INVESTIGATIONS ON THE GENETICS OF YEAST

(SACCHAROMYCES CEREVISIAE)

A thesis presented for the degree of Doctor of Philosophy

of the University of Glasgow

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Investigations on the Genetics of Yeast (Saccharomyces cerevisi

General Summary

The main object of the work reported was to investigate th possibilities of (a) carrying out in yeast a type of genetic analysis of the same kind as that of random sampling of gametes in higher organisms, and (b) using intact incomplete asci for this purpose, i.e. containing only one, two, or three instead o the normal four spores.

The incomplete asci utilized were derived from three genotypically different diploid strains. One strain was heterozyg for adenine requirement (AD/ad), another for tryptophane requir ment (TRYP/tryp), and the third for both these requirements. Each strain was heterozygous for the mating type gene (α/a).

A study of individual spores dissected from two- and three spore asci showed that each spore was haploid.

Intact one-spore asci germinated to give haploid colonies each of whose cells were all of one mating and nutritional phen type. Intact two-spore asci gave either diploid colonies whos cells were all of one nutritional phenotype, or haploid colonie whose cells were either of one or two nutritional phenotypes. The haploid colonies arose when the two spores were of the same mating type, and the diploid colonies when they were of complementary mating type and fused at germination.

Approximately one half of the intact three-spore asci gave colonies made up of both haploid and diploid cells. The latte

arose following fusion of spores of complementary mating type, and the former from the odd spore which was invariably of a mati type. Presumably the odd spores of a mating type failed to germinate. This conclusion is supported by evidence from the analysis of intact one- and two-spore asci where approximately 80% of such spores failed to germinate.

The results show that both one-spore asci and haploid cell arising from two- and three-spore asci are analagous to random samples of gametes in higher organisms and can, therefore, be genetically analysed in the same manner. Such an analysis showed that the three genes AD/ad, TRYP/tryp, and α/a freely recombined with one another.

The possibility of using the diploid cells arising from intact two- and three-spore asci for genetic analysis was also studied. This type of analysis, however, required the working out of an appropriate theory and the first steps in the develop ment of this are presented.

Information obtained from this 'diploid' analysis confirms the genetic results obtained from the analysis of one-spore asc and the haploid cells derived from two- and three-spore asci. In addition, it yielded information of linkage between the gene loci and their centromeres.

When taken jointly, the two types of genetic analysis of yeast, demonstrated for the first time in the present work, achieve all the advantages of random sampling and tetrad analy:

with the exceptions of directly detecting any abnormalities of meiosis and providing evidence of chromatid interference.

Both the 'total isolation' and the 'delayed enrichment aft starvation' techniques were employed to provide genetically marked (auxotrophic) strains necessary for the above investigat In reporting the results emphasis is laid on a comparison of th spectrum of strains obtained with those obtained by various workers from other species of fungi.

This comparison revealed the following points of interest: the parallelism of yeast, Neurospora, Aspergillus, Ophiostoma a Penicillium in the high proportions of auxotrophs requiring arginine, lysine, and adenine; the high proportion of methioni requiring auxotrophs common in yeast and Neurospora but not in Aspergillus and Ophiostoma where a correspondingly high proport of parathiotrophic types have been isolated; and the occurrenc in yeast of a greater variety of amino acid requiring types tha occur in either Aspergillus or Ophiostoma.

Types of auxotrophs isolated which have not hitherto been recorded as having arisen in some or all of the other four spec include those requiring tyrosine, histidine, serine or glycine, arginine or ornithine (not citrulline), and tryptophane (only, i.e. not responding to any known precursor).

Types of auxotrophs isolated which may be of particular interest to biochemists are those requiring tyrosine and that requiring arginine or ornithine. The former do not respond t

phenylalanine and may therefore involve the blockage of a similar biochemical reaction as is supposed to be blocked in human phenylketonuries. The latter does not respond to citrulline which is an intermediate between arginine and ornith in the Krebs cycle. Also of interest may be the auxotrophs requiring arginine and adenine which are competitively inhibite by lysine and guanine respectively.

The 'delayed enrichment after starvation' technique did no prove any more efficient than the 'total isolation' technique for the isolation of yeast auxotrophs. This was partly due to the vast amount of labour necessary to weed out the diauxotroph from the slow growing strains which, presumably, were selected by this technique. The results of the present work do not exclude the possibility that this technique would be successful should a different monoauxotrophic strain be used.

P R E F A C E

This thesis is divided into three parts.

Part I deals with the genetic analysis of intact incomplete asci of yeast, together with a report on the first steps in the development of a theory for this special type of analysis.

The work reported in Part II was undertaken prior to, and as a necessary prerequisite for the investigations of Part I. It deals with the production of genetically 'marked' mutant (auxotrophic) strains of yeast, and includes a comparison of the spectrum of mutants obtained with those obtained by various workers from other fungi.

Part III records the results of an unsuccessful attempt to develop an efficient technique for the isolation of mutant strains of yeast.

ACKNOWLEDGEMENTS

The author wishes to thank Dr. G. Pontecorvo of the Genetics Department, Glasgow University, for suggesting and supervising this work.

Part of this work was carried out while under tenure of a Postgraduate Maintenance Allowance from the Agricultural Research Council herewith acknowledged with thanks.

CONTENTS

Statement of Problem

Genetic analysis of *Saccharomyces cerevisiae*[*] is usual based on tetrad analysis, that is, on the analysis of all t four products of meiosis of each diploid cell which has undergone meiosis. In yeast the four products are include into a specialised cell (ascospore) and kept together in an ascus. The isolation of the four ascospores from one ascu and subsequent analysis of the haploid cells which arise mitotically from each of these four ascospores has certain distinct advantages: it permits to locate the centromeres, to detect any abnormalities of meiosis, and to study certai details of crossing over (e.g. chromatid interference).

In the classical organisms of genetic study, such as maize and *Drosophila*, tetrad analysis has not yet been possible; genetic analysis is based on pooled random samples of the haploid products of many diploid cells which have gone through meiosis. In other organisms, such as *Neurospora*, *Aspergillus*, *Coprinus*, *Chlamydomonas*, some mosses, etc., both types of analysis are possible.

In yeast the situation is different; random samples o ascospores cannot be obtained since the ascus wall is very tough and not easily broken without microdissection. Such a disadvantage has probably accounted for the fact that, at present, very few workers use yeast as an organism for

[*] for short in the present work the term "yeast" is used for *Saccharomyces cerevisiae*.

genetic study compared with the number using other micro-organisms. This, in spite of the fact that yeast has many distinct advantages from the biochemical point of view (Lindegren, 1952); it is in fact the organism the biochemistry of which is best known.

The development of a method which would dispense with the laborious process of tetrad analysis in yeast and thus facilitate speedier genetical analysis constituted the main object of the work reported in this thesis.

An abundance of asci containing the full complement of four spores is necessary for tetrad analysis. However, in practically all strains of yeast spore formation results in many one-, two-, and three-spore asci with an abundance of two-spore asci and very few complete asci. Even after prolonged selection to increase the frequency of complete asci, incomplete ones are still formed. Such incomplete asci in fact constituted a large proportion of the asci produced by the diploid strains available to the author. The occurrence of this high proportion prompted the attempt to make use of them in fulfilling the main object of the work reported.

Thus, the present work aimed at elucidating (a) the nature of the ascospores present in incomplete asci, and (b) whether or not these asci could be utilized, without dissection, for genetical analysis.

The results indicate that, within limits, such asci can be successfully utilized without dissection. This requires however, the development of a theory for this special type of analysis, and a first step in this direction is reported here.

In order to pursue the investigations outlined above, genetically marked haploid strains had to be produced. The results obtained in isolating these are of sufficient interest to justify reporting here (Parts II and IIIA of this thesis). In particular, it is of some interest to compare the spectrum of mutants obtained in yeast with those of Neurospora, Aspergillus, Ophiostoma and Penicillium. In these four fungi various workers have accumulated a large body of data.

An attempt to develop an efficient technique (based on differential survival under specific starvation) for the isolation of auxotrophic mutants was unsuccessful. The results and the possible reasons for failure are discussed.

General Introduction and Historical Account

The genetics of yeasts, especially strains of Saccharomyces cerevisiae, have been the subject of many investigatio since the discovery of their sexual cycle and its use for experimental breeding by Winge and Laustsen (1938, 1939).

The analytical procedure has followed the same lines as that first employed by these workers, namely, that of tetrad analysis. This type of analysis has shown clearly that inheritance in yeasts follows the classical Mendelian rules.

Genes determining mating type specificity (Lindegren, 1943a, 1943b, 1943c), affecting fermentative and vitamin synthesizing ability (Lindegren, 1945, 1949a; Lindegren, Spiegleman and Lindegren, 1944; Lindegren and Lindegren, 19 Winge, 1949; Winge and Roberts, 1948, 1950; Gilliland, 194 growth requirements (Lindegren, 1949a, 1949b; Pomper and Burkholder, 1949; Pomper, 1950; Leupold and Hottinguer, 1954), morphology of the cells and colonies (Winge and Laustsen, 1939; Ditlevsen, 1944), have all been utilized an shown to be inherited in a more or less simple Mendelian manner. Further the genetics of a respiratory deficiency (Chen, Ephrussi and Hottinguer, 1950), of flocculence (Thorn 1951), and of the ability of haploid cells to diploidize spontaneously (Winge and Roberts, 1949), have been worked ou A case of complementary gene action in yeasts has also been reported (Magni, 1949).

Several cases of linkage between genes and between them and their centromeres have been reported by Lindegren (1949a, 1949b, 1952) in Saccharomyces cerevisiae, and maps of at least four chromosomes have been constructed. The statistical treatments for calculating linkage relationships from data obtained by tetrad analysis of unordered asci have been developed by Lindegren (1949a, 1949b), Perkins (1949, 1953), Whitehouse (1950, 1954), Papazian (1952a, 1952b), and Spiegelman (1952). By means of these treatments map distances between genes and between genes and centromeres can be calculated.

Obviously the production of large numbers of four-spore asci is necessary for tetrad analysis of yeast. That it is not easy to obtain them is shown by the many investigations on the effect of environmental factors on spore formation (Rees, 1869; Engel, 1872; Hansen, 1883; Beijerinck, 1894; Garodowka, 1908; Welton, 1914; Baltatu, 1939; Graham and Hastings, 1941; Nickerson and Thimann, 1941, 1943; Mrak, Phaff and Douglas, 1942; Lindegren and Lindegren, 1944; Fowell, 1952). The technique of Lindegren and Lindegren (see page 13) has been used with considerable success in the present work. But even this technique leads to the production of a high percentage of incomplete asci and in some strains almost exclusively so.

Studies on the nature of ascospores within these incom-

plete asci and their possible use in genetical analysis have been extremely limited. In view of this the present author has investigated the nature of the ascospores within incomplete asci of Saccharomyces cerevisiae, and the possible utilization of these asci for genetical analysis; the findings are reported here. The first steps in the development of a theory for this special type of analysis are also reported.

For the studies on incomplete asci it was necessary to cross strains differing in genetic markers. The choice fell on nutritionally mutant strains (auxotrophs) produced by ultra violet irradiation. These mutants were isolated by the 'total isolation' technique first evolved by Beadle and Tatum (1941, 1945), using species of Neurospora. This technique has since been used with success to produce auxotrophs of other moulds such as Ophiostoma multiannulatum (Fries, 1947), Aspergillus terreus (Raper et al., 1945), Penicillium notatum-chrysogenum (Bonner, 1946; Pontecorvo, 1947), Aspergillus nidulans (Pontecorvo, 1949a; Hockenhull, 1948), Ustilago maydis (Perkins, 1949), and bacteria such as Escherichia coli (Anderson, 1944; Gray and Tatum, 1944; Tatum, 1945, 1946; Roepke et al., 1944; Demerec, 1946; Lederberg and Tatum, 1946), Acetobacter melanogenum (Gray and Tatum, 1944), Bacterium aerogenes (Devi, Pontecorvo and Higginbottom, 1947, 1951), Bacterium subtilis (Burkholder

and Giles, 1947), Azotobacter agilis (Karlsson and Barker, 1948) and Salmonella typhimurium (Plough, 1950).

This technique was chosen to produce mutants in the present work because it was of interest to compare the spectrum of yeast auxotrophs obtained with those of Neurospora, Aspergillus, Ophiostoma and Penicillin isolated by various other workers. Since most of these auxotrophs were isolated by the 'total isolation' technique, the selection of different kinds of yeast auxotrophs owing to different techniques was excluded (see Fries, 1947; Lein Mitchell and Houlahan, 1948; and Pontecorvo, 1950).

For the same reason the spectra of yeast auxotrophs isolated by Reume and Tatum (1949), and Pomper and Burkholder (1949, 1950) were not included in the comparison. They used the 'double plating technique' (Lederberg, 1946) which, as can be seen by comparing their results, involves a highly selective personal factor. Out of a total of 14 auxotrophs characterized by the former workers, only 3 were monoauxotrophs; of 19 characterized by the latter, all except one were monoauxotrophs. These differences are probably in part due to the enormous scatter in the germination times of the irradiated cells. Such a scatter was shown to occur by Devi, Pontecorvo and Higginbottom (1951) who used this technique for isolating auxotrophs of Aerobacter aerogenes.

During this work on the production of auxotrophs the employment of a more efficient technique than that of 'total isolation' was investigated. Beadle and Tatum's 'total isolation' technique has been modified and improved upon in several ways by later workers using microorganisms other than yeast. Such modifications have resulted both in increasing the number of auxotrophs recovered per number of tested isolates, and in an opportunity to select those auxotrophs requiring a specific nutrillite for growth (Lederberg and Tatum, 1946; Fries, 1947, 1948a, 1948b; Lein Mitchell and Houlahan, 1948; Davis, 1948, 1949a, 1949b Lederberg and Zinder, 1948; Pontecorvo, 1946; Macdonald and Pontecorvo, 1950; Adelberg and Myres, 1952; Woodward, De Zeeuw and Srb, 1952; Forbes, 1952; Lederberg and Lederberg, 1952; Margalith, Sermonti, and Silvestri, 1953).

Among the more efficient of these methods are those of Fries (1947) and Davis (1948, 1949a). But neither of these methods can readily be applied to the isolation of yeast auxotrophs. Fries's method depends on the differential filtration of prototrophic mould spores, which produce hyphae on germination in minimal medium, and auxotrophic spores unable to do so. Yeast cells give no such hyphae on germination. Davis's method which has been applied to species of bacteria susceptible to penicillin depends on the fact that this antibiotic will only kill proliferating cells

and not affect the non-proliferating cells. No substance i known which kills dividing yeast cells but not non-dividing ones in the same manner as penicillin kills bacteria.

A technique which was thought may be applicable to increase the efficiency in isolating yeast auxotrophs was that evolved by Macdonald and Pontecorvo (1950, 1953) in Aspergillus nidulans. This work was prompted by the work of Fries (1948a, 1948b) who demonstrated that polyauxotrophi conidia of Ophiostoma multiannulatum survive longer than monoauxotrophic (having one growth requirement) in minimal medium. Macdonald and Pontecorvo bearing this fact in mind combined a delayed enrichment technique with that of differential starvation and obtained a yield of almost 70% polyauxotrophs among the total colonies isolated.

In view of these results it was decided to investigate the possibility of using this technique for the isolation of polyauxotrophs of yeast.

General Materials and Methods

1. Media and other substrata used

The principal media used in the isolation, maintenance and comparison of all strains is given below. The complete medium was first used by Pontecorvo and Roper (unpublished). It contained most of the known growth factors and was used throughout the studies for the isolation and maintenance of strains whose genotypes were unknown.

The minimal medium, containing a mixture of mineral salts, glucose and three accessory growth factors, was a modification of the minimal medium evolved by Ephrussi (personal communication). The medium was modified after some preliminary studies on the growth requirements of the strains employed in the present work.

Both haploid and diploid auxotrophs were maintained on minimal medium supplemented with the growth factors which they required. This was done to decrease selection of strains of diverse genotypes, requiring additional growth factors, which could arise from the maintained strains by mutation.

Composition of complete medium:

Glucose	10 gms.
Peptone	3 gms.
Difco yeast extract	5 gms.
Hydrolysed yeast nucleic acid	3 ml.
Hydrolysed thymus nucleic acid	2 ml.
Hydrolysed casein	5 ml.
Vitamin solution	2.5 ml.
Casein digest	2 ml.
Tap water	1 litre

Buffered at pH 6

Concentration of various constituents of complete medium:

Constituent of complete medium	Concentration of solution use
Hydrolysed yeast nucleic acid	Equivalent to 100 mgms. of yea nucleic acid per ml.
Hydrolysed thymus nucleic acid	Equivalent to 100 mgms. of thymus nucleic acid per ml.
Hydrolysed casein	20% with respect to casein
Casein digest	Equivalent to 125 mgms. of casein per ml.

Constituents of mixed vitamin solution:

D-biotin	25 gamma	Nicotinic acid	10 mg.
i-inositol	50 mg.	Riboflavin	10 mg.
Calcium D-pantothenate	10 mg.	p-amino benzoic acid	5 mg.
Aneurin	10 mg.	Distilled water	10 ml.

Composition of minimal medium:

Glucose	20 gms.
Ammonium chloride	2 gms.
Potassium dihydrogen phosphate	1.5 gms.
Magnesium sulphate	0.5 gms.
Calcium chloride	0.5 gms.
Solution of trace elements	0.25 ml.
Special vitamin solution	2 ml.
Distilled water	1 litre

Composition of trace element solution:

Ferrous sulphate 7 H_2O	30 mgms.
Potassium permanganate	3 mgms.
Zinc sulphate 7 H_2O	42 mgms.
Cobalt nitrate 6 H_2O	5 mgms.
Potassium iodide	10 mgms.
Distilled water	25 ml.

Composition of special vitamin solution:

D-biotin	25 gamma
i-inositol	50 mgms.
Calcium D-pantothenate	10 mgms.
Distilled water	10 ml.

When solidified medium was needed 20 gms. of agar were added per litre of liquid medium.

Sporulation media

Two kinds of presporulation media were used to induce sporulation of the diploid strains. The use of modified Garodkawa's medium (1908) resulted in a very high percentage of incomplete asci and very few complete ones. Lindegren a Lindegren's (1944) presporulation medium was used when a hi percentage of complete four-spore asci were needed.

Engel's (1872) gypsum block method as modified by Greham and Hastings (1941) was used to reduce the time required to obtain mature spores and to decrease the danger o contamination. The addition of 3 ml. of a dilute solution of acetic acid (pH 4) to the gypsum blocks was made after inoculation of the yeast, thus following the recommendation of Baltatu (1939).

Modified Garodkawa's presporulation medium:

Peptone	10 gms.
Glucose	2.5 gms.
Sodium chloride	5 gms.
Difco yeast extract	20 gms.
Agar	20 gms.
Distilled water	1 litre

Lindegren and Lindegren's presporulation medium:

Beet leaf extract	10 ml.
Beet root extract	20 ml.
Grape juice	16.5 ml.
Apricot juice	35 ml.
Dried yeast	2 gms.
Glycerin	2.5 ml.
Calcium carbonate	3 gms.
Agar	2 gms.
Distilled water	added to give a final volume of 100 ml.

The beet leaf extract was prepared by grinding approximately 200 gms. of beet leaves in a meat grinder and squeezing the juice out of the ground mass through a cheese cloth. The beet root extract was prepared in a similar fashion. Both the apricot and grape juice were commercial products.

Preparation of gypsum slopes:

Equal weights of distilled water and plaster of Paris were rapidly mixed in a 250 ml. flask and the mixture poured into test-tubes where it was allowed to solidify in a slanting position. Before sterilizing the slopes were dried for 24 hours at 50° C.

Saline solution:

Suspensions and dilutions of yeast cells were made in normal (0.85%) saline solution. To facilitate dilution of cells the saline solution was prepared in 9 ml. amounts, pipetted into and sterilized in standard containers. Dilution by a factor of ten could then be done easily by transferring 1 ml. of the solution to be diluted into the prepared 9 ml. of saline solution.

Sterilization:

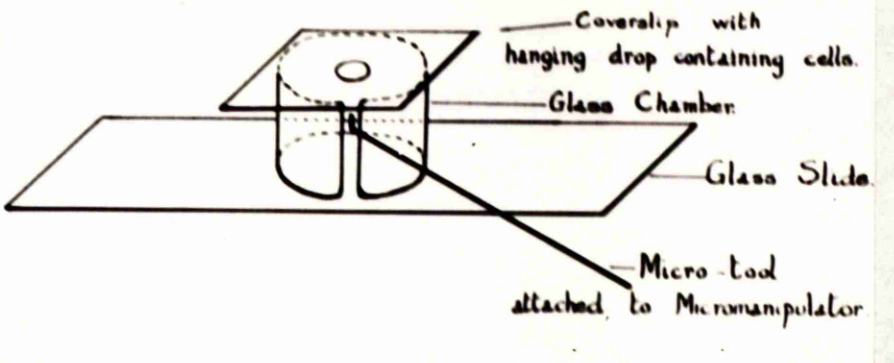
All media, gypsum slopes and saline were sterilized by autoclaving at 10 pounds per square inch pressure for a period of 15 minutes. All glassware was sterilized by dry heat in an oven maintained at 160° C., for 1.25 hours.

2. Method of Isolation of Asci and Ascospores

Single ascospore cultures were always used in order to guarantee as far as possible the purity of each strain. Th isolation and dissection of all asci, together with the isolation of ascospores was carried out with the aid of a de Fonbrune micromanipulator, using micro-needles and micro-loops prepared with the aid of the de Fonbrune microforge. (de Fonbrune, 1934, 1937; Comandon and de Fonbrune, 1938)

Using aseptic techniques asci were removed from the gypsum slope or presporulation medium and suspended in salin together with the accompanying diploid vegetative cells whic had not sporulated. A droplet of such a suspension was transferred to a 3/4 inch square coverslip and inverted over a modified van Tieghem chamber. Such a chamber was constructed by placing a 1/4 inch length of tubing, 5/8 inch in diameter, in the middle of a glass slide. Before placing the tubing in position, about 1/4 inch of its circumference was cut out. This was done to allow the insertion of micro-tools into the chamber. Before inversion of the

coverslip over the chamber, a drop of sterile water was placed in the base; this prevented the droplet containing the yeast asci drying out.



MICRODISSECTION CHAMBER.

With the aid of the low power lens of a compound microscope both droplet and micro-tool were centred in the field of vision, and by means of the coarse adjustment screw on the nose of the micromanipulator, the micro-tool was brought into approximately the same plane as the cells in the droplet of saline before changing to the high power lens. Dissection of an ascus was performed by transferring it very quickly from the fringe of the droplet to a dry part of the coverslip. The rapidity of this transfer is essential since surface tension forces operate to prevent the separation of the ascus from the fringe of the droplet.

To rupture the wall of the ascus, it was gently rolled

about on the dry part of the coverslip by a micro-loop until the wall collapsed. Direct pressure on the ascus resulted in a lower percentage germination of the ascospores, probably due to injury. The liberated spores were lifted away individually from the coverslip within the micro-loop which was then lowered from the surface of the coverglass. The coverglass containing the droplet was then replaced by another coverglass carrying on the underside four separated spots of melted sterile complete medium agar. The micro-loop was then gently raised toward the surface of one of these agar droplets and the ascospore deposited on its surface. This operation was repeated until all the ascospores of an ascus had been transferred each to a different agar droplet. The coverglass was then removed from the chamber and placed, agar downward, over plastic concavity slides into the cavities of which had been placed a drop of sterile water to prevent drying out of the agar droplets. The concavity slides were incubated until microcolonies of yeast visible to the naked eye appeared. Such microcolonies could usually be seen after 24 hours incubation.

