THE GENETIC CONTROL OF CARBOHYDRATE UTILISATION BY THE MOULD *ASPERGILLUS NIDULANS*

A THESIS

submitted for the degree of

Doctor of Philosophy,

University of Glasgow

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SEPTEMBER 1961

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ACKNOWLEDGEMENTS

I wish to thank Prof. G. PONTECORVO F.R.S. for his criticism and encouragement and for the hospitality extended to me by members of the Department of Genetics.

The work presented in this thesis was done with the support of the Nuffield Foundation and the Agricultural Research Council.

CONTENTS

General Introduction.

In 1895 Buchner discovered enzymes and five years later, when the work of Mendel became generally known, the gene concept was established. The possibility that the action of genes may be mediated by enzymes was quickly recognised and discussed more-or-less speculatively by a number of authors in the period 1900-1920 (Wheldale, 1909; Goldschmidt, 1916; Troland, 1917; Wright,1917) while the importance of combining biochemical and genetical techniques in the investigation of gene action was later emphasised particularly by Haldane (1937; 1942).

The first demonstration of the genetic control of a metabolic process came from the example of alcaptonuria in Man described by Garrod (1923). The abnormality is inherited as a Mendelian recessive and is detected by blackening of the urine on exposure to air due to the accumulation of homogentisic acid. It was found that the serum of normal individuals contains an enzyme degrading the acid whereas the enzyme could not be detected in the serum of alcaptonurics, Garrod concluded that the activity of the enzyme is genetically controlled but this concept was not widely appreciated until revised by Beadle (1945).

Considerable interest developed in physiological genetics in the 1930's, particularly in pigment formation in plants (Lawrence and Price, 1940), the guinea pig (Wright, 1941) and Drosophila (Euphrussi, 1942). The aim of these investigations was to discover the chemical basis of genetically determined differences, but on the whole progress was slow due to the unsuitability of the organisms

and difficulties in the isolation of intermediates in pigment synthesis. The success of the new approach to physiological genetics begun by Beadle and Tatum (1941) with the isolation of nutritionally deficient mutants of the mould Neurospora can be attributed to re-evaluation of the problem in switching the line of enquiry to investigation of the possible genetic control of known, or at least part known, metabolic processes in an organism suitable for both biochemical and genetical investigation.

The main findings of the early Neurospora work were that the majority of the induced nutritional mutants require single nutrients for growth, the requirements are determined by single genes and different stages in a series of synthetic reactions are controlled by different genes (Horowitz, 1950). These results, together with a reappraisal of the earlier work, were summarised by Beadle to support the general hypothesis that the ability to bring about a specific metabolic reaction is controlled by a specific gene (Beadle, 1945). Two important concepts were advanced in the hypothesis, one was that genes determine the structure of proteins thereby controlling the activity of enzymes, the second that genes have single primary functions and that the structure of one enzyme is determined by one gene.

The validity of the results derived from the use of induced nutritional mutants was criticised because the mutants tested may represent a class of repairable mutants specially selected by the isolation procedure employed (Delbruck, 1946). However, analysis of temperature sensitive mutants of Neurospora

and Escherichia shows that selection does not occur (Horowitz and Leupold, 1951) and it can be argued from the data for these mutants that at least 70% of the genes tested have single functions (Horowitz, 1950). Mutants with multiple requirements presented an apparent contradiction of single gene function but this was resolved by the finding that in many cases they fail to synthesise a common precursor (Davis, 1955; Horowitz and Fling, 1956) and it was generally accepted by about 1950 that the best working hypothesis available was that of primary gene function (Beadle, 1950; Horowitz, 1950; Lederberg, 1950B), although reservations were expressed on the problem of identifying the function (Emerson, 1950; Lederberg, 1950b). Basically two questions underlie subsequent studies in gene action; first is the hypothesis of 'one gene - one protein' a correct description of the formal relationship of genes and proteins, second what ~~were~~ are the mechanisms of gene action?

Studies of the genetic control of enzyme activity are mainly based on the investigation of induced nutritional mutants of micro-organisms and the number of reports in which mutants are shown to differ from the wild type in the activity of one enzyme has steadily increased since the late 1940's to totals of eleven in 1956 (Horowitz, 1956) and over fifty in a recent list (Fincham, 1959a), and the analysis of some gene - enzyme systems has been brought to a high degree of refinement, particularly in Neurospora (Pateman and Fincham, 1958; Yanofsky, 1960; Catcheside, 1960a) and Escherichia (Monod, 1956; Pardee et al., 1959;

Yanofsky and Crawford, 1959; Garen, 1960). Two situations are found, the most common is that all mutations affecting a particular enzyme occur at the same locus, the second that the enzyme is affected by mutations at more than one locus (Lederberg, 1956).

The first situation is that postulated by Beadle (1945; 1950) in which the activity of an enzyme is controlled by one gene. Mutation of the gene usually reduces or abolishes the enzymes activity (Horowitz, 1956; Fincham, 1959a) which is regained by prototrophic revertants. In some systems reversion occurs only by back-mutation (Giles et al., 1957; Pateman, 1957; Yanofsky and Crawford, 1959) but in others results from either back-mutation or mutation of suppressor genes. The function of non-specific suppressors is generally interpreted in terms of alternate metabolic pathways or release from inhibition (Strauss, 1955; Suskind and Kurek, 1959) but no mechanism has been proposed for the action of allele specific suppressors (Yanofsky, 1956). Mutants that form enzymes with altered kinetic properties (Fincham, 1957; Horowitz and Fling, 1953; Maas and Davis, 1952) or synthesise proteins closely related immunologically to the normal enzyme but without enzymic activity (Suskind, 1957; Yanofsky and Stadler, 1958) may be interpreted as producing altered proteins (Horowitz, 1956).

The first demonstration of the genetic control of the structure of a protein came from the study of sickle-cell anaemia in Man. The trait is characterised by the presence of two types

of haemoglobin molecule which can be separated electrophoretically (Pauling et al., 1949) and are determined by difference at a single locus, heterozygotes form both normal and sickle haemoglobins whereas the homozygotes form only normal or sickle haemoglobins (Neel, 1949). Similar differences have been detected in a number of mammalian proteins (Aschaffenburg and Drewery, 1955; Ashton and McDougall, 1958) and where investigated are found to be determined by single gene differences (Smithies and Hickman, 1958; Hunt and Ingram, 1959; Cohen, 1960).

The formation of a number of inducible enzymes, notably tyrosinase in Glomerella (Markert, 1950), beta-galactosidase in Escherichia (Lederberg, 1950b) or Neurospora (Landman and Bonner, 1952) is controlled by more than one gene. These exceptional cases have provided the basis of criticisms of the one gene - one enzyme hypothesis for oversimplification (Lederberg, 1956), and have led to the discovery of a second way in which genes may act in enzyme forming systems. If a number of genes control the formation of an enzyme and yet only one determines its structure the remaining genes must act at stages in enzyme formation not concerned with specification of structure, and their function in controlling rates of enzyme formation is suggested by the findings that induction involves a great increase in the rate of synthesis of the induced enzyme and that single gene mutation can lead to the constitutive formation of an enzyme not detectably different in structure or specificity from the induced enzyme (Hogness, 1959). In the cases of

beta-galactosidase (Jacob and Monod, 1959; Pardee et al., 1959), tryptophane synthetase (Cohen and Jacob, 1959) and ornithine transcarbamylase in Escherichia (Gorini, 1960) and tyrosinase in Neurospora (Horowitz et al., 1960) the role of genes in determining either the structure of the enzyme or its rate of formation may be separated and genes have been classified as either structural or regulatory (Jacob and Monod, 1959; 1961). It is convenient to regard these two aspects of gene action separately.

Analysis of the beta-galactosidase system in Escherichia also led to the discovery of permeases as stereo-specific catalytic sites in cell membranes effecting the uptake of metabolites (Cohen and Monod, 1957). Some mutants which fail to ferment lactose are found to lack the permease function and fail to accumulate the sugar though forming the enzyme (Monod, 1956; Rickenburg et al., 1956). However as permeases are probably proteins (Cohen and Monod, 1957) control of their activity comes within the scope of the general problem of the genetic control of protein structure.

In the chemical analysis of protein structure the techniques first worked out and applied to insulins by Sanger have been extended to a variety of other proteins, both enzymic and non-enzymic (Anfinsøn, 1959). The fundamental finding is that each protein has an unique sequence of amino-acid residues in the one or more polypeptides which make up the molecule and it is generally agreed that the superstructure of protein molecules, the helical coiling of the primary chains (Pauling, Gorey and Branson, 1951) their folding and aggregation are dependant on

the amino-acid sequence of the polypeptides (Crick, 1958; Kendrew, 1959).

The development of the 'finger printing' technique for the analysis of amino-acid sequences has resulted in identification of the precise alteration in the structure of a protein occurring as the result of mutation. It was found that in sickle cell anaemia the normal and sickle cell haemoglobins differ in only one residue in one of the pairs of polypeptides of which the molecules are composed (Ingram, 1957). Analysis of a number of other haemoglobin variants shows that each differs from the normal in substitution of single residues (Hunt and Ingram, 1959) while two haemoglobins which have different substitutions at the same place in the polypeptide are determined by allelic genes (Hunt and Ingram, 1958). In the two cases in which it has been possible to examine the genetic control of protein molecules composed of dissimilar polypeptides it is found that the structure of each polypeptide is determined by different genes. The four polypeptides which, pair at a time, comprise various haemoglobins are determined by four non-allelic genes only two of which are linked (Ingram, 1961), and the two components of Escherichia tryptophane synthetase are determined by a pair of closely linked genes (Yanofsky and Crawford, 1959). It seems possible that isozymes, different protein molecules with the same enzymatic properties (Markert and Moller, 1959) may result from aggregation of different polypeptides in an analogous way to the haemoglobins (Itano et al., 1959; Ingram, 1961).

The results of the investigations of the genetic control of enzyme activities and of protein structure show that the general concepts advanced by Beadle (1945) for the genetic specification of protein structure are substantially correct and that the primary action of many genes is to determine the order in which amino-acids are arranged in polypeptides.

A model of gene structure developed in the past ten years (Pontecorvo, 1959) provides the central feature of theories of gene action and molecular mechanisms by which genes determine the structure of proteins.

The model stems from the ideas of Muller (Raffel and Muller, 1940; Muller, 1947) and Goldschmidt (1951), and experiments of Lewis (1950; 1951) in which a series of anomalous position effects were described. The gene is visualised as a segment of chromosome determining a specific function within which there are many sites at which mutation can occur and impair this function, and within which recombination can occur between mutants at different sites to yield an undamaged gene (Pontecorvo, 1952a).

The increase in the resolving power of genetic analysis (Pontecorvo, 1952b) resulting from the use of micro-organisms in genetics provides much evidence to support the model (Pontecorvo, 1959; Demerec and Hartman, 1959). The numbers of functionally allelic mutants separable by recombination are generally large (Pritchard, 1955; Pontecorvo and Roper, 1956; Benzer, 1957) and recombination between allelic mutants is sufficiently widespread

to be the rule rather than the exception (Pontecorvo, 1954;1956). The reciprocal product of intra-genic recombination, the double mutant in coupling, has been recovered in Aspergillus as the result of mitotic crossing over (Roper and Pritchard, 1955).

An unexpected finding was that the mutant sites are linearly arranged in the gene (Pritchard, 1955; 1960a). Linearity has since been found in almost all cases in which it has been sought (Pontecorvo, 1959) and is maintained at levels of analysis that are probably close to absolute resolution (Benzer, 1957; Pritchard, 1960b).

The role of ribonucleic acid (R.N.A) as an essential component in protein synthesis is well established (Brachet, 1955; Chantrenne, 1958), and the identification of deoxy-ribonucleic acid (D.N.A) as the genetic material of many micro-organisms (Hotchkiss, 1955; Levinthal, 1959a) and discovery of its molecular structure (Watson and Crick, 1953) provide the basis for a number of similar theories of gene action of which Crick's (1958) is the most comprehensive. The theory draws on the established concept of the role of templates in the synthesis of polymeric molecules (Dounce, 1952), the findings that proteins and nucleic acids are essentially linear polymers and that genes have linear structure. It is visualised that genetic information is contained in the base sequence of the paired complementary D.N.A. chains within the nucleus. Instructions for protein synthesis are then transferred to R.N.A. templates within cytoplasmic ribosomes, perhaps by a

special form of R.N.A. (Brenner et al., 1961), and specify the sequence in which activated amino-acid residues are assembled on the template prior to polymerisation (Hoagland et al., 1959).

The theory predicts co-linearity between mutant sites in a gene and the points of amino-acid substitution in the corresponding polypeptides, and the development of suitable experimental systems in micro-organisms in which combined intra-genic mapping and amino-acid sequences can be done is being actively pursued in a number of laboratories (Garen, 1960; Brenner and Barnett, 1959; Yanofsky, 1960).

Two general mechanisms by which genes determine the rates of synthesis of proteins have been suggested. In one, based mainly on consideration of the effects of mutation on human haemoglobin, it is postulated that the rate of synthesis is a function of protein structure (Brenner, 1959). The hypothesis turns on the idea that the structure of a polypeptide determines the rate of dissociation of the polypeptide - template complex and thus the rate of protein synthesis (Vogel, 1957b). Individuals heterozygous for genes determining altered haemoglobins are often found to synthesise relatively small amounts of the unusual protein (Itano, 1957), and it has been suggested that the thalassaemia diseases, a group of haemoglobin deficiency diseases, may be due to alterations in the structure of the haemoglobin polypeptides not detectable electrophoretically but affecting their rates of synthesis (Ingram and Stretton, 1959).

A second and radically different hypothesis has developed from analysis of enzyme formation in bacteria, in which a special class of genes, 'regulators', are supposed to control rates of enzyme formation (Jacob and Monod, 1959). A break-through in the problem of the role of the inducer in induced enzyme formation (Pollock, 1953) resulted from the discovery of enzyme repression (Vogel, 1957a) a process antithetical to induction in which the accumulation of a metabolite specifically inhibits the formation of an enzyme controlling a stage in the synthesis of the metabolite. It is generally accepted that induction and repression are different aspects of a single control mechanism based on the specific inhibition of enzyme formation by repressor substances (Vogel, 1957a and b; Pardee, 1959; Magasanik et al., 1959), and that the inducer releases the synthesis of the induced enzyme by antagonising an endogenous repressor. Haploid constitutive mutants isolated from inducible strains (Lederberg, 1950b; Jacob and Monod, 1959) or non-repressible mutants isolated from repressible strains (Cohen and Jacob, 1959; Gorini, 1960) are recessive and they are supposed to be 'loss' mutants that either fail to synthesise a repressor substance endogenously or to bring about its formation from an exogenous metabolite. The mutants identify regulator genes which function in the synthesis of repressors, they map at different loci from genes controlling enzyme structure (Jacob and Monod, 1959; Pardee et al., 1959; Gorini, 1960).

Results indicating a repressor substance as a cytoplasmic factor in the formation of partial heterozygotes in reciprocal

crosses between constitutive and inducible strains of Escherichia have been reported (Pardee et al., 1959) while the immunity of lysogenic bacteria to bacteriophage can also be interpreted on the basis of cytoplasmic repressors (Jacob et al., 1960b). Further support for the repressor theory comes from the demonstration that the rate of synthesis of an enzyme is controlled by the intra-cellular level of a repressing metabolite (Gorini and Maas, 1957) and the balance between substrate and product (Gorini, 1960). The repressor of the beta-galactosidase system in Escherichia is not a protein (Pardee and Prestidge, 1959).

There is no generally accepted hypothesis for the mode of action of repressors. A cytoplasmic function has been discussed (Vogel, 1957b; Magasanik et al., 1959) in which repressors are supposed to affect the activity of protein synthesising templates, but this model is difficult to reconcile with recent observations of multiple effects in enzyme regulation. More than one enzyme may be formed in response to one inducer (Monod, 1956; Robichon-Schulzmajster, 1958; Kurahashi, 1957), a situation distinguishable from sequential induction (Stanier, 1950) because mutants unable to form the first enzyme of a series are able to form the remaining enzymes. Similarly in co-ordinate repression (Ames and Garry, 1959) the formation of a number of enzymes is repressed by one metabolite. A genetic function of the repressor is postulated in the operon theory (Jacob et al., 1960a; Jacob and Monod, 1961). The repressor is supposed to control the activity of a corresponding operator gene which in turn controls the co-

ordinate expression of a number of linked genes determining the structures of sequentially acting enzymes. The theory is associated with development of the concept of 'messenger' R.N.A. (Brenner et al., 1961) and implies that the repressor may be a form of messenger R.N.A.

The possible significance of enzyme regulation in the evolution of micro-organisms has been discussed by Pardee (1961). Control of enzyme formation provides a mechanism of metabolic regulation important in cellular economy and the long term adjustment of organisms to changes in their environment (Pardee, 1959). It is complementary to the concept of 'pacemakers' (Krebs, 1957) as enzymes controlling reactions that limit the overall activity of metabolic pathways. On the other hand, the short term regulation and integration of metabolism results from the operation of 'feed-back' mechanisms which automatically control enzyme function and are inherent in enzyme systems (Krebs, 1957; Pardee, 1959). Permeases may also function in regulating metabolic processes either by determining the concentration of substrates within cells or by affecting the capacity of cells to respond to changes in their environment (Novick and Weiner, 1959).

The Aims of the Investigation.

The filamentous fungus Aspergillus nidulans is an organism exceptionally favourable for genetic studies at both the inter-genic and intra-genic levels of analysis (Pontecorvo, 1959) yet it has been the subject of few biochemical investigations (Hockenhull, 1949; Shepherd, 1956), while the loci that have been subjected to fine genetic analysis so far control stages in the synthesis of the purine adenine (Pritchard, 1955; 1960a; Martin-Smith, unpublished) and do not provide a suitable system for enzymatic studies. On the other hand knowledge of carbohydrate metabolism is relatively advanced, methods for the identification of enzymes degrading carbohydrates are well established (Colowick and Kaplan, 1955) and have been applied in the investigation of bacterial mutants defective in carbohydrate utilisation (Monod, 1956; Kalckar, Kurahashi and Jordon, 1959; Gross and Englesberg, 1959). Mutants of this type are also used extensively in both yeast and bacterial genetics to provide markers (Winge, 1952; Lederberg, 1950a) but they have been sought in only a few studies of mould genetics and have not been used previously as markers.

The work presented in the thesis centres on investigation of the genetic control of carbohydrate utilisation by A.nidulans. It has two principal aims, first to evaluate the use of differences in carbohydrate utilisation as genetic markers in the mould, and second to attempt the fine genetic analysis of the most suitable of the new loci as a step in the development of an experimental system in which combined genetic analysis and enzymatic studies can be undertaken.

Part 1. The Isolation and Genetic Analysis of Sugar Mutants of Aspergillus nidulans.

Introduction.

It has been known for some time that both yeasts (Armstrong, 1905) and bacteria (Lewis, 1934) differ considerably in their ability to ferment specific carbohydrates, and this variation is employed in classifying the organisms (Henrici, 1941; Lodder and Van Rij, 1952; Wilson and Miles, 1955). The development of methods for the controlled hybridisation of yeasts (Winge and Laustsen, 1938) or bacteria (Tatum and Lederberg, 1946) have resulted in demonstration of the genetic control of carbohydrate fermentation in these organisms and subsequently fermentation characters have been widely used as genetic markers in studies with Saccharomyces (Lindegren, 1949; Winge, 1952; Hawthorne and Mortimer, 1960) and several bacteria including Escherichia coli (Lederberg, 1949; Lederberg et al., 1951; Morse, Lederberg and Lederberg, 1956a and 1956b; Lederberg, 1960), Salmonella (Zinder and Lederberg,1952) and Pneumococcus (Austrian and Colowick, 1953; Hotchkiss and Marmur, 1954). The techniques for differentiating between fermenting and non-fermenting organisms are available from the taxonomic use of the characters, but while the fermentation markers used in yeast genetics are mostly derived from naturally occurring strains, (Lindegren, 1949; Winge, 1952) most of those used in bacterial genetics have been selected as the result of spontaneous or induced mutations (Lederberg, 1949 and 1950a).

In principle two kinds of metabolic defect may result in an organism failing to ferment a particular carbohydrate, they may fail to synthesise an active enzyme concerned in the metabolism of the carbohydrate or they may not be able to accumulate the carbohydrate from the medium. Failure to form specific carbohydrases has been inferred in comparative studies with yeasts (Winge, 1952) but there is little direct evidence for the absence of specific enzymes in non-fermenting yeasts apart from studies with mutants failing to ferment galactose (Robichon-Szulmajster, 1958). A number of examples in which non-fermenting bacteria have been found to lack specific carbohydrases are well documented (Monod, 1956; Gross and Englesberg, 1959; Kurahashi, 1957) while it has also been shown that bacteria gaining the ability to ferment a carbohydrate, either as the result of transformation (Hotchkiss and Marmur, 1954; Marmur and Hotchkiss, 1955) or mutation (Englesberg, 1957; Kline and Baron, 1957), also gain the ability to form specific enzymes controlling the metabolism of the carbohydrate.

Some mutants of E.coli which fail to ferment lactose have been shown to be unable to accumulate the sugar from the medium, mutants of this type are described as cryptic mutants and are defective in an inducible permease (Cohen and Monod, 1957) for the uptake of beta-galactosides (Monod, 1956). Other mutants lacking an alpha-glucoside permease have been described (Cohen and Monod, 1957) and a constitutive permease for the uptake of galactose by E.coli has now been demonstrated (Horeker, Thomas and Monod, 1960). No evidence for carbohydrate permeases in

moulds has been reported but it has been shown that the uptake of histidine by Neurospora depends upon a specific permease (Mathieson and Catcheside, 1956).

It is generally true, excepting the yeasts, that little use is made of physiological characters in Fungal Taxonomy although attempts are being made in this direction with the lower Phycomycetes (Cantino, 1955). (An intriguing but inaccessible paper by Sakaguchi and Kodama (1934) cited by Thom and Raper (1945) apparently discusses the taxonomic use of fermentation characters in the Aspergilli). However moulds exhibit considerable physiological variation including variation in the ability to assimilate different carbohydrates (Foster, 1949; Cochrane, 1958). Most moulds assimilate a number of different carbohydrates and it may be expected that mutants lacking either specific permeases or carbohydrases may occur and could be isolated by their failure to grow when the carbohydrate is supplied as sole carbon source. It is a conspicuous omission in mould genetics that mutants of this kind have been sought in only very few instances (Emerson, 1944; Landman, 1950) and that characters based on carbohydrate assimilation are not employed as genetic markers. This omission is curious for the methodology required to isolate mutants of this type is clearly described in Beadle and Tatum's (1941) classic paper on induced nutritional deficiency and is repeated in the Neurospora literature (Ryan, 1950). The two investigations with moulds in which mutants failing to assimilate carbohydrates were sought employed Neurospora. In early work Emerson (1944)

isolated a series of mutants with reduced growth rates on any one of a number of carbohydrates following treatment with antisera. Mutants with reduced growth on lactose have also been isolated (Landman, 1950) and were shown to have reduced beta-galactosidase activities, though analysis revealed multigenic control of the enzyme for the mutants identified at least six loci affecting its formation (Landman and Bonner, 1952). None of the mutants reported in either of these investigations appear in the lists of mapped mutants of Neurospora (Barratt et al., 1954).

The most comprehensive genetic investigations of moulds apart from Neurospora, include those with Aspergillus nidulans (Pontecorvo, et al., 1953; Kafer, 1958), Ustilago maydis (Perkins, 1949), Glomerella cingulata (Wheeler, 1956) and Venturia inaequalis (Boone et al., 1956). In each case nutritionally deficient auxotrophic mutants have been used extensively, but in none of the investigations do attempts appear to have been made to isolate mutants failing to utilise specific carbohydrates for growth. Differences in the utilisation of L-sorbose by naturally occurring strains or induced mutants of Ustilago have been reported more recently (Matsushima and Klug, 1958).

The work described in Part 1 of the thesis specifically explored the possibility that mutants unable to utilise specific carbohydrates for growth may be isolated in the filamentous fungus Aspergillus nidulans. The growth of the organism with a variety of carbon sources was tested and a technique developed by which mutants of the type sought were isolated. The genetic analysis

of the mutants is described together with evaluation of their suitability as markers in mould genetics. One series of mutants exhibited exceptional genetic behaviour (Chapter 6). The first chapter deals briefly with techniques for A.nidulans and is based entirely on the results of other workers.

There is no general term in microbial genetics which can be applied to the mutants described here and it is proposed to call them 'sugar mutants'. A sugar mutant is defined as a mutant impaired in its ability to utilise a specific carbohydrate as sole carbon source for growth. The mutants cannot be termed 'fermentation mutants' or, for example,'sucrose negative mutants' for they are not recognised by a fermentation test; the term 'assimilation mutant' is ambiguous. The term adopted is misleading for the interpretation that the mutants require a specific sugar for growth is possible, but in fact organisms of this type are not known.

Chapter 1. Materials and Methods.

The filamentous fungus *Aspergillus nidulans* has been the subject of intensive genetic investigation by a number of workers in the University of Glasgow during the past fourteen years. The following outline of the biology of the organism and the techniques of genetic analysis available in it briefly summarise some of their findings (Pontecorvo et al., 1953; Pontecorvo, 1959). The mycology of the organism is described in detail in Thom and Raper's (1945) Manual.

1. *Aspergillus nidulans* is a homothallic ascomycete. The mycelium consists of slender branching hyphae in which occasional cross-walls separate coenocytic cells containing many minute nuclei. The nuclei are typically haploid. The mycelium is propagated asexually by conidiospores produced in aerial sporing heads each of which develops from a single erect hypha. The hypha forms a terminal vesicle on which a densely packed layer of columna shaped cells develop each giving rise to several chains of uninucleate conidia. The occurrence of these uninucleate haploid cells in the life-cycle of the organism greatly facilitates the purification of strains and the isolation of mutants (Pontecorvo et al., 1953).

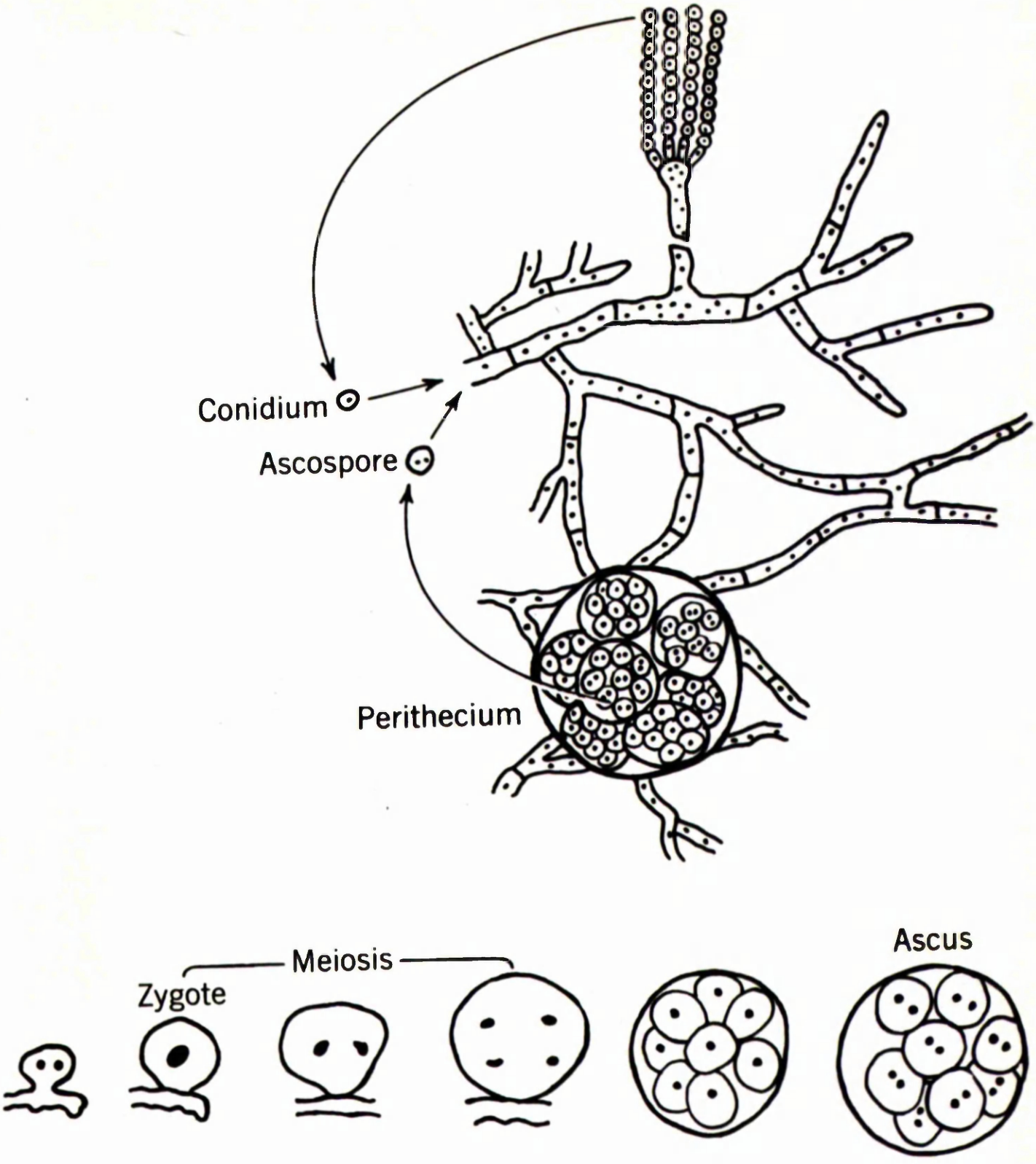
The organism is propagated sexually by ascospores that are produced in fruiting bodies (perithecia) which develop embedded in the surface of the mycelium. A young perithecium contains a mass of hyphae on which many short lateral cells develop. These cells are ascus primordia and each contains

two nuclei which fuse to yield the zygotic nucleus of the ascus. The zygotic nucleus undergoes an immediate reduction division, after which the four nuclei divide mitotically and each of the eight resulting nuclei is enclosed in an ascospore. The mature ascospores are binucleate due to a further mitotic division which occurs after formation of the ascospore walls, and they are irregularly arranged in the ascus. The life-cycle of A.nidulans is shown schematically in Plate 1.

In crosses between strains of A.nidulans both selfed and hybrid perithecia are formed. Individual perithecia may be collected, tested for hybridity by sampling the ascospores, and meiotic data obtained by analysis of the colonies isolated by plating dilute suspensions of random ascospores. This is the technique of Perithecium Analysis and depends on the fact that in a hybrid perithecium the vast majority of the zygotic nuclei result from fusion of a nucleus of each of the parental types (Hemmons, Pontecorvo and Bufton, 1953). Asci can also be isolated for the purpose of tetrad analysis by use of the micro-manipulator (Strickland, 1958).

All of the strains of Aspergillus nidulans employed in genetic studies in Glasgow are isogenic and have been derived from one original single conidium isolate of the wild type organism by either mutation or meiotic recombination. This practice has been successful in maintaining fertility between strains and yielding consistent linkage data (Pontecorvo et al., 1953; Kafer, 1958).

Three principal types of mutant of A.nidulans have


Conidium
Ascospore
Perithecium
Meiosis
Zygote
Ascus

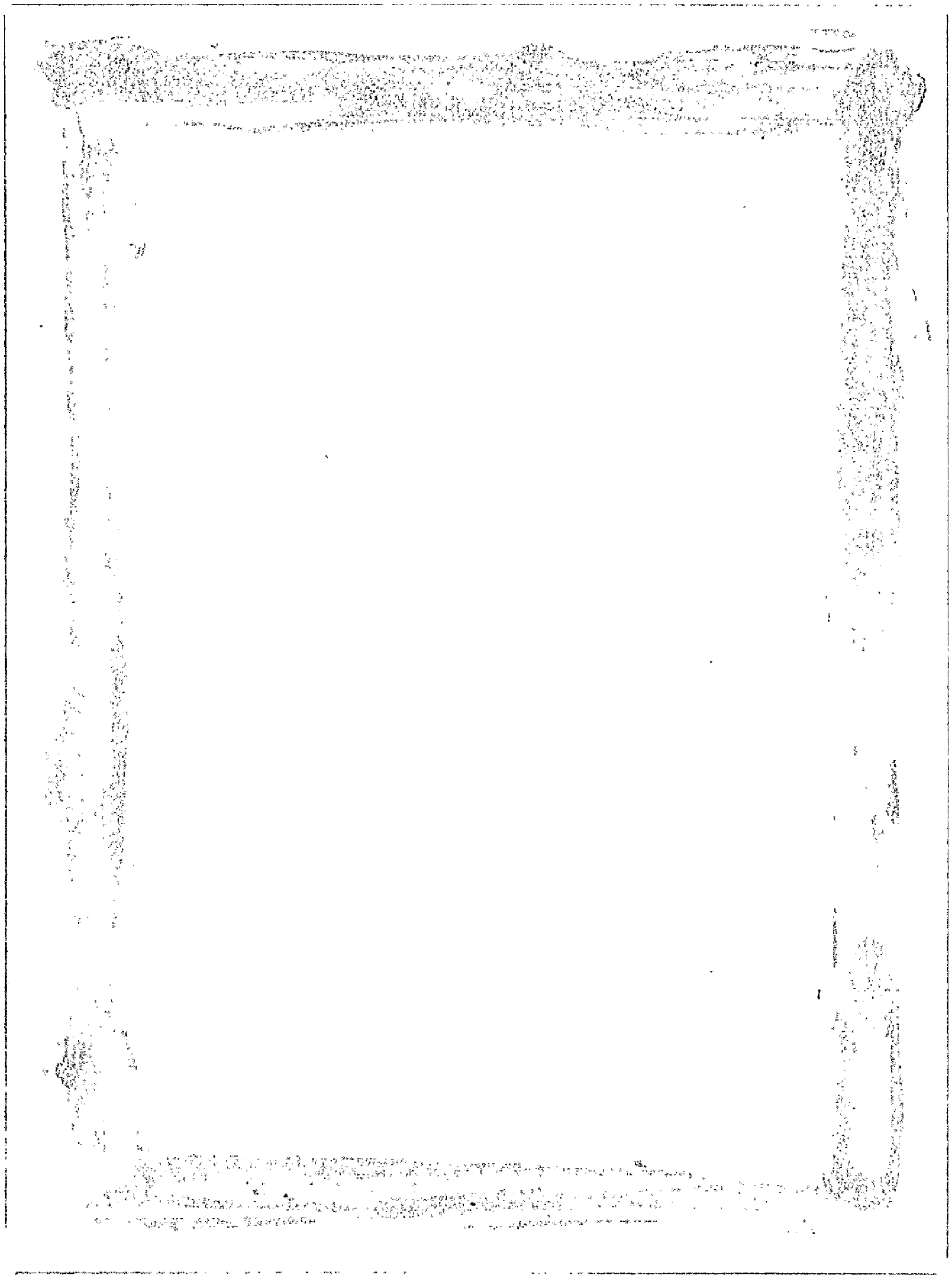


PLATE 1 The Life Cycle of Aspergillus nidulans

been isolated for use in genetic studies. The first are morphological mutants and include variants for conidial colour or colonial morphology. The second type are auxotrophic mutants which fail to grow on the simple inorganic salts-carbohydrate medium suitable for the wild type unless specific nutrients are added. A large number of auxotrophic mutants have been isolated, particularly since the development of the Starvation Technique (Macdonald and Pontecorvo, 1953), the majority were isolated after U.V. irradiation but a few after X-ray treatment. The third type of mutant are a series of spontaneous mutants resistant to the drug acriflavine (Roper and Kafer, 1957).

All three types of mutant are used in selective techniques to recover segregants appearing as sectors in growing colonies or as rare recombinant progeny in crosses.

Pairs of strains of A.nidulans with complementary nutritional requirements form balanced heterokaryons in which nuclei of the component strains occupy a common cytoplasm permitting growth of the hyphae on unsupplemented medium (Pontecorvo et al., 1953). Within heterokaryotic cells nuclei of each of the component haploid types occasionally fuse to yield a diploid nucleus. Some of the nuclei of the resulting clone may be isolated as heterozygous diploid strains either as sectors growing out of the heterokaryotic mycelium or by selection applied to the conidia formed by the heterokaryon and in which the several types of nuclei are separated (Roper, 1952). Conidia of diploid strains are of greater diameter

than those of haploids and the difference is sufficient to provide a reliable criterion to distinguish the strains (Pontecorvo, Tarr Gloor and Forbes, 1954). Both heterokaryons and heterozygous diploids are used to perform complementarity tests for the allelism of pairs of recessive mutants that are pheno-typically alike (Pontecorvo, 1952a; 1956).

Heterozygous diploid strains of A.nidulans are relatively stable but undergo infrequent nuclear changes constituting the parasexual cycle (Pontecorvo, 1954) in which recombination and segregation of genetic characters takes place in mitotically dividing nuclei (Pontecorvo, Tarr Gloor and Forbes, 1954; Pontecorvo and Kafer, 1958). Two characteristic processes occurring in mitotic recombination are somatic crossing over, in which nuclei become homozygous for markers for which they were previously heterozygous, and mitotic haploidisation, in which diploid nuclei give rise to haploid nuclei without a meiotic division. Mitotic recombination may be employed to locate genes in linkage groups, to order genes relative to centromeres and to locate centromeres (Pontecorvo and Kafer, 1958). The parasexual cycle thus provides the organism with an alternative mechanism to the sexual cycle for the generation and conservation of variation, while the prolonged diploid phase provides the opportunity for Heterosis.

2. Linkage Maps of A.nidulans

The 1957 linkage maps of A.nidulans based on the work of Kafer, (1958) are given to provide reference for the

markers employed in the course of the genetic analysis (Figure 1). The map describes eight linkage groups, a result which correlates with recent cytological evidence for eight chromosomes (Elliot, 1960).

A list of the markers used in the investigation, their symbols, phenotypes and origins is also drawn up (Table 1).

3. Media

Standard media routinely prepared in the Department of Genetics have been used throughout the investigation. The chemicals were of Analytical Reagent grade unless stated otherwise.

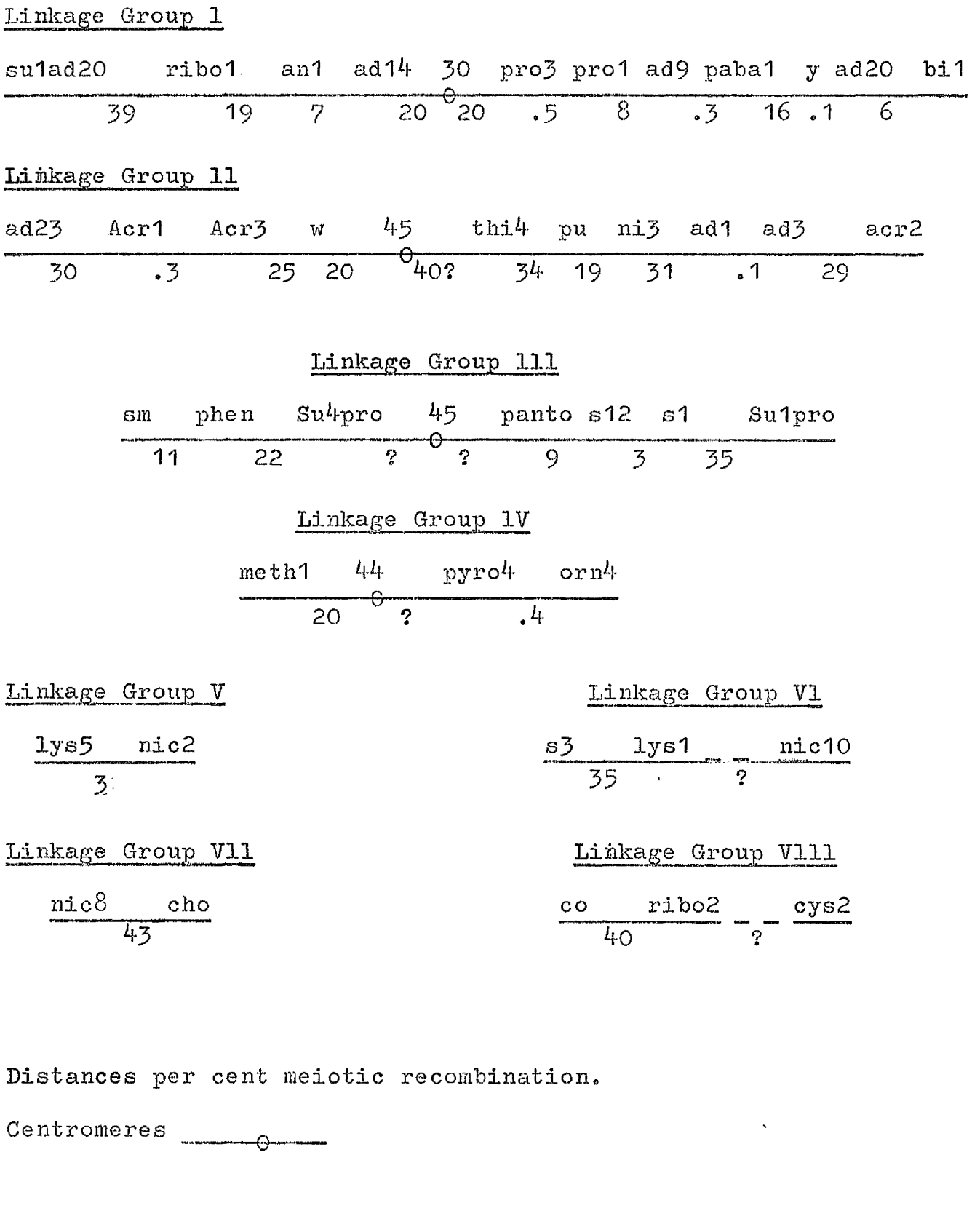
A. Minimal Medium (M.M.)

