STUDIES ON THE SELECTION OF MUTANTS IN *ASPERGILLUS NIDULANS*

by

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A thesis submitted to the University of Glasgow for the degree of Doctor of Philosophy.

April, 1963.

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ACKNOWLEDGEMENTS

I would like to express my sincere gratitude to Professor G. Pontecorvo, F.R.S. for the inspiration, guidance and encouragement which I have received from him.

I also wish to thank Mr. E. Forbes who introduced me to the genetics of *Aspergillus nidulans*, and offered many helpful suggestions. I am indebted to Dr. O.H. Siddiqi as well as to other members of the department for fruitful discussions, and offer my sincere thanks to Miss M. McKinlay who drew my attention to many a linguistic fault in the manuscript.

During the course of this work I was supported by grants from the 'Alexander Milman' and the 'Sir Maurice Bloch' funds, made available by the Hebrew University of Jerusalem.

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LIST OF ABBREVIATIONS

A.M.	acetate medium
B.M.	basal medium
C.M.	complete medium
F.A.	fluoracetate
F.A.M.	fluoroacetate medium
M.M.	minimal medium
N.A.	nitrous acid
P.F.P.A.	para-fluorophenylalanine
S.M.	succinate medium
S.F.A.M.	succinate fluoroacetate medium

I GENERAL INTRODUCTION

In endeavouring to understand genetic material, its nature, and behaviour in function, recombination and mutation, geneticists have made extensive use of selective techniques. These techniques permit the selective recovery of the results of rare events (Pontecorvo, 1958). They are particularly suitable for work with micro-organisms where such techniques have found extensive use; for example, Benzer's (1961) mapping of the rII region of bacteriophage T4.

Most of the existing selective techniques have one limitation in common. This is the selection of one type only, e.g. selection of a few organisms with a wild type phenotype from a large population with a mutant phenotype (prototrophs from auxotrophs) or selection of a few organisms with a mutant phenotype from a large population of organisms with a wild type phenotype (resistants from sensitives).

Thus while systems which select in one direction only have been very useful in mapping (Benzer, 1961) and have sufficed to establish certain characteristics of recombination (Pritchard, 1955, 1960a, 1960b; Siddiqi, 1962a; Siddiqi and Putrament, 1963), mutation (Luria and Delbrück, 1943) and mutagenesis (Freese, 1959a, 1959b), they have not assisted greatly in elucidating the role of the genetic material in function.

For most critical studies of the phenomenon of recombination the

analysis of all the products involved in a single recombinational event is necessary. At present this can only easily be done by 'selective tetrad analysis'. One way of selecting specified tetrads for analysis has been given by Lissouba and Rizet (1960). These authors crossed heteroallelic colourless ascospore mutants of the fungus Ascobolus immersus and selected for further analysis asci (tetrads) in which wild type coloured ascospores appeared.

While 'selective tetrad analysis' is essential for the study of the phenomenon of recombination it seems unlikely that it will become a major tool for the study of the nature of genetic material in mutation and function. For this purpose any technique would be of great value which enables selection of mutations in the same cistron in both directions, i.e. mutant from wild type and vice-versa. Such a technique will be referred to as 'two-way selection'.

A 'two-way selection' technique would be particularly useful for the study of mutation as such, as it would allow the analysis in parallel of the patterns (both induced and spontaneous) of 'forward' and 'back' mutation within the same gene (Auerbach, 1962).

Furthermore, for studies of the mechanism of mutagenesis such a technique might also prove rewarding. This applies to both the 'base analogue type' of mutation (base changes in the coding DNA, Freese, 1959a, 1959b, or re-arrangements in the genetic material, Tessman, 1962), and the 'acridine-type' of mutation (believed due to deletion or insertion

of a single nucleotide Crick et al., 1961 Lerman, 1963).

A 'two-way selection' system has been the major tool used by Crick et al. (1961) in mapping intra cistron suppressors in the rII region of bacteriophage T4. In this system the 'forward'- mutants are selected on inspection because r mutants differ morphologically from r^+ when plated on Escherichia coli B, while 'back'-mutants (or wild type recombinants) are selected from a large excess of rII 'forward'-mutants because they form plaques on Escherichia coli K12 (Benzer, 1961) on which rII 'forward'-mutants cannot develop plaques.

Using the rII 'two-way selection' system Crick et al. (1961) showed that true 'back'-mutants hardly ever occur. Similar findings were made by Jinks (1961) by mapping of intra-cistron suppressors in the h region of bacteriophage T4 where a restricted 'two-way selection' based on host range is possible. Furthermore, the work of Crick et al. (1961) has made possible the study of the nature of the genetic code by purely genetic analysis.

It is essential to have suitable 'two-way selection' systems available if one wishes to extend the investigation of intra-cistron suppression to organisms other than phage.

In pursuing the aims discussed above an attempt was first made to obtain in the ascomycete Aspergillus nidulans mutants amenable to 'selective tetrad analysis'. As described in Part III of this thesis, colonies were screened for ascospore colour mutants and strains with

colourless (cl) ascospores, and strains with blue (bl) ascospores were isolated. Unfortunately, all the mutants isolated proved to be 'non-autonomous' (Sturtevant, 1920; Ephrussi, 1938). However, from the results obtained a model to explain the origin of peritheoia in Aspergillus nidulans could be constructed.

When it was evident that 'selective tetrad analysis' was not facilitated by these ascospore colour mutants, a search for a 'two-way selection' system was begun and was successful (Part IV). The principle of the system is the correlation of resistance and auxotrophy. In the present work 'forward'-mutants unable to grow on acetate as the sole carbon source were selected by plating conidia on a medium containing fluoroacetate and glucose while 'back'-mutants were selected by plating conidia on medium containing acetate as the sole carbon source. In addition, information on the genetics and behaviour of these mutants was gathered. They were found to behave particularly interestingly in complementation, as all tested combinations between any two 'forward' mutants (in trans arrangement) which complemented in heterozygous diploids failed to complement in heterokaryons. Revertants of the 'forward'-mutants and an enhancer mutant were also studied.

(Throughout the present work 'revertant' and 'back'-mutant are used synonymously).

II MATERIALS AND METHODS

1) Life cycle of Aspergillus nidulans

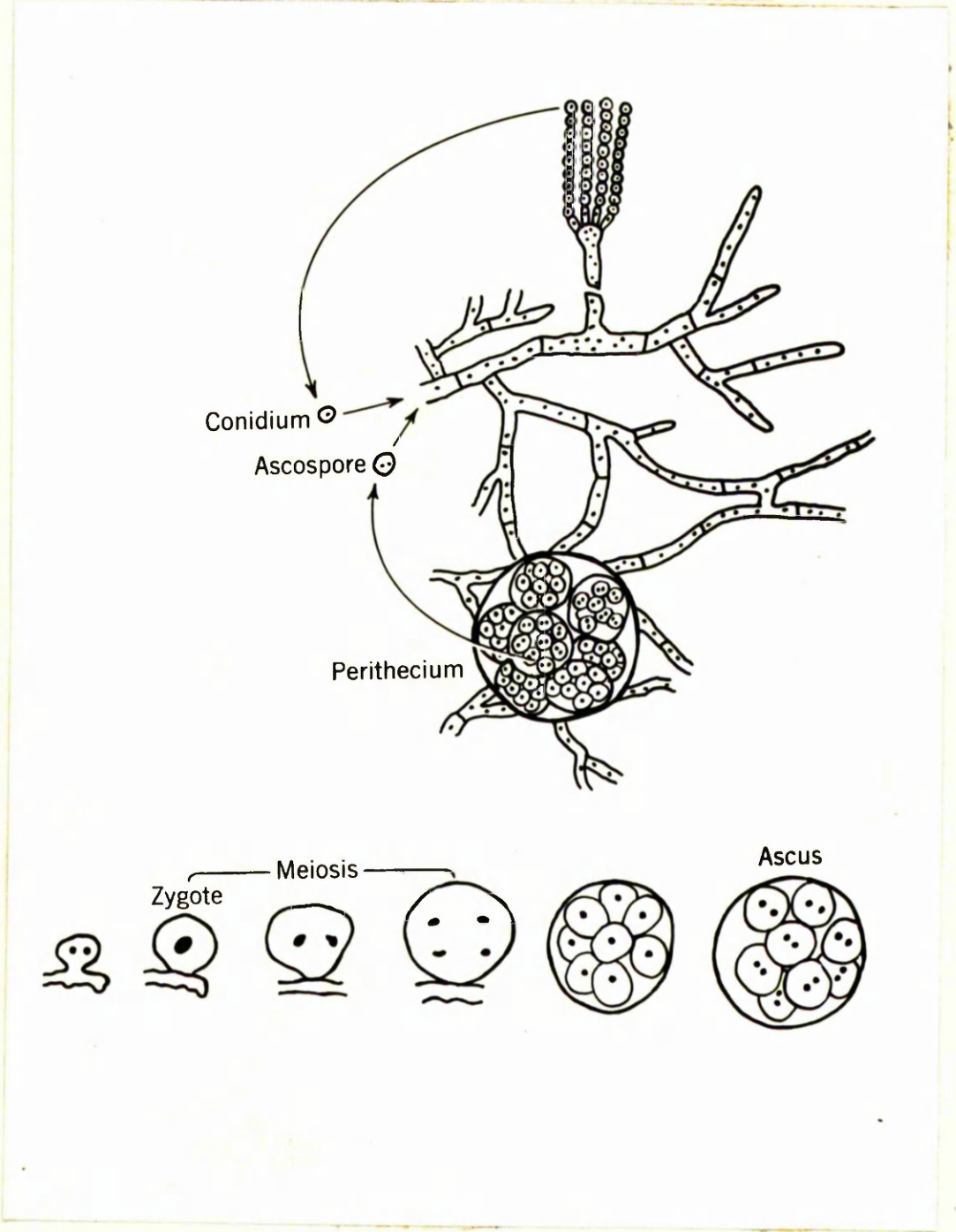
Aspergillus nidulans (Eidam) Winter, is an ascomycete belonging to the family Aspergillaceae of the order Plectascineae. The details of its life history have been described elsewhere (Thom and Raper, 1945; Pontecorvo et al., 1953). The cytology of Aspergillus nidulans has been re-investigated by Elliott (1960) and some findings relevant to its sexual reproduction have been recently described (Apirion, 1963b, Part III). only the salient features of its life cycle (Fig. 1) will be briefly redescribed here.

The mycelium consists of branched septate hyphae, each 'cell' in the hyphae being multinucleate. Anastomosis followed by nuclear migration between hyphae occurs readily. The fungus propagates vegetatively by means of asexual spores or conidia which are produced in columnar heads borne on aerial hyphae called conidiophores. The head of the conidiophore bears primary and secondary sterigmata, each of which has a single nucleus from which all the conidia in one chain derive their nuclei. Different chains in one head produced by a heterokaryotic mycelium however, may carry genetically different nuclei.

The fungus reproduces sexually by means of ascospores. The asci are found in closed fruiting bodies or cleistothecia. It has been the practice in this laboratory to refer to these as perithecia and this

Figure 1

Life cycle of Aspergillus nidulans



Taken from Pontecorvo et al., 1953

usage will be adhered to. A perithecium may contain up to about 10,000 asci, each containing eight binucleate ascospores which are unordered. Perithecia and asci may easily be ruptured to liberate the ascospores.

Genetic analysis has shown that the asci in one perithecium, produced by a heterokaryotic mycelium, tend to be of exclusively crossed or exclusively selfed origin (Pontecorvo et al., 1953). It now seems likely that all the asci in a single perithecium originate from a pair of nuclei which enter into conjugated divisions to give rise to dikaryotic ascogenous hyphae from which the asci originate (Pontecorvo et al., 1953; and Part III).

2) Media

All chemicals used are of analytical grade unless otherwise stated.

a) Minimal medium (M.M.)

Ingredients per litre:	(Pontecorvo et al., 1953)
D-glucose	10 g.
$NaNO_3$	6 g.
KCl	0.52 g.
$MgSO_4$	0.52 g.
KH_2PO_4	1.52 g.
$FeSO_4 \cdot 7H_2O$)	traces
$ZnSO_4 \cdot 7H_2O$)	
Agar	10 g.

adjusted to pH 6.5 by NaOH or HCL.

b) Basal medium (B.M.)

As M.M. but without glucose.

c) Complete medium (C.M.)

The medium in use at present is similar to that given in Pontecorvo et al. (1953) with some modifications. It consists of M.M. supplemented with the following ingredients per litre:-

Difco bacto peptone	2 g.
Yeastrel (Brewers' Food Supply Company Ltd., Edinburgh)	1 g.
Difco bacto casamino acids technical	1.5 g.
Nucleic acid hydrolyzate; acid and alkaline hydrolized	0.4 g.

(for details see Pontecorvo et al., 1953).

Vitamins:

Riboflavin	1 mg.
Nicotinamide	1 mg.
Para-aminobenzoic acid	0.5 mg.
Pyrodoxin.HCl	0.5 mg.
Aneurin.HCl	0.5 mg.
Biotin	0.02 mg.

d) Acetate medium (A.M.)

Ingredients per litre:

CH_3COONH_4	12 g.
$MgSO_4 \cdot 7H_2O$	0.5 g.
KH_2PO_4	3 g.
NaCl	2 g.
$FeSO_4 \cdot 7H_2O$) $ZnSO_4 \cdot 7H_2O$)	traces
agar	12 g.

adjusted to pH 6.1 by NH_4OH or HCl.

e) Fluoroacetate medium (F.A.M.)

Ingredients per litre:

D-glucose	5 g.
$NaNO_3$	4 g.
KCl	1 g.
$MgSO_4 \cdot 7H_2O$	0.5 g.
KH_2PO_4	3 g.
$FeSO_4 \cdot 7H_2O$) $ZnSO_4 \cdot 7H_2O$)	traces
CH_2FCOOH (technical)	30 g.
agar	15 g.

adjusted to pH 6.1 by concentrated NH_4OH.

In later work 40 g. fluoroacetic acid were used instead of 30 g.

f) Succinate medium (S.M.)

B.M. plus 1%* succinic acid.

g) Succinate fluoroacetate medium (S.F.A.M.)

S.M. plus F.A.; final concentration of F.A. 0.5% pH 4-4.5 adjusted by NH_4OH.

h) Liquid media

Any of the various media in a liquid form (i.e. without agar). Unless liquid medium is specifically mentioned in this thesis, agar medium was used.

3) Growth factor supplements and sugar utilization tests

For the concentrations of the various nutrients added to M.M. to enable growth of the various mutants, see Pontecorvo et al. (1953); Kafer (1958). For classification of sugar mutants (mutants unable to utilize a particular sugar as a carbon source), B.M. plus the sugar concerned was used, the sugar usually in concentrations of 1% (Roberts, 1961).

