avoided. Isolates are made from the colonies which develop and the growth factor requirements of the isolates characterised in the usual manner.

2. Material and Methods.

Materials.

Fine Chemical Solutions: These were all made up in double distilled water, under acid clean conditions, and sterilised by autoclaving for 10 minutes at 10 lbs pressure.

Inositol: A stock solution containing 3 mg/ml was held. 5 ml of this solution was used per litre of minimal medium.

D.L. Tryptophane: The stock solution contained 10 mg/ml. 10 ml was used per litre of minimal medium and ensured good growth of tryptophane requiring strains.

Guanosine: 10 mg guanosine/ml distilled water gave a suspension. On heating to 60-80°C the suspension dissolved. 5 ml of this solution was added per litre of minimal medium and assured good growth of the strain requiring guanosine.

The preparation of other materials used has already been described.

Methods.

These were similar to the methods which have been described earlier in this thesis.

U/v irradiation was carried out for 8 minutes at a distance of 45 cms. (see Material and Methods, Part II). However, wide variations in mains voltage meant that the killing rates varied in different experiments. In each case the u/v dosage is expressed, as before, in terms of killing rate calculated from viable counts after irradiation, relative to conidia plated (haemocytometer estimate).

3. Elimination of Certain Types of Mutants.

Conidia from a six day old sub culture of the biotin requiring mutant were u/v irradiated (96% kill) and were then suitably diluted and layer plated on two series of plates; one series of minimal and the other of minimal supplemented with thiosulphate (By using thiosulphate instead of sulphite there will also be a selective elimination of those mutants requiring thiosulphate only as well as those able to utilise either sulphite or thiosulphate). Control plates from each series had complete medium added as a third layer immediately and were then incubated at 37°C with the starvation plates. Complete medium was added to plates from each series after 139 hours starvation at 37°C and the plates reincubated at 37°C. Colony counts and isolates were made in the usual manner and the isolates characterised as to their growth factor requirements.

The results are shown in Table 33.

It will be seen that, when starvation is conducted on minimal medium, sulphite requiring mutants preponderate as usual, but on minimal supplemented with thiosulphate they have almost been completely eliminated. However, the other types of mutants come up in the same proportions as can be seen in the table below.

Starvation for 139 hours on:	No. of Isolates made.	Mutants responding to:-				Total Mutants (excluding SO_3)
		SO_3	Adenine	Amino Acids	Vitamins	
Minimal	400	24	8	1	2	11
Minimal plus Thiosulphate	400	1	8	3	5	16

It would appear, therefore, that the addition of thiosulphate to the starvation medium only affects survival of conidia from those mutants requiring sulphite or thiosulphate, in addition to biotin.

TABLE 33 (cont).

Starvation on minimal medium supplemented with thiosulphate.

Plate Series	Plate Nos.	Starv^{n}. period in Hours.	Haem. Estim. per Plate.	Colony Count per Plate.	Total Colony Count.	Correction Factor.	Comparative Colony Counts.	%age Viability taking control as 100%	Isolates made per Plate	Total Isolates	Among Isolates	
											No. of nutritional mutants	%age of nutritional mutants
A (control)	1	0	1080	53	584	1	584	100	25	100	1	1
	2			60					25			
	3			47					25			
	4			54					25			
	5		2160	101					0			
	6			97					0			
	7			95					0			
	8			77					0			
B	1	139	54000	69	1336	$\frac{200}{3}$	20.04	3.44	26	400	17*	4.25
	2			100					52			
	3			110					62			
	4			79					50			
	5			46					25			
	6			68					35			
	7			69					30			
	8			130					80			
	9		108000	133					10			
	10			154					10			
	11			187					10			
	12			191					10			

* Of these 17 mutants: 1 requires SO_3^{--}, 3 require Amino acids, 8 require adenine, and 5 require vitamins.

TABLE 33. Conidia of the biotinless strain u/v irradiated (95.7% kill).

Starvation on minimal medium.