Composition:-

$NaNO_3$	6.0 g.	KCl	0.52 g.
$MgSO_4\ 7H_2O$	0.52 g.	KH_2PO_4	1.52 g.
$FeSO_4$	trace	$ZnSO_4$	trace
Glucose	10.0 g.	Agar (B.D.H)	15.0 g.
Distilled water to 1.0 l.		pH 6.5	

The agar was dissolved by steaming when the salts and glucose were added and the solution made up to volume. The pH was adjusted to 6.5 with NaOH or HCl and the solution filtered through paper pulp, dispensed in quantities of 100 ml or 200 ml in conical flasks and autoclaved at 15 lbs. pressure for 15 minutes.

FIGURE 1. Linkage Maps of Aspergillus nidulans



Distances per cent meiotic recombination.

Centromeres ——o——

After Kafer, 1958.

TABLE 1. Mutants of *Aspergillus nidulans* Employed in the Present Investigation

Symbol	Designation	Phenotype	Origin of Mutant		Reference
			Strain	Agent	
Morphological Mutants					
w3	white 3	Colourless conidia	paba1 bi1	Spontaneous	Pontecorvo et al. 1953
y2	yellow 2	Yellow conidia	wild type	X-rays	"
sm	small	Small colony	bi1; lys5	Spontaneous or U.V.	"
Drug Resistant Mutant					
Acr1	acriflavine 1	Resistance to acriflavine, (semi-dominant)	paba1y; co	Spontaneous	Roper and Kafer, 1957

Table 1. (continued)

Nutritional Mutants	Growth response to:-				
ab1	aminobutyric 1	Amino-butyrate	bi1	U.V.	
ad3	adenine 3	Adenine	y; thi1	X-rays	Pontecorvo et al. 1953
ad14	adenine 14	Adenine	bi1	U.V.	Pritchard, 1955
ad20	adenine 20	Adenine	bi1	U.V.	"
ad23	adenine 23	Adenine	bi1	U.V.	Pontecorvo et al. 1953
an1	aneurine 1	Aneurine	bi1	U.V.	"
arg2	arginine 2	Arginine	bi1	U.V.	
bi1	biotin 1	Biotin	wild type	X-rays	Roper, 1950
lys5	lysine 5	Lysine	bi1	U.V.	Pontecorvo et al. 1953
meth1	methionine 1	Methionine	bi1	U.V.	"
meth2	methionine 2	Methionine	bi1	U.V.	
ni3	nitrite 3	Nitrite	bi1; w3	U.V.	
nic8	nicotinic 8	Nicotinic acid/ anthranilic acid/ tryptophane	bi1	U.V.	"
nic10	nicotinic 10	Nicotinic acid/ anthranilic acid	bi1; Acr1w3	U.V.	

Table 1. (continued)

paba1	p-aminobenzoic 1	p-aminobenzoic acid	bi1	X-rays	Pontecorvo et al. 1953
panto1	pantothenic 1	Pantothenic acid	y; thi1	X-rays	"
phen2	phenylalanine 2	Phenylalanine/ phenylpyruvic	bi1	U.V.	"
pro1	proline 1	Proline/ arginine	bi1	U.V.	Forbes, 1956
pu1	putrescine 1	Putrescine / spermidine	bi1; w3	U.V.	Sneath, 1955
pyro4	pyridoxine 4	Pyridoxine	bi1	U.V.	Pontecorvo et al. 1953
ribo1	riboflavine 1	Riboflavine	bi1	U.V.	
ribo2	riboflavine 2	Riboflavine	bi1; w3Acri1	U.V.	
ribo6	riboflavine 6	Riboflavine	bi1; w3	U.V.	Roberts, 1959
s3	sulphite 3	Sulphite	bi1	U.V.	Pontecorvo et al. 1953
s12	sulphite 12 (formerly s8 and sd)	Sulphite	wild type	Nitrogen mustard	Hockenhull, 1949

Suppressor Mutation

su1ad20	suppressor 1 adenine 20	Suppresses ad20	ad20; pyro4	Spontaneous	Pritchard, 1955

B. Complete Medium (C.M.)

Composition:-

$NaNO_3$	6.0 g.	KCl	0.52 g.
$MgSO_4.7H_2O$	0.52 g.	KH_2PO_4	1.52 g.
Difco Bacto Peptone		2.0 g.	
Yeast Extract (Yeastrel)		1.0 g.	
Casamino acids		1.5 g.	
Yeast Nucleic Acid hydrolysate (see below)		3.0 ml.	
Vitamin solution (see below)		1.0 ml.	
Glucose	10.0 g.	Agar (B.D.H)	15 g.
Tap water to 1 l.		pH 6.0	

The medium was prepared in the same way as the minimal medium except that the pH was adjusted to 6.0 and the vitamin solution not added until after filtration.

Liquid Complete Medium.

This was prepared in the same way as C.M. but without the addition of agar. It was dispensed in 100 ml. quantities and autoclaved at 15 lbs. pressure for 15 minutes.

Preparation of the Yeast Nucleic Acid hydrolysate:-

2.0 g. Yeast Nucleic Acid + 15 ml N.NaOH
2.0 g. Yeast Nucleic Acid + 15 ml N.HCl

The mixtures were separately boiled for 20 minutes, carefully mixed, brought to pH 6.0 and filtered hot. The volume was made up to 40 ml and the solution stored over chloroform at 4° C.

Preparation of the Vitamin solution:-

Composition:-

Riboflavin	10 mg.	Nicotinamide	10 mg.
p.aminobenzoate	1 mg.	Pyridoxin HCl	5 mg.
Aneurin HCl	5 mg.	Biotin	0.02 mg.
Calcium pantothenate	20 mg.	Choline chloride	20 mg.
Inositol	40 mg.	Folic acid	1 mg.

Distilled water to 10 ml.

The solution was filtered and autoclaved at 10 lbs. pressure for 10 minutes.

C. Nutritional Supplements

In the preparation of test media the following quantities of nutrients were added to 200 ml of M.M.

Nutrient	Amount	Stock Solution
gamma-aminobutyric acid	1.0 ml	1% (w/v)
para-aminobenzoic acid	1.0 ml	0.001 M
Adenine	2.0 ml	0.05 M
Aneurin	1.0 ml	20 microg/ml
Arginine	1.0 ml	0.2 M
Biotin	1.0 ml	4 microg/ml
Lysine	1.0 ml	0.2 M
Methionine	1.0 ml	15 mg/ml
Nitrite ($NaNO_2$)	1.0 ml	0.5% (w/v)
Nicotinic Acid	1.0 ml	10 microg/ml
Pantothenate (Ca salt)	1.0 ml	20 microg/ml
Phenylalanine	1.0 ml	1% (w/v)
Proline	1.0 ml	0.1% (w/v)
Putrescine	1.0 ml	200 microg/ml
Pyridoxin	1.0 ml	10 microg/ml
Riboflavin	1.0 ml	20 microg/ml
Sulphite ($Na_2S_2O_3$)	1.0 ml	15% (w/v)

D. The Use of Acriflavin

A stock solution of 0.5% (w/v) acriflavin was prepared. It was employed in the following ways.

(1) Selection of acriflavin resistant sectors:-
Selection on M.M. 0.5 ml Stock Solution per 200 ml medium.
Selection on C.M. 1.0 ml " " " " " "

(2) Classification of progeny for acriflavin resistance:-
2.0 ml Stock Solution per 200 ml C.M.

4. Special Media used in the Classification of the Sugar Mutants.

The use of these media is described in detail in Chapters 2 and 3.

Basal Medium.

The medium was prepared in precisely the same way as Minimal Medium except that no glucose was added. It was dispensed in 190 ml quantities.

Solutions of Carbohydrates

A 20% (w/v) solution of the carbohydrate was prepared in distilled water, dispensed in 10 ml quantities in 1 oz. screw-cap bottles and autoclaved at 15 lbs. pressure for 15 minutes.

Preparation of Test Media.

10 ml of the carbohydrate solution was added aseptically to 190 ml of molten Basal Medium (final concentration of 1% Carbohydrate), mixed and test plates poured.

Sources of the Carbohydrates.

The carbohydrates most extensively used in the investigation were of the following grades and obtained from the sources shown.

Analytical Reagents (B,D.H)	D.Glucose Lactose Sucrose
Biochemical Reagent (B.D.H)	Fructose
Bacteriological Grade (T. Kerfoote)	D.Galactose Maltose D.Sorbitol

5. Standard Methods

In all work the practice of normal aseptic routine was observed. All experiments were done at 37° C. unless stated otherwise.

A. Maintenance of Cultures

Departmental Stock Cultures are maintained on slopes of standard C.M. and stored at 4° C. Subcultures are made by transferring a mass of conidia at six monthly intervals, the new cultures are incubated for a few days and then stored in the refrigerator until their next subculture.

Mutants, recombinants and other strains employed in the present work were maintained in the same way except that they were stored in the laboratory. Strains were purified by repeated single colony isolation.

B. Plating Conidia or Ascospores

Normal Saline: 0.85 NaCl in distilled water distributed in 9.0 ml quantities in 1 oz. screw-cap bottles and autoclaved at 15 lbs. pressure for 15 minutes.

Calzolene Solution: A 1 in 1,000 dilution (vol/vol) of calzolene oil in distilled water was prepared and autoclaved for 15 minutes at 15 lbs. pressure.

Conidia were collected by touching a wire loop containing a drop of saline on the surface of the mycelium. The conidia are non-wettable but the addition of the calzolene solution (0.1 ml per 9.0 ml saline) which is a wetting agent allows them to be suspended in saline. Conidia were partly

separated by shaking the bottle vigorously, but if a suspension of mainly single conidia was required the suspension was repeatedly passed through the narrow bore of a Pasteur pipette until the chains of conidia were broken up. The total conidia per unit volume of suspension was estimated by counting a sample of the suspension under the microscope using a haemocytometer. The suspension was diluted to contain the desired numbers of conidia, not more than 0.2 ml pipetted on to the surface of the plating medium and dispersed with a glass spreader that had been sterilised by dipping in alcohol and igniting. Ascospores were plated in the same way except that no calzolene oil was required as the spores are wettable.

C. Crossing

A thick layer of M.M. was poured in a petri dish. A heavy inoculum of conidia of the parental types was streaked on the surface of the medium with a wire loop, thoroughly mixed and 'dug' into the surface of the agar. One loop of liquid C.M. was touched along the streak, the dish sealed with 'sellotape' to make it anaerobic (this suppresses formation of conidia and also prevents the agar drying up) and incubated for three weeks by which time abundant perithecia were formed.

The Classification of Progeny:-

Ascospores from a known hybrid perithecium were plated on C.M. at dilutions yielding not more than 50 colonies per dish. A second set of C.M. plates was prepared and each dish marked with a pattern of 26 positions. Each position

was then inoculated with conidia taken from a different discrete colony on the spread plates and the second set of plates incubated until the colonies spored. An inoculating device (Forbes, unpublished) with 26 metal prongs set in the same pattern as the sporing colonies was then used to replicate conidia from the colonies on to test media which identify the characters present in the parent strains and permit the progeny to be classified according to these characters.

D. Establishment of Balanced Heterokaryons and the Selection of Heterozygous Diploid Strains.

Liquid C.M. was heavily inoculated with conidia of the component strains and incubated overnight. The resulting mycelial pellet was washed with three changes of saline to remove the nutrient solution and then teased out into small pieces on the surface of a plate of M.M. The pieces were set apart from each other and the plate incubated until heterokaryotic mycelium grew out of some of the pieces. The heterokaryon was maintained by transfer on M.M. (Pontecorvo et al., 1953).

To isolate the corresponding heterozygous diploid strain a dense suspension of conidia harvested from the heterokaryon was mixed with cool molten M.M., plates poured and incubated. Colonies of the heterozygous diploid developed and were clearly distinguishable from the background growth of the haploid conidia (Roper, 1952).

Chapter 2. Carbohydrate Utilisation by A.nidulans

The Aspergilli are in general both vigorous and omnivorous in their attack of carbon compounds and utilise many different carbohydrates as carbon sources for growth (Steinberg, 1939; Lilly and Barnett, 1953; Cochrane, 1958). There are no published data concerning carbohydrate utilisation by A.nidulans but A.rugulosus, a member of the nidulans group (Thom and Raper, 1945) has been shown to utilise any one of a dozen simple carbohydrates supplied separately as carbon sources for growth in static liquid cultures, and though the rates of growth varied considerably between the different carbohydrates eight of them yielded similar dry weights of mycelium after prolonged incubation (Lilly and Barnett, 1953). These findings strongly suggested that A.nidulans would utilise a variety of carbohydrates for growth, and this was confirmed in the case of the few carbohydrates that had been tested previously (Forbes, personal communication). Qualitative experiments were therefore done to discover which of a number of common carbohydrates and related organic compounds are assimilated by A.nidulans.

1. Auxanographic Tests.

A prototrophic strain (y2) was chosen as the test organism and an auxanographic technique used to examine the effectiveness of a number of compounds (carbohydrates, sugar alcohols and organic acids) as carbon sources for growth.

Auxanographic plates were prepared by mixing a dense suspension of conidia of the test strain in cool molten basal medium, pouring the medium while still liquid and incubating the plates for 18 hours to germinate the conidia (Beyerinck, 1889; Pontecorvo, 1949). Each compound was tested by placing a small quantity of the crystals (about 0.01 grm.) at a point on the surface of the medium and observing the growth resulting after incubation for 24 hours. Four different compounds were tested on each plate while similar amounts of glucose were added to provide controls. Growth was estimated visually in comparison to the glucose controls.

The results (Table 2) show that A.nidulans utilised most of the compounds tested about one third of which yielded the same density of growth as glucose.

2. Growth of Colonies

Further experiments were done to compare the growth of colonies originating from point inocula on media containing different carbohydrates.

Nine of the carbohydrates yielding good growth in the auxanographic tests were chosen and plates containing basal medium and 1% (w/v) of each carbohydrate prepared. The plates were inoculated with conidia of the wild type on the prototrophic strain y2. The plates were inspected at intervals during three days incubation.

TABLE 2

Auxanographic Tests for the Utilisation of Carbohydrates by *A.nidulans*, strain y2

C.source	Growth	C.source	Growth	C.source	Growth	C.source	Growth
D.Glucose	++	Sucrose	++	Raffinose	+	Glycerol	++
D.Fructose	++	Maltose	++	Soluble starch	+	Erythritol	(+)
Mannose	+	Cellobiose	++			Adonitol	0
				Acetate	++	Sorbitol	++
D.Galactose	+	Melibiose	+	Tartrate	+	Mannitol	(+)
L.Sorbose	0	Lactose	+	Citrate	0		
Rhamnose	+	Trehalose	+				

Growth is recorded after 24 hours incubation in comparison to the growth resulting from glucose. Carbohydrates yielding the same density of growth as glucose are indicated by ++, those yielding less growth by +, and those yielding no growth by 0.

Different rates of growth were observed with different carbon sources. Both the wild type and y2 grew at the same rate on one group of carbohydrates (glucose, fructose, sorbitol*, sucrose, maltose and raffinose) but less rapidly at first on the other three carbohydrates (galactose, lactose and cellobiose) apparently requiring a period of adaptation before growth began.

3. Rates of Growth of Colonies of Strain bi1;w3 on the Carbohydrates Used in the Investigation.

Five carbohydrates (sorbitol, galactose, sucrose, maltose and lactose) were eventually chosen for mutation experiments in which the strain bi1;w3 was most extensively used. It is therefore of interest to record the rates of growth of colonies of this strain on the five carbohydrates and also on glucose, the standard carbon source, fructose and C.M. Duplicate plates were prepared containing basal medium with 1% (w/v) of the carbohydrate and biotin (0.02 microgrm. per ml). Conidia were inoculated in the centre of each plate and the radii of the resulting colonies recorded daily during seven days incubation. The results are shown in Table 3.

The strain grew more rapidly on C.M. than on the defined media, but little difference in the rate of extension of the mycelium on the different carbohydrates was detected though a lag in growth on galactose and lactose was again observed after the first days incubation. The appearance

*It is convenient to regard the sugar alcohol sorbitol as a carbohydrate for descriptive purposes.

TABLE 3

Rates of Growth of Strain bi1; w3 on the Carbohydrates Used in the Investigation.

Period of incubation	Carbon Source							
	Glucose	Fructose	Galactose	Sucrose	Maltose	Lactose	Sorbitol	Complete medium
18 hours	2.0	2.0	0.5	2.0	1.5	1.0	2.0	3.0
1 day	3.5	3.0	1.5	3.5	3.0	2.5	3.0	4.0
2 days	8.5	8.0	7.0	8.5	8.0	8.5	8.0	10.0
3 days	13.5	12.5	13.0	14.0	12.5	14.0	13.5	18.5
4 days	18.0	16.5	19.5	19.0	17.5	20.0	18.5	26.0
5 days	24.0	20.0	24.5	24.0	21.0	25.0	23.0	32.0
7 days	36.0	29.0	37.0	36.5	33.5	37.5	35.0	-

Colonies grow from point inocula and their radii are measured (mms.) after the periods of incubation indicated. Each figure in the table is the average for two colonies growing on different plates.

of the mycelium and the degree of sporulation was roughly equal for all the carbohydrates.

Discussion

As expected A.nidulans was found to utilise most of the compounds tested as carbon sources for growth. The pattern of growth on the different sugars is similar to that reported for A.rugulosus (Barnett and Lilly, 1953) and some of the carbohydrates appear to be utilised adaptively.

A.nidulans is thus shown to assimilate a number of different carbohydrates and therefore the organism must possess the ability to accumulate the carbohydrates from the medium and to synthesise the enzymes concerned in their metabolism. It follows that mutants may occur that have either lost the ability to accumulate a specific carbohydrate (Cohen and Monod, 1957) or fail to synthesise an enzyme concerned in the metabolism of the carbohydrate. The frequency at which such mutants occur may be increased by mutagenic treatment and they may be detected by their failure to utilise specific carbohydrate for growth (Beadle and Tatum, 1941).

In selecting the carbohydrates for the mutation experiments the most obvious and important requirement was that the wild type organism should make good growth on the carbohydrate and the rate of growth should be reasonably high. This not only facilitates all stages of the experimental work but also adds to the likelihood of isolating mutants clearly

distinguishable from the wild type. Such mutants are desirable because the mutant phenotype will probably be clearly distinguishable in subsequent genetic experiments while a large difference between the rates of growth of the mutant and wild type on the carbohydrate may provide the basis for selective techniques. The five carbohydrates were also chosen with regard to the interest of comparing the mechanism of the genetic control of their metabolism in A.nidulans with that in other organisms. The sugars that appear to be metabolised adaptively (lactose and galactose) were selected because of the intrinsic interest of adaptive processes and also in view of the extensive studies on the induction of beta-galactosidase in E.coli (Monod, 1956; Cohen and Monod, 1957) and the recent studies on the metabolism of galactose (Kalckar, 1958). Sorbitol and maltose are inexpensive carbohydrates and maltose is widely employed in fermentation tests in yeast and bacterial genetics (Winge, 1952; Lederberg, 1949). The choice of sucrose was in one respect unwise for the sugar may be degraded by any one of several enzyme systems (Summer and Myrbach, 1950; Dixon and Webb, 1958) and the likelihood of isolating mutants defective in all these systems is small. However should mutants be isolated they are likely to be defective in a permeability function (Cohen and Monod, 1957) and could be extremely interesting. As a matter of economy it was advisable to use sugars that are not expensive for addition to media at the rate of 1% (w/v) could become costly particularly in a mutant screening programme.

Summary

1. The ability of A.nidulans to utilise a number of carbohydrates, organic acids or sugar alcohols as carbon sources for growth was tested.

2. Most of these compounds supported growth after 24 hours incubation though in some cases a period of adaptation was required before growth began.

3. Mutants may occur that have lost the ability to metabolise specific carbohydrates and may be detected by their failure to utilise these carbohydrates for growth.

4. A group of five carbohydrates (sorbitol, galactose, sucrose, maltose and lactose) were selected for use in mutation experiments. Two of these sugars (galactose and lactose) are apparently metabolised adaptively.

Chapter 3. Sugar Mutants of Aspergillus nidulans

It is shown in the preceding chapter that A.nidulans utilises a number of different carbohydrates for growth and must therefore possess the ability to synthesise enzymes effecting the metabolism of these carbohydrates. It follows that Mutants failing to synthesise one of these enzymes in an active state may occur and may be detected by their inability to utilise a particular carbohydrate for growth. A search was therefore made for mutants of this type are called "sugar mutants".

The search for mutants raises two practical problems, a method for the induction of the mutants and a method by which the mutants, assuming that they are present, may be isolated. The postulated sugar mutants of A.nidulans belong to the general class of nutritionally deficient mutants in which an alteration of the nutritional characteristics of the organism occurs that may be attributed to the failure to produce an active enzyme (Beadle and Tatum, 1941). It is therefore reasonable to assume that the methods by which auxotrophic mutants are induced and isolated will also be effective in the induction and isolation of sugar mutants, and this was adopted as a working hypothesis in the experiments designed to isolate sugar mutants of A.nidulans.

A replica plating technique for A.nidulans was developed during the course of the investigation and used to isolate mutants.

Neither the use of a replica plating technique nor the search for sugar mutants had been previously attempted with A.nidulans and therefore auxotrophic mutants were isolated in the experiments to provide a test of the effectiveness of the technique and a basis of comparison for the frequency at which sugar mutants are isolated. It may be noted here that the use of the replica plating technique placed some restrictions on the design of the irradiation experiments.

1. The Induction of the Mutants.

A. Choice of the Mutagenic Agent.

Nutritionally deficient mutants of A.nidulans have been isolated following exposure of conidia to X-rays or U.V. light (Pontecorvo et al., 1953) or treatment with mustard-gas (Hockenhull, 1949). In this investigation U.V. light was employed as the mutagenic agent in preference to a chemical agent or X-rays. Dosage, and thus the rate of killing, may be closely controlled during irradiation but this is not the case with chemical agents which were therefore ruled out as control over the rate of killing was of importance in the isolation of mutants.

Treatment with U.V. light was preferred to X-ray because of the difference in the genetic effects produced by the two agents and also on practical considerations. In both Drosophila (Muller and Mackenzie, 1939) and Maize (Stadler, 1941) the survivors of X-ray irradiation show a high frequency of gross chromosomal aberrations whereas no increase in the frequency of such aberrations was detected among the survivors of U.V. irradiation in which mainly minute chromosomal rearrangements or terminal deficiencies are found (Swanson and Stadler, 1955). It has also been observed that in Neurospora the survivors of X-ray irradiation are more frequently sterile than are the survivors of U.V. irradiation (Lindegren and Lindegren, 1945). Sterility is probably the

result of gross chromosomal abnormalities (Hollaender et al., 1945b). In this work gross chromosomal aberrations, particularly translocations which are commonly associated with X-ray damage and lead to spurious linkage relationships, would be a considerable hindrance to genetic analysis at a later stage of the investigation. While in view of the object of the second part of the investigation, the fine genetic analysis of a series of sugar mutants, U.V. light was obviously to be preferred to X-rays for it is known that U.V. light induces a higher proportion of "point" mutations than X-rays (Swanson and Stadler, 1955). Loss of fertility is, of course, undesirable.

B. Factors in the Design of the U.V. Irradiation Experiments.

The replica plating technique used to isolate the mutants is shown to work at an optimal efficiency when the initial plates, prepared by spreading suspensions of irradiated conidia, bear between 100 and 150 colonies each. It was therefore desirable to achieve relatively constant survival rates in the irradiation experiments in order that initial plates could be prepared without multiple platings of the irradiated suspensions at different dilutions.

The effectiveness of U.V. light in killing and producing mutations is a function of the wavelength of the light employed and of the dose given (Hollaender and Emmons, 1941), while experimental conditions before and after irradiation affect both the survival rates and the proportion of mutants among the

survivors. These factors were considered in designing the U.V. experiments and in attempting to select conditions most favourable for the induction of mutants while keeping control of the rate of killing.

(1) Pre-irradiation treatment.

No pre-irradiation treatment was attempted though treatments such as the exposure of cells to non-mutagenic doses of nitrogen-mustard (Swanson et al., 1949) or incubation with nucleic acid precursors (Haas and Doudney, 1953) have been reported to increase the frequency at which mutants occur among the survivors of U.V. irradiation. In the case of A.nidulans any treatment which permits nuclear divisions will be of limited value for the conidia of A.nidulans are uninucleate and such treatments may substitute a multi-hit target for a single-hit target and thus reduce the rate at which nutritionally deficient mutants are isolated due to the growth of mixed clones containing both unaltered and mutant nuclei. In the preparation of conidial suspensions for irradiation attention was paid to the standardisation of procedure. The conidia were harvested from cultures grown from single colony isolates of the parental strains to reduce the likelihood of repeated isolation from mutant clones that may be already present in the culture. They were not wetted long enough before irradiation to permit germination or nuclear divisions.

(2) U.V. irradiation.

The effectiveness of U.V. light as a mutagenic agent varies with the wavelength of the light, the most effective

wavelengths are in the range 2500 to 2650 Å and this is true in the irradiation of wet fungal spores for which an optimal dose is observed for the induction of mutants (Hollaender and Emmons, 1941; Pomper and Atwood, 1955). The optimal doses, measured as the corresponding survival fractions, observed for some fungi are collected in Table 4 which shows that doses permitting about 5 - 10% survival are generally optimal. (Pontecorvo et al., 1953) suggest an optimal dose for the induction of auxotrophic mutants in A. nidulans at 5% survival of viable conidia and this suggestion has been followed in the present investigation. Irradiation experiments were done under standard conditions to determine the survival curves for the parent strains and to find the exposures required under these conditions for 5% survival.

(3) Post-irradiation treatment.

The most important factor in the treatment of cell suspensions after irradiation is the action of visible light in reversing the effects of U.V. light (Kelner, 1949a). Photoreactivation has been demonstrated in all the organisms in which it has been sought (Dulbecco, 1955; Jagger, 1958), including yeasts (Swanson and Giese, 1950; Kelner, 1952; Sarachech, 1954), a pathogenic fungus Ustilago maydis (Brown, 1952), and the moulds Neurospora crassa (Brown, 1951); Goodgal, 1950) and Penicillium species (Roegner, 1951; Kelner, 1952). It is generally found that visible light not only reverses killing by U.V. (Kelner, 1949a) but also reduces the proportion of mutants among the surviving cells (Zelle et al., 1958).

TABLE 4

Optimal Doses for the Production of Mutations in Fungal Spores by U. V. Light.

Organism	Type of Mutation	Optimal Dose ※	Proportion of mutants among survivors	Reference
Trichophyton mentagrophytes	Colonial morphology and pigmentation	4%	42%	Emmons and Hollaender, 1939.
Aspergillus terus	Colonial morphology	40%	20%	Hollaender et al., 1945 (a)
Neurospora crassa	Colonial morphology	9%	13%	Hollaender et al., 1945 (b)
Penicillium notatum	Colonial morphology	Not reported	20%	Hollaender and Zimmer, 1945
Penicillium notatum	Colonial morphology	3.6%	23%	Stahman and Stauffer, 1947.
Aspergillus nidulans	Nutritional deficiency	5%	1.25%	Pontecorvo et al., 1953

※ Dose expressed as percentage survival of spores.

In this work it was assumed that irradiated conidia of A.nidulans are sensitive to photoreactivation by visible light and in fact reversal of killing was observed in a single test made to detect it. Irradiation was done in a dark room to avoid photoreactivation and the irradiated suspensions exposed to light for the minimum time necessary for plating.

A second important factor in the treatment of irradiated suspensions is the effect of growth conditions immediately after irradiation. It has been shown that for certain reverse mutations in Escherichia coli conditions in the first hour after irradiation that are favourable for growth, particularly protein synthesis, increase the yield of mutants (Whitkin, 1956). Such conditions apparently "fix" the effect of U.V. light and render the cells insensitive to photoreactivation (Kelner, 1949b). These conditions were satisfied for it was necessary to plate the irradiated conidia on C.M. in order that auxotrophic mutants might be isolated. The irradiated conidia were plated immediately after treatment and the plates put into the incubator without delay.

C. U.V. Irradiation Experiments.

(1) Method

Conidial suspensions of stock strains y2; pyro4 or bi1; w3 were spread on C.M. at dilutions yielding about 25 colonies per plate and the plates incubated for three days, by which time good sporulation had occurred. Conidia were taken from discrete single colonies and inoculated onto C.M. slopes

which were then incubated for 5 to 7 days at 37° C. Each slope originated from a different single colony and a different slope was used for each of the irradiation experiments. The conidia were harvested from the slope cultures with a wire loop within 7 days of completing incubation (the cultures standing at bench temperatures meanwhile) and suspended in saline with calzolene oil. The suspension was vigorously agitated by alternate sucking and blowing from a Pasteur pipette until it contained mainly separate conidia, when it was diluted to a density of 10^6 conidia per ml. A sample of 10 mls. of this suspension was pipetted into a large petri- dish (9.5 cms. diameter) for irradiation, and a control plating to determine the proportion of initially viable conidia made by spreading 0.1 mls. of a 10^{-4} dilution of the suspension on C.M. Irradiation was done in a dark room at the same time of day, to avoid as far as possible the effects of fluctuation in mains voltage, and the U.V. lamp (Hanovia Xl, output 90% at 2537 Å) allowed to stabilise by switching it on at least one hour before use. The open petri-dish was placed 45 cms. below the lamp and rocked gently throughout irradiation. In the experiments to determine the survival curves of the original strains y2;pyro4 and bi1; w3 samples of 0.1 ml. (1/100 of total volume) were taken at intervals throughout 20 minutes irradiation. In the mutation experiments a single sample of 1.0 ml. was taken at the end of the period of irradiation yielding 5% survival, diluted by 1/100 and 0.2 ml. plated on C.M.

In all of the experiments the irradiated suspensions were diluted and plated away from the darkroom but with as little exposure to light as possible (a maximum of 2 minutes) and the plates put into the incubator within 15 minutes of completing irradiation.

(2) Results.

(a) Survival curves.

The data obtained in the survival experiments with the original strains y2; pyro4 and bi1; w3 are recorded in Table 5 and the survival curves drawn in Figure 2. The survival curves show an exponential killing of the conidia of both strains on exposure to U.V. but the strains differ in their sensitivity to U.V. light the strain with white conidia being more sensitive than the strain with yellow conidia. Under standard conditions of irradiation 50% inactivation of the white conidia occurs after $2\frac{1}{2}$ minutes irradiation while the yellow conidia are 50% inactivated after $5\frac{1}{2}$ minutes. The 5% survival levels are estimated at exposures of 9 minutes for bi1; w3 and $18\frac{1}{2}$ minutes for y2; pyro4 though in fact somewhat shorter exposures were generally used in the mutation experiments (8 and 16 minutes respectively).

(b) A single photoreactivation experiment.

In the course of the determination of the survival curve of y2;pyro4 a single test was made to detect reversal of U.V. killing by visible light. A suspension of conidia that had been irradiated for 20 minutes was sampled to determine

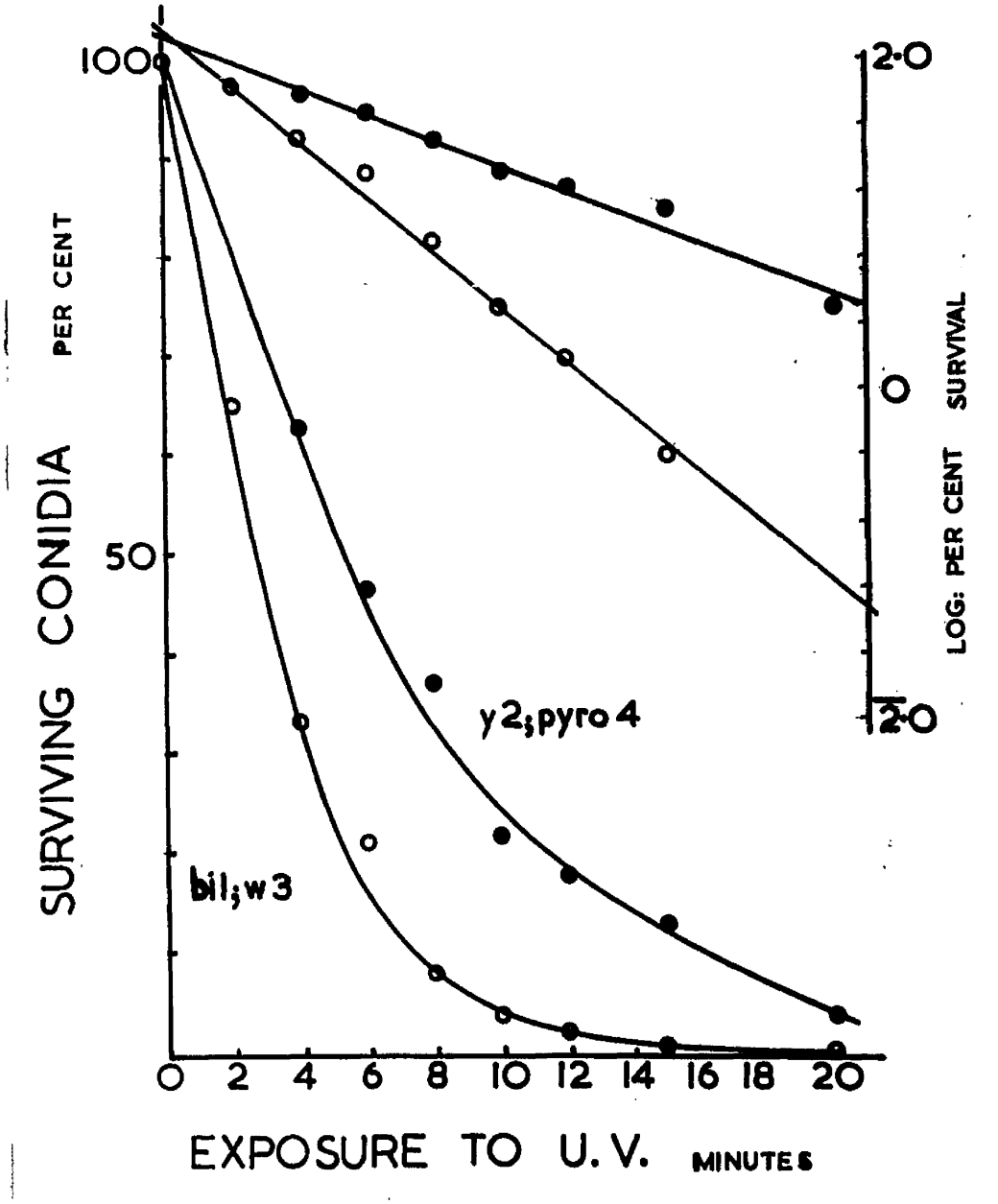


FIGURE 2 The Survival of Conidia of Aspergillus nidulans under U.V. Irradiation.

TABLE 5

The Survival of Conidia of Aspergillus nidulans under U.V. Irradiation.

y2; pyro4

Exposure to U.V (Mins)	Plating* Dilution	Vol:(ml.)	Colonies per plate		Survival (%)	(log)
0	4×10^2	0.1	120) 130) 134)	128	100	2.00
2	-	-	-		-	-
4	4×10^2	0.1	78) 84) 87)	81	63.2	1.80
6	3×10^2	0.1	75) 79) 83)	79	46.3	1.67
8	1×10^2	0.1	187) 188) 195)	190	37.1	1.57
10	1×10^2	0.1	110) 114)	112	21.9	1.34
12	1×10^2	0.1	78) 90) 101)	89	17.4	1.24
15	1×10^2	0.2	115) 128) 137)	127	12.5	1.10
20	1×10^2	0.2	27) 32) 36)	32	3.0	0.48

* Initial suspension = 0.78×10^6 conidia per ml.
Viability = 65.6%

TABLE 5 (Continued)

bi1; w3

Exposure to U.V. (Mins)	Plating* Dilution	Vol:(ml.)	Colonies per plate		Survival (%)	(log)
0	2×10^3	0.2	82) 82) 96)	87	100	2.00
2	2×10^3	0.2	46) 57) 68)	57	65.6	1.82
4	2×10^3	0.2	28) 29) 30)	29	33.3	1.52
6	2×10^3	0.2	18) 18)	18	20.7	1.32
8	1×10^2	0.2	125) 134) 141)	133	7.7	0.89
10	1×10^2	0.2	44) 58) 64)	55	3.2	0.51
12	1×10^2	0.2	23) 32) 36)	30	1.7	0.23
15	1×10^2	0.2	6) 6) 8)	7	0.4	$\bar{1}.60$
20	1×10	0.2	3) 11) 15)	10	0.06	$\bar{2}.78$

* Initial suspension = 1.04×10^6 conidia per ml.
Viability = 83.6%

the surviving conidia. It was then exposed to daylight and after 10 minutes another sample taken and plated in the same way as the first. The result was:-

Sample	Plating		Colonies per plate	
	Dilution	Volume	Replicates	Mean
20 mins.U.V.	1/100	0.2 ml.	27, 32, 36	32
20 mins.U.V. + 10 minutes visible light	1/100	0.2 ml.	51, 62, 75	63

The result shows that conidia of y2; pyro4 are photoreactivated by visible light after exposure to U.V.

(c) Mutation experiments.

In the work described in Part 1 of this thesis four mutation experiments were done with y2; pyro4 and three with bi1; w3 and in these experiments survival rates of 3.1, 4.4, 5.9, 6.1 and 10.7% obtained (Table 6). (Omitting experiments O and A with y2; pyro4 in which various doses of U.V. were given.) A series of 12 experiments done with bi1; w3 (Part 2 Table 21) yielded survival rates varying between 4.9% and 9.0% with a mean of 5.75% and a standard error of 1.7% and it is evident from these results that a reasonable degree of control over the rate of killing was achieved.

2. Isolation of Mutants.

A. Methods.

(1) A replica plating technique for A.nidulans

The isolation of nutritionally deficient mutants can be both tedious and time consuming, particularly when no selective

technique is available which automatically differentiates the mutants from the parental type. A number of selective techniques that are effective in isolating auxotrophic mutants of filamentous fungi have been described for Ophiostoma (Fries, 1947), Aspergillus (MacDonald and Pontecorvo, 1953) and Neurospora (Catcheside, 1954; Woodward et al., 1954) but for all of these techniques it is necessary to possess at least one mutant so that the technique can be tested and its efficiency evaluated before it is used. No sugar mutants of A.nidulans were available to begin with so that it was not possible to test any of the selective techniques and a conventional method of isolation had to be employed. The obvious method was that of total isolation in which all the survivors of mutagenic treatment are tested individually, but as this method is laborious it was decided to attempt a replica plating technique (Lederberg and Lederberg, 1952) for A.nidulans. The design and testing of the technique developed is described in the published paper together with its use in isolating auxotrophic mutants and sugar mutants. The results summarised in the paper for the isolation of auxotrophic mutants are presented in full here, while those for the isolation of sugar mutants are superseded by the data presented below, and Table 4 of the paper is now out of date.

The tests and criteria employed in the isolation of the sugar mutants which are only briefly outlined in the paper are described in full in the section following the off-print.

Reprinted from THE JOURNAL OF GENERAL MICROBIOLOGY
Vol. 20, No. 3, June 1959
 PRINTED IN GREAT BRITAIN

ROBERTS, C. F. (1959). *J. gen. Microbiol.* **20**, 540–548

A Replica Plating Technique for the Isolation of Nutritionally Exacting Mutants of a Filamentous Fungus (*Aspergillus nidulans*)

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SUMMARY: This paper describes a replica plating technique for a filamentous fungus and shows its effectiveness in the isolation of nutritionally-exacting mutants, including mutants unable to grow with certain sugars as sole carbon source.

The two main applications of the replica plating technique are in the classification of colonies differing from each other in a number of known nutritional requirements, and in the isolation of new mutant strains. The first application is mainly in experiments on gene recombination where nutritional requirements are used as genetic characters. Organisms derived by recombination are plated so that each can give rise to a colony, and then each of the colonies is classified according to its combination of nutritional characters. It is not usually necessary to test more than a few hundred colonies for their response to a small number of nutrients. A convenient method for doing this (Mr E. C. Forbes, personal communication) is to prepare initial plates with a number of the colonies, perhaps 30, set in a standard pattern on each plate. These colonies are then replicated on to a series of test media by means of a multi-point inoculator, the points of which are also arranged in the standard pattern. The colonies are classified by their failure to grow on test media lacking particular nutrients. Such an application is now well known and has been used routinely in this laboratory for several years.

In the second application of replica plating, i.e. the isolation of mutants, the preparation of initial plates with an ordered pattern of colonies is impracticable because it is necessary to test many thousands of colonies, since mutants occur very infrequently. Lederberg & Lederberg (1952) resolved this difficulty by using a velveteen pad to replicate rapidly from initial plates bearing large numbers of randomly distributed colonies. Their technique and its variations have found wide usage in microbiology, and have been successfully applied to bacteria (Lederberg & Lederberg, 1952), to actinomycetes (Braendle & Szybalski, 1957) and to unicellular algae (Eversole, 1956).