* Percentages will always be expressed as weight/volume unless otherwise stated.

4) Stock cultures

The cultures were incubated at 37°C. This incubation temperature was used throughout the work. The strains were maintained on slopes of C.M. or M.M. supplemented with the required nutrients, and were sub-cultured approximately every six months.

Purified cultures were obtained by plating suspensions of well broken chains of conidia (single conidia plating) and sub-culturing from well isolated colonies. All cultures, from which conidia were harvested for selection work, were maintained on slopes of C.M. To obtain cultures of independent origin, slopes were inoculated with conidia from different colonies which arose from 'single conidia plating'.

5) Auxanographic techniques

The general principles were described by Pontecorvo (1949).

a) Testing for ability to grow on different carbon sources - see Roberts (1961).

b) Testing for optimum pH for growth on carbon sources

Since the pH 6.5 of M.M. was found to be unsuitable for growth of Aspergillus nidulans on a medium containing succinate as the sole

carbon source, a suitable pH was sought as follows: Conidia of a suitable strain were embedded in B.M. and incubated overnight; blocks of medium at each end of one diameter of the Petri dish were removed. At one of these points was placed succinic acid plus ammonium hydroxide at a high pH (about 9) and at the other point was placed succinic acid plus ammonium hydroxide at a low pH (about 3). The plate was then incubated for 2-3 days. Gradients of pH from the low to the high were created on the dish and when a certain zone of growth appeared, the pH of this zone was measured by indicator paper. This method, which is especially suitable for acids was also applied to citric and malic acids.

c) Testing the response of various strains to a certain chemical in one Petri dish

A Petri dish with suitable medium minus the chemical in question was prepared. The chemical, in a solution, was pipetted into a trough cut out in the agar medium on one side of the dish. The strains were inoculated along parallel lines (4-6 point inocula per line) at right angles to the trough. In this way varying degrees of response of strains to a particular chemical can be detected.

6) Plating

Suspensions of spores were made in sterile saline or distilled water. Chains of conidia were broken by adding the wetting agent Tween 80 and sucking up and down through a Pasteur pipette. Density of spore suspensions was estimated by haemocytometer counts, and viable counts were obtained by plating suitable dilutions of a suspension on C.M.

a) Spreading

A measured amount of suitable diluted suspensions of spores were dispersed with a glass spreader on the surface of agar medium.

b) Embedding

A suspension of spores was added to melted, cooled agar medium and poured into dishes.

c) Top layer

A volume of 4-5 ml. of agar medium plus spores was poured into dishes containing a bottom layer (20-25 ml.) of the same agar medium and was spread rapidly to form a thin top layer.

The last two methods were used for handling large quantities

of spores. The plating techniques are in common use in the genetic study of Aspergillus nidulans.

7) Nitrous acid treatment

The method is described by Siddiqi (1962b). In the present work the method was slightly modified.

1 ml. of a suspension, in distilled water of conidia, of the strain to be tested was added to 8 ml. of 0.1M acetate buffer pH 4.4 and kept in a water bath or in an incubator for 10 minutes at 37°C. 1 ml. of 0.125-0.250M* $NaNO_2$ was added and the incubation mixture was stirred every 2-3 minutes. The treatment was stopped after 10-20 minutes by transferring 1 ml. of the incubation mixture into 9 ml. of 0.066M phosphate buffer pH 7.1 at room temperature. For viability counts suitable dilutions, in distilled water, were plated on C.M.

8) Selective techniques

8) Selective techniques

One of the purposes of this work was to establish a suitable technique for estimation of the proportion of mutant nuclei, of a

* $NaNO_2$ is oxidized slowly in solution to $NaNO_3$, with consequent decrease in mutagenic activity of the preparation.

particular kind in a given population of spores: selection of 'forward'-mutants as part of a 'two-way selection' system (see Part I). As some of the mutants resistant to F.A. were found to be suitable for a 'two-way selection' system by virtue of their inability to grow on A.M. (Part IV, B), and as these mutants (designated f) were found to grow much more rapidly on S.M. than the wild type strains (see Part IV, E 2 c i), various combinations of glucose, succinate, and fluoroacetate in media were tried, in order to discover the 'spectrum' of the possible mutants, and to establish the best technique for the estimation of the ratio of mutant to non-mutant nuclei in a given population. The various techniques are described below (for further details see Part IV, B and C).

a) Sectoring

Conidia from a strain sensitive to F.A. were inoculated (26 points per dish) into F.A.M.; resistant sectors appeared after 3-5 days.

b) Incubation in liquid B.M. plus F.A.

Spores of a strain sensitive to F.A. were incubated for 16 days in liquid B.M. plus 2.5% F.A. at pH 6.5 plus the requirements necessary for the particular strain.

c) Sandwiching in fluoroacetate medium

See Apirion (1962)

d) Sandwiching in succinate medium

Spores of a strain sensitive to F.A. were plated on dishes containing 20-25 ml. S.M. and covered by a top layer of 4-6 ml. S.M.

e) Sandwiching between F.A.M. and S.F.A.M.

On a basal layer of 20-25 ml. F.A.M. (F.A. 4%, glucose 1%) in a Petri dish, spores of a strain sensitive to F.A. were plated and covered with a top layer of 4-5 ml. of S.F.A.M.

f) Sandwiching between S.M. and S.F.A.M.

On a basal layer of S.M. (20-25 ml.), spores of a strain sensitive to F.A. were plated in a top layer of 4-5 ml. of S.M. and covered with 5-6 ml. of S.F.A.M.

9) Crossing

Crosses were made on M.M., or, in cases of crosses between two non-complementary mutants, on M.M. supplemented by the relevant growth factor. Dishes containing specially thick layers of M.M. were streaked with a dense mixture of conidia from the two strains, which in all cases carried at least one pair of complementary nutritional requirements. The streaked surface was broken up by means of a sterile wire loop to form a roughly oblong area of 1 x 3 cm., and a few drops of liquid C.M. were added to allow some initial growth. The dishes were sealed with sellotape after the first day of incubation and were incubated for a further 9-19 days. This method is now in common use for crossing strains of Aspergillus nidulans, and resulted from the accumulated experience of various workers in this department.

10) Analysis of crosses

Two methods are available (Pontecorvo et al., 1953).

a) Recombinant selection

Ascospores from several perithecia were collected and plated

on a selective medium on which only recombinant ascospores could grow. This method requires that the two parental strains carry complementary genes determining nutritional requirements, and are unlinked to the markers whose segregation is being studied. Segregation analysis was performed by transferring recombinant colonies ...to master plates of C.M., 26 to a plate; from there, the colonies were replicated, using a multiple wire replicator, to various media to reveal their genotypes.

b) Perithecium analysis

Analysis of a perithecium is based on the fact that the 10,000 or so asci of an individual perithecium are almost invariably of either exclusively selfed or exclusively crossed origin. The three types of perithecia in a cross between two strains can be distinguished easily if the two strains involved have different conidial colour.

A small sample of an ascospore suspension prepared from a single perithecium is streaked on C.M. and incubated until conidial colour develops. The conidia of each streak may be purely of a parental colour or a mixture of two or three colours (depending on whether the parental strains differ in one or in two genes affecting conidial colour). Streaks of the first type indicate a selfed

peritheciun and streaks of the second type indicate a hybrid peritheciun. Ascospores of the hybrid peritheciun, stored at 4°C., are plated on C.M. and their further analysis carried out as above (Part II, 10a).

11) Synthesis of heterokaryons and diploids

a) Heterokaryons

For synthesis of heterokaryons, strains were so chosen that each had at least one growth factor requirement not possessed by the other. A mixture of conidia from the two strains was inoculated into tubes with liquid M.M. (6 ml.) plus a few drops of liquid C.M. and these tubes were incubated for 24-48 hours to allow growth of heterokaryotic mycelium. This mycelium was teased out on dishes of M.M. On further incubation a balanced heterokaryon grew out of the teased mycelium. To confirm that growth was due to establishment of a balanced heterokaryon further transfers of vigourously growing hyphal tips were made. When the two strains carried non-complementing requirements, suitable nutrients were added. Certain combinations which failed to form heterokaryons under these conditions were grown on liquid C.M. overnight (Pontecorvo et al., 1953) and the mycelium was harvested and treated as above till growth of a balanced

heterokaryon was achieved.

b) Diploids

To obtain diploids (Roper, 1952), conidia from a balanced heterokaryon were embedded in M.M. at a density of approximately $10^6 - 10^7$ conidia per dish. Diploid colonies which arose were isolated and purified by single conidia plating (Part II, 4). The diploids were identified by reason of their being prototrophic while the parental strains were auxotrophic, and by reason of their having a larger conidial diameter than haploid strains. (For further details see Roper, 1952; Pontecorvo et al., 1953).

12) Haploidization of diploid strains

Haploid strains were isolated after spontaneous haploidization or after treatment with DL-para-fluorophenylalanine (Morpurgo, 1961; Lhoas, 1961; Forbes, 1963). Dishes of C.M. with 0.0061% P.F.P.A. were inoculated with conidia by a sterile needle at 30 points, and the dishes were then incubated for 3-4 days. While the growth of the colonies as a whole was reduced and non sporulating, conidiating sectors appeared. When conidia from these sectors were streaked on

C.M. and colonies from there were re-isolated, they were found to be mainly haploid. When the diploid was green but heterozygous for conidial colour mutants a proportion of the sectors had the mutant colour. These haploids, after purification, were analysed as before (see Part II, 10a).

13) Allocation of a marker to its linkage group (Forbes, 1959, 1963)

A diploid between MSD (master strain D) and a haploid strain carrying the desired marker was synthesized. This diploid was haploidized and the genotypes of the haploid segregants were examined (see Part II, 10a). MSD is a strain carrying markers in each of its linkage groups:

Linkage group	Marker	Linkage group	Marker
I	su_7ad20 y_4, ad20	V	lys5
II	Acr1	VI	s3
III	phe2	VII	nic8
IV	pyro4	VIII	ribo2

Since in haploidization there is recombination between markers in different linkage groups but not within linkage groups (Pontecorvo et al., 1953; Pontecorvo et al., 1954; Pontecorvo and Käfer, 1958),

the marker in question will recombine with the markers of seven linkage groups, not with the marker of its own linkage group.

14) Strains

All strains used in this work belong to the Glasgow University collection, with the exception of the strain bi1;orn9 cha, which was obtained from Dr. E. Kafer (McGill University).

Table I lists all mutants used as genetic markers. The new mutants obtained during the course of this work are described in the appropriate sections. The known linkage relationships of the loci referred to in this thesis are shown in Fig. 2.

Table I

Mutants used as genetic markers.

Symbol of mutant and locus*	Phenotype determined by mutant
Acr1	acriflavine resistant
ad1	adenine requiring
ad3	adenine requiring
ad4	adenine requiring
ad8, ad20	adenine requiring
ad23	adenine requiring
an1	aneurine requiring
arg3	arginine requiring
bi1	biotin requiring
cha	chartreuse conidia
fr1	unable to utilize fructose
gal4, gal7	unable to utilize galactose
lac3, lac5	unable to utilize lactose
lys5	lysine requiring
meth1	methionine requiring
ni3	unable to utilize nitrate
nic2	nicotinic acid requiring

Table I continued

nic8	nicotinic acid requiring
orn7, orn9	ornithine requiring
paba1	para aminobenzoic acid requiring
paba22	para aminobenzoic acid requiring
palA1	alkaline phosphataseless
palB7	alkaline phosphataseless
phe2	phenylalanine requiring
pro1	proline requiring
pu	putrescine requiring
pyro4	pyridoxine requiring
ribo1	riboflavin requiring
ribo2	riboflavin requiring
ribo3	riboflavin requiring
ribo5	riboflavin requiring
ribo6	riboflavin requiring
s1	unable to utilize sulphate
s3	unable to utilize sulphate
s12	unable to utilize sulphate
sm	small colony
su-ad20	suppressor of ad20
thi1, thi4	thiazole requiring

Table I continued

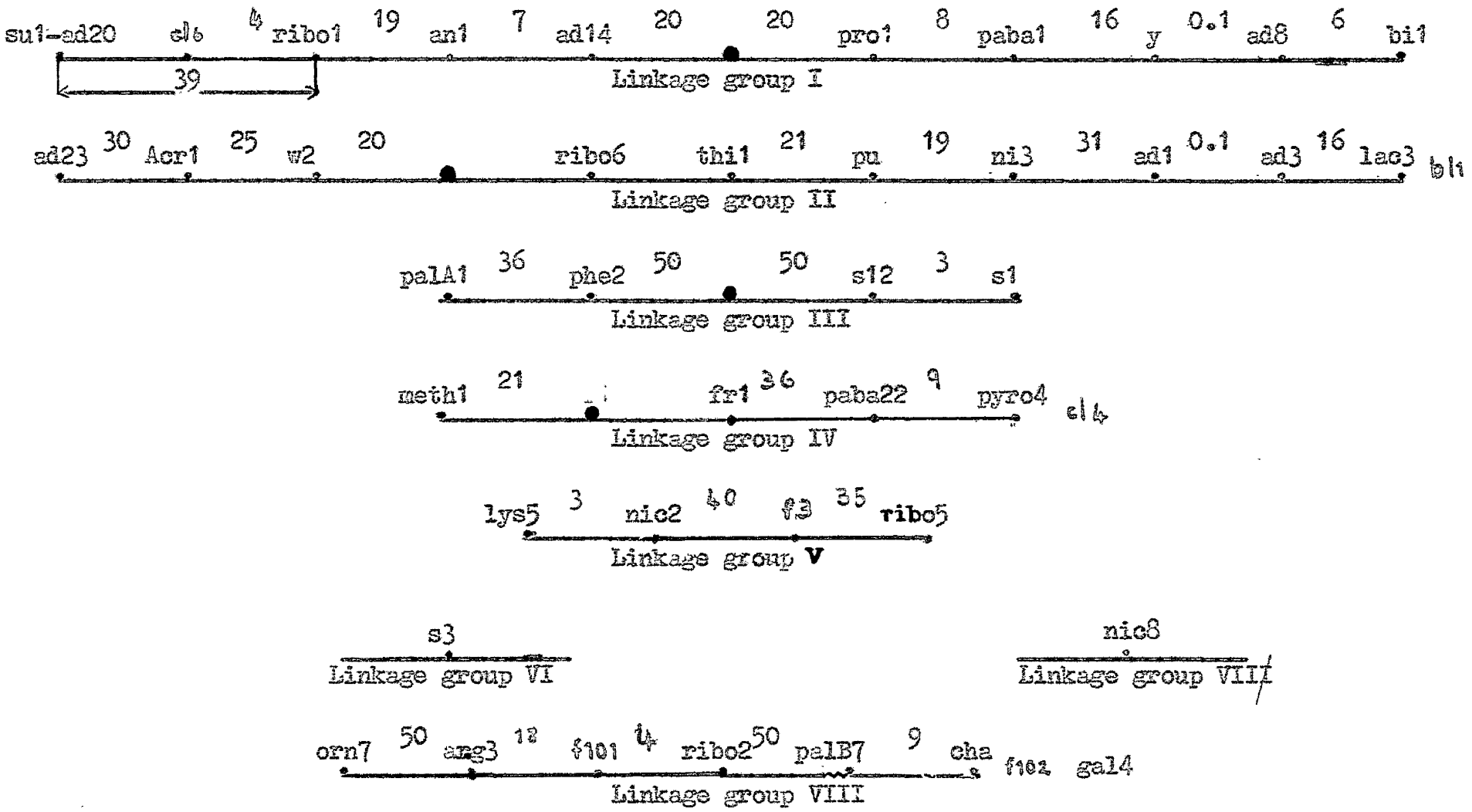
y	yellow conidia
w2, w3	white conidia

* Allelic mutants are placed after the locus symbol.