Plate Series	Plate Nos.	Starv^{n}. period in Hours.	Haem. Estim. per Plate.	Colony Count per Plate.	Total Colony Count.	Correction Factor.	Comparative Colony Counts.	%age Viability taking control as 100%	Isolates made per Plate	Total Isolates	Among Isolates: No. of nutritional mutants	Among Isolates: %age of nutritional mutants
A (control)	1	0	1080	40	528	1	528	100	25	100	2	2
	2			57					25			
	3			45					25			
	4			36					25			
	5		2160	94					0			
	6			86					0			
	7			87					0			
	8			83					0			
B	1	139	54000	25	1128	$\frac{200}{3}$	16.92	3.19	15	400	35*	8.75
	2			32					25			
	3			44					38			
	4			82					52			
	5			57					40			
	6			116					50			
	7			118					60			
	8		108000	115					60			
	9			109					15			
	10			146					15			
	11			143					15			
	12			141					15			

* Of these 35 mutants: 24 require SO_3^{--}, 1 requires Amino acid, 8 require adenine, and 2 require vitamins.

4. Isolation of Mutants which do not come up with other techniques.

In these experiments a search was made for mutants requiring guanosine, tryptophane or inositol.

In the first experiment conidia from a 6 day old sub culture of the biotin requiring mutant were u/v irradiated (87.5% kill) suitably diluted, layer plated on minimal medium, and incubated at 37°C. The plates were separated into two series and two procedures were followed as described below:

1. After starvation, the plates had 0.1 ml of a biotin solution (0.02 microgms/ml) spread on top. The tiny colonies which developed after 24 hours incubation at 37°C were marked and a third layer of minimal medium supplemented with biotin, tryptophane, guanosine and inositol was added. Isolates were then made from among those colonies which developed after a further period of incubation at 37°C. This method followed that of delayed enrichment of Lederberg and Tatum (1946) (see Part II, Introduction).

2. After starvation minimal medium supplemented with biotin, tryptophane, guanosine, and inositol was added and the colonies which developed were counted and isolated in the usual manner.

The isolates from both procedures were then tested and their growth factor requirements characterised. The experimental results are shown in Table 34.

Among the 420 isolates made, one mutant requiring tryptophane (in addition to biotin) was found. Nicotinic acid, nicotinamide, anthranilic acid, 3-OH anthranilic acid, serine, indole, or kynurenine which are substances known to be metabolically related to tryptophane, did not support the growth of this mutant.

Several mutants requiring sulphite, and one requiring adenine, were also found among the isolates made. They must have been mutants able to grow slightly on impurities present in the media.

The data available do not allow a comparison between the two procedures which were followed in this experiment. The application of Lederberg and Tatum's method should lessen the labour involved in isolating and testing colonies, by allowing some of the mother strain colonies to grow up first, which then of course do not have to be subsequently isolated.

In the second experiment, conidia from a 6 day old sub culture of the biotin requiring strain were u/v irradiated (96.2% kill) and layer plated on minimal medium after suitably diluting. The control plates had complete medium added as a third layer and were incubated

Procedure 2. Normal Starvation Method.

Plate Series	Plate Nos.	Haem. Estim. Spores per Plate.	Period of Starv^{n}. in Hours.	No. of Colonies which grew on each Plate.	No. of Colonies isolated from each Plate.	Total No. of Colonies isolated and tested.	No. of Nutritional Mutants among Isolates.	%age of Nutritional Mutants among Isolates.
A	1	50,000	124	10	9	112	12 (including 1 tryptophaneless)	10.7
	2	50,000		9	9			
	3	50,000		4	4			
	4	50,000		32	29			
	5	50,000		34	17			
	6	100,000		20	21			
	7	100,000		24	23			
B	1	100,000	168	32	21	234	12 (including 1 adenineless)	5.1
	2	100,000		29	25			
	3	100,000		58	28			
	4	200,000		59	40			
	5	200,000		48	20			
	6	200,000		54	20			
	7	200,000		61	40			
	8	200,000		81	40			

except where stated, all the mutants were sulphiteless.