Lederberg's replica plating technique has not been previously described in a form that can be applied readily to a filamentous fungus. Among the filamentous fungi *Aspergillus nidulans* has been extensively subjected to detailed genetic investigation (Pontecorvo *et al.* 1953; Käfer, 1958; Pontecorvo & Käfer, 1958) and it was desirable to have a straightforward replica plating technique for it. This paper describes the technique developed and its use in isolating nutritionally-exacting mutants of two types. One type are the usual

auxotrophic mutants which can only grow when a particular nutrient (e.g. an amino acid, vitamin, etc.) is present in the medium. The other type are unable to utilize particular sugars as sole carbon sources for growth and are called 'sugar mutants'.

METHODS

Design of technique. The general method of replica plating was described by Lederberg & Lederberg (1952); it is useful to recall here the three essential stages. (1) Spreading a suspension of mainly separate organisms on a solid medium so that after incubation most of the colonies on the initial plate have grown from single organisms. (2) The use of a pad of sterile velveteen or similar rough material (e.g. filter-paper) to make replica inoculations from the initial plate on to a series of plates with different media ('test plates'). (3) Visual inspection of the series of replica plates to compare the responses of individual colonies to different media. There are a number of essential conditions for replica plating and factors which affect the efficiency of the technique. An obvious condition is that the organism should form discrete colonies on agar media. It is an advantage if these colonies are derived from uninucleate cells, and with fungi they should also sporulate reasonably well.

In the preparation of initial plates it is necessary to strike a balance between the number of colonies tested on each plate and the fact that with increased crowding of colonies the proportion of mutants that will not be detected because of mixed growth (syntrophy) also increases. Obviously organisms with nuclei which determine a nutritional requirement will only be detected when growing in a pure clone, and not when mixed with organisms which have unaltered nuclei and consequently are not exacting.

Choice of material for a replica pad is largely governed by the organism. In general a velveteen pad or a filter-paper disk are suitable for wettable spores or vegetative cells, especially bacteria, while dry spores are most effectively transferred on some type of metal prong.

In *Aspergillus nidulans* uninucleate haploid dry conidia are formed in profusion on solid media and suspensions of mainly separate conidia can easily be obtained. When conidia germinate close together on agar their hyphae tend to grow together and form mixed colonies. However, this does not occur between hyphae from conidia which are more than 0·5 cm. apart, and these conidia give rise to discrete colonies separated by zones of no growth. A Petri dish of 9·5 cm. internal diameter would accommodate *c.* 300 colonies if the conidia were at regular intervals of 0·5 cm., but in practice the upper limit, in order to avoid too many mixed colonies, is about 200 conidia on each initial plate.

Velveteen and filter-paper have proved only partly suitable for a replica pad (Dr E. Calef, personal communication). Moreover, with this type of pad only a single inoculum can be taken from the initial plate and this does not allow more than three replicas to be made. Eventually a 'replicator' similar to the multi-point inoculators used for *Aspergillus* to classify colonies by their nutritional requirements was chosen, but having many more points

like the instrument for use with Streptomyces mentioned by Braendle & Szybalski (1957).

Construction of the replicator. One of the replicators is shown in Pl. 1, fig. 1. The base-plate is made from Perspex sheet in which holes that allow easy fitting of the steel pins (dressmaker's pins 2·5 cm. long) without letting their heads through are drilled in a regular pattern at 0·5 cm. intervals. It is important that the points of the pins should be in one plane, therefore the pins should be of equal lengths and the base-plate flat. The pins are held in position by Perspex cement, and a reference point to orientate the replica is provided by omitting a few of them from the pattern.

General method of application. To prepare the initial plates a dilute spore suspension is spread on solid medium to yield 100–200 colonies distributed evenly on each plate, and the plates incubated until good sporulation has taken place. The series of test plates are prepared with media chosen according to the purpose of replication (examples below) and dried to remove excess moisture. After sterilizing the replicator by dipping the pins in ethanol and igniting, the first initial plate is opened with the colonies upwards and the replicator lowered on to them. It is then withdrawn, laid on the bench with the pins uppermost and the first test plate inoculated by touching it against the points. The replicator can be recharged without further sterilization, and the sequence is repeated to inoculate each test plate of the series.

It is, of course, essential that all inocula are taken from exactly the same positions on the initial plate. This is readily achieved if the holes made in the initial plate when taking the first inoculum are used as guides for succeeding inoculations. Eight replica plates can be made from one initial plate without difficulty. With attention to normal aseptic routine contamination is negligible.

Strains and media. Strains of *Aspergillus nidulans* held in the Genetics Department of Glasgow University were used. Media and the general technique for handling *A. nidulans* given by Pontecorvo *et al.* (1953) were followed unless otherwise stated. Minimal medium (MM) was a solution of inorganic salts, including nitrate and sulphate as main nitrogen and sulphur sources, with 1% (w/v) glucose and 1·5% agar. Complete medium (CM) was a complex medium containing casein hydrolysate, yeast extract, various vitamins, a hydrolysate of yeast nucleic acid and the same concentrations of inorganic salts, glucose and agar as in MM.

RESULTS

A reconstruction experiment to illustrate the general application of the technique

Serial dilutions of a mixed suspension of conidia from three strains of *Aspergillus nidulans* with different conidial colours and nutritional requirements were spread on CM, incubated and plates with suitable numbers of colonies selected as initial plates. The replicator was used to inoculate a series of Petri dishes containing test media which in turn allowed only one of the three

strains to grow, and finally on to CM on which they all grew. Plate 1, fig. 2, shows one of the sets of dishes; Table 1 gives details of strains, media and description of the photograph.

Table 1. *A reconstruction experiment to illustrate the general application of replica plating to* Aspergillus nidulans (*see also Pl.* 1, *fig.* 2)

Colonies of the three strains were grown together on the initial plate and replicas made on three test plates, each of which allowed only one of the three strains to grow, and finally on to CM on which they could all grow.

Strain*	Conidia	Nutritional requirement
*y*2; *pyro*4	Yellow	Pyridoxin
*bi*1; *w*3 *ad*3	White	Biotin, adenine
*paba*1; *ad*30	Green	*p*-Aminobenzoate, adenine

Plate		Medium	Incubation	Colonies growing
Initial	0	CM	4 days at 37°	All three strains
Replica	1	MM + pyridoxin	1 day at 37°	*y*2; *pyro*4
	2	MM + biotin + adenine	1 day at 37°	*bi*1; *w*3 *ad*3
	3	MM + adenine + *p*-aminobenzoate	1 day at 37°	*paba*1; *ad*30
	4	CM	2 days at 37°	All three strains

* Symbols of mutant alleles: (*a*) Requirements, *pyro*4 = pyridoxin; *bi*1 = biotin; *ad*3 and *ad*30 = adenine (different loci); *paba*1 = *p*-aminobenzoate. (*b*) Conidial colours, *y*2 = yellow; *w*3 = white; the wild type is green. (See Pontecorvo *et al.* 1953, for further details.)

In Pl. 1, fig. 2, orientation of the replicas is shown by three spots near the lower edge of the initial plate and three corresponding stabs or colonies on the other plates. Green, yellow and white colonies can be distinguished on plates 0 and 4 by their different shades. A number of mixed colonies on plate 4 have grown on more than one of the test plates, while plate 3 shows slight growth of strain *bi*1; *w*3 *ad*3 (point-like colonies) where close to colonies of other strains from which biotin was diffusing into the medium. It is clear from Pl. 1, fig. 2, that colonies with different nutritional requirements can be identified by their different growth responses when replicated on to the appropriate test media. Combination of nutritional requirements with different conidial colours allows the identification to be verified and provides a good illustration of the general application of replica plating to *Aspergillus nidulans*.

Reconstruction experiments with increasing numbers of colonies on the initial plates

Reconstruction experiments were done to investigate to what extent the efficiency of the technique decreases with increasing total number of colonies on the initial plates when, as in the actual isolation of mutants, the mutants sought represent a small proportion of all colonies. Mixed platings of two auxotrophic strains were used to simulate the situation when isolating nutritionally-exacting mutants, and to provide an estimate for the optimal density of colonies on initial plates when using the technique for this purpose. The two auxotrophic strains chosen had differently coloured conidia, to facilitate scoring. Suspensions of conidia of *Aspergillus nidulans* strains *y*2; *pyro*4 and

w3 ad3 were mixed and plated on CM to give a series of initial plates with *c.* 10 colonies of *w3 ad3* and 50–500 colonies of *y2; pyro4* on each plate. (See Table 1 for explanation of symbols.) Replication was performed to see how many of the initial *w3 ad3* colonies could be identified on the replica plates by their failure to grow on an adenine-deficient test medium. Replicas were therefore made from each initial plate on to MM + pyridoxin (no adenine) and on to CM (containing adenine). The test plates were compared after incubation for 24 hr. at 37° and the positions of colonies growing on CM but not on MM + pyridoxin marked. Incubation was continued and the scoring finally checked by inspection of conidial colours on the CM plates. A high proportion of colonies marked after 24 hr. as possible colonies of strain *w3 ad3* proved to have been scored correctly.

Table 2. *Reconstruction experiments to show the loss of efficiency of the replica plating technique as the number of colonies on the initial plates increases*

Initial plates were prepared with about 10 colonies of *Aspergillus nidulans*, strain *w3 ad3* and from 50 to 500 colonies of strain *y2; pyro4*. Replicas were made on to MM + pyridoxin and on to CM and compared after incubation for 24 hr. to identify colonies of strain *w3 ad3*.

Initial plates				Replica plates
		Total no. of colonies		
No. of plates	Mean no. of colonies/ plate	*y2; pyro4*	*w3 ad3*	No. of colonies of *w3 ad3* identified on the replica plates
4	45	139	41	39 (95 %)
4	79	290	27	21 (78 %)
8	110	798	78	67 (86 %)
4	162	612	37	25 (68 %)
2	205	396	14	6 (43 %)
3	305	870	44	18 (41 %)
2	*c.* 500	*c.* 1000	28	6 (21 %)

Further experiments of the same type were performed by replicating directly on to CM and scoring by conidial colour alone. Those colonies which showed only white conidia under the binocular microscope were counted as recoveries. When *w3 ad3* colonies on the replica plates formed obvious groups each group was counted as one as it probably represents a single colony on the initial plate. The data are presented in Table 2. It is apparent that as the total number of colonies on the initial plates increased fewer *w3 ad3* colonies were identified on the test plates. At a density of 100–200 colonies on each initial plate (the average proportion of the rare component was 0·06 for these plates) identification was of the order of 70 % of the *w3 ad3* colonies on the initial plates. In the isolation of nutritional mutants described below, the mutagenic treatment used is known from previous work to yield a proportion of 0·01 auxotrophs among the colonies growing from spores which survive the treatment. Therefore, though the reconstruction experiment does not exactly parallel the conditions used when isolating auxotrophs after mutagenic treatment, the figures indicate that about 70 % of nutritional mutants present on initial plates may be isolated by replica plating.

Isolation of auxotrophic mutants by replica plating

Screening experiments were done primarily to isolate sugar mutants, but auxotrophic mutants were isolated at the same time to provide some idea of the efficiency of the replica plating technique and to compare the frequencies of auxotrophic and sugar mutants.

Suspensions in saline of mainly separate conidia of *Aspergillus nidulans*, strains *y*2; *pyro*4 or *bi*1; *w*3, were irradiated under a u.v. lamp until the viable count was only 5% of the untreated controls, then diluted and plated on CM to yield 100–200 colonies/plate. After incubation for 3 days good sporulation had occurred and replicas were made on to MM + pyridoxin (or MM + biotin) and on to CM. Plates were compared after incubation for 24 hr. and isolates of colonies which failed to grow on MM + pyridoxin (or biotin) but which grew on CM were made from the CM plates. These isolates were retested and those which failed to grow at all on MM + pyridoxin (or + biotin) were purified by single colony isolation and classified for nutritional requirement by routine methods (Pontecorvo *et al.* 1953).

Table 3. *Auxotrophic mutants of* Aspergillus nidulans *isolated by replica plating, grouped into nutritional classes and compared with those obtained by other workers by total isolation*

Class of nutrient requirement	Replica plating		Total isolation*	
Nitrite or ammonia†	3	5%	17	18%
Thiosulphate or sulphite†	1	2%	7	8%
Amino acids	11	18%	12	13%
Components of yeast nucleic acid	6	10%	11	12%
Vitamins	17	31%	22	23%
Not tested	20	34%	24	26%
	58		93	
	6422 colonies on initial plates		5408 colonies tested	

* Data from Pontecorvo *et al.* (1953). The figures are not strictly comparable as different starting strains were used and they include strains obtained after irradiation with X-ray, and with u.v. radiation at survival rates other than 5%.

† The discrepancy between the two methods here is probably due to the 20 partial mutants obtained by replica plating which were not classified. Mutants unable to utilize nitrate or sulphate characteristically give partial growth on MM.

In this experiment 110 isolates were made from 49 initial plates bearing a total of 6422 colonies (4033 of strain *y*2; *pyro*4 and 2389 of strain *bi*1; *w*3). After retesting the yield was 58 mutants (0·9%) with a new nutritional requirement (36 from strain *y*2; *pyro*4 and 22 from strain *bi*1; *w*3). Twenty of these mutants were not tested further because the additional requirements for growth were only partial. The absolute requirements of the remaining 38 strains (0·6%) are given in the second column of Table 3, grouped by nutritional classes.

In a second experiment with strain *bi*1; *w*3 only auxotrophic mutants with a total nutritional requirement were isolated and the yield was therefore

diminished, namely, 36 auxotrophs from *c.* 7400 colonies tested (0·5%). These auxotrophs have not been tested for their additional nutrient requirements.

The isolation of sugar mutants by replica plating

It was thought worth examining the possibility that strains of *Aspergillus nidulans* might occur which are unable to use certain sugars as sole carbon source. The screening experiments were carried out in conjunction with the isolation of auxotrophic mutants described above. Colonies on the initial plates were replicated on to series of seven test plates; one was minimal medium + 1% (w/v) glucose and the growth factors required by parent strain (the control), five were similar media but with glucose replaced by 1% galactose, lactose, maltose, sorbitol or sucrose, respectively; a final plate was complete medium. The auxotrophic mutants were detected by failure to grow on the control plate though growing on CM. The sugar mutants were detected by failure to grow on one or more of the five plates with the unusual sugars, though growing on the control plate (with glucose), the remaining sugars, and on CM. Strains presumed to be sugar mutants were isolated from the control plates, retested and purified by single colony isolation. The pure strains were finally tested by plating suspensions of separate conidia on control plates with glucose, and on media with the glucose replaced by the given sugar as commercially supplied or by this sugar twice recrystallized. Only those strains were retained which gave parent growth with glucose as carbon source but little or no growth with the sugar (as received or recrystallized) when colonies arose from single conidia.

Table 4. *Sugar mutants in* Aspergillus nidulans *isolated by replica plating*

The sugar mutants were isolated by replica plating and were detected by their failure to grow on a given sugar as sole carbon source.

Carbon source	No. of mutants	Minimum no. of loci
Sucrose	0	—
Maltose	2	1
Lactose	9	1
Galactose	8	1
Sorbitol	7	2
Fructose*	3	1
	29	6

* Mutants first isolated because of their failure to grow on sucrose or sorbitol.

In the experiments with *Aspergillus nidulans* strains *y*2; *pyro*4 and *bi*1; *w*3 9 sugar mutants (0·14%) were isolated from 6422 colonies tested by replica plating. In the second experiment with strain *bi*1; *w*3 20 sugar mutants (0·27%) were isolated from *c.* 7400 colonies. The distribution of these mutants is shown in Table 4, together with the number of loci that they are so far known to involve. The frequency of sugar mutants of types detectable by the tests in the two screening experiments was thus 29/13,800 (0·2%). In the same

experiments the frequency of auxotrophic mutants with a clear-cut nutritional requirement was 74/13,800 (0·5 %). One or more mutants have been obtained for each of the sugars tested, except sucrose.

DISCUSSION

This paper describes the application of a replica plating technique to a filamentous fungus for the isolation of nutritionally-exacting mutants. The efficiency of the technique in recovering auxotrophic mutants of *Aspergillus nidulans* is about the same as that of the much more laborious method of total isolation (Pontecorvo *et al.* 1953). There should not be any difficulty in adapting the technique for work with other filamentous fungi. The main advantage of replica plating in the isolation of mutants is the saving of time and labour which enables a far larger population to be tested in a given time than could be done by the total isolation method. However, replica plating cannot be readily employed for quantitative studies and when an estimate of the numbers of a rare component in a population is required the total isolation method is preferable because every colony obtained after plating is known to have been tested once. In both replica plating and total isolation a fraction of the rare component may not be detected because of the growth of mixed colonies.

The replica plating procedure adopted for *Aspergillus nidulans* yielded auxotrophic mutants at the rate of 0·5–0·9 % of the colonies growing from conidia after 95 % killing by u.v. radiation; yields of the same order are obtained by total isolation after similar u.v. treatment. Reconstruction experiments to test the effectiveness of the procedure indicate a recovery of *c.* 70 % of colonies of the rarer type used on the initial plates. The procedure appears, therefore, to isolate almost as high a proportion of auxotrophic mutants as would the total isolation method under the same conditions. The distribution of mutants among the major nutritional classes does not show any obvious differences between the two methods, though the numbers investigated were small.

The author wishes to thank Professor G. Pontecorvo, F.R.S., for advice and criticism in the work and in the preparation of this paper, Mr E. C. Forbes for introduction to the techniques for *Aspergillus nidulans*, and Dr E. R. B. Shanmugasundaram for classifying the auxotrophic mutants. This work is part of a programme supported by the Nuffield Foundation.

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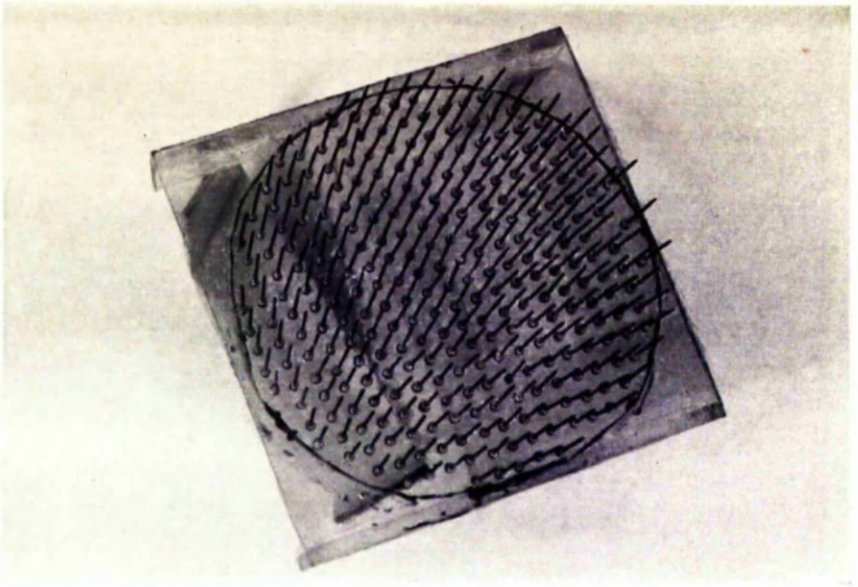


Fig. 1

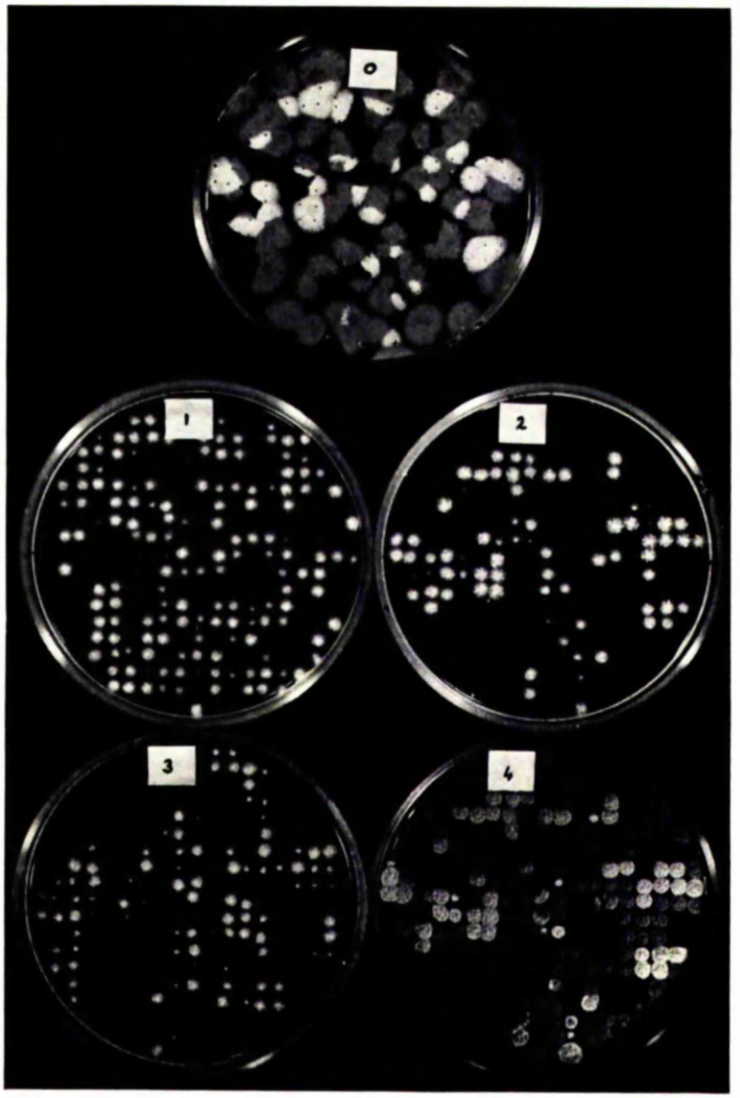


Fig. 2

C. F. ROBERTS—A REPLICA PLATING TECHNIQUE. PLATE 1

(*Facing p.* 348)

PONTECORVO, G. & KÄFER, E. (1958). Genetic analysis based on mitotic recombination. *Advanc. Genet.* **9**, 71.

PONTECORVO, G., ROPER, J. A., HEMMONS, L. M., MACDONALD, K. D. & BUFTON, A. W. J. (1953). The genetics of *Aspergillus nidulans*. *Advan. Genet.* **5**, 141.

EXPLANATION OF PLATE

Fig. 1. The replicator.

Fig. 2. Illustration of the application of replica plating to *Aspergillus nidulans*. Three strains with different nutritional requirements and with green, yellow or white conidia were grown together on the initial plate (0). Replicas were made on test media which allowed the strains with yellow (1), with white (2) or with green conidia (3) to grow. The last plate (4) contained medium on which all three strains grew. Colonies with differently coloured conidia can be distinguished on plates 0 and 4. Plates 1–3 distinguished colonies by their nutritional requirements and the correlation of conidial colour with nutritional requirement can be seen. (Table 1 gives details of the strains and media used.)

(*Received* 21 *November* 1958)

(2) Tests employed in the isolation of the sugar mutants.

The definition of a mutant is usually more-or-less arbitrary and though the experimental procedure employed in isolating the sugar mutants and the criteria adopted in testing them may be described in detail, it is recognised that some degree of selection which cannot be described concisely must have been practiced by the experimenter. The basic test used to define a sugar mutant is comparison of the growth made by the original and the mutant strains on solid media containing either the particular sugar or D glucose as sole carbon source. The mutant fails to yield the same growth as the original strain on a specific sugar but grows as well as the original on glucose, which was chosen as the standard carbon source. It is important for genetic analysis that the mutant and the original type should be clearly distinguishable phenotypically. This has been constantly borne in mind and only those mutants obviously different from the original type in their growth on a specific sugar have been isolated and retained for genetic analysis.

The screening routine used in testing the mutants.

Nutrients required by the original strains were added to all of the test media at the usual concentrations, and the sugar mutants isolated by the following procedure.

1. Replica plating. This is described in the published paper.

2. Presumptive mutants were retested for growth on the relevant sugar or on glucose. Many of them were discovered

to be of the original type at this stage and were discarded. This may have been due to isolation of strains with temporary radiation damage or to testing colonies containing a mixture of original and mutant nuclei. However it is most likely that the colonies were mistaken as mutants due to uneven inoculation of the replica plates, a light inoculum on a test plate and a heavy inoculum on the control plates simulating the result for a mutant.

3. Mutants were purified either by micro-manipulation and single colony isolation. Purification by single colony isolation was repeated at least once, generally twice, and the conidia spread in the second and third platings were taken directly from the single colonies isolated previously. The strains were again tested for their mutant phenotypes.

4. The possibility that the presumptive sugar mutants may have an additional nutritional requirement, which is not manifest on the glucose control medium because a mass of conidia are inoculated, was tested by plating suspensions of conidia of the mutants on media with either glucose or the relevant carbohydrate as sole carbon source and on C.M. (to test for possible differences in viability on the different media). In a few instances the presumed mutants were found to yield poor growth on the glucose control plate compared with the original type when the colonies originated from single conidia and these mutants were discarded. The mutants retained grew as well as the original strain on the plates with glucose but

yielded a mutant phenotype on the relevant sugar and did not show a gross difference in viability between the glucose medium and C.M.

5. The possibility that the mutants had reduced growth on the sugar because they had become sensitive to a substance present as an impurity in it was tested in the following way. The sugars that are not available in an 'analytical reagent' grade (galactose, maltose and sorbitol) were twice re-crystallised from water-alcohol mixtures (about equal volumes of ~~each~~ solvent were used), washed with alcohol and then with ether before drying under vacuum. The re-crystallised sugars were used in the preparation of media on which suspensions of conidia of the presumptive mutants were plated as in test 4. In no case was a difference detected between the growth of the mutants on the sugars as received or after re-crystallisation. The sugars available in A.R. grades (lactose and sucrose) were not re-crystallised and the mutants failing to grow on these sugars were subjected to test 4 only.

The mutants isolated and analysed genetically were all screened by this routine. About 1/10 of the initial isolates were finally retained as sugar mutants, most of those discarded being rejected at the second test.

B. Results.

In isolating mutants it was sometimes found that more than one mutant was obtained from the same initial plate. In such cases only one of the mutants was retained unless there

was a clear phenotypic difference between the mutants, that is total as ppposed to partial nutritional requirement, or failure to grow on different sugars.

(1) The isolation of auxotrophic mutants.

The small proportion of the auxotrophic mutants isolated that have been classified for their nutritional requirements are described in the published paper.

The yield of auxotrophic mutants isolated by replica plating in the work described in Part 1 of the thesis is shown in the last two columns of Table 6. A total of 65 mutants were isolated from 98 initial plates bearing 14,318 colonies, that is 4.5 mutants for each 1,000 colonies tested. In a second series of mutation experiments described in Part 2 of the thesis (Part 2 Table 29) a further 97 mutants were isolated from 120 initial plates bearing 16,517 colonies, an average of 5.9 mutants for each 1,000 colonies tested. The grand total for all the mutation experiments is thus 162 mutants isolated by replica plating from 218 initial plates bearing 30,835 colonies at an average of about 140 $\pm$ 30 colonies per plate, and yielding 5.25 mutants for each 1,000 colonies tested (one mutant per 190 colonies). This result compares favourably with the yields obtained by Pontecorvo et al. (1953) using the total isolation method and recovering 5 to 10 mutants per 1,000 colonies tested.

Among the unclassified mutants having a total requirement 10 have been found to respond to p.aminobenzoate

TABLE 6

Mutation Experiments and the Isolation of Auxotrophic Mutants of *A. nidulans* by Replica Plating

Experiment	Culture		U.V. Irradiation			Replica Plating				
						Initial Plates			Auxotrophic mutants	
	Age* (days)	Viability of conidia (%)	Total conidia (x 10^7)	Exposure (Mins)	Survival (%)	No.	Total	Colonies per plate	Total	per 1000 colonies
y2;pyro4										
0	6+0	65.0	0.78	8-15	37.4-12.6	9	1108	123±40	4	3.6
A	6+3	42.5	0.74	15-18.5	8.7-1.6	5	511	102±46	2	1.2
B	6+6	33.3	1.10	16.5	4.4	14	2182	156±25	15	6.9
C	7+5	32.8	1.11	16	6.1	4	678	170±45	4	5.9
					Totals	32	4479	140±42	25	5.6
bi1;w3										
Alpha	6+0	82.0	1.22	9	5.9	16	1841	115±15	10	5.4
Beta	6+0	100.0	1.03	9	3.1	6	648	108±34	1	1.5
Gamma	6+0	68.6	0.90	8	10.7	15	3000	c. 200	9	3.0
						29	4350	c. 150	20	4.6
					Totals	66	9839	149	40	4.1

*The first figure is the period of incubation of the culture and the second the period of storage before harvesting the conidia.

(Obaid Siddiqui, personal communication). The rate at which mutants requiring this vitamin were isolated by replica plating was thus 11 in 30,835 colonies tested (1 in 2,803) and again is of the same order as was obtained by total isolation, 2 from 5,408 colonies tested (1 in 2,700) (Pontecorvo et al., 1953).

(2) The isolation of sugar mutants.

In the mutation experiments with y2; pyro4 colonies growing from conidia surviving U.V. irradiation were tested for their ability to utilise sucrose, sorbitol, maltose and lactose, while in the experiments with bi1; w3 they were tested for their growth on D. galactose in addition to these sugars. The mutants isolated and retained after testing are listed in Table 7. The three mutants failing to utilise fructose were isolated due to their failure to grow on sucrose or sorbitol (see next section). No mutants failing to utilise only sucrose were isolated among over 13,000 colonies tested. Mutants failing to utilise maltose were isolated least frequently (1 in 6,600 colonies tested), then the fructose mutants (1 in 4,400 colonies) and the lactose and maltose mutants (1 in 1,890 colonies), while mutants failing to utilise galactose were isolated most frequently (1 in 1,230 colonies). From the same series of initial plates auxotrophic mutants were isolated at the rate of 1 in 217 colonies tested (4.6 mutants per 1,000 colonies).

The growth of the original strains and the sugar mutants on the different carbohydrates is shown in Plates 2a to 2e.

TABLE 7

Sugar Mutants of *A. nidulans* Isolated by Replica Plating

Mutation Experiment	Total colonies on initial plates	Auxotrophic mutants	Sugar mutants						Designation of the mutants
			Fructose	Galactose	Lactose	Maltose	Sorbitol	Sucrose	
y2;pyro4									
A	511	2	0	-	0	0	0	0	
B	2182	15	2	-	1	1	0	0	fr1; fr2; lac1; mal 1.
C	678	4	0	-	0	0	0	0	
	3371	21	2	-	1	1	0	0	
bi1;w3									
Alpha	1841	10	1	1	0	0	2	0	fr3;gal 1 sb3, sb4.
Beta	648	1	0	0	1	0	0	0	lac2
Gamma	7350	29	0	7	5	1	5	0	gal2-8; lac3-7 mal2; sb5-9
	9839	40	1	8	6	1	7	0	
Totals	13,210	61	1	8	7	2	7	0	
Proportion of mutants among colonies tested		1:217	1:4,400	1:1,230	1:1,890	1:1,600	1:1,890	0	

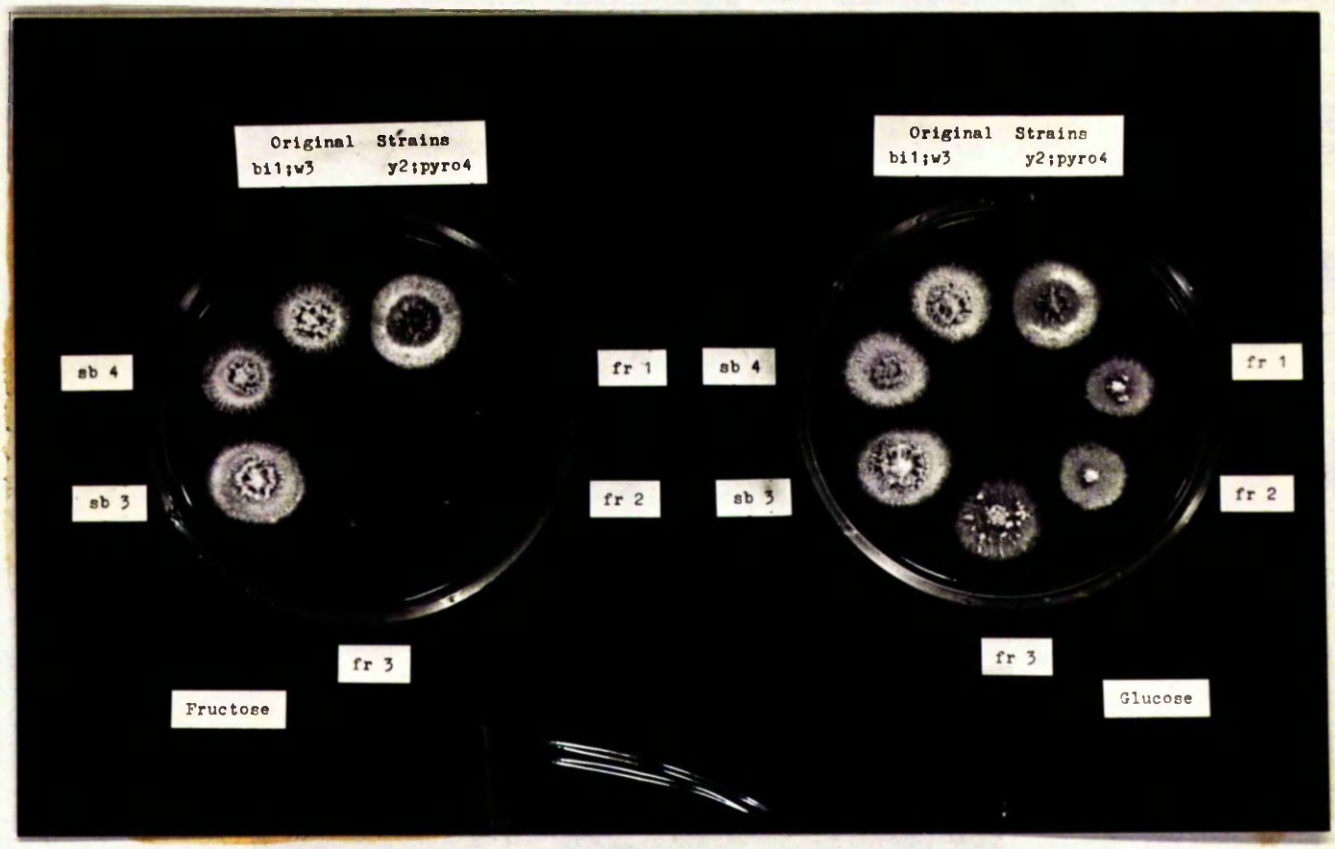
PLATE 2a Growth of the original strains and fructose mutants on fructose or glucose. The plate also shows that the sorbitol mutants are able to utilise fructose for growth.

Colonies originate from stab inocula of a mass of conidia of the strains indicated. Media are Basal Medium + fructose or glucose + biotin and pyridoxin.

Three days incubation at 37° C.

PLATE 2b Growth of bi1;w3 and the sorbitol mutants on sorbitol or glucose. Two of the mutants are total mutants (sb3 and sb5) and the remainder partial mutants.

Basal Medium + sorbitol or glucose + biotin. After 3 days at 37° C.


Original Strains
bi1;w3 y2;pyro4
sb 4
sb 3
fr 3
Fructose
fr 1
sb 4
fr 2
sb 3
Original Strains
bi1;w3 y2;pyro4
fr 1
fr 2
fr 3
Glucose

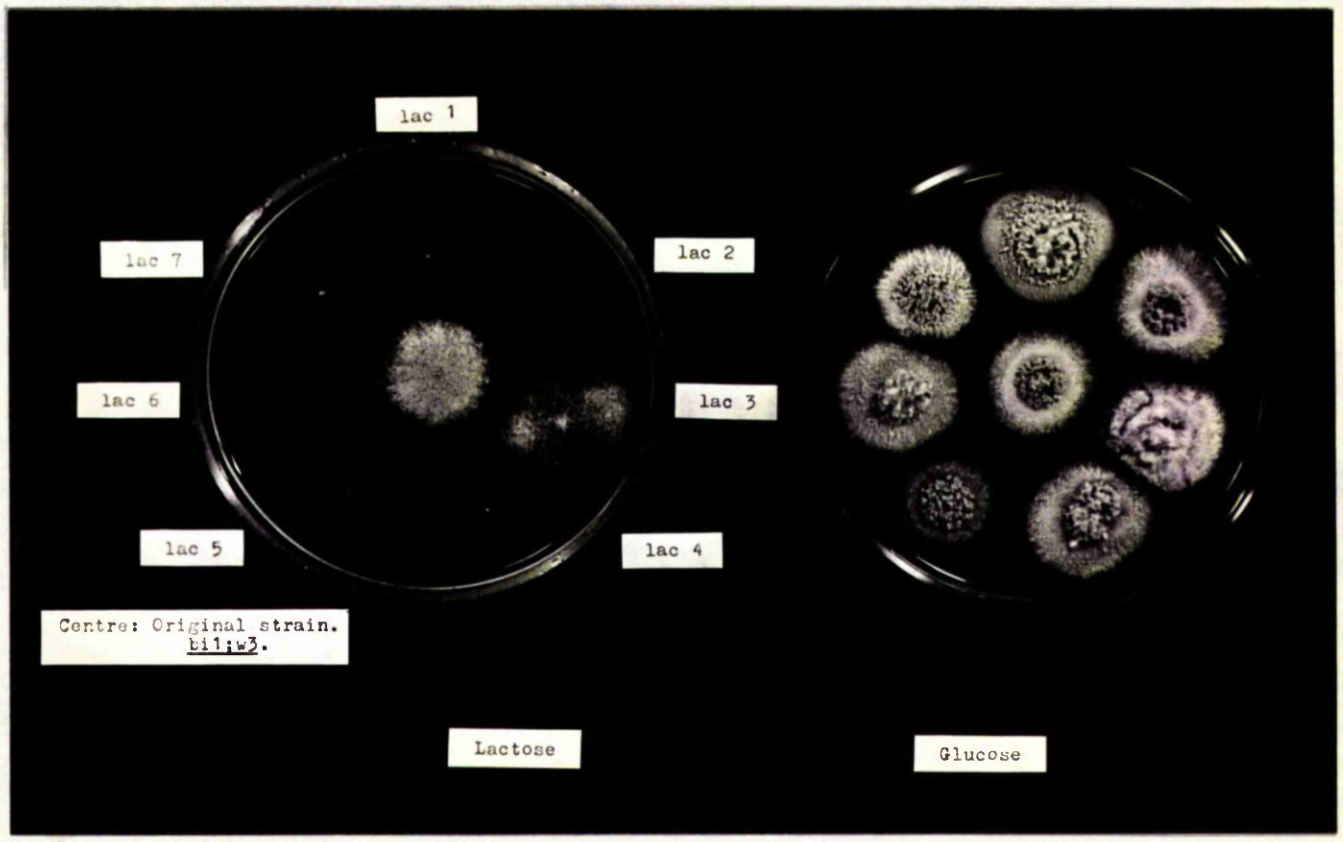

Original strain
bi1;w3.
sb 9
sb 8
sb 7
sb 6
Sorbitol
sb 3
sb 4
sb 5
Glucose

PLATE 2c Growth of bi1;w3 and the Lactose mutants on lactose or glucose. Sparse mycelium is formed by all of the mutants but is not visible for some of them in the photograph.

Basal Medium + lactose or glucose + biotin. After 4 days at 37° C.

PLATE 2d Growth of the original strains and maltose mutants on maltose or glucose.