Figure 2

Linkage relationships of loci* referred to in this thesis

Data of other workers in black; own data in red.



*Map distances are given in percent recombination here and throughout the thesis.

III FORMAL AND PHYSIOLOGICAL GENETICS OF ASCOSPORE COLOUR IN ASPERGILLUS NIDULANS

This Part is presented in the form of the manuscript of a paper accepted for publication in "Genetical Research, Cambridge" with the addition of Tables 8-12.

Formal and physiological genetics of ascospore colour in *Aspergillus nidulans*

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INTRODUCTION

A combination of fine genetic analysis with tetrad analysis is desirable for an understanding of recombination at the intragenic level. Tetrads in which intragenic recombination has occurred are very rare however, and "blind" dissection of huge numbers of tetrads in order to identify these few is not very rewarding. A practical solution to this difficulty is a system in which the rare tetrads can be selected from the mass and the analysis limited to them. Such a method was described by Lissouba & Rizet (1960); Rizet, Lefort, Engelmann, Lissouba & Rousseau (1960), and Lissouba (1961). In heteroallelic crosses between mutants of *Ascobolus immersus* with colourless ascospores rare tetrads containing coloured spores could be identified visually among the many thousands of colourless tetrads.

The present paper reports the isolation of ascospore colour mutants in *Aspergillus nidulans* with the

object of applying to this organism, with its more versatile and better explored genetics, the approach used with Ascobolus immersus. Unfortunately, the ascospore colour mutants of Aspergillus nidulans found so far, and described in the present paper, turned out to be unsuitable for the initial purpose. The genes identified which affect pigment production in the ascospores produce effects which are not cell localized, i.e. they are, in the classical terminology, "non-autonomous" in action (Sturtevant, 1929; Ephrussi, 1938). The results reported here are, however, interesting in other respects.

Aspergillus nidulans is a homothallic ascomycete multiplying asexually by means of uninucleate haploid conidia and sexually by means of ascospores. The four products of meiosis divide mitotically in the ascus and each of the eight haploid nuclei is included in one of the eight dark red ascospores. There are about 10,000 asci in one fruiting body (perithecium). The perithecium is spherical, about 0.5 mm. in diameter, and has a dark red outer wall.

MATERIALS AND METHODS

Techniques and symbols not described here may be

found in the papers by Pontecorvo, Roper, Hemmons, Macdonald & Buxton (1953); Pontecorvo & Käfer (1958) and Käfer (1958).

Ascospore colour mutants were obtained by treating conidial suspensions of the strain y; w2; s12 with UV or HNO_2 (Siddiqi, 1962). Treated conidia were plated on complete medium to give 30-50 colonies per dish. The colonies produced mature perithecia after 10-14 days. A few perithecia from each colony were squashed between a slide and a cover slip in a drop of lysol and examined with the naked eye, and with the microscope when necessary.

RESULTS

1. Mutants (Table 8)

Eleven mutants were isolated; six differed from the wild type in having dark blue ascospores, and five in having white (colourless) ascospores. The blue mutants were given the general symbol bl and the white mutants the general symbol ol. Four bl mutants (Nos. 1-4) and five ol mutants (Nos. 1 and 3-6) were studied in the work described here. The dark blue colour of bl mutants slowly changed to red over a period

of a month or more. The ol mutants varied from completely colourless in the case of ol4 to distinctly pink in the case of ol1. The wall of the perithecia of ol strains was pale pink, so that under a low-power dissecting microscope they were easily distinguishable on the surface of a colony even without picking them up.

2. Formal genetics of the mutants

bl mutants (blue ascospores)

In crosses with strains having wild type ascospores all four of the bl mutants showed a 1:1 segregation of blue versus red ascospores among the colonies originating from ascospores of crossed origin (Table 1).

In crosses of bl1, bl3 and bl4 with bl2 no colonies with red ascospores, that is wild type recombinants, were obtained among 200 colonies examined from each cross. Thus these mutants are closely linked or allelic. Tests of bl mutants in diploid heterozygotes showed that bl mutants are recessive to bl^+ (Table 4). Tests of two of them (bl1 and bl2, Table 4) in diploids showed that these two do not complement in the trans arrangement, i.e. they are allelic. The other two by two combinations of bl mutants have not been tested.

Table 1. Crosses between blue ascospore mutants (bl^x) and wild type (bl$^+$)

Mutant used in the cross	Number of colonies with blue ascospores	red ascospores	Total
bl1	46	45	91
bl2	36	43	79
bl3	42	35	77
bl4	38	43	81
Total	162	166	328

Crosses were of the type: y;w2;a12;blx x nabal. In each case ascospores from a single hybrid perithecium were plated on complete medium.

By haploidization of a diploid between bl1 and tester strain MSD carrying markers in each of the eight chromosomes (Forbes, 1959 and unpublished), bl1 was located in linkage group II, but crosses involving other markers of this linkage group covering most of it (ad22, Aor1, w2, ribo6, th14, pu, n13, ad3, lac5) did not reveal meiotic linkage between bl1 and any of them. So the bl locus adds at least 50 units to this already long linkage group. (Table 9)

o1 mutants (colourless ascospores)

In crosses with a strain having wild type ascospores, the colourless mutants o14, o15 and o16 gave a 1:1 segregation of red versus white ascospores (Table 2). Crosses of each of the mutants o11, o13, o15 with o16 gave no wild type recombinants in about 100 colonies originating from ascospores of crossed origin, while all four gave a 3:1 segregation of white to red ascospores in crosses with o14 (Table 3). It was concluded that o14 identifies one locus and the other four mutants another locus or closely linked loci. Mitotic haploidization from diploids with tester strain MSD located o14 on linkage group IV and o16 on linkage group I. Two further crosses, (Table 10) (Table 11)

Table 2. Crosses between colourless ascospore mutants (*olx*) and wild type (*ol*$^+$)

Mutant used in the cross	Number of colonies with colourless ascospores	Number of colonies with red ascospores	Total
ol4	33	29	62
ol5	45	41	86
ol6	56	60	116
Total	134	130	264

Crosses were of the type: *y;y2;ol2;olx* X *bi1;meth1*. In each case ascospores from a single hybrid peritheclum were plated on complete medium.

Table 3. Recombination between independently arisen colourless ascospore mutants (cl)

Mutants used in the cross	Number of colonies with colourless ascospores	Number of colonies with red ascospores	Total
cl4 x cl1	134	60	194*
cl4 x cl3	204	58	262*
cl4 x cl5	201	70	271*
cl4 x cl6	121	45	166*
cl6 x cl1	89	0	89
cl6 x cl3	132	0	132
cl6 x cl5	117	0	117

*Not significantly different from the results (3:1) expected in the case of independent segregation. Thus cl4 identifies one locus and the other four identify another unlinked locus.

Crosses were of the type: bi1;el x cly; y; w2; cl2. Ascospores from a number of perithecia from each cross were plated on minimal medium on which only bi^+; a^+ recombinants can grow.

o16 bi1; w2 x *pro1 paba1 y* and *o16 bi1; w2* x *ribo1 an1 ad14 y; meth1 pyro4*, located *o16* on the left arm of linkage group I about 4 units from *ribo1*. (Table 12) Whether *o16* was proximal or distal to *ribo1* could not be determined from the data obtained from these crosses. *o14* was tested for linkage with all the known loci in linkage group IV (*meth1, fr1, pyro4* and *paba22*). The results suggested loose linkage distal to *meth1*.

Dominance and epistasis of mutants

Some of the dominance relationships of ascospore colour mutants with the wild type alleles and with one another were investigated by synthesizing diploids (Roper, 1952) heterozygous and homozygous for *b1* and *o1* mutants in combination with wild type and among themselves. The results (Table 4) showed that the *b1* and *o1* mutants tested are recessive to wild type.

Two crosses between a blue (*b12*, linkage group II) and two colourless mutants (*o13* and *o11*, linkage group I) showed the expected independent segregation. Both colourless mutants were epistatic to the blue mutant, as indicated by a 1:2:1 ratio of colonies with red:colourless: blue ascospores in these crosses (Table 5). Epistasis

Table 4. Ascospore colour of diploids heterozygous and homozygous for ascospore colour mutants

Combination	Type of diploid	Colour of the ascospores of the diploid
*MSD / paba1; w2 bl1	bl+ / bl1	red
MSD / paba1; w2 bl4	bl+ / bl4	red
MSD / cl6 bl1; w2	cl+ / cl6	red
MSD / bl1; w2; cl4	cl+ / cl4	red
bl1; w2; s12; cl4 / paba1 y; bl1; s12	cl4 / bl1	red
bl1; w2; s12; cl4 / cl6 an1 ad14 paba1 y	cl4 / cl6	red
y; bl1; s12 / paba1; w2 bl1	bl1 / bl1	blue
y; bl1; s12 / paba1; w2 bl2	bl1 / bl2	blue
cl6 bl1; w2; s12 / cl6 an1 ad14 paba1 y	cl6 / cl6	colourless

*MSD is a strain which has markers on each of its eight linkage groups (Forbes, unpublished).

Table 5. Crosses between blue (bl) and colourless (cl) ascospore mutants

Type of cross	Number of colonies with colourless ascospores	blue ascospores	red ascospores	Total
bl2 × cl3*	232	166	120	518
bl2 × cl1	38	15	19	72

*Many colonies did not form mature ascospores and were therefore classified by the colour of the perithecial wall; such a classification is liable to error.

Crosses were of the type: paba1; bl2 × clx y; w2; s12.

Ascospores from a number of perithecia from each cross were plated on a minimal medium, on which only paba$^+$; s$^+$ recombinants can grow.

was confirmed by crossing each of four colourless isolates which also required thiosulphate (*s12*) from the cross *pabaA; b12 x a13 y; w2; s12* with strain *pabaA*, which has wild type (red) ascospores. From these crosses *paba*$^{+}$ *a*$^{+}$ recombinants were selected by plating suspensions of ascospores on minimal medium. One of these four crosses yielded recombinant colonies with blue ascospores. This indicated that the isolate used in this cross had the double mutant genotype: *a13; b12*. Among the 78 colonies of this cross, 45 had colourless ascospores, 20 red and 13 blue.

3. Physiological controls of ascospore colour

The technique used in analysing crosses between strains differing in ascospore colour is based on the fact that the 10,000 or so asci of an individual perithecium are almost invariably either all of crossed origin or all of selfed origin, a fact which has been utilized in the technique of "perithecium analysis" (Pontecorvo et al., 1953). The three types of perithecia can be distinguished easily if one parent has the genotype *w;y* and the other *w*$^{+}$ *y*$^{+}$. White (*w*) is epistatic to *y*$^{+}$/*y* (yellow conidia).

If a small sample of an ascospore suspension prepared from a single perithecium is streaked on complete medium and incubated until conidia colour is developed, it normally gives only one of three patterns, either pure green, pure white, or mixed yellow, white and green. Streaks of the first two types indicate a selfed perithecium and streaks of the third type a hybrid perithecium.

In the present experiments the two parents also differed in genotype with respect to ascospore colour, and in addition to the test described above, a sample of ascospores was examined microscopically to determine the phenotype of the ascospores.

The ascospores analysed in this way were from crosses of types bl x bl^+, al x al^+ and bl x al. Segregation of wild type and mutant ascospores was never found, either in crossed or, understandably, in selfed perithecia. The ascospores of a perithecium were all of the same colour, irrespective of their genotypes. Thus, the genes tested so far determining ascospore colour variation are "non-autonomous" in action. Moreover, the wall of each perithecium (crossed or selfed) is of the same colour as the walls of the ascospores contained in it,

and this colour can be wild type or that of either parental strain - even in the case of crossed perithecia (Tables 6 and 7).

In detail, the results (Table 7) of crosses between strain al3 pabal (with green conidia and colourless ascospores) and strain y; w2 bl2; s12 (with white conidia and blue ascospores) were as follows:

1. All three classes of perithecia - with colourless or red or blue ascospores - occurred. Their proportions varied in replicates of the same cross.

2. Both crossed and selfed perithecia were found among all three classes.

3. Among selfed red perithecia, both parental genotypes (paba; al3 and y; w2 bl2; s12) were found, while among selfed colourless or selfed blue perithecia only one parental genotype occurred, in each case the parental type with the corresponding ascospore genotype. That only parental phenotypes occur in selfed perithecia, and that this phenotype always corresponds to the genotype of the selfed ascospores, was supported by classifying selected red perithecia from a cross of type al3 x al^+ (al3 y; w2; s12 x al^+ bl1; methl). This cross gives,

Table 5. Colour of ascospores of peritheeia of selfed and crossed origin in crosses of type bl x bl$^+$ and cl x cl$^+$

Cross	Type of cross	Type of peritheclum	Phenotype of ascospores in peritheeium			Total No. of peritheeia
			white	red	blue	
y; w2 bl1; s12 x bl1; bl$^+$; meth1	bl1 x bl$^+$	selfed*	0	3	5	9
		crossed	0	5	2	7
cl6 y; w2; s12 x cl$^+$ bl1; meth1	cl6 x cl$^+$	selfed*	51	13	0	64
		crossed	2	22	0	24

*The genotype of the selfed peritheeia corresponded in all cases to the genotype of the parent with the same colour of ascospores.

Table 7. Colour of ascospores and perithecia of selfed and crossed origin from the cross o13 paba1 x y; w2 b12; s12 (o13 x b12)

Phenotypo of perithecium and ascospores	Selfed o13	Selfed b12	Crossed	Total*
colourless	42	0	16	58
blue	0	17	39	56
red	10	4	39	53

*About equal numbers of perithecia of each phenotype were tested. The actual proportions in this cross were: red about 10%, colourless about 40%, and blue about 50%.

of course, white and red peritheoia. 172 red peritheoia were picked and analysed (using the technique mentioned above). 108 of these 172 peritheoia were of crossed origin and 64 were of selfed origin. All the 64 of selfed origin were selfed of the parental genotype (al^+) producing red peritheoia. There were no selfed red peritheoia having the al parental genotype.