The isolation of whole intact asci was carried out as described above except that the whole ascus was transferred to the agar droplets instead of the individual ascospores.

To facilitate the later transfer of the vegetative cells arising from the ascospores to another medium for propagation,

the agar droplets deposited on the under side of the coverglass were prepared according to a method of Hemmons (unpublished - personal communication). This method consisted of using thin permeable cellophane strips approximately 1/8 inch square, which after sterilization were dipped into molten complete agar medium and immediately transferred to the under side of a coverglass where the agar solidified around the cellophane and stuck to the coverglass. This cellophane-agar slip was transferred after the deposition of the ascospore or ascus, or after the vegetative cells had arisen, by lifting the slip from the coverglass using a sterile forceps.

3. Method of Producing Heterozygous Diploid Strains

The procedure adopted for the crossing of two genetically different haploid strains, in order to produce a diploid heterozygous strain, was that used by Chen (1950). This combined the methods adopted by Lindegren and Lindegren (1943) Winge and Laustsen (1938) and Pomper and Burkholder (1949).

Single haploid vegetative cells derived from single ascospore cultures of each of the strains to be crossed were placed side by side on a cellophane-agar slip by a micro-loop attached to the de Fonbrune micro-manipulator. The coverglass containing the cellophane-agar slip was then transferred to a van Teigham cell and the cells relocated and centred in the low power field of vision of a compound micro-

scope. The cells were examined under the high power lens every 10 minutes for evidence of fusion. Should one or both of the haploid cells reproduce vegetatively before fusion, the cells were discarded and another similar attemp made at crossing the strains. Cell fusion took place usually from 4 to 8 hours after placing the cells side by side, the average time for fusion being 4.25 hours.

Most haploid strains used in the experiments to be described were auxotrophs, lacking the ability to synthesiz different growth factors. The diploid strain resulting from the fusion of two such auxotrophic haploid strains, each with different requirements, should therefore be a prototroph, i.e. able to grow on minimal medium which lacks the growth factors required by each of the haploid auxotroph from which it was derived. To ensure that the diploid zygote had actually arisen from the fusion of the two auxotrophic haploid cells, a sample of cells arising by vegetative propagation from the diploid fusion cell was tested for growth on minimal medium. Their growth showed that the cross had been successful.

Such a method of hybridization, as well as ensuring as far as possible that the heterozygous diploid is formed, has the distinct advantage over Winge and Laustsen's method (193 of crossing ascospores in that the only cell of the strain i not consumed in the mating process; cells of each haploid

strain are therefore available for other crosses. Further, there is no gamble with regard to the mating type of the cells to be crossed because a sample of each haploid strain can be tested for mating type before the cross is undertaken. The method also overcomes Winge and Roberts' objections (1948) to Lindegren's (1943b) mass mating procedure of vegetative cells.

The diploid cells used in the experiments were prevented from forming asci by transfer to a new minimal medium agar slope every 7 days.

4. Origin of Strains Used

All the strains of yeast used in the following experiments originated from four single ascospore cultures of Saccharomyces cerevisiae kindly supplied by Professor B. Ephrussi. Two of these cultures, namely B15 and C53-8d, were of 'a' mating type, the other two, B26 and C24-15b, of 'α' mating type. All four strains originated from two strains, namely, "Boulangerie II" which was made available to Professor Ephrussi from the yeast collection of the Laboratoire de Fermentations of L'Institut Pasteur de Paris, and "Yeast Foam" a strain of American bakers yeast supplied by Professor Ö. Winge of the Carlsberg Laboratories, Copenhagen. All auxotrophs used were isolated and identified in a series of experiments described later (Part II of this thesis).

PART I

The Genetic analysis of Intact, Incomplete Asci of Yeast

Introduction

The formation by different yeast species of asci containing less than four ascospores, i.e. incomplete asci, has been mentioned casually by a number of workers. Certain species of Saccharomyces and Nadsonia normally produce only one spore per ascus (Kostka, 1926; Stelling-Dekker, 1931), but genetical investigations on the nature of the ascospores of these species have been few and incomplete.

Winge (1944) showed that the ascospores of Saccharomyces unisporus gave colonies which differ in form from the mother type, presumably owing to segregation. These one-spore cultures diploidized early in development and the cells were capable of producing asci. Similarly, Nadson and Konokotina (1926) working with Nadsonia elongata and N. fluvescens, and Skovsted (1943) working with N. Richetri considered that meiosis and segregation took place at spore formation which was followed by the degeneration of three nuclei.

Investigations of ascospores within incomplete asci of species capable of producing complete asci have been even fewer. Lindegren and Lindegren (1944) and Lindegren (1949b) studied the ascospores within two- and one-spore asci of a diploid strain of S. cerevisiae which they regarded as illegitimate, i.e. formed by fusion of haploid cells of the

same mating type. The two-spore asci were inviable. The one-spore asci produced diploid colonies which resembled the mother diploid; the spore was therefore not a product of meiosis.

Three-spore asci have occasionally been utilized for genetical analysis of S. cerevisiae (Lindegren, 1949a, 1949b Pomper and Burkholder, 1949). These workers assumed that the missing spore, if it had been present, would have corresponded to the fourth genotype.

The present study is concerned with finding out the nature of the spores within incomplete asci of S. cerevisiae and whether or not these asci can be used, without dissection, for genetical analysis. This type of analysis required the development of a special type of theory and this is presented.

In view of certain mistakes of previous workers, it was necessary to be sure that the diploid cells, from which the asci were derived, were legitimate zygotes. The use of suitable markers took care of this.

Materials and Methods

The diploid cells used in the experiments were produced by crossing haploid strains of opposite mating type which were completely heterothallic, practically never producing asci in cultures derived from single ascospores. The genetic markers utilized, besides mating type, were either

or both of the following monogenic differences in nutritional requirements. AD/ad, independence of versus requirement for adenine; TRYP/tryp, independence of versus requirement for tryptophane. The actual haploid strains used, together with their derivation, are shown in the following table.

Haploid strain	Derivation	Mating type	Nutritional requirements
C24-13b	Received from Prof. B. Ephrussi	a	none
B15	Received from Prof. B. Ephrussi	a	none
19/20, 1b	A single ascospore culture from a cross A7 x B15 (strain A7 which requires adenine for growth was obtained by u.v. treatment of C24-13b)	a	adenine
19/20.8	A single ascospore culture from a cross D14/18 x C24-15b (strain D14/18 which requires tryptophane for growth was obtained by u.v. treatment of B15)	a	tryptophane

Crosses were made with the de Fonbrune micromanipulator as described on page 17 of this thesis. If both haploid strains used in a cross were auxotrophs of different types, the resulting diploid was tested for prototrophy by plate-testing on minimal medium; this provided an extra check that the cross was legitimate. If only one of the strains crossed was an auxotroph, the resulting diploid cells were

induced to form four-spore asci. A few of these asci were dissected and the cells resulting from each of the four ascospores tested for nutritional requirement. A resulting ratio of 1 requirer to 1 non-requirer per ascus indicated that the cross was legitimate.

The method of inducing the formation of one-, two- and three-spore asci from diploid strains is described under "General Material and Methods". Incomplete asci were isolated individually on to cellophane-complete agar medium slips and allowed to germinate intact at 28°C. The cells of the colonies obtained after 24 to 48 hours incubation were analysed for ploidy and genotype. The method of analysis of cells arising from one-spore asci differed from that of cells arising from two- and three-spore asci. Each method of analysis is described below.

Method of analysis of cells arising from germination of intact one-spore asci.

Nutritional requirements:

Each cellophane-agar slip containing the colony which had arisen from a one-spore ascus was removed from the coverglass and placed, colony upward, on a petri-dish of solidified complete agar medium. Twenty such strips were placed on each dish and the dish incubated for two days to allow further growth of the colonies. A small inoculum of cells from each colony was then plate-tested for nutritional

requirement.

A small inoculum of cells from each colony was also transferred to each of two tubes of complete medium, one containing a suspension of haploid vegetative cells of strain C24-15b, mating type α, the other a suspension of B15, mating type a. Such tubes were examined after 18 hours incubation. If the tested cells were haploid, mating would have taken place in one of the tubes. If mating did not occur in either, the colony was assumed to be diploid. Two criteria were adopted to distinguish whether or not mating had occurred. Mating was assumed to have taken place if there could be seen (a) a characteristic flocculation of cells on shaking the tube and (b) characteristic fertilisation bridges between cells of opposite mating type on microscopic examination of a drop of suspension. The morphology of colonies and cells was also characteristic of ploidy.

If any doubt arose as to the ploidy of the cells after such tests, an inoculum of the cells was given an opportunit of forming four-spore asci by transferring them to Lindegren and Lindegren's presporulation medium and then to gypsum slants. Formation of any four-spore asci confirmed that th cells were diploid.

Method of analysis of cells arising from germination of intact two- and three-spore asci:

The colony of cells resulting from germination of an individual two- or three-spore ascus (on the cellophane-aga slip) was transferred to 0.1 ml. of complete liquid medium deposited in the form of a drop on the surface of complete-agar medium in a petri-dish. The cellophane-agar slip was agitated in this drop and the drop, now containing the separated cells of the colony, spread over the surface of t complete-agar medium. The dish was incubated for three days after which a sample of the colonies which had grown on the plate was first tested for ploidy and then nutrition requirement in the same manner as described for the analysi of colonies arising from one-spore asci.

A modified method of analysis of colonies arising from two-spore asci was adopted after the first two experiments. The cellophane-agar slip on which the colony had grown was transferred to the surface of complete-agar medium containe in a petri-dish; thirty such transfers were made per dish and each dish incubated for three days to allow the colonie to become larger. Each colony was then tested for ploidy and nutritional requirement. Those found to be haploid 'non-requirers' by these tests could be composed either of both 'requiring' and 'non-requiring' cells or of 'non-requiring' cells only. A further analysis of such haploid colonies was not undertaken and therefore no distinction wa made between those containing both 'requiring' and 'non-requiring' cells and those containing only 'non-requiring'

cells.

The diploid colonies were assumed to be of one type of cell only, an assumption based on the fact that no diploid colonies of 'mixed cells' (different nutritional phenotype) were detected in the first two experiments.

Theoretically Expected Results

The following calculations of the results expected from the analysis of intact incomplete asci are based on the fact that each ascospore within them is a haploid product of meiosis, and on the assumption that there is no differential viability of nuclei of certain genotypes after meiosis.

That the ascospores within two- and three-spore asci ar haploid was shown by a preliminary series of experiments in which twenty-five asci of each type were dissected. Each of the dissected ascospores gave rise to a haploid culture (see experimental results, page 72).

a. One-spore Asci

Consider a single gene B/b and the mating type gene a/α 1. Independent assortment of these two genes would be expected to yield a genotypic (and therefore a phenotypic) ratio of asci of 1 a B : 1 α B : 1 a b : 1 α b from both the cross a B x α b and the reciprocal cross a b x α B. Such a ratio would also be expected if both these genes were situated on the same chromosome and showed free recombinatio

with one another.

2. Complete linkage between the two genes would be indicated when the combination of alleles entering the cross is always similar to those recovered from the asci; only tw types of asci would therefore be expected per cross.

3. Partial linkage between the two genes is indicated when the phenotypes of cells arising from a series of one-spore asci deviates from 1:1:1:1, the ratio expected in the case of free recombination. The calculation of the amount of linkage is similar to that employed in the analysis of random spores.

These considerations also apply to the analysis of the haploid cells of colonies arising from the germination of three-spore asci (see page 70).

b. Two-spore Asci

It is expected that intact two-spore asci would give on germination (a) colonies of diploid cells all of one kind or (b) colonies of haploid cells which could be all of one type or (c) of two types. Diploid cells (case a) arise when the two spores are of opposite mating type and fuse at germination, and haploid cells (cases b and c) when the two spores are of the same mating type. The expected ratio of different types of asci with regard to the mating type of the two spores is 1 a and a : 4a and α : 1 α and α.

The haploid colonies may consist of cells all of the

same genotype or of two different genotypes (other than for mating type). The ratio of such different kinds of haploid colonies depends on the number and linkage relationships of the genes segregating from a heterozygous diploid, e.g. in the case of a one gene segregation a genotypic ratio of 1 : 4 : 1 would be expected among the colonies, viz.,

Gene B/b segregating:

Asci	((B)) ( ) ((B))		((B)) ( ) ((b))		((b)) ( ) ((b))
Expected ratio	1	:	4	:	1
Haploid cells of colony	B only		B and b		b only

Linkage relationships between two or more genes can be calculated if each of the haploid colonies is considered to consist of cells derived from two spores of the same mating type. Colonies having two types of haploid cells ("di-type colonies") derive from asci with two spores of different genotypes, apart from mating type. The genotype of each spore is deduced by isolating and classifying the two kinds of cells. Colonies of haploid cells all of the one type only ("mono-type colonies") are assumed to derive from asci having two genotypically identical spores. In the calculations to follow the mono-type colonies must be counted twice.

On the basis of this spore classification linkages are calculated by treatment such as is used for random sampling

of gametes in higher organisms (Mather, 1955). The greater the number of genes segregating the higher the expected proportion of colonies with the two types. The use of more than one gene segregating makes it possible to detect and estimate the misclassification due to the fact that sometime only one spore germinates. In our calculations a mono-type colony is taken as derived from two identical spores. No information with regard to the linkage relationships of gene to their centromeres can be obtained by analyzing data from haploid colonies in this way.

The phenotypic ratios of diploid colonies arising from intact two-spore asci give information on the linkage relationships both between genes and between genes and their centromeres.

The phenotypic ratios of the diploid cells of colonies from intact three-spore asci can also give this information. Such information, however, depends upon the assumption that in the three-spore ascus the two spores which fuse and give diploid colonies do not first give rise to haploid vegetativ cells which fuse later to give two possible combinations. This source of misclassification could be avoided by isolating a single diploid cell taken at random after germination of a three-spore ascus. However, the possibili of differential survival or multiplication of cells of different genotypes within the colony arising from the

germination of the asci would have to be considered. Finally, four-spore asci could also be used by isolating a single diploid cell from the colony derived from each ascus.

In the following calculations of the results expected from analysis of the diploid colonies arising from intact two-spore asci under different genotypic conditions, the following points should be borne in mind:

1. Each segregating gene, except that for mating type, is represented by a fully dominant and fully recessive allele.

2. In all calculations x represents the relative frequency of second division segregation between the mating type gene a/α and its centromere, y the relative frequency between gene B/b and its centromere and z the relative frequency between gene C/c and its centromere; 1-x, 1-y and 1-z represent the relative frequencies of first division segregation.

Calculation 1

Result expected when a. gene B/b freely recombines with centromere and is independent of the mating type gene, a/α, or b. when gene B/b freely recombines with its centromere an is on the same chromosome as gene a/α, but on the opposite side of the centromere

These two situations cannot be distinguished from one another without the use of further 'markers'.

From Table 1 (page 31) the expected proportion of phenotype b colonies is equal to $(2x + 2y - 3xy)/8$.

When gene B/b shows free recombination with its centro-

TABLE 1

The Types and Frequencies of Four-spore Asci, Together with the Proportions of Diploid Phenotypes, Expected When the Mating Type Gene, a/α, and Gene B/b are On Different Chromosomes.

Cross: $\frac{a}{\alpha}\ \frac{B}{B} \times \frac{\alpha}{a}\ \frac{b}{b}$ or $\frac{\alpha}{\alpha}\ \frac{B}{B} \times \frac{a}{a}\ \frac{b}{b}$

1st. Division Segregation For Gene(s)	2nd. Division Segregation For Gene(s)	Types of Asci I	II	III	Frequency	Proportion of Phenotypes B	b
		A					
a/α, B/b	-	B a B a b α b α	B α B α b a b a		(1-x)(1-y)	All	0
		B					
B/b	a/α	B a B α b a b α	B α B a b a b α		x(1-y)	3/4	1/4
		C					
a/α	B/b	B a b a B α b α	B a b a b α B α		(1-x)y	3/4	1/4
		D					
-	a/α, B/b	B a b α B a b α	B a b α b a B α	B a b α b α B a	xy	7/8	1/8
		1 :	2	: 1			

more than $y = \frac{2}{3}$ (Mather, 1935). Therefore, the proportion of different phenotypes expected under the above conditions is equal to:-

$$1 - [(2x + \tfrac{4}{3} - 2x)/8]\ \underline{B} \text{ and } (2x + \tfrac{4}{3} - 2x)/8\ \underline{b}$$

$$= \tfrac{5}{6}\ \underline{B} \text{ and } \tfrac{1}{6}\ \underline{b}$$

Calculation 2

Result expected when genes B/b and a/α freely recombine with one another but are each completely linked to their centromeres

In this situation only asci of types IA and IIA of Table 1 are expected. Thus, no diploid phenotypes of constitution b are expected.

The result would be the same if the two genes were on the same chromosome, either on the same side or on opposite sides of the centromere.

Calculation 3

Result expected when gene B/b is completely linked to its centromere and the frequency of second division segregation of gene a/α(x) is known

In this situation only asci of types IA, IIA, IB and IIB of Table 1 are expected. The expected proportion of phenotype b diploids is therefore equal to $\frac{1}{4}x(1-y)$.

Complete linkage of gene B/b to its centromere (i.e. $y = 0$) is therefore indicated when the proportion of b phenotypes is equal to $\frac{1}{4}x$.

Calculation 4

Calculation of the linkage relationship between gene B/b and its centromere when the frequency of second division segregation (x) of the mating type gene, a/α, is known, and both genes freely recombine with one another.

In this situation all types of asci in Table 1 are expected. The expected proportion of phenotype b diploids is, therefore, equal to $(2x + 2y - 3xy)/8$.

If both x and the proportion, p, of diploid b phenotypes are known, then y, the frequency of second division segregation of gene B/b, may be calculated using this equation.

This is most simply done using the alternative quantities $X = 1 - \frac{3}{2}x$, and $Y = 1 - \frac{3}{2}y$, which are related to the proportion, p, thus:

$$1 - 6p = XY$$

It will be seen that this relationship is fundamental in work of this kind and will occur again. It is worth noting that the quantities X and Y take the minimum values of zero for free recombination of the genes a/α and B/b with their centromeres, and maximum values of 1 for complete linkage to their centromeres.

Calculation 5

Calculation of the linkage relationship between gene B/b and the mating type gene, a/α, when they are on the same chromosome but on opposite sides of the centromere.

Table 2 (page 34) shows the types and frequencies of asci, together with the proportion of phenotype b diploids expected under the above conditions.

TABLE 2

The Types and Frequencies of Four-spore Asci, Together with the Proportions of Diploid Phenotypes, Expected When the Two Linked Genes a/α and B/b are On Opposite Sides of the Centromere.

Cross: a . B x α . b

1st. Division Segregation For Gene(s)	2nd. Division Segregation For Gene(s)	Types of Asci I	II	III	Frequency	Proportion of Phenotypes B	b
		A					
a/α, B/b	-	B a	B α		(1-x)(1-y)	All	0
		B a	B α				
		b α	b a				
		b α	b a				
		B					
B/b	a/α	B a			x(1-y)	3/4	1/4
		B α					
		b a					
		b α					
		C					
a/α	B/b	B a			y(1-x)	3/4	1/4
		b a					
		B α					
		b α					
		D					
		St.					
		2	3	4			
		B a	B a	B α			
-	a/α, B/b	b α	b α	b a	xy	7/8	1/8
		B a	B α	B α			
		b α	b a	b a			
		1	: 2	: 1			

St. / 2 3 4 denotes asci resulting after two, three and four strand crossing over.

This proportion, p, is equal to $\frac{1}{4}(x + y - \frac{3}{2}xy)$, or, if $X = 1 - \frac{3}{2}x$, and $Y = 1 - \frac{3}{2}y$, it may be derived from,

$$1 - 6p = XY$$

It is convenient at this stage to introduce a measure, u, of the relative frequency of tetratype and ditype asci with regard to the genes A/a and B/b. Thus of the three types of asci

a B	a b	a B
a B	a b	a b
a b ,	a B ,	a B ,
a b	a B	a b

the first two are taken to comprise a proportion, 1-u, and the latter a proportion, u.

In Table 2, the quantity u is derived directly from: $u = x + y - \frac{3}{2}xy$, or, if $U = 1 - \frac{3}{2}u$, from $U = XY$. Thus it may be directly estimated from:

$$U = 1 - 6p.$$

The appendix shows (page 67) that u (or U) is related to the mean number of chiasmata between the loci of the two genes, and, through this, to the recombination frequency, f, between them. The fundamental relationship here is:

$$f = \frac{1}{2}(1 - U^{\frac{2}{3}}).$$

Consequently, the recombination frequency between the two loci may be directly derived from:

$$f = \frac{1}{2}\left[1 - (1 - 6p)^{\frac{2}{3}}\right]$$

$$= 2p + 2p^2 + \frac{20}{3}p^3 \ldots\ldots$$

The first two terms of this expansion will usually give

f to a sufficient degree of accuracy. For instance, if p = 0.1, the accurate formula gives:

$$f = \tfrac{1}{2}(1 - 0.4^{\frac{2}{3}})$$
$$= 0.229$$

The series expansion to two terms gives:

$$f = 0.2 + 0.02$$
$$= 0.22$$

Calculation 6

Calculation of the linkage relationship between gene B/ and the mating type gene, a/α, when they are on the same sid of the centromere.

Tables 3 and 4 (pages 37 and 38) show the types and frequencies of asci expected from heterozygotes derived from the crosses . B a x . b α (gene order: centromere-B/b-a/α) and . a B x . α b (gene order: centromere-a/α-B/b).

In both instances, u is used to denote the frequency o tetratype asci. In the first instance, u is related to x and y by the relationship:

$$x = y + u - \tfrac{3}{2}uy$$
or
$$X = YU$$

and, in the second instance, by the relationship:

$$y = x + u - \tfrac{3}{2}ux$$
or
$$Y = UX$$

In both instances, the proportion, p, of phenotype b diploids expected is equal to $\frac{1}{4}$ u, and hence

$$u = 4p$$
or
$$U = 1 - 6p.$$

TABLE 3

The Types and Frequencies of Four-spore Asci, Together with the Proportions of Diploid Phenotypes, Expected When the Two Linked Genes a/α and B/b are On the Same Side of the Centromere.

Cross: $\frac{\cdot\ B\ a}{}$ x $\frac{\cdot\ b\ \alpha}{}$ (gene B/b nearest the centromere)

1st. Division Segregation For Gene(s)	2nd. Division Segregation For Gene(s)	Types of Asci I	II	III	Frequency	Proportion of Phenotypes B	b
		A					
a/α, B/b	-	B a	B α		(1-y)(1-u)	All	0
		B a	B α				
		b α	b a				
		b α	b a				
		B					
B/b	a/α	B a			(1-y)u	3/4	1/4
		B α					
		b a					
		b α					
		C					
-	(a/α), B/b	B a	B α		y(1-u)	All	0
		b α	b a				
		B a	B α				
		b α	b a				
		D					
		St. 2	3	4			
-	a/α, B/b	B α	B a	b a	yu	3/4	1/4
		B a	B α	B α			
		b a	b a	B a			
		b α	b α	b α			
		1 :	2 :	1			

(a/α) denotes that second division segregation of gene a/α results from crossing over between gene B/b and the centromere and not from crossing over between genes B/b and a/α.