Basal Medium + maltose or glucose + biotin and pyridoxin. After 3 days at 37° C.


lac 1
lac 2
lac 3
lac 4
lac 5
lac 6
lac 7
Centre: Original strain.
bi1;w3.
Lactose
Glucose

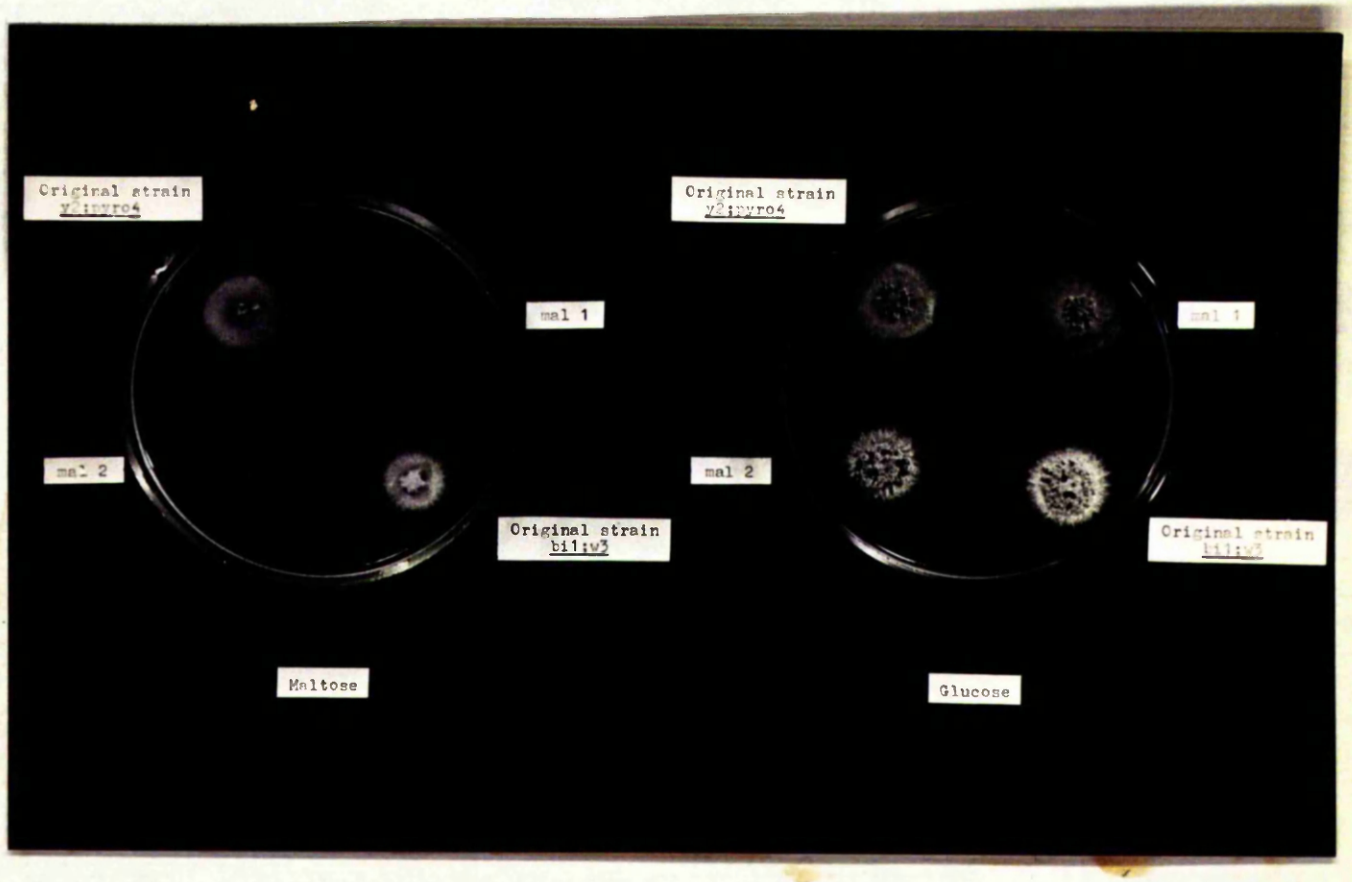
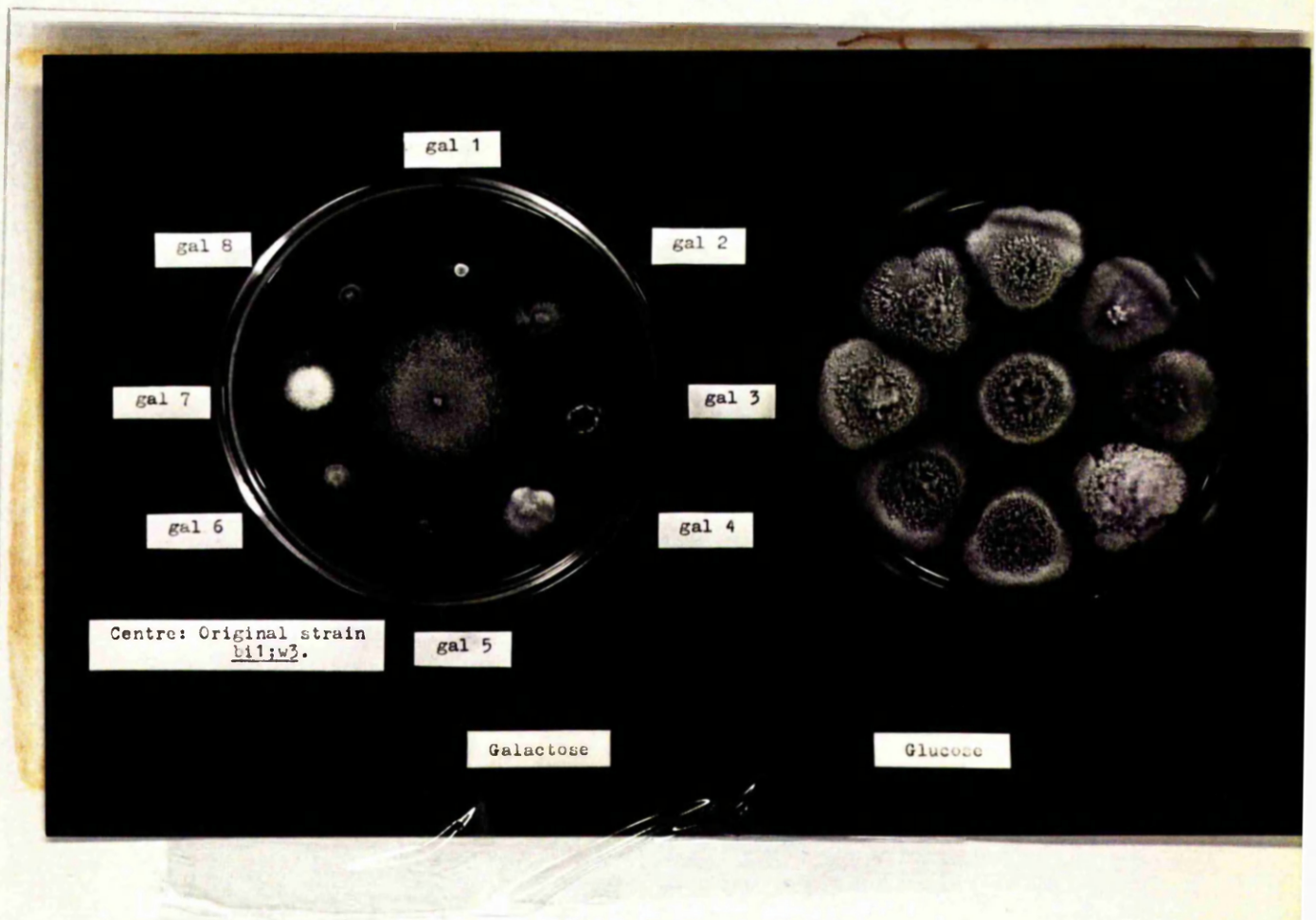

Original strain
y2;pyro4
mal 1
mal 2
Original strain
bi1;w3
Maltose
Original strain
y2;pyro4
mal 1
mal 2
Original strain
bi1;w3
Glucose

PLATE 2e Growth of bi1;w3 and the galactose mutants on galactose or glucose.

Basal Medium + galactose or glucose + biotin. After 4 days at 37° C.


gal 1
gal 8
gal 2
gal 7
gal 3
gal 6
gal 4
Centre: Original strain
bi1;w3.
gal 5
Galactose
Glucose

(3) The growth of the sugar mutant with different sugars as carbon source.

In attempting to separate the mutants phenotypically and to test for possible multiple mutants a number of the mutants were examined for their ability to utilise a range of different carbohydrates for growth. These tests were not extended beyond the range of carbohydrates immediately available. Conidia of the mutants were inoculated on to plates containing basal medium with 1% carbohydrate and the nutrients required by the original strain. The results are qualitative and are shown in Table 8. The mutants either grow like wild type on the sugars tested, though sometimes yielding slightly less vigorous growth, or show one of three characteristic phenotypes. In one the conidia germinate but fail to grow beyond this stage, these are termed total mutants. (In no case did conidia of a mutant fail to germinate). In a second the conidia germinate and form a very sparse colony which produces few conidial heads, such 'starvation' growth is typical of Aspergillus when growth is limited by a nutrient, and these mutants are called partial mutants. Only a few instances of a third type of mutant were met and these are all mutants for galactose utilisation, in which the conidia germinate and produce a conidiate colony but the growth of the colony is slow compared with the original type and its morphology is abnormal. These mutants are termed slow growing mutants. Generally the mutants only showed a mutant phenotype when grown on one particular sugar, but three exceptions were recorded.

TABLE 8

The Growth of the Sugar Mutants with Different Sugars as Carbon Source.

Sugar mutants	Carbon Source						
	Galactose	Lactose	Maltose	Sorbitol	Sucrose	Fructose	Glucose
Fructose							
fr1;fr2;fr3;	(+)	(+)	+	-T	-T	-T	+
Sorbitol							
sb3	+	+	+	-T	(+)	+	+
sb4	+	+	+	-P	(+)	+	+
sb5				-T		+	+
sb6)							
sb7)				-P		+	+
sb8)							
sb9)							
Maltose							
mal 1	+	+	-P	+	(+)	+	+
mal 2			-P				+
Lactose							
lac 1	+	-P	+	+	(+)	+	+
lac 2	+	-P	+	+	+	+	+
lac 3)							
lac 4)	+	-P					
lac 6)							
lac 7)							
lac 5	+	-P					
Galactose							
gal 1	-T	(+)	+	+	+	+	+
gal 6	-T	(+)					+
gal 2)							
gal 4)	-S	+					+
gal 7)							
gal 3	-S	(+)					+
gal 5; gal 8	-T	-P					+

Key + Wild type growth (+) Grows, but not as well as wild type.
- Mutants -S Slow growing mutants.
-T Total mutant, no growth after germination
-P Partial mutant, partial growth yielding sparse colonies which form few conidia.

Fructose mutants.

Three mutants, one initially isolated and described as a sucrose mutant and two as sorbitol mutants, were found to yield similar mutant phenotypes when tested for growth on sucrose or sorbitol. It is probable that sorbitol is normally metabolised by reduction to fructose which suggested that the three mutants are defective in their metabolism of fructose and this was found to be the case for the three mutants all failed to grow on fructose which is a good carbon source for wild type. It appears that the mutants are inhibited by fructose (or a metabolic product of fructose) for they fail to grow on media containing glucose to which small amounts of fructose are added.

Sugar per plate (about 20 ml. medium)		Growth of the fructose mutants
Glucose	+ Fructose	
0.20 g.	0 g.	+
0.18	0.02	- T
0.10	0.10	- T
0.02	0.18	- T
0	0.20	- T

It appears that growth is inhibited on media containing carbohydrates that either give rise to fructose or contain fructose as part of the molecule. It is not clear why these mutants should yield reduced growth on galactose or lactose.

Maltose mutant.

It was noted that one of the maltose mutants (mal 1)

did grow as well as wild type on sucrose though growing normally on lactose. This could indicate that mal 1 is defective in an alpha-glucosidase (maltose and sucrose are alpha-glucosides; lactose a beta-glucoside).

Galactose mutants.

Most of the galactose mutants grow somewhat less vigorously than the original type on lactose and two of them (gal5 and gal8) yield partial growth. This may be due to failure to utilise the galactose residue contained in lactose. It is apparently not due to inhibition of the mutants by galactose or a galactose derivative for all the mutants grow normally on mixtures of glucose + galactose and glucose + lactose.

It was also noted that all of the lactose mutants grow normally on galactose and on mixtures of galactose + glucose, galactose + lactose or lactose + glucose. These observations indicate that the mutants probably lack beta-galactosidase.

4. Morphological abnormalities of the sugar mutants.

The recent observations that certain strains of bacteria lyse when grown in the presence of galactose (Fukasawa and Nikaido, 1959) though closely related organisms grow normally with galactose in the medium and can utilise the sugar as a carbon source, prompted investigation of the mycelium formed by the sugar mutants of A.nidulans. Conidia of the mutant strains were inoculated on basal medium with either

1% glucose or 1% of the sugar that reveals the mutant phenotype as carbon source and also the nutrients required by the strains. The mycelium growing on the two carbon sources were examined after 3 days incubation using fresh squash preparation mounted in lacto-phenol. The mutants bi1;w3;fr1, bi1;w3;sb3 and sb4, and bi1;w3;gal1 to gal5 were examined, but the remaining mutants (maltose and lactose mutants) were not investigated. (The investigation was done after the genetic analysis had been completed and one mutant representing each of the loci discovered was selected for examination).

bi1; w3; sb3 and bi1; w3; sb4

Both strains produce hyphae which appear similar with either glucose or sorbitol as carbon source. These hyphae do not obviously differ from those of bi1;w3 on sorbitol, though the colonies do, of course, differ markedly.

bi1; w3; fr1

The hyphae of this strain growing with glucose as carbon source were indistinguishable from those of the original strain. The original strain bi1;w3 also produced normal hyphae with fructose as carbon source but the hyphae of the mutant were strikingly abnormal and a photomicrograph of the abnormal hyphae is reproduced (Plate 3a). Normal hyphae were not photographed, they are of uniform diameter, except where hyphal tips are developing into conidial heads, and branch repeatedly. The hyphae growing on fructose were of the same dimensions and general appearance as those on glucose, but many swellings were

PLATE 3a Abnormal mycelium formed by bi1;w3;fr1 when inoculated on Basal Medium + fructose + biotin. The swollen vesicles are not formed by the original strain on ~~galactose~~ fructose or by the mutant when grown on glucose.

Magnification x 1500

PLATE 3b Abnormal mycelium formed by bi1;w3;gal5 when inoculated on Basal Medium + galactose + biotin. Terminal and inter-calary vesicles are typical of growth of the mutant on galactose.

Magnification x 1500

observed both along the length of the hyphae (inter-calary) and terminally. The vesicles formed are of 3 to 5 times the width of normal hyphae when inter-calary, and from 5 to 15 times the width when terminal. They are fragile and apparently empty for no visible material was released when they were broken by pressure on the cover slip. The terminal vesicles could represent abortive conidiophores, while the inter-calary vesicles often appeared to have been formed terminally and become sub-terminal due to development of a hypha from the swelling.

bi1; w3; gal1 to gal5

The galactose mutants differ considerably among themselves in the structure of the hyphae formed during growth with galactose. The mycelium of the slow growing mutant bi1;w3;gal4 was but little different from the original strain, while that of the total mutant bi1;w3;gal5 was markedly abnormal (see photomicrograph, Plate 3b). One of the mutants (gal3) produced unusual hyphae in growth on glucose. The abnormalities can be described under four headings:-

1. Diameter of the hyphae. A micrometer eyepiece was used to measure the diameter of the hyphae formed by the mutants on glucose or galactose. It was generally found that the hyphae formed on galactose were about twice the diameter of those of the mutants on glucose or of the original strain on galactose.

2. Vesicles. Vesicles, similar to those formed by bi1;w3;fr1 on fructose were observed in growth on galactose. The vesicles were about 3 times the diameter of the hyphae.

3. In some mutants hyphae were seen consisting of many short cells, that is, there was an apparent excess of cross wall formation or the cells had failed to elongate.

4. In some mutants a brown pigment was observed which was cell localised and occurred mainly in the short cells. The pigment is apparently associated with formation of the cross walls.

The observations made on each of the galactose mutants are listed in Table 9.

TABLE 9

Abnormal Hyphae Formed by Galactose Mutants of A. nidulans in Growth on Galactose

Mutant	Growth * on galactose	Conidium formation	Hyphae formed on galactose			
			Diameter ** of hyphae	Vesicles	Cross-walls	Brown Pigment
gal 1	-T	Very few	Normal	Present, mainly intercalary	Normal	0
gal 2	-S	Few	x2	Present, mainly intercalary	Many	+
gal 3	-S	None	x2	Few, intercalary	Many	+
gal 4	-S	Few	x2	Very few, intercalary	Normal	0
gal 5	-T	None	x2	Present, intercalary and terminal	Normal	0

Key:- * T = total mutant; S = slow growing mutant

** Compared to diameter of hyphae of mutant growing on glucose.

Discussion.

The time course for the killing of conidia of A.nidulans by U.V. irradiation follow exponential curves which fall from zero time without initial plateaux. Curves of this sort are typical for killing by inactivation of a single-hit target (Norman, 1951), and this result agrees with the known uninucleate and haploid condition of the conidia (Pontecorvo et al., 1953). It is interesting to note that differently coloured conidia are inactivated by U.V. light at different rates. The resistance of the yellow conidia relative to the white probably results from a masking effect of the yellow pigment which may serve to reduce the amount of radiant energy penetrating the conidia. This is supported by data of Kilby (1960) which show that pigments extracted from yellow conidia absorb in the U.V. region.

Standardisation of the procedure for the irradiation experiments has provided reasonable control over the rate of killing in different experiments which has in turn contributed to the efficiency of the replica plating technique. The technique is discussed in the published paper. The main conclusions were that it isolates a similar spectrum of mutants as does the method of total isolation and with comparable efficiency. (This is assuming that the populations tested were themselves of comparable composition). Further results have supported these conclusions and in the case of one particular type auxotroph (requiring para-aminobenzoate) it

was found that the rate of isolation was the same by both techniques.

The sugar mutants were isolated at rates of between 1 in 1,000 and 1 in 5,000 among the conidia surviving U.V. irradiation. There are few published data on the rates at which fermentation mutants of yeast or bacteria are recovered, in E.coli non-fermenting mutants were isolated at rates of the order of 10^{-4} or less (Lederberg, 1947) but in one case with Saccharomyces mutation from non-fermenter to fermenter was as high as 10^{-2} after X-ray irradiation (Winge and Roberts, 1948). However data are highly heterogenous with respect to the induction of mutants and their isolation so that comparison of rates has little validity.

Mutants suffering a general defect in carbon metabolism (Lederberg et al., 1951) were not isolated but they probably would not have been distinguishable from auxotrophic mutants in the screening procedure employed and would have been scored accordingly. Mutants defective in the utilisation of glucose were not recovered either though they should be detected by the screening technique if they had occurred. It was pointed out earlier that it is unlikely that mutants failing to grow on sucrose should be isolated unless they are defective in a specific 'sucrose permease'. If such a system exists in A.nidulans the present experiments failed to detect mutants defective in this function.

It is possible that the mutants isolated may fail

to grow with particular sugars as carbon source for reasons other than failure to utilise the sugar. It is unlikely that any of the mutants have become sensitive to substances present as impurities in the sugars for the mutants (apart from the fructose mutants) grow on mixtures of glucose and the sugar in question while they retain the mutant character when tested on media containing the re-crystallised sugars. Other indirect effects could account for an apparent failure of the mutants to utilise the sugars and though unlikely they cannot be entirely discounted until the absence of specific enzymes of carbohydrate metabolism is demonstrated in the mutants.

The mycelium of moulds grown under conditions of nutritional deficiency or low oxygen tension often show morphological abnormalities similar to those described for the fructose and galactose mutants (Foster, 1949 page). Anaerobic conditions cannot, of course, exist in plate cultures and the production of abnormal hyphae due to carbohydrate starvation also seems unlikely for not all of the mutants showed the effect. It is possible that the mutants suffer an impairment of wall synthesis analogous to galactose induced lysis in certain bacteria (Fukasawa and Nikaido, 1959; Yarmolinsky and Weismeyer, 1959). The cell walls of Aspergilli are known to contain chitin an acetyl-glucosamine polymer (Cochrane, 1958) but it is not known if other carbohydrate residues are present. This is not unlikely in consideration of the complex composition of bacterial cell walls (Salton, 1956).

Summary

1. A replica plating technique for A.nidulans is described.

2. The technique isolates the same general spectrum of auxotrophic mutants as the method of total isolation and is of equal efficiency.

3. Altogether 25 mutants failing to utilise specific carbohydrates for growth were isolated by replica plating following U.V. irradiation.

Chapter 4 Segregation of the Ability to Utilise Specific Carbohydrates for Growth.

The first step in the genetic analysis of a mutant is to test if the difference between mutant and wild phenotypes is determined by a difference in Mendelian factors. In a haploid organism such as A.nidulans which possesses a normal sexual cycle this may be done as the result of a single cross between the mutant and wild type strains. The cross is analysed by the technique of perithecium analysis which is necessary because the organism is homothallic and some of the perithecia are formed by selfing (Hemmons, Pontecorvo and Bufton; 1953).

Crosses were set up between the sugar mutants and suitable stock strains to test the segregation of the mutant phenotypes and also to isolate recombinants required in further analysis. All of the crosses were fertile and individual perithecia were tested for hybridity by sampling the ascospores and observing the appearance of recombinant progeny for conidial colours. Ascospores from hybrid perithecia were plated at high dilution on C.M. and the resulting colonies classified for their nutritional requirements and ability to utilise the relevant sugars for growth.

The results for the segregation of the sugar markers are shown in Table 10. In two sets of crosses only sufficient progeny were tested to isolate the desired recombinants and the results of these crosses have been pooled, this is not properly valid for the data are not homogeneous, but it is sufficient to

TABLE 10

Segregation of the Ability to Utilise Carbohydrates in *A.nidulans*

Cross	Growth of progeny on sugars		Test of 1:1 segregation		
	Mutant	Wild type	X^2	D.F.	P
y2;pyro4;lac1 x bi1;w3	66	60	0.276	1	0.70-0.50
* y2;pyro4 fr1 x ribo1 bi1; Acr1 w3 thi4 ad3	78	100	2.719	1	0.10-0.05
y2;pyro4 fr2 x bi1;w3 ad3	48	60	1.333	1	0.30-0.20
y2;pyro4;mal1 x bi1;w3 ad3	44	52	0.666	1	0.50-0.30
bi1;w3;sb3 x ad14 paba1 y	7	3			
bi1;w3;sb4 x ad14 paba1 y	6	5			
bi1;w3;sb5 x ad14 paba1 y; meth1	7	7			
" " sb6 x "	3	5			
" " sb7 x "	7	12			
" " sb8 x "	10	16			
" " sb9 x "	12	11			
Totals	52	59	0.441	1	0.70-0.50
bi1;w3;gal 1 x paba1 y ad20	7	18			
" " gal 2 x "	9	6			
" " gal 3 x "	8	19			
" " gal 4 x "	14	11			
" " gal 5 x "	11	13			
" " gal 6 x "	5	9			
" " gal 7 x "	3	8			
" " gal 8 x "	9	7			
Totals	66	91	3.981	1	0.05-0.02

* Linkage detected between fr1 and pyro4

demonstrate 1 : 1 segregation which was confirmed in further analysis. Linkage was detected between a sugar mutant and a nutritional marker in one of the crosses.

All of the mutant phenotypes segregate as if determined by Mendelian factors and the 1 : 1 segregation of mutant to wild phenotypes for growth on specific carbohydrates indicates segregation of pairs of alleles.

Discussion.

The results obtained in the preliminary crossing experiments demonstrate that the ability to metabolise certain carbohydrates is genetically determined in the mould A.nidulans and the mutants failing to utilise specific carbohydrates are all shown to differ from wild type at single genetic loci.

This is essentially the same result as observed in bacteria in which strains fermenting a specific carbohydrate differ from non-fermenting strains at a single locus (Lederberg, 1947; Lederberg et al., 1951; Zinder and Lederberg, 1952; Hotchkiss and Marmur, 1954; Morse, Lederberg and Lederberg, 1956; Lederberg, 1960) and stands in distinction to the results obtained for yeasts in which the ability to ferment different carbohydrates is controlled by a number of series of polymeric genes (Winge, 1952; Roberts, Ganesan and Haupt, 1959). This difference between the genetic control of carbohydrate metabolism in yeasts or in the bacteria and A.nidulans results from the origin of the strains employed in the recombination experiments. In bacteria and in A.nidulans the non-metabolising strains have

been derived by single step mutations, whereas the strains employed in yeast genetics are of natural origin and are regarded as species of yeasts (Lodder and Van Rij, 1952).

Demonstration of the genetic control of carbohydrate utilisation in the mould A.nidulans opens the possibility of using such characters as genetic markers. The full genetic analysis of the sugar mutants was therefore undertaken to explore this possibility and to characterise the sugar mutants as genetic markers.

Chapter 5. Genetic Analysis of the Sugar Mutants.

1. Outline of Procedure.

The first step in the genetic analysis of a number of mutants which fail to utilise a specific carbohydrate is to separate the mutants into groups according to the loci that they occupy. The mutants have then to be located precisely in the genetic maps of the organism and this is most economically done in two stages. The first makes use of the parasexual cycle (Pontecorvo, 1954) and the technique of mitotic haploidisation (Pontecorvo, Tarr Gloor and Forbes, 1954; Pontecorvo and Kafer, 1958) to allocate each of the mutants to a linkage group. The second stage is standard analysis through the sexual cycle to map the mutants in relation to other markers already known in each of the linkage groups. Usually the mutants are 'loss' mutants and they are therefore likely to be recessive to wild type, the cases in which this assumption is wrong will be quickly revealed in the course of the analysis.

2. Methods.

A. Tests of allelism.

Allelism is the relationship between genetic elements which may be specified either in terms of genetic structure or in terms of function (Pontecorvo, 1952a; 1956). Recessive mutants determining similar phenotypes may be subjected to two tests which investigate their structural relationship by testing for recombination between the mutants or their functional relationship by testing for complementarity of the mutants. Mutant alleles at loci which recombine at a readily detectable frequency (more

than 5%) and are therefore not located close together in one linkage group are almost always complementary, that is organisms heterozygous at both loci have a non-mutant phenotype. This was the general result of classical genetic analysis at low resolving power where allelic mutants do not recombine and are non-complementary, while non-allelic mutants recombine and are complementary.

The functional test for allelism of mutants located within less than one map unit was developed by Roper and Pontecorvo in the analysis of genetic fine structure (Roper, 1950; Pontecorvo, 1952a; 1956; 1959) based on the ideas of Muller (Raffel and Muller, 1940) and the results of experiments of Lewis (1950, 1951) with Drosophila. Recessive mutants which determine similar phenotypes and are located close together in the chromosome are tested for the phenotype of the heterozygote resulting from combination of the mutants either in a cis arrangement x1x2/++ or in a trans arrangement x1+/+x2 (Haldane, 1942). The cis arrangement serves as a control and in all cases determines a non-mutant phenotype for one chromosome is non-mutant and the result of the test depends upon the phenotype of the trans heterozygote. If this phenotype is nearer to wild type than those of the two homozygotes, the mutants are supposed to be defective in different functions and are therefore classified as non-allelic. However if the phenotype of the trans heterozygote is mutant or close to mutant the mutants are supposed to be defective in the same function and are therefore classified as allelic. A number of mutants which are non-complementary when tested a pair at a time define a functional

unit of allelism and the use of the complementarity test in this way was of vital importance to the development of the modern concept of gene structure (Pontecorvo, 1952a; 1956; 1959). The use of the test was the basis of Benzer's definition of the 'cistron' as a region of chromosome within which mutants are non-complementary (Benzer, 1957).

The 'cistron' concept breaks down as the result of an inconsistency first described by Calef (1956) in Aspergillus nidulans and confirmed by Martin-Smith (1958). In these cases it was found that particular pairs of mutants, all non-complementary to several other mutants, were nevertheless complementary to one-another. Several other instances of complementation of alleles (intra-locus complementation) were described soon after (Giles, Partridge and Nelson, 1957; Fincham and Pateman, 1957) and as larger numbers of combinations of mutants have been tested it has become clear that intra-locus complementation is the rule rather than the exception (Catcheside and Overton, 1958; Catcheside, 1960a; 1960b). Some loci probably do not exhibit complementation of alleles (Catcheside, 1960b; Hartman et al., 1960b) but typically one third of the mutants at a locus complement in at least one combination (Catcheside, 1960b). Non-complementarity of the majority of the mutants at a locus relates all the mutants to a single functional deficiency and can be used to rescue the cistron concept (Fincham, 1959; Catcheside, 1960b). However the term 'functional unit', defined as a region of the chromosome within which two mutants are non-complementary, either directly or indirectly through a third mutant, is perhaps preferable (Pritchard, 1960b).

A further possible failure of the complementarity test is suggested by a preliminary report (Lewis, 1960) in which two loosely linked mutants were non-complementary.

(1) Complementarity tests.

Both heterokaryons and heterozygous diploids of A.nidulans are readily synthesised (Pontecorvo et al., 1953; Roper, 1952) and may be used to perform complementarity tests. The results of tests done in both ways for one pair of mutants are generally the same though it is expected that differences may exist (Pontecorvo, 1952b).

Heterozygous diploids are isolated via the corresponding heterokaryons by means of Roper's technique (1952) and in testing pairs of sugar mutants for complementarity both the heterokaryon and the trans heterozygote were tested for their growth on the relevant carbohydrate. Where the test yielded ambiguous results further recombination tests were done.

(2) Recombination tests.

If x1 and x2 represent two mutants that fail to grow on the same sugar a cross between the mutants will yield progeny that are distributed among four classes:-

Class	Genotype		Phenotype Growth on carbohydrate	Frequency
Parental	x1	+	-	$\frac{1}{2}$ (1-p)
Parental	+	x2	-	$\frac{1}{2}$ (1-p)
Recombinant	x1	x2	-	$\frac{1}{2}$ p
Recombinant	+	+	+	$\frac{1}{2}$ p

The wild type recombinants (++) are distinguishable from the remaining classes by their ability to grow on the sugar and the frequency at which they occur may be used to estimate the recombination fraction (p) between x1 and x2 or to distinguish qualitatively whether x1 and x2 are closely linked or not.

The sugar mutants were out-crossed and recombinants with additional nutritional markers isolated. Crosses were then set up between the sugar mutants using strains with complementary nutritional markers. Generally the strains also had the markers for conidial colour arranged to permit identification of hybrid perithecia or the fraction of viable ascospores by the appearance of recombinants for conidial colour. In preliminary tests a suspension of ascospores from a single hybrid perithecium was plated on C.M. and about 200 of the resulting colonies classified for their ability to grow on the relevant carbohydrate. This method will detect recombinants occurring at a rate of more than about 1 in 100, and it is sufficiently sensitive for the purpose of the preliminary grouping of the mutants. It was also possible to estimate recombination frequencies of less than 1% by selection experiments in which wild type recombinants were detected by their growth on media in which the relevant carbohydrate was sole carbon source.

B. Location of Sugar Mutants in Linkage Groups by Mitotic Haploidisation.

Heterozygous diploid strains of A.nidulans undergo processes of mitotic segregation and recombination outside the sexual cycle (Pontecorvo, 1954) and two of these processes, somatic crossing over and mitotic haploidisation, can be used in formal

genetic analysis (Pontecorvo, Tarr Gloor and Forbes, 1954; Pontecorvo and Kafer, 1958). During haploidisation whole chromosomes segregate as units and homologous chromosomes are distributed at random among the resulting haploid strains, the process may thus be employed to assign genes to their linkage groups (Pontecorvo and Kafer, 1958) and an effective technique has been developed for this purpose (Forbes, 1959). Somatic crossing over permits deduction of the order of gene loci in a chromosome arm.

The technique of Forbes' (1959) depends upon the use of multiply marked 'tester strains' which have one marker in each of the known linkage groups and also markers permitting selection of hyphae containing haploid nuclei. Selection is required because mitotic segregation normally occurs infrequently and also as haploid segregants must be distinguished from diploid segregants. The efficiency of selection is considerably increased by 'double selection' for haploids containing two particular markers.

A diploid strain is synthesised between the tester strain and a suitable strain carrying the unlocated mutant x. Haploid strains arising from this diploid by mitotic haploidisation are isolated by the appropriate selective techniques, checked for ploidy by measurement of the diameter of their conidia (Pontecorvo, Tarr Gloor and Forbes, 1959) and classified for the markers included in the diploid. If the new mutant is located in one of the chromosomes for which selection is made the haploids will all be of one type with respect to x, but if x is located in one of the unselected chromosomes it will show strictly alternative

segregation with the marker defining that chromosome and random recombination with the remaining markers. This may be illustrated by consideration of a hypothetical diploid with three pairs of chromosomes defined by markers a, b, and c, and in which selection is made for a or a^+.

Diploid:- Tester strain

New mutant

Case 1		Case 11	
x in repulsion to a		x in repulsion to b	
$\frac{a \quad +}{+ \quad x}$	$\frac{b \quad c}{+ \quad +}$	$\frac{a}{+}$	$\frac{b \quad +}{+ \quad x} \quad \frac{c}{+}$
Selected	Unselected	Selected	Unselected

Consider the first case in which x is in repulsion to a. If selection is made for a^+ all of the haploids will be x, while they will all be x^+ if a is selected.

In the second case x is in repulsion to b. Selection of either a or a^+ will yield haploids segregating at random with respect to x/x^+ or c/c^+, but b will segregate only with x^+ and b^+ only with x.

Mitotic crossing over and haploidisation are rare events and thus the probability of both occurring in the same nuclear lineage in a limited period of growth is very small and may be ignored for practical purposes.

In locating the sugar mutants two tester strains (kindly made available to me by Mr. E.C. Forbes) have been used which together contain markers in all the eight linkage groups known in A.nidulans. The strains have the genotypes described below, the Roman numerals indicating the linkage groups (Kafer, 1958).

Tester strain A (Tester A)

I	II	III	IV	V	VII
su1ad20 y ad20	Acr1	phen2	pyro4	lys5	nic8

Tester strain C (Tester C)

I	II	VI	VIII
su1ad20 y ad20	Acr1	s3	ribo2

A diploid was synthesised between Tester A and bi1;w3;(x) (x representing the unlocated sugar marker) which had the genotype shown in Table 11 (Diploid 1), was prototrophic and formed green conidia. This diploid is not suitable for the selection of haploid segregants but an effecient double selection was made in either of two ways using a mitotic recombinant of Diploid 1 * which is homozygous for y ad20 and consequently requires adenine for growth and forms yellow conidia (Diploid 2, Table 11).

* Diploid 1 is used to test the dominance relationship of the sugar mutant to wild type.

TABLE 11

Location of Sugar Mutants by Mitotic Haploidisation.

(x represents the sugar mutant).

Diploid 1 (Green conidia: prototrophic)

1				11		111	1V	V	V11	?
su1ad20	y	ad20	+	+	Acr1	phen2	pyro4	lys5	nic8	+
+	+	+	bi1	w3	+	+	+	+	+	x

Diploid 2 (Yellow conidia: adenine requiring)

$$\frac{\text{su1ad20 y ad20 +}}{\text{+ y ad20 +}}$$

Linkage Groups 11, 111, 1V, V and V11 as above.

Selection	Haploid segregants	Location of x
Diploid 2		
su1ad20 y ad20	1. All x^+	Group 1 or 11
Acr1	2. Two class segregation of x with phen2, pyro4, lys5 or nic8	Group 111, 1V, V or V11.
	3. Segregation of x at random to all markers.	Not in groups tested.
Diploid 2		
su1ad20 y ad20	4. All x^+	Group 1
w3	5. All x. As 2 or 3 above.	Group 11
Diploid 1		
y sectors	6. All $bi1^+$ x^+. As 2 or 3 above.	Group 1
w sectors	7. All (y) $bi1^+$ x^+ or (y^+) bi1x	Group 1
	8. All x^+. As 2 or 3 above.	Group 11

Diploid 1 was synthesised, purified by plating for single colonies and replated on C.M. Diploid 2 was isolated by visual selection of yellow conidial heads appearing as sectors in the green colonies, it was also purified and checked for ploidy by measurement of the diameter of its conidia. Haploids were selected from Diploid 2 by two methods the first stage of which was common to both and was selection on an adenineless medium containing phenylalanine, pyridoxin, lysine and nicotinic acid. (Sugar markers are, of course, non-selective on glucose media and no additions were required with respect to these markers). A dilute suspension of conidia of Diploid 2 was spotted on the surface of the medium with a wire loop so that each spot contained only a few conidia. The conidia grow slowly on adenineless medium because su1ad20 is partially recessive, but hyphae with haploid nuclei containing the chromosome su1ad20 y ad20 are adenine independant and will grow at the normal rate. After two days incubation sparse aconidiate colonies had formed and a top layer of the same medium was added to select for adenine independant strains (Pritchard, 1955). In selection for adenine independance (su1ad20 y ad20) and acriflavine resistance (Acr1), acriflavine was included in the top layer and resistant colonies grew through to the surface after a further 5-7 days incubation. In selection for adenine independance and white conidia (w3) no acriflavine was included in the top layer but white conidial heads were selected visually from the colonies appearing at the surface of the medium after a further 3-4 days incubation. The segregants were streaked on C.M. and tested for ploidy (with experience most

of the haploid segregants may be recognised by direct examination). In both methods of selection a proportion of the segregants were diploid and prototrophic, having undergone further mitotic crossing over to become homozygous for su1ad20 and Acr1 or w3, but between one and two thirds of the isolates were haploid. The haploids were classified for x and the nutritional markers, and x located according to the distribution of markers (Table 11).

If all the haploids isolated by adenine-acriflavine selection were x^+ a second adenine-white selection was done to distinguish between location in group 1 or 11. If the analysis with Tester A showed that x was not located in any of the groups tested exactly the same procedure was repeated with Tester C, with the addition of thiosulphate and riboflavine to the selective media, to locate x in group V1 or V111.

In the cases when x was apparently located in group 1, that is when all the haploids obtained by both methods of selection were x^+, a third selection was done with Diploid 1 to confirm the location of x. Conidia of Diploid 1 were plated on C.M. and segregants appearing as yellow or white conidial heads sectoring in the green colonies selected visually. The segregants were streaked on C.M. tested for ploidy and the haploids classified. If x is located in group 1 it will segregate in coupling to bi1 (Table 11). With experience a surprisingly high proportion of haploid segregants (up to 80% of total isolates) were recovered in this way, though some diploids failed to yield many sectors. It appeared that incubation of the plates for five days or more favoured the appearance of the colour sectors.

The method of mitotic haploidisation is both powerful and economical in locating new genes in contrast to the establishment of linkage groups by meiotic analysis. However, like analysis through meiosis, the method is subject to a number of difficulties, the most important of which result from viability effects. If the new marker x has very poor viability on the selective media it may not appear among the selected haploids and all the haploid segregants will be x^+ with either type of selection. However the marker in coupling to x will be selected against and thus all the haploids will be of one type with respect to this marker as well and x^+.

Diploid $\frac{b\ +}{+\ x}$ Haploids $b\ x^+$ or $(b^+\ x)$ not recovered

Where viability effects occur caution is necessary in the interpretation of the segregations observed and recovery of haploids of the type $b\ x^+$ without the complementary type $b^+\ x$ is not sufficient evidence to finally locate x and b in the same chromosome.

C. Mapping Sugar Mutants by Meiotic Analysis.

The final stage of analysis was mapping the sugar mutants meiotically and this was done by the routine technique of perithecium analysis (Pontecorvo, Roper et al., 1953).

In some cases location was achieved by crossing strains containing most of the markers known in the linkage group under investigation, but with the larger linkage groups it was more

convenient to do several crosses each involving a few of the markers in the group. The strains employed were from the stock held in the Department of Genetics or were isolated from preliminary crosses. In certain instances new unmapped nutritional markers, located by other workers in the linkage group under investigation, were included in the crosses.

(1) Detection and estimation of linkage.

Methods given by Mather (1951) were followed. Deviation of the allele ratio from 1:1 was tested by X^2 and different procedures used in testing for linkage and estimation of recombination fractions according to whether the alleles show normal or disturbed segregations. The expressions used in making these tests and estimates are given below.

If x:+ and y:+ represent segregating factors a cross involving these factors will yield four classes of progeny and the numbers of progeny in each class may be represented as a1, a2, a3, and a4 and the total progeny by n.

Class.	x y	x +	+ y	+ +	
No. in class	a 1	a 2	a 3	a 4	Total = n.

Deviation of the segregation of the alleles from 1:1 was tested by expression:-

Segregation x:+ $X^2 = \frac{(a1 + a2 - a3 - a4)^2}{n}$ D.F. = 1

y:+ $X^2 = \frac{(a1 - a2 + a3 - a4)^2}{n}$ D.F. = 1

(a) Normal segregation

When both x:+ and y:+ did not deviate significantly from 1:1 (P = 0.05) linkage between x and y was detected by use of expression:-

$$\text{Linkage } x - y \qquad X^2_L = \frac{(a1 - a2 - a3 + a4)^2}{n} \qquad \text{D.F.} = 1$$

If linkage was detected (P = 0.05) the recombination fraction and standard deviation of the fraction were calculated.

$$\text{Recombination fraction, } p\ (\%) = \frac{(a2 + a3)}{n} \times \frac{100}{1} \quad \text{(Coupling)}$$

$$\text{or} \qquad = \frac{(a1 + a4)}{n} \times \frac{100}{1} \quad \text{(Repulsion)}$$

$$\text{Standard deviation of } p\ (\%) = \sqrt{p\left(\frac{1 - p}{n}\right) \times \frac{100}{1}}$$

(b) Disturbed segregations (One factor)

When either x:+ or y:+ deviated significantly from 1:1 segregation (P = 0.05) linkage was detected between x and y by use of the expression:-

$$\text{Linkage } x - y \qquad X^2_L = \frac{(a1\ a4 - a2\ a3)^2\ n}{(a1 + a2)\ (a3 + a4)\ (a1 + a3)\ (a2 + a4)}$$

$$\text{D.F.} = 1$$

If linkage was detected the recombination fraction and its standard deviation was calculated as for normal segregation.

(c) Disturbed segregations (Two factors)

When both x:+ and y:+ deviate from 1:1 it is necessary to analyse crosses with markers in both coupling and repulsion to test for linkage.