DISCUSSION

Aspergillus nidulens is homothallic and has differentiated male and female organs like heterothallic Aspergilli (Adams, unpublished) and the asci originate from dikaryotic ascogenous hyphae (Elliott, 1960). Furthermore, practically all the asci (10,000 or so) in each peritheoium are either of crossed or of selfed origin (Pontecorvo et al., 1953). To account for this it was assumed that one "male" nucleus and one "female" nucleus enter into conjugated divisions to give rise to all the dikaryotic ascogenous hyphae and therefore to all the asci (Pontecorvo et al., 1953). On the basis of the preceding facts and of what is known from other ascomycetes (e.g. Martens, 1946), let us see how the observations of the present work can be interpreted.

It will be assumed that:

1. The primordium of the female organ - protoperithecium - begins with only a few nuclei.

2. The male organ contributes only one nucleus. This nucleus and one of the female nuclei enter into conjugated divisions to give rise to the dikaryotic ascogenous hyphae.

3. Side by side with the development of the ascogenous hyphae, the other nuclei - exclusively female - of the protoperithecium multiply and ultimately give rise to the other parts of the perithecium, such as the perithecium wall, which are therefore female.

Thus, while the crossed or selfed origin of the asci in a perithecium is determined by the particular pair of nuclei - one male and one female - which entered into conjugated divisions, the colour of the ripe perithecium wall and of the ripe ascospores (in the particular system of genes investigated here) is determined by the genotypes of the nuclei present in the protoperithecium.

In a cross between blue and colourless strains (bl x +bl) in which the hyphae were largely heterokaryotic, the protoperithecial primordium might contain nuclei of either or both types present in the heterokaryon. Thus a ripe perithecium, derived from a heterokaryotic

protoperithecium, would usually be red (wild type), while the asci of this perithecium could be either crossed or selfed, and if selfed, of either parental type depending on the genotype of the nuclei which entered into conjugated divisions. On the other hand, a ripe perithecium, derived from a homokaryotic protoperithecium, would have a parental phenotype (colourless or blue), but the asci of such a perithecium could again be either crossed or selfed depending on the male nucleus, but if selfed they must be of the corresponding genotype, i.e. the same genotype as the nuclei in the protoperithecium.

The same considerations are valid and compatible with the observations in crosses of the type bl x bl^+ and gl x gl^+.

A protoperithecium could, conceivably, be heterokaryotic but with only one nucleus of one parental type and all the others of the other parental type. If this single nucleus were the female contribution to the ascogenous hyphae, and therefore to all the asci, and if in addition the male nucleus contributed to the ascogenous hyphae were of the same parental type, the maternal parts of the perithecium would be left with nuclei all of one

parental type while the asci would all be selfed of the other parental type. The fact that peritheoia of this type were not found in this investigation can be explained by assuming, either that the number of female nuclei in the protoperitheoium is considerable, or that between the formation of the primordium of the protoperitheoium and the time when the male nucleus is contributed all nuclei divide once or more.

SUMMARY

By nitrous acid or UV treatment ascospore colour mutants of two kinds, blue and colourless, were obtained in Aspergillus nidulans (wild type has red ascospores). Four blue mutants were located in linkage group II within 0.5 unit of one another (locus symbol: bl1). Of the colourless mutants, four were located in linkage group I within 1 unit of one another (locus symbol: cl6), and one in linkage group IV (locus symbol: cl4). In diploids the mutants were recessive. Colourless was epistatic to blue.

In crosses these characters behaved as "non-autonomous" both in the ascospores and in the asci; all the ascospores of the asci in one peritheoium as well as the peritheoium

wall were of the same colour. In crosses between strains having blue peritheoia and strains with colourless peritheoia, red, blue and colourless peritheoia were found; each type could contain either crossed or selfed peritheoia. Selfed red peritheoia were of either parental type but selfed blue or colourless peritheoia always had the corresponding genotype.

The phenotype of the peritheoium (peritheoial wall and ascospores) is considered to be determined by the homo- or heterokaryotic constitution of the protoperitheoium which gave origin to it.

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Table 8. Origin, isolation and designation of ascospore colour mutants

Symbol	Mutagen	Molarity of nitrous acid	Time of treatment (mins.)	Percent. survival	Colonies tested
b11	Nitrous acid	0.025	15	3.6	1500
b12	"	0.025	10	7.1	800
b13	"	0.0125	15	11.5	1000
b14	"	0.025	10	7.3	1900
b15	"	0.0125	20	2.3	3300
b16	"	0.0125	20	4.2	1800
c11	"	0.025	10	7.1	2200
c13	"	0.0125	15	11.5	2700
c14	"	0.0125	22	4.2	5000
c15) c16)	UV	-	9	8.5	5800

The starting strain had the genotype y;w2;s12.
All mutants except the last two were isolated by squashing mature perithecia between a slide and a cover slip, and colour was determined with the naked eye. The last two mutants (c15, c16) were isolated after screening colonies with mature perithecia under a low-power dissecting microscope.

Table 9. Location of bl1 in linkage group II by haploidization, after treatment with F.F.P.A., of diploid MSD/paba1; w2 bl1

Linkage group	Tester marker	bl^+	bl
I	$paba^+$	7	6
	paba	2	1
II	Acr^+	0	7
	Acr	9	0
II	w^+	9	0
	w	0	7
III	phe^+	9	7
	phe	0	0
IV	$pyro^+$	3	5
	pyro	6	2
V	lys^+	6	6
	lys	3	1
VI	s^+	4	5
	s	5	2
VII	nic^+	7	5
	nic	2	2

Table 9 continued

Linkage group	Tester marker	bl$^+$	bl
VIII	ribo$^+$	2	6
	ribo	7	1

Table 10. Location of cl4 in linkage group IV by spontaneous haploidization of diploid MSD/bi1; w2; cl4

Linkage group	Tester marker	cl$^+$	cl
I	bi$^+$	7	8
	bi	4	4
II	Acr$^+$	8	9
	Acr	3	3
III	phe$^+$	6	4
	phe	5	8
IV	pyro$^+$	0	12
	pyro	11	0
V	lys$^+$	8	10
	lys	3	2
VI	s$^+$	6	6
	s	5	6
VII	nic$^+$	9	8
	nic	2	4
VIII	ribo$^+$	7	5
	ribo	4	7

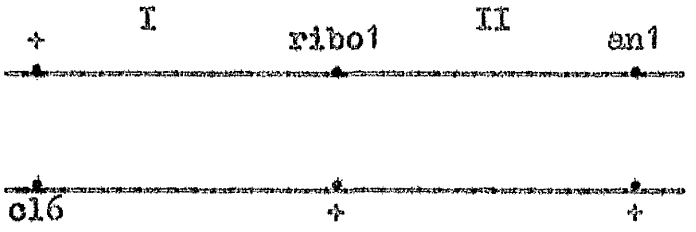
Table 11. Location of cl6 in linkage group I by spontaneous haploidization of diploid MSD/cl6 bi1; w2

Linkage group	Tester marker	cl$^+$	cl
I	bi$^+$	22	0
	bi	0	13
II	Acr$^+$	11	11
	Acr	11	2
III	phe$^+$	15	9
	phe	7	4
IV	pyro$^+$	10	10
	pyro	12	3
V	lys$^+$	11	4
	lys	11	9
VI	s$^+$	12	7
	s	10	6
VII	nic$^+$	14	4
	nic	8	9
VIII	ribo$^+$	9	5
	ribo	13	8

Table 12. Location of cl6 by meiotic analysis

Cross - cl6 bi1; w2 x ribo1 an1 ad14 y; meth1 pyro4

The data are tabulated only in respect of the markers cl6 ribo1 an1 as in a three point cross.



Crossover regions	Genotypes		
none	+ ribo an	27)	60
	cl + +	33)	
I	cl ribo an	0)	2
	+ + +	2)	
II	+ ribo +	5)	11
	cl + an	6)	
I & II	cl ribo +	0)	1
	+ + an	1)	

Linkage map. cl6 4 ± 4.6 ribo1 16.2 ± 8.6 an1

IV MUTANTS UNABLE TO UTILIZE ACETATE

(A) Introduction

Aspergillus nidulans grows on acetate as the sole carbon source and is sensitive to fluoroacetate. Strains resistant to F.A. were selected as described in chapter B. Some of the resistant strains were unable to grow on a medium containing acetate as the sole carbon source (f strains). This was exactly as predicted by the theory of 'two-way selection' discussed in chapter B.

In chapter C various methods for the selection of 'forward'-mutants are described, compared and discussed.

The ability of f strains to 'back'-mutate is described and discussed in chapter D.

Chapter E contains a description of the growth and characteristics of wild type and f strains on various media.

The formal genetics of some f mutants, revertants, and an enhancer mutant are described in chapter F, where it is shown that the f mutants occur at three unlinked loci.

Chapter G deals with complementation. The results of complementation tests between f mutants at different loci in the

trans configuration in heterokaryons and in heterozygous diploids are described. The implications of the observation that all pairs tested complement in the diploid and none in the heterokaryon are discussed and some possible explanations put forward.

Finally, in chapter H, certain possibilities of the system and the findings are discussed.

B) A general system for the automatic selection of auxotrophs from prototrophs and vice versa in micro-organisms

The principles of a 'two-way selection' are outlined, and techniques for selecting fluoroacetate resistant mutants are described. Some of the mutants were found to be unable to utilise acetate. Studies on the ability of some of the mutants to revert are mentioned. This section is in the form of a paper published in "Nature".

(*Reprinted from Nature, Vol.* 195, *No.* 4845, *pp.* 959–961, *September* 8, 1962)

A GENERAL SYSTEM FOR THE AUTOMATIC SELECTION OF AUXOTROPHS FROM PROTOTROPHS AND VICE VERSA IN MICRO-ORGANISMS

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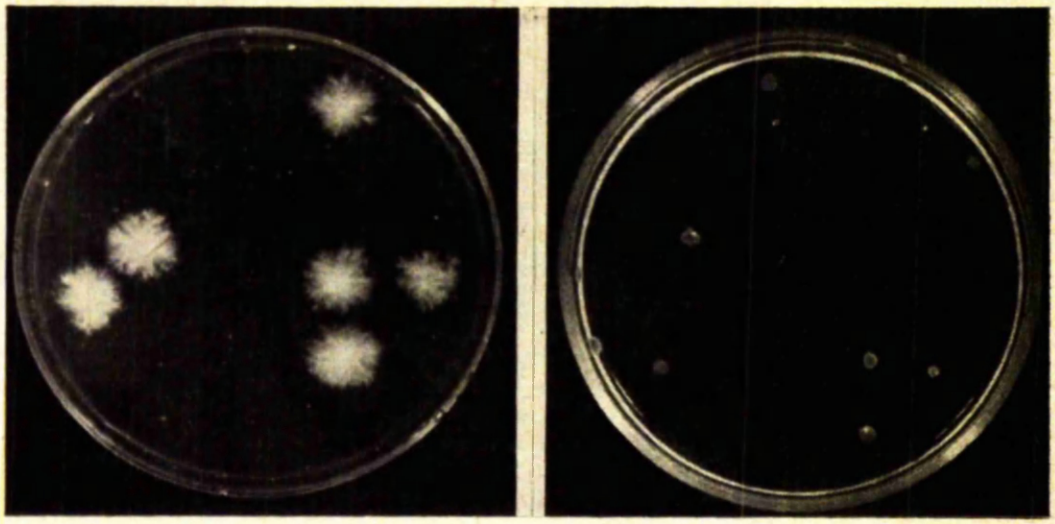
IN microbial genetics there have long been available techniques of high resolving power for the automatic selection of 'forward'-mutants, for example, in respect of resistance to drugs[1] and parasites[2], and of 'back'-mutants, for example, from auxotrophy to prototrophy[3,4]. The automation is based on establishing conditions such that cells of the parent strain —sensitive in the case of 'forward'-mutation to drug resistance, and auxotrophic in the case of 'back'-mutation to prototrophy—cannot grow while the 'forward'-mutants, or the 'back'-mutants, respectively, do grow.

It has been obvious for a long time that it would be very useful to be able to apply some such automation to selection in both directions in one and the same system. Most of the systems in which selection in both directions is possible have, so far, been only partially successful, mainly because of inadequate resolving power in either or both directions. To give a few examples: (1) Methods based on visual selection (for example, colour or morphology of the colony, enzymatic or other colour reaction, type of plaque, etc.) do select in both directions, but with low resolving power in both. (2) Methods based on drug resistance[1] or on host–parasite relations[5,6] or on reversion from auxotrophy[3,4] have a high resolving power but only in one direction. (3) Methods based on gradual enrichment[7–9] are not suitable for precise quantitative work. (4) Methods based on the fact that reversion from auxotrophy in respect of one growth factor sometimes involves mutation to auxotrophy in respect of another[10–13] are not sufficiently general.

The lack of a technique with high resolving power for the selection of mutants or recombinants in both directions in one system has been one of the main bottlenecks in genetic analysis at the intragenic level.



a *b*

Fig. 1. *a*, 5×10^6 conidia of a strain of *Aspergillus nidulans*, sensitive to fluoroacetate, plated on fluoroacetate medium (3 per cent, see text): six resistant colonies have developed. About 20 per cent of the resistants so obtained are auxotrophic, that is, unable to utilize acetate as sole source of carbon. *b*, 5×10^6 conidia of a strain resistant to fluoroacetate and unable to utilize acetate, plated on medium with acetate as sole source of carbon: eight prototrophic colonies have developed

Yet, the principles on which some such technique could have been based have been well known for a long time, and it is surprising that they have not been applied deliberately. There are examples in which these principles have been accidentally or incidentally used[14], and others[15,16] in which the results are likely to be interpretable on the basis of these principles. The principles are the following: consider a toxic analogue A' of a growth factor or metabolite A. Resistance to A' may—and often will—be based on failure to take up or further metabolize A', and therefore A. Resistant mutants of either type, that is, unable to take up or to utilize A, will be auxotrophic for one or more metabolites—for short, B—which the normal strain synthesizes from A. Thus, on a medium containing A' and B (or a substance which can replace B), 'forward'-mutants can be selected because they are resistant to A' (and by hypothesis, auxotrophic for B). 'Back'-mutants, capable of utilizing A and sensitive to A' can then be selected in the usual way, that is, by inoculating the auxotrophs in the absence of B but in the presence of A.