TABLE 34. Conidia of the biotinless strain u/v irradiated (87.5% kill). Minimal medium supplemented with biotin, tryptophane, guanosine and inositol added after starvation on minimal medium.

Procedure 1. Starvation followed by Delayed Enrichment.

Plate Series	Plate Nos.	Haem. Estim. Spores per Plate.	Hrs. from innoculation to addition of Biotin	Hrs. from innoculation to addition of further growth factors.	No. of Colonies which grew on addition of Biotin	No. of Colonies delayed till addition of growth Factors.	No. of delayed Colonies isolated and tested.	Total No. of Colonies isolated	No. of Nutritional Mutants among Isolated	%age of Nutritional Mutants among Isolates.
A	1	50,000	100	124	12	6	6	36	1	2.8
	2	100,000			36	14	14			
	3	200,000			34	16	16			
B	1	50,000	144	168	10	14	11	38	10	26.3
	2	100,000			21	19	18			
	3	200,000			33	38	9			

at $37^{o}C$ with the starvation plates. Minimal supplemented with biotin, tryptophane, guanosine and inositol was added as a third layer after starvation and colony counts and isolates were made as before, the growth factor requirements of the isolates being determined in the normal fashion.

The results are detailed in Table 35.

Among 1000 isolates made, one guanosine requiring mutant was found and there were also four mutants requiring sulphite. The growth of the guanosine requiring mutant was inhibited by hypoxanthine.

The two mutants which required tryptophane and guanosine respectively (in addition to biotin) and which were isolated by the selective starvation method, are mutant types which have never been isolated from complete medium in *Aspergillus nidulans.* However, the numerical results do not appear to be significant. With selective starvation, one tryptophaneless mutant and one guanosineless mutant was found out of 1346 isolates (Tables 34.2 and 35). For the whole of the remainder of the starvation series 1825 isolates were tested from 96 hours or more starvation (Table 17a).

TABLE 35. Conidia of the biotinless strain u/v irradiated (96.2% kill). Minimal medium supplemented with biotin, inositol, guanosine and tryptophane added after starvation on minimal medium.

Plate Series	Plate Nos.	Starv^{n}. period in Hours.	Haem. Estim. per Plate.	Colony Count per Plate.	Total Colony Count.	Correction Factor.	Comparative Colony Count.	%age Viability taking control as 100%	No. of Isolates per Plate	Total Isolates	Among Isolates.		
											No. of non mutants	No. of nutritional mutants	%age of nutritional mutants
A (control)	1	0	2100	97	1194	1	1194	100%	30	300	292	8	2.67%
	2			73					30				
	3			85					20				
	4			74					20				
	5			100					20				
	6			70					20				
	7			81					20				
	8			89					20				
	9			99					20				
	10			70					20				
	11			78					20				
	12			90					20				
	13			105					20				
	14			83					20				

B	1	112	105000	124	3502	100	35.02	2.93%	60	1,000	995	5‡	0.5%
	2			147					60				
	3			200					60				
	4			193					60				
	5			141					60				
	6			120					32				
	7			84					35				
	8			211					48				
	9			127					45				
	10			56					20				
	11			64					20				
	12			138					40				
	13			102					60				
	14		210000	171					60				
	15			192					60				
	16			255					60				
	17			287					60				
	18			321					40				
	19			259					60				
	20			133					30				
	21			177					30				

‡ 1 mutant required guanosine, and 4, sulphite.

	Isolated from complete medium	Isolated from supplemented minimal	Totals
No. of strains other than tryptophaneless or guanosineless.	1825	1344	3169
No. of tryptophaneless and guanosineless.	0	2	2
Totals	1825	1346	3171

The observed distribution could occur in 18% of the cases so that the results are clearly not significant apart from the fact that the data are not homogenous because the percentage of mutants in the controls (0 Hrs starvation) varied considerably.