St. 2 3 4 denotes asci obtained after two, three and four strand crossing ove

TABLE 4

The Types and Frequencies of Four-spore Asci, Together with the Proportions of Diploid Phenotypes, Expected When the Two Linked Genes B/b and a/α are On the Same Side of the Centromere.

Cross: . a B x . α b (gene a/α nearest the centromere)

1st. Division Segregation For Gene(s)	2nd. Division Segregation For Gene(s)	Types of Asci I II III	Frequency	Proportion of Phenotypes B	b
		A			
a/α, B/b	-	a B a b a B a b α b α B α b α B	(1-x)(1-u)	All	0
		B			
-	a/α, (B/b)	a B a b α b α B a B a b α b α B	x(1-u)	All	0.
		C			
a/α	B/b	a B a b α B α b	(1-x)u	3/4	1/4
		D St. 2 / 3 / 4			
-	a/α, B/b	a B a b a b α B α B α b a b a B a B α b α b α B 1 : 2 : 1	xu	3/4	1/4

(B/b) denotes that second division segregation of gene B/b results from crossing over between gene a/α and the centromere, not from crossing over between genes a/α and B/b.

St. 2 / 3 / 4 denotes asci obtained after two, three and four strand crossing over.

The same relationship for the recombination fraction, f, as was obtained in Calculation 5 thus holds, namely:

$$f = \tfrac{1}{2}\left[1 - U^{\frac{2}{3}}\right]$$
$$= \tfrac{1}{2}\left[1 - (1 - 6p)^{\frac{2}{3}}\right]$$
$$= 2p + 2p^2 + \tfrac{20}{3}p^3 \ldots\ldots$$

A note on Calculations 5 and 6

Neither Calculation 5 nor 6 allow the derivation of either x or y (the frequencies of second division segregation for genes a/a, and B/b), and, even when either x or y is known, two possible values always exist for the other.

The following cases need to be considered. Suppose that x is known in each instance and y is required to be found. A precisely similar situation exists in the reverse case.

a. $x \geq 4p$

Here, $X < 1 - 6p = U$, and hence the possible solutions are $Y = UX$, and $Y = X/U$, i.e. either

$$y = u + x - \tfrac{3}{2}ux$$

and the gene B/b lies on the same side of the centromere but further away than gene a/a, or

$$x = u + y - \tfrac{3}{2}uy$$

and the gene B/b lies on the same side of the centromere but nearer than gene a/a.

b. $x < 4p$

Here $X > 1 - 6p = U$, and hence either $Y = XU$, or $Y = U/X$

i.e. either

$$y = u + x - \tfrac{3}{2}ux$$

and the gene B/b lies on the same side of the centromere but further away than gene a/a,

or $$u = x + y - \tfrac{3}{2}xy$$

and the gene B/b lies on the opposite side of the centromere to gene a/a.

The Detection and Measurement of Linkage between Two Genes, B/b and C/c, and between these Genes and their Centromeres.

The proportions of diploid phenotypes arising from two-spore asci, formed from a diploid heterozygous for the two linked genes B/b and C/c, differ according to how the initial cross is made, i.e. whether the alleles of these genes enter the heterozygote in the coupling or in the repulsion phase. Such is not the case if the genes are assorting independently

Rare colonies of phenotype b c are expected from the asci of a heterozygote in which the alleles are in the repulsion phase, whereas when they are in the coupling phase such colonies are common. This situation is independent of the relationship of the genes to their centromeres, viz.,

a. a B C x a b c
or
a B C x a b c ------ Diploid b c phenotypes common

b. a b C x a B c
or
a b C a B c ------ Diploid b c phenotypes rare

Information with regard to the amount of linkage between two genes and between these genes and their centromeres is

therefore more efficiently obtained from the asci arising from a heterozygote in which the alleles are in the coupling phase.

Calculation 7

Results expected when genes B/b, C/c and the mating type gene, a/α, undergo free recombination with one another but not necessarily with their centromeres

Table 5 (page 43) illustrates the types and frequencies of complete asci which are expected from a heterozygote in which the genes B/b, C/c and the mating type gene a/α, are each linked to their centromeres but undergo free recombination with one another.

a. Results expected when the three genes recombine freely both with one another and with their centromeres

When the three genes segregate independently of their centromeres and of one another then y, z, and x are each equal to 2/5. The proportions of diploid colonies expected under these conditions are:

$$\frac{25}{36}\ \underline{B}\ \underline{C},\quad \frac{5}{36}\ \underline{B}\ \underline{c},\quad \frac{5}{36}\ \underline{b}\ \underline{C},\quad \frac{1}{36}\ \underline{b}\ \underline{c}.$$

b. Results expected when the three genes undergo free recombination with one another but are either completely linked or show free recombination with their centromeres.

These results, calculated in a similar manner to those above, are shown in the following table:

Genes completely linked to their centromeres (no 2nd division segregation)	Genes undergoing free recombination with their centromeres	Ratios of phenotypes expected			
		BC	Bc	bC	bc
1. a/α, B/b and C/c	-	1	0	0	0
2. a/α and B/b	C/c	5	1	0	0
3. a/α and C/c	B/b	5	0	1	0
4. B/b and C/c	a/α	9	1	1	1
5. a/α	B/b and C/c	25	5	5	1
6. B/b	a/α and C/c	25	5	5	1
7. C/c	a/α and B/b	25	5	5	1
8. -	a/α, B/b and C/c	25	5	5	1

It can be seen from this table that the ratio of 25 BC : 5 Bc : 5 bC : 1 bc will be expected in every case where any one of the genes (including that for mating type) is completely linked to its centromere and the other two show free recombination with their centromeres (cases 5, 6 and 7); this ratio will also be expected when all three genes show free recombination with their centromeres (case 8).

As for the two gene case (Calculation 4, page 33), the equations derived in Table 5 may be most simply expressed in terms of the alternative quantities:

$$X = 1 - \tfrac{3}{2}x$$
$$Y = 1 - \tfrac{3}{2}y$$
$$Z = 1 - \tfrac{3}{2}z$$

If p_b and p_c then represent the proportions of pheno-

TABLE 5

The Types and Frequencies of Four-spore Asci, Together with the Proportions of Diploid Phenotypes, Expected When the Three Genes a/α, B/b and C/c Show Free Recombination With One Another but Not Necessarily With Their Centromeres.

Cross: $\frac{a\ B\ C}{}$ x $\frac{\alpha\ b\ c}{}$
or
$\frac{\alpha\ B\ C}{}$ x $\frac{a\ b\ c}{}$

1st. Division Segregation For Gene(s)	2nd. Division Segregation For Gene(s)	Combinations of Alleles* 1 2 3 4 5 6 7 8	Combinations of Alleles Giving Types of Asci Expected	Frequency	Proportion of Phenotypes Bc	bC	bc
a/α, B/b, C/c	-	a α B C c a α B C c α a b c C α a b c C	(1,3,4), (1,3,5), (2,3,4), (2,3,5)	(1-x)(1-y)(1-z)	0	0	0
a/α, B/b	C/c	a α B C C a α B c c α a b c C α a b C c	(1,3,4), (1,3,5), (2,3,4), (2,3,5)	(1-x)(1-y)z	1/4	0	0
a/α, C/c	B/b	a α B B C a α b b C α a B b c α a b B c	(1,3,5), (1,4,5), (2,3,5), (2,4,5)	(1-x)y(1-z)	0	1/4	0
B/b, C/c	a/α	α B C c a B C c α b c C a b c C	(1,2,3), (1,2,4)	x(1-y)(1-z)	1/8	1/8	1/8
a/α	B/b, C/c	α B C C c c α b c c C C a B C c c C a b c C C c	(1,2,3), (1,2,4), (1,2,5), (1,2,6)	(1-x)yz	3/16	3/16	1/16
B/b	a/α, C/c	α B C C c c a B c c C C α b C c C c a b c C c C	(1,2,3), (1,2,4), (1,2,5), (1,2,6)	x(1-y)z	1/8	1/4	0
C/c	a/α, B/b	α B B b b C a b b B B C α B b B b c a b B b B c	(1,2,6), (1,3,6), (1,4,6), (1,5,6)	xy(1-z)	1/4	1/8	0
-	a/α, B/b, C/c	α a B B C c c C a α b b c C C c α a B b c C c C a α b B C c C c	(1,3,5), (1,4,5), (1,3,6), (1,4,6), (1,3,7), (1,4,7), (1,3,8), (1,4,8), (2,3,5), (2,4,5), (2,3,6), (2,4,6), (2,3,7), (2,4,7), (2,3,8), (2,4,8)	xyz	3/32	3/32	1/32

*When read vertically represent the alleles within each of the four spores of an ascus.

types b and c respectively (i.e. bC + bc, and Bc + bc), and p_{bc} represents the proportion of phenotypes b c, the equations may be written:

$$\begin{aligned} 1 - 6p_b &= XY \\ 1 - 6p_c &= XZ \\ 36(p_{bc} - p_b p_c) &= YZ(1 - X)(2 + X) \end{aligned}$$

These may be solved directly for X, Y, and Z, and hence for x, y, and z.

If $g = \frac{36(p_{bc} - p_b p_c)}{(1-6p_b)(1-6p_c)}$, then the solutions are given by:

$$1 - \tfrac{3}{2}x = X = \frac{\sqrt{[8g+9]} - 1}{2(g+1)}$$

$$1 - \tfrac{3}{2}y = Y = \frac{1-6p_b}{X}$$

$$1 - \tfrac{3}{2}z = Z = \frac{1-6p_c}{X}$$

These equations give unique values for x, y, and z (the frequencies of second division segregation of each of the three genes, a/a, B/b, and C/c).

Necessary and sufficient conditions that these solutions should have a real meaning are that:

$$p_b \leq \tfrac{1}{6}$$

$$p_c \leq \tfrac{1}{6}$$

and, $$p_{bc} \leq \frac{p_b}{2} \frac{1-2p_b-4p_c}{1-6p_b}, \text{ if } p_c > p_b,$$

or, $$p_{bc} \leq \frac{p_c}{2} \frac{1-4p_b-2p_c}{1-6p_c}, \text{ if } p_b > p_c.$$

As an illustration of the application of these formulae,

consider the case where the following proportions of phenotypes occur: $Bc = \frac{1}{12}$, $bC = \frac{1}{12}$, and $bc = \frac{1}{48}$.

$$p_b = p_c = \frac{5}{48}, \text{ and } p_{bc} = \frac{1}{48}$$

$$\mathcal{E} = \frac{36\left(\frac{1}{48} - \frac{25}{48 \times 48}\right)}{(3/8)^2} = \frac{23}{9} = 2.56$$

$$X = \frac{\sqrt{[8 \times 2.56 + 9]} - 1}{2 \times 3.56} = 0.622$$

$$Y = Z = \frac{\frac{3}{8}}{0.622} = 0.600$$

$$x = \frac{2}{3}(1 - 0.622) = 0.25$$

$$y = z = \frac{2}{3}(1 - 0.600) = 0.27$$

Neither p_b nor p_c may theoretically exceed a value of $\frac{1}{6}$, but as the table on page 42 shows, the value of $\frac{1}{6}$ may be attained when one or more genes freely recombine with their centromeres. The following special cases may be distinguished:

a. If $p_b = \frac{1}{6}$ and $p_c < \frac{1}{6}$, then $y = \frac{2}{3}$, i.e. gene B/b freely recombines with its centromere. Here, further information is required before unique values can be obtained for x and z, which satisfy:

$$1 - 6p_c = (1 - \tfrac{3}{2}x)(1 - \tfrac{3}{2}z).$$

b. If $p_c = \frac{1}{6}$ and $p_b < \frac{1}{6}$, then $z = \frac{2}{3}$, i.e. gene C/c freely recombines with its centromere. Further information is required before unique values can be obtained for x and y which satisfy:

$$1 - 6p_b = (1 - \tfrac{3}{2}x)(1 - \tfrac{3}{2}y).$$

c. If $p_b = p_c = \frac{1}{6}$, then either $x = \frac{2}{3}$ or $y = z = \frac{2}{3}$. In both these instances further information is needed to obtain a complete solution.

In all three of these instances, a knowledge of one of the values x, y or z (other than $\frac{2}{3}$) is needed before all recombination values can be estimated.

The modified equations, derived from those of Table 5, are worth re-examination:

$$1 - 6p_b = XY$$

$$1 - 6p_c = XZ$$

$$36(p_{bc} - p_b p_c) = YZ(1-X)(2+X)$$

The first two of these are repetitions of the equation obtained in Calculation 4 (where only the mating type gene, a/α, and gene B/b were dealt with), and may have been written down directly. The third equation represents something new: it gives the extent to which the proportion, p_{bc}, of double recessive phenotypes differs from what would be expected from a consideration of the proportions, p_b and p_c, of phenotypes b (bc + bC) and c (bc + Bc).

This equation shows, for instance, that, if either gene B/b or C/c freely recombines with its centromere (i.e. if Y or Z is zero), the proportion of double recessive phenotypes, bc, is the product of the proportions of phenotypes b and c (i.e. $p_{bc} = p_b p_c$). This is also true if gene a/α is completely linked to its centromere (i.e. if X = 1) irrespective

of the relationship of genes B/b and C/c and their centromeres.

In other instances the proportion, p_{bc}, of double recessive phenotypes will always exceed the proportion expected from a consideration of the proportions p_b and p_c.

Table 5a may be used to simplify the calculation of x, y, and z from any set of data giving p_b, p_c and p_{bc}. The main steps in the calculations are as follows:

1. Calculate $g = \frac{36(p_{bc} - p_b p_c)}{(1-6p_b)(1-6p_c)}$

2. Enter this value of g in Table 5a to obtain values for 1/X and x.

3. Calculate Y and Z using the formulae:

$$Y = \frac{1}{X} \times (1-6p_b)$$

$$Z = \frac{1}{X} \times (1-6p_c)$$

4. Calculate y and z from the formulae:

$$y = \tfrac{2}{3}(1-Y)$$
$$z = \tfrac{2}{3}(1-Z)$$

For example, taking the data analysed on page , g = 2.56, and hence from Table 5a, 1/X = 1.60 and x = 0.25. Since (1-6pc) = (1-6pb) = 3/8, Y = Z = $\frac{3}{8} \times 1.60 = 0.60$, and hence y = z = 0.27.

Calculation involving three or more independent genes in addition to that for mating type

The above equations (page 44) also show how it is possible to deal with three or more freely recombining genes

TABLE 5a

Table to simplify calculations of second division segregation frequencies

g	1/x	x
0.0	1.000	0.000
0.2	1.064	0.040
0.4	1.123	0.073
0.6	1.179	0.101
0.8	1.231	0.125
1.0	1.281	0.146
1.5	1.396	0.189
2.0	1.500	0.222
2.5	1.596	0.249
3.0	1.686	0.271
4.0	1.851	0.306
5.0	2.000	0.333
6.0	2.157	0.355
7.0	2.266	0.372
8.0	2.386	0.387
9.0	2.500	0.400
10.0	2.608	0.411
12.0	2.836	0.432
14.0	3.000	0.444
16.0	3.176	0.457
20.0	3.500	0.476
30.0	4.195	0.508
40.0	4.784	0.527
50.0	5.306	0.541
100.0	7.321	0.576
200.0	10.028	0.600
∞	∞	0.667

(in addition to that for mating type). For example, with genes B/b, C/c, and D/d, the frequencies of second division segregation may be represented as y, z, and t, with corresponding parameters:

$$Y = 1 - \tfrac{3}{2}y$$

$$Z = 1 - \tfrac{3}{2}z$$

$$T = 1 - \tfrac{3}{2}t$$

If the values of p_b, p_c, p_d, p_{bc}, p_{bd}, and p_{cd} are then measured, the above equations may be repeated, giving:

$$1 - 6p_b = XY$$

$$1 - 6p_c = XZ$$

$$1 - 6p_d = XT$$

$$36(p_{bc} - p_b p_c) = YZ(1-X)(2+X)$$

$$36(p_{bd} - p_b p_d) = YT(1-X)(2+X)$$

$$36(p_{cd} - p_c p_d) = ZY(1-X)(2+X)$$

A seventh equation may be added involving the proportion, p_{bcd}, of triple recessives.

Any four of these equations may be used to solve for x, y, z, and t. Probably, the simplest procedure would be to calculate 'g' for each of the phenotypes, bc, bd, and cd, and to use the average of these three g's in calculating X. The values of Y, Z, and T then follow from the first three equations.

Here, the frequencies of second division segregation for all genes (i.e. x, y, z, and t) may be estimated

TABLE 6

The Types and Frequencies of Four-spore Asci, Together with the Proportions of Diploid Phenotypes, Expected When The Two Linked Genes B/b and C/c are On the Same Side of the Centromere and Freely Recombine with the Mating Type Gene a/α.

Cross: a . B C x α . b c or α . B C x a . b c (gene order, centromere - B/b - C/c.)

1st. Division Segregation For Gene(s)	2nd. Division Segregation For Gene(s)	Combinations of Alleles*	Combinations of Alleles Giving Types of Asci Expected	Frequency	Proportion of Phenotypes Bc	bC	bc
		1 2 3 4 5 6					
a/α, B/b, C/c	-	α a B C α a B C a α b c a α b c	(1,3,4), (2,3,4)	(1-x)(1-y)(1-½u-f)	0	0	0
		α a B c α a B c a α b C a α b C	(1,3,4), (2,3,4)	(1-x)(1-y)(f-½u)	0	0	0
a/α, B/b	C/c	α a B C α a B c a α b C a α b c	(1,3,4), (2,3,4)	(1-x)(1-y)u	1/4	0	0
a/α	B/b, (C/c)'	α a B C α a b c a α B C a α b c	(1,3,4), (2,3,4)	(1-x)y(1-½u-f)	0	0	1/4
		α a B c α a b C a α B c a α b C	(1,3,4), (2,3,4)	(1-x)y(f-½u)	1/4	1/4	0
a/α, (C/c)"	B/b, C/c	St ** 2 3 4 a α B C c c a α b C C c α a B c C C α a b c c C 1:2:1	(1,3,4), (2,3,4), 2x(1,3,5), 2x(2,3,5), (1,3,6), (2,3,6)	(1-x)yu	1/8	1/4	0

B/b, C/c	a/α	a α B C α a B C a a b c α α b c	(1,3,4), (2,3,4)	x(1-y)(1-½u-f)	0	0	-
		a α B c α a B c a a b C α α b C	(1,3,4), (2,3,4)	x(1-y)(f-½u)	1/4	1/4	0
B/b	a/α, C/c	a α B C α a B c a a b C α α b c	(1,3,4), (2,3,4)	x(1-y)u	1/8	1/4	0
-	a/α, B/b, (C/c)'	a α B C α a b c a a B C α α b c	(1,3,4), (2,3,4)	xy(1-½u-f)	0	0	1/8
		a α B c α a b C a a B c α α b C	(1,3,4), (2,3,4)	xy(f-½u)	1/8	1/8	0
(C/c)"	a/α, B/b, C/c	St** 2 3 4 a α B C c c α a b C C c a a B c C C α α b c c C 1:2:1	(1,3,4), (2,3,4), 2x(1,3,5), 2x(2,3,5), (1,3,6), (2,3,6)	xyu	3/16	1/8	0

*when read vertically represent the alleles within each of the four spores of an ascus.
**the products of two, three, and four strand crossing over.
(C/c)' denotes that second division segregation of gene C/c results from a cross-over between gene B/b and the centromere.
(C/c)" denotes that first division segregation of gene C/c results from two and four strand crossing over between the centromere, gene B/b and gene C/c.

provided that not more than one of the genes freely recombines with its centromere: if only one gene freely recombines with its centromere a complete solution may still be obtained.

Calculation 8

Calculation of the linkage relationship between two genes, B/b and C/c, when they are independent of the mating type gene, a/α, and are on the same side of the centromere

Table 6 (page 50) shows the types and frequencies of complete asci expected from a heterozygous diploid in which the genes B/b and C/c are linked in the coupling phase, and are independent of the mating type gene a/α.

For simplicity, the gene order has been taken as centromere-B/b-C/c. The alternative case where the gene order is centromere-C/c-B/b can be similarly derived by interchanging the appropriate symbols.

As previously, y and z represent the frequencies of second division segregation of the genes B/b and C/c respectively, and u is taken to represent the frequency of tetratype combinations with regard to these two genes. The three quantities y, z, and u are then related by the formula:

$$z = y + u - \tfrac{3}{2}uy$$ .....See appendix (page 67)

or, if $Y = 1 - \tfrac{3}{2}y$, $Z = 1 - \tfrac{3}{2}z$, and $U = 1 - \tfrac{3}{2}u$, by:

$$Z = YU$$

It is therefore sufficient to work in terms of any two of

these values; Table 6 employs y and u. The quantity, f, in this table represents the frequency of recombination between the genes B/b and C/c, which may be derived from u using, as previously, the formula:

$$f = \tfrac{1}{3}\left[1 - (1 - \tfrac{3}{2}u)^{\frac{2}{3}}\right] = \tfrac{1}{3}(1 - U^{\frac{2}{3}}).$$

If it were possible to solve for x, y, and u, then z could be derived. However, in this instance, only u may be completely derived (and hence f). The quantity u comes from the following formula:

$$u = \frac{4(p_c - p_b)}{1 - 6p_b}, \text{ where of necessity } p_c > p_b.$$

If $p_b > p_c$, then the gene B/b lies further from the centromere than C/c, and u is given by:

$$u = \frac{4(p_b - p_c)}{1 - 6p_c}.$$

This formula could have been directly derived by noting that, as previously,

$$1 - 6p_b = XY$$
$$1 - 6p_c = XZ$$

Hence,

$$\frac{1 - 6p_c}{1 - 6p_b} = \frac{Z}{Y} = U = 1 - \tfrac{3}{2}u, \text{ or } u = \frac{4(p_c - p_b)}{1 - 6p_b}$$

An additional fact, however, comes from the full analysis. From the equations derived from Table 6 it may be shown that:

$$p_{bc} = p_b \frac{1 - 2p_b - 4p_c}{1 - 6p_b} \times \frac{1 - \frac{1}{2}u - f}{1 - u}, \text{ provided } p_c > p_b, \text{ i.e. } z > y.$$

or,

$$p_{bc} = p_c \frac{1 - 2p_c - 4p_b}{1 - 6p_c} \times \frac{1 - \frac{1}{3}u - f}{1 - u}, \text{ provided } p_b > p_c, \text{ i.e. } y > z.$$

Since both u and f may be derived from p_b and p_c using the above formulae, this gives a basis for the prediction of the proportion of double recessive phenotypes, b c, on the assumption of no interference of one chiasma by another. It can thus be used as a measure of chiasma interference.

A comparison of the above formula with the conditions in Calculation 7 shows that the cases where the genes B/b and C/c are on different chromosomes, and on the same chromosome on the same side of the centromere may be readily distinguished.