These very obvious considerations show how wide the field of search can be for systems in which selection in both directions at high resolving power may be possible. There is no need to stress how useful this possibility is for a variety of purposes: fine recombination analysis, specific mutagenesis at the intra-

cistron level, etc. The search for suitable systems can start either from sensitive wild-type strains, selecting from them auxotrophs by virtue of their resistance to an analogue, or from auxotrophic strains, selecting those which are resistant to an analogue of a metabolite coming before the block which causes the auxotrophy. Clearly, as resistance can be achieved in a number of ways—of which the inability to take up or metabolize the toxic substance is only one—we should expect neither every resistant to a toxic substance to be auxotrophic in respect of a metabolite related to that toxic substance, nor every auxotroph to be resistant to a toxic substance related to the growth factor required by it.

The following is an example of a system of the first kind. It was based on selection of 'forward'-mutants by means of their resistance to an analogue (fluoroacetic acid). Some of these resistant mutants are auxotrophic, and more precisely unable to utilize acetate as the only source of carbon. 'Back'-mutants of these auxotrophic resistant mutants can be selected by plating them on a medium with acetate as the only source of carbon.

Most *Aspergillus nidulans* strains can grow on acetate as the only source of carbon. For the purpose of the present work a medium of the following constitution (per 1,000 ml.) was used: ammonium acetate, 12 gm.; sodium chloride, 2 gm.; magnesium sulphate (7 H_2O), 0·5 gm.; potassium dihydrogen phosphate, 3 gm.; ferrous and zinc sulphate, traces; agar, 12 gm.; *p*H adjusted to 6·1 by ammonium hydroxide or hydrochloric acid.

Fluoroacetate at high concentration prevents the growth of most *A. nidulans* strains. The fluoroacetate medium used for obtaining resistant mutants was as follows (per 1,000 ml.): glucose, 5 gm.; sodium nitrate, 4 gm.; potassium chloride, 1 gm.; magnesium sulphate (7 H_2O), 0·5 gm.; potassium dihydrogen-phosphate, 3 gm.; ferrous and zinc sulphate, traces; fluoroacetic acid (technical), 30 gm.; agar, 15 gm.; *p*H adjusted to 6·1 by concentrated ammonium hydroxide. On this medium sensitive strains do not grow, while resistant strains grow well (Fig. 1).

To select resistant mutants, suspensions of up to about 10^8 conidia/ml. from fluoroacetate-sensitive strains capable of utilizing acetate as the only source of carbon were spread in volumes of 0·1–0·2 ml. on the surface of the fluoroacetate agar medium (25 ml./dish). When mutagenic treatment was used, for example, nitrous acid[17], part of the suspension was treated before plating. After a few hours incubation a further thin layer of the same fluoroacetate medium (3–5 ml./dish) was poured on top. Resistant colonies

Table 1. SELECTION OF 'FORWARD'-MUTANTS RESISTANT TO FLUOROACETIC ACID

Exp.	Treatment	Conidia per dish ($\times 10^3$)	Total conidia plated ($\times 10^3$)	Resistant mutants: No.	Resistant mutants: per 10^6 plated conidia
I	None	30	30	0	0
		300	300	0	0
		3,000	6,000	16	2·6
		17,000	85,000	105	1·2
	Total untreated		91,330	121	1·3
	Nitrous acid *	30	30	4	133
		150	300	26	87
	Total nitrous acid treated		330	30	91
II	None	5	5	0	0
		50	50	0	0
		500	500	2	4
		5,000	5,000	6	1·2
		10,000	10,000	11	1·1
			15,555	19	1·2

* 7 min. in: $NaNO_2$ (M 0·02) in buffer acetate pH 4·4: survival about 45 per cent.

began to appear 3–4 days later, and by the sixth or seventh day they were isolated on a complex medium with glucose as the main carbon source. These resistant mutants were then tested for their ability to grow on acetate medium. Of the 30 resistant mutants obtained after nitrous acid treatment (Table 1) 6 were also auxotrophic, that is, not able to grow on acetate as only source of carbon (Fig. 1), and required an alternative source, for example, succinate or glucose.

Back-mutants from these resistant auxotrophs were selected by plating the conidia on medium with acetate as the only carbon source. Table 2 shows, as an example, the results of plating on such medium three different suspensions of untreated conidia from one resistant auxotrophic strain (f.10). Of the 168 back-mutants from this strain obtained in this way, 24 were tested on fluoroacetic acid medium; all were sensitive.

Table 2. SELECTION OF SPONTANEOUS 'BACK'-MUTANTS ABLE TO UTILIZE ACETATE AS SOLE CARBON SOURCE FROM ONE 'FORWARD'-MUTANT (f.10) RESISTANT TO FLUOROACETIC ACID AND UNABLE TO UTILIZE ACETATE

Exp.	Conidia per dish ($\times 10^6$)	Total conidia plated ($\times 10^6$)	Back mutants: No.	Back mutants: per 10^6 plated conidia
I	45	135	19	0·14
II	120	240	112	0·47
III	60	180	37	0·20
			168	

As expected, not all resistant auxotrophs back-mutate, and those which do so may back-mutate both in respect of the auxotrophy and of the resistance, or only in respect of the auxotrophy. So far, 29 resistant auxotrophs of independent origin have been tested for back-mutation (minimum per strain: 2×10^8 conidia). Of these, six did back-mutate, and one of them back-mutated in respect of the auxotrophy but remained resistant to fluoroacetic acid.

This attempt in one specific case shows that the general principles mentioned at the beginning are valid and that forward and back selection, based on resistance determined by auxotrophy, is possible with high resolving power in both directions.

I thank Prof. G. Pontecorvo for guidance, Mr. E. Forbes for advice and Dr. O. H. Siddiqi for a discussion which led to this work.

This work was supported by a grant from the Hebrew University of Jerusalem out of the Sir Maurice Bloch Fund.

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Printed in Great Britain by Fisher, Knight & Co., Ltd. St. Albans.

(c) Selection and isolation of 'forward'-mutants

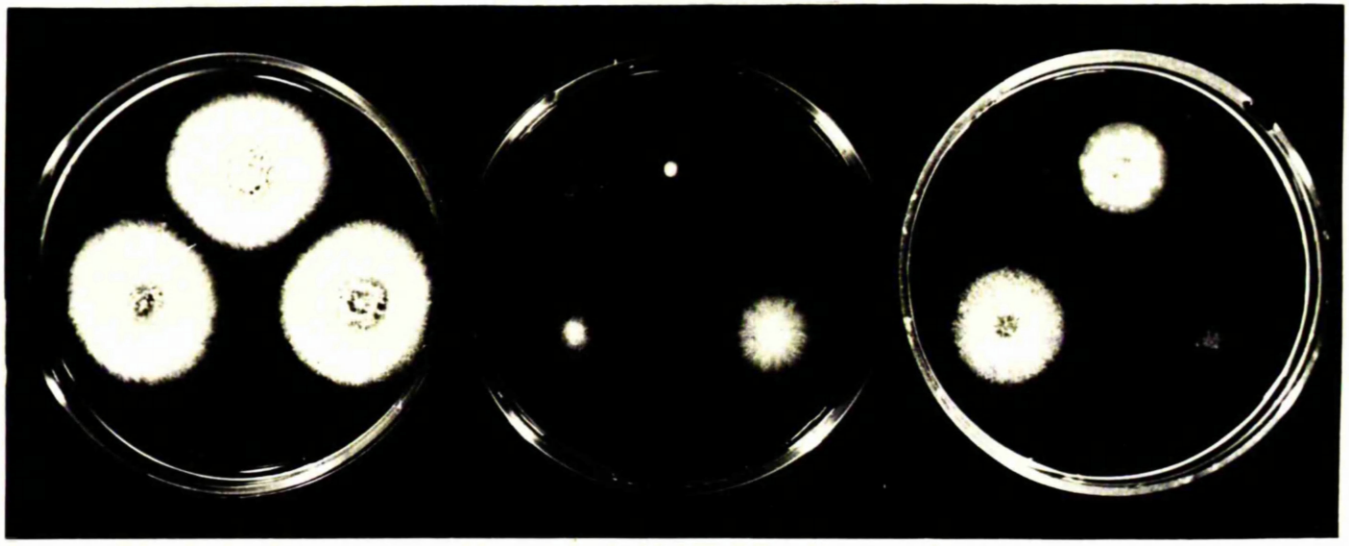
A metabolite and its analogue, namely acetate and fluoroacetate, were used in the search for a 'two-way selection' system. First, an attempt was made to work out the system qualitatively, and for this purpose conidia of a strain sensitive to F.A. were inoculated with a needle into F.A.M. Resistant sectors were isolated and some of them were found to be auxotrophic (unable to grow on A.M. - Fig.3). This method of selection was possible because strains sensitive to F.A. grow to a very limited extent on F.A.M., thus allowing the emergence of resistant sectors.

One of the objects of these investigations was to establish a technique for the estimate of the proportion of mutant nuclei in a given population of conidia. As a 'sectoring' technique is not suitable for this purpose, various other methods were tried. Another interest was to determine what kinds of mutants are isolated under different selective conditions.

As f strains were found to grow better on succinate medium than f^+ strains (Part IV, E2ei), an attempt was made to select f mutants on various media containing combinations of glucose, succinate and fluoroacetate.

Five different methods were tested. In one method spores were

Figure 3



WILD TYPE: ABOVE

RESISTANT PROTOTROPH: LEFT RESISTANT AUXOTROPH: RIGHT

Note: the middle dish contains fluoroacetate 2%, glucose 0.5%.

incubated in liquid B.M. containing F.A., while in the other four, spores were plated on various combinations of F.A. glucose, and succinate. The results obtained by each technique, and its suitability for estimating the proportion of mutant nuclei, are discussed in the following five sections.

1) Incubation of conidia in liquid B.M. plus F.A.

As fluoroacetate was found to be poisonous for Aspergillus nidulans (Part IV, E1a) it was assumed that incubation of spores in medium containing F.A. as the sole carbon source would result in the wild type conidia utilizing it and dying, while mutant conidia (unable to utilize acetate) would survive. Hence, 10^7 conidia from the strain w3;pyro4 were incubated in this medium (10^6/ml.) in a universal container. Samples were withdrawn at intervals and plated on C.M. for viability counts (Table 13). After 16 days of incubation the contents were added to melted cooled C.M. and poured into Petri dishes. Thirty colonies were recovered, of which twenty-eight grew on A.M. while two did not. These two were found to be more resistant to F.A. than the parental strain w3;pyro4, and were designated w3;pyro4;f101 and w3;pyro4;f102.

Table 13. Viability test of conidia of the strain w3;pyro4 after incubation in liquid B.M. + 2.5% F.A. at pH 6.5

Incubation time in days	No. of conidia plated*	No. of colonies	Percentage of viable conidia
0	350	384	109.71
2	2,000	332	16.60
3	1,000	129	12.90
4	1,000	97	9.70
5	1,000	63	6.30
6	1,000	40	4.00
7	10,000	60	0.60
8	10,000	18	0.18
10	100,000	15	0.015
12	100,000	11	0.011

* Estimate from haemocytometer counts.

Because of the length of time required for the selective elimination of the sensitive conidia, this technique was not further used.

2) Sandwiching conidia in fluoroacetate medium

As resistant sectors were obtained from point inoculation of conidia of strains sensitive to F.A. into F.A.M., conidia of sensitive strains were plated on the same medium to discover whether or not isolated mutant conidia would develop into colonies under these conditions. (The medium, technique, and results are described in Part IV, B.) In further work the percentage of F.A. was increased from 3% to 4%. The results obtained are presented in Table 14. Most of the resistant mutants could utilize acetate which is in agreement with results previously obtained (Part IV, B). This technique was not completely satisfactory for estimating the frequency of mutant (f) to non-mutant (f^+) nuclei in a given population of spores, as shown by a reconstruction experiment (Table 15).

Table 14. Selection of spontaneous 'forward'-mutants resistant to F.A. by plating conidia of the strain bi1 on F.A.M.

F.A. 4% glucose 0.5%.

Experiment*	No. of conidia per dish ($x10^6$)	Total conidia plated ($x10^6$)	Resistant mutants No.	Resistant mutants per 10^6 plated conidia	No. of resistant mutants tested for auxotrophy	No. of auxotrophs**
1	18	36	8	0.22	4	3
2	40	120	38	0.32	25	0
3	15	30	3	0.10	3	2
4	21	42	21	0.50	7	1
5	19.5	39	19	0.49	5	2
6	20	40	11	0.27	5	1
7	16	32	17	0.53	5	0
8	17	34	2	0.06	2	0
9	20	40	12	0.30	8	2
10	17.5	35	10	0.29	7	1
11	20	40	9	0.22	4	2
12	18	36	8	0.22	7	0
13	16	32	10	0.31	8	2
14	17	34	12	0.35	5	1
15	14	42	6	0.14	5	2

Table 14 continued

16	25	50	6	0.12	5	0
17	25	50	27	0.54	10	1
18	22	44	5	0.11	5	0
19	24	48	11	0.23	9	0
20	21	42	39	0.93	10	0
21	23	46	9	0.20	9	4
22	25	50	28	0.56	10	5

* The experiment denotes batches of conidia of different origin (Part II, 4).

** In this table as well as in other tables of this chapter this number is a minimum level for auxotrophic mutants, as the resistant mutants were transferred to C.M. and tested for auxtrophy without any further purification. The fact that the vast majority of the resistant mutants were prototrophic cannot be attributed to contamination with wild type conidia, since the prototroph resistant mutants obtained differ in appearance from wild type on acetate medium and grow less well.

Table 15. Reconstruction experiment for 'forward' selection of f mutants by sandwiching conidia in F.A.M.

F.A. 3% glucose 0.5%

No. of conidia per dish		Total conidia plated		No. of resistant colonies	
y;pyro4 ($x10^6$)	w3;pyro4;f3	y;pyro4 ($x10^6$)	w3;pyro4;f3	y;pyro4 f & fa*	w3;pyro4;f3
0.01	7	0.04	28	0	23
0.5	7	2	28	3	21
1	7	4	28	4	12
10	7	40	28	9	2

* f = resistant to fluoroacetate unable to utilize acetate

fa = resistant to fluoroacetate able to utilize acetate

3) Sandwiching conidia in succinate medium

3) Sandwiching conidia in succinate medium

Conidia of the strains bi1 and paba1 were plated on dishes containing S.M.; after 3-5 hours, a top layer of 4-6 ml. of S.M. was added. The dishes were examined after four days. In this way rapidly growing colonies on S.M. were selected (Table 16). (Strains of Aspergillus nidulans grow very slowly on S.M. Part IV, E2oi.) Two morphologically different types of colonies were distinguished among those selected; one type of colony grew compactly while the other grew in a spidery fashion on S.M. Colonies of both types were isolated and tested for resistance to F.A. and for ability to utilize acetate. All the 35 compact type colonies tested were sensitive to F.A. and utilized acetate, while all the spidery type colonies were resistant to F.A. Among 17 colonies of the latter type, two utilized acetate and 15 did not.