To find out whether or not the tryptophane and guanosine requiring mutants were capable of growth on complete medium, conidia from each were spot inoculated on a plate of complete medium with conidia from two other mutants, one requiring adenine and the other tryptophane or nicotinic acid. The latter two mutants were originally isolated from complete medium. As a control, a plate of minimal medium supplemented with biotin, tryptophane, guanosine and adenine was spot inoculated with conidia from all four strains. The growth of these four strains on both media is shown in Plate 13. It is clear then that, on complete medium, the growth of both mutants isolated by selective starvation is inhibited, the

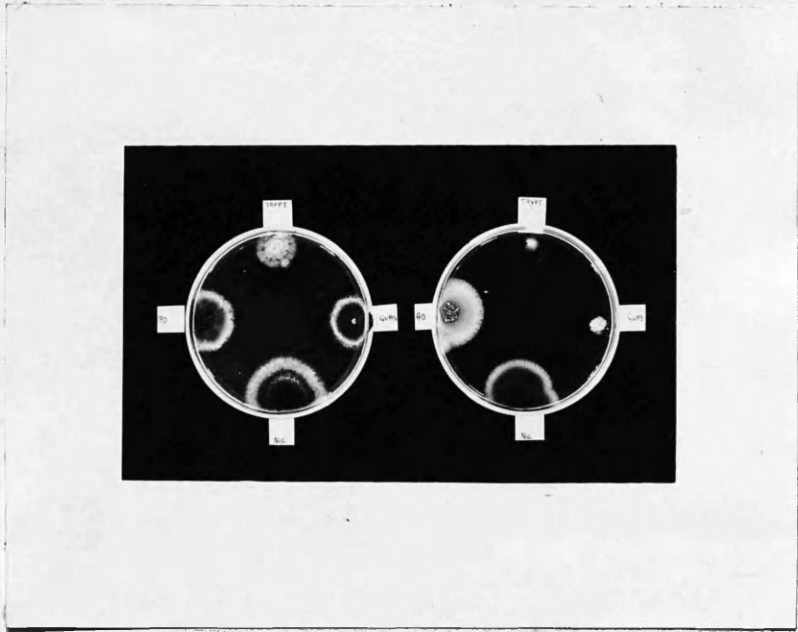


Plate 13. Four strains are shown on each plate: top: tryp (tryptophane requiring; bottom: nic (tryptophane or nicotinic acid requiring); left: ad (adenine requiring); right: guas (guanosine requiring) :- grown on :-

Left, minimal medium supplemented with growth factors,

Right, complete medium.

Complete medium contains inhibitor substances for guas. and tryp.

guanosine requiring mutant by hypoxanthine and the tryptophane requiring mutant by unknown substances present in complete medium. Thus, even though the statistical comparison made above is not significant, the properties of the two strains isolated by means of the selective technique make it very probable that this technique is effective.

5. Discussion and Conclusions.

It has been found possible to adapt the starvation technique in two ways.

First, unwanted nutritional mutants can be selectively eliminated by supplementing the starvation medium with their growth factor requirements (other than biotin). After starvation, these mutants will then tend to occur only as often as they do after Total Isolation. By using this method it may now be possible to adjust the quantitative spectrum of mutants found after starvation so that only certain groups of mutants have a selective advantage as starvation progresses.

Secondly, nutritional mutants whose growth is inhibited on complete medium can be isolated by the starvation method, when, at the end of the starvation period, minimal medium supplemented with biotin and the growth factors of the particular strains desired, is added instead of complete medium. In employing this method in a search for nutritional mutants, care should of course be taken that the supplemented medium, added after starvation, does not contain substances which may antagonise each other. The method is not restricted to the isolation of nutritional mutants which cannot grow on a complete medium and can be used for the

isolation of nutritional mutants of any desired type.

In a search for three types of nutritional mutants, previously not obtained, two were in fact found among 1346 isolates (Tables 34.2 and 35). The third type not so far found is that with a growth factor requirement for inositol. Inositol requiring mutants are of frequent occurence in other fungi. Since they have never been found in Aspergillus nidulans, not even with the selective starvation technique just mentioned, their absence raises interesting problems. In bacteria, inositol requiring mutants have also never been found (Pontecorvo 1950a).