It must first be noted that:

$$\frac{1 - \frac{1}{3}u - f}{1 - u} = \frac{1}{2} + \frac{1 - 2f}{2(1-u)} \text{ is always greater than } \frac{1}{3}.$$

It then follows that, if $p_c > p_b$, the ratio:

$\frac{p_{bc}(1 - 6p_b)}{p_b(1 - 2p_c - 4p_c)}$ is always less than $\frac{1}{3}$ when the genes are on different chromosomes, and greater than $\frac{1}{3}$ when they are on the same chromosome, on the same side of the centromere. The corresponding ratio:

$\frac{p_{bc}(1 - 6p_c)}{p_c(1 - 4p_b - 2p_c)}$ must be used if $p_b > p_c$.

Should a further independent gene D/d be introduced (on a third chromosome), whose frequency of second division segregation, t, is less than $\frac{2}{3}$, complete solutions for x, y,

z, and t are possible.

In this instance the appropriate formulae are:

$$1 - 6p_b = XY$$

$$1 - 6p_c = XZ$$

$$1 - 6p_d = XT$$

$$36(p_{bd} - p_b p_d) = YT(1 - X)(2 + X)$$

$$36(p_{cd} - p_b p_d) = ZT(1 - X)(2 + X)$$

with the solutions:

$$1 - \tfrac{3}{2}x = X = \frac{\sqrt{[(8g + 9)]} - 1}{2(g + 1)}$$

$$1 - \tfrac{3}{2}y = Y = \frac{1 - 6p_b}{X}$$

$$1 - \tfrac{3}{2}z = Z = \frac{1 - 6p_c}{X}$$

$$1 - \tfrac{3}{2}t = T = \frac{1 - 6p_d}{X} .$$

Here, g may be calculated from: $\frac{36(p_{bd} - p_b p_d)}{(1 - 6p_b)(1 - 6p_d)}$, from $\frac{36(p_{cd} - p_c p_d)}{(1 - 6p_c)(1 - 6p_d)}$, or from any average (e.g. arithmetic, weighted, or geometric) of these two values. One of the simplest and most accurate is:

$$g = \frac{18(p_{bd} + p_{cd} - p_b p_d - p_c p_d)}{(1 - 3p_b - 3p_c)(1 - 6p_d)}$$

The value g can be used in calculating X, and hence Y, Z, and T. Thus, x, y, z, and t, the frequencies of second division segregation for each of the genes A/a, B/b, C/c, and D/d may be derived.

Calculation 9

Calculation of the linkage relationship between two genes B/b and C/c when they show free recombination with the mating type gene, a/a, and are on opposite sides of the centromere

Table 7 (page 57) shows the types and frequencies of complete asci expected from a heterozygous diploid in which the above conditions prevail and the two genes B/b and C/c are in the coupling phase.

The situation here appears to be the most complicated of those so far encountered in that it has characteristics in common both with the situation existing when the genes B/b and C/c are on different chromosomes, and with the situation existing when these genes are on the same chromosome on the same side of the centromere.

For instance, when x 0, i.e. when the mating type gene a/a is completely linked to its centromere, the situation is exactly the same as for genes on different chromosomes. On the other hand, when y and z are both small, the situation appears similar to that when the genes are on the same chromosome on the same side of the centromere.

In general it is theoretically possible to solve for x, y and z under the present assumptions (B/b and C/c on opposite sides of the centromere), although this is rather involved computationally. It would appear that any set of data which might be subjected either to Calculation 7 (B/b

TABLE 7

The Types and Frequencies of Four-spore Asci, Together with the Proportions of Diploid Phenotypes, Expected When the Two Linked Genes B/b and C/c are On Opposite Sides of the Centromere and Freely Recombine with the Mating Type Gene a/α.

Cross: $\frac{\alpha\ B\ .\ C}{}$ x $\frac{a\ b\ .\ c}{}$ or $\frac{a\ B\ .\ C}{}$ x $\frac{\alpha\ b\ .\ c}{}$

1st. Division Segregation For Gene(s)	2nd. Division Segregation For Gene(s)	Combinations of Alleles*	Combinations of Alleles Giving Types of Asci Expected	Frequency	Proportions of Phenotypes Bc	bC	bc
		1 2 3 4 5 6 α a B C α a B C a α b c a α b c	(1,3,4), (2,3,4)	$(1-x)(1-y)(1-z)\frac{(1-ku-f)}{1-u}$	0	0	0
a/α, B/b, C/c	-	α a B c α a B c a α b C a α b C	(1,3,4), (2,3,4)	$(1-x)(1-y)(1-z)\frac{(f-ku)}{1-u}$	0	0	0
a/α, B/b	C/c	α a B C C α a B c c a α b C c a α b c C	(1,3,4), (2,3,4), (1,3,5), (2,3,5)	$(1-x)(1-y)z$	1/4	0	0
		a α B C α a B C α a b c a α b c	(1,3,4), (2,3,4)	$x(1-y)(1-z)\frac{(1-ku-f)}{1-u}$	0	0	1/4
B/b, C/c	a/α	a α B c α a B c α a b C a α b C	(1,3,4), (2,3,4)	$x(1-y)(1-z)\frac{(f-ku)}{1-u}$	1/4	1/4	0
a/α, C/c	B/b	α a B C α a b C a α B c a α b c	(1,3,4), (2,3,4)	$(1-x)y(1-z)$	0	1/4	0
a/α	B/b, C/c	St. 2 3 4 α a B C C c α a b c c C a α B C c c a α b c C C 1:2:1	(1,3,4), (2,3,4), 2x(1,3,5), 2x(2,3,5), (1,3,6), (2,3,6)	$(1-x)yz$	3/16	3/16	1/16
B/b	a/α, C/c	a α B C α a B c a a b C α α b c	(1,3,4), (2,3,4)	$x(1-y)z$	1/8	1/4	0
C/c	a/α, B/b	a α B C α a b C a a B c α α b c	(1,3,4), (2,3,4)	$xy(1-z)$	1/4	1/8	0
-	a/α, B/b, C/c	St. 2 3 4 a α B C C c α a b c c C α α B C c c a a b c C C 1:2:1	(1,3,4), (2,3,4), 2x(1,3,5), 2x(2,3,5), (1,3,6), (2,3,6)	xyz	3/32	3/32	1/32

*when read vertically represent the alleles within each of the four spores of an ascus.

St. / 2 3 4 : combinations of alleles resulting from two, three, and four strand crossing over.

and C/c on different chromosomes), or to Calculation 8 (B/b and C/c on the same side of the centromere), may also be explained under the hypothesis of linked genes situated on opposite sides of the centromere; this, unless further independent information was available.

The equations which may be derived from Table 7 may be simplified into the two familiar equations:

$$1 - 6p_b = XY$$
$$1 - 6p_c = XZ$$

together with one other equation giving the proportion, p_{bc}, of double recessive phenotypes. This may be put in the alternative forms:

$$p_{bc} = \frac{1}{4}\,\frac{1 - \frac{1}{2}u - f}{1 - u}\,x\,(1 - y)(1 - z) + \tfrac{1}{16}(1 - x)yz + \tfrac{1}{32}xyz$$

or,

$$36(p_{bc} - p_b p_c) = YZ(1 - X)(2 + X + h),$$

where

$$h = \frac{(1 + 2Y)(1 + 2Z)}{Y^{\frac{1}{2}}Z^{\frac{1}{2}}\ (1 + 2YZ)}\ .$$

Consequently, three equations are available which may be solved for X, Y, and Z.

The actual solution is most easily carried out by using a rough value for h, solving for X, Y, and Z to get a more exact value for h, resolving to get a still more exact value and so on. The basis of this solution is the equations:

$$X = \frac{\sqrt{[(3 + h)^2 + 4(2 + h)g]} - (1 + h)}{2(1 + g)}$$

$$Y = \frac{1 - 6p_b}{X}$$

$$Z = \frac{1 - 6p_c}{X} ,$$

where, as previously, $g = \frac{36(p_{bc} - p_b p_c)}{(1 - 6p_b)(1 - 6p_c)}$.

As an illustration, we may calculate x, y, and z from the same data used in demonstrating Calculation 7 (on page 45), but now upon the assumption that the genes B/b and C/c are on different sides of the centromere.

A first approximation for h may be obtained using the values for Y and Z calculated in that example. Taking Y = Z = 0.6, a first rough estimate of h is:

$$h = \frac{2.2 \times 2.2}{(0.6)^{\frac{1}{3}} \times 1.48} = 4.0$$

and g = 2.56.

Thus the first approximation to the solutions are given by:

$$X = \frac{\sqrt{(110.44)} - 5}{7.12} = 0.773$$

$$Y = Z = \frac{\frac{3}{8}}{0.773} = 0.49$$

A better value for h is consequently:

$$h = \frac{1.98 \times 1.98}{(0.49)^{\frac{1}{3}} \times 1.48} = 4.26$$

which gives as a second approximation:

$$X = \frac{\sqrt{(116.3)} - 5.26}{7.12} = 0.779$$

$$Y = Z = \frac{\frac{3}{8}}{0.779} = 0.481.$$

This is near the first approximation and therefore may

be taken to give the final solutions:

$$x = \tfrac{2}{3}(1 - 0.779) = 0.147$$

$$y = z = \tfrac{2}{3}(1 - 0.481) = 0.346.$$

Thus, on the assumption that genes B/b and C/c are on the same chromosome on opposite sides of the centromere, the above values suffice to explain the observed proportions p_b, p_c, and p_{bc}.

To determine what the situation may be, i.e. whether the genes are linked or on different chromosomes, it is necessary to have a further gene D/d, preferably known to be on an independent chromosome. With four genes it is possible to calculate three values for g:

$$g_{bc} = \frac{36(p_{bc} - p_b p_c)}{(1 - 6p_b)(1 - 6p_c)}$$

$$g_{bd} = \frac{36(p_{bd} - p_b p_d)}{(1 - 6p_b)(1 - 6p_d)}$$

$$g_{cd} = \frac{36(p_{cd} - p_c p_d)}{(1 - 6p_c)(1 - 6p_d)}$$

If the three genes B/b, C/c, and D/d are all on different chromosomes, then these values of g will be the same.

If, on the other hand, two genes are linked and the third is on a different chromosome, then one value for g will be greater than the other two. This value will show which two genes are linked. For example, if $g_{bc} > g_{bd} = g_{cd}$ then genes B/b and C/c are linked.

Finally, if all of the values of g are different the

three genes are on the same chromosome.

For example, suppose, as before, that:

$p_b = p_c = \frac{5}{48}$ and $p_{bc} = \frac{1}{48}$, and that the introduction of the gene D/d gives $p_d = \frac{1}{8}$, and $p_{bd} = p_{cd} = \frac{1}{64}$. Then

$$\mathcal{G}_{bc} = \frac{23}{9} = 2.56$$

and

$$\mathcal{G}_{bd} = \mathcal{G}_{cd} = \frac{36\left[\frac{1}{64} - \left(\frac{5}{8 \times 48}\right)\right]}{\frac{3}{8} \times \frac{1}{4}} = 1.00$$

We should therefore conclude that genes B/b and C/c are linked and that gene D/d is on a different chromosome. Also, since the ratio:

$\frac{p_{bc}(1 - 6p_b)}{p_b(1 - 2p_b - 4p_c)} = \frac{1}{5} < \frac{1}{3}$, the possibility of the genes B/b and C/c being on the same side of the centromere can be immediately ruled out. (See page 54)

It is possible to solve for X using the proportions of diploid recessive phenotypes for any two of the genes B/b, C/c, and D/d. In the above example the solutions for X are 0.779, 0.781, and 0.781 respectively. The difference between the first solution and the last two might be used as a measure of chiasmata interference.

Taking X = 0.781, the overall solution for the above example is:

$$x = \frac{2}{3}(1-X) = 0.146$$

$$Y = Z = \frac{1 - 6p_b}{X} = 0.480 \text{ and therefore } y = z = 0.347$$

The value t (the frequency of second division segregation of gene D/d) is derived thus:

$$T = \frac{1 - 6pd}{X} = 0.320, \text{ and therefore } t = 0.453.$$

Map Distance and Interference

The mean number of chiasma, m, between any two loci is related, by the formula given in the appendix, to the frequency of tetratype asci, or, when one locus is the centromere, to the frequency of second division segregation, say x, as follows:

$$e^{-\frac{3}{2}m} = X = 1 - \frac{3}{2}x$$

$$\text{or,} \qquad m = -\frac{2}{3}\log_e X.$$

This gives twice the map distance between the two loci on the assumption of no interference and, on the same assumption map distances calculated in this manner are directly additive (since XY = U).

If, however, interference occurs, this relationship no longer holds and it is necessary to consider how this will affect the previous calculations and the conclusions drawn from them.

First, if the calculations deal with genes situated on different chromosomes, there will be no change in the estimates of x, y, z, etc. The relation between these estimates and the map distances will, however, have changed. To derive the map distances, further information of the type considered by Haldane (1918) and Mather (1938) will need to be employed, the true value m being conditioned in

all instances by the inequalities:

$$x \leq m \leq -\tfrac{2}{3}\log_e X.$$

If, in fact, the additive relation

$$u = x + y - \tfrac{3}{2}rxy \qquad (0 \leq r \leq 1)^{\ddagger}$$

may be shown to hold, then

$$m = -\tfrac{2}{3r}\log_e(1 - \tfrac{3}{2}rx).$$

Secondly, if two or more genes are linked then the results given in the tables will still hold, but they need to be simplified using different substitutions for u or f. For example, in Table 2 the proportion of phenotype b diploids expected is still $\frac{1}{4}(x + y - \frac{3}{2}xy)$ but this is no longer equal to $\frac{1}{4}u$. In fact, $4p_b$ now provides a lower limit for the value of u. In practice, however, unless m is greater than $\frac{1}{3}$, the difference is unlikely to be great.

For instance, if $x = y = \frac{1}{3}$ and $u = x + y - xy$, the map distance between the genes a/a and B/b calculated on the assumption of no interference would be $\frac{2}{3}\log_e 2 = 0.46$, compared with the true distance of $\log_e \frac{3}{2} = 0.40$, while if $x = y = \frac{1}{6}$, the corresponding values are $\frac{2}{3}\log_e \frac{4}{3} = 0.19$ and $\log_e \frac{6}{5} = 0.18$.

Similar situations exist in respect of the other tables and no solution can be obtained unless either the manner in which the quantities x, y, and u are related is known or, at least, genes situated on three different chromosomes are involved in the calculation. In the former instance, direct

(‡ r represents the extent of interference; it takes a value of 0 when interference is complete, and 1 when there is no interference)

substitution provides a solution. In the latter instance, the formulae derived from Table 5 may be used to provide a solution for x, y and z etc. (the frequencies of second division segregation of each gene). These may then be used to estimate the proportions of double recessive phenotypes with respect to genes situated on the same chromosomes, which by comparison with the experimentally observed proportion would give a measure of chiasma interference.

Summary and Discussion of Calculations 1 - 9

It appears from these calculations that if the frequencies of second division segregation for genes a/c, B/b, C/c ..... are denoted by x, y, z ..... , the fundamental relationships are most simply expressed in terms of:

$$X = 1 - \tfrac{3}{2}x$$
$$Y = 1 - \tfrac{3}{2}y$$
$$Z = 1 - \tfrac{3}{2}z \quad .....$$

These values can take any value between 0 and 1 and can be directly related to the proportions of tetratype asci, (and hence to the recombination frequencies), with respect to linked genes.

The first set of fundamental formulae gives the proportions, p_b, p_c, p_d ..... of diploid phenotypes exhibiting the single recessive characters b, c, d ..... These are:

$$1 - 6p_b = XY$$
$$1 - 6p_c = XZ \qquad \text{(Formulae 'A')}$$
$$1 - 6p_d = XT$$

The second set of formulae gives the proportions p_{bc},

pbd..... of diploid phenotypes exhibiting the double recessive characters b and c, b and d ..... For genes on different chromosomes, these are:

$$36(P_{bc} - P_bP_c) = YZ(1-X)(2+X)$$
$$36(P_{bd} - P_bP_d) = YT(1-X)(2+X) \quad \ldots\ldots \text{(Formulae 'B')}$$

When the genes B/b and C/c are linked, then formulae B need to be modified and different results are obtained according to whether the two genes are on the same side of the centromere or not. Calculations 8 and 9 show how the analysis may be carried out on the assumption of non-interference.

Correspondingly more complicated results may be obtained when interference is assumed to occur. However, in this instance, further parameters need to be introduced as measures of this interference, and genes on at least three different chromosomes are needed in order to obtain a complete solution.

Such a solution is most easily obtained using formulae A and B to estimate x, y, and z etc. Further analysis then yields estimates of quantities such as u and f, used to indicate the frequencies of tetratype and ditype asci, which in turn may be used to investigate the extent of chiasma interference.

When no interference occurs, the measurements X, Y, Z ..... may be directly translated into mapping distances, M, using the formulae:

$$M = -\tfrac{1}{3}\log_e X.$$

With complete interference the corresponding formula is:

$$M = \tfrac{1}{2}x,$$

and with partial interference the mapping distance lies between these two values:

$$\tfrac{1}{2}x \leq M \leq -\log_e X$$

The formulae A are important in that if the value of x is known, the corresponding values of y, z, etc. can always be directly calculated. Formulae B are important in that they provide a method of deriving such a value for x. Also, if three chromosomes (including that on which the mating type gene is situated) are included, they provide a means by which any further linkages can be detected and measured. With only three genes (including that for mating type) there appears however to be no general method to determine the existence of linkage. In this instance, only if

$$p_{bc} > \frac{p_b}{2}\,\frac{1 - 2p_b - 4p_c}{1 - 6p_b} \qquad (p_c > p_b)$$

can it definitely be stated that the genes B/b and C/c are linked.

Theoretically it is also possible to calculate the formulae to predict the triple and higher order recessive phenotypes. These formulae might be used to further increase the accuracy of estimation. However, since the proportions of such phenotypes will usually be very small, the gain of information by this method is also likely to be very small.

Appendix

Let m, n, and m + n be the mean numbers of chiasmata between gene B/b and its centromere, between gene B/b and gene C/c, and between gene C/c and its centromere respectively.

The actual numbers of chiasmata then follow Poisson distributions and, for example, the probability of r chiasmata occurring between gene B/b and its centromere is given by:

$$P(r) = \frac{e^{-m} m^r}{r!}$$

Now, let A_r and $1 - A_r$ represent the frequencies of first and second division segregation of the gene B/b when r chiasmata occur between this gene and the centromere.

It is not difficult to show by employing these values that if a further chiasma occurs, one half of the second division segregants become first division segregants, while all the first division segregants become second division segregants.

Hence,

$$A_{r+1} = \tfrac{1}{2}(1 - A_r).$$

This equation has to be solved with the condition that $A_0 = 1$

The solution of this gives:

$$A_r = \tfrac{1}{3} + \tfrac{2}{3}(-\tfrac{1}{2})^r$$

so that, as r becomes large, A_r tends to $\frac{1}{3}$.

It is now possible to write down the overall frequency

of first division segregation using the probabilities of different numbers of chiasmata occurring. This is:

Frequency of first division segregation of gene B/b = 1-y =

$$\sum_{r=0}^{\infty} A_r P(r)$$

$$\sum_{r=0}^{\infty} \frac{e^{-m} m^r (\frac{1}{3} + \frac{2}{3}(-\frac{1}{2})^r)}{r!}$$

$$= \frac{1}{3} + \frac{2}{3} e^{-\frac{3}{2}m}$$

Hence,

$$y = \frac{2}{3}(1 - e^{-\frac{3}{2}m})$$

or

$$Y = 1 - \frac{3}{2}y = e^{-\frac{3}{2}m}.$$

By precisely similar reasoning, it follows that:

$$Z = 1 - \frac{3}{2}z = e^{-\frac{3}{2}(m+n)}$$

and,

$$U = 1 - \frac{3}{2}u = e^{-\frac{3}{2}n} \qquad (\text{.....Formula A})$$

and hence that

$$Z = YU$$

or

$$(1 - \tfrac{3}{2}z) = (1 - \tfrac{3}{2}y)(1 - \tfrac{3}{2}u)$$

or

$$z = y + u - \tfrac{3}{2}yu.$$

Mather (1935) gives the formula corresponding to A (above) for the recombination frequency, f, between two genes in terms of the mean number of chiasmata, n. This formula is:

$$f = \tfrac{1}{2}(1 - e^{-n})$$

which in our case is equivalent to:

$$f = \tfrac{1}{2}(1 - U^{\frac{2}{3}}).$$

The quantity, u, above is not expressible in terms of

first and second division segregation since it involves the linkage relationship of the genes B/b and C/c. It gives instead the relative frequencies of the ditype asci as compared with the tetratype asci. Thus the asci of

constitution: B C / B C / b c / b c and b C / b C / B c / B c occur with a total

frequency of 1 - u, while the asci of constitution: B C / B c / b C / b c

occur with frequency u. The frequencies of the two types

B C / B C / b c / b c and b C / b C / B c / B c are not however specified. These may

be easily derived if it noted that, for one or more chiasmata, these two types occur with equal frequency. The only difference in frequency thus arises from those instances in which no chiasma occurs. These represent a proportion e^{-n} of the total.

Consequently the frequencies of the two types

B C / B C / b c / b c and b C / b C / B c / B c are $\frac{1}{2}(1 - u + e^{-n})$ and $\frac{1}{2}(1 - u - e^{-n})$

respectively. The quantity e^{-n} is, of course, related to u by the formula A above, i.e. by:

$$U = 1 - \tfrac{3}{2}u = e^{-\frac{3}{2}n}.$$

A simple form for the above two frequencies is:

$$(1 - \tfrac{1}{2}u - f) \text{ and } (f - \tfrac{1}{2}u).$$

c. Three-spore Asci

Individual colonies from intact three-spore asci are expected to contain two kinds of cells: diploid from fusion of two ascospores of complementary mating type, and haploid resulting from the odd ascospore.

The expected phenotypic ratios among the diploid cells arising from these asci are similar to those expected among the diploid cells arising from intact two-spore asci, under similar conditions (Calculations 1 - 9). The haploid cells may be analysed in the same manner as those arising from intact one-spore asci.

Experimental Results

The following results show how the theory of the previous sections may be applied in practice. As such they are not intended to derive highly accurate estimates of map distances (for which many more observations would be needed) so much as to illustrate the practical applicatio of these special types of genetical analyses.

1. One-spore Asci

Strain

The diploid strain used in this experiment was heterozygous for mating type and adenine requirement. It was obtained by crossing strain 19/20 1b with strain C24-13b and designated a ad // a AD.

Experimental analyses

Number of asci isolated = 40

Number of asci which germinated = 22

Percentage mortality of asci = 45

a. Ploidy and mating type

Number of asci producing haploid colonies = 22

Number of asci producing diploid colonies = 0

	Number of asci giving colonies of a mating type	Number of asci giving colonies of α mating type
Obtained:	18	4
Expected (1:1 ratio):	11	11

Deviation highly significant due to shortage of asci producing colonies of α mating type.

b. Nutritional phenotype

1. Independent of mating type

	Phenotype of colonies	
	AD	ad
Number of colonies Obtained:	13	9
Expected (1:1):	11	11

$X^2 = 0.73$
$P = 0.4$

2. Colonies of α mating type only

	Phenotype of colonies	
	AD	ad
Number of colonies Obtained:	2	2
Expected (1:1):	2	2

3. Colonies of a mating type only

	Phenotype of colonies	
	AD	ad
Number of colonies Obtained:	11	7
Expected (1:1):	9	9

$X^2 = 0.89$
$P = 0.35$

c. Mortality of α mating type spores

If it is assumed that each of the one-spore asci which did not produce colonies contained ascospores of α mating type, it can be calculated, as follows, that about 80% of such asci are inviable.