As this method yielded predominantly mutants sensitive to F.A. it was not further investigated.

4) Sandwiching of conidia between fluoroacetate medium and succinate fluoroacetate medium

Conidia from the strain paba1 were spread on F.A.M. (F.A. 4%,

Table 16. Selection of rapidly growing colonies by plating conidia on succinate medium

Strain	No. of conidia per dish ($x10^6$)	Total conidia plated ($x10^6$)	No. of compact colonies	No. of spidery colonies	No. of compact colonies per 10^6 plated conidia	No. of spidery colonies per 10^6 plated conidia
bi1	15	60	27	14	0.45	0.23
paba1	3.8	3.8	8	3	2.10	0.79

glucose 1%) and covered with S.F.A.M. After six days of incubation, resistant colonies arose (Table 17). Of 36 resistant colonies tested, two utilized acetate, while 34 did not. This technique was not further investigated because the mutants obtained were found to differ considerably from the f mutants isolated by other methods (see Part IV, E2e).

5) Sandwiching conidia between succinate medium and succinate fluoroacetate medium

As f strains grow better than f^+ strains on S.M. (see Part IV, E2ei), attempts were made to combine the two characteristics of f mutants, -resistance to F.A. and rapid growth on succinate-, for selection of 'forward'-mutants. However, when conidia were plated on S.M. containing F.A. no resistant colonies arose even when very low concentrations of F.A. were used. Also, strains inoculated into such a medium failed to grow. However, young f colonies on S.M. in contact with F.A. continued to grow while young f^+ colonies were inhibited, (this was found by using the technique described in Part II, 5e). Therefore, conidia of a strain sensitive to F.A. were plated in a top layer on top of S.M., incubated overnight, and then covered with S.F.A.M. Resistant colonies arose after 4-6 days

Table 17. Selection of fluoroacetate resistant mutants by plating conidia of the strain pabaI on F.A.M. plus S.F.A.M.

No. of conidia per dish ($x10^6$)	Total conidia plated ($x10^6$)	Resistant mutants No.	Resistant mutants per 10^6 plated conidia
2.5	20	77	3.85

(Table 18). The majority of resistant mutants tested (86 out of 89) failed to utilize acetate and were resistant on F.A.M. The three colonies that utilized acetate may have been leaky mutants.

The efficiency of this technique was tested by means of a reconstruction experiment (Table 19) and was found satisfactory as the 'Grigg effect' (Grigg, 1952) was almost negligible up to 10^7, conidia per dish.

Conidia of the strain bi1 were treated with nitrous acid (0.02 M. for fifteen minutes, survival about 2.3%). The conidia were plated in dishes containing C.M. to give 30-50 colonies per dish, and replicated on A.M. (Roberts, 1959). Of about two thousand colonies tested in this way two acetate non utilizing mutants were found (Dolezilova, unpublished results). One of these two was found to be resistant to F.A. while the other was sensitive.

Thus there are strains with all the four possible combinations of phenotypes with regard to ability to grow on acetate and resistance to fluoroacetate:

a) Wild type, i.e. able to grow on acetate (A.M.) as the sole carbon source but sensitive to F.A.

b) strains which can grow on A.M. and are resistant to F.A. designated fa

c) a strain which cannot grow on A.M. and is sensitive to F.A. designatedd ace

Table 18. Selection of fluoroacetate resistant mutants by sandwiching conidia of the strain bi1 between S.M. and S.F.A.M.

Experiment*	No. of conidia per dish ($x10^6$)	Total conidia plated ($x10^6$)	Resistant mutants No.	Resistant mutants per 10^6 plated conidia
1	10	30	18	0.6
2	7.5	15	21	1.4
3	2.5	10	9	0.9
4	20	60	31	0.52
5	6	12	17	1.42
6	12	24	93	3.87
7	3.5	7	13	1.86
8	8	24	46	1.92

*See first foot note in Table 14.

Table 19. Reconstruction experiment for the 'forward' selection of mutants by sandwiching conidia between S.M. and S.F.A.M.

No. of conidia per dish		Total conidia plated		No. of resistant colonies	
y;pyro4 ($\times 10^6$)	w3;pyro4;f3	y;pyro4 ($\times 10^6$)	w3;pyro4;f3	y;pyro4;f	w3;pyro4;f3
0.01	5	0.05	25	0	22
0.4	5	2	25	2	28
1	5	5	25	4	29
10	5	50	25	37	21
30	5	150	25	84	15
100	5	500	25	78	6

d) strains which cannot grow on A.M., and are resistant to F.A. designated f.

It is to be noted that all the fa mutants which were tested grew less well on A.M. than the strains from which they were derived.

The origin, method of isolation and designation of all the mutant strains which have been isolated during the course of this work, by means of the various techniques are given in Table 20.

Of all the various methods used for isolation of mutants, only three appeared suitable for the development of a 'two-way selection' system. As one of thesemethods (sandwiching between F.A.M. and S.F.A.M.) seemed to select mutants differing in phenotype from the mutants selected by the other techniques (Part IV, E2e), there remained only two techniques to be analysed thoroughly.

The first of these, selection of mutants by sandwiching conidia in F.A.M., appeared at first quite suitable, but further examination revealed a 'Grigg effect' at plating densities of 10^6 or more conidia per dish (Table 15). The second technique, on the other hand, showed a 'Grigg effect' only at or above 10^7 conidia per dish (Table 19). Considering that resistant mutants selected by the first technique are mainly prototrophic (fa), while resistant mutants selected by the second technique are mainly auxotrophic, the difference in these two techniques for selection of f mutants becomes substantial.

Table 20. Origin and designation of f and fa mutants

f resistant to F.A. unable to utilize acetate

fa resistant to F.A. able to utilize acetate

Symbol	Parent strain treated	Mutagen	Method of isolation
f1	bi1	none	sandwiching in F.A.M.
f2	w3;pyro4	"	sectoring on F.A.M.
f3	w3;pyro4	"	"
f4	bi1;w3	"	"
f6	bi1	N.A.	sandwiching in F.A.M.
f7	bi1;w2	none	sectoring on F.A.M.
f8	bi1;w2	"	"
f9	bi1	N.A.	sandwiching in F.A.M.
f10	bi1	"	"
f11	bi1;w2	"	"
f12	bi1;w2	N.A.	"
f13	y bi1	none	"
f14	bi1;w2	N.A.	"
f15	bi1;w2	"	"
f16	bi1;w2	none	"
f17	bi1;w2	"	"
f18	bi1	"	"

Table 20 continued

f19	bil	none	sandwiching in F.A.M.
f20	bil	"	"
f21	bil	"	"
f22	bil	"	"
f23	bil	"	"
f24	bil	"	"
f26	bil	"	"
f27	bil	"	"
f28	bil	"	"
f29	bil	"	"
f30	bil	"	"
f31	bil	"	"
f32	bil	"	"
f33	bil	"	"
f34	bil	"	"
f35	bil	"	"
f36	bil	"	"
f38	bil	N.A.	"
f39	bil	"	"
f40	bil	"	"
f41	bil	"	"

Table 20 continued

f45	bi1	N.A.	sandwiching in F.A.M.
f51	bi1	"	replica plating
f101	w3;pyro4	none	incubation in liquid B.M.+F.A.
f102	w3;pyro4	"	"
f201	paba1	"	sandwiching between F.A.M. & S.F.A.M.
f301	bi1	"	sandwiching between S.M. & S.F.A.M.
f302	bi1	"	"
f303	bi1	"	"
f305	bi1	"	"
f306	bi1	"	"
f307	bi1	"	"
f308	bi1	"	"
f309	bi1	"	"
f401	bi1	"	sandwiching in S.M.
f402	bi1	"	"
f403	bi1	"	"
f404	bi1	"	"
fa1	y bi1	"	sandwiching in F.A.M.
fa2	y bi1	"	"
fa3	y bi1	"	"
fa4	bi1	"	sectoring on F.A.M.

Table 20 continued

fa5	bi1	none	sectoring on F.A.M.
fa6	bi1	"	sandwiching in F.A.M.
fa7	bi1	"	"
fa8	bi1	"	"
fa9	bi1	"	"
fa10	bi1	"	"
fa11	bi1	"	"
fa12	bi1	"	"
fa13	bi1	"	"
fa14	bi1	"	"
fa15	bi1	"	"
fa16	bi1	"	"
fa17	bi1	"	"
fa18	bi1	"	"

Furthermore the 'S.M. plus S.F.A.M.' technique is cheaper and less dangerous to use as the quantities of F.A. used are much less than in the F.A.M. technique. Thus the 'S.M. plus S.F.A.M.' technique seems the obvious choice for further work.

It is of note that the f mutants vary considerably in their degree of resistance to F.A. Mutants isolated by incubation in liquid B.M. + F.A. (Part IV, C1) and by use of the 'S.M. plus S.F.A.M.' technique are among the least resistant.

Mutants requiring acetate and mutants unable to grow on acetate as the sole carbon source are not very common in the literature. Those requiring acetate are known to occur in Aspergillus nidulans (Käfer, unpublished) and Neurospora crassa (Lein et al., 1951), whilst mutants probably unable to grow on acetate as the sole carbon source have been studied in Escherichia coli by Gilvarg and Davis (1956) and by Reeves and Ajl (1962).

Summary

1) The combination of resistance and auxotrophy was achieved by using the metabolic analogue fluoroacetate.

2) Various techniques for the isolation of mutants resistant to F.A. and unable to utilize acetate were tried.

3) Sandwiching conidia between a basal layer of S.M. and an upper layer of S.F.A.M. proved to be the best technique for a 'two-way selection', using fluoroacetate, among those tried.

(D) Selection and isolation of 'back'-mutants

For the detection of 'back'-mutants, conidia from f strains were embedded in A.M. plus the necessary growth factor requirements, and the dishes were scored after 3-5 days. Results are presented in Table 21. As expected, not all resistant auxotrophs 'back'-mutated, and those which did so might have 'back'-mutated in respect of the auxotrophy and the resistance, or only in respect of the auxotrophy. From forty-four f mutants tested for 'back'-mutation only thirteen 'back'-mutated spontaneously (Table 21). 'Back'-mutants of eight of these thirteen mutants were also tested for resistance to F.A., and while all of the 'back'-mutants of seven of these strains were found to be sensitive to F.A., all the 'back'-mutants of one strain (f3) remained resistant to F.A. (Table 21).

On the basis of growth on A.M., two types of 'back'-mutants are observed: 'back'-mutants forming large colonies, and 'back'-mutants forming small colonies (even the larger type of colony is slightly smaller than, and different in its growth pattern from, the wild type strains on A.M.). The 'back'-mutants of each mutant fell into one of these two categories, with the exception of the 'back'-mutants of the strains bi1 f307 and bi1 f309 which fell into both categories.

There seems to be an inverse correlation between the frequency

Table 21. Selection of spontaneous 'back'-mutants able to utilize acetate as sole carbon source from forward f mutants resistant to F.A. and unable to utilize acetate

f mutant* tested	No. of conidia per dish ($x10^6$)	Total conidia plated ($x10^6$)	Back mutants No.	Back mutants per 10^6 plated conidia	Back mutants tested for resistance to F.A. No. tested	Back mutants tested for resistance to F.A. No. resistant
f1	40	320	0	0		
f2	15	15	8	0.53	8	0
f3	21	84	12	0.14	12	12
f3	15	60	19	0.32	19	19
f4	20	80	0	0		
f4	50	1,000	0	0		
f4	60	600	0	0		
f4	80	1,200	0	0		
f6	60	240	0	0		
f7	35	280	0	0		
f8	25	200	117	0.58	52	0
f9	50	300	0	0		
f10	40	320	164	0.51	52	0
f11	35	320	0	0		
f12	45	225	0	0		
f13	50	300	0	0		
f14	45	340	0	0		

Table 21 continued

f15	35	280	0	0		
f16	50	500	0	0		
f17	75	375	0	0		
f18	48	288	0	0		
f19	70	350	0	0		
f20	64	448	0	0		
f21	30	270	0	0		
f22	25	250	0	0		
f23	45	360	0	0		
f24	55	275	0	0		
f26	35	350	0	0		
f27	65	260	0	0		
f28	70	280	68	0.24	-	-
f29	65	325	0	0		
f30	80	320	0	0		
f31	75	300	0	0		
f32	50	200	0	0		
f33	50	200	4	0.02	-	-
f34	40	320	0	0		
f35	35	210	2	0.0095	-	-
f36	50	300	0	0		

Table 21 continued

f101	35	70	1	0.014	1	0
f101	45	225	3	0.013	3	0
f101	35	70	2	0.029	2	0
f102	15	30	24	0.8	-	-
f102	25	100	43	0.43	-	-
f102	30	240	95	0.39	-	-
f301	50	250	236	0.94	-	-
f302	60	240	0	0	0	0
f303	60	300	0	0	0	0
f305	45	225	3	0.01	3	0
f306	45	270	0	0	0	0
f307**	60	300	448	1.49	15	0
f307	20	40	85	2.12	6	0
f307	15	30	51	1.70	-	-
f308	55	220	0	0	0	0
f309**	60	240	8	0.03	4	0
f309	100	400	51	0.13	8	0
f309	100	400	18	0.04	12	0

* Where the same mutant is given more than once, the conidia for each experiment were of independent origin (Part II, 4).

** Two very distinct types among the revertants: large and small colonies on acetate medium.

of 'back'-mutants and their size. Mutants which revert with a low frequency give 'back'-mutants which produce large colonies (f33, f35, f101 and f305), while mutants which revert with a high frequency produce small colonies (f2, f3, f8, f10, f28, f102, and f301). Again, among the revertants of f307 and f309, where two classes of size of colonies are found, the small outnumber the large by about five to one.

This might be explained by assuming two different mechanisms for the origin of small and large 'back'-mutants; the small might arise by extra-cistron suppressors while the large might arise by intra-cistron suppressors, or be genuine 'back'-mutants. Intra-cistron suppressed mutants are expected to resemble the original state more than extra-cistron suppressed mutants, since only in the first case is the original function of the affected cistron supposed to be repaired. As such a repair is possible only by mutations in certain sites of the affected cistron, while in the other case mutation of any site in a cistron will probably repair the original metabolic effect, it is expected that 'forward'-mutants which can revert by extra-cistron suppressors do so more frequently than those which revert by intra-cistron suppressors.

As previously mentioned, most of the f mutants failed to 'back'-mutate spontaneously. That this is a genuine failure of 'back'-mutation and not merely a failure of detection of 'back'-

mutants under the conditions of the test is indicated by the fact that nitrous acid is an effective mutagen for reversion in this system (Table 22).