Classes for both coupling and repulsion data were summed and the table constructed

$A1 = a1 + a4$ $\qquad\qquad$ $A2 = a2 + a3$

	A2	A1	
Repulsion	AR2	AR1	
Coupling	AC2	AC1	Total = n

Linkage was detected using the expression

$$X^2_L = \frac{(AR2 \times AC1 - AR1 \times AC2)^2 n}{(AR2 + AR1)(AC2 + AC1)(AR2 + AC2)(AR1 + AC1)}$$

If linkage was detected the recombination fraction (p) was estimated from the expression

$$\frac{p^2}{(1-p)^2} = \frac{AR1 \times AC2}{AR2 \times AC1}$$

The standard deviation of p was calculated from

$$V_p = \frac{p^2 (1-p)^2}{h} \qquad \frac{1}{h} = \frac{1}{4} \times \left[\frac{1}{AR1} + \frac{1}{AR2} + \frac{1}{AC1} + \frac{1}{AC2}\right]$$

Standard deviation of $p = \sqrt{V_p}$

3. Results.

A. Fructose Mutants.

Three fructose mutants were isolated, one (fr1) was detected by failure to grow on sucrose, it was originally called suc1, and the other two (fr2 and fr3) by failure to grow on sorbitol, originally called sb1 and sb2 respectively. All three mutants are identical phenotypically failing to grow on either fructose, sucrose or sorbitol (Chapter 3, Table 8) and complementarity tests show that the mutants are allelic to one another but not allelic to either sb3 or sb4. (Table 12). The mutants therefore define a locus controlling fructose utilisation and it is called the fr1 locus.

In outcrossing one of the mutants y2;pyro4fr1 linkage was detected between pyro4 and fr1 and mitotic haploidisation confirmed location of fr1 in the same linkage group as pyro4 (Group 1V). Haploid strains isolated from the diploid Tester A/bi1;w3;fr1 by selection for adenine independance and acriflavine resistance showed the following segregation:-

Tester A/bi1;w3;fr1 Segregation of markers in 25 haploids.

	fr1	+		fr1	+
phen2	8	6	pyro4	0	9
+	8	3	+	16	0

	fr1	+		fr1	+
lys5	6	4	nic8	9	3
+	10	5	+	7	6

The diploid Tester A/bi1;w3;fr1 grows on fructose and therefore fr1 is recessive.

TABLE 12

Complementarity Tests of the Fructose Mutants.

Component strains	Heterokaryons		Diploids	
	Growth on		Growth on	
	Glucose	Sucrose* or Sorbitol	Glucose	Sucrose or Sorbitol
bi1;w3;*fr1* + y2;pyro4 *fr2*	+	-	Not isolated.	
bi1;w3;*fr2* + y2;pyro4 *fr1*	+	-	Not isolated.	
y2;pyro4*fr1* + bi1;w3; *fr3*	+	-	+	-
y2;pyro4*fr2* + bi1;w3; *fr3*	+	-	+	-
y2;pyro4*fr1* + bi1;w3; *sb3*	+	+	Not synthesised	
y2;pyro4*fr1* + bi1;w3; *sb4*	+	+	Not synthesised	
y2;pyro4*fr2* + bi1;w3; *sb3*	+	+	+	+
y2;pyro4*fr2* + bi1;w3; *sb4*	+	+	+	+

KEY: + = Growth; wild type phenotype

\- = No growth; mutant phenotype

* The same result was observed with or without the addition of nutrients required by the component strains.

A cross was made between the strains shown in Table 13 and the fr1 locus mapped meiotically by single perithecium analysis. The result is shown below.

Group IV

meth1		fr1		pyro4
	21.0 ± 2.3		40.0 ± 2.8	

Therefore it is shown that three allelic mutants isolated which fail to utilise fructose for growth identify a locus concerned with the control of fructose metabolism. The locus is called the fr1 locus and is located in linkage group IV. No further analysis of the mutants was attempted.

TABLE 13 Location of fr1 by Meiotic Analysis.

Cross	pro1	paba1	y	+	+	meth1	+	pyro4
	+	+	+	bi1	w3	+	fr1	+
						a		b

Genotypes of ascospores			Totals		Cross-overs
meth1	+	pyro4	70	147	None
+	fr1	+	77		
+	+	pyro4	22	38	Singles, a
meth1	fr1	+	16		
meth1	+	+	54	97	Singles, b
+	fr1	pyro4	43		
meth1	fr1	pyro4	10	27	Doubles a and b
+	+	+	17		
		Total		309	

Table of values of X^2

	X^2	D.F.	Probability
Segregation:-			
meth1 : +	0.262	1	0.70 - 0.50
fr1 : +	0.935	1	0.50 - 0.30
pyro4 : +	1.168	1	0.30 - 0.20
Linkage:-			
meth1 - pyro4	4.922	1	0.05 - 0.02
meth1 - fr1	20.197	1	< 0.01
fr1 - pyro4	12.042	1	< 0.01

Recombination fractions

meth1 - fr1	21.0 ± 2.3%
fr1 - pyro4	40.0 ± 2.8%

B. Sorbitol Mutants.

The sorbitol mutants are distinguished from the fructose mutants by their ability to grow on fructose. The seven mutants isolated are of two distinct phenotypes, spores of two of the mutants (sb3 and sb5) germinate on sorbitol but fail to grow, while those of the remaining five mutants (sb4, sb6, sb7, sb8 and sb9) yield partial growth forming characteristic sparse colonies with little sporulation. This phenotypic difference and complementation of sb3 and sb4 in the heterozygote suggested that the mutants are at different loci and the first experiments done to locate sb3 and sb4 were based on this possibility. Diploids synthesised between Tester A and bi1;w3;sb3 or bi1;w3;sb4 failed to locate either of the mutants, but mitotic haploidisation of the diploids with Tester C located both sb3 and sb4 in group VI (Table 14). (sb9 was also located in group VI by mitotic analysis). All of the diploids grow normally on sorbitol showing that sb3 and sb4 (and also sb9) are recessive.

A cross between sb3 and sb4 was analysed (the parent strains are shown in Table 15). Ascospores contained in a single hybrid perithecium were plated on C.M. and the resulting colonies tested for their ability to grow on sorbitol. The two mutants are closely linked for of 189 colonies tested none grew as wild type, 91 were phenotypically sb3 and 98 phenotypically sb4.

The same cross was used in a selection experiment (Table 15). A number of perithecia were selected, cleaned by rolling on hard agar and tested for hybridity. The ascospores contained

TABLE 14

Location of sb3 and sb4 by Mitotic Haploidisation.

Distribution of markers in haploid segregants.

Tester A/bi1;w3;sb3

17 haploids

	sb3	+
phen 2	6	9
+	0	2
pyro4	4	5
+	2	6
lys5	5	4
+	1	7
nic8	0	0
+	6	11

Tester A/bi1;w3;sb4

19 haploids

	sb4	+
phen 2	7	9
+	2	1
pyro4	5	5
+	4	5
lys5	7	4
+	2	6
nic8	0	2
+	9	8

Tester C/bi1;w3;sb3

11 haploids

	sb3	+
s3	0	5
+	6	0
ribo2	4	4
+	2	1

Tester C/bi1;w3;sb4

46 haploids

	sb4	+
s3	0	25
+	21	0
ribo2	12	17
+	9	8

TABLE 15

A Selection Experiment to Estimate the Recombination Fraction Between sb3 and sb4

Cross $\frac{ad14\ paba1\ y\ +}{+\ +\ +\ bi1} \times \frac{+}{w3} \times \frac{sb3\ +}{+\ sb4}$

Control platings

Perithecium	Plating		Colonies per plate	
	Dilution	Volume	Replicates	Mean
1	10^{-1}	0.1	30, 39, 41	37
2	10^{-1}	0.1	39, 43, 57	47

Selective platings

Perithecium	Plating		No. of Plates	Estimated No. viable spores	Colonies growing on sorbitol
	Dilution	Volume			
1	10^{-1}	1.0	7	2562	5
	10^{-2}	1.0	21	790	12 (+ 1 diploid)
2	10^{-1}	0.1	8	373	7
	10^{-2}	0.5	8	186	1
			Totals	3911	25

Recombination fraction $= \frac{25 \times 2}{3911} \times \frac{100}{1} = 1.27\%$

in two hybrid perithecia were separately suspended in about 10 ml. of saline. Samples of 0.1 ml. of 1/10 dilution of the spore suspensions were mixed with about 20 ml. of cool molten M.M. containing the nutrients required by the parent strains and plated. These plates served as controls to estimate the number of viable ascospores in each suspension. Further samples were plated in the selective medium which was of the same constitution as the control medium except that the glucose was replaced by 1% sorbitol.

Altogether 26 colonies growing like the wild type in the selective medium were isolated and were classified for their nutritional requirements. One of the colonies was prototrophic and proved to be diploid on measurement of the diameter of its conidia. The remaining 25 colonies were haploid, segregated 1:1 for the unselected markers, and yield an estimate 1.27% for the recombination fraction between sb3 and sb4. This fraction is a rough estimate for the data are not homogeneous, moreover it is assumed that the 25 strains growing on sorbitol are haploids though in fact some may be disomic for chromosome VI and consequently able to utilise sorbitol because sb3 and sb4 are complementary. In this case the estimated recombination fraction will be too high.

The seven mutants were tested for complementarity using heterokaryons and heterozygotes (Table 16). The result for the heterozygote test is ambiguous and it is not possible to decide if the three complementing groups of mutants represent

TABLE 16 Complementarity Tests of the Sorbitol Mutants.

Diploids

ad14	paba1	y	+	+	sbx	+
+	+	+	bi1	w3	+	sby

ad14 paba1 y; sbx

bi1;w3; sby	3	4	5	6	7	8	9
3	-T*	+	-T**				
4		-P*					
5	-T**	+					
6	+	+	+				
7	+	-P	+	+			
8	-P	-P	-P	-P	-P		
9	+	+	+	-P	+	-P	

Key

\+ Wild type growth. Complementary mutants.

\- Mutant growth. Non-complementary mutants.

-T No growth.

-P Partial growth.

Complementation Groups

sb3; sb5	sb4; sb7	sb6; sb9

sb8

* Diploid not synthesised. The heterokaryon fails to grow sorbitol

** Diploids not obtained. Heterokaryons fail to grow on sorbitol with or without nutrients required by component strains.

different cistrons spanned by a deletion (sb8) or if the mutants represent a single functional unit defined by sb8 and within which intra-locus complementation occurs. For convenience the region is termed the sb3 locus. An apparent difference noted between complementation of the mutants in heterokaryons or heterozygotes and the possible relationship of the mutants is discussed in Chapter 6.

Crosses which were made between strains with sb3 or sb4 and other markers in linkage group V1, including the sugar marker lac1, are described in the section on lac1 (Table 18). No linkage was discovered between sb3 and lac1 or between sb3 and two (s3 and nic10) of the three markers previously known in group V1. The third marker lys1 an X-ray induced mutant was not tested.

The seven sorbitol mutants therefore define a complex locus which is allocated to linkage group V1 on the basis of mitotic haploidisation although linkage was not detected between the locus and three of four other markers known in the group.

C. Lactose Mutants.

Seven phenotypically similar mutants which make partial growth on lactose were isolated.

Complementarity tests of the mutants (Table 17) show that lac1 is non-complementary to lac2, lac4, lac6 and lac7, but complementary to lac3 and lac5, while lac3 and lac5 are non-complementary when tested in a heterokaryon. These results indicate that the mutants represent two loci concerned with the control of lactose metabolism and the loci are designated lac1 and lac3.

TABLE 17

Complementarity Tests of the Lactose Mutants.

Component Strains	Heterokaryons		Diploids	
	Growth on Glucose	Lactose	Glucose	Lactose
y2;pyro4; lac1 + bi1;w3; lac2	+	-P	+	-P
ad14 paba1y; lac1 + bi1;w3; lac3	+	+	+	+
" lac1 + " lac4	+	-P	+	-P
" lac1 + " lac5	+	+	+	+
" lac1 + " lac6	+	-P	+	-P
" lac1 + " lac7	+	-P	+	-P
bi1;w3; lac3 +y2;pyro4; lac5	+	-P	+	?

Key

\+ Wild type growth.

-P Partial growth. Mutant.

(1) lac1 locus.

Haploid strains isolated from the diploid Tester A/bi1;w3;lac1 following adenine-acriflavine selection show that lac1 segregates at random with respect to the markers in Tester A.

Tester A/bi1;w3;lac1 Segregation of markers in 31 haploids.

	lac1	+		lac1	+
phen2	13	11	pyro4	5	6
+	3	4	+	11	9

	lac1	+		xlac1	+
lys5	8	10	nic8	7	5
+	8	5	+	9	10

However, haploids isolated from the diploid Tester C/bi1;w3;lac1 show alternate segregation of lac1 and s3 indicating that lac1 is located in group V1. Adenine-acriflavine selection yielded 28 haploids segregating:-

Tester C/bi1;w3;lac1 Segregation of markers among 28 haploids.

	lac1	+		lac1	+
s3	0	9	ribo2	8	3
+	19	0	+	11	6

The growth of both diploids on lactose was wild type indicating that lac1 is recessive.

The two closely linked mutants sb3 and sb4 were also located in group V1 by mitotic analysis and consequently crosses were made between these markers and lac1 but no linkage was detected between the markers.

Cross 1. $\frac{ad14\ paba1\ y\ +}{+\ +\ +\ bi1}$ $\frac{+}{w3}$ $\frac{sb3\ +}{+\ lac1}$

Cross 2. $\frac{ad14\ paba1\ y\ +}{+\ +\ +\ bi1}$ $\frac{+}{w3}$ $\frac{sb4\ +}{+\ lac1}$

Segregation of sb3, sb4 and lac1

Genotypic classes	Cross 1 (sb3)	Cross 2 (sb4)
sb lac1	19	31
sb +	23	37
+ lac1	23	34
+ lac1	25	36
Totals	90	128

Table of values of X^2

	Cross 1 (sb3)			Cross 2 (sb4)		
	X^2	D.F.	P.	X^2	D.F.	P.
Segregation:-						
sb : +	0.400	1	0.7 - 0.5	0.500	1	0.5 - 0.3
lac1 : +	0.400	1	0.7 - 0.5	0.031	1	0.9 - 0.8
Linkage:-						
sb - lac1	0.044	1	0.8 - 0.7	1.542	1	0.3 - 0.2
Totals	0.844	3	0.9 - 0.8	2.073	3	0.7 - 0.5

Strains with sb3 or sb4 and lac1 in coupling were isolated and crossed to a strain carrying two markers in group VI, s3 and nic10. The only linkage detected between any of the four markers sb, lac1, s3 and nic10 was between nic10 and lac1. The data for the crosses are homogeneous for the two markers and the pooled results give an estimate of the recombination fraction between nic10 and lac1 as 37.1 $\pm$ 2.8% (Table 18).

TABLE 18

Detection of Linkage between lac1 and nic10

Cross 1. $\frac{ad14 \quad + \quad + \quad bi1}{+ \quad y \; ad20 \quad +} \quad \frac{w3}{+} \quad \frac{+ \quad + \quad sb3 \quad lac1}{s3 \; nic10 \quad + \quad +}$

Cross 2. $\frac{ad14 \quad paba1 \quad + \quad + \quad bi1}{+ \quad + \quad y \; ad20 \quad +} \quad \frac{w3}{+} \quad \frac{+ \quad + \quad sb4 \quad lac1}{s3 \; nic10 \quad + \quad +}$

Genotype of ascospores				Cross 1 (sb3)	Cross 2 (sb4)
+	+	+	+	4	9
+	+	+	lac1	7	18
+	+	sb	+	6	12
+	+	sb	lac1	7	21
+	nic10	+	+	12	10
+	nic10	+	lac1	1	11
+	nic10	sb	+	8	18
+	nic10	sb	lac1	6	9
s3	+	+	+	8	5
s3	+	+	lac1	8	14
s3	+	sb	+	4	11
s3	+	sb	lac1	8	11
s3	nic10	+	+	12	11
s3	nic10	+	lac1	9	6
s3	nic10	sb	+	7	14
s3	nic10	sb	lac1	1	8
			Totals	108	188

TABLE 18 (continued)

Allele ratios

Cross 1. $\frac{s3}{+} = \frac{57}{51}$ $\frac{nic10}{+} = \frac{56}{52}$ $\frac{sb3}{+} = \frac{47}{61}$ $\frac{lac1}{+} = \frac{47}{61}$

Cross 2. $\frac{s3}{+} = \frac{80}{108}$ $\frac{nic10}{+} = \frac{87}{101}$ $\frac{sb4}{+} = \frac{104}{84}$ $\frac{lac1}{+} = \frac{98}{90}$

	Cross 1	(sb3)	Cross 2	(sb4)
Table of values of	X^2	P.	X^2	P.
Segregation:-				
s3:+	0.333	0.70-0.50	4.160	0.05-0.02
nic10:+	0.148	0.70	1.043	0.50-0.30
sb:+	1.813	0.20-0.10	2.124	0.20-0.10
lac1:+	1.813	0.20-0.10	0.341	0.70-0.50
Linkage:-				
s3 - nic10	0.148	0.70	0.344	0.70-0.50
s3 - sb	3.710	0.10-0.05	0.006	0.99-0.98
s3 - lac1	0.148	0.70	0.638	0.50-0.30
nic10 - sb	0.093	0.80-0.70	0.021	0.90-0.80
nic10 - lac1	8.325	0.01	11.225	<0.01
sb - lac	0.593	0.50-0.30	2.126	0.20-0.10

All X^2 are for 1 degree of freedom

Recombination fraction

nic10 - lac1	36.1 ± 4.6 %	38.7 ± 4.9 %

Combined data 37.1 ± 2.8 %

(2) lac3 locus

Analysis was done entirely with lac5 before non-complementarity of lac5 and lac3 was discovered. It is desirable to have data on the frequency of recombination between the two mutants to confirm their location.

Diploids Tester A/bi1;w3;lac5 and Tester C/bi1;w3;lac5 were synthesised; all of the haploids isolated from these two diploids following selection for adenine independance and acriflavine resistance were $lac5^+$ but segregated at random with respect to the unselected markers, a result which indicates location of lac5 in either group 1 or 11. The wild type growth of both diploids on lactose indicates that lac5 is recessive.

Tester A/bi1;w3;lac5 Segregation of markers among 13 haploids.

	lac5	+		lac5	+
phen2	0	7	pyro4	0	6
+	0	6	+	0	7

	lac5	+		lac5	+
lys5	0	6	nic8	0	6
+	0	7	+	0	7

Tester C/bi1;w3;lac5 Segregation of markers in 38 haploids.

	lac5	+		lac5	+
s3	0	16	ribo2	0	30
+	0	22	+	0	8

Visual selection of white sectors from Tester A/bi1;w3;lac5 (Diploid 1) on adenineless medium yielded 21 haploids, 20 lac5 (w3 lac5) and 1 $lac5^+$ (w3 $lac5^+$).

This result indicates location of lac5 in group 11. The one lac5^{+} strain probably arose by mitotic crossing over between lac5 and w3 followed by haploidisation.

Tester A/bi1;w3;lac5 Segregation of markers in 21 white haploids.

	lac5	+
phen2	13	1
+	7	0

	lac5	+
pyro4	12	0
+	8	1

	lac5	+
lys5	13	1
+	7	0

	lac5	+
nic8	9	1
+	11	0

In addition to the 21 haploid strains with white conidia that were isolated from Tester A/bi1;w3;lac5 a further 14 white spored diploid strains were also isolated. Of these 14 strains 2 were of identical phenotype to lac5 on lactose media and as lac5 is recessive these two diploids must have been either homozygous for both w3 and lac5 or aneuploids monosomic for the white chromosome. The 12 diploids homozygous for w3 and (presumably) heterozygous for lac5 probably arose by mitotic crossing over between w3 and the centromere and this suggests that lac5 is proximal to w3 or not in the same arm as w3. The two diploids apparently homozygous for w3 and lac5 may have arisen either by double mitotic crossing over in both arms of the white chromosome or by non-disjunction of the white chromosome. Non-disjunction is the more likely explanation for mitotic crossing over is an infrequent event and the probability of the two cross-overs in one chromosome in one nuclear lineage is very small.

In attempting meiotic mapping of lac5 in group 11 three crosses were analysed (Table 19). These crosses included the nutritional markers ribo6 (requirement for riboflavine) and ab1 (requirement for gamma-aminobutyric acid) located by other workers in group 11 but not mapped. The nutritional markers were mapped successfully but lac5 was not located with certainty. Crosses 1 and 2 show that lac5 is not linked to ad23, Acr1, w3, ribo6 or pu1. In cross 3 the allele ratios were markedly disturbed and as only one set of data is available no more than crude estimates can be made of the linkage values between the markers involved. The data obtained in analysis of the three crosses is summarised in Table 20.

An estimate of the recombination fraction between ad3 and lac5 using the data from cross 3 can be made if it is assumed that the poor viability of ad3 and lac5 act independantly in the double mutant ad3 lac5. (I am indebted to Dr. Gale of the Genetics Department for showing me this method of calculating the recombination fraction). This method yields an estimate for the recombination fraction between ad3 and lac5 of 23.7%

Let:-

p = recombination fraction ad3 - lac5

q = 1-p

v = viability factor for ad3

m = " " " lac5

Class	+ lac5	ad3 +	+ +	ad3 lac5	
Observed	71	47	82	4	Total 204
Expected	q m	q v	p	p v m	

Then $\frac{p}{q} = \sqrt{\frac{4 \times 82}{71 \times 47}} = 0.312$ $\frac{p}{1-p} = 0.312$ $p = 0.237$

And $v = \sqrt{\frac{47 \times 4}{71 \times 82}} = 0.177$ $m = \sqrt{\frac{71 \times 4}{47 \times 82}} = 0.271$

Check

Class			Segregation Expected	Observed
+	lac5	qm = 0.206	71.2	71
ad3	+	qv = 0.135	46.7	47
+	+	p = 0.237	82.0	82
ad3	lac5	pvm = 0.011	3.8	4
		0.589	203.7	204

It is desirable that further crosses involving the markers nic3, ad3, lac5 and acr2 should be analysed to map lac5 with greater confidence than can be done on the results described here.

TABLE 19

Location of lac5 by Meiotic Analysis

Cross 1. $\frac{y2 \quad +}{+ \quad bi1} \quad \frac{+ \quad ribo6 \quad pu1 \quad +}{w3 \quad + \quad + \quad lac5}$

		w3		+		
		ribo6	+	ribo6	+	
pu1	lac5	8	14	10	7	39
	+	8	18	19	9	54
+	lac5	4	26	15	7	52
	+	6	14	23	15	58
		26	72	67	38	203

Allele ratios $\frac{w}{+} = \frac{98}{105}$ $\frac{ribo6}{+} = \frac{93}{110}$ $\frac{pu1}{+} = \frac{93}{110}$ $\frac{lac5}{+} = \frac{91}{112}$

Table of values of X^2		D.F.	Probability
Segregation:-			
w3:+	0.241	1	0.70 - 0.50
ribo6:+	1.424	1	0.30 - 0.20
pu1:+	1.424	1	0.30 - 0.20
lac5:+	2.172	1	0.20 - 0.10
Linkage:-			
w3 - pu1	0.833	1	0.50 - 0.30
w3 - ribo6	27.709	1	< 0.01
w3 - lac5	2.606	1	0.20 - 0.10
pu1 - ribo6	0.833	1	0.50 - 0.30
pu1 - lac5	0.399	1	0.70 - 0.50
lac5 - ribo6	1.424	1	0.30 - 0.20

Recombination fraction w3 - ribo6 = 31.5 ± 3.3 %

TABLE 19 (continued)

Cross 2. $\frac{y2}{+} \quad \frac{+}{bi1} \qquad \frac{+}{ad23} \quad \frac{+}{Acr1} \quad \frac{+}{w3} \quad \frac{ribo6}{+} \quad \frac{pu1}{+} \quad \frac{lac5}{+} \qquad \frac{+}{nic8}$

		ad23								+								
		Acr1				+				Acr1				+				
		w3		+		w3		+		w3		+		w3		+		
		ribo6	+	ribo6	+	ribo6	+	ribo6	+	ribo6	+	ribo6	+	ribo6	+	ribo6	+	
pu1	lac5	2	6	2	2	0	2	4	1	3	5	2	1	1	2	12	5	50
	+	1	8	0	2	1	2	5	2	2	2	3	1	0	0	11	3	43
+	lac5	4	11	3	2	0	0	2	3	2	3	2	1	1	4	6	5	49
	+	4	8	4	1	1	0	4	2	2	7	0	0	3	5	8	3	52
		11	33	9	7	2	4	15	8	9	17	7	3	5	11	37	16	194

Allele ratios

$$\frac{ad23}{+} = \frac{89}{105} \qquad \frac{Acr1}{+} = \frac{96}{98} \qquad \frac{w3}{+} = \frac{92}{102}$$

$$\frac{ribo6}{+} = \frac{95}{99} \qquad \frac{pu1}{+} = \frac{93}{101} \qquad \frac{lac5}{+} = \frac{99}{95}$$

TABLE 19 (continued)

Cross 2.

Table of values of X^2	X^2	D.F.	Probability
Segregation:-			
ad23:+	1.318	1	0.30 - 0.20
Acr1:+	0.023	1	0.90 - 0.80
w3:+	0.516	1	0.50 - 0.30
ribo6:+	0.046	1	0.90 - 0.80
pu1:+	0.329	1	0.70 - 0.50
lac5:+	0.046	1	0.90 - 0.80
Linkage:-			
lac5 - ad23	0.185	1	0.70 - 0.50
lac5 - Acr1	0	1	1.0
lac5 - w3	0.046	1	0.90 - 0.80
lac5 - ribo6	0.516	1	0.50 - 0.30
lac5 - pu1	0.516	1	0.50 - 0.30
Acr1 - ribo6	10.436	1	< 0.01
w3 - ribo6	26.722	1	< 0.01
pu1 - ribo6	1.009	1	0.50 - 0.30

Recombination fractions

ad23 - Acr1	33.5 ± 3.4 %
Acr1 - w3	24.7 ± 3.1 %
w3 - ribo6	31.4 ± 3.3 %
Acr1 - ribo6	47.4 ± 3.6 %

TABLE 19 (Continued)

Cross 3. $\frac{y2 \quad +}{+ \quad bi1}$ $\frac{+ \quad + \quad ribo6 \quad pu1 \quad + \quad + \quad + \quad lac5}{Acr1 \quad w3 \quad + \quad + \quad ab1 \quad ni3 \quad ad3 \quad +}$

				w3				+				
				ribo6		+		ribo6		+		
				pu1	+	pu1	+	pu1	+	pu1	+	
ab1	ni3	ad3	lac5									0
			+		4		7		9		5	25
		+	lac5		2		8		10		4	24
			+		2		3		6		2	13
	+	ad3	lac5									0
			+		1							1
		+	lac5						3			3
			+		3		2		2		1	8
+	ni3	ad3	lac5			1						1
			+			2		2				4
		+	lac5							3		3
			+	1		1		1		1		4
	+	ad3	lac5					3				3
			+	3		6		7		1		17
		+	lac5	4	1	11		15	3	6	1	41
			+	10	1	10	3	24	1	6	2	57
				18	14	31	23	52	34	17	15	204

Allele ratios

$$\frac{w3}{+} = \frac{86}{118} \quad \frac{ribo6}{+} = \frac{118}{86} \quad \frac{pu1}{+} = \frac{118}{86}$$

$$\frac{ab1}{+} = \frac{74}{130} \quad \frac{ni3}{+} = \frac{74}{130} \quad \frac{ad3}{+} = \frac{51}{153} \quad \frac{lac5}{+} = \frac{75}{129}$$

TABLE 19 (continued)

Cross 3.

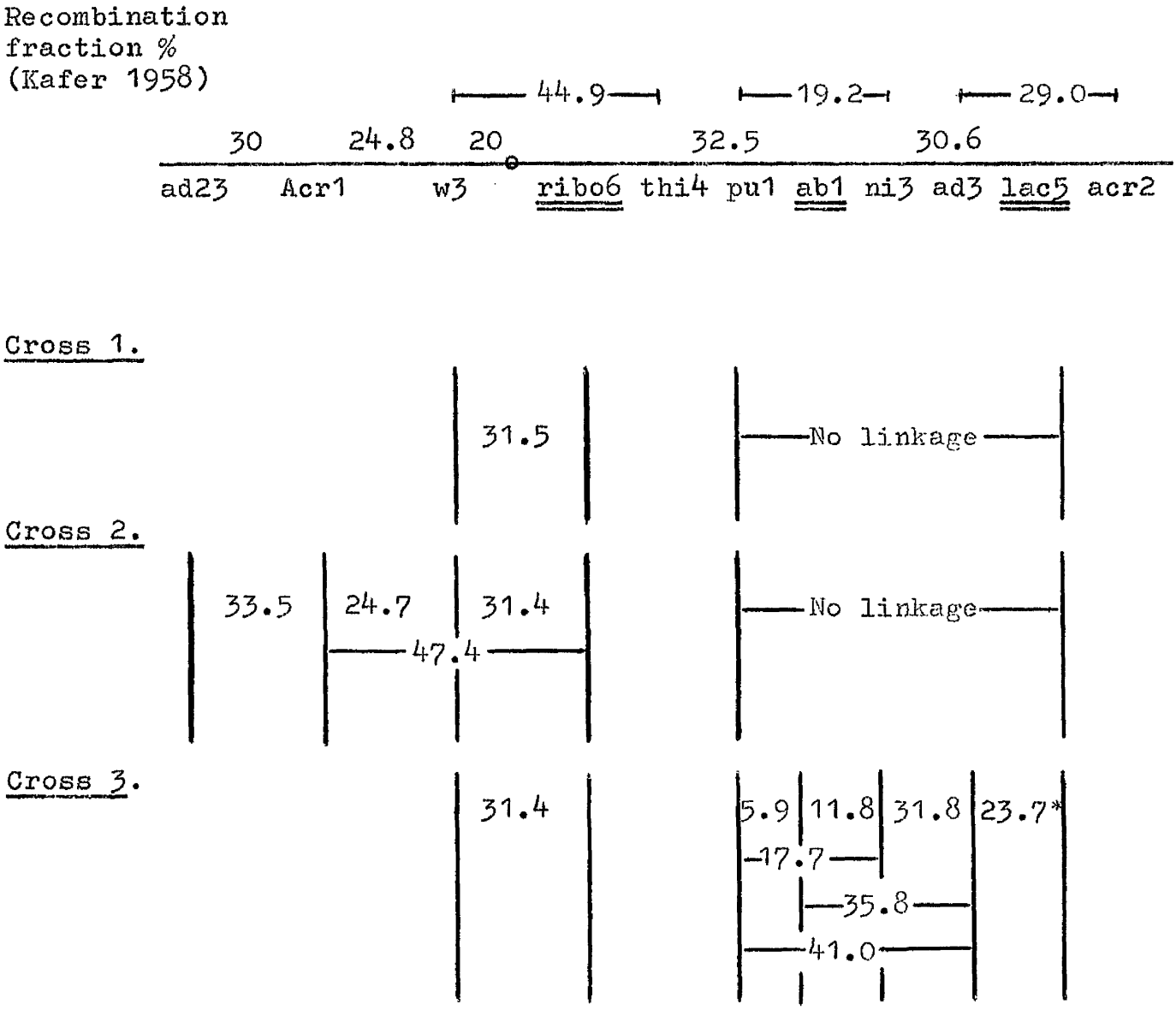
All the allele ratios show disturbed segregation and therefore apparent recombination fractions are calculated.

Recombination fractions

	Number of recombinants (Total progeny = 204)	Recombination fraction
lac5 - ab1	109	50 %
lac5 - ni3	111	50 %
lac5 - ad3	86	41.2 %
ribo6 - w3	64	31.4 %
pu1 - ab1	12	5.9 %
pu1 - ni3	36	17.7 %
pu1 - ad3	85	41.0 %
ab1 - ni3	73	35.8 %
ni3 - ad3	65	31.8 %

TABLE 20

Probable Location of lac5 in Linkage Group 11



New Data, including previously mapped markers.

The vertical bars indicate markers included in the crosses.

Markers previously unmapped or incompletely mapped are underlined.

* This recombination fraction is calculated assuming that the viability factors for ad3 and lac5 act independantly in the double mutant.

D. Maltose mutants.

Two mutants that yield partial growth on maltose were isolated. A heterokaryon synthesised between y2;pyro4;mal1 and bi1;w3;mal2 grew well on glucose but like the mutant on maltose or maltose plus pyridoxin and biotin and the mutants are therefore non-complementary. The heterozygous diploid was not isolated.

Mitotic analysis of the diploid Tester A/bi1;w3;mal1 locates mal1 in linkage group VII, haploids isolated by adenine-acriflavine selection segregating in the following manner:-

Tester A/bi1;w3;mal1 Segregation of markers in 53 haploids.

	mal1	+		mal1	+
phen2	19	11	pyro4	15	10
+	15	10	+	17	11

	mal1	+		mal1	+
lys5	19	11	nic8	0	21
+	13	10	+	32	0

The diploid Tester A/bi1;w3;mal1 grew like the wild type on maltose, therefore mal1 is recessive.

A cross between bi1;w3;mal1 and ribo1y;nic8 was analysed, the marker nic8 defines group VII. Linkage between nic8 and mal1 was detected and the recombination fraction estimated as $41.3 \pm 3.4\%$. (Table 21). In this cross the marker mal1 had poor viability. No attempt was made to map mal1 in relation to the marker cho1 (requirement for choline) which involves a group VII. translocation (Kafer, 1958).

TABLE 21 Location of mal1 in Linkage Group VII by Meiotic Analysis

Cross $\frac{\text{ribo1} \quad y \quad +}{+ \quad + \quad bi1} \quad \frac{+}{w3} \quad \frac{nic8 \quad +}{+ \quad mal1}$

		+		w3	
		y	+		
nic8	mal1	9	11	15	35
	+	20	12	37	69
+	mal1	9	14	30	53
	+	8	19	25	52
		46	56	107	209

Allele ratios

$$\frac{y}{+} = \frac{46}{56} \quad \frac{w3}{+} = \frac{107}{102} \quad \frac{nic8}{+} = \frac{104}{105} \quad \frac{mal1}{+} = \frac{88}{121}$$

Table of values of X^2	X^2	D.F.	Probability
Segregation:-			
nic8:+	0.0005	1	0.95 - 0.90
mal1:+	5.211	1	0.05 - 0.02
Linkage:-			
nic8 - mal1	5.861	1	0.02 - 0.01

Recombination fraction

nic8 - mal1 = 41.6 $\pm$ 3.4 %

E. Galactose Mutants.

Eight mutants failing to grow like the original type on galactose were isolated and may be separated into four phenotypic groups on the basis of their growth on galactose or lactose.

Mutants			Carbon Source	
			Galactose	Lactose
gal1	gal6		Fail to grow	Near wild type growth
gal2	gal4	gal7	Slow growing	Wild type growth
gal3			Slow growing	Near wild type growth
gal5	gal8		Fail to grow	Partial growth

Complementarity tests were done with balanced heterokaryons or the corresponding heterozygotes synthesised between the mutants taken in all possible pairs. No differences were detected between the results of the tests done with either the heterokaryons or the diploids and the results are presented in Table 22. Only two pairs of mutants were clearly non-complementary (gal1 and gal6; gal5 and gal8) and the test thus divides the eight mutants into six complementing groups. However the mutant gal6 was 'partially' complementary (the growth of the heterozygote on galactose was intermediate between that of the mutant and of wild type) with all of the mutants except gal1 while gal4 and gal7 were also partially complementary. The test is thus inconclusive for the partially complementary mutants could equally well be regarded as either allelic or non-allelic and the mutants grouped in different ways. Crosses were therefore set up to test for recombination between the complement-

TABLE 22 Complementarity Tests of the Galactose Mutants

Diploids.	paba1	y	ad20	+	+	galx	+
	+	+	+	bi1	w3	+	galy

bi1;w3; galy	paba1 1	y 2	ad20 3	+ 4	+ 5	galx 6	7	8
1	-							
2	+		+	+	+	(+)	+	+
3	+			+				
4	+							
5	+		+	+				
6	-		(+)	(+)	(+)			
7	+		+	(+)	+	(+)		
8	+		+	+	-	(+)	+	

Key + Wild type growth. Complementary mutants.

(+) Partial growth. Partially complementary mutants.

- No growth. Non-complementary mutants.

Complementation Groups:-

(gal1;gal6) (gal2) (gal3) (gal4) (gal5;gal8) (gal7)

ing mutants (gal1 and gal5) were selected to represent the two pairs of non-complementary mutants.

The crosses were all fertile, single perithecia were tested for hybridity and ascospores from hybrid perithecia plated on C.M. About 100 ascospores from each of the crosses were tested for their growth on galactose. In a number of crosses between mutants that differ phenotypically segregation of the two mutant phenotypes among the progeny could be observed, in other crosses the progeny failing to grow on galactose were all alike. The results are presented in Table 23. The recombination fractions between the mutants are calculated from the appearance of wild type recombinants for growth on galactose.

$$\text{Recombination fraction (\%)} = \frac{2 \times \text{Wild type progeny}}{\text{Total progeny}} \times \frac{100}{1}$$

In all of the crosses except one wild type recombinants were recovered at a high frequency. The exceptional cross was between gal4 and gal7 in which no recombinants growing on galactose were detected among 113 progeny tested indicating that the frequency of recombination between gal4 and gal7 is less than 1 in 100. The recombination fractions between the mutants were generally near 50% and the mutants are therefore not genetically linked. However in two crosses, gal5 x gal2 and gal7 x gal2 segregation indicating linkage was observed and when tested for divergence from 3:1 segregation ratio (mutant : wild) linkage between gal2 and gal7 was confirmed.

TABLE 23 Recombination between Groups of Complementing Galactose Mutants.

Crosses $\frac{paba1 \quad y \quad ad20 \quad +}{+ \quad + \quad + \quad bi1} \quad \frac{+}{w3} \quad \frac{galx}{+} \quad \frac{+}{galy}$

Cross			Segregation of ascospores			
			Mutant		Wild type	Recombination fraction (%)
galx	galy	Total	galx	galy	gal+	
1	2	124	60	31	33	50
1	5	68	53		15	46
3	2	130	36	53	41	50
5	2	103	85		18	35
5	3	77	34	24	19	49
7	2	104	91		13	25
7	3	104	72		32	50
7	4	113	113		0	< 1.0
7	5	67	33	17	19	50

Recombination fractions (%) between complementing mutants

		gal1 gal6	gal2	gal3	gal4	gal5 gal8
gal7;		?	25	50	< 1.0	50
gal5;	gal8	46	35	49	?	
gal4;		?	?	?		
gal3;		?	50			
gal2;		50				

	Segregation of ascospores		Test of 3:1 segregation		
Cross	Mutant	Wild type	X^2	D.F.	P.
gal5 x gal2	85	18	3.12	1	0.3 - 0.2
gal7 x gal2	91	13	8.68	1	0.01

Recombination fraction:- gal7 - gal2 = 25%

The combined results of the recombination and complementation tests therefore show that the eight mutants define five loci controlling galactose metabolism, two of the loci are in the same linkage group. The loci and allelic mutants are indicated below:-

(gal1, gal6) (gal2) (gal3) (gal4, gal7) (gal5, gal8)

Of the four phenotypic groups three are shown to differ genotypically while the fourth is subdivided genetically.

(1) gal1 locus.

Analysis of haploids derived mitotically from Tester A/bi1;w3;gal1 locates gal1 in group III, haploid segregants selected for adenine independance and acriflavine resistance showing the following phenotypes.

	gal1	+
phen2	0	16
+	4	0

	gal1	+
pyro4	1	7
+	3	9

	gal1	+
lys5	0	4
+	4	12

	gal1	+
nic8	1	1
+	3	15

TABLE 24 Location of gal1 in Linkage Group III by Meiotic Analysis

Cross 1. $\frac{+ \quad + \quad +}{\text{paba1} \quad y \quad \text{ad20}}$ $\frac{+ \quad \text{sm} \quad \text{phen2} \quad \text{panto1} \quad \text{s12}}{\text{gal1} \quad + \quad + \quad + \quad +}$ (a) (b)

		gal 1				+				
		sm		+		sm		+		
		phen2	+	phen2	+	phen2	+	phen2	+	
panto1	S12	0	1	1	3	5	2	0	0	12
	+	0	2	0	4	0	0	0	0	6
+	S12	0	0	1	13	6	0	0	1	21
	+	9	0	6	28	32	0	0	5	80
		9	3	8	48	43	2	0	6	119
Cross-over		a	a&b	b	0	0	b	a&b	a	

Allele ratios $\frac{\text{gal1}}{+} = \frac{68}{51}$ $\frac{\text{sm}}{+} = \frac{57}{62}$ $\frac{\text{phen2}}{+} = \frac{60}{59}$ $\frac{\text{panto1}}{+} = \frac{18}{101}$ $\frac{\text{S12}}{+} = \frac{33}{86}$

Table of values of X^2

Segregation:-	X^2	D.F.	Probability
gal 1:+	2.416	1	0.20 - 0.10
sm:+	2.100	1	0.20 - 0.10
phen2:+	0.008	1	0.90 - 0.80
panto1:+	57.800	1	< 0.01
S12:+	23.620	1	< 0.01
Linkage:-			
gal 1 - sm	57.800	1	< 0.01
gal 1 - phen2	40.006	1	< 0.01
gal 1 - panto1	8.136	1	0.50 - 0.30
sm - phen2	72.581	1	< 0.01

Recombination fractions

gal1 - sm	15.1 ± 3.3 %
gal1 - phen2	23.5 ± 3.9 %
sm - phen2	10.9 ± 2.9 %

TABLE 24 (continued)

Cross 2. $\frac{+ \quad + \quad +}{\text{paba1} \quad y \quad \text{ad20}}$ $\frac{\text{meth2} \quad \text{arg2} \quad + \quad + \quad +}{+ \quad + \quad \text{gal1} \quad \text{sm} \quad \text{phen2}}$

(a) (b)

		gal1				+				
		sm		+		sm		+		
		phen2	+	phen2	+	phen2	+	phen2	+	
meth2	arg2	17	2	2	1	4	0	9	77	112
	+	4	0	0	0	0	0	1	3	8
+	arg2	0	1	0	0	1	0	1	4	7
	+	60	8	2	2	1	1	5	54	133
		81	11	4	3	6	1	16	138	260
Cross-overs		(o)	(b)	(a&b)	(a)	(a)	(a&b)	(b)	(o)	

Allele ratios $\frac{\text{meth2}}{+} = \frac{120}{140}$ $\frac{\text{arg2}}{+} = \frac{119}{141}$ $\frac{\text{gal1}}{+} = \frac{99}{161}$ $\frac{\text{sm}}{+} = \frac{99}{161}$ $\frac{\text{phen2}}{+} = \frac{107}{153}$

Table of values of X^2

Segregation:-	X^2	D.F.	Probability
meth2:+	0.385	1	0.70 - 0.50
arg2:+	1.860	1	0.20 - 0.10
gal1:+	14.775	1	< 0.01
sm:+	14.775	1	< 0.01
phen2:+	8.128	1	< 0.01
Linkage:-			
gal 1 - meth2	24.452	1	< 0.01
gal 1 - arg2	32.610	1	< 0.01
gal 1 - sm	204.013	1	< 0.01
gal 1 - phen2	131.927	1	< 0.01

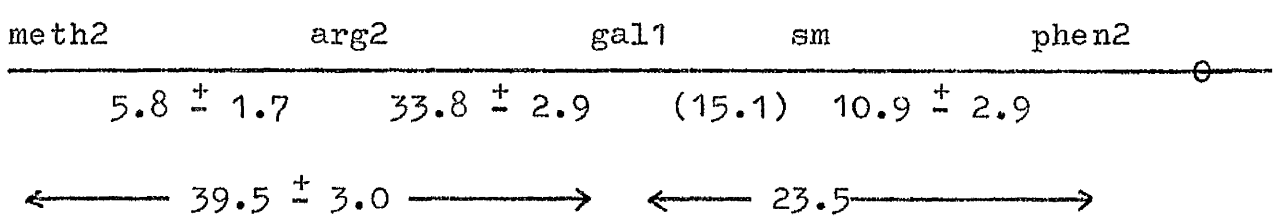
Recombination fractions

gal 1 - meth2	39.5 ± 3.0 %
gal 1 - arg2	33.8 ± 2.9 %
gal 1 - sm	5.4 ± 1.6 %
gal 1 - phen2	15.8 ± 2.3 %
sm - phen2	12.3 ± 2.0 %
meth2 - arg2	5.8 ± 1.7 %

The heterozygote Tester A/bi1;w3;gal1 grows like the wild type on galactose and thus gal1 is recessive.