It has been recognised that the methods by which nutritional mutants have been isolated in microorganisms have tended to select special types (see Lein, Mitchell and Houlahan 1948, Pontecorvo 1950a). The use of complete media obviously plays some part in this selection. Considering the encouraging results, the selective starvation method might be profitably employed in a search for other nutritional mutants not so far isolated from complete medium as well as for discovering unsuspected relationships between growth factors.

Summary:

1. Unwanted nutritional mutants of certain types can

be selectively eliminated in the starvation technique by the addition to the starvation medium of the growth factors required by the unwanted types (apart from biotin).

2. By adding, after starvation, minimal medium supplemented with biotin, tryptophane, guanosine, and inositol, instead of complete medium, one mutant requiring tryptophane and one requiring guanosine were isolated. These are types which had never been obtained before. These two mutants grow well on supplemented minimal medium but they grow poorly on complete medium suggesting the presence in it of specific inhibitors.

General Discussion and Conclusions.

The question of differences in the spectra of nutritional mutants, which occur in any one microorganism following mutagenic treatment, when different methods of isolation are used, has already been mentioned in the discussion to Part II of this thesis. Selective methods of isolation, such as the starvation technique, do undoubtedly cause variations in the spectra of mutants and there may be qualitative differences as well as quantitative ones, as indeed there is between the Starvation and Total Isolation methods (Tables 17 and 17a). It follows that, if comparisons are made between the distribution of mutant types occuring in different microorganisms, then of course care has to be taken that these have a validity by ensuring in the first instance that the mutants have been isolated by the same method. Although, even then, it cannot be assumed that, if different mutagenic agents are used, these will be aspecific in their action in a quantitative fashion, nor that the same method of isolation will not select differently in different organisms.

These microorganisms in which most data are available on the quantity and type of nutritional mutants

which have been isolated following mutagenic treatment are Aspergillus nidulans, Neurospora crassa, Ophiostoma multiannulatum and Penicillium notatum-chrysogenum (Tables 17, 17a, Fries 1946, 1947, Mitchell and Houlahan 1946, and Bonner 1946). In Penicillium all the mutants have been isolated by Total Isolation as have most of the nutritional mutants in Neurospora. However, the majority of the mutants in Aspergillus were isolated by the starvation method and in Ophiostoma about one half were isolated by a filtration method (see Introduction to Part II) and the remainder by Total Isolation.

Bonner (1946) has stated that the types and frequencies of nutritional mutants which occur in Penicillium are on the whole similar to those found in Neurospora. But it is felt that, since the methods of isolation are not comparable in every case, it would be inopportune to attempt to assess the significance of the spectra of mutants obtained in these different fungi, on a strictly quantitative basis. However, it is apparent that there are striking similarities between the types of nutritional mutants found in different microorganisms, although there are also some interesting qualitative differences.

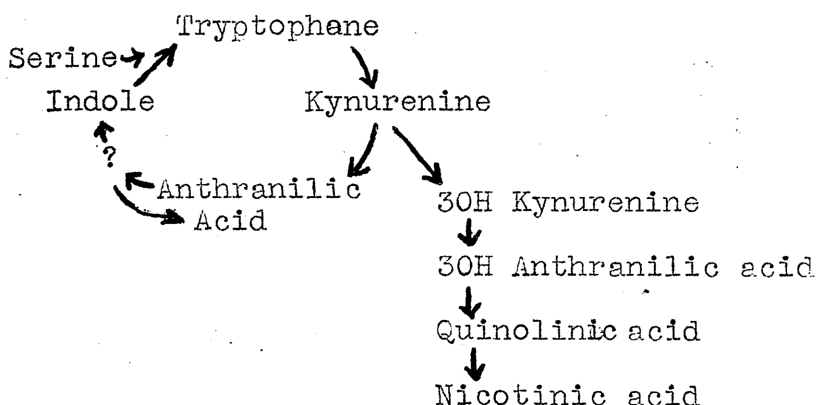
For example, mutants having a requirement for inositol have never been isolated in Aspergillus nidulans although they are found in other fungi, but not however

in bacteria (Pontecorvo 1950a). The non-occurence of inositol requiring mutants in _Aspergillus_ is probably significant since even a special search for them involving a selective starvation method has failed (see Part IV). While these results do not necessarily suggest that inositol is not an essential metabolite in _Aspergillus_ as it is not in bacteria, it would at least appear that an external source of this vitamin does not compensate for the failure of the organism to synthesise it. This in turn might suggest differences in the properties of membranes and perhaps in the seat of specific synthesis in the spatial organisations of the cells of different microorganisms.