No. of asci not producing colonies = 18
" " " which gave colonies of α mating type = 4

Therefore, total number of asci containing α mating type spores on the above assumption = 22.

Percentage mortality of such asci = 18 x 100/22 = 81.8.

2. Two-spore Asci

A. Dissection of asci to show ploidy of the ascospores.

Strain

No marked strains were available when this experiment was carried out. Consequently, the 'wild type' diploid

strain obtained by crossing strains B15 and C24-13b was used to produce the asci.

Experimental analysis

No. of asci dissected = 25

" " " giving no viable ascospores = 1

" " " " one " " = 3

" " " " two " " = 21

Mating type of ascospores

All ascospores gave haploid colonies. All three of the ascospores derived from the asci which gave only one viable spore were of of a mating type. The following ratio was obtained among those asci which produced two viable ascospores.

	Two a type spores	One a and one α type spore	Two α type spores
No. of asci:			
Observed	6	12	3
Expected (1:4:1)	3.5	14	3.5

Overall ratio of ascospores of a mating type to those of α mating type = 24 a : 18 α

B. Analysis of intact asci

Strains

Four experiments were undertaken. The diploid strain

utilized in the first and second experiments was heterozygous for adenine requirement as well as mating type; it was obtained by crossing strain B15 with strain 19/20 1b and designated α ad // a AD. The diploid strain utilized in the third experiment was heterozygous for tryptophane requirement and mating type; it was obtained by crossing strain C12-24b with strain 19/20.8 and designated α TRYP // a tryp. In the fourth experiment a diploid heterozygous for both adenine and tryptophane requirements besides mating type was used; it was obtained by crossing strain 19/20.8 with strain 19/20 1b and designated a AD tryp // α ad TRYP.

Experimental analyses

Experiment 1 and 2 (results combined).

Cross: α ad x a AD.

Number of asci isolated = 125

Number of asci producing colonies = 100

Percentage mortality of asci = 20

a. Ploidy and mating type

Number of asci producing diploid colonies = 77

Number of asci producing haploid colonies = 23

Mating type of haploid colonies:

	Number of asci giving colonies of *a* mating type	Number of asci giving colonies of *α* mating type
Obtained:	19	4
Expected (1:1 ratio):	11.5	11.5

∴ Significant shortage of asci giving colonies of *α* mating type.

The ratio of asci giving haploid colonies to those giving diploid colonies:

a. Overall ratio

	Two *a* type spores (haploid colonies)	One *α* type and one *a* type spore (diploid colonies)	Two *α* type spores (haploid colonies)
Number of asci:			
Obtained:	19	77	4
Expected (1:4:1 ratio):	16.67	66.67	16.67

$X^2 = 11.6$
$P = 0.0035$

Deviation highly significant.

b. Ratio of asci giving diploid colonies to those giving haploid colonies of *a* mating type only.

	Two *a* type spores (haploid colonies)	One *α* type and one *a* type spore (diploid colonies)
Number of asci:		
Obtained:	19	77
Expected (1:4)	19.2	76.8

Therefore, deviation in overall ratio is due to a shortage of asci containing spores of *a* mating type.

c. Percentage mortality of asci containing two *a* mating type spores

The following calculation is based on the assumption that all asci not producing colonies contained <u>two</u> spores of *a* mating type.

Number of asci not producing colonies = 25

Number of asci producing *a* mating type colonies = 4

Total number of *a* mating type asci = 4+25 = 29

Percentage mortality of such asci = 25 x 100/29
= 86.2

b. Nutritional phenotypes of cells derived from asci

Cells of diploid colonies:

	Phenotypes of cells AD	ad	Total
Number of colonies:			
Obtained:	66	11	77
Expected (5:1)*:	64.2	12.8	

Cells of haploid colonies: (only *a* mating type cells analysed

	Phenotypes in sample of cells all AD	AD and ad	all ad	Total
Number of colonies:				
Obtained:	5	10	4	19
Expected (1:4:1)*:	3.2	12.7	3.2	

$X^2 = 1.8$
$P = 0.4$

* Expected ratios are based on the assumption of free recombination between all the markers and between them and their centromeres.

Experiment 3

Cross: α TRYP x a tryp

Number of asci isolated = 93

Number of asci producing colonies = 50

Percentage mortality of asci = (93-50) x 100/93
= 46.3

a. Ploidy and mating type

Number of asci producing diploid colonies = 42

Number of asci producing haploid colonies = 8

Mating type of haploid colonies:

	Number of asci giving colonies of a mating type	Number of asci giving colonies of α mating type
Obtained:	8	0
Expected (1:1):	4	4

Deviation highly significant due to shortage of asci containing α mating type spores.

Ratio of asci giving diploid colonies to those giving haploid colonies of a mating type:

	Two a type spores (haploid colonies)	One a type and one α type spore (diploid colonies)
Number of asci:		
Obtained:	8	42
Expected (1:4):	10	40

$X^2 = 0.5$
$P = 0.45$

b. Nutritional Phenotype

Asci giving diploid colonies:

	Phenotypes TRYP	tryp	Total
Number of asci:			
Obtained:	36	6	42
Expected (5:1)*:	35	7	

Asci giving haploid colonies:

	Either TRYP only or TRYP and tryp**	All tryp	Total
Number of asci:			
Obtained:	5	3	8
Expected (5:1)*:	6.67	1.33	

$X^2 = 2.5$
$P = 0.1$

Experiment 4

Cross: a AD tryp x α ad TRYP

Number of asci isolated = 50

Number of asci producing colonies = 44

Percentage mortality of asci = (50-44) x 100/50
= 12

a. Ploidy and mating type

Number of asci producing diploid colonies = 37

* Expected ratios are based on the assumption of free recombination between the markers and between them and their centromeres.

** not differentiated.

Number of asci producing haploid colonies = 7

Mating type of haploid colonies:

	Number of asci giving colonies of a mating type	Number of asci giving colonies of α mating type
Obtained:	5	2
Expected (1:1)*:	3.5	3.5

Ratio of asci giving diploid colonies to those giving haploi colonies:

	Two a type spores (haploid colonies)	One a type and one α type spore (diploid colonies)	Two α type spores (haploid colonies)
Number of asci:			
Obtained:	5	37	2
Expected (1:4:1)*:	7.33	29.33	7.33

$X^2 = 6.6$
$P = 0.035$

b. Nutritional phenotypes

Asci giving diploid colonies**:

	Phenotypes			
	AD TRYP	AD tryp	ad TRYP	ad tryp
Number of asci:				
Obtained:	30	3	3	1
Expected (25:5:5:1)*:	25.7	5.1	5.1	1

$X^2 = 2.5$
$P = 0.48$

* Expected ratios are based on the assumption of free recombination between all the markers used and between them and their centromeres.

** Asci giving haploid colonies not analysed for nutritional requirement.

3. Three-spore Asci

A. Dissection of asci to show ploidy of ascospores.

Strain

As for the two-spore asci, the 'wild type' diploid strain obtained by crossing strains B15 and C24-15b was used because no 'marked' strains were available when this experiment was undertaken.

Experimental analysis

No. of asci dissected				= 25
" " "	giving no viable spores			= 0
" " "	" only one	"	"	= 3
" " "	" " two	"	"	= 4
" " "	" three	"	"	= 18

Mating type of ascospores

All spores gave haploid colonies. The three spores which were the only viable products of the asci from which they were derived were all of a mating type. Of the four asci which produced only two viable ascospores, three yielded one a and one α type spore, and the other, two a type spores. The following ratio was obtained among those asci giving three viable spores:

	Two a type and one α type spore	Two α type and one a type spore	Total
Number of asci:			
Observed:	11	7	18
Expected (1:1)	9	9	

The overall ratio of α mating type ascospores to those of a mating type = 28 α : 37 a.

B. Analysis of intact asci.

Strain

The diploid strain utilised in this experiment was heterozygous for mating type and adenine requirement. It was obtained by crossing strain 19/20 1b with strain C24-13b, and designated α ad // a AD.

Experimental analysis

Number of asci isolated = 16

Number of asci producing colonies = 16

a. Ploidy and nutritional phenotype

Phenotypes		
Haploid cells	Diploid cells	Number of asci
a AD	AD	5
	ad	0
a ad	AD	4
	ad	0 (n.e.)
α AD	AD	0
	ad	0
α ad	AD	0
	ad	0 (n.e.)
Not present	AD	5
	ad	2
	Total:	16

(n.e.) denotes that such asci are not expected.

b. Nutritional phenotype only

		Phenotypes AD	ad	Total
Number of asci:				
With diploid cells:	Obtained:	14	2	16
	Expected (5:1):	13.3	2.7	
With haploid cells:	Obtained:	5	4	9
	Expected (1:1):	4.5	4.5	

4. Analyses of Pooled Results from Intact One-, Two- and Three-spore Asci

A. The pooled phenotypes of haploid cells produced by one-, two- and three-spore asci which arose from the heterozygous diploid a ad // a AD.

Type of ascus producing cells	Phenotypes a AD	a ad	α AD	α ad	Total
One-spore	11	7	2	2	22
Two-spore*	20	18	(na)	(na)	38
Three-spore	5	4	0	0	9
Totals:	36	29	2	2	69
Expected (1:1/1:1)**:	32.5	32.5	2	2	

$x^2 = 0.76$
$P = 0.38$

* both spores of all two-spore asci are assumed to have germinated; if the cells of a colony arising from such an ascus are all of one phenotype the two spores are assumed to have possessed that phenotype.

** the 1:1:1:1 ratio is not realised because of the high mortality of α mating type spores. Expected ratios are based on free recombination between gene AD/ad and the mating type gene.

(na) denotes not fully analysed.

B. The pooled phenotypes of diploid cells arising from two- and three-spore asci.

1. Only gene AD/ad segregating besides mating type.

Type of ascus producing cells	Cross from which asci taken	Phenotypes AD	Phenotypes ad	Total
Two-spore	α ad x a AD	66	11	77
Two-spore	α ad TRY x a AD try	33	4	37
Three-spore	α ad x a AD	14	2	16
	Totals:	113	17	130
	Expected (5:1)*:	108.3	21.7	

$X^2 = 1.2$
$P = 0.28$

2. Only gene TRYP/tryp segregating besides mating type.

Type of ascus producing cells	Cross from which asci taken	Phenotypes TRYP	Phenotypes tryp	Total
Two-spore	α TRY x a try	36	6	42
Two-spore	α ad TRY x a AD try	33	4	37
	Totals:	69	10	79
	Expected (5:1):	65.8	13.2	

$X^2 = 0.93$
$P = 0.35$

Summary and Conclusions

a. One-spore asci

Of forty one-spore asci isolated from the diploid α ad // a AD, twenty-two germinated all producing haploid colonies, eighteen of which were of a mating type. If it is assumed that those asci not producing colonies contained

* expected ratio is based on free recombination between gene AD/ad and the mating type gene and between each of these genes and their centromeres.

ascospores of α mating type, it can be calculated that about 80% such asci are inviable. It is not yet known whether this low viability is due to the mating type allele itself or to some closely linked locus.

Of the 22 haploid colonies, 13 were AD and 9 ad, that is, the two alleles segregated in a ratio not significantly different from 1 : 1. There is no evidence of linkage between this gene and the mating type gene; deviation from the 1 a AD : 1 a AD : 1 α ad : 1 α ad ratio is clearly due to the low viability of the asci containing spores of α mating type.

These results demonstrate that a genetic analysis similar to that of random sampling of gametes in higher organisms is possible in yeast.

b. Two-spore asci

All colonies produced by ascospores dissected from two-spore asci were haploid. Further, the overall ratio of α mating type ascospores to those of a mating type did not differ significantly from the expected 1 : 1 (18 α; 24 a).

Intact two-spore asci produced either colonies made up of only diploid cells all of one nutritional phenotype, or colonies of haploid cells which were all of one type or of two types. Presumably diploid colonies arose when the two spores were of complementary mating type, and haploid colonies when the spores were of the same mating type. The

normal formation of diploid colonies from two-spore asci shows that the low viability of a mating type spores, detected upon analysis of one-spore asci and confirmed by the present results, is not manifest in the heterozygote or in the haploid ascospore if the latter fuses with a spore of complementary mating type.

Phenotypes in samples of cells taken from haploid colonies of a mating type originating from two-spore asci of the cross in which only gene AD/ad was segregating (in addition to mating type), gave a ratio among the colonies of 5 all AD : 10 both AD and ad : 4 all ad; this does not differ significantly from the expected 1 : 4 : 1 ratio.

Analysis of the diploid colonies obtained from two-spore asci in the two crosses in which only gene AD/ad and only gene TRYP/tryp were segregating besides mating type gave respective ratios of 66 AD : 11 ad, and 36 TRYP : 6 tryp These ratios do not differ significantly from the 5 : 1 ratio expected when each of the nutritional genes and the mating type gene show free recombination with one another and when at least one gene in each cross shows free recombination with its centromere, i.e. it is 33.3 or more map units away from its centromere (Calculation 1, page 30).

Analysis of the diploid colonies obtained from two-spore asci in which both the AD/ad and TRYP/tryp genes were segregating gave a ratio of 30 AD TRYP : 3 ad TRYP : 3 AD

tryp : 1 ad tryp, which does not differ significantly from the 25 : 5 : 5 : 1 ratio expected when there is free recombination of the two genes, of each of these and the mating type gene, and of at least two and possibly three of the genes and their centromeres (Calculation 7, page 41. See, however, the conclusions on the analysis of pooled results).

c. Three-spore asci

All the ascospores dissected from three-spore asci were similar to those of one- and two-spore asci in that they gave haploid colonies. A slight but not significant shortage of a mating type spores resulted (overall ratio: 37 a : 28 α). This degree of shortage, however, was not anywhere near as great as that evident in the experiments on intact asci where 'marked' strains were used. Taking into consideration the overall results of these experiments, it would appear that both the ad allele and, to a lesser degree, the tryp allele enhanced the mortality of the a mating type spores.

Half the individual colonies arising from intact three-spore asci contained two kinds of cells; diploid cells resulting from the fusion of two ascospores of complementary mating type, and haploid cells resulting from the odd ascospore. The other half of the colonies contained only diploid cells, presumably because the odd ascospore which should have given haploid cells was of a mating type and

failed to germinate. The diploid cells from each ascus were invariably of one type, showing that two ascospores of complementary mating type fuse on germination without first dividing.

Analysis of the haploid cells of the colonies yielded a phenotypic ratio of 5 AD : 4 ad, a result not significantly different from that expected.

Analysis of the diploid colonies yielded a phenotypic ratio of 14 AD : 2 ad. The small number of asci analysed is not enough to draw any definite conclusions. The most which can be said of these results, taken alone, is that they do not provide any evidence of linkage either between the mating type gene and the AD/ad gene, or between these genes and their centromeres.

d. Pooled results of one-, two-, and three-spore asci.

The pooled phenotypes of haploid cells produced by one-, two-, and three-spore asci from the diploid α ad // a AD clearly indicate that the two genes freely recombine with one another. The observed ratio of phenotypes, 36 : 29 : 2 : 2, deviated from the expected 1 : 1 : 1 : 1 ratio for two reasons: the haploid cells of α mating type arising from two-spore asci were not analysed, and most of the α mating type spores did not germinate.

The analysis of pooled diploid phenotypes arising from

two- and three-spore asci of both crosses in which the gene AD/ad was segregating do not refute the hypothesis that this gene and that for mating type freely recombine with one another, and that at least one of them freely recombines with its centromere (113AD : 17ad).

Similarly, the ratio of diploid phenotypes from two- and three-spore asci of both crosses in which gene TRYP/tryp was segregating do not refute that this gene freely recombines with the mating type gene, and that at least one of them freely recombines with its centromere (69TRYP : 10tryp).

It will have been realised, however, that these results might equally well be explained on the basis of the model used in Table 5 (page 43): the genes a/α, AD/ad, and TRYP/tryp show free recombination with one another but not with their centromeres.

From the pooled results the best estimates which can be obtained for $p_{ad} = \frac{17}{130} = 0.131$, for $p_{tryp} = \frac{10}{79} = 0.127$, and for $p_{ad,\ tryp} = \frac{1}{37} = 0.027$. Hence,

$$g = \frac{36(0.027 - [0.131 \times 0.127])}{(1 - [6 \times 0.131])(1 - [6 \times 0.127])} = 7.33$$

From Table 5a: $1/X = 2.306$, and $x = 0.377$, and hence

$$Y = 2.306(1 - [6 \times 0.131]) = 0.4935$$
$$Z = 2.306(1 - [6 \times 0.127]) = 0.5496$$

and,

$$y = \tfrac{2}{3}(1 - 0.4935) = 0.338$$
$$z = \tfrac{2}{3}(1 - 0.5496) = 0.300$$

Consequently, this data might be explained on the assump-

tion of free recombination of each of the three genes with one another, and linkage with their centromeres (frequencies of second division segregation of 0.377, 0.338, and 0.300).

On the assumption of complete interference map distances of the three genes from their centromeres are: a/a = 19, AD/ad = 17, and TRYP/tryp = 15 units. On the assumption of no interference these values are 28, 24 and 20 units respectively.

Of course, without a very large number of diploid phenotypes (about 1000), it is impossible either to determine accurately which explanation is best (that of free recombination with their centromeres or the above), or to obtain good estimates of map distances. The above analyses are therefore more of a demonstrative nature than of a conclusive one.

The results on intact asci confirm those of dissected asci in showing that the ascospores within each of the types of incomplete asci each contain one of the four haploid products of meiosis.

It should also be noted that (a) none of the intact two-spore asci gave both haploid and diploid cells, and (b) none of the intact three-spore asci gave colonies containing haploid and diploid cells both of which were of 'recessive' nutritional phenotype. Both types of such asci would have been expected should two ascospores of complemen-

tary mating type within the same ascus divide mitotically before fusing.

Discussion

Genetic analysis of yeast, of the type reported in the present work, is intermediate in technical difficulty between tetrad analysis and the analysis of random gametes. The last type of analysis, not hitherto carried out in yeast, has been shown to be possible by the utilization of either one-spore asci or haploid cells arising from intact two- or three-spore asci. This method gives more information than tetrad analysis but fails to provide direct evidence of irregular segregations, does not locate the centromeres and does not give information about chromatid interference. Thus, the technical difficulty of dissection of spores from an ascus, inherent in tetrad analysis, can be overcome without serious loss except for special purposes.

Should the object of the present work have been to carry out formal genetic analysis, i.e. locating genes, work would have continued on one-spore asci. Clearly, this is a more efficient way of mapping than tetrad analysis. However, having shown that an analysis of random ascospores is possible by utilizing one-spore asci, the author was more interested in finding whether the diploid colonies arising from intact two- and three-spore asci could also be utilized in genetic analysis. It has been shown that such

colonies can be utilized and that their analysis provides information as to the location of centromeres.

The present results suggest that analysis of intact two-spore asci may be suited to strains of homothallic yeasts, where it would be expected that every such ascus would on germination produce diploid cells all of one kind if, as in the present work, the ascospores fused immediately on germination without first dividing.

Although analysis of intact four-spore asci has not been undertaken in the present work, there is no obvious reason why it could not be done. If progenies from diploid cells isolated at random from each of a series of colonies arising from the germination of individual four-spore asci were analysed, the same results would be expected as those from the analysis of diploid cells arising from intact two- and three-spore asci of the same heterozygote. A simplified technique for such an analysis, which would avoid the trouble of dissection, may be visualised. Such a technique would include streaking the colony produced by each ascus on nutrient agar and later isolating and testing a single colony arising as a result of the streak.

The genetic analysis of two-spore asci from a cross involving the inheritance of a single 'nutritional' gene could be simplified by ignoring the ploidy of the colonies arising from such asci. In the case of complete dominance, phenotypic classification could then be carried out directly.

Consider a colony arising from an ascus containing two ascospores of the same mating type, one carrying the dominant allele, the other the recessive allele. On plate-testing (growth of cells on minimal medium), such a colony, even though containing haploid cells of either one or two types, would give the same response as a colony containing only diploid cells, such as would have resulted if the ascospores had been of complementary mating type. A similar situation would exist if the two ascospores each carried the recessive allele. An example illustrating this type of analysis is shown in the following table. It should be borne in mind, however, that if an analysis ignoring the ploidy of the cells was undertaken, the theoretically expected results would be different from those worked out for diploid cells alone: the effect of the mating type gene would be excluded.

Illustration of the type of genetic analysis of intact two-spore asci in which the ploidy of the cells arising at germination of such asci is ignored

The mating type gene, a/α, and the 'nutritional' gene B/b segregating from the diploid a B // α b:

Sample of different types of asci expected*:	((a B)) ( ) ((a B))	((a B)) ( ) ((a b))	((a B)) ( ) ((α B))	((a b)) ( ) ((a b))	((a b)) ( ) ((α b))
Ploidy of cells produced by ascus:	n	n	2n	n	2n
Growth of cells on minimal medium:	+	+	+	-	-
Genotypes of cells which grow on minimal medium	a B	a B	a B//α b	-	-

* for simplicity not all types of asci are shown, e.g., those containing two α type spores are omitted.

If a semi-lethal allele closely linked to the a mating type allele was discovered and utilized in crosses with strains carrying the α mating type allele, used in the present crosses, the analysis of intact two- and three-spore asci would yield more information per tested colony: two-spore asci from such crosses would yield a higher percentage of diploid colonies per number of asci germinating, and three-spore asci would yield a higher percentage of colonies containing only diploid cells. It should be remembered, however, that very few of the more easily analysed one-spore asci from such crosses would germinate.

A Comparison of Diploid Gametic and Tetraploid Zygotic Ratios expected from Autotetraploid Organisms with the Phenotypic Ratios expected among Diploid Colonies derived from Intact Incomplete Asci of Yeast.

In a proportion of cases varying from species to species, the four alleles of a gene present in autotetraploids segregate randomly into the gametes in pairs during meiosis; in a yeast tetrad, one allele is present in each of the four spores which later fuse in pairs in four out of the six possible ways. Diploid products result in both cases: in the former diploid gametes, in the latter diploid zygotes.

The diploid gametic ratios of autotetraploids and those of diploid colonies arising from intact two- and three-spore yeast asci are the same when (a) a single gene is segregating, and (b) two genes which are situated on different chromosomes are segregating; this, provided at least one gene in the case of yeast, shows free recombination with its centromere and both show free recombination with the mating type gene.

Compare the following two cases:

Case 1

Autotetraploid genotype: B B b b, C C c c

(duplex and heterozygous for the two genes which show free recombination with each other)

Diploid gametic ratios from this autotetraploid are derived in the following table:

	1 BB	4 Bb	1 bb
1 CC	1 BBCC	4 BbCC	1 bbcc
4 Cc	4 BBCc	16 BbCc	4 bbCc
1 cc	1 BBcc	4 Bbcc	1 bbcc

Diploid gametic 'phenotypic' ratio (with full dominance):

25 BC : 5 Bc : 5 bC : 1 bc

The tetraploid zygotic phenotypic ratio will be the same when this autotetraploid is crossed with a multiplex autotetraploid (diploid gametes of constitution aabb).

Case 2

Genotype of diploid yeast producing asci: a a, B b, C c, (all genes independent of their centromeres).