Analysis of two crosses (Table 24) confirms the location of gal1 in linkage group III and permits the gal1 marker to be ordered relative to previously mapped markers. In the second cross the allele ratios are markedly disturbed but as the data for gal1 - sm and gal1 - phen2 in coupling and in repulsion are not homogeneous they cannot be combined to estimate the recombination fractions between the markers. The data are consistent with the location of gal1 indicated below. The recombination fractions for gal1 - sm and gal1 - phen2 are those obtained in the first cross.

Linkage Group III.



(2) gal2 locus

The diploid Tester A/bi1;w3;gal2 was synthesised and haploids arising mitotically selected for adenine independance and acriflavine resistance. All of the haploids isolated were gal2^{+} and showed random segregation of the unselected markers. This result indicated location of gal2 in group I or II.

Tester A/bi1;w3;gal2 Segregation of markers in 45 haploids.
(Selection su1ad20 y ad20; Acr1)

	gal2	+		gal2	+
phen2	0	18	pyro4	0	23
+	0	27	+	0	22

	gal2	+		gal2	+
lys5	0	9	nic8	0	17
+	0	36	+	0	28

Haploids were then selected from the same diploid but for white conidia and adenine independance and of the 21 haploids isolated 20 were gal2$^+$ and 1 gal2, again showing random segregation of the unselected markers.

Tester A/bi1;w3;gal2 Segregation of markers in 21 haploids.

	gal2	+		gal2	+
phen2	1	16	pyro4	0	13
+	0	4	+	1	7

	gal2	+		gal2	+
lys5	0	8	nic8	0	7
+	1	12	+	1	13

This result indicates that gal2 is located in group 1 (if the single gal2 haploid is discounted), or that gal2 is located in some other group, but does not appear among the

haploid segregants due to poor viability on the selective medium.

To test the first possibility conidia of the prototrophic green spored diploid Tester A/bi1;w3;gal2 (Diploid 1) were plated on C.M. and recombinants with yellow or white conidial heads selected visually from the green colonies. The recombinants were purified by streaking on C.M. and tested for ploidy by measurement of the diameter of the conidia. Altogether 16 haploids were isolated, again these were all gal2$^+$ but showed random segregation with respect to the markers defining groups 1 and 11 (bi1 and w3), thus eliminating the possibility that gal2 is located in group 1 and confirming that the marker is not in group 11. The unselected markers segregated at random with respect to gal2$^+$.

Tester A/bi1;w3;gal2 Segregation of bi1 and w3 among 16 haploids. (Selection of y or w recombinants from Diploid 1).

	gal2	+		gal2	+
y bi1$^+$; w3$^+$	0	6)	w3	0	10
(y)bi1$^+$; w3	0	3)	+	0	6
(y$^+$)bi1; w3	0	7			

It therefore appears that failure to recover gal2 haploids is due to a viability factor associated with the marker and operating on both the selective medium and C.M. If gal2 were located in one of the groups represented in Tester A by the markers phen2, pyro4, lys5 or nic8, the diploid synthesised between Tester A and bi1;w3;gal2 would have gal2 in repulsion to one of these markers and the gal2$^+$ segregants would then all

be of one type with respect to the marker defining the group in which gal2 is located. That is, all phen2$^+$ or pyro4$^+$ etc. However the gal2$^+$ haploid segregants were of both types with regard to the four markers and gal2 cannot be located in the groups defined by phen2, pyro4, lys5 and nic8, and gal2 is thus located by elimination in either group V1, or group V111, the groups represented in Tester C by s3 and ribo2. Mitotic analysis with Tester C was not attempted.

It is noted that evidence was obtained for linkage between gal2 and gal7 and that gal7 is probably located in group V111 (Section (4)). It is therefore most likely that gal2 is also located in group V111, but this requires confirmation by analysis of diploids with Tester C, while the nature of the viability effect associated with gal2 should also be investigated.

All of the heterozygous diploids grew like the wild type on galactose and therefore gal2 is recessive.

(3) gal3 locus

The diploids Tester A/bi1;w3;gal3 and Tester C/bi1;w3;gal3 both yield haploids all gal3$^+$ on selection for adenine independance and acriflavine resistance and these haploids segregate at random with respect to the unselected markers. (The wild type growth of the two diploids on galactose indicate that gal3 is recessive).

Tester A/bi1;w3;gal3 Segregation of markers among 36 haploids.

	Gal3	+
phen2	0	28
+	0	8

	gal3	+
pyro4	0	17
+	0	19

	gal5	+
lys5	0	20
+	0	16

	gal3	+
nic8	0	10
+	0	26

Tester C/bi1;w3;gal3 Segregation of markers among 31 haploids.

	gal3	+
s3	0	18
+	0	13

	gal3	+
ribo2	0	26
+	0	5

These results suggest location of gal3 in group 1 or 11. Conidia of the green spored prototrophic diploid Tester A/bi1;w3;gal3 (diploid 1) were therefore plated onC.M. to isolate colonies growing from single conidia and white or yellow conidial heads appearing as sectors in the green colonies selected visually, streaked on C.M. and the ploidy of the purified colour segregants established by measurement of the diameters of their conidia. Not more than one white or one yellow sector was picked from each diploid colony. The 10 haploids isolated in this way showed linkage of w3 and gal3 and therefore gal3 is located in group 11. The unselected markers segregated at random with respect to gal3.

Tester A/bi1;w3;gal3 Segregation of markers among 10 hapoids.

Selection of yellow or white sectors.

	gal3	+			gal3	+
y bi1$^+$; w3$^+$	0	4	)	w3	6	0
(y) bi1$^+$; w3	6	0	)	+	0	4
(y$^+$) bi1; w3	0	0				

The marker gal3 has not been mapped meiotically in group 11 but a cross involving this marker and w3 yielded no evidence of linkage between them.

Cross	ribo1	an1	ad14	pro1	+	+	+	bi1	w3	+
	+	+	+	+	paba1	y	ad20	+	+	gal3

Segregation of progeny for w3 and gal3

w3	gal3	32
w3	+	23
+	gal3	37
+	+	31
Total progeny		123

Table of values of X^2

	X^2	D.F.	Probability
Segregation:-			
w3 : +	1.364	1	0.30 - 0.20
gal3 : +	1.823	1	0.30 - 0.20
Linkage:-			
w3 - gal3	0.073	1	0.98 - 0.97

(4) gal4 locus

The two mutants gal4 and gal7 are closely linked for no wild type recombinants were detected among a sample of 100 of the progeny resulting from a cross between them. The mutants are partially complementary, the double heterozygote makes growth intermediate between that of mutant or wild type when tested on galactose, and it is possible that the mutants represent two closely linked but functionally distinct loci. The data available do not permit choice between the possibilities of one or two loci but for the purpose of location the mutants are regarded as representing a single locus. Much of the analysis was done with gal7 which is more readily classified, especially when segregating meiotically, although preliminary mitotic analysis employed gal4.

The diploid Tester A/bi1;w3;gal4 was synthesised and strains arising by mitotic haploidisation isolated following selection for adenine independance and acriflavine resistance. The haploids were all $gal4^+$ and showed random segregation of the unselected markers. (The diploid grew like wild type on galactose indicating that gal4 is recessive).

Tester A/bi1;w3;gal4 Segregation of markers among 17 haploids.

	gal4	+
phen2	0	12
+	0	5

	gal4	+
pyro4	0	4
+	0	13

	gal4	+
lys5	0	5
+	0	12

	gal4	+
nic8	0	6
+	0	11

This result indicates location of gal4 in group 11 or 11 and location in group 1 was apparently confirmed by analysis of 8 haploids isolated from the green diploid Tester A/bi1;w3;gal4 (diploid 1) by visual selection of yellow or white sectors. Eight haploid strains were isolated, these segregated at random with respect to the unselected markers phen2, pyro4, lys5 and nic8 and also w3 but showed coupling of gal4 and y^+ bi1 which is consistent with location of gal4 in group 1.

Tester A/bi1;w3;gal4 Segregation of markers among 8 haploids.

	gal4	+		gal4	+
y $bi1^+$; $w3^+$	0	5			
(y) $bi1^+$; w3	0	1	w3	2	1
(y^+) bi1; w3	2	0	+	0	5

Following the results obtained for gal4 which indicate location in group 1, a cross between gal7 and group 1 markers was analysed (Table 25). Linkage was not detected between gal7 and any of the markers tested, and gal7 is therefore not located between su1ad20 and bi1 in chromosome 1. The possibility that gal7 is distal to su1ad20 or to bi1 but recombines freely with these markers was then tested by mitotic analysis.

A diploid homozygous for ad20 and with su1ad20 and gal7 in coupling was synthesised and adenine independant diploid sectors which arise by mitotic crossing over between su1ad20 and

TABLE 25 Failure to Detect Linkage of gal7 to Group I Markers

Cross 1. $\frac{+ \quad + \quad \circ \quad paba1 \quad y \quad ad20 \quad + \quad gal7}{su1ad20 \quad ribo1 \quad + \quad + \quad ad20 \quad bi1 \quad +}$

			gal7				+				
			+		su1ad20		+		su1ad20		
			ribo1	+	ribo1	+	ribo1	+	ribo1	+	
paba1	y	bi	3		3	1	1	1	2		11
		+	3	12	6	13	3	12	7	10	66
	+	bi	1	1	4			1	1	1	9
		+									0
+	y	bi	2								2
		+		1	1		1	1	1		5
	+	bi	7	6	13	5	5	2	12	8	58
		+	1	2						1	4
			17	32	27	19	10	17	23	20	155

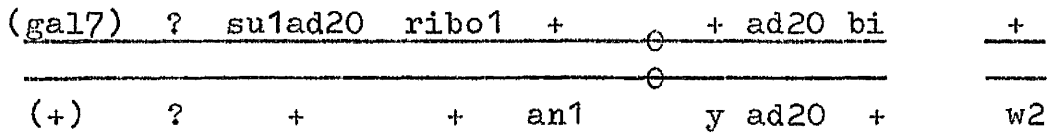
Allele ratios

$\frac{gal7}{+} = \frac{70}{85}$ $\frac{su1}{+} = \frac{89}{66}$ $\frac{ribo1}{+} = \frac{77}{78}$ $\frac{paba1}{+} = \frac{86}{69}$ $\frac{y}{+} = \frac{84}{71}$ $\frac{bi1}{+} = \frac{80}{75}$

Table of values of X^2

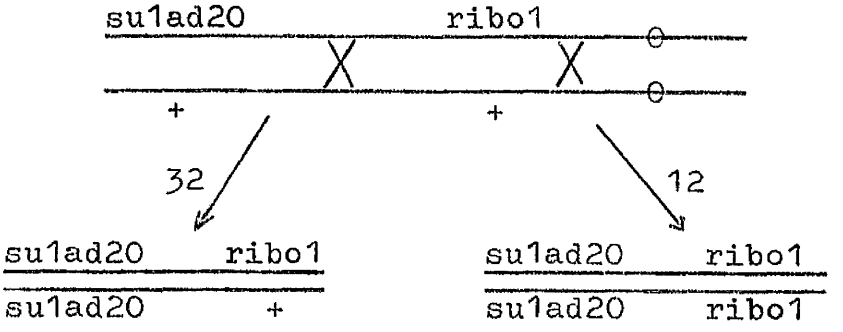
Segregation:-	X^2	D.F.	Probability
gal7:+	1.462	1	0.30 - 0.20
su1ad20:+	3.420	1	0.10 - 0.05
ribo1:+	0.006	1	0.99 - 0.98
paba1:+	1.862	1	0.20 - 0.10
y:+	1.010	1	0.50 - 0.30
bi1:+	0.161	1	0.70 - 0.50
Linkage:-			
gal7 - su1ad20	0.523	1	0.50 - 0.30
gal7 - ribo1	0.316	1	0.70 - 0.50
gal7 - paba1	0.006	1	0.99 - 0.98
gal7 - y	0.057	1	0.90 - 0.80
gal7 - bi1	0.523	1	0.50 - 0.30

and the centromere selected by growth on adenineless medium (su1ad20 is a partial suppressor in the heterozygote but a full suppressor when homozygous). If gal7 were distal to su1ad20 then the homozygous su1ad20 sectors should also be homozygous for gal7 and yield a mutant phenotype on galactose.
The diploid synthesised was:-

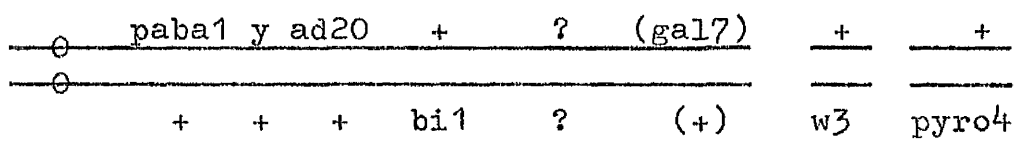


The circles indicate the location of the centromere.

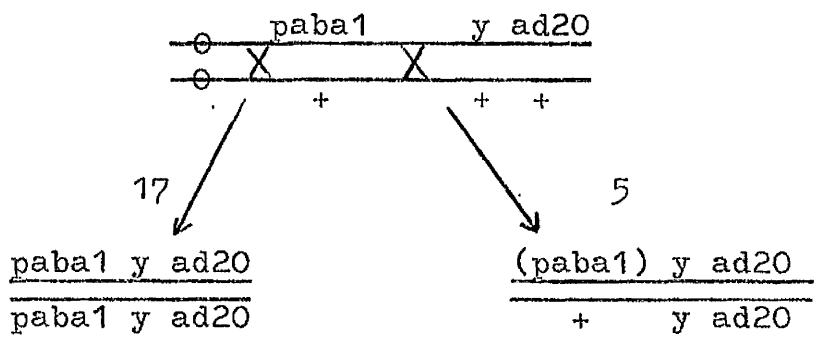
The diploid grew like wild type on galactose and therefore gal7 is recessive. A suspension of conidia taken from a single colony of the diploid was spotted with a wire loop on an adenineless medium containing riboflavine and 47 diploid adenine independant sectors isolated from the resulting slow growing colonies. None of the diploids had a mutant phenotype in growth on galactose (none were homozygous gal7) and therefore gal7 cannot be distal to su1ad20. Of the 44 diploids 32 resulted from crossing over between su1ad20 and ribo1 and 12 from crossing over between ribo1 and the centromere.



The possibility that gal7 is located distal to bi1 was then investigated using a second diploid synthesised with y and gal7 in coupling. The diploid synthesised was:-



A suspension of conidia of the diploid was plated on C.M. and yellow spored recombinants, selected visually from the green colonies, purified and tested for ploidy by measurement of the diameter of their conidia. A total of 22 diploid strains homozygous for y were isolated, not one of these was homozygous for gal7 and therefore gal7 cannot be distal to bi1 and is not located in group 1. Of the 22 homozygous y/y diploids 17 resulted from mitotic crossing over between the centromere and paba1, and 5 by crossing over between paba1 and y.



Both of the diploids were plated on C.M. and white spored haploid ~~sectors~~ recombinants isolated by visual selection. In each case the distribution of gal7 in the haploids confirms that the mutant is not located in group 1 for gal7 segregates at random with respect to the group 1 markers.

Diploid $\frac{su1\ ad20\quad ribo1\quad +}{+\quad +\quad an1}$ ⊗ $\frac{+\ ad20}{y\ ad20}$ $\frac{gal7}{+}$ $\frac{+}{w2}$

Genotypes of white spored haploids	Numbers recovered
su1 ad20 ribo1 ad20; w2; gal7	9
su1 ad20 ribo1 ad20; w2; gal7$^+$	4
an1 (y) ad20; w2; gal7	18
an1 (y) ad20; w2; gal7$^+$	16

Diploid $\frac{paba1\quad y\quad ad20\ +}{+\quad +\quad +\quad bi1}$ ⊗ $\frac{gal7}{+}$ $\frac{+}{w3}$ $\frac{+}{pyro4}$

Genotypes of white spored haploids	Numbers recovered
paba1 (y) ad20; w3; gal7	9
paba1 (y) ad20; w3; gal7$^+$	2
bi1; w3; gal7	9
bi1; w3; gal7$^+$	4

The results obtained with gal7 therefore clearly show that the mutant is not located in Linkage group 1 and the segregation observed for gal4 which indicated location in this group possibly resulted from a chance segregation in the 8 haploids

analysed. (An alternative possibility is that gal4 involves a translocation of a fragment of chromosome VIII to chromosome I this would account for the apparent linkage of gal4 with group I markers and also failure of gal4 and gal7 to recombine). To locate gal7 the diploid was synthesised between Tester D (containing the markers of both Tester A and Tester C) and bi1;w3;gal7. The green spored prototrophic diploid (Diploid 1) was plated on C.M. and haploid segregants isolated by selection of white or yellow spored sectors. Only 8 haploids were isolated and these show alternative segregation of ribo2 (group VIII) and gal7 and it is tentatively suggested that gal7 is located in group VIII but this result requires confirmation.

Tester D/bi1;w3;gal7 Segregation of markers among 8 haploids.

		gal7	+
I	(y) bi1$^+$; w3	0	0
	y bi1$^+$; w3$^+$	5	1
	(y$^+$) bi1; w3	2	0

		gal7	+
II	w3	2	0
	+	5	1

		gal7	+
III	phen2	4	1
	+	3	0

		gal7	+
IV	pyro4	5	1
	+	2	0

		gal7	+
V	lys5	1	0
	+	6	1

		gal7	+
VI	s3	5	0
	+	2	1

		gal7	+
VII	nic8	3	1
	+	6	1

		gal7	+
VIII	ribo2	0	1
	+	7	0

The results described here are interpreted in the simplest fashion that the two mutants gal4 and gal7 represent a single locus which is possibly located in linkage group VIII; it is clear however that alternative interpretations are possible and further investigation is required to establish the relationship of the mutants and their location.

(5) gal5 locus

Haploid strains were isolated from the diploid Tester A/bi1;w3; gal5 by selection of sectors with white conidia growing on adenineless medium, all the haploids were gal5 which would be expected if gal5 is located in group 1.

Tester A/bi1;w3;gal5 Segregation of markers among 30 haploids.

	gal5	+
phen2	0	19
+	0	11

	gal5	+
pyro4	0	17
+	0	13

	gal5	+
lys5	0	13
+	0	17

	gal5	+
nic8	0	5
+	0	25

The diploid grew like the wild type on galactose and thus gal5 is recessive.

Further evidence for location of gal5 in group 1 was obtained by visual selection of white, yellow and green sectors arising in colonies of the green spored prototrophic diploid Tester A/bi1;w3;gal5 (Diploid 1) for the 16 haploids all segregated with bi1 and gal5 in coupling.

Tester A/bi1;w3;gal5 Segregation of the markers bi1 and gal5 among 16 haploids.

	y	y^+	w3	Totals
bi1;gal5	0	2	1	3
bi1; +	0	0	0	0
+ ; gal5	0	0	0	0
+ ; +	9	0	4	13

Three crosses were analysed and the results of these confirm location of gal5 in group 1. The first cross (Table 26) located gal5 distal to ribo1 with a recombination fraction of 33.6 ± 3.2% between the markers. The second and third crosses (Table 26) included the markers su1ad20, gal5 and ribo1, with gal5 in coupling or repulsion to su1ad20 or ribo1. The allele ratios show disturbed segregation of su1ad20 but not gal5 or ribo1. The combined data for gal5 and ribo1 are homogeneous and the recombination fraction between the markers is estimated as 36.6 ± 2.8% from the combined data. The data for su1ad20 and gal5 are heterogeneous, separate estimates of the recombination fraction are 19.3 ± 1.1% (Cross 2, markers in repulsion) and 15.9 ± 2.6% (Cross 3, markers in coupling). In both crosses there is a shortage of su1ad20^{+} (adenine requiring strains) suggesting that the media were deficient in adenine and selection for su1ad20 (adenine independance) occurred but that the degree of selection was different in the two experiments. In both crosses one of the single cross-over classes between su1ad20 and gal1 are markedly deficient and in each case it is the adenine requiring strains (su1ad20^{+}) that are poorly represented.

Products of crossing-over

Cross 2			Cross 3		
su1ad20	gal5	16.	su1ad20	+	17
+	+	1.	+	gal5	1.

This result cannot be due to a chromosomal aberration (inversion) as there is a shortage of only one

of the cross-over types and the reciprocal types are present, i.e., there is no suppression of recombination.

Mapping of the gal5 locus is shown below. The recombination fraction quoted for su1ad20 - gal5 is an average value of those obtained in Crosses 2 and 3.

Group 1

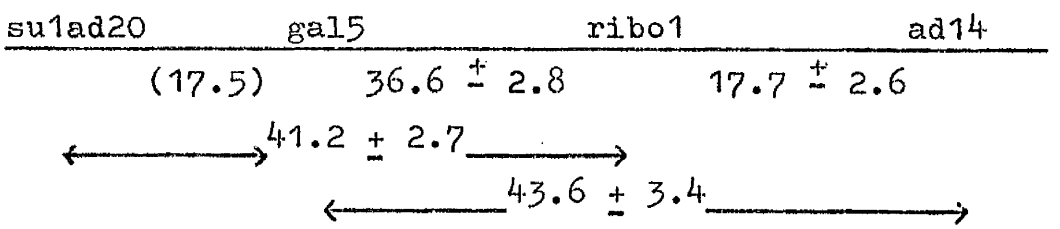


TABLE 26 Location of gal5 in Linkage Group I by Meiotic Analysis

Cross 1. $\frac{+}{gal5}$ $\frac{ribo1}{+}$ $\frac{ad14}{+}$ $\frac{pro1}{+}$ $\frac{paba1}{+}$ $\frac{y}{+}$ $\frac{+}{bi1}$ $\frac{+}{w3}$

				gal5				+				
				ribo1		+		ribo1		+		
				ad14	+	ad14	+	ad14	+	ad14	+	
pro1	paba1	y	bi1					1	2		1	4
			+	5	4	4	11	18	1	1	1	45
		y+	bi1	1		1	2	5		1		10
			+									0
		w3	bi1	2	1			5		1		9
			+	9		2	2	16	4	2	1	36
	+	y	bi1									0
			+	1								1
		y+	bi1		1		1	1				3
			+					1			1	2
		w3	bi1				1					1
			+									0
+	paba1	y	bi1									0
			+	1	1	1	2	1	1		1	8
		y+	bi1									0
			+									0
		w3	bi1									0
			+	1				1			1	3
	+	y	bi1									0
			+	2			3				3	8
		y+	bi1	1		1	18	4			11	35
			+						1			1
		w3	bi1	3	2		21	5	4	2	9	46
			+				2	3		1	2	8
				26	9	9	63	61	13	8	31	220

TABLE 26 (continued)

Cross 1.

Allele ratios:- $\frac{gal5}{+} = \frac{107}{113}$ $\frac{ribo1}{+} = \frac{109}{111}$ $\frac{ad14}{+} = \frac{104}{106}$ $\frac{pro1}{+} = \frac{111}{109}$

$\frac{paba1}{+} = \frac{115}{105}$ $\frac{y2}{+} = \frac{66}{51}$ $\frac{bi1}{+} = \frac{108}{112}$ $\frac{w3}{+} = \frac{108}{112}$

Table of values of X^2

Segregation:-	X^2	D.F.	Probability
gal5:+	0.1656	1	0.70 - 0.50
ribo1:+	0.0182	1	0.90 - 0.80
ad14:+	0.0182	1	0.90 - 0.80
pro1:+	0.0182	1	0.90 - 0.80
paba1:+	0.4545	1	0.70 - 0.50
y2:+	1.0224	1	0.30 - 0.20
bi1:+	0.0727	1	0.80 - 0.70
Linkage:-			
gal5 - ribo1	23.612	1	< 0.01
gal5 - ad14	17.708	1	< 0.01
gal5 - pro1	2.186	1	0.20 - 0.10
gal5 - paba1	2.200	1	0.20 - 0.10
gal5 - y2	2.470	1	0.20 - 0.10
gal5 - bi1	1.674	1	0.20 - 0.10

Recombination fractions

gal5 - ribo1	33.6 ± 3.2 %
gal5 - ad14	43.6 ± 3.4 %
ribo1 - ad14	17.7 ± 2.6 %

TABLE 26 (continued)

Cross 2 $\frac{\text{su1 ad20} \quad + \quad \text{ribo1} \quad \text{pro1} \quad + \quad + \quad \text{ad20 bi1}}{+ \quad \text{gal5} \quad + \quad + \text{ paba1} \quad y \quad \text{ad20} \quad +} \quad \frac{\text{pyro4}}{+}$

a b

Genotype of ascospores	Total	Cross-overs
su1ad20 gal5 ribo1	4	Double a and b
su1ad20 gal5 +	16	Single a
su1ad20 + ribo1	34	None
su1ad20 + +	20	Single b
+ gal5 ribo1	17	Single b
+ gal5 +	29	None
+ + ribo1	1	Single a
+ + +	3	Double a and b
Total	124	

Allele ratios $\frac{\text{su1ad20}}{+} = \frac{74}{50}$ $\frac{\text{gal5}}{+} = \frac{66}{58}$ $\frac{\text{ribo1}}{+} = \frac{56}{68}$

Table of values of X^2	X^2	D.F.	Probability
Segregation:-			
su1ad20 : +	4.648	1	0.05 - 0.02
gal5 : +	0.516	1	0.50 - 0.30
ribo1 : +	1.161	1	0.30 - 0.20
Linkage:-			
su1ad20 - gal5	88.767	1	<0.01
gal5 - ribo1	5.891	1	0.02 - 0.01
su1ad20 - ribo1	5.038	1	0.05 - 0.02

Recombination fractions

su1ad20 - gal5 $19.3 \pm 1.1\%$

gal5 - ribo1 $35.4 \pm 4.2\%$

TABLE 26 (continued)

Cross 3 $\frac{\text{su1 ad20 gal5}}{+} \quad \frac{\text{ribo1}}{+} \quad \frac{\text{pro1}}{+} \quad \frac{\text{y ad20}}{+ \text{ ad20 bi1}} \quad \frac{+}{\text{pyro4}}$

a b

Genotype of Ascospores	Total	Cross-overs
su1 ad20 gal5 ribo1	55	None
su1 ad20 gal5 +	38	Single b
su1 ad20 + ribo1	7	Double a and b
su1 ad20 + +	17	Single a
+ gal5 ribo1	1	Single a
+ gal5 +	7	Double a and b
+ + ribo1	24	Single b
+ + +	52	None
Total	201	

Allele ratios $\frac{\text{su1 ad20}}{+} = \frac{117}{84}$ $\frac{\text{gal5}}{+} = \frac{101}{100}$ $\frac{\text{ribo1}}{+} = \frac{87}{114}$

Table of values of X^2	X^2	D.F.	Probability
Segregation:-			
su1 ad20 : +	5.418	1	0.02 - 0.01
gal5 : +	0.049	1	0.98 - 0.95
ribo1 : +	3.627	1	0.10 - 0.05
Linkage:-			
su1 ad20 - gal5	93.378	1	< 0.01
gal5 - ribo1	11.945	1	< 0.01
su1 ad20 - ribo1	8.363	1	< 0.01

Recombination fractions

su1 ad20 - gal5	$15.9 \pm 2.6\%$
gal5 - ribo1	$37.8 \pm 3.4\%$
su1 ad20 - ribo1	$46.7 \pm 3.5\%$

Discussion.

The result of the analysis of the 27 sugar mutants isolated is summarised in Table 27 and the positions of the loci identified shown in a genetic map (Figure 3). The mutants define ten new loci of which nine have been located in linkage groups by mitotic analysis while six of the locations have been confirmed by meiotic mapping.

Fermentation mutants of Saccharomyces (Winge and Laustsen, 1939; Winge, 1952) or E.coli (Lederberg, 1949) are recessive 'loss' mutants which fail to form specific enzymes of carbohydrate metabolism. The sugar mutants of A.nidulans are also found to be recessive and this result is consistent with the hypothesis that the mutants lack specific enzymes concerned in carbohydrate metabolism.

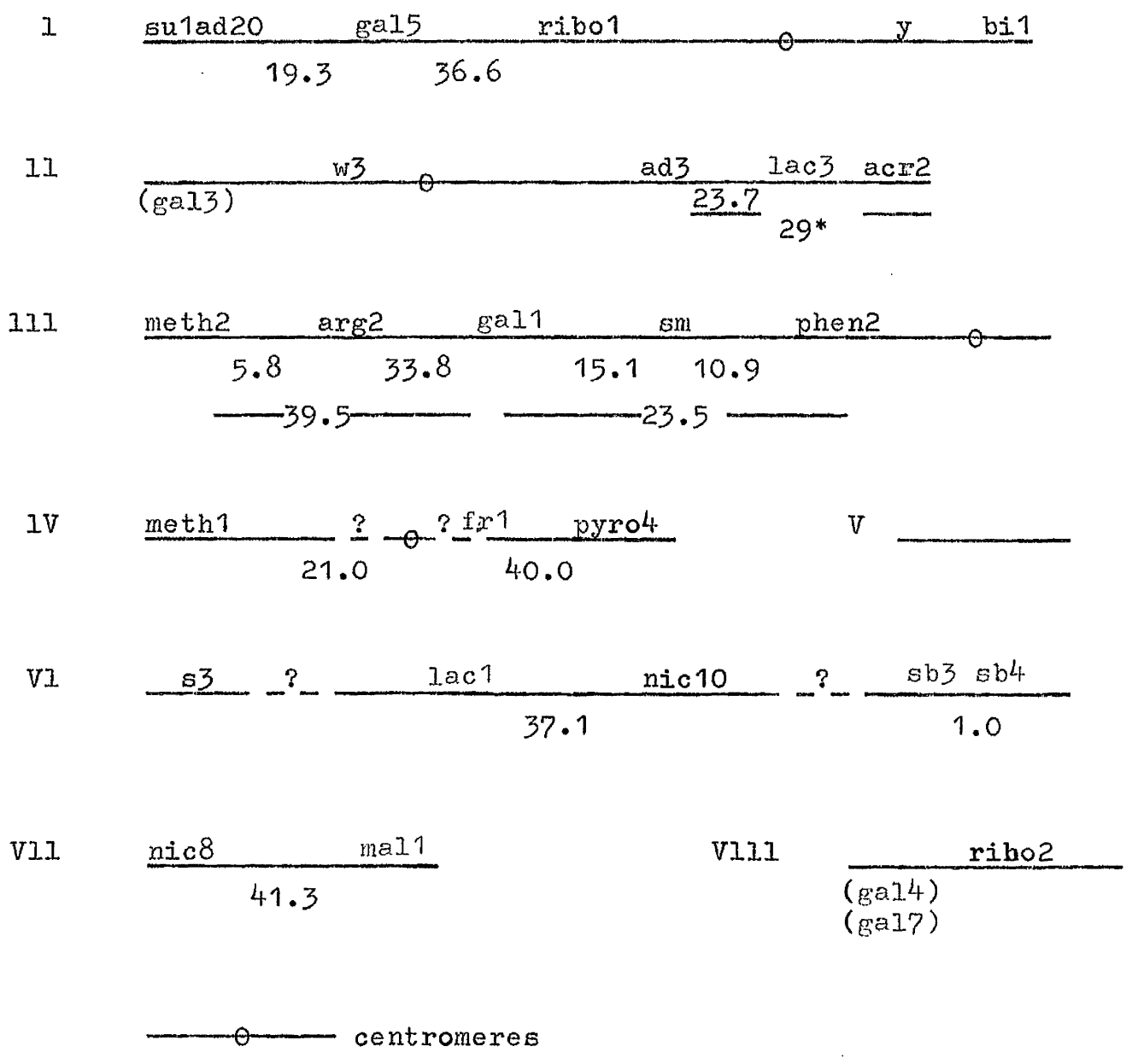
The ten loci concerned with carbohydrate utilisation by A.nidulans which have been identified in this investigation are apparently distributed at random among the linkage groups. None of the six meiotically mapped loci are closely linked to previously known markers although analysis of auxotrophic mutants of A.nidulans has shown an excess of close linkage between non-allelic mutants (Pontecorvo, 1956). No close linkage between loci concerned with the control of the metabolism of different carbohydrates has been discovered while two cases of close linkage between phenotypically similar mutants analysis has failed to establish the relationship of the mutants. The sorbitol mutants may represent a single complex locus or three closely linked but functionally distinct loci (these mutants are discussed fully in

TABLE 27

Genetic Analysis of Sugar Mutants of *A.nidulans*

Locus	Phenotype	Linkage Group	No. of Alleles	Allelic Mutants
fr1	Fails to utilise fructose, sucrose or sorbitol	IV	3	fr1 - 3
sb3	Fails to utilise sorbitol	VI	7	sb3 - 9
lac1	Fails to utilise lactose	VI	5	lac1, 2, 4, 6 and 7
lac3	" lactose	II	2	lac3, 5
mal1	" maltose	VII	2	mal1, 2
gal1	" galactose	III	2	gal1, 6
gal2	" galactose	VI or VIII	1	-
gal3	" galactose	II	1	-
gal4	" galactose	VIII	2 (?)	gal4, 7 (?)
gal5	" galactose Partial growth on lactose.	I	2	gal5, 8

Figure 3. Location of Sugar Markers in A.nidulans



Linkage groups after Kafer, 1958.

Distances in percent meiotic recombination.

The markers in parenthesis have not been located meiotically.

* Recombination fraction from Kafer, 1958.

the following Chapter), while the simplest interpretation of two partially complementary and closely linked galactose mutants (gal4 and gal7) is that they are within the same locus although alternative interpretations are not excluded.

Mutants failing to utilise maltose or fructose, and perhaps sorbitol, each result from mutation within single loci but as the numbers of mutants of each type tested are small, it is possible that further undetected loci are also concerned in the metabolism of these sugars. A single locus is known for the control of maltose fermentation in E.coli (Lederberg, 1949; Hayes, 1960) while in Saccharomyces four unlinked loci at which polymeric genes are situated determine the fermentation of the sugar (Winge, 1952). There are apparently no previous reports of induced mutants which fail to utilise or to ferment sorbitol or fructose.

The seven lactose mutants are separated into two groups by complementarity tests, mutants representing each of the groups are found to be unlinked and therefore two unlinked genes controlling lactose utilisation by A.nidulans have been identified. Growth of the organism on lactose suggests that the sugar is utilised adaptively. The genetic control of the metabolism of lactose has been extensively studied in E.coli (Monod, 1956; Cohen and Monod, 1957; Pardee, Jacob and Monod, 1959; Jacob and Monod, 1959). Lactose is metabolised by an inducible system in E.coli which has been shown to comprise two components, a 'permease' for the uptake of the sugar and the enzyme beta-galactosidase by which it is hydrolysed. Both components are

inducible and each is controlled by complementary genes which are closely linked. One of the genes determines the permease and the second the structure of the enzyme. Other groups of mutants failing to ferment lactose and unlinked to the 'lac' region have been described, one forms both permease and enzyme although a non-fermenter, while the second forms reduced amounts of the two components (Pardee, Jacob and Monod, 1959). It is possible that the two lactose loci described in Aspergillus correspond to the permease and beta-galactosidase loci of E.coli and if this is the case it is of interest to note that the loci are not closely linked.

In both Saccharomyces (Spiegelman, 1950) and E.coli (Kalckar, Kurahashi and Jordon, 1959) the enzymes concerned in galactose metabolism are inducible. In Saccharomyces galactose metabolism is controlled by at least four unlinked loci (Hawthorne, 1956; Hawthorne and Mortimer, 1960), while in E.coli five groups of complementing galactose mutants reveal five loci controlling galactose metabolism, four are closely linked to each other and the site for lambda prophage while the fifth is unlinked to lambda (Lederberg, 1960). The utilisation of galactose by Aspergillus also occurs adaptively* and the eight mutants isolated define five (perhaps six) loci controlling the metabolism of galactose by the mould. The five loci recombine freely and thus there is no close linkage between genes controlling galactose metabolism in Aspergillus or Saccharomyces but some of the corresponding genes in Escherichia are closely linked.

*Later biochemical studies confirm that galactose is metabolised adaptively by Aspergillus.

Genetic analysis of auxotrophic mutants in moulds and yeast has shown that in general genes determining enzymes concerned with different stages in the same metabolic pathway are not linked or ordered in any obvious way (Harowitz, 1956; Pontecorvo, 1959), whereas in a number of bacteria, particularly Salmonella and Escherichia it is found that in some, but not all cases, genes determining enzymes in the same pathway are linked (Demerec, 1956; Demerec and Hartman, 1956; Demerec and Hartman, 1959) while in one example five linked genes are ordered in the same sequence as the enzymic reactions which they determine (Hartman, Loper and Serman, 1960). It has been argued that linkage of this type may have a selective value for bacteria in which mating characteristically involves only fragments of the genome (Demerec and Hartman, 1956; Clowes, 1960). The finding that genes determining lactose or galactose metabolism by Aspergillus are unlinked parallels the general result for moulds and yeasts.

Analysis of constitutive 'lac' mutants of E.coli which are recessive and involve a cytoplasmic factor has led to development of the concept of induction by displacement of endogenous repressor substances (Pardee, Jacob and Monod, 1959; Pardee, 1959), while dominant constitutive mutants and 'point' mutants which fail to form several different enzymes determined by linked loci are interpreted as mutations of 'operator' genes which control the function of linked structural genes and are the site of action of repressor substances (Jacob and Monod, 1959; Jacob, Perrin, Sanchez and Monod, 1960). Mutants of E.coli

which fail to form several enzymes of galactose metabolism determined by linked genes (Kalchar, Kurahashi and Jordon, 1959) may be interpreted as the result of mutation of operator genes. Inducibility of typosinase in Neurospora has been investigated and it is found that the loci determining enzyme structure are unlinked to those for enzyme formation (Horowitz et al., 1960). The possibility that constitutive lactose or galactose mutants of Aspergillus may occur has not been investigated but clearly such mutants could be of considerable interest in testing the 'operan' hypothesis in this organism.

In general the sugar mutants of A.nidulans have provided suitable markers for genetic analysis, the characters segregate sharply in both meiotic and mitotic recombination and allele ratios indicate good viability of the mutants on the media used. The markers are convenient in that no nutritional supplements are required for growth on media containing a utilisable carbohydrate, while the markers may also be used in selective techniques in which the relevant sugars are supplied as sole carbon source.

The numbers of genetic markers available in Aspergillus could be extended by use of other sugars in further mutation experiments. There is no obvious reason against the use of sugar markers in genetic studies with other colonial moulds.

Summary

1. Genetic analysis of 27 sugar mutants of A.nidulans has revealed 10 new loci, 9 have been located in linkage groups and 6 mapped meiotically.

2. The mutants are all recessive and none are closely linked to previously known markers.

3. Two loci controlling lactose metabolism and five controlling the metabolism of galactose recombine freely.

4. The sugar mutants are shown to provide suitable markers for genetic analysis in Aspergillus and similar markers would probably be of value in genetic studies of other moulds.