The occurence of mutants requiring either arginine, ornithine or proline; arginine or ornithine; or arginine only, suggests that the biosynthesis of arginine proceeds from proline through the intermediacy of ornithine to arginine, in _Aspergillus nidulans_ (Pontecorvo 1950b). However, in _Neurospora_ it has been shown, by utilising mutants blocked at different steps, that arginine is synthesised through the ornithine cycle which was originally shown to occur in mammalian liver by Krebs (1932) (Srb and Horowitz 1944, Srb 1946). In this reaction citrulline is an intermediate between ornithine and arginine. _Penicillium notatum-chrysogenum_ and

certain lactic acid bacteria have also been shown to synthesise arginine by way of ornithine and citrulline (Bonner 1946, Volcani and Snell 1948). Either the reaction series is different in Aspergillus and the other microorganisms, or possibly citrulline is not an intermediate but exists in equilibrium with an intermediate between arginine and ornithine (Horowitz and Mitchell 1951).

In the biosynthesis of amino acids leading to the formation of nicotinic acid, the following interrelationships are proposed in Neurospora following the study of different mutants (Haskins and Mitchell 1949)



Although the same cycle is probably present in Aspergillus nidulans, it would appear that an alternative biosynthetic pathway exists in this organism since several mutants which utilise, anthranilic acid, 3OH anthranilic acid or nicotinic acid, but not kynurenine, tryptophane, or indole are known (Pontecorvo

1950a) side by side with mutants analogous to those of _Neurospora_.

There are further examples in the biochemical genetics of microorganisms which illustrate that there are probably differences in the detail of certain other syntheses. Nevertheless, it has been emphasised that comparative biochemistry of the most diverse organisms illustrates that the same sequence of reactions are common to many of them (Horowitz 1950). It has been suggested that this emphasises not only genetic relationships among living organisms, but also the great age of the biosynthesis of, for example, amino acids and vitamins (Horowitz 1950) which offers some support for Horowitz's conception of the evolution of biochemical synthesis (see below). Most precursors of essential metabolites (amino acids, vitamins, nucleosides, etc.,) can only have doubtful biological value. On this account it is rather difficult to visualise how biosynthetic sequences could have evolved step by step from simple to more complex. However, Horowitz (1945) has suggested that evolution might have taken a course which was the reverse of the steps occuring in biosyntheses. His reasoning is based on the assumption that the appearance of anything so intricate as living matter implies that the environment must have consisted of

chemicals of high complexity. The first "living" forms would have to be capable of utilising these chemical complexes and, as the latter gradually become used up, survival would have become dependent on these forms acquiring mutations allowing them capabilities of synthesising them from what are now the immediately preceding precursors, and so on.

On these premises, the evolution of living forms would proceed from heterotrophy towards autotrophy and probably then from autotrophy towards certain degrees of heterotrophy again, for it is possible, according to Lwoff (1943) to follow evolutionary series in certain microorganisms, plants and animals which show the gradual loss of synthetic functions. In bacteria, for example, one can trace from coliform through typhoid to dysentry bacilli, the loss of certain synthetic abilities, resulting in increased growth factor requirements.

While the selection which would operate on primitive living matter, as discussed by Horowitz, is of an obvious nature in the postulated environment, the selection of, for example, mutant microorganisms which have increased growth factor requirements over the parent strain, is less obvious.