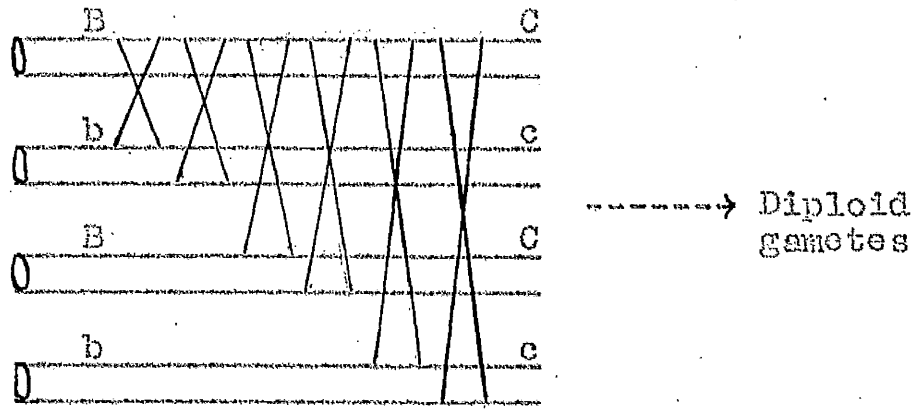
Phenotypic ratios among diploid cells expected from two- and three-spore asci: 25 BC : 5 Bc : 5 bC : 1 bc (Calculation 7a, page 41).

If only one gene is segregating a ratio of 5:1 would be expected in each case.

The above ratios would differ as between tetraploid and yeast when, in the latter, the mating type gene is linked to either or both of the genes B/b and C/c. They will also differ when these two genes are linked to one another; this is due to the special features of tetraploid meiosis.

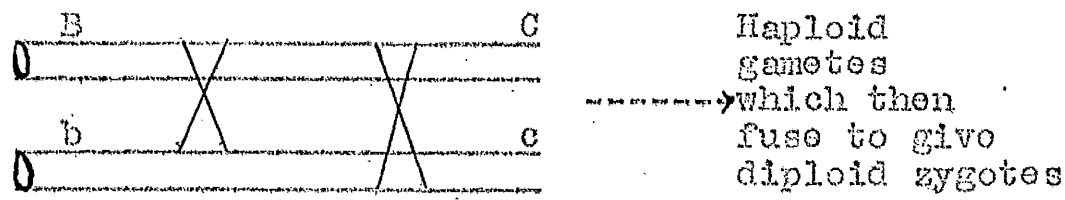
In an autotetraploid eight chromatids are present during diplotene of meiosis and each chromatid can cross over with

any of its six non-sister chromatids, viz:



(The diagram does not mean to imply that all chiasma shown can occur in the same 'octad', or that octads regularly occur)

In yeast only four chromatids are present during diplotene of meiosis; each can cross over with two non-sister chromatids, viz:



In the case of linkage, therefore, the theory of the formation of diploid gametes in polysomic inheritance as developed by Mather (1936) and Fisher (1947) cannot be applied as it stands to the formation of diploid zygotes from individual yeast asci.

PART II

The Production, Isolation and Characterization of Auxotrophic Strains of Saccharomyces cerevisiae

Introduction

Genetically marked strains were necessary for the studies on incomplete asci already described; the following experiments were undertaken to obtain them. The mutagenic agent used was ultra violet light.

It was also of interest to compare the data obtained with similar data obtained by other workers in Neurospora, Aspergillus, Ophiostoma and Penicillium. Such a comparison has been made and the more interesting points of similarity and dissimilarity between the sets of data have been discussed.

Methods

a. Preparation of cell suspension

An inoculum from a single ascospore culture of haploid cells maintained in minimal medium (or minimal medium plus the growth factor(s) needed if an auxotroph was used as starting strain), was transferred to a slope of complete medium and incubated for 3 days. Two or three platinum wire loopfuls of the resulting culture were added to 1.5 ml. of saline and the clumped cells of this suspension broken up by drawing it into and forcing it out of a Pasteur pipette

Very little further separation of cells was accomplished after approximately fifty such 'in and out' movements. The number of cells per ml. of this suspension was determined by a haemocytometer count. Each cluster of cells was recorded as a single cell. The presence of clusters probably decreases the efficiency of the technique since mixed colonies resulting from clusters of auxotrophic and prototrophic cells are classified as prototrophs. However, at the doses of irradiation used (survival of the order of 10^{-3}) the chances of survival of two cells in a cluster is low.

From an original density of 10^7 cells per ml., serial dilutions were made to a working density of not more than 2×10^5, and not less than 7×10^4 cells were ml. For an estimate of the percentage viability, a further dilution to between 7×10^2 and 10^3 cells per ml. was made. One tenth ml. of this last suspension was spread on each of five petri-dishes of complete medium. Percentage viability was estimated from a colony count after three days incubation.

b. Irradiation of cell suspension

Nine ml. of the suspension, containing not more than 2×10^5 and not less than 7×10^4 cells per ml., were poured into a petri-dish (inside diameter of base = 3.5 inches) and the suspension irradiated with ultra violet for 3 minutes 30 seconds at a distance of 40 centimetres from the tube. During exposure the dish was agitated for three seconds every

fifteen seconds. This dose gave approximately 0.25% survival. The source of the ultra violet was a Hanovia XI low pressure jacketted mercury lamp with 90% output at 2537 A.

c. The recovery and isolation of auxotrophs

After irradiation the suspension was thoroughly mixed with a 1 ml. pipette. 0.1 ml. of this suspension was spread on each of 6 to 12 petri-dishes of complete medium. After 4 days incubation there were between 25 and 100 colonies per dish.

At first, colonies resulting from cells surviving the irradiation were tested individually as follows: very small inocula from each colony were transferred in duplicate to correspondingly marked positions on one dish of complete medium and one of minimal medium. Thirty colonies could be tested in each pair of dishes. The inocula were always very small (straight platinum wire) and were taken from the upper part of the colony in order to avoid as far as possible the carrying over of any complete medium to the minimal medium plates.

As in the first experiments (Experiments B, C, and D) (Table 10, page 105) no vitamin requiring auxotrophs were obtained, there was the possibility that this failure would be the result of carrying over vitamins with the inoculum. A very small quantity of vitamin carried over in this way might be ample to support enough growth on minimal medium as

to lead to misclassification. Accordingly, in a second series of experiments (Experiments D1 and E, Table 9), instead of direct transfer of cells, inocula from saline suspensions were used. These suspensions were made by transferring a small inoculum of the colony to be tested to approximately 0.15 ml. of saline in a concavity slide. Even with these 'semi-washed' inocula, containing less contaminating nutrionts, no vitamin requiring auxotrophs were isolated nor were more auxotrophs of other types recovered; this more laborious procedure was therefore discontinued.

d. Characterization of auxotrophs

Two methods were employed to identify the growth requirements of the auxotrophs. When a small number were to be identified the auxanographic method (Beijerinck, 1889; Pontecorvo, 1949b) was adopted to determine their broad and specific growth requirements. When the number of auxotrophs exceeded five, their broad requirements were determined by the plate-test method (Fries, 1948a), and their specific requirements by the auxanographic method.

A. Auxanographic method

Dishes were prepared, each having some 10^7 cells suspended in 20 ml. of minimal agar medium. At marked point on each dish one loopful of each of the following substances were added: 1) casein hydrolysate plus tryptophane, 2) yeas

nucleic acid hydrolysate, 3) a dilute solution of yeast extract "yeastrel", and 4) complete medium as control. The petri-dishes were then incubated for 18 to 24 hours. The appearance of a turbid zone of cell growth around any of the added substances indicated the broad nature of the growth factor(s) required by the auxotroph. The following table shows the broad characterization of auxotrophs by this test.

Growth response to:	Indicated growth factor:
Casein hydrolysate plus tryptophane	An amino acid
Hydrolysed yeast nucleic acid	A nucleoside or nucleotide
'Yeastrel'	A vitamin of the B group
Complete medium	Control response

Further characterization of an auxotroph was carried out by the use of single substances belonging to the group indicated by the first test. For example, if a growth response was obtained from casein hydrolysate plus tryptophane, individual amino acids (a few crystals of each) were tested by the auxanographic technique. A turbid zone of growth around the inoculum of any one of these amino acids indicated a requirement for a particular amino acid. Similar additional tests were then undertaken to determine as far as possible the alternative growth factor requirements

Alternative growth factors which were tested are shown in Table 8.

TABLE 8

Further tests which were carried out to find the alternative growth factor requirements of auxotrophs.

If auxotroph responded to:	Also tested for growth response were:
Arginine	Ornithine, citrulline, proline
Tryptophane	Indole, serine, anthranilic acid, kynurenine, nicotinic acid
Methionine	Sodium sulphite, potassium thiosulphate
Tyrosine	Phenylalanine, para-hydroxy-phenylpyruvic acid, phenyl-pyruvic acid, dihydroxyphenyl-alanine
Isoleucine plus leucine (natural mixture)	Isoleucine, leucine, valine
Serine	Glycine

B. Plate-test method

The broad requirements of a group of auxotrophs were determined by the plate-test method (Fries, 1948a). Requirements were determined simultaneously by inoculating each auxotroph on to a series of petri-dishes containing (a) minimal medium, (b) minimal medium plus casein hydrolysate, plus tryptophane (0.1 ml. of a standard solution per plate), (c) minimal medium containing yeast nucleic acid

hydrolysate (0.1 ml. of a standard solution per plate), (d) minimal medium plus vitamins (0.05 ml. of a standard solution per plate), and (e) complete medium. After three days incubation the growth of an auxotroph on any one or more of the supplemented minimal medium plates indicated that such plates contained the growth factor(s) required. Further characterization of the auxotroph with regard to its specific growth requirements was determined by the auxanographic method.

Experimental Results and Observations

Table 9 (page 104) gives the viable counts before and after irradiation in 7 out of 8 experiments.

Although known conditions of irradiation were the same for each experiment, the viable counts varied vastly between experiments, giving survival rates between 0.017% and 0.525%. The variation was probably due to (a) changes in the mains voltage and (b) variation of cell densities in different experiments. Variations due to photoreactivation could be excluded because in every case exposure to strong light was avoided.

Table 10 (page 105) summarises results of the isolation and broad testing of colonies for auxotrophy. Table 11 (page 107) summarises results of finer characterization. The pooled results gave 48 auxotrophs out of a total of 2,117 isolates, i.e. 2.25% of the isolates tested were auxotrophic.

TABLE 9

Cells giving origin to colonies before and after irradiation with U.V.

Expt.	Culture irradiated	No. of cells per ml. irradiated (viable count)	No. of colonies after irradiation		Viable count as percent of initial viable count
			per plate	total	
C	C53-8d prototroph	1.4×10^5	1.62 2.54 3.78 4.98 5.62 6.82 7.79 8.76 9.77 10.67	735	0.53
D	C53-8d prototroph	2.05×10^5	1.27 2.35 3.30 4.29 5.27 6.28 7.18 8.35 9.18	247	0.31
D1	C53-8d prototroph	7.7×10^4	1.18 2.18 3.16 4.23 5.18 6.13 7.18 8.14 9.24 10.27 11.30	219	0.26
E	B7/20 adenineless	1×10^5	1.17 2.11 3.19 4.27 5.14 6.12 7.16		0.02

			9.25 10.16 11.15 12.17 205	
H	D1/2 tryptophane-less	2.15×10^5	1.21 2.18 3.18 4.19 5.23 6.20 7.20 8.24 9.25 10.26 11.27 12.20 13.31 319	0.12
J	D1/2 tryptophane-less	2.33×10^5	1.11 2.13 3.16 4.12 5.14 6. 9 7.11 8. 7 9. 7 10. 9 11.12 12.11 132	0.05
K	D1/2 tryptophane-less	2.88×10^5	1.76 2.82 3.54 4.64 5.71 6.101 7.82 8.78 9.67 10.91 11.94 12.97 957	0.27
B	C24-13b prototroph	not recorded	not recorded	-
			Mean*:	0.22

* for seven of the eight experiments.

TABLE 10

Auxotrophs obtained

Experiment (survival rates after U.V. in brackets)	Strain treated	Isolates (no.)	Auxotrophs*						
			Total						
			(no.)	(%)	S	A.A.	N.A.	Vt.	Uk.
B (?)	C24-13b prototroph	210	6	2.9	0	4	1	0	1
C (0.5)	C53-8d prototroph	330	8	2.4	2	4	1	0	1
D (0.3)	C53-8d prototroph	200	4	2.0	0	4	0	0	0
D1 (0.3)	C53-8d prototroph	150	5	3.3	0	4	1	0.	0
E (0.02)	B7/20 adenineless	193	4	2.1	0.	4	0	0	0
H (0.12)	D1/2 tryptophaneless	254	7	2.8	0	5	2	0	0
J (0.05)	D1/2	120	3	2.5	0	1	1	0	1

of the 48 auxotrophs, 45 were fully tested. Each of the 43 required a single specific growth factor. In the case of 5 auxotrophs no success was obtained in determining a single specific requirement by means of the tests used for all the others. If all five were cases of multiple requirements, this would constitute 10% of the total.

The most frequent requirements are for amino acids and of these methionine (9), tryptophane (7), leucine and isoleucine (5), arginine (4) and lysine (3) are in the majority. However, these quantitative data are not homogeneous because in half of the experiments (E, H, J and K) the strain irradiated was either tryptophane- or adenine-requiring. Only two types of auxotrophs requiring nucleic acid components were isolated, namely adenine-requiring (5) and uracil-requiring (2).

No vitamin-requiring auxotrophs were isolated in spite of the modified technique (page 100) adopted in experiments D1 and E which supposedly would have made possible the isolation of such auxotrophs should the reason for their previous non-discovery have been due to contamination by growth factors from complete medium. It should be borne in mind, however, that the minimal growth requirements of yeast include pantothenate, biotin and inositol. In other micro-organisms such as Neurospora, Aspergillus nidulans, Ophiostoma multiannulatum and Escherichia coli, auxotrophic

TABLE 11

Substances to which auxotrophs gave a growth response.

Experiment	Total number of auxotrophs recovered	Substance(s) to which auxotroph responds	Number of auxotrophs responding	Designation of auxotroph(s)
B	6	adenine monohydrochloride	1	A7
		L lysine monohydrochloride	1	B4
		DL methionine	1	B14a
		DL tryptophane	1	B3
		DL tyrosine	1	B7/28
		unknown	1	B6
C	8	adenine monohydrochloride	1	C2
		L arginine monohydrochloride	2	C5 & C6
		potassium thiosulphate	2	C1 & C4
		DL serine/glycine	1	C3
		DL tryptophane/anthranilic acid/indole	1	C7/27
		unknown		
D	4	leucine and isoleucine (natural mixture)	1	D3
		DL tryptophane	2	D14/18 & D1
		DL tryptophane/indole	1	D11/9
D1	5	adenine monohydrochloride	1	D1/7
		L lysine monohydrochloride	1	D1/6
		DL methionine	2	D1/8 & D1/3
		DL tryptophane	1	D1/2
E	4	adenine monohydrochloride plus:		
		DL methionine	2	C2/E1 & C2/E5
		DL tryptophane	1	C2/E3
		L arginine monohydrochloride/ DL ornithine monohydrochloride (not citrulline)	1	C2/E4
H	7	DL tryptophane plus:		
		uracil	2	D1/H4 & D1/H6
		DL methionine	1	D1/H5
		L histidine	2	D1/H1 & D1/H7
		leucine and isoleucine (natural mixture)	2	D1/H3 & D1/H11
J	3	DL tryptophane plus:		
		adenine monohydrochloride	1	D1/2, J2
		leucine and isoleucine (natural mixture)	1	D1/2, J9
		unknown	1	D1/2, J4
K	11	DL tryptophane plus:		
		L arginine monohydrochloride	1	D1, K4
		DL methionine	3	D1, K2; D1,K6 & D1,K9
		L lysine monohydrochloride	1	D1, K5
		L histidine	1	D1, K10
		adenine monohydrochloride	1	D1, K11
		leucine and isoleucine (natural mixture)	2	D1, K12 & D1, K15
		unknown	2	D1, K8 & D1, K1

mutants requiring one or other of these vitamins arise frequently.

A direct comparison of the qualitative yields of auxotrophs obtained in yeast with those obtained by the 'total isolation technique' in other microorganisms reveals some striking differences and similarities. Tables 12 and 13 (pages 109 and 111) show lists of auxotrophs which have been isolated in Aspergillus nidulans (Pontecorvo et al., 1953), Ophiostoma multiannulatum (Fries, 1947), Neurospora crassa (Tatum, Barratt, Fries and Bonner, 1950), and Penicillium notatum-chrysogenum (Bonner, 1946), together with those isolated in yeast. Unfortunately there is no general survey published of all the auxotrophs of Neurospora. The most comprehensive list of Neurospora auxotrophs is that published by Tatum, Barratt, Fries and Bonner (1950). These investigators studied the mutagenic effect of different agents, including ultra violet, X-rays, methyl-bis-(-chloroethyl) amine, methylcholanthrene and tris-(-chloroethyl) amine, on microconidia of Neurospora and found no significant difference among either the qualitative or quantitative spectra of auxotrophs isolated after treatment with these different agents. A comparison of the results of Tatum et al. (1950) with those of other workers indicates that their list is fairly typical of the spectrum of auxotrophs generally obtained in Neurospora, except in the percentage of

TABLE 12

A comparison of the types of auxotrophs obtained by 'total isolation' from Saccharomyces cerevisiae (present work) with those obtained from Aspergillus nidulans (Pontecorvo et al., 1953), Ophiostoma multiannulatum (Fries, 1947) and Penicillium notatum-chrysogenum (Bonner, 1946).

Type of auxotroph	Organism			
	S. cerevisiae	A. nidulans	O. multiannulatum	P. notatum-chrysogenum
Not fully tested	5	24	9	43
Parathiotrophic	2	7	34	0
Sulphite	0	5	34	0
Thiosulphate	2	2		0
Paranitrotrophic	0	17	0	31
Nitrite	0	13	0	[31
Ammonium	(x)	4	0	
Amino Acids	34	12	89	250
Arginine	3	1	[	[
Arginine/ornithine	1	5	42	65
Arginine/ornithine/proline	0	1	]	]
Proline	0	0*	0	0
Ornithine	0	0*	0	0
Lysine	3	3	32	54
Methionine	[	1	[	50
Methionine/cystine	9	1	9	0
Tyrosine	1	0	0	0
Phenylalanine	0	0*	0	4
Isoleucine	0	0*	0	1
Leucine	0	0	0	12
Leucine/Isoleucine (nat. mixt.)	6	0	0	0
Tryptophane	5(x)	0*	0	2
Tryptophane/indole	1(x)	0	0	0
Tryptophane/indole/anthranilic acid	1(x)	0	0	0
Serine/glycine	1	0	0	0
Histidine	3	0	0	16
Aspartic acid	0	0	1	0
Nucleic acid components	7	11	81	19 (not further classified)
Adenosine/adenine	5(x)	10*	4*	
Guanosine/guanine	0	0*	0*	
Cytidine/uridine	0	1	1	
Uracil	2	0	26	
Hypoxanthine	0	0	51	

TABLE 12 (continued)

Type of auxotroph	Organism			
	S. cerevisiae	A. nidulans	O. multiannulatum	P. notatum-chrysogenum
Vitamins	0	20	32	55
Biotin	(x)	4(x)	2*	5
p-aminobenzoic acid	0	2	0*	6
Pantothenic acid	(x)	1	6	0
Nicotinic/anthranilic acid	0	4	[6	[5
Nicotinic/anthranilic/tryptophane	0	3	[	[
Riboflavin	0	1	0	0
Pyridoxin	0	1	(x)	6
Choline	0	1	0	9
Aneurin	0	3(x)	(x)	18
Inositol	(x)	0	18	6
Multiple	0	2	0	0
Totals	48	93	245	245	398
From Isolates	2117	5408	13405	85595

(x) denotes either that the growth factor was normally present in minimal medium or that in some experiments a starting strain requiring such a factor was used; the data for auxotrophs of the kind so indicated are therefore not comparable between all organisms.

* denotes that auxotrophs requiring such a factor have been isolated by special selective techniques (Pontecorvo et al., 1953; Fries, 1947).

[denotes that tests to distinguish between these types of auxotrophs have not been undertaken or have not been published.

TABLE 13

Types of auxotrophs isolated from Neurospora crassa by Tatum, Barratt, Fries and Bonner (1950) by means of the 'total isolation' technique. Those types not isolated by these workers but which have been isolated by other workers are also indicated.

Type of auxotroph	Number isolated	Whether isolated by workers other than Tatum et al. (1950)
Not fully tested	18	
Parathiotrophic	0	
Sulphite	0	
Thiosulphate	0	
Paranitrotrophic	0	
Nitrite	0	+
Ammonium	0	+
Amino Acids	81	
Arginine	2	
Arginine/ornithine	0	+
Arginine/citrulline	0	+
Arginine/ornithine/proline	0	+
Lysine	19	
Proline	0	+
Cysteine	0	+
Methionine	23	
Methionine/cysteine	19	
Methionine/homocysteine	0	+
Methionine/cystathionine	0	+
Tyrosine/phenylalanine	see multiple	
Phenylalanine	1	
Isoleucine	0	
Isoleucine/valine	2	
Valine	0	+
Threonine	0	+
Leucine	6	
Tryptophane	4	
Tryptophane/indole	0	+
Tryptophane/nicotinic/indole	0	+
Tryptophane/nicotinic acid	3	
Serine/glycine	1	
Homoserine	0	+
Histidine	0	+
Aspartic/glutamic acid	1	
Nucleic acid components	25	
Adenine	21	
Cytidine/uridine	0	+
Uracil	4	
Vitamins	17	
Biotin	(x)	
p-aminobenzoic acid	3	
Pantothenic acid	1	
Nicotinic acid	3	
Nicotinic/anthranilic acid	1	
Nicotinic/tryptophane/anthranilic	0	+
Riboflavin	1	
Pyridoxin	5	
Choline	2	
Aneurin	1	
Inositol	0	+
Multiple* and Others**	4	
Totals	145	
From isolates	19,218	

(x) denotes presence of growth factor in minimal medium.

* two multiples, one requiring para-aminobenzoic acid and uracil, the other para-aminobenzoic acid, anthranilic acid, phenylalanine and tyrosine.

** two others, one requiring acetate and the other asparagine.

arginine-requiring auxotrophs which, for some unknown reason, is lower than that attained by other authors (Horowitz, Bonner, Mitchell, Tatum and Beadle, 1945).

To complete the qualitative spectrum of auxotrophs already obtained in Neurospora, Table 13 indicates those types of auxotrophs not isolated by Tatum et al. (1950). This table also refers to those types of auxotrophs not isolated by the 'total isolation' technique in Aspergillus or Ophiostoma; such auxotrophs were isolated in both organisms by selective techniques and include in Aspergillus both a guanine and a tryptophane-requiring auxotroph and in Ophiostoma a para-aminobenzoic acid and guanine-requiring auxotroph (Fries, 1947; Pontecorvo et al., 1953).

Examination of Tables 12 and 13 reveals that the high proportions of arginine-, lysine- and adenine-requiring auxotrophs are common to all five species. The high proportion of parathiotrophic auxotrophs isolated from Aspergillus and Ophiostoma is not paralleled by yeast or Neurospora. In the case of Penicillium it is not clear from the published accounts whether or not the high proportion of auxotrophs which respond to methionine were tested for response to simpler compounds such as thiosulphate or sulphite. Should they not respond to such compounds, Penicillium would resemble yeast in the high proportion of methionine-requiring auxotrophs recovered. It should be

noted that the complete medium used in the isolation of auxotrophs in yeast and Aspergillus was identical. The differences in the qualitative yields of auxotrophs between these two species cannot therefore be due to any selective effect of the medium used.