Chapter 6 A Difference in Complementation between Pairs of Mutants when Tested in Heterokaryons or Heterozygotes.

Recessive mutants are tested for complementarity by the combination of pairs of mutants in a double (trans) heterozygote the phenotype of which determines if the mutants are non-complementary (a mutant phenotype) or complementary (a phenotype equal or close to the wild type). The complementarity test which detects the functional homology of genes, is of particular value in the analysis of phenotypically similar mutants in defining functional units of allelism (Pontecorvo, 1952a) and has been used extensively in this way in a number of organisms, particularly Drosophila (Green and Green, 1949; Lewis, 1951; Carlson, 1960), Aspergillus (Pritchard, 1955; Pontecorvo, 1956; 1959; Roper and Pritchard, 1955), bacteriophage (Benzer, 1957), Neurospora (Beadle and Coonradt, 1944), and more recently Salmonella (Hartman et al., 1960b) and Escherichia coli (Lederberg, 1960).

The test can be applied to all organisms in which a prolonged diploid phase occurs in the life-cycle, among micro-organisms this is the case with the sporing yeasts (Winge and Laustsen, 1938), Aspergillus nidulans (Roper, 1952) and a number of other moulds (Pontecorvo, Roper and Forbes, 1953; Pontecorvo and Sermonti, 1954; Ishitani, Ikeda and Sakaguchi, 1956; Buxton, 1956).

In Neurospora, in which true heterozygotes have not been obtained (Pateman and Fincham, 1958), strains disomic for a single chromosome ('pseudo-wild types'; Pittenger, 1954) can be

used for complementarity tests (Pateman and Fincham, 1958; Case and Giles, 1960), and in E.coli the formation of clones of relatively stable partial heterozygotes in transduction ('heterogenotes'; Morse, Lederberg and Lederberg, 1956[b]) can also be used for the same purpose (Lederberg, 1960). The complementarity or 'cis-trans' test in bacteriophage (Benzer, 1957) involves an association of phage genetic elements which is analogous to the formation of a heterozygote.

In those micro-organisms in which a diploid phase is either transient or does not occur, complementarity tests may be done by the formation of balanced heterokaryons between pairs of mutants (Beadle and Coonradt, 1944; Pontecorvo, 1946). The heterokaryon test is extensively used in Neurospora and other moulds. In Salmonella unilinear inheritance of transducing particles that do not become incorporated into the recipient genome ('abortive transduction'; Stocker, Zinder and Lederberg, 1953) has provided a system in which complementarity tests may be made by an essentially heterokaryotic association of genetic elements (Ozeki, 1956; Hartman, et al., 1960b).

The application of complementarity tests to micro-organisms and particularly to large numbers of phenotypically similar auxotrophic mutants has shown that complementation between mutants can result from one of two distinct processes, namely inter-locus (inter-genic) complementation or intra-locus (intra-genic) complementation (Pontecorvo, 1956; Catcheside and Overton, 1958; Demerec and Hartman, 1959; Fincham, 1960; Yanofsky and St.Lawrence, 1960; Catcheside, 1960b).

The characteristic features of inter-locus complementation are that mutants fall into discrete groups, all the mutants within a group complementing all mutants in all other groups while the majority of mutants within a single group are characteristically non-complementary. The groups may or may not be closely linked (Pontecorvo, 1956) and rare exceptional mutants which fail to complement mutants in two or more adjacent groups are the result of deletions overlapping adjacent chromosome segments corresponding to different functional units (Benzer, 1957; Hartman et al., 1960a, 1960b).

Intra-locus complementation was at first regarded as an exception to the rule of non-complementarity of alleles (Calef, 1956; Pontecorvo, 1956) but it is now clear that it is the rule rather than the exception in those organisms in which it has been sought (Catcheside and Overton, 1958). Most work has been done with Neurospora (Fincham and Pateman, 1957; Pateman and Fincham, 1958; Giles, Partridge and Nelson, 1957; Woodward, Partridge and Giles, 1958; Catcheside, 1960b) and Salmonella (Hartman et al., 1958; 1960a; 1960b; Demerec and Hartman, 1959) but intra-locus complementation has also been detected in Saccharomyces (Roman, 1956; Woods, unpublished) and Aspergillus (Calef, 1956; Martin-Smith, 1958). The main features of intra-locus complementation are that it occurs between mutants which are not deletions (Catcheside, 1960b) and map as single genetic sites (Pateman and Fincham, 1958; Case and Giles, 1960; Hartman et al., 1960a and 1960b). It occurs at most, but not all, of the loci tested and only a minor fraction of the mutants at a locus

complement and then only in certain combinations (Catcheside and Overton, 1958; Catcheside, 1960b). The major fraction of mutants at a locus which are non-complementary in all combinations serve, together with the relationships of the complementing mutants, to identify all of the mutants with a common function, that is to identify a 'cistron' or a functional unit (Pritchard, 1960b). The relationship of complementing mutants can be described by complementation maps which are constructed by a method analogous to mapping by overlapping deletions (Benzer, 1957). The maps are characteristically linear though with some exceptions (Catcheside, 1960b) and divide the whole unit of allelism into a number of sub-units according to the relationship of the groups of complementing mutants (Giles, Partridge and Nelson, 1957; Catcheside, 1960b). The numbers of sub-units may be large (Catcheside, 1960b).

Complementarity tests can only be made with both heterokaryotic and heterozygotic association of genetic elements in a few organisms and in only one, A.nidulans, has this been done extensively. It has been general experience in A.nidulans that the results of tests done with either heterokaryons or heterozygotes are the same, although Pontecorvo's (1952b) analysis of cell organisation implies that mutant alleles may have different functional relationships when separated in different nuclei or combined within the same diploid nucleus.

In the course of testing pairs of sorbitol mutants for complementarity a difference was found between the growth of balanced heterokaryons on sorbitol compared to the growth of the

heterozygotes synthesised between the same strains. Although a situation like this has been expected in prihciple (Pontecorvo, 1952b) it has not been found previously. It was therefore necessary to discover if the apparent failure of certain heterokaryons to complement and establish wild type growth on sorbitol, although the corresponding heterozygotes complement and yield wild type growth on sorbitol, was due to intrinsic factors or to factors introduced by the method of testing. The possibility of forcing pairs of mutants that are expected to complement to form wild type heterokaryons on sorbitol by variation of the environmental conditions was also investigated.

Methods.

The principle of the method for testing mutants for complementarity in heterokaryons was first described by Beadle and Coonradt (1944). In its present application heterokaryons were established between pairs of sorbitol mutants by balance for complementary nutritional requirements when growing with glucose as carbon source. The heterokaryons were then tested for their ability to utilise sorbitol by transferring them to media containing sorbitol as sole carbon source. The composition of the sorbitol medium and the physical environment were varied in attempting to force the heterokaryons to form wild type growth on sorbitol, while the hyphae growing on sorbitol were tested for heterokaryosis by returning them to glucose media.

Heterokaryons were synthesised by the routine procedure for A.nidulans in which conidia of the two component strains are

mixed together on the surface of liquid C.M. and incubated overnight. The resulting pellet of mycelium was washed free of nutrients in sterile saline and transferred to the surface of a plate of M.M. where it was dissected into small pieces with mounted needles. On further incubation heterokaryotic mycelium was isolated as sectors growing out of some of these pieces and was maintained by continued transfer on M.M. The heterokaryons were balanced by requirement of the component strains for adenine and p.amino-benzoic acid or for biotin. Heterozygous diploids were isolated by harvesting conidia from the heterokaryons and plating them in M.M. Diploid spores gave rise to colonies which were recovered by visual inspection and subsequently purified by single colony isolation.

Results.

Seven sorbitol mutants were isolated, two of them (sb3 and sb5) are absolute mutants and the remaining five (sb4, sb6, sb7, sb8 and sb9) partial mutants. The initial complementarity tests (Chapter 5, Table 16) were made with heterokaryons or heterozygotes synthesised between strains of the type:-

ad14 paba1 y; sbx and bi1; w3; sby

All of the 21 possible combinations between the 7 sorbitol mutants taken a pair at a time were tested in heterokaryons. Each of the heterokaryons grew well with glucose as carbon source but not one established wild type growth when tested on sorbitol whether or not the medium was supplemented with the nutrients

required by the component strains. The results were that heterokaryons between two total mutants failed to grow at all on sorbitol, while heterokaryons between either a total and a partial mutant or between two partial mutants in all cases yielded partial (mutant) growth which was clearly different from that of the wild type.

A total of 20 combinations of pairs of mutants in heterozygous diploid strains were isolated from the 21 heterokaryons and tested for their growth on sorbitol minimal medium. Of the 20 heterozygotes 12 were complementary growing like the wild type on sorbitol while the remaining 8 were non-complementary and yielded partial (mutant) growth only (Chapter 5, Table 16). (The heterozygous diploid which was not isolated was that between the two total mutants sb3 and sb5. Repeated attempts to isolate this heterozygote and also the two homozygotes sb3/sb3 and sb5/sb5 were unsuccessful). Some of the colonies of complementing wild type diploids were observed to occasionally form sectors with the mutant phenotype when growing on sorbitol minimal medium. These sectors probably result from mitotic segregation of the sorbitol markers and indicate that the diploids were heterozygous for these markers and were not contaminants isolated in error. The high frequency at which the diploids appeared on the selective plates also argues against accidental contamination.

In later investigations two new sets of heterokaryons were synthesised between sb3 (a total mutant) or sb4 (a partial mutant) and the remaining sorbitol mutants using the same strains

as in the initial experiments. The heterozygous diploids were not isolated from this second set of heterokaryons.

All of the heterokaryons grew well on M.M. with glucose as carbon source though tending to sector out the component types. They were transferred three times on M.M. before testing for growth on sorbitol and only vigorously growing mycelium forming an homogeneous mixture of conidial heads of the two component types were used in making the tests. Pieces of mycelium from each heterokaryon were transferred to unsupplemented sorbitol medium or to sorbitol medium supplemented with the nutrients required by the parental strains. The inocula were taken from either younger or older growth on M.M. and in either larger (c 4 m.m. cube) or smaller (c 1 m.m. cube) blocks of medium. A total of 12 different inocula were tested for each of the heterokaryons. Precisely the same results were observed as in the initial tests and are summarised in Table 28. All of the heterokaryons yielded mutant (partial) growth on sorbitol although eight of the corresponding heterozygotes were previously found to be complementary and grow like the wild type on sorbitol. Heterokaryons between total mutants (sb3 + sb3; sb3 + sb5) failed to grow on both supplemented and unsupplemented sorbitol media, while heterokaryons between a total and a partial mutant, or between two partial mutants in all cases yielded partial growth on sorbitol. On supplemented sorbitol medium the heterokaryons segregated out the component strains.

TABLE 28

An Apparent Difference between the Ability of Pairs of Sorbitol Mutants to Complement in Heterokaryons or in Heterozygotes.

ad1^{4} paba1 y; sbx + bi1; w3; sby

Components	Growth on Sorbitol		Components	Growth on Sorbitol	
	H.K.	H.Z.		H.K.	H.Z.
sb3 + sb3	- T	?*	sb4 + sb3	- P	+
sb3 + sb4	- P	+	sb4 + sb4	- P	- P
sb3 + sb5	- T	?*	sb4 + sb5	- P	+
sb3 + sb6	- P	+	sb4 + sb6	- P	+
sb3 + sb7	- P	+	sb4 + sb7	- P	- P
sb3 + sb8	- P	- P	sb4 + sb8	- P	- P
sb3 + sb9	- P	+	sb4 + sb9	- P	+

Key

+ Wild type growth

- Mutant growth

?* The diploids could not be isolated.

T Fails to grow

P Partial growth

H.K. = Heterokaryon

H.Z. = Heterozygote

The results for the growth of the heterokaryons on sorbitol are those obtained in experiments with heterokaryons synthesised on two different occasions but between the same series of strains. The results for the heterozygotes are those obtained in the initial experiments described in Chapter 5.

Partial growth of the mycelium on unsupplemented sorbitol medium was maintained for at least three serial transfers on this medium, while the mycelium always immediately yielded vigorously growing heterokaryons on return to unsupplemented medium containing glucose. These two observations suggest that the mycelium growing on sorbitol was indeed heterokaryotic.

A number of experiments were done in attempting to force heterokaryons between pairs of mutants that complement in the heterozygote to form wild type heterokaryotic growth on sorbitol by varying the environmental conditions. Repeated transfer on unsupplemented sorbitol media only yielded growth of type first obtained, while incubation of the heterokaryons on unsupplemented media at 25° C. or at 30° C. rather than 37° C. also only produced growth of the mutant type. Transfer to sorbitol media supplemented with 0.1% glucose or 0.1% maltose (1/10 normal concentrations) yielded slightly better growth, but this could be attributed to the presence of an alternate carbon source and no wild type heterokaryons were observed. Transfer to media with higher concentrations of sorbitol than usual (5.5% final concentration as compared with 1.0%) again only produced heterokaryons of the mutant type. Supplementing the medium with either p.aminobenzoate or adenine (that is maintaining balance by requirement for adenine or p.aminobenzoate and biotin) also yielded only mutant heterokaryotic growth.

No experiments were done in which the pH of the test medium was varied from the usual value of about pH 6.5.

It therefore appears that for the strains and experimental conditions tested here, all combinations of sorbitol mutants in balanced heterokaryons fail to complement for growth on sorbitol, though certain pairs of mutants form complementary growth when tested in heterozygous diploids. In no case did a heterokaryon that might be expected to grow like the wild type on sorbitol produce growth other than that of the component with the less severe mutant phenotype.

Discussion.

It is reasonably certain that the balanced heterokaryons synthesised between pairs of sorbitol mutants remain heterokaryotic in their growth on sorbitol for it is unlikely that non-heterokaryotic mycelium could maintain characteristic partial growth during repeated subculture on unsupplemented sorbitol medium or immediately yield vigorous heterokaryotic growth when returned to media containing glucose. It is necessary, however, to isolate and test single hyphal tips to verify heterokaryosis of the mycelium.

The failure of the mutants to complement in heterokaryotic association could result from unsuitability of the nuclear balance established for growth of the heterokaryons on glucose minimal medium for growth on sorbitol minimal medium. However, if this were the cause of failure supplementing the sorbitol minimal medium with the nutrients required by the component strains, or combinations of these nutrients, should make some difference to the growth of the heterokaryons but no differences were detected.

On the other hand if the partial growth of the mutants was sufficiently rapid to prevent expression of any selective advantage that may be enjoyed by the complementing mutants in heterokaryotic association, it may still be expected that sectors of wild type growth should occur in the partially growing colonies. However no such sectors were observed among many colonies inspected although they would be distinguishable from growth of the mutant type.

Therefore it appears justifiable, on present evidence, to regard the differences observed between the heterokaryons and the heterozygotes as a function of the sorbitol mutants and not as the result of a spurious nutritional interaction. Further evidence on this point could be obtained by testing the mutants in heterokaryons synthesised between strains with different sets of complementary nutritional markers. This was not possible with the strains available.*

Accepting the results at their face value it becomes necessary to enquire into the relationship of the mutants. The evidence available is tabulated below.

* Heterokaryons and heterozygotes between strains ribo1 pro1 bi1; w3; sbx and ad14 paba1 y; sby have since been tested and found to yield exactly the same results as described here.

Phenotypes: Total mutants, sb3, sb5
Partial mutants, sb4, sb6, sb7, sb8, sb9

Complementarity Tests:

Result for Heterokaryons: all mutants non-complementary.

Result for Heterozygotes: (non-complementing mutants are in the same group or overlapping groups, complementing mutants are in groups which do not overlap).

sb3 sb5 sb4 sb7 sb6 sb9

sb8

Location:

Three of the mutants, sb3, sb4 and sb9 were independantly located in the same chromosome as s3 (Chromosome VI) by mitotic analysis but in meiotic analysis no evidence of linkage was detected between either sb3 or sb4 and s3 or two other markers located in this chromosome (Chapter 5, page 87).

Two of the mutants, sb3 and sb4, are apparently separable by recombination but the recombination fraction of 1% measured in selection experiments may be an over-estimate because sb3 and sb4 are complementary in heterozygotes and ascospores disomic for the sixth chromosome would be selected along with haploid recombinants but may not be distinguishable from the true haploids on the basis of their conidial diameters. In the absence of closely linked markers little progress can be made in recombination analysis but it would be interesting to determine qualitatively if recombination

occurs between other mutants particularly sb8 and the remaining mutants. All of the mutants should be tested for reversion.

The functional relationships of the mutants are ambiguous, the number of mutants tested is small and the data do not permit an unequivocal choice between inter-locus or intra-locus complementation. The pattern of complementation revealed by the heterozygotes is not typical of intra-locus complementation in which it is generally found that some mutants overlap several but not all other groups (Woodward, Partridge and Giles, 1958; Catcheside, 1960b). Moreover the proportion of complementing mutants is higher than usually observed, 6 of 7 mutants tested whereas 2 or 3 among 7 would be usual (Catcheside, 1960b; Crick and Orgel, 1960), but this may be a feature of testing heterozygotes for the fraction of one third complementing mutants generally quoted is derived from the results of heterokaryon tests in Neurospora. The three pairs of non-complementing mutants could be regarded as mutants at different but closely linked loci spanned by a single deletion (sb8) or as alleles of a single locus defined by sb8 and between which complementation occurs. On the whole the second hypothesis is the more economical but it is emphasised that critical evidence either way is lacking.

It is felt that the main interest of the observations reported here lies in the clear difference that has been consistently found in the ability of certain pairs of mutants to complement in heterozygotes but not in heterokaryons. The fact of this difference is independant of the precise formal

relationship of the mutants though its possible significance for theories of complementation depends on the relationship of the mutants.

Inter-locus complementation can be visualised as resulting from one of two kinds of mechanism. One, which is generally accepted, is that complementing mutants are defective in the ability to form different enzymes and complementation is due to the presence of both the enzymes in the cytoplasm of heterokaryons or double heterozygotes between the mutants.

A second mechanism is suggested by the results of recent studies on human haemoglobin and would involve enzyme sub-units rather than whole enzymes. The haemoglobin molecule is composed of two different pairs of identical polypeptide chains, one pair of identical chains are known as the alpha chains and the second pair as the beta chains. At acid pH the molecule dissociates assymetrically to yield separate pairs of alpha and beta chains and these pairs of chains will reassociate to form the original molecule upon neutralisation. As the result of mutations individuals produce haemoglobins with single defects in either the alpha or beta chains (Ingram, 1959; Hunt and Ingram, 1959), and in mixing experiments <u>in vitro</u> reassociation of pairs of alpha and beta chains of different origin has been shown to occur and to yield new types of 'hybrid' molecules (Itano and Singer, 1958; Singer and Itano, 1959).

This may be illustrated:-

Original Molecules				Products of Reassociation	
A*A* B B -----	A*A* + B B	)		A*A*B B	A A B B
		)	-----		
A A B*B* -----	A A + B*B*	)		A A B*B*	A*A* B*B*

The structures of the alpha and beta chains are determined by two unlinked loci (Smith and Torbert, 1958) and an individual doubly heterozygotic for genes determining defects in both the alpha and beta chains has been found to have red blood cells containing all four types of haemoglobin molecule that would be expected from random association of normal and defective alpha and beta chains (Itano, Singer and Robinson, 1959; Itano and Robinson, 1960). The second mechanism for inter-locus complementation suggested by these results postulates that complementing mutants are defective at loci determining different sub-units of a single enzyme and complementation is due to the association of undamaged sub-units (Fincham, 1960). A mechanism of this type probably applies in the case of tryptophane synthetase in E.coli in which the physiologically active enzyme is composed of two separable components determined by two closely linked genetic loci (Yanofsky and Crawford, 1959), and may also account for complementation of mutants at other pairs of closely linked loci determining similar phenotypes and of which there are many examples (listed in Demerec and Hartman, 1959).

If the sorbitol mutants represent three complementary loci (and sb8 is a deletion) explanation of the failure of complementation in the heterokaryons as a failure of the process of reassociation (be it of enzymes, parts of enzymes or even a permease and an enzyme) is not acceptable for if reassociation can occur in the cytoplasm of heterozygotic cells there is no apparent reason why it should not occur in the heterokaryon. However, a reasonable hypothesis can be advanced to account for the difference between heterokaryons and heterozygotes if the relative proportions of the re-associating units is considered. Thus if there is an excess of one type of nucleus in the heterokaryon an extreme disproportion of gene products may result and shortage of one of the re-associating units may result in such a small effective increase in enzymic activity due to complementation that it is not detectable against the growth of the partial mutants. Such disproportion could not occur in the heterozygotes.

Some evidence against nuclear imbalance was obtained, i.e. supplementing the sorbitol minimal medium with nutrients required by the component strains did not alter the growth of the heterokaryons, but better evidence would be provided if the results were confirmed in testing heterokaryons balanced for different nutritional requirements.

A number of mechanisms for intra-locus complementation have been suggested (Roman, 1958; Woodward, Partridge and Giles, 1958; Catcheside, 1960b; Crick and Orgel, 1960). It is unlikely

that intra-locus complementation results from direct inter-action of genes for somatic diploid nuclei have not been detected in Neurospora (Pateman and Fincham, 1958) while in Salmonella inter-action of genes (i.e. recombination) would result in effective incorporation of the transduced fragment and it is precisely the failure of incorporation which permits the detection of complementation (Ozeki, 1956). It is generally accepted that intra-locus complementation results from inter-action of gene products and although the possibility of inter-action at intermediate stages between D.N.A. and protein have been recognised by several authors (Catcheside and Overton, 1958; Catcheside, 1960b; Crick and Orgel, 1960), theories of complementation are all based on a protein-protein inter-action (Woodward, Partridge and Giles, 1957; Catcheside, 1960b; Crick and Orgel, 1960). The principal support for protein-protein theories comes from the demonstration of the recovery of specific enzymic activities in heterokaryons between complementing mutants (Woodward, Partridge and Giles, 1958; Pateman and Fincham, 1958; Yanofsky, 1960) and the case of tryptophane synthetase of Neurospora in which only mutants able to form an enzymically inactive protein closely related to the enzyme (C.R.M.) are able to complement, while in complementing heterokaryons C.R.M.'s corresponding to those of the two components are present together with the active enzyme (Yanofsky, 1960).

A theory of complementation based on non-overlapping functions (Roman, 1958) became untenable when the numbers of sub-units into which a locus can be divided by complementation became large (Catcheside, 1960a). A re-association theory

(Woodward, Partridge and Giles, 1958) based on analogy to *in vitro* re-association of alpha and beta haemoglobin polypeptides was particularly attractive in accounting for the observed maximum recoveries of 25 - 30% of the wild type enzymic activity (Woodward, Partridge and Giles, 1958; Pateman and Fincham, 1958). But this theory is also not consistent with large numbers of complementing sub-units within the locus or the formation of an enzyme qualitatively different from the wild type enzyme as the result of complementation (Fincham, 1959). Moreover the quantitative basis for the estimation of specific enzymic activities in heterokaryons is open to question for the proportion of homokaryotic mycelium is not known.

A current theory (Crick and Orgel, 1960) is based upon the hypothesis that loci at which complementation occurs determine the formation of polypeptides which aggregate to yield an active enzyme molecule. Mutation alters the amino-acid sequence in such a way that the configuration of the active site is disturbed and the enzyme inactivated. Complementing mutants are then a special class of mutants which determine alteration of amino-acids at places in one of the polypeptide chains such that alteration in the other chain allows the formation of an aggregate in which the correct configurations of the active site is restored. This theory derives from the observed linearity of complementation maps (Catcheside, 1960b) and the approximate co-linearity of the genetic and complementation maps for the pan 2 locus in *Neurospora* (Case and Giles, 1960). It also predicts the formation of an active enzyme qualitatively different from the wild type enzyme

as observed by Fincham (1959). On the point of aggregation of differently altered but like polypeptide chains in the cytoplasm of a heterokaryon the theory is not supported by the observation that individuals heterozygotic for genes determining defective alpha or beta haemoglobin polypeptides do not form mixed aggregates of the type alpha + defective alpha (Itano, Singer and Robinson, 1959) or beta + defective beta (Vinograd and Hutchinson, 1960).

If the sorbitol mutants comprise an allelic series the failure of mutants to complement in heterokaryons could be accounted for in two ways:-

(1) Imbalance of nuclei and nuclear products results in the formation of few active aggregates.

(2) Inter-action of gene products is not at the protein-protein level but at a stage of synthesis intermediate between D.N.A. and protein.

Nuclear imbalance has been discussed above, the arguments are the same for failure of either inter- or intra-locus complementation in heterokaryons.

The difference between the ability of a pair of mutants to complement in the heterozygote and their inability to do so in a heterokaryon clearly implies that complementary inter-action cannot take place in or across the cytoplasm of the heterokaryon but only within the heterozygotic nucleus. Since complementing mutants recover the ability to grow like the wild type on sorbitol

it is most probable that they synthesise a fully active enzyme protein, but as the synthesis of cytoplasmic proteins does not generally occur in the nucleus (Brachet, 1955) the inter-action taking place in the nucleus probably does not involve proteins and may involve some other gene product, possibly R.N.A. Failure of the gene products to act across the cytoplasm could be due to instability outside the nucleus, or the product becoming 'enclosed' once in the cytoplasm and not available for inter-action. It is of interest in this connection that evidence has been obtained for unstable intermediates between D.N.A. and enzymic protein (Riley, Pardee, Jacob and Monod, 1960). The mechanism of inter-action within the nucleus is entirely conjectural, but possible mechanisms would be 'copy-choice' in the synthesis of R.N.A. templates or re-association of R.N.A. sub-units.

It would be of great interest to know more of the enzymology of the mutants, the enzymic defect could well be a sorbitol dehydrogenase (Shockley and Pride, 1959), but the present lack of closely linked markers for fine genetic analysis is most unfortunate.

Observations similar to the ones reported here have been made by Case and Giles, (1960) with mutants at the pan 2 locus in Neurospora. They note that complementation was more consistent and clear-cut in pseudo-wild types than heterokaryons and attribute this to a nuclear ratio effect. They also report a few cases of failure to detect complementation between mutants in

heterokaryons while the corresponding pseudo-wild types complement.

Summary

1. A difference has been observed between complementation of pairs of sorbitol mutants in heterokaryons or in heterozygotes. The difference is, on present evidence, a function of the mutants.
2. An unequivocal choice cannot be made between inter-locus or intra-locus complementation on the data available.
3. It is suggested that complementing mutants form an active enzyme in the heterozygote which permits growth on sorbitol but fail to form this enzyme in heterokaryons at a level great enough to be detectable against the partial growth of the mutants.
4. Two hypotheses are advanced to account for the differences observed between the heterokaryons and heterozygotes.
   a. The difference is due to imbalance of the nuclei in the heterokaryon which leads to disproportion of enzyme units involved in complementation. Disproportion does not occur in the heterozygotes. This hypothesis is equally applicable to inter-locus or intra-locus complementation.
   b. If complementation is intra-locus it occurs only as the result of inter-action within the heterozygotic nuclei and probably does not involve a protein-protein inter-action.

Part II The Genetic Analysis of Galactose Utilisation by Aspergillus nidulans.

Introduction

Among the sugar mutants of A.nidulans isolated in the first part of the investigation those failing to utilise galactose for growth occurred most frequently. Genetic analysis shows that the eight mutants isolated define five genes concerned with the control of galactose metabolism, two of the genes were located in well marked chromosomes although neither was found to be closely linked to known markers. In view of recent progress in knowledge of pathways of galactose metabolism (Kalckar, 1958) and the identification of specific enzymic deficiencies in mutants of Saccharomyces (Robichon-Szulmajster, 1958;) E.coli (Kurahashi, 1957; Kalckar, Kurahashi and Jordon, 1959) and Salmonella (Fukasawa and Nikaido, 1959b) which fail to metabolise galactose it seemed likely that the galactose mutants of A.nidulans might provide a suitable experimental system in which combined genetic and biochemical analysis could be carried out. The object of the second part of the investigation was to develop the genetic analysis of the mutants, particularly to attempt to map a series of mutant alleles at one of the galactose loci.

The model of gene structure proposed by Pontecorvo in 1952 has been strikingly confirmed by the results of genetic analysis at high resolution in organisms ranging from bacteriophage to Drosophila (Pontecorvo, 1959). It is found that the majority of independantly arising allelic mutants, mutants which fail to

complement when combined in trans heterozygotes, are separable by recombination and therefore result from mutation at different sites within the same gene or cistron. The number of sites within the gene at which mutation can occur is large (Pontecorvo and Roper, 1956) and in many organisms it has been found that these sites may be arranged in a unique linear array. Analysis of intra-genic recombination reveals linearity of mutant sites in organisms ranging from those without chromosomal apparatus such as phage (Benzer, 1957; Streisinger and Franklin, 1956; Campbell, 1959), Salmonella (Demerec and Hartman, 1956; Hartman et al., 1960a), E.coli (Pardee, Jacob and Monod, 1959; Yanofsky and Crawford, 1959; Gross and Englesberg, 1959), and the yeast Schizosaccharomyces (Leupold, 1958) to organisms such as Aspergillus (Roper, 1950; Pritchard, 1955; Martin-Smith, unpublished), Neurospora (Case and Giles, 1958 and 1960; Yanofsky, 1960) and Drosophila (Green and Green, 1949; Carlson, 1959).

In Drosophila and Aspergillus the results of intra-genic analysis are consistent with recombination by a mechanism of crossing over. Reciprocal products, the double mutant in the cis arrangement, have been recovered at a number of loci in Drosophila including lozenge (Green and Green, 1949), star-asteroid and bitharax (Lewis, 1951) and dumpy (Carlson, 1959), and also the ad8 (Roper and Pritchard, 1955) and ad9 (Martin-Smith, unpublished) loci in Aspergillus as the result of mitotic crossing over. In both organisms intra-genic recombination is accompanied by recombination of adjacent markers while the distribution of the markers among cross-over and non-crossover classes permits

deduction of the order of the alleles (Roper, 1950; Pritchard, 1955 and 1960a; Martin-Smith, unpublished). In Aspergillus the results of three point crosses between alleles are also consistent with recombination by crossing-over (Pritchard, 1955).

In Saccharomyces analysis of half tetrads resulting from intra-genic mitotic recombination has failed to demonstrate reciprocal products at the ad3 and ad6 loci (Roman, 1956) or the isolencine locus (Roman and Jacob, 1958). In Schizosaccharomyces reciprocal products were not demonstrated at the ad7 locus while the apparent recovery of reciprocal products at the mating type locus is open to doubt in the interpretation of phenotypes (Leupold, 1958). Tetrad analysis in crosses between a pair of allelic pyridoxin mutants in Neurospora showed that three asci in which wild type pyridoxin independant ascospores were recovered did not contain the expected double mutants (Mitchell, 1955), while in similar analysis of the pan2 locus wild type pantothenic independant recombinant spores were isolated from asci some of which contained the expected double mutants and some of which did not (Case and Giles, 1958). Analysis of intra-genic recombination in Neurospora using random ascospores generally yields prototrophic recombinants in which there is an equal distribution of adjacent markers between cross-over and non-crossover classes (St.Lawrence, 1956; Freese, 1957; Mitchell, 1957) though alleles at the pan2 locus have been ordered using adjacent marker recombination (Case and Giles, 1960). Mitotic half tetrads in yeasts failed to demonstrate recombination of markers adjacent to the ad6 locus (Roman, 1956) but showed a high frequency of recombination

of markers adjacent to the inositol (Roman and Jacob, 1958) and mating type loci (Leupold, 1958).

Failure to detect reciprocal products of intra-genic recombination in yeasts and Neurospora and the tendency for adjacent markers to yield an excess of parental combinations has led a number of authors to conclude that the mechanism of intra-genic recombination is fundamentally different from that of inter-genic recombination by crossing-over (Mitchell, 1955; Roman and Jacob, 1958; St.Lawrence, 1956). Mechanisms based on analogy to the theory of gene conversion developed by Lindegren (1949) to explain irregular 3:1 segregation ratios in yeast tetrads and using the idea of non-synchronous replication in a copy-choice mechanism (Lederberg, 1955) have been supported by several authors (Beadle, 1957; Mitchell, 1957; St.Lawrence and Bonner, 1957; Roman, 1956). Although there is no doubt that 'genuine' irregular tetrads do occur (Mitchell, 1955a and 1955b; Strickland, 1958) these are exceptional while proof of intra-genic recombination by crossing-over is satisfactory in Drosophila and Aspergillus. A hypothesis which is gaining general acceptance is that recombination may have a common mechanism in all organisms and that high resolution analysis is revealing features of recombination not suspected from the results of classical analysis (Pontecorvo, 1959; deSerres, 1958; Pritchard, 1960a and 1960b).

The most striking feature of intra-genic recombination revealed so far is the occurrence of intense localised negative interference, that is a marked increase in recombination in the intervals immediately adjacent to the region within which

recombinants are being selected (Pritchard, 1955 and 1960a). The intensity of interference increases as the selected interval becomes smaller. Interference occurs in both meiotic and mitotic recombination and also when recombination is selected between closely linked non-allelic mutants, that is in inter-genic recombination (Calef, 1957; deSerres, 1958; Giles, deSerres and Barbour, 1959). Analysis of negative interference has led to the proposal that recombination only occurs in a small proportion of dividing cells in which homologous chromosomes are effectively paired while in the effectively paired segment crossing-over occurs frequently (Pritchard, 1960a).

The first step in further analysis of galactose utilisation by A.nidulans was the isolation of more mutants. The number of mutants was increased to 36 and most of these grouped in preliminary experiments to detect recombination between the new mutants and located galactose mutants. An attempt was made to order a series of alleles at one of the galactose loci but this was not successful.

Chapter 7 The Isolation and Preliminary Location of Additional Mutants Failing to Utilise Galactose for Growth.

1. The Isolation of the Mutants.

A. Method.

The method of U.V. irradiation and replica plating described in Chapter 3 was employed in the induction and isolation of the mutants.

The strain used was again bi1;w3 and in each of twelve irradiation experiments the conidia treated were harvested from slope cultures grown from different single colony isolates of the strain. In each experiment 10 initial plates were prepared bearing a total of between 1,000 and 1,500 colonies derived from conidia surviving U.V. irradiation (5% survival) and the colonies replicated onto Basal Medium (plus biotin) with either galactose or glucose as carbon source and also onto C.M. Strains failing to grow on galactose but growing on glucose were isolated and subjected to the testing and purification procedure described in Chapter 3. Auxotrophic mutants were also isolated.

B. Results.

A total of 97 auxotrophic mutants were isolated by replica plating from initial plates bearing 16,517 colonies, the rate of isolation of auxotrophs, 5.9 per 1,000 colonies tested, was of the same order as observed previously. Details of the irradiation experiments and yield of auxotrophic mutants are recorded in Table 29

TABLE 29 Twelve Irradiation Experiments to Isolate Additional Galactose Mutants

Experiment	Culture		U.V. Irradiation			Replica Plating				
						Initial plates			Auxotrophic mutants	
	Age (days)	Viability of conidia (%)	Total conidia ($\times 10^7$)	Exposure (mins)	Survival (%)	Colonies			Total	per 1,000 colonies.
						No.	Total	Per plate		
bi1; w3										
a	5 + 0*	97	1.12	8	5.0	10	1424	142 ± 17	8	5.6
b	6 + 0	99	0.91	8	5.1	10	1110	111 ± 19	9	8.1
c	6 + 2	95	1.08	8	9.0	10	1859	186 ± 23	4	2.2
d	6 + 3	80	1.14	8	8.3	10	1513	151 ± 17	7	4.6
e	6 + 5	108	1.12	8	5.0	10	1508	151 ± 27	7	4.6
f	6 + 6	105	1.08	8	4.9	10	1516	152 ± 22	7	4.6
g	6 + 6	97	1.03	8	6.1	10	1101	110 ± 32	11	10.0
h	6 + 7	78	0.98	8	5.8	10	1172	117 ± 19	9	7.7
i	5 + 0	87	1.12	8	5.0	10	1154	115 ± 13	9	7.8
j	5 + 1	78	1.16	8	5.4	10	1216	122 ± 21	10	8.2
k	5 + 2	94	0.86	8	6.0	10	1355	136 ± 18	6	4.5
l	5 + 3	99	1.24	8	5.4	10	1589	159 ± 21	10	6.3
					Totals	120	16517	138 ± 31	97	5.9

* The first figure is the period of incubation of the culture and the second the period of storage before harvesting the conidia.

A further 28 mutants failing to grow on galactose were isolated, 19 of these are total mutants and the remaining 9 slow growing mutants. The yield of galactose mutants, 1.7 per 1,000 colonies tested, is about twice that in the initial experiments, this possibly results from increased experience in the replica plating technique and the closer attention that is given when screening for a single type of mutant. The origin of the new mutants, numbered serially gal9 to gal36, is shown in Table 30 together with description of their phenotypes.

2. The Preliminary Location of the Mutants.

The most convenient method for grouping the new mutants is to test for recombination between them and located mutants. An accurate measure of the frequency of recombination is unnecessary for the purpose of preliminary location and a qualitative test based on the appearance of recombinant strains with wild type growth on galactose was employed. The test will detect recombinants occurring at a rate of 5% or greater, it was checked in crosses between pairs of located mutants and found to be satisfactory.

A. Method

Crosses were set up between each of the new mutants (bi1;w3;gal x) and either paba1 y ad20; gal1 or gal5 paba1 y ad20, later crosses were also made between the mutants remaining unlocated and paba1 y ad20; gal7. A single large perithecium was selected from each cross cleaned of contaminating hyphae and conidia and crushed in a small volume of saline. Samples of the resulting suspension containing about 200 viable ascospores

TABLE 30 The Origin and Phenotypes of 28 Additional Galactose Mutants

Irradiation experiment	No. of Colonies tested.	Galactose mutants	Growth on galactose
a	1424	gal 9	- T
		gal 10	- T
		gal 11	- S
		gal 12	- T
b	1110	gal 13	- T
		gal 14	- T
c	1859	gal 15	- T
d	1513	gal 16	- S
e	1508	gal 17	- T
		gal 18	- T
		gal 19	- T
f	1516	gal 20	- S
g	1101	gal 21	- S
		gal 22	- S
		gal 23	- T
h	1172	gal 24	- T
i	1154	gal 25	- S
		gal 26	- S
j	1216	gal 27	- T
		gal 28	- S
		gal 29	- T
		gal 30	- T
		gal 31	- S
k	1355	gal 32	- T
l	1589	gal 33	- T
		gal 34	- T
		gal 35	- T
		gal 36	- T
Totals	16517	28	19 Total Mutants 9 Slow growing mutants

-T Total mutant, fails to grow on galactose

-S Slow growing mutant.

were streaked with a wire loop on C.M. and on Basal Medium + galactose + the nutrients required by the parental strains. The plates were inspected after three days incubation.

The growth resulting on C.M. distinguishes between hybrid and selfed perithecia by the appearance of green spored recombinant strains among the progeny of a hybrid. The test was repeated if a selfed perithecium had been selected. On the medium with galactose spores from hybrid perithecia produced either wild type growth or failed to grow, distinguishing between freely recombining mutants which yield many wild type recombinants and pairs of closely linked mutants which yield few wild type recombinants.

B. Results.

(1) Test of Method

The method was applied to five crosses between located mutants.

Cross	Growth of ascospores streaked on galactose.
paba1 y ad20 x bi1; w3	
gal6 x gal1	-
gal8 x gal5	-
gal6 x gal5	+
gal2 x gal5	+
gal3 x gal5	+

The recombination test differentiates clearly between the allelic mutants (gal1 and gal6, gal5 and gal8) and the non-allelic mutants.

(2) Location of new mutants.

The results of the crosses with gal1, gal5 or gal7 are

described in Table 31. Of the 28 new mutants 10 are closely linked to gal1, 5 to gal5 and 3 to gal7, the remaining 10 mutants recombine freely with the galactose markers tested. No conflicting results were obtained. The results for seven of the crosses with gal1 were checked by plating spores from hybrid perithecia on C.M. and testing the resulting colonies individually for growth on galactose, or by selection experiments in which spores from a hybrid perithecium were plated in a medium with galactose as sole carbon source and scored for the appearance of colonies capable of growing on galactose (samples of the suspensions were also spread on C.M. to estimate the numbers of viable ascospores tested). The results of these checks all confirmed the results of the qualitative tests.

Cross	Qualitative test for recombination	Total progeny tested.	gal$^+$ recombinants	Estimated recombination fraction.
Plating on C.M.:				
gal1 x gal12	+	26	9	50%
x gal22	+	58	15	50%
x gal27	-	84	0	$<$ 2.0%
Selective plating:				
gal1 x gal15	-	7200	7	0.2%
x gal23	-	1900	0	$<$ 0.05%
x gal24	-	2200	0	$<$ 0.05%
x gal36	-	6000	2	0.07%

The five new mutants closely linked to gal5 were all shown to be non-complementary to gal5 (Chapter 9). The result of the analysis of the galactose mutants is summarised in Table 32.

TABLE 31

Location of the New Galactose Mutants by Recombination Tests.