The theoretical aspects of the long term selective value which such nutritional mutants may exhibit have

been discussed by Luria (1947) and others. A genetic block in a reaction series which results in a growth factor requirement (obtainable from the environment) may mean that genes controlling the chain of reaction steps prior to the block will become free of metabolic function and will be able to mutate without deleterious effects to the organisms. Houlahan and Mitchel (1947) have reported that a pyrimidine requiring mutant of Neurospora can have this growth factor requirment compensated for by the mutation of a non-allelic gene. If the mutated form of one gene can take over the function of another, then conceivably the 'freed' gene, or at least its mutant allele, can itself become functional in some other way as has been suggested by Haldane (1932) and Harland (1936). However, as Catcheside (1950) has pointed out, a suppressor gene does not necessarily take over the function of another gene but may act by altering the cellular environment so that perhaps an enzyme inactivated by a gene mutation in a normal environment, is reactivated in the changed environment. Nevertheless, this does not invalidate the hypothesis that genes 'freed' by the loss of metabolic function can mutate and may ultimately be of new use to an organism.

With regards to the immediate selective value which nutritional mutants might have among a wild type popu-

lation, Lwoff (1946) has suggested that the advantage might be an 'energetic' one, wherebye on losing certain synthetic abilities, they are able to carry out other metabolic functions more successfully. This assertion was supported by reference to a mutant of *Aerobacter*. The methionine requiring parent strain of this bacterium has been shown to have an increased growth rate over a non-methionine requiring mutant which arose spontaneously when both strains were grown on a methionine supplemented medium (Monod 1946). Roepke, Libby and Small (1944) have reported that some nutritional mutants of *Escherichia coli* grow faster than the parent strain, although these differences in growth rates may not be significant (Ryan and Schneider 1949). In *Neurospora*, a spontaneously occuring mutant with an additional block in the path of adenine synthesis, outgrew the parental adenine requiring strain with a single block (Mitchell and Mitchell 1950). Increased growth rates may in fact play some part in the selection of nutritional mutants, but experimental procedures designed to investigate differences in growth rates should take into account that the behaviour of pure cultures may be different from those of mixtures (Ryan and Schneider 1949).

Braun (1947) has emphasised that increased viability could form the basis of a positive

selection. For example, if among cells, only 50% of which remain viable long enough to reproduce, a mutation arises to increase this viability then there would be a selection in favour of the mutant cells, if growth rates, etc., are equal.

It has been shown in this thesis that conidia from nutritional mutants of Aspergillus nidulans vary in their dying off rates on a starvation medium. The suggestion is that conidia from nutritional mutants in which there is a failure of more fundamental energy - exchange processes survive starvation longer by virtue of their lowered rates of metabolism. It is well known that conidia in ageing cultures show a progressive loss of viability. Conidia in such cultures will be in the dormant state and metabolism will be at a low rate. It could be suggested that the metabolism of certain nutritional mutants might proceed at a rate lower than others and than wild type conidia. If so, then over a long period of time, as the conidia of a wild type population, in the dormant state, becomes inviable, certain nutritional mutant conidia among them (present as the result of spontaneous mutation) might survive longer because of their lowered metabolism, and also since the metabolic processes which lead to inviability might take longer, due to their lowered metabolic rates. There would then be a selection in favour of these nutritional

mutants as the period of time increased up until they, themselves, became inviable. This suggestion would be open to experimental test, simply by measuring the proportion of nutritional mutant conidia occuring among survivors in progressively ageing cultures.

It has been seen that in the evolution of micro-organisms, increased growth rates of nutritional mutants in the vegetative state may play some part in their selection. It is suggested here that increased viability of nutritional mutants in the resting stage might also offer some basis for their selection under arid, starvation, or other conditions in which the dormancy of resting cells was prolonged.

Summary.

1. The comparative biochemistry of different micro-organisms as illustrated by the nutritional mutants which occur, indicate close similarities but differences in detail of certain biosyntheses.

2. Differential survival under starvation may have evolutionary significance. In a wild type population of dormant resting cells, certain nutritional mutants which have arisen as the result of spontaneous mutation, may be positively selected as the period of dormancy extends, by virtue of the slower rate of dying off arising from lowered metabolic rates.

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