Whereas mutagens applied to spores just before plating may be effective in inducing 'forward'-mutation (which probably entails a loss of function), the same mutagens may appear to be ineffective in inducing 'back'-mutation (which probably entails recovery of a lost function or gain of a new function) because of the lag between mutation induction and expression, which may require a number of divisions of the mutated nucleus (Auerbach, 1951). In the case of the f mutants, which are capable of growing slightly on A.M. (Part IV, E2a) a mutation induced in a conidium might go through the nuclear divisions necessary for mutation expression.

It is interesting to note that the vast majority of mutants which were selected by sandwiching of conidia in F.A.M. did not revert, while among the mutants isolated by sectoring of sensitive colonies on F.A.M., or by incubating conidia sensitive to F.A. in liquid B.M. plus F.A., or by sandwiching conidia in S.M. plus S.F.A.M., a good proportion did revert. The mutants isolated are designated by b together with the number of the f allele from which they originated (Table 23).

As in the case of the 'forward'-selections, an experiment was designed to test for the 'Grigg effect' (Table 24). From this

Table 22. Selection of 'back'-mutants able to utilize acetate as sole carbon source from 'forward' f mutants after treatment of conidia with nitrous acid

f mutant tested	Treatment	No. of Conidia per dish ($x10^6$)	Total conidia plated	Back mutants No.	Back mutants per 10^6 plated conidia
f101	none	70	280	3	0.01
	nitrous acid*	4	32	24	0.75
f4	none	30	300	-	-
	nitrous acid**	5	40	-	-

* N.A. 0.0145M. 10 minutes, survival 58%

** N.A. 0.0145M. 10 minutes, survival 51%

Table 23. Origin and designation of reverse mutants able to utilize acetate as sole carbon source

All revertants were isolated after embedding conidia in acetate medium

Symbol of suppressor	f mutant strain used	Mutagen
b1-f3	w3;pyro4;f3	none
b1-f8	bi1;w2;f8	N.A.
b2-f8	bi1;w2;f8	none
b1-f10	bi1;f10	"
b2-f10	bi1;f10	"
b1-f101	w3;pyro4;f101	"
b2-f101	w3;pyro4;f101	"
b3-f101	w3;pyro4;f101	"
b4-f101	w3;pyro4;f101	"
b5-f101	w3;pyro4;f101	"
b1-f102	w3;pyro4;f102	"
b2-f102	w3;pyro4;f102	"
b3-f102	w3;pyro4;f102	N.A.
b1-f301	bi1;f301	none
b1-f305	bi1;f305	"
b1-f307	bi1;f307	"
b2-f307	bi1;f307	"
b1-f309	bi1;f309	"
b2-f309	bi1;f309	"

Table 24. Reconstruction experiment for 'back'-mutation*

No. of conidia per dish		Total conidia plated		Prototrophic colonies	
y;pyro4	w3;pyro4;f3 ($\times 10^6$)	y;pyro4	w3;pyro4;f3 ($\times 10^6$)	y;pyro4	w3;pyro4;f3;b-f3
10	0.005	60	0.03	71	0
10	0.05	60	0.3	58	0
10	0.5	60	3	63	0
10	5	60	30	52	8
10	50	60	300	69	65
10	100	60	600	48	82

* Conidia of a strain (y;pyro4) able to grow on acetate medium and conidia of a strain (w3;pyro4;f3) unable to grow on acetate medium were mixed in different proportions and embedded in acetate medium.

table, in which the embedding technique was used, it can be seen that few wild type spores grew out of 10^8 mutant spores and that this selection method is efficient up to a plating density of about 5×10^7 conidia per dish.

Summary

1) Revertants able to utilize acetate were selected from various *f* mutants.

2) Most of the *f* mutants tested did not revert, and this failure is thought to be genuine.

3) The ability of 'forward' *f* mutants to revert depended on the method of their isolation. There was an inverse correlation between the frequency of reversion of *f* forward mutants and the size of the revertant colonies.

(E) Characteristics of wild type and of mutants

1) Wild type

a) Competitive inhibition between fluoroacetate and various carbon sources in the growth of the wild type

An auxanographic test for carbon sources was made with conidia of the strain w3;pyro4 (about 10^7 conidia per dish were embedded in B.M.). Two holes were made in the B.M. approximately one inch apart. One was filled with a solution of F.A. (5%) adjusted to pH 6.5 with NH_4OH, and the other with a solution of the carbon source (10%). The carbon sources tested were acetate, glucose, fructose, sucrose and lactose, all of which support the growth of Aspergillus nidulans (Roberts, 1961). The growth of the strain w3;pyro4 on these carbon sources was found to be inhibited by F.A. and the inhibition seemed to be competitive since the boundary between the zone of growth and the zone of inhibition appeared as a straight line (Pontecorvo, 1949).

Competitive inhibition between fluoroacetate and the sources tested might be accounted for in two ways. All the carbon sources tested are known to be degraded to acetate via glycolysis and the competition may arise between acetate and fluoroacetate; alternatively,

they may all be converted to some other common derivative such as citrate, where again the fluoroanalogue might act competitively. It was shown that when mammals are fed with F.A. they synthesize from it fluorocitrate and this metabolite was shown to impair the functioning of the enzyme aconitase (Morrison and Peters, 1954).

b) Growth of Aspergillus nidulans on acetate as the sole carbon source

Suitable growth conditions were found by using auxanographic techniques. A viability test of conidia of the strain w3;pyro4 on C.M. and on A.M. did not reveal differences. In most organisms studied (Kornberg and Elsden, 1961) growth on acetate necessitates the operation of the 'glyoxalic cycle' and involves the induction of at least one enzyme, isocitritase, which is inhibited and repressed by various carbon sources including succinate. The compounds citrate and succinate are poor carbon sources for Aspergillus nidulans and inhibit its growth on A.M. Attempts were made, therefore, to select mutants which overcame this inhibition by adding to A.M. citrate (0.4%) or succinate (0.2%), concentrations which inhibit growth on A.M. completely. In each case about 10^9 conidia of the strain bi1 were tested but no mutant colony which overcame this inhibition was recovered.

The growth of fa strains (fluoroacetate resistant, utilizing

acetate) on acetate medium is more severely affected by citrate or succinate than that of wild type strains. Concentrations of 0.2% citrate or 0.1% succinate allow growth of wild type strains on A.M. but not of fa strains. This information could widen the application of the 'two-way selection' technique, using F.A., to more loci.

Growth on acetate was not only sensitive to various carbon source inhibitions but also to certain genotypic interactions. The growth of strains carrying the mutants lys5, ad1, ad23, orn9 and arg3 was reduced to varying degrees. None of the vitamin-requiring or sugar mutant strains which were tested were found to be affected in this way; nor were all of the amino acid and adenine mutant strains affected.

Mycelium of f^+ strains grown on C.M. or M.M. when transferred to A.M. failed to grow. This was also the case when mycelium of resistant strains (fa) was transferred from F.A.M. to A.M. Therefore, all transfers were made as far as possible via conidia, or from one medium through C.M. to another medium. Especially interesting in this context is the phenomenon that many colonies resistant to F.A., which arose after plating conidia on F.A.M., when transferred to C.M. developed conidia which failed to grow on A.M. However, after one more transfer through C.M. their conidia did grow on acetate. This is akin to adaptation which is 'memorized' for one vegetative generation during which fa strains mimic f strains completely.

2) f mutants

a) Residual growth on acetate

All f mutants isolated show some degree of growth on A.M. and vary from one another in this respect. This residual growth is due to utilization of acetate and not to utilization of impurities in the agar or of the agar itself as a carbon source, since sparse residual growth of f mutants is also observed in liquid A.M. This residual growth could be due to 'leakiness' of the mutants, or, if they are non-'leaky', to the existence of a different and inefficient pathway for the utilization of acetate other than that which is blocked by the f mutants. The fact that all of the f mutants obtained are 'leaky' can be taken as weak evidence supporting the second possibility, and, if this is the case, then the variability of growth on acetate of the f mutants, above the basic level attributable to the alternative pathway, could be due to varying degrees of 'leakiness'.

Some of the more 'leaky' mutants are less resistant to F.A., a correlation which is expected from the hypothesis for the mechanism of resistance to F.A. described in Part IV, B.

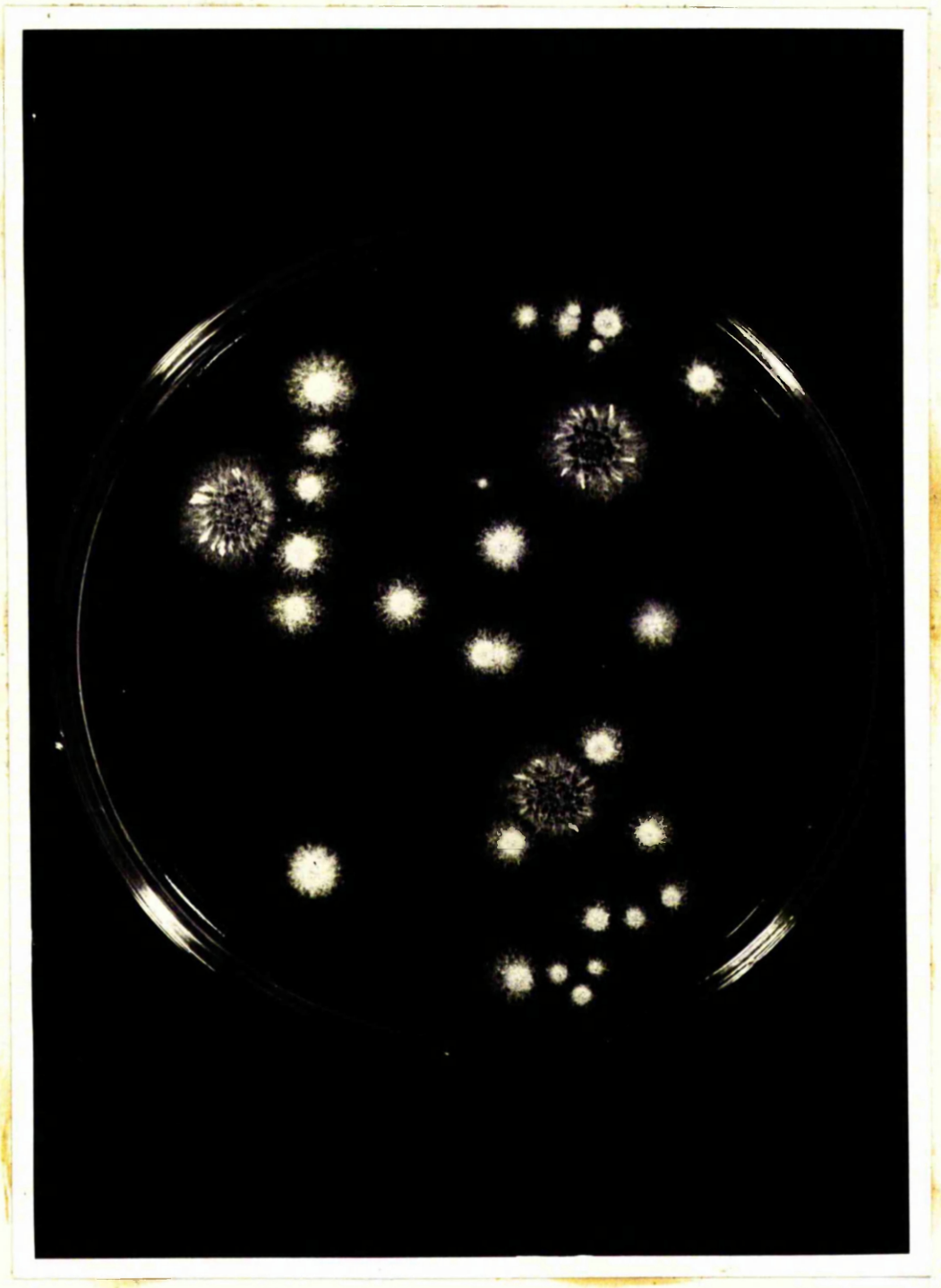
b) The 'proline effect'

An attempt was made to discover whether or not the inability of the f mutants to grow on acetate as the sole carbon source could be circumvented. For this purpose various growth factors were tested auxanographically on A.M. using conidia of the strain w3;pyro4;f101. The strain responded to casein hydrolysate and, when tested with individual amino acids, responded to proline and glutamate (glutamate and proline are interchangeable in the metabolic pathways of various organisms). The response of f strains to proline seemed to be quantitatively related to the amount of proline added. However, it is not merely utilization of proline as a carbon source for a clear 'sparing effect' was seen when proline and acetate were tested auxanographically on the same dish. It is more likely that proline activates the alternative inefficient pathway for acetate utilization, mentioned in the previous section, rather than repairs the metabolic lesion impaired by an f mutation. This phenomenon can, however, be successfully used in analysis of crosses. By plating spores of an f strain on A.M. plus proline 0.02%, colonies are obtained which can easily be distinguished from f^+ colonies (Fig.4).

The viability of conidia of f strains on A.M. plus proline

Figure 4

Growth of f and f^+ colonies on acetate medium & proline



Note: the three larger colonies are f^+.
The medium contains 0.02% L-proline.

is comparable to their viability on C.M. All the f strains tested, except f201 and strains selected in a similar manner (F.A.M. plus S.F.A.M., Part IV, 04), showed the 'proline effect'.