TABLE 14

Types of auxotrophs isolated in yeast but not in some or all of the other four species

Type of auxotroph isolated in yeast	A. nid.	O. multi.	N. crassa	P. not.-chry.
Tyrosine	-	-	-	-
Serine/glycine	-	-	+	-
Parathiotrophic	+	+	-	-
Tryptophane	+*	-	+	+
Histidine	-	-	+	+
Arginine/ornithine (not citrulline)	+	?	-	-

* isolated only by selective techniques

Mention of those types of auxotrophs not found in yeast but found comparatively frequently in some or all of the other four species does not seem to be justified at this time in view of the number of auxotrophs isolated in yeast compared with the numbers isolated in the other species.

However, the reverse does not hold, that is, mention is

justified of those types of auxotrophs found in yeast and not in some or all of the other species. Examination of Table 14 (page 113) reveals that among these types are tyrosine, serine/glycine, histidine, and tryptophane (only, i.e., not responding to other known related growth factors, for example indole or anthranilic acid).

Whether the differences shown in Table 14 are real or are simply due to the range of nutrients used to characterize auxotrophs is not certain from the published accounts.

One type of auxotroph, that requiring L-tyrosine for growth, shows novel properties. Two auxotrophs of this type have been isolated, one by the 'total isolation' technique, the other in the experiments on the 'starvation' technique (Part IIIA). Both these strains were tested, without success, for growth responses to each of the following substances: L-phenylalanine, para-hydroxyphenylalanine, para-hydroxyphenylpyruvic acid, phenylpyruvic acid and di-iodotyrosine. These mutants could be biochemically analogous to phenylketonuria in man, in which the synthesis of tyrosine from phenylalanine is supposed to be partially blocked (Følling, 1934; Penrose and Quastel, 1937; Jervis, 1947).

The occurrence of auxotrophs requiring tyrosine as well as other aromatic compounds in Neurospora has been reported by Tatum et al. (1950). The biochemical situation has been

cleared by Davis (1950) for Escherichia coli by the demonstration that shikimic acid is a precursor of all the aromati compounds required by these mutants. Clearly these types of auxotrophs are not similar to the tyrosine requiring ones isolated in yeast. No biochemical investigations were carried out on these mutants because the investigation of sequences of biochemical reactions was outside the scope of the present work. For the same reason, none of the other mutants was investigated beyond the minimum necessary for using them as markers in genetic analysis.

It is interesting to note that the arginine requiring auxotroph which responds also to ornithine does not respond to citrulline which is an intermediate between ornithine and arginine in the Krebs cycle. In this, yeast resembles Aspergillus nidulans (Pontecorvo et al., 1953) but differs from Neurospora and Escherichia coli.

Most of the auxotrophs requiring arginine / ornithine, or arginine alone were examined auxanographically for interactions with lysine. In nearly all cases (Table 15) lysine competitively inhibits growth on exogenous arginine (Doermann 1944; Pontecorvo et al., 1953). Only one strain, B7/20, requiring arginine or ornithine, which was used in the 'starvation' experiments of Parts III and IIIA, was fully tested for the ratio of molar concentrations at which this competitive inhibition takes place. It was found that a

slight inhibition occurred at molar ratios of 2 lysine : 1 arginine and that complete inhibition occurred at molar ratios of 3 lysine : 1 arginine, ratios slightly higher than those found in arginine requiring auxotrophs of Aspergillus by Pontecorvo et al. (1953). No precise tests were undertaken to discover whether or not the lysine requiring auxotrophs were competitively inhibited by arginine. However, there was no indication of such inhibition on auxanographic plates.

TABLE 15

Competitive inhibition of arginine requiring auxotrophs by lysine

Strain	Requirement(s)	Competitively inhibited by lysine
B7/20[#]	arginine/ornithine	+
C5	arginine	+
C6	arginine	+
D1/2,K4	arginine	?(not noted)

[#] a single ascospore culture from a cross C2/E4 (arginine/ornithine plus adenine requiring) x C24-13b (prototrophic).

Of the five auxotrophs requiring adenine for growth, three were competitively inhibited by guanine on auxanographic plates; the other two were not. No tests were undertaken to discover the ratio of molar concentrations at which these competitive inhibitions takes place.

TABLE 16

Competitive inhibition of adenine-requiring auxotrophs by guanine

Strain	Requirements	Competitively inhibited by guanine
A7	adenine	+
C2	adenine	+
D1/7	adenine	+
D1/2,J2	adenine and tryptophane	-
D1/2,K3	adenine and tryptophane	-

Summary and Conclusion

Haploid vegetative cells of Saccharomyces cerevisiae treated with ultra violet and screened by 'total isolation' yielded 48 auxotrophs. This number was sufficient to provide 'markers' for further genetic work. The types and yields of these auxotrophs are described and a comparison made with those isolated by a similar technique by other workers in Aspergillus nidulans, Ophiostoma multiannulatum, Neurospora crassa and Penicillium notatum-chrysogenum. This comparison has revealed similarities and differences between the types and yields of auxotrophs recovered in Saccharomyces and those recovered in the other four species. In particular:

a. the variety in the types of amino-acid-requiring

auxotrophs recovered in Saccharomyces is greater than that recovered in Aspergillus and Ophiostoma but less than that recovered in Neurospora and Penicillium.

b. no vitamin-requiring auxotrophs have been recovered in Saccharomyces in spite of the adoption in some experiments of a modified technique; a high proportion of such auxotrophs are common to all four other species.

c. the high proportions of arginine-, lysine-, and adenine-requiring auxotrophs are common in all five species.

d. the high proportion of auxotrophs responding to methionine recovered in Saccharomyces is common in both Neurospora and Penicillium but not in Aspergillus and Ophiostoma where a correspondingly high proportion of parathiotrophic auxotrophs has been recovered. In Penicillium, however, the published accounts to not permit to decide whether this high proportion denotes a requirement for methionine or for simpler substances such as thiosulphate.

e. the high proportion of auxotrophs in Saccharomyces responding to tryptophane but not to other intermediates in tryptophane or nicotinic acid synthesis is not paralleled by any of the other four species.

f. only Penicillium is similar to yeast in the high proportion of auxotrophs requiring histidine.

The types of auxotrophs isolated from Saccharomyces but not from some or all of the other four species have been

listed; these include those requiring tyrosine, histidine, tryptophane only, serine/glycine and arginine/ornithine (not citrulline).

A number of points are of interest: these include the occurrence of a tyrosine-requiring auxotroph which does not respond to the known precursors of tyrosine and which up to the present has not been isolated in any one of the above mentioned other species; the competitive inhibition by lysine of exogenous arginine supplied to arginine-requiring auxotrophs; and the competitive inhibition by guanine of exogenous adenine supplied to adenine-requiring auxotrophs.

PART III

The Determination of the Differences between the Survival Rates of a Monoauxotrophic and a Diauxotrophic Strain of Saccharomyces cerevisiae under Starvation Conditions

Introduction

Fries (1948a, 1948b) and Macdonald and Pontecorvo (1950, 1953), working with Ophiostoma multiannulatum and Aspergillus nidulans respectively, found that conidia with two additional nutritional requirements (diauxotrophic), arising as a consequence of induced mutation among irradiated conidia with only one requirement (monoauxotrophic), survived longer under starvation conditions than the parent monoauxotrophic conidia. By appropriate periods of starvation in minimal medium the latter workers obtained yields of up to 69% diauxotrophs among colonies arising from irradiated monoauxotrophic conidia.

It was hoped that this technique could be applied to Saccharomyces cerevisiae if monoauxotrophic cells were found to die off quicker than diauxotrophic cells under starvation conditions (S. cerevisiae has no conidial stage). To determine this, 'reconstruction' experiments, similar to those of Aspergillus and Ophiostoma, were carried out. Cells of both a monoauxotrophic strain, and of a diauxotrophic strain derived from it, were subjected to starvation over different periods to determine whether there was a differential survival

with the same trend as in the other two species.

In A. nidulans the colour of the conidia of the mono-auxotrophic strain differed from that of the diauxotrophic strain; the numbers of cells of each strain surviving starvation could, therefore, be scored visually. No coloured cells of S. cerevisiae were available to serve the same purpose. To avoid the laborious process of scoring surviving colonies by testing their growth requirements, a modified technique was evolved; this is described below.

Following the choice of auxotrophic strains, three experiments were undertaken: the first to determine the survival rate of the monoauxotrophic strain; the second and third, utilizing the knowledge gained from the first, to determine the period of starvation at which the greatest difference existed between the survival percentages of the monoauxotrophic and diauxotrophic strain.

Choice and origin of strains:

A personal communication from Professor B. Ephrussi indicated that the 'wild type' strain B15 required three vitamins for normal growth. It was reasoned that if one of these requirements was absolute, a 'ready made' monoauxo-troph would be available. To determine this, washed cells of a single spore culture of this strain, harvested after three days incubation on complete medium, were plated on a series of modified minimal agar media. Each medium was

similar to that usually used except that it lacked either one, two or all three of the following normal constituents of minimal medium: D-biotin, i-inositol, calcium D-pantothenate. Approximately 1 x 10^2 cells were spread on each of four dishes of each medium. The dishes were incubated for four days after which time any visible colonies were counted.

Table 17 (page 123) gives the results of the tests. It shows that, in spite of each of the factors being necessary for normal growth, a small amount of growth will occur without their presence. Strain B15 could not, therefore, be used as either the monoauxotrophic or diauxotrophic strain in the 'reconstruction' experiments.

It had been noted previously that, in order to survive, strain B7/20, requiring arginine or ornithine for growth, needed subculturing more often than any other strain routinely subcultured in the laboratory. For this reason it was used as the monoauxotrophic strain in the 'reconstruction' experiments. The diauxotrophic strain, C12/29, arose as an induced mutant among ultra violet irradiated cells of B7/20; it required adenine in addition to arginine or ornithine.

'Reconstruction' Experiments

Methods

Known numbers of washed auxotrophic cells, harvested

TABLE 17

The number and comparative size of colonies arising after incubation of strain B15 on minimal agar media from which were omitted one or more of the usual vitamin supplements.

Series	Growth factors omitted from minimal medium[x]	No. of colonies after 4 days incubation (per plate)	(total)	Approximate size of colonies relative to those of series 1
1	none	82 76 89 86	333	normal
2	D-biotin i-inositol Ca. D-pantothenate	not counted		a few pin-point colonies visible to the naked eye
3	Ca. D-pantothenate	90 70 77 85	322	colonies two-thirds normal size
4	i-inositol	70 92 78 83	323	colonies two-thirds normal size
5	D-biotin	not counted		pin-point colonies on the average twice the size of those on dishes of series 2

[x] the amount of each of the growth factors per plate when added was as follows:

D-biotin, 0.05 ml. of a solution of 25 gamma per 10 ml. distilled water; i-inositol, 0.05 ml. of a solution of 50 mgms. per 10 ml. distilled water; calcium D-pantothenate, 0.05 ml. of a solution of 10 mgms. per 10 ml. of distilled water.

from three-day old cultures on complete agar medium, were spread on the surface of 15 ml. amounts of minimal agar medium in petri dishes; this medium lacked arginine, ornithine and adenine, the growth factors required by the auxotrophs. Four ml. of molten minimal agar medium, cooled to 40°C., were poured over the spread cells to prevent their death by desiccation and to ensure that any prototrophic colonies arising by back mutation would not be spread over the plate when the enrichment was added.

Enrichment in the form of eight discs of modified complete agar medium[x], $\frac{1}{8}$ inch in diameter and $\frac{1}{4}$ inch thick were placed at equidistant points on the surface of the minimal agar medium. These enrichment discs, used instead of a layer of complete agar medium, prevented cracking of the agar when colonies arose after enrichment. A top layer of complete agar medium would, it was thought, encourage rather than prevent such cracking. Further, the use of discs made it easier to isolate samples of the colonies which arose after enrichment.

Colony counts were made five to seven days after enrichment depending on the period of starvation. Survival rates

[x] stock complete medium modified by the addition of each of the following per 100 ml. : 2 ml. L-arginine monohydrochloride (equivalent to 24 mg. per ml.), 0.1 gm. of yeastrel, 4 ml. of casein hydrolysate (equivalent to 125 mgms. of casein per ml.), and 2 ml. of hydrolysed yeast nucleic acid (equivalent to 100 mgms. of yeast nucleic acid per ml.).

were calculated from the differences between the numbers of colonies appearing after enrichment and the number of cells plated. The numbers of viable cells plated were known from the numbers of colonies appearing on the immediately enriched series of dishes.

In Experiments 2 and 3, monoauxotrophic and diauxotrophic cells were plated on different dishes and counts of colonies made directly. This avoided testing the growth requirements of each colony arising after enrichment; such testing would have to be done if only mixed suspensions were plated. It was not known, however, whether this procedure would give the same results as those obtained from plating mixed suspensions. It was possible that one strain influenced the dying off of the other when both were plated together. To find whether this was so, additional series of dishes were plated with mixed suspensions and starved for periods similar to those plated with single strains. The numbers of colonies which appeared on the mixed suspension dishes were then compared with those on the single strain dishes starved for a similar period. If one strain influenced the survival rate of the other when both were plated together, either more or fewer colonies would have resulted on the mixed suspension dishes than expected by calculation from the results of single strain dishes.

A brief summary of the three experiments is as follows:

Experiment	Suspensions(s) of cells starved	Periods of starvation (days)	Number of dishes (per suspension per period)
1	Monoauxotrophic	3, 6 and 7	4
2	Monoauxotrophic, Diauxotrophic, and Mixed	3 and 6	5
3	Monoauxotrophic, Diauxotrophic, and Mixed	1, 2 and 3	5

Experimental Results

The results are shown in Tables 18 - 21 (pages 127, 128, 129 and 131) and Figures 1 - 3 (pages 132 and 133). They may be summarised as follows:

1. The majority of monoauxotrophic cells died off within the first three days of starvation in minimal medium. (Survivals of 4.8% in the 1st, 6.1% in the 2nd, and 25% in the 3rd experiment.) The factors accounting for the differences in the results of the three experiments remain unknown.

2. The diauxotrophic cells died off at a slower rate than the monoauxotrophic ones. (Survivals after three days starvation of 25% versus 6% in the 2nd, and 53% versus 16% in the 3rd experiment.) The results are therefore in full agreement with those of Fries, and Macdonald and Pontecorvo.

TABLE 18

The percentage survival of monoauxotrophic cells of strain B7/20 (arginine requiring) after periods in minimal medium of 3, 6 and 7 days.

Period of starvation* (days)	No. of cells plated (haemocytometer count)	No. of colonies counted 5 days after enrichment (per plate)	(total)	% survival of cells**
0 (control)	1.7×10^2	130 134 139 179	582	100
3	1.7×10^2	3 8 8 7	28	4.8
6	1.7×10^2	0 1 0 0	1	0.17
7	1.7×10^2	0 0 5 0	5	0.86
7	5.4×10^2	1 1 0 2	4	0.34

* period of starvation means the length of time elapsing before enrichment was added.

** based on 100% survival of cells after immediate enrichment.

TABLE 19

The percentage survival of monoauxotrophic cells of strain B7/20 (arginine requiring: of strain C12/29 (arginine and adenine requiring: ad arg) after 3 and 6 days in minimal me

Strain or mixture	Days of starvation	Colonies expected with no starvation (based on control viable cell counts) (no. per plate)	Colonies counted after enrichment (no. per plate)	(mean)
arg	0 (control)	-	214 228 197 c 232	218
arg ad	0 (control)	-	152 150 134 141 149	145
arg - arg ad	0	-	159 144 168 151 158	156
arg	3	4,360	242 270 185 363 c	265
arg ad	3	2,900	785 664 510 c 853	582
arg - arg ad	3	3,630	472 500 473 307 585	468
arg	6	65,380	736 912 740 615 755	752
arg ad	6	48,450	1,303 1,184 1,178 938 1,176	1156
arg - arg ad	6	46,490	715 1,079 677 788 1,423	936

* Calculated from the number of colonies obtained from each of the simultaneously enriche dishes.

'c' denotes that the plate became contaminated.

TABLE 20

The percentage survival of monoauxotrophic cells of strain B7/20 (arginine requiring: arg) and diauxotrophic cells of strain C12/29 (arginine and adenine requiring: arg ad) after 1, 2 and 3 days in minimal medium.

Strain or mixture.	Days of starvation	Colonies expected with no starvation (based on control viable cell counts) (no. per plate)	Colonies counted a after enrichment (no. per plate)	(mean)	% survival of cells	Number of colonies expected on mixed suspension (arg - arg ad) plates[x] (per plate)
arg	0 (control)	-	139 114 141 139 110	129	100	-
arg ad	0 (control)	-	131 141 143 155 162	146	100	-
arg - arg ad	0	-	121 124 155 136 131	133	100	140
arg	1	257	176 153 183 155 192	172	66.8	-
arg ad	1	293	265 298 289 272 267	278	94.8	-
arg - arg ad	1	275	176 183 214 187 170	186	67.6	217

(continued overleaf)

TABLE 20 (continued)

Strain or Mixture	Days of starvation	Colonies expected with no starvation (based on control viable cell counts) (no. per plate)	Colonies counted after enrichment (no. per plate)	(mean)	% survival of cells	Number of colonies expected on mixed suspension (arg - arg ad) plates* (per plate)
arg	2	257	86 91 75 84 73	82	31.8	-
arg ad	2	293	249 186 213 220 200	214	72.8	-
arg - arg ad	2	275	144 126 143 132 c	136	49.6	144
arg	3	517	68 87 93 93 73	81	15.6	-
arg ad	3	587	310 286 313 359 279	291	52.7	-
arg - arg ad	3	522	225 163 172 183 c	186	33.7	170

* calculated from the number of colonies obtained from each of the simultaneously enriched single strain series of plates.

'c' denotes plate became contaminated.

TABLE 21

A comparison of the survival percentages of monoauxotrophic and diau
after various periods in minimal medium.

Strain*	Period of starvation (days)	% survival	% survival arg ad / % survival arg	The nc observ that e suspen (obs.)	(
Experiment 2:					
arg.	0	100	1.0	156	
arg. ad.		100			
arg.	3	6.1	4.1	468	4
arg. ad.		25.2			
arg.	6	1.5	1.5	936	7
arg. ad.		2.3			
Experiment 3:					
arg.	0	100	1.0	133	1
arg. ad.		100			
arg.	1	66.8	1.4	186	2
arg. ad.		94.8			
arg.	2	31.8	2.3	136	1
arg. ad.		72.8			
arg.	3	15.6	3.4	186	1
arg. ad.		52.7			

* arg. denotes monoauxotrophic strain B7/20 requiring arginine; arg. a
trophic strain C12/29 requiring arginine and adenine.

** dishes plated with both the monoauxotrophic and diauxotrophic strains
colonies expected on these dishes is calculated from the results of singl
starved for a similar period.

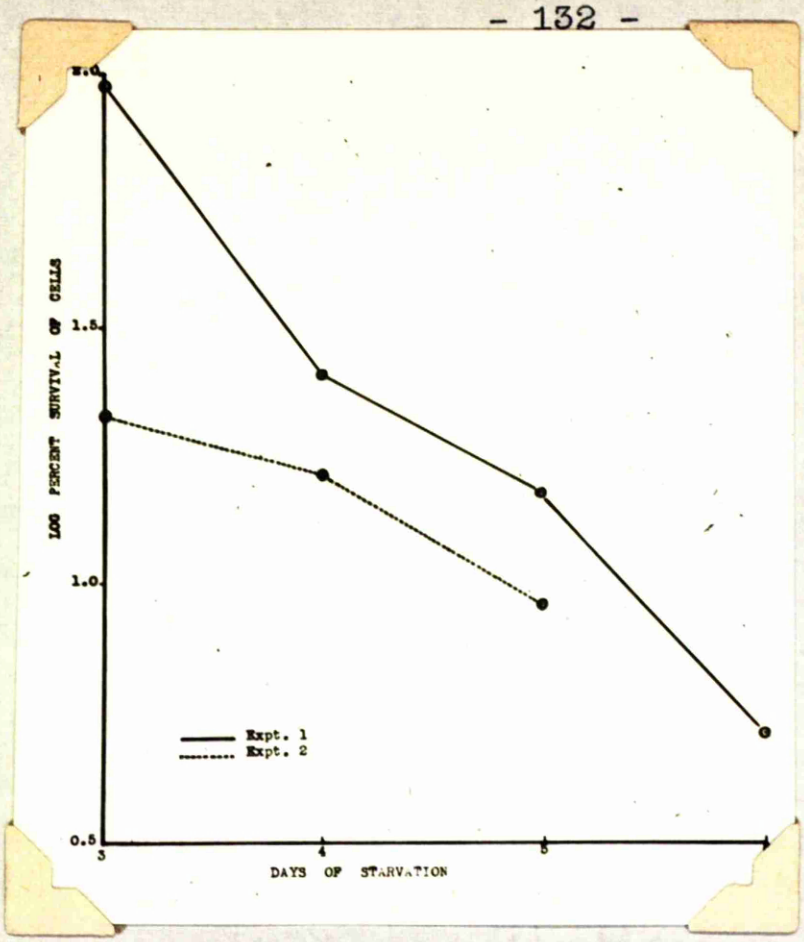


Figure 1. Rate of dying off in minimal medium of monoauxotrophic cells of strain B7/20 which require arginine for growth.

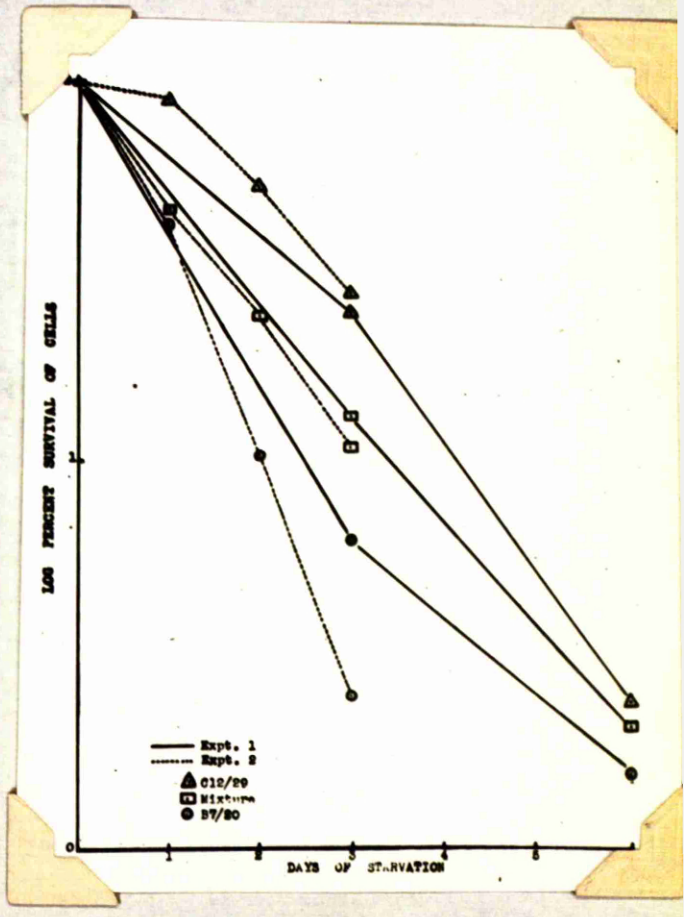


Figure 2. The rates of dying off in minimal medium of the monoauxotrophic strain B7/20 (arginine requiring) and those of the diauxotrophic strain C12/29 (arginine and adenine requiring). For comparison the rate of dying off of a mixture of the two strains is also shown.