Mutant	Crossed to gal1	gal5	gal7	Location confirmed
gal9	+	+	+	
10	+	-		Non-complementary to gal5
11	+	+	-	
12	+	+	+	
13	+	-		Non-complementary to gal5
14	+	-		Non-complementary to gal5
15	-	+		c 0.2% recombination with gal1
16	+	+	+	
17	-	+		
18	+	+	+	
19	+	-		Non-complementary to gal5
20	+	+	+	
21	+	+	+	
22	+	+	-	
23	-	+		0.05% recombination with gal1
24	-	+		0.05% recombination with gal1
25	+	+	+	
26	+	+	-	
27	-	+		2.0% recombination with gal1
28	+	+	+	
29	-	+		
30	-	+		
31	+	+	+	
32	-	+		
33	+	-		Non-complementary to gal5
34	+	+	+	
35	-	+		
36	-	+		c 0.07% recombination with gal1

The table shows the growth of a suspension of ascospores from a hybrid perithecium streaked on galactose.

Key:- - Mutant. + Wild type.

TABLE 32

The Genetic Analysis of Galactose Mutants of A.nidulans.

Locus	Closely linked mutants	Non-complementary mutants	Total
gal1	gal1; 6; 15; 17; 23; 24; 27; 29; 30; 32; 35; 36;	gal1; 6	12
gal2			1
gal3			1
gal4, gal7 ?	gal4; 7; 11; 22; 26;		7
gal5	gal5; 8; 10; 13; 14; 19; 33;	*gal5; 8; 10; 13; 14; 19; 33;	7
Not located	gal9; 12; 16; 18; 20; 21; 25; 28; 31; 34;		10
		Total	36

* The results were obtained in the experiments described in Chapter 9.

Discussion

The replica plating technique was effective in isolating more mutants which fail to grow on galactose and the total number of mutants available was increased to 36. The rate at which auxotrophic mutants were isolated in these experiments was about the same as in the earlier experiments but the galactose mutants were isolated at double the previous rate (1.7 mutants in 1,000 survivors tested, compared to 0.8 mutants per 1,000 survivors). Examination of the replica plates may have been more critical in the second series of mutation experiments in which a single type of mutant was sought.

The purely qualitative method employed to locate the new mutants was successful for in all cases in which the provisional result was checked it was found to have been correct. A total of 12 mutants (all total mutants) are available at the gal1 locus and 7 (also total mutants) at the gal5 locus. Of the remaining mutants three slow growing mutants are closely linked to gal7, another slow growing mutant. The remaining mutants were not tested for recombination with gal2 or gal3 and may include mutants unlinked to either of these markers and defining further loci concerned with the control of galactose metabolism by A.nidulans.

Summary.

1. A further 28 mutants failing to utilise galactose for growth were isolated by replica plating following U.V. irradiation, 19 are total mutants and 9 slow growing mutants.

2. Qualitative recombination tests located all but 10 of the new mutants. Ten of the mutants are closely linked to gal1, five are closely linked to gal5, and three to gal7.

Chapter 8. The Suitability of the Galactose Mutants for Selective Plating Techniques.

The development of high resolution genetic analysis in Micro-organisms (Pontecorvo, 1952a) has depended upon the use of induced auxotrophic mutants (Beadle and Tatum, 1941) and the application of selective techniques (Tatum and Lederberg, 1946) to isolate rare prototrophic recombinants from large populations of mutants. Selective techniques were first used successfully for the analysis of intra-genic recombination in Aspergillus by recovering prototrophes formed in crosses between allelic mutants (Roper, 1950; Pritchard, 1955) and later to isolate prototrophic diploids arising by recombination in mitotically dividing heterozygous diploid organisms (Roper and Pritchard, 1955; Pritchard, 1955 and 1960a). The use of selective techniques for intra-genic analysis has since been extended to a variety of other micro-organisms (see Pontecorvo, 1959 and Demerec and Hartman, 1959 for recent reviews).

In moulds and yeasts almost all of the loci subjected to fine genetic analysis determine nutritional requirements but in principle selective techniques are equally applicable to loci controlling the metabolism of carbohydrates and in the bacterium Escherichia coli mutants which fail to assimilate lactose (Pardee, Jacob and Monod, 1959) or arabinose (Gross and Englesberg, 1959) have been used for fine genetic analysis. The experiments described in this chapter investigate the suitability of the galactose mutants for selective techniques when screening

populations of cells for recombinants formed either meiotically or mitotically. Only the two loci with known linked markers (gal1 and gal5) were tested.

1. Reconstruction Experiments to Test for the Grigg Effect.

When a mixed population of cells is plated in a medium in which only a small proportion of the cells can grow it has been observed that in certain cases the growth of the colony forming cells depends upon the density of plating, the recovery of colony forming cells falling off as the total number of cells per plate increases. This phenomenon may be called the Grigg effect (Grigg, 1952) and results from inhibition of the cells capable of growth by those that cannot grow, inhibition may be due to accumulation of antagonistic substances produced by the non-colony forming cells or exhaustion of a nutrient required by the growing cells. In experiments with some mutants of Neurospora the limiting factor has been shown to be exhaustion of carbohydrate (Grigg, 1960). This type of effect could well occur in selection experiments with the galactose mutants in which rare recombinants able to utilise galactose are selected from heterogeneous populations of mutants and reconstruction experiments were done to examine this possibility and to test if the gal+ organisms are distinguishable by selective techniques.

Method.

A number of reconstruction experiments were done in which large numbers of conidia of strains with the gal1 or gal5 markers were plated in media containing galactose as carbon source together

with small numbers of conidia of strains capable of utilising galactose (gal$^+$ strains). Generally strains with contrasting conidial colours were used to facilitate scoring. The media all contained galactose at a final concentration of 1% (w/v) and were supplemented with the nutrients required by the strains under test at the usual concentrations.

Conidia were carefully harvested with a wire loop from the surface of C.M. slope cultures, suspended in saline + calsolene and the chains of conidia broken by passage through a Pasteur pipette. The concentration of conidia in the suspension was then estimated by haemocytometer counts and adjusted to the desired level when a sample of the suspension was diluted and plated on C.M. to estimate the proportion of viable conidia. Further samples at different dilutions were used in the experimental platings and the numbers of viable conidia plated calculated from the control platings on C.M.

Results.

(1) Exhaustion of Biotin in the Medium.

In an early experiment in which conidia of the strain bi1 were plated with gal5 bi1; w3 the number of bi1 colonies recovered decreased as the numbers of gal5 bi1; w3 conidia increased. In this case inhibition of growth was shown to result from the depletion of biotin in the medium for the expected numbers of bi1 colonies were recovered when the level of biotin was increased (Table 33). Colonies of the gal$^+$ strain were readily distinguished from the background growth before they had

TABLE 33

The Effect of Biotin in the Recovery of Colonies of bi1 from Mixed Platings of Conidia of gal5 bi1;w3 and bi1.

Biotin (micro-grm per plate)	Viable Conidia per plate		Number of bi1 colonies observed.	
	gal5 bi1;w3	bi1	Replicates	Mean
Experiment 1.				
0.2	8×10^7	90	15,17,13,29	19
0.2	8×10^6	90	50,63,61,56	58
0.2	8×10^2	90	78,76,79,88	80
Experiment 2.				
8	2×10^7	15	15, 17	16
80	2×10^7	15	11, 15	13
500	2×10^7	15	13, 16	15

It is shown in Experiment 1 that recovery of biotin requiring gal$^+$ colonies decreases at higher plating densities. Experiment 2 shows that failure to recover gal$^+$ colonies is due to the exhaustion of biotin in the medium.

started to spore.

(2) Delayed growth of the gal$^+$ strains.

Further experiments were done with a variety of strains in which galactose markers were combined with linked markers determining nutritional requirements. In all of these experiments the gal$^+$ colonies could be distinguished from the background growth. The expected numbers of gal$^+$ colonies were recovered even at the highest plating densities, but it was observed that in some cases the growth of the gal$^+$ strains was considerably slower at the higher plating densities than at lower densities. This was at first thought to be characteristic of the gal1 marker but it became clear on further experiments that the lag in growth was not correlated with gal1 but only occurred if no alternative carbon source (an amino acid) was present in the medium. The lag is probably a result of depletion of carbohydrate in the medium by the germinating conidia at higher plating densities (Table 34).

2. The Selection of Apparent Recombinants between Allelic Mutants.

The reconstruction experiments show that rare gal$^+$ strains may be selected from populations of galactose mutants by plating in media containing galactose as carbon source and that up to 10^7 cells can be tested in each dish without seriously reducing the recovery of the gal$^+$ strains. If pairs of allelic galactose mutants yield gal$^+$ strains as a result of intra-genic recombination (either meiotic or mitotic) the recombinants should also be detectable by selective plating and experiments were done to

TABLE 34

Recovery of Colonies of gal$^+$ Strains in Mixed Platings of Conidia.

Strains Plated	Total viable conidia plated.		Recovery of gal$^+$ colonies		Lag in growth of gal$^+$ colonies **	Alternate carbon source
	gal$^-$	gal$^+$	Replicates	Mean		
bi1;w3;gal1 and bi1	3×10^7	25	32,24,37,28	30	2 days	No
	3×10^2	25	17,22,21,22	20	-	
y;meth2 arg2 gal1 sm phen2 and y; meth2 arg2	4×10^6	100	95,90,105	95	None	Yes
	4×10^5	100	85, 126	106	None	
	4×10^4	100	107,112,78	99	None	
	4×10^3	100	117,88,89	101	-	
gal5 paba1 y ad20 and bi1; w3	4×10^6	100	61,77,86	75	2 days	No
	4×10^5	100	82,130,85	99	2 days	
	4×10^4	100	127,137,102	122	None	
	4×10^3	100	122,95,86	101	-	
su1ad20 gal5 ribo1 pro1 y ad20 and su1ad20 pro1 paba1 ad20	10^6	25	22, 25	24	None	Yes
	10^5	25	28, 25	27	None	
	10^4	25	26, 15	20	None	
	10^3	25	21, 25	23	-	
su1ad20 gal5 ribo1 pro1 y ad20 and ribo1 pro1 paba1 ad20 bi1; w3	10^6	80	86, 89	88	None	Yes
	10^5	80	81, 52	67	None	
	10^4	80	74, 83	79	None	
	10^3	80	91, 94	93	None	
	10^2	80	89, 84	87	-	

** The lag in growth is the increased incubation period required at higher plating densities to yield the same growth as observed after 3 days incubation at the least dense plating.

test this possibility. Only a few strains with markers linked to the gal1 or gal5 loci were available for these preliminary experiments and the experiments therefore do not provide critical evidence for recombination between allelic mutants particularly as the reversion rates of the mutants were not estimated. The experiments do show, however, that if gal$^+$ recombinants occur they will be isolated by the selective techniques used.

Methods.

(1) Meiotic analysis.

Crosses between allelic galactose mutants were fertile forming abundant perithecia, these were harvested en masse without cleaning and crushed against the side of a test tube with a glass rod to release the ascospores. Saline was added and the suspension, which contains ascospores, conidia and debris, filtered through sterile (autoclaved) nylon mesh (stocking material supported on a wire grid) to remove the larger debris including the empty perithecia. Samples of the resulting suspension were diluted and plated on C.M. to estimate the numbers of viable spores (ascospores and conidia) in the suspension. In each cross the parental strains carried the unlinked markers for white or yellow conidia, and green spored recombinant colonies appearing on the C.M. control plates provide a method of estimating the proportion of viable recombinant ascospores in the suspension. Known volumes of the suspension were mixed in cool molten Basal Medium containing 1% galactose as carbon source and the nutrients required by the parental

strains and poured at about 20 ml per dish. The plates were inspected after 3 to 5 days incubation.

(2) Mitotic analysis.

Heterokaryons balanced for complementary nutritional requirements were synthesised between pairs of allelic galactose mutants and diploids selected by the routine procedure. The diploids were purified by plating on C.M. and conidia taken from isolated single colonies (to avoid isolating from existing clones) plated at a low density on a variety of selective media containing galactose as the principal carbon source. The media were supplemented with small amounts of glucose to permit limited growth of the heterozygotes and provide conditions under which recombinants able to utilise galactose might be selected as rapidly growing sectors.

Results.

(1) Meiotic analysis. gal1 locus.

The crosses tested, gal1 x gal6 and gal1 x gal15, included markers linked to gal1, the recombination fractions between the distal marker (arg2) and gal1 being 34% and between gal1 and the proximal marker (sm) about 10%. Perithecia were harvested but on plating the resulting suspensions of spores in a selective medium containing galactose and supplemented with methionine, arginine, phenylalanine and biotin, a dense background growth resulted which prevented the confident selection of gal^+ strains. Heavy background growth was not observed in the reconstruction experiments with gal1, including one in which methionine and arginine were

TABLE 35

The Selection of Apparent Meiotic Recombinants between Allelic Mutants at the gal1 Locus.

Cross. $\frac{y \quad +}{+ \text{ bi1}} \quad \frac{+}{w3} \quad \frac{\text{meth2 arg2 (gal1 +)} \quad \text{sm} \quad \text{phen2}}{+ \quad + \quad (+ \text{gal6}) \quad + \quad +}$

	Cross gal1 x gal6	Cross gal1 x gal15
No.of selective plates	22	15
Total viable cells per plate (conidia and ascospores)	4.9×10^6	8.3×10^6
Total viable recombinant ascospores tested	15.4×10^6	25.5×10^6
gal$^+$ colonies	7	16
Apparent recombination fraction	0.98×10^{-6}	1.24×10^{-6}

present in the selective medium.

The most clear gal$^+$ colonies were picked from the selective plates and purified by plating for single colonies. Each of the strains had conidia of haploid dimensions, but all were non-requiring for methionine, arginine or phenylalanine, while of the 23 strains isolated all but two were also sm$^+$ (sm is a morphological marker for small colony). If the strains had arisen by crossing over they must have all resulted from either double (21 strains) or triple cross-overs (2 strains). However as no single cross-overs were detected and the rates at which the gal$^+$ strains were isolated were very low it seems most likely that they were reversions of either gal16 or gal15.

gal5 locus.

Crosses between two pairs of allelic mutants which did not include linked markers were fertile and on plating mixtures of ascospores and conidia in a selective medium supplemented with p.amino benzoate, adenine and biotin clear gal$^+$ colonies were isolated. The gal$^+$ strains were purified and had conidia of haploid dimensions. The strains showed normal segregation of the unselected markers (Table 36). (The reversion rate of some of the alleles of the gal5 locus were later estimated, see next Chapter, and found to be not unduly high).

The results obtained here indicate the possibility of achieving a fine analysis of the gal5 locus meiotically but this could not be attempted as the recombinant strains with linked markers were not available.

TABLE 36

The Selection of Apparent Meiotic Recombinants between Allelic Mutants at the gal5 Locus.

Cross $\frac{(gal5 \quad + \quad) \quad paba1 \quad y \quad ad20 \quad + \quad +}{(\ + \quad gal13\ ) \quad + \quad + \quad + \ bi1 \quad w3}$

	Cross gal5 x gal13	Cross gal5 x gal33
No.of selective plates	6	2
Total viable cells per plate (ascospores & conidia)	2.9×10^6	3.5×10^6
Total viable recombinant ascospores tested	2.4×10^6	1.50×10^6
gal$^+$ colonies	72	3
Apparent recombination fraction	56.8×10^{-6}	4.0×10^{-6}

Allele segregation among the apparent recombinants isolated in the Cross gal5 x gal13

paba1 = 33/39 y = 15/+ 15 ad20 = 33/+ 39 bi1 = 36/+ 36 w3 = 42/+ 30

(2) Mitotic Analysis

Equal volumes of dilute suspensions of conidia of the double heterozygotes (gal1 +/+ gal6 or gal5 +/+ gal8) were plated on Basal Medium containing 1% galactose without added glucose or supplemented with 1/100 or 1/1000 the normal amounts of glucose. The same volumes of the suspensions were also plated on C.M.

Roughly equal numbers of colonies were recovered on all of the plates in both experiments. On the selective plates with added glucose the colonies grew evenly without forming sectors, whereas on the medium with no added glucose the colonies grew very slowly and occasionally produced rapidly growing wild type sectors.

In both experiments the sectors were diploid and prototrophic.

Diploid $\frac{paba1\ y\ ad20\quad +}{+\quad +\quad +\quad bi1}\quad \frac{+}{w3}\quad \frac{gal1\quad +}{+\quad gal6}$

Medium	Colonies observed (Replicate dishes)	Diploid gal^+ sectors.
C.M.	23, 24, 20	-
B.M. + 1% Galactose	22, 29, 18, 25	5
" " + 0.01% glucose	15, 19, 25, 23	0
" " + 0.001% "	35, 33, 21, 18	0

Diploid $\dfrac{(gal5\ +\ )\ paba1\ y\ ad20\ +}{(\ +\ gal8)\ +\ +\ +\ bi1}\quad \dfrac{+}{w3}$

Medium	Colonies observed (Replicate dishes)	Diploid gal^+ sectors.
C.M.	18, 11, 20, 17	-
B.M. + 1% Galactose	17, 17, 17, 19, 15, 17, 16, 20	6
" " + 0.01% glucose	18, 12, 15, 17	0
" " + 0.001% "	20, 11, 12, 16	1

Discussion

The experiments described above show that the galactose mutants are suitable for selective plating techniques and that rare organisms capable of growth on galactose may be selected from populations of mutants. In reconstruction experiments gal^+ conidia form easily recognised colonies when plated with large numbers of conidia of mutants in media containing 1% galactose as carbon source. The vitamin biotin, normally added at very high dilution, may be exhausted from the medium and thus reduce the recovery of gal^+ biotin requiring organisms, while in some selective media the growth of gal^+ organisms may be slowed but it was generally found in the reconstruction experiments that the expected numbers of gal^+ organisms were recovered.

Selective plating of ascospores, together with parental conidia, derived from crosses between allelic galactose mutants led to the isolation of haploid gal^+ strains, while double heterozygotes synthesised between pairs of allelic mutants in the

trans arrangement form slow growing colonies when plated on galactose and give rise to rapidly growing wild type gal$^+$ diploid sectors at a reasonable rate.

Two crosses between allelic mutants at the gal1 locus included linked markers but gal$^+$ organisms were isolated at a low rate (of the order of 10^{-6}) and had apparently arisen without recombination of the linked markers. It seems most likely that these organisms were revertants but the reversion rates of the mutants involved were not estimated. A heavy background growth was experienced in these experiments, preventing confident selection of gal$^+$ colonies, the growth may have been the result of the inclusion of three amino acids in the selective medium or perhaps was due to the presence of unreduced ascospores for the corresponding heterozygotes are known to yield partial growth on galactose. Unreduced ascospores can occur in crosses at a rate of 1 in 1,000 (Pritchard, Pontecorvo, 1953) and may thus have been present at numbers of 500 to 1,000 per dish.

In crosses between gal5 alleles and also in experiments to detect mitotic recombination between gal1 or gal5 alleles strains with linked markers were not available and it is therefore not possible to distinguish if the gal$^+$ organisms isolated had arisen by reversion or intra-genic recombination.

Summary

1. Organisms capable of growing on galactose and present as a small proportion of heterogeneous populations of mutants not growing on galactose may be isolated by selective plating on media containing galactose as carbon source.

2. By use of selective plating techniques haploid *gal*$^+$ organisms are isolated from populations of ascospores derived from crosses between pairs of allelic galactose mutants, and diploid *gal*$^+$ organisms are isolated from mitotically dividing cells of the double heterozygotes synthesised between pairs of mutants.

Chapter 9. An Attempted Mitotic Analysis of the gal5 Locus.

Fine genetic analysis of the gal5 locus was attempted through the parasexual cycle employing the methods to detect mitotic recombination developed by Roper and Pritchard (1955) and Pritchard (1955; 1960a). Four allelic mutants were selected for study and their reversion rates estimated. The mutants were outcrossed to isolate recombinant strains with linked markers, the recombinants combined in double heterozygotes with pairs of mutant alleles in the trans arrangement and wild type (gal^+) diploid sectors isolated from the heterozygotes during growth on a selective medium containing galactose as sole carbon source. The heterozygotes produced many sectors whereas controls homozygous for each of the alleles produced few sectors. The analysis failed to yield information about the possible order of mutant sites in the gal5 locus for the gal^+ diploid sectors appeared to result from a process not involving crossing over but by apparent reversion of the mutants.

1. Reversion Rates of Mutants at the gal5 Locus.

A. Method.

Single colonies of the four strains tested (gal5 bi1;w3 and similar strains with the alleles gal8, gal10 or gal13) were isolated by plating and used to establish slope cultures. Conidia were collected from the slopes after 7 days incubation, suspended in saline + calzolene and the conidial chains broken up. Samples of each suspension were then plated at high dilution on C.M. to estimate the numbers of viable conidia, and further samples plated without dilution in Basal Medium + 1% galactose and biotin (40 microgrms per plate). A top layer of the same medium without conidia was added. Revertants growing on galactose formed identifiable colonies after 4 days incubation, some of them were purified by plating and retested for growth on galactose. The revertants were not out-crossed to test for suppressor mutations.

B. Results.

The results of the experiments are described in Table 37. The reversion rates of three of the mutants are each less than 10^{-8} while the high figure obtained in one experiment with gal13 was apparently the result of sampling a clone for no revertants were detected in a second experiment.

TABLE 37

The Reversion Rates of Mutants at the gal5 Locus.

Mutant	Total viable conidia plated	Total gal$^+$ revertants	Frequency of reversion
gal5	16.15×10^8	0	-
gal8	48.90×10^8	2	0.04×10^{-8}
gal10	72.22×10^8	7	0.10×10^{-8}
gal13	17.34×10^8	903*	52.0×10^{-8}
	6.76×10^8	0	-

* The large number of revertants in this experiment was apparently the result of sampling a clone.

2. Attempted Mitotic Analysis

A. Method.

The gal5 locus is in Linkage Group 1 between the markers su1ad20 and ribo1; su1ad20 is distal to gal5 and the distances between the loci are 19.3% meiotic recombination between su1ad20 and gal5 and 36.6% between gal5 and ribo1. The location of these markers together with the other Group 1 markers involved in the analysis is shown in the following map.

su1ad20	gal5	ribo1	lu1		pro1	paba1	y	ad20	bi1
	19.3	36.6	19	?	20	7.9	15.7	0.1	5.7

centromere

Distances in meiotic recombination percent.

The original mutants were each crossed to stock strains to isolate two sets of recombinants with the arrangement of linked markers required for the analysis. The two series of crosses and the recombinant strains isolated are described below.

Cross 1 $\dfrac{\text{su1ad20} + \quad \text{pro1} \quad \text{paba1} \quad \text{ad20} \quad + \quad +}{+ \quad \text{galx} \quad + \quad + \quad + \quad \text{bi1} \quad \text{w3}}$

Isolate galx pro1 paba1 ad20; w3

Cross 2 $\dfrac{\text{su1ad20} \quad + \quad \text{ribo1} \quad \text{lu1} \quad \text{y} \quad \text{ad20} \quad + \quad +}{+ \quad \text{galx} \quad + \quad + \quad + \quad + \quad \text{bi1} \quad \text{w3}}$

Isolate su1ad20 galx ribo1 lu1 y ad20

A single perithecium was selected from each cross, tested for hybridity and the ascospores plated on C.M. The resulting colonies were tested individually to isolate the required recombinants which were then replated twice to purify the strains by single colony isolation and the final stock cultures retested for their phenotypes. Close linkage between y and ad20 has been used in the second set of crosses to determine the presence of the suppressor of ad20 in the recombinants, strains that have yellow conidia but are adenine independant are of the genotype su1ad20 y ad20 with 0.999 probability.

Balanced heterokaryons were established between the recombinants and the corresponding heterozygous diploids isolated. In establishing the heterokaryons and in selecting the diploids the minimal medium was supplemented with an excess of adenine (5 ml 0.05 M per 200 ml medium) as the suppressor su1ad20 is recessive and the heterokaryons or diploids consequently require adenine for good growth.

Three types of diploid were synthesised with respect to the galactose alleles. The first type were homozygous for each of the four alleles tested and serve as controls. The second and third types were both double heterozygotes with pairs of alleles in trans and had each pair of alleles in either of two possible reciprocal arrangements with respect to the flanking markers. The four alleles were combined in all possible pairs in the heterozygotes, that is six pairwise combinations with each

combination in two arrangements. The three types of diploid synthesised and the notation used to describe each of them are illustrated below:-

Diploid 1 Homozygotes.

$$\frac{su1\,ad20 \quad gal5 \quad ribo1 \quad lu1 \quad \overset{\circ}{\underset{\circ}{}} \quad + \quad + \quad y \; ad20}{+ \quad gal5 \quad + \quad + \quad pro1 \; paba1 \; + \; ad20} \quad \frac{+}{w3}$$

Diploid 2 Trans heterozygotes.

$$\frac{su1\,ad20 \; (gal5 \quad + \;) \; ribo1 \; lu1 \quad \overset{\circ}{\underset{\circ}{}} \quad + \quad + \quad y \; ad20}{+ \quad (\; + \; gal8) \quad + \quad + \quad pro1 \; paba1 \; + \; ad20} \quad \frac{+}{w3}$$

Diploid 3 Trans heterozygotes.

$$\frac{su1\,ad20 \; (\; + \quad gal8) \; ribo1 \; lu1 \quad \overset{\circ}{\underset{\circ}{}} \quad + \quad + \quad y \; ad20}{+ \quad (gal5 \; + \;) \quad + \quad + \quad pro1 \; paba1 \; + \; ad20} \quad \frac{+}{w3}$$

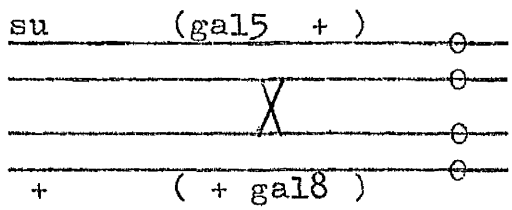
Notation:- Diploid 1 written gal5/gal5
Diploid 2 " gal5/gal8
Diploid 3 " gal8/gal5

If crossing over occurs between the galactose alleles in mitotically dividing nuclei one of the resulting strands will be $gal^+ \; gal^+$ and hyphae containing nuclei with a strand of this type may be recovered by selection on a medium containing galactose as carbon source. Crossing over may be detected by segregation of the distal marker su1ad20, for one of the phenotypes produced by the three possible diploids is distinguishable from the other two.

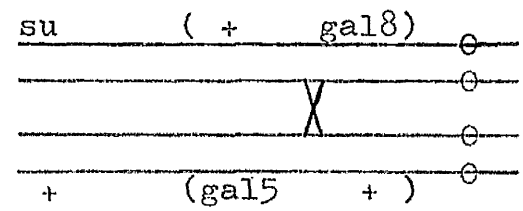
Genotype	Phenotype	Growth on adenineless medium
su1ad20/+	Partial growth	(Mutant su1ad20 is recessive)
su1ad20/su1ad20	Good growth	(Wild type)
+/+	Partial growth	(Mutant ad20 is a partial mutant)

The relative distribution of the distal marker in recombinants arising from the two types of heterozygote may permit the alleles to be ordered according to the following scheme:-

Diploid 2



Diploid 3



Products of mitotic crossing over:-

1 su (gal5 +)
2 + + +

1 su (+ gal8)
2 + (gal5 gal8)

3 su (gal5 gal8)
4 + (+ gal8)

3 su (+ +)
4 + (gal5 +)

Chromatid segregation:-

Select strand 2

2 + (+ +)
3 su (gal5 gal8)

or

2 + (+ +)
4 + (+ gal8)

Select strand 3

3 su (+ +)
1 su (+ gal8)

or

3 su (+ +)
2 + (gal5 gal8)

If the order of the mutant sites is the same as that in the scheme, Diploid 2 will only yield gal$^+$ diploids of the original type with a partial requirement for adenine but Diploid 3 will yield some gal$^+$ diploid sectors with no requirement for adenine (su/su). If the order of the sites is the opposite of that suggested in the scheme gal$^+$ adenine independant sectors would be derived from Diploid 2 but not Diploid 3.

A feature of intra-genic recombination is the occurrence of a greater number of cross-overs in intervals near the region within which recombination has been selected and this localised negative interference (Pritchard, 1955; 1960b) is known to occur in both meiotic and mitotic recombination (Pritchard, 1960a). It cannot be predicted in the present analysis if negative interference will be sufficiently severe to cause random distribution of the distal marker and thus prevent ordering the alleles. Previous analyses by mitotic recombination have been successful (Pritchard, 1955; 1960a; Martin-Smith, personal communication) although the distal markers were much more closely linked (5% and 0.3% meiotic recombination) than in the present case (20% recombination).

Each of the diploids synthesised were plated on C.M. to isolate single colonies and conidia taken from an individual colony of each of the diploids suspended in saline + calsolene and plated at low density on basal medium containing 1% galactose and adenine at a final concentration of 0.0025 M (5 times the normal supplement). After 16 days incubation the slow growing diploid colonies had produced many wild type sectors. Conidia

picked from these sectors were replated on C.M. for purification and the resulting strains examined for ploidy by measurement of the diameter of their conidia and tested for adenine requirement.

B. Results.

The heterozygotes synthesised between all possible combinations of the four galactose mutants all have mutant phenotypes in their growth on galactose. The mutants are therefore allelic and defining a single functional unit. They do not show intra-locus complementation (Catcheside and Overton, 1958).

In addition to the four mutants discussed here heterozygotes were also synthesised between gal5 and the remaining mutants closely linked to gal5. These mutants (gal14, gal19 and gal33) were all non-complementary to gal5.

(1) Frequency of sectoring.

Diploids homozygous for gal5 alleles rarely produce gal^+ sectors, whereas the heterozygotes formed many sectors and apparently different pairs of alleles form sectors at different rates (Table 38). All of the sectors observed had conidia of diploid dimensions.

Measurement of the rate of sectoring involves difficulties of both method and interpretation. If it is assumed that the sectors arise from nuclei in which mitotic intra-genic recombination has occurred the rate of sectoring may be related to the rate of recombination. The most important single problem is to ascribe a value to that proportion of nuclei potentially capable of giving rise to sectors which do in fact form visible sectors.

This problem is apparently imponderable and it is therefore impossible to make any absolute estimate of the rate of sectoring. Other difficulties in making an absolute estimate would be to count the number of nuclei per unit volume of colony and to know in what part of the final colony nuclei must have arisen to have had time to produce a sector. Presumably recombinants occurring late in the growth of the colony will not be expressed as visible sectors.

Many of these difficulties disappear if a relative estimate of the rate of sectoring is made and if it is assumed that in an experiment done under standard conditions the numbers of nuclei tested and the critical colony volumes are equal. It is also assumed that the probability of expression of sectors is the same for all colonies of the same diploid and for all colonies of different diploids. A minor assumption is that the colonies are of equal size, this is not valid in the present experiments but is probably acceptable as a first approximation.

The average number of sectors per colony (m) may be estimated from the proportion of colonies without sectors (Po) by the method of Luria and Delbruck (1943).

$$Po = e^{-m}$$
$$m = -\log_e Po$$

The relative values of m (Table 38) are not altered appreciably if they are corrected to a value for a colony of standard size. There is no correlation between mean colony radius and the rate of sectoring in different diploids and therefore it seems unlikely that the rate of sectoring is a function of the colony size.

TABLE 38

Diploid gal$^+$ Sectors Produced by Heterozygotes Between gal5 Alleles.

Diploid	No.of Colonies plated	Mean radius (m-m)	No.of gal$^+$ sectors	Mean No.of sectors per colony. (m x 10^{-3})	
Homozygotes.					
5/5	235	2.5	1	4.7	
8/8	370	2.0	0	0	
10/10	251	2.4	1	14.1	
13/13	310	2.4	0	0	
Heterozygotes					
5/8	1007	1.5	12	22.1	
8/5	828	1.7	50	62.3	
	1835	1.6	62		34.4
5/10	625	1.9	2	3.6	
10/5	93	3.3	0	0	
	718	2.1	2		2.8
5/13	1131	1.9	82	75.3	
13/5	639	2.5	17	26.8	
	1770	2.0	99		57.5
8/10	133	2.8	0	0	
10/8	149	2.8	1	6.7	
	282	2.8	1		5.5
8/13	376	2.5	0	0	
13/8	134	2.7	3	130.8	
	510	2.6	3		5.9
10/13	806	1.8	3	3.7	
13/10	439	1.7	2	6.6	
	1245	1.8	5		4.0

Three points may be made about the data.

1. The rate of sectoring of homozygotes is low.

2. The rate of sectoring of two pairs of heterozygotes (gal5 and gal8; gal5 and gal13) is greater than those of either the controls or the remaining heterozygotes by a factor of ten.

3. Diploids between the same pair of alleles but in reciprocal arrangements yield sectors at rates of the same order (with the exception of gal8 and gal13) for both arrangements.

(2) Distribution of the distal marker.

All the 172 gal$^+$ diploid sectors isolated from the heterozygotes were of the same phenotype as the original diploids in their growth on adenineless medium, that is either su1ad20/+ or +/+, and had apparently arisen without recombination of the distal marker.

A number of gal$^+$ diploids originating from the heterozygotes gal5/gal13 or gal13/gal15 were analysed to recover the Group 1 chromosomes in haploid segregants arising by mitotic haploidisation. The diploid conidia were plated at low density on C.M. and white or yellow conidial heads appearing as sectors in the green colonies selected visually. Only one segregant was picked from each colony, these were purified by replating, tested for ploidy, and the haploids classified for the Group 1 markers. Each diploid analysed yielded a majority of haploids in which all of the Group 1 markers were in the same combination as in the original diploids, a small number of haploid segregants which were recombinant for some of the markers were interpreted as secondary segregants. The

FIGURE 4 Genotypes of the gal$^+$ Diploid Strains

Diploid 2

su1 (gal5+) ribo1 lu1 + + y ad20

\+ (+gal13) + + pro1 paba1 + ad20

gal$^+$ Diploids

Haploids	Totals
su1 gal ribo1 lu1 ... and + + + + ...	4
or	
su1 + ribo1 lu1 ... and + gal + + ...	6
	10

Diploid 3

su1 (+gal13) ribo1 lu1 + + y ad20

\+ (gal5+) + + pro1 paba1 + ad20

gal$^+$ Diploids

Haploids	Totals
su1 gal ribo1 lu1 ... and + + + + ...	6
or	
su1 + ribo1 lu1 ... and + gal + +	3
	9

A total of 19 gal$^+$ diploid strains derived from the heterozygotes (gal5/gal13 and gal13/gal5) were analysed by mitotic haploidisation. The genotypes of the gal+ diploids are inferred from two types of haploid that each produces.

results are shown in Figure 4. It is clear that each of the 19 gal$^+$ diploid sectors analysed had arisen without recombination of the distal marker and that the diploids apparently result from reversion of either of the galactose alleles. The limited data indicate that the two alleles 'revert' at about the same rate.

Discussion

Two aspects of the analysis are discussed, first the mechanism by which the galactose utilising sectors arise and second the possible significance of the rate of sectoring.

The gal$^+$ character of the diploid sectors is stable, it is inherited via the conidia and segregates sharply in one half of the haploids derived from the gal$^+$ diploids by mitotic haploidisation. These results are consistent with the hypothesis that the sectors arise as the result of a nuclear rather than a cytoplasmic change. It is generally accepted that changes of this sort are the result of a process of recombination (Pritchard and Roper, 1955; Roman, 1956; Leupold, 1958) and the fact that sectoring occurs in heterozygotes but very rarely in homozygotes supports this interpretation. However tha analysis suggests that in the present case recombination is not by a process of crossing-over for no recombination of markers adjacent to the gal5 locus was detected, either by segregation of the distal marker or by analysis of the genotypes of 19 of the 172 gal$^+$ diploids isolated.

Two processes which may prevent detection of crossing-over can be eliminated as unlikely. Crossing-over followed by

preferential chromatid segregation could account for recovery of diploids all heterozygous for the distal marker, but it does not predict failure to detect recombination of the distal marker in all of the 19 gal$^+$ diploids analysed by haploidisation. Secondary selection could also prevent detection of segregation of the distal marker, but again recombination of the marker should still have been detected in the mitotic haploids. Moreover, although selection against diploid strains with a partial requirement for adenine (su/+; +/+) might be expected it is unlikely that selection should also operate against strains not requiring adenine (su/su). Failure to detect recombination of the distal marker would result if there were an excess of double cross-overs in the selected region but it is unlikely that doubles should exceed singles so that no single cross-overs are detected in a sample of 19 of the 172 diploids isolated. Localised negative interference would result in random distribution of the distal marker among the products of crossing-over and in the case of markers closely linked to the selected segment it is found that although recombination is increased, single cross-overs still exceed doubles (Pritchard, 1960a).

The 19 gal$^+$ diploids analysed by mitotic haploidisation are all formally explicable by reversion of either of the mutant alleles, but if reversion were the origin of the sectors it should also occur in homozygotes as well as heterozygotes. Moreover if reversion were due to suppressor mutations (dominant suppressors would be selected) mutation of either a single non-specific locus in Chromosome 1 or two loci also in Chromosome 1 would have to

be postulated to account for the genotypes of the haploids isolated by mitotic haploidisation, a process in which it is known that non-homologous chromosomes segregate at random (Pontecorvo and Kafer, 1958).

No test was made for the occurrence of the reciprocal product of crossing-over at the gal5 locus, that is the double mutant in the cis arrangment. This might be done by application of a 'homo-allele' test (Roman, 1956) to the haploids with a mutant phenotype isolated from the gal$^+$ diploids, but the demonstration of the double mutant would depend upon negative results in the test. The double mutant would fail to yield sectors when recombined with either of the two mutants in further diploids, whereas the single mutants would yield sectors when recombined with the non-identical mutant but not the identical mutant.

The two principal features of the formation of the gal$^+$ diploid sectors are therefore that they result from a nuclear change which apparently does not involve recombination of adjacent markers and occurs much more frequently in heterozygotes than homozygotes. These features are characteristic of gene conversion rather than crossing-over (Lindegren, 1949; Mitchell, 1955a and 1955b; Strickland, 1958).

The results of previous intra-genic mitotic analysis are detailed in Table 39. The result for the gal5 locus parallels those previously observed in yeasts rather than Aspergillus, particularly the results for the ad6 locus in Saccharomyces (Roman, 1956). It appears that failure to detect recombination

TABLE 39 The Results of Analysis of Intra-genic Mitotic Recombination

Organism	Locus	Distance to * distal marker	Analysis demonstrates			Reference
			Reciprocal products	Recombination of distal marker	Order of alleles	
Aspergillus	ad8	5.7 %	Yes	Yes	Yes	Pritchard 1955 1960a
Aspergillus	ad9	0.3 %	Yes	Yes	Yes	Martin-Smith Unpublished.
Aspergillus	gal5	19.3 %	?	No	No	Present results
Saccharomyces	ad6	50 %	No	No	No	Roman, 1956
	ad3	50 %	No	?	No	"
	i	9 %	No	Yes	No	Roman & Jacob 1958
Schizo-saccharomyces	ad7	None	No	-	Yes	Leupold, 1958
	h	1.1 or 5.2 %	(Yes)	Yes	Yes	"

* Meiotic recombination fraction.

of distal markers may be correlated with loose linkage of the marker but there is no obvious mechanism to account for this. The analysis failed to yield information about the order of the alleles.

If it is accepted that the diploid *gal*$^{+}$ sectors result from recombination between alleles it follows that the rate of sectoring will be a function of the rate of recombination and it may be possible to order the alleles by their relative rates of sectoring when taken a pair at a time. In analysis of the *ad7* locus in *Schizosaccharomyces* (Leupold, 1958), it was shown that 6 alleles were ordered by their frequency of mitotic recombination in precisely the same sequence as by meiotic recombination. The number of alleles of the *gal5* locus tested is small but the results suggest that the frequency of sectoring cannot be used in this way in *A.nidulans*.

There are two consequences of the hypothesis that the rate of sectoring is related to the rate of recombination. One is that the rate of sectoring between a pair of alleles is constant, the second that the rates between different alleles are additive. The limited data tend to support the first point in that the rates of sectoring between pairs of alleles are of the same order when tested in either reciprocal arrangement (Table 38). The data do not support the second point. The mutant *gal10* yields few sectors with any of the other three mutants but is probably not a deletion for it reverts at a measurable rate. Three alleles yielded sectors at a detectable frequency in two pairwise

combinations (gal5 and gal8, gal5 and gal13) and it might be expected that the rate of sectoring of the third combination between them (gal8 and gal13) should be either the sum or the difference of the first two. In fact this pair of alleles yielded a heterozygote which formed few sectors although the difference in rate of sectoring of the first two pairs of alleles is roughly equal to the rate at which one of the pairs sectors. The significance of the rate of sectoring has therefore not been determined but apparently it is not related in any simple way to the frequency of recombination between alleles. The experiments with Schizosaccharomyces which were successful in ordering alleles by frequency of mitotic recombination were based on selection of vegetatively growing single cells, this suggests that failure to obtain consistent rates in the present experiments is a feature of selection in growing colonies. The large number of assumptions which have to be made, even in attempting relative estimates, reflects the inherent unsuitability of the technique for quantitative work. A better technique might be achieved in testing conidia formed by doubly heterozygous diploids of A.nidulans though in this case a fluctuation test would be required to account for sampling clones.

Summary

1. Double heterozygous diploids synthesised between pairs of allelic mutants at the gal5 locus produce gal$^+$ diploid sectors at a detectable rate whereas the corresponding homozygous diploids form sectors very rarely.

2. The gal$^+$ diploids arise without recombination of the marker distal to the gal5 locus.

3. These results may be interpreted as indicating mitotic gene conversion at the locus.

4. The rate at which sectors are produced apparently varies between pairs of alleles but the significance of the rate of sectoring has not been determined.

5. The analysis failed to yield information about the order of the alleles at the locus.

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