On the assumption that proline somehow enabled acetate molecules to enter the cell or allows them to participate in the metabolism of the f mutants, it was predicted that it might have the same effect on f mutants when they are grown on F.A.M., thereby rendering them sensitive. Hence proline was added to F.A.M. and the strains carrying one of the mutants f1, f6, f10, f20 respectively, tested. All of them remained resistant just as in the absence of proline. This, however, does not rule out the role of proline as suggested above as usually only acetate and proline were present in the medium, while in this case F.A., proline and glucose were present, and glucose might inactivate the pathway through which proline exerts its action.

f mutants become sensitive to F.A. by interactions of other genes. f strains carrying the mutants orn7, orn9, arg3, or lys5 were found to be sensitive to F.A.

c) f mutants and intermediates of the Krebs' cycle

As metabolism of acetate is intimately related to the Krebs'

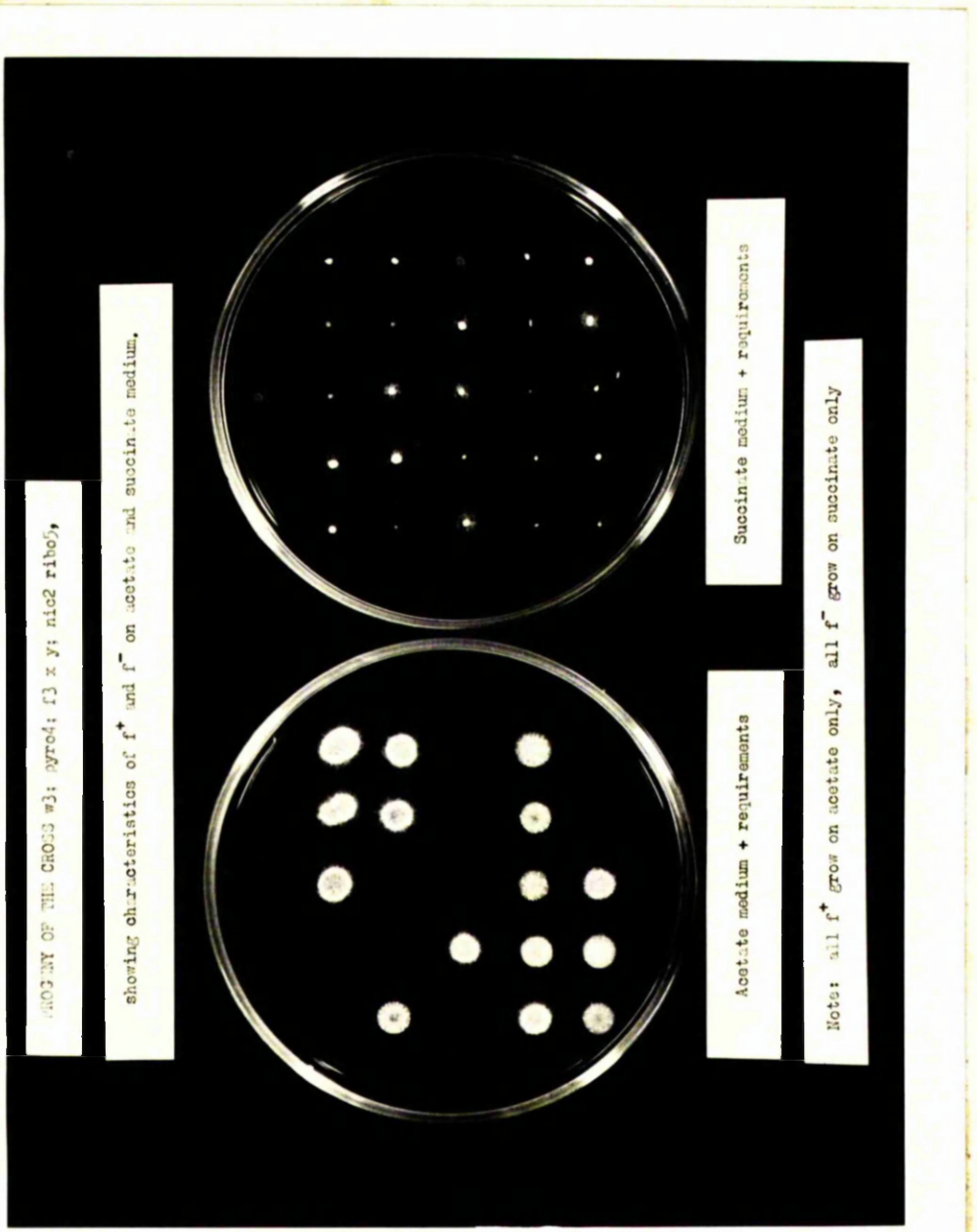
cycle, an attempt was made to compare growth of f strains and wild type strains on metabolites of this cycle.

i) Succinate

Wild type strains of Aspergillus nidulans grow very poorly on succinate (S.M.) as the sole carbon source and on other metabolites of the Krebs' cycle such as fumarate or malate. However, f mutants utilize succinate, fumarate and malate as sole carbon sources much better than the wild type strains (succinate was used extensively during this work). The difference in utilization of succinate is so great that it can be used as a routine for scoring progeny of crosses in which f mutants are involved (Fig.5). All f strains tested showed this phenomenon while none of the fa strains did.

Among the f mutants two groups can be distinguished on the basis of intensity of growth on S.M. after incubation for 4-5 days. This grouping might prove to be significant, and might identify alleles of one cistron, as is seen in Part IV, F1a). As yet, f mutants cannot be grouped on any other phenotypic criterion.

Figure 5


AUOGRAY OF THE CROSS W3: gyrA4: f3 x y: nic2 rib5,
showing characteristics of f+ and f- on acetate and succinate medium.
Acetate medium + requirements
Succinate medium + requirements
Note: all f+ grow on acetate only, all f- grow on succinate only

ii) Malate-malonate

f mutant strains were found to be more inhibited by malonate than an f^+ strain. On B.M. containing 7% malonic acid and 1% DL-malic acid pH 4 adjusted by NH_4OH, the strains paba1 grew well while the strains paba1 y; f3, paba1; w3; f101 and paba1; f102 were completely inhibited (these three strains were chosen to represent three loci (Part IV, F1a).

iii) Cis-aconitic acid

On cis-aconitic acid (B.M., cis-aconitic acid 1%, pH 5, adjusted by NH_4OH) strains paba1, paba1 y; f3, paba1; w3; f101 and paba1; f102 all grew very poorly but to approximately the same extent.

d) Fatty acids as the sole carbon source for f and f^+ strains

As conversion of acetate and fatty acids to an anhydride with CoA (coenzyme A) might involve the same enzymes, strains w3; pyro4 and w3; pyro4; f2 were tested auxanographically on B.M. for ability to utilize propionate and butyrate (pH 6.5, adjusted by NH_4OH). While both strains failed to respond to propionate, w3; pyro4 responded slightly to butyrate but the strain w3; pyro4; f2 failed to do so.

e) The nature of the f mutants

What is the actual block in the f mutants, or what is the difference between f and f^+ strains? None of the growth tests

on various media succeeded in revealing qualitative differences among the f mutants although they map at three distinct loci (Part IV, F1a). Thus their similar behaviour suggests that they all may be defective in the same primary function (in the sense that only one protein is involved). For instance, they could be defective in the uptake of acetate and the fact that they can utilize acetate under certain conditions (the 'proline effect') does not argue either in favour or against this.

The fact that the f mutants grow much better on metabolites of the Krebs' cycle probably implies that all the Krebs' cycle enzymes are active in the f mutants. If, as in mammals, F.A. exerts its toxic effect by interfering with aconitase, the f mutants might lack this enzyme. However, their growth on cis-aconitate which is a specific substrate for aconitase (Anfinsen, 1955), suggests that aconitase is present.

That the strain w3; pyro4; f2 failed to respond to butyrate while the strain w3; pyro4 did, might indicate that f strains lack an enzyme for the activation of acetyl moities, i.e. they cannot activate acetate to acetyl CoA, and this enzyme might well be acetyl-thiokinase. An organism lacking such an enzyme should not be affected in its growth on metabolites containing more than two carbons, as it should still possess all the enzymes necessary for formation of acetyl CoA from pyruvate. In the case of the f mutants there is no detectable difference between f and f^+

strains growing on glucose or glycerol except that f strains tend to form more perithecia than f^+ strains.

The f mutants isolated on the basis of resistance to F.A. revealed, on closer examination, various pleiotropic effects, some of which, such as rapid growth on succinate, proved useful in the genetic analysis of f mutants. Rapid growth on succinate is also interesting in the sense that the f mutants here demonstrate loss of one function (failure to grow on acetate) and gain of another function (rapid growth on succinate) both due to the mutation f^+ to f.

The f mutants collected by various techniques had the same properties in all tested conditions with the exception of the strain f201 and strains isolated by the same method (F.A.M. plus S.F.A.M. Part IV, 04). These differed from all other f strains in three ways:

1) by the absence of the 'proline effect'
2) by having a different type of residual growth on A.M.
3) by being easily 'breast-fed' (Pontecorvo, et al., 1953) on A.M. by f^+ colonies growing on the same plate.

Why the particular technique by which these mutants were isolated selects f mutants which differ from all other f mutants collected is not very clear. It is, however, the only technique in which glucose, succinate, and fluoroacetate are used together. This adds to the common knowledge that change of selection conditions affects the kinds of mutants isolated.

Summary

1) Fluoroacetate seems to inhibit competitively the growth of Aspergillus nidulans on various carbon sources.

2) Growth of Aspergillus nidulans on acetate as the sole carbon source is sensitive to other carbon sources.

3) Residual growth of f mutants on acetate is not considered to be due to 'leakiness' of the f mutants but to a different pathway which the f mutants do not affect.

4) Proline and glutamate have a 'sparing effect' on strains carrying an f mutant and probably enable them to utilize acetate to a certain extent.

5) f mutants utilize succinate malate and fumarate more rapidly than wild type strains.

6) f mutant strains tested are more sensitive than a wild type strain to malonate.

7) f mutant strains and a wild type strain utilize cis-aconitate equally but poorly.

8) An *f* mutant strain cannot utilize butyrate whereas a wild type strain can.

9) The three *f* loci are considered to be responsible for the same primary function which affects the uptake or the further utilization of acetate.

(F) Formal genetics of mutants

For further genetic studies only certain of the *f* mutants were chosen. Mutants which 'back'-mutate were considered, as one of the main interests in searching for a 'two-way selection' was the study of intra-cistron suppressors. Also studied were the mutants selected by using S.M. plus S.F.A.M. - the technique which proved most suitable for 'two-way selection' using F.A. - and one mutant which did not revert (*f4*).

1) 'Forward'-mutants (f)

a) Genic origin, number of loci, recessivity

The following heterokaryons were synthesized:

1) *w3; pyro4; f3* / *paba1 y* (*f3* / *f⁺*)
2) *w3; pyro4; f101* / *paba1 y* (*f101* / *f⁺*)
3) *w3; pyro4; f102* / *paba1 y* (*f102* / *f⁺*).

From each of these heterokaryons conidia were harvested and plated on

C.M., and 104 colonies (52 white and 52 yellow) from each heterokaryon were tested. The genotypes of the colonies were determined by replication to various media; in each case only the two corresponding parental types were found.

In crosses of the type $\underline{f}$ x $\underline{f}^+$ a 1:1 ratio of $\underline{f}$ to $\underline{f}^+$ progeny (Table 25), as expected in crosses involving a single Mendelian factor, was always obtained.

In all cases tested (about 800 progeny) the correlation between resistance to F.A. (rapid growth on S.M.) and auxtrophy on A.M. was complete. In no case were progeny of such a cross found to be either sensitive to F.A. (not rapid growers on succinate) and auxotrophic on A.M., or to have the reciprocal phenotype, i.e. resistant to F.A. (rapid growers on succinate) and prototrophic on A.M. It would seem, therefore, that all the characteristics by which $\underline{f}$ strains are distinguished from corresponding $\underline{f}^+$ strains are due to a single Mendelian factor.

All $\underline{f}$ mutants chosen - on the basis of the criteria given before - for linkage studies (14 out of 55) were crossed in certain pairs. The results show clearly that these mutants map at three different unlinked loci (Table 26). These loci are designated f3, f101 and f102 and the known mutants mapping at these loci (Table 26) are as follows:

Table 25. Crosses between f mutant strains and wild type strains

Analysed by 'perithecium analysis'

Cross*	Type of cross	No. of colonies with phenotype		Total	Test for 1:1 ratio	
		f	f^+		χ^2_1	P
w3; pyro4; f2 x paba1 y; ad23	f2 x f^+	108	87	195	2.26	0.13
w3; pyro4; f3 x y; nic2 ribo5	f3 x f^+	148	152	300	0.05	0.92
w3; pyro4; f101 x y; ad3; s1	f101 x f^+	91	119	210	3.73	0.05
w3; pyro4; f102 x paba1 y; ad23	f102 x f^+	72	83	155	0.78	0.37
bi1; f301 x y; ad1; s12	f301 x f^+	43	61	104	2.09	0.15
bi1; f302 x y; ad1; s12	f302 x f^+	42	55	97	1.74	0.19
bi1; f303 x y; nic2 ribo5	f303 x f^+	47	54	101	0.48	0.50
bi1; f307 x y; ad1; s12	f307 x f^+	94	79	173	1.30	0.25

* In crosses involving a strain carrying an ad mutant only ad^+ progeny were scored.

Table 26. Crosses between f mutants

Analysed by 'perithecium analysis'

Cross*			Type of cross	No. of colonies with phenotype		Total	Test for 3:1 ratio	
				f	f+		χ^2_1	P
w3; pyro4; f2	x	paba1 y; ad23; f3	f2 x f3	82	0	82	-	
paba1 y; ad23; f3	x	bi1; w3; f4	f3 x f4	53	0	53	-	
w3; pyro4; f3	x	bi1; w2; f8	f3 x f8	83	17	100	3.41	0.06
w3; pyro4; f3	x	paba1; w3; f101	f3 x f101	77	23	100	0.12	0.68
paba1 y; ad23; f3	x	w3; pyro4; f102	f3 x f102	80	20	100	0.48	0.46
w3; pyro4; f3	x	bi1; f303	f3 x f303	78	0	78	-	
y; pyro4; nic2 f3 ribo5	x	bi1; f305	f3 x f305	104	0	104	-	
y; pyro4; nic2 f3 ribo5	x	bi1; f306	f3 x f306	78	0	78	-	
bi1; w2; f8	x	pyro4; f101	f8 x f101	58	18	76	0.07	0.78
bi1; w2; f8	x	w3; pyro4; f102	f8 x f102	100	0	100	-	
paba1; w3; f101	x	w3; pyro4; f102	f101 x f102	80	20	100	1.33	0.25

Table 26 continued

y; s1; f101	x	bi1; f301	f101 x f301	69	35	104	4.15	0.04
y; s1; f101	x	bi1; f302	f101 x f302	104	0	104	-	
y; s1; f101	x	bi1; f305	f101 x f305	74	30	104	0.82	0.36
y; s1; f101	x	bi1; f306	f101 x f306	71	29	100	0.85	0.35
y; s1; f101	x	bi1; f307	f101 x f307	70	32	102	2.21	0.14
y; s1; f101	x	bi1; f308	f101 x f308	102	0	102	-	
y; s1; f101	x	bi1; f309	f101 x f309	104	0	104	-	
w3; pyro4; f102	x	bi1; f301	f102 x f301	104	0	104	-	
w3; pyro4; f102	x	bi1; f307	f102 x f307	104	0	104	-	
bi1; f301	x	y; ad1; s12; f307	f301 x f307	104	0	104	-	

* In crosses involving a strain carrying an ad mutant only ad$^+$ progeny were scored.

locus *f3*	*f2*, *f4*, *f303*, *f305*, *f306*
locus *f101*	*f302*, *f308*, *f309*
locus *f102*	*f8*, *f301*, *f307*

Since each of the three loci contains mutants selected by different techniques, it is unlikely that further loci determining the same phenotypic differences exist.

All the mutants located in one of the loci (designated *f3*) are of the type which grows well on S.M., while all the mutants located in the other two loci (designated *f101* and *f102* are of the type which grows less well on S.M. (see Part IV, E2oi). It should be noted that the mutants *f101* and *f102*, the only mutants which were isolated in the same experiment (incubation in liquid B