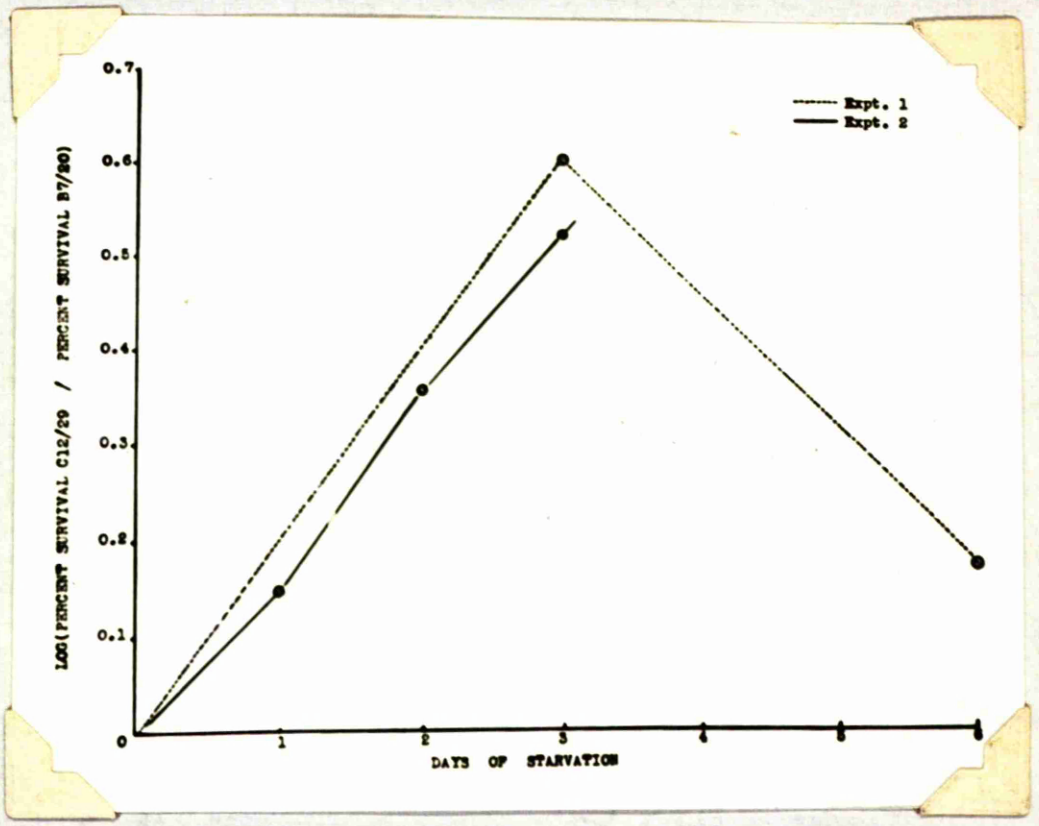


Figure 3. The relative rates of dying off in minimal medium of cells of the monoauxotrophic strain B7/20 (requiring arginine) and those of the diauxotrophic strain C12/29 (requiring arginine and adenine).

3. Thus, four times as many diauxotrophic as monoauxotrophic cells survived three days in minimal medium in experiment 2, and three and one-half times as many in experiment 3. Neither after one, two nor six days starvation (times 1.4, 2.3, and 1.5 respectively) did the differential survival rate exceed that manifest at three days (Table 21).

4. When both types of cells were plated together as a mixed suspension, up to at least three days starvation each type of cell died off at a similar rate as when plated on its own. It was therefore to be expected that in the experiments to follow (Part IIIA) the diauxotrophic cells, arising as a consequence of induced mutation among monoauxotrophic cells, also would die off at a slower rate under starvation than the monoauxotrophic cells; this in spite of both types being necessarily plated together.

Discussion

The knowledge that a differential survival rate did exist between the two strains and that it was highest sometime between three and six days starvation was considered sufficient for the purpose of undertaking the next series of investigations in which the cells would be irradiated before starvation (Part IIIA). A further experiment to determine whether or not a higher differential survival rate existed

sometime between three and six days starvation would, it was thought, contribute very little additional information; this in view of the delay in the germination of ultra violet irradiated cells. If this delay is caused by factor(s) independent of the starvation factor(s), then, for the same differential survival rate a longer period of starvation would be necessary for irradiated than for non-irradiated cells.

PART IIIA

The Starvation Rates in Minimal Medium of Ultra Violet induced Diauxotrophic Cells and their parent Monoauxotrophic Cells.

Introduction

Two experiments were undertaken to determine whether or not diauxotrophic cells, arising by mutation from ultra violet irradiated monoauxotrophic cells, behave in the same manner as those used in the 'reconstruction' experiments (Part III), i.e. die off more slowly than their parent monoauxotrophic cells under starvation conditions. This would be shown by a progressive rise in the percentage of induced diauxotrophs recovered after successive periods of starvation.

It had been noted in previous experiments (Part II) that the growth of ultra violet irradiated cells is delayed; colonies arising from such cells appear on the average 48 hours after those from non-irradiated cells. If this delay is caused by a decrease in the general metabolic rate of the cells, it is probable that their rate of dying off under starvation is also slowed down. Bearing this in mind, starvation in the following experiments was not limited to a three-day period (when the maximum differential survival rate was recorded between monoauxotrophic and diauxotrophic cells in the 'reconstruction' experiments of Part III), but was

also extended to 4, 5 and 6 days respectively.

Methods

The monoauxotrophic strain used was the same as that used in the 'reconstruction' experiments, namely, B7/20 requiring arginine or ornithine for growth. The methods of starvation and enrichment of cells were also the same.

Prior to starvation 9 ml. suspensions in saline of approximately 2 x 10^6 cells per ml. were irradiated for 3.5 minutes at 40 cms. from the ultra violet tube.

In the first experiment, the percentage of viable cells surviving irradiation was determined from pre-irradiation platings on complete agar medium and post-irradiation platings (embedded) in immediately enriched minimal medium.

It became known before the second experiment that irradiated cells embedded in immediately enriched minimal medium gave lower viable counts than cells plated on complete medium. Thus, in the second experiment, the percentage of cells surviving irradiation was determined from pre- and post-irradiation embedding in immediately enriched minimal medium. The mortality effect due to embedding was thus excluded.

At the same time, known dilutions of pre- and post-irradiation cells were embedded in complete agar medium. This involved very little extra labour and served to determine whether mortality due to embedding in minimal medium

followed by enrichment was independent of that due to irradiation. If it was not, the percentage mortality of cells due to irradiation as calculated from minimal medium platings would be different from that calculated from complete medium platings.

In both experiments cells were starved for 3, 4, 5, and 6 days. Samples of colonies arising 6 to 9 days after enrichment were transferred to complete medium and later tested for their growth requirements. The unchanged mono-auxotrophs were identified by their ability to grow on minimal medium plus L-arginine monohydrochloride (0.2 ml. of a 0.2 M. solution/20 ml. medium). The isolates which did not grow on such medium were, presumably, diauxotrophs. These were plate-tested for their additional requirement by the same technique as that used in Part II of this thesis, except that for minimal medium read here minimal medium plus arginine.

In addition, plate-tests were carried out on the following two media: (a) minimal medium plus arginine plus DL-methionine (0.1 ml. of a 0.25 M. solution), and (b) minimal medium plus arginine plus DL-tryptophane (0.2 ml. of a 0.025 M. solution). These media served to 'screen' methionine and tryptophane-requiring auxotrophs, which constituted the largest proportion of all the auxotrophs in the 'total isolation' experiments.

In the first experiment, diauxotrophs other than those requiring these two substances were identified auxanographically. In the second experiment diauxotrophs, other than those requiring methionine, were not characterized further.

Experimental Results and Observations

The results of these experiments are shown in Tables 22 - 26 (pages 140, 141, 142, 145 and 147) and Figures 4 and 5 (page 143). They may be summarised as follows:

1. The mortality of cells due to embedding in minimal medium (as compared with embedding in complete medium) is independent of their mortality due to irradiation. This was shown in the second experiment by a comparison of the results of pre- and post-irradiation platings in minimal medium (enriched) with those in complete medium. 0.7% of the cells survived irradiation as calculated from minimal medium platings and 0.8% as calculated from complete medium platings (Table 23, page 141). 76% mortality ensued owing to embedding the cells in minimal medium (Table 22, page 140).

2. Survival percentages of cells following periods in minimal medium of 4, 5, and 6 days in the first experiment (25.5, 14.8, and 4.7 respectively) closely resembled those after 3, 4, and 5 days in the second experiment (22, 16.7, and 11 respectively). Survival percentages after three days, however, differed greatly: 95 in the first and 22 in

TABLE 22

The percentage mortality of non-irradiated cells of strain B7/20 (arginine requiring) when embedded in immediately enriched minimal agar medium (Experiment 2)[*].

Series of plates and medium in which cells embedded	Haemocytometer count of no. of cells plated (per dish)	No. of colonies following 4 days incubation (per dish)	(mean)	% mortality of cells
C1-C3 Complete	39	41 50 58	50	100-(121x100/ 50 x 10) = 76
M1-M3 Enriched minimal	391	112 128 123	121	

[*] assuming 100% viability of cells when embedded in complete agar medium.

TABLE 23

The percentage survival of monoauxotrophic cells of strain B7/20 (arginine requiring) following u.v. (Experiments 1 and 2).

Series of plates and medium in which cells embedded	Colonies expected with no irradiation (per series)	Colonies counted after incubation (per plate)	Colonies counted after incubation (per series)	Percentage survival of cells following u.v.
Experiment 1:				
AI1 - AI5 Enriched minimal	1.06×10^5	12 9 13 12 17	63	0.06
Experiment 2:				
CI1 - CI6 Complete	3.0×10^4	43 42 33 50 36 34	238	0.8
MI1 - MI6 Enriched minimal	$7.3 \times 10^{4*}$	99 92 72 112 64 61	510	0.7

* calculated from pre-irradiation platings in minimal agar medium.

TABLE 24

The percentage survival of irradiated monoauxotrophic cells of strain B7/20 (arginine requiring) after periods in minimal medium of 3, 4, 5 and 6 days (Experiment 1), and 3, 4, 5, 6 and 7 days (Experiment 2).

Series of plates and period of starvation*	(days)	No. of viable irradiated cells plated (per series)	Colonies counted after enrichment (per plate	(per series)	% survival of cells
Experiment 1:					
AI1 - AI5	0 (control)	6.3×10	12 9 13 12 17	63	100
A1 - A5	3	3.2×10^2	56 62 51 88 42	299	95.0
B1 - B5	4	6.3×10^2	37 32 24 38 32	163	25.5
C1 - C5	5	6.3×10^2	24 17 11 22 20	94	14.8
D1 - D5	6	1×10^3 (4 dishes)	13 10 10 14 c	47	4.7
Experiment 2:					
MI1 - MI6	0 (control)	5.1×10^2	99 92 72 112 64 61	510	100
M3/1 - M3/6	3	1×10^3	38 28 36 38 39 40	219	22.0
M4/1 - M4/6	4	2×10^3	52 59 71 48 53 50	333	16.7
M5/1 - M5/6	5	2.1×10^3 (5 dishes)	46 39 36 68 41	230	11.0
M6/1 - M6/6	6	1.1×10^3	no colonies		0
M7/1 - M7/6	7	6.8×10^3	-	3	0.04

* period of starvation means the length of time elapsing before enrichment is added.

'c' means that plate became contaminated.

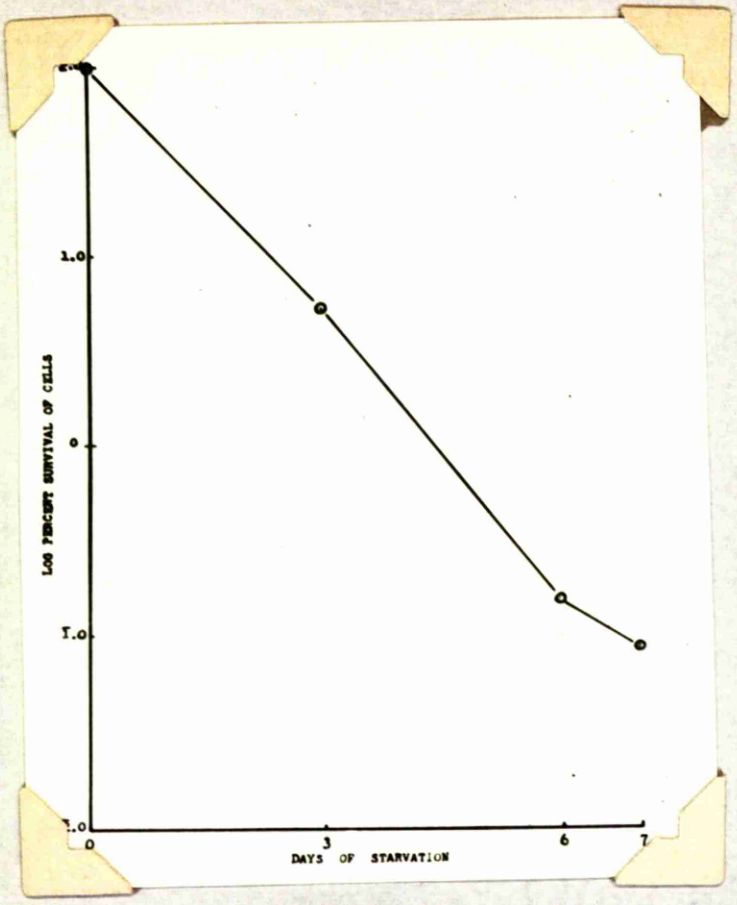
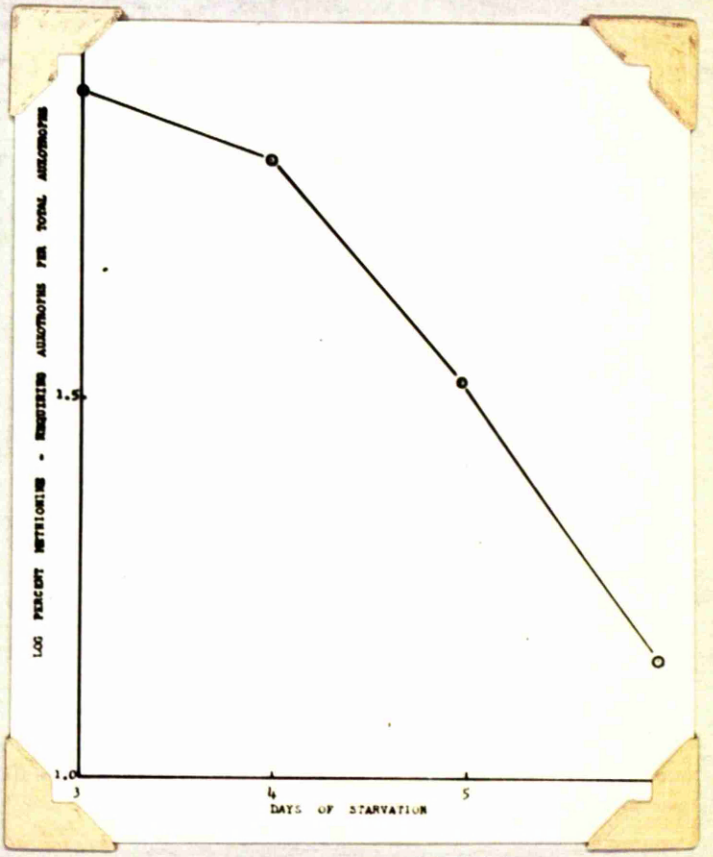


Figure 4. The rates of dying off in minimal medium of u.v. irradiated cells of the monoauxotrophic strain B7/20 (requiring arginine or ornithine for growth).

Figure 5. The decrease in the percentages of methionine-arginine requiring auxotrophs among the total diauxotrophs isolated after successive periods of starvation in minimal medium.



the second experiment (Table 24, page 142).

3. Out of a total of 2163 isolates tested, 60 proved to be diauxotrophs. The number of isolates tested per starvation period are given in Table 25 (page 145). Many of these isolates were slow growing strains (presumably selected by this technique); they were screened from true diauxotrophs by a further plate-test.

4. The first experiment gave a totally unexpected result, namely, the isolation of 9.5% diauxotrophs from control (immediately enriched) platings, a drop to 1.3% from plates starved for 3 days, and a progressive rise to 3.8%, 7.5% and 6.4% after 4, 5, and 6 days starvation respectively.

These results were not confirmed by those of the second experiment. Here the percentages of diauxotrophs recovered after different starvation periods did not differ significantly from one another or from the control platings. They were within approximately the same range (1.34 - 3.81%) as those obtained by the 'total isolation' technique (Part II of this thesis).

5. In the first experiment 5 out of the six diauxotrophs isolated from the control (immediately enriched) plates required DL-methionine. Further, 2 out of 3 of those isolated after three days starvation, and 2 out of 6 after four days starvation required this growth factor (Table 26,

TABLE 25

The percentages of diauxotrophs isolated from ultra violet irradiated cells after starvation periods of 0, 3, 4, 5 and 6 days (Experiment 1), and 0, 3, 4, 5, 6 and 7 days (Experiment 2

Series of plates and period of starvation		Colonies isolated and plate-tested	Diauxotrophs isolated after retest	Percentage of diauxotrophs
	(days)	(no.)	(no.)	
Experiment 1:				
AI1 - AI5	0	63	6	9.5
A1 - A5	3	239	3	1.3
B1 - B5	4	159	6	3.8
C1 - C5	5	94	7	7.5
D1 - D5	6	47	3	6.4
Experiment 2:				
CI1 - CI6[x]	0	137	3	2.2
CI7 - CI12[xx]	0	210	8	3.8
MI1 - MI6	0	444	6	1.4
M3/1 - M3/6	3	218	5	2.3
M4/1 - M4/6	4	333	7	2.1
M5/1 - M5/6	5	216	6	2.8
M6/1 - M6/6	6	0	0	0
M7/1 - M7/6	7	3	0	0
	Totals:	2163	60	

[x] cells plated on the surface of complete agar medium.

[xx] cells embedded in complete agar medium.

page 147). Presumably, therefore, a mutation affecting the ability to synthesize DL-methionine had occurred within the clone of monoauxotrophic cells prior to their irradiation.

This hypothesis could have been tested by isolating and testing colonies from pre-irradiation control platings. In view of the low spontaneous gene mutation rate of most organisms, however, colonies from such plates were not tested for diauxotrophy, and by the time the above result was discovered such plates had been discarded.

Should the occurrence of such a mutation be the true explanation of this result, it is surprising to note that such diauxotrophic cells died off at a faster rate than the monoauxotrophic cells from which they arose. This is shown graphically in Figure 5 (page 143).

Out of a total of 100 colonies isolated from pre-irradiation platings in the second experiment no diauxotrophs were recovered. This does not refute the mutation hypothesis of the first experiment; the mutation could have arisen on the three-day old culture of monoauxotrophic cells used only for the first experiment.

6. It is not apparent that this technique selects at all and if it does selects specific types of auxotrophs (Table 26, page 147). The spectrum of types was similar to those isolated by the 'total isolation' technique with the exception of those which when first isolated were stimulated by a

TABLE 26

Substances to which diauxotrophs gave a growth response

Series of plates and period of starvation (days)	Total number of diauxotrophs recovered (per series)	Substances to which diauxotrophs respond*, and number responding	(no.)	Designation of diauxotroph(s)
Experiment 1:				
AI1 - AI5 0	6	L-lysine monohydrochloride	1	XA1
		DL-methionine	5	XA2, 3, 6, 8, 9
A1 - A5 3	3	Adenine monohydrochloride	1	XA14
		DL-methionine	2	XA11, 13
B1 - B5 4	6	Adenine monohydrochloride	1	XB4
		Aneurin	1	XB7
		Leucine & isoleucine (N.M.)	1	XB9
		Unknown (C.H.)	1	XB5
		DL-methionine	2	XB1, 2
C1 - C5 5	7	DL-tyrosine	1	XC2
		L-lysine monohydrochloride	2	XC4, 5
		Uracil	1	XC7
		DL-methionine	1	XC8
		Adenine monohydrochloride	1	XC10
		Unknown (C.M.)	1	XC9
D1 - D5 6	3	Uracil	1	XD3
		Leucine & isoleucine (N.M.)	2	XD4, XD6
Experiment 2**:				
CI1 - CI6 0	3	Casein hydrolysate	2	XE1, 3
		Yeast nucleic acid hyd.	1	XE2
CI7 - CI12 0	8	DL-methionine	2	XF1, 5
		Casein hydrolysate	3	XF2, 6, 8
		Yeast nucleic acid hyd.	1	XF3
		Complete medium	1	XF7
		Mixture of vitamins (stim.)	1	XF4
MI1 - MI6 0	6	DL-methionine	2	XG2, 5
		Casein hydrolysate	2	XG3, 4
		Yeast nucleic acid hyd.	1	XG1
		Complete medium	1	XG6
M3/1 - M3/6 3	5	Casein hydrolysate	3	XH1, 4, 5
		Yeast nucleic acid hyd.	1	XH2
		Mixture of vitamins (stim.)	1	XH3
M4/1 - M4/6 4	7	DL-methionine	1	XI3
		Casein hydrolysate	2	XI1, 2
		Yeast nucleic acid hyd.	2	XI5, 6
		Complete medium	1	XI4
		Mixture of vitamins (stim.)	1	XI7
M5/1 - M5/6 5	6	DL-methionine	1	XJ3
		Casein hydrolysate	3	XJ1, 2, 6
		Complete medium	2	XJ4, 5

* in addition to arginine/ornithine

** diauxotrophs, other than those requiring DL-methionine, were not fully characterised.

(N.M.): natural mixture. (C.H.): responds to casein hydrolysate. (C.M.): responds only to complete medium. (stim.): slow grower stimulated to normal growth by a mixture of vitamins.

vitamin mixture. One such auxotroph was isolated in the first experiment and three in the second. When further tested for their specific requirements, three showed growth over each individual test plate and the other responded to aneurin. Presumably the three had adapted between tests to grow without the vitamin initially required. Such adaptation may account for the fact that no such auxotrophs were isolated by the 'total isolation' technique in which germination takes place on complete medium on which they could adapt before they were tested.

The tyrosine requiring auxotroph was similar to the one isolated by the 'total isolation' technique (Part II) in that it did not respondto any known precursor of tyrosine.

Discussion

No increase in the efficiency of isolating auxotrophs was shown even in the first of these experiments. The labour involved in screening the slow growing strains from the true diauxotrophs greatly decreases the efficiency.

Clearly, the differences in survival rates shown in the 'reconstruction' experiments of Part III between non-irradiated monoauxotrophic and diauxotrophic cells were not manifest to the same extent in these experiments. The differences could have been masked by the enormous scatter in the germination times of the cells after irradiation (Devi, Pontecorvo, and Higginbottom, 1951).

The results of the present work do not exclude the possibility that in further experiments using different monoauxotrophic strains, increased efficiency in the isolation of auxotrophs by this technique may be realised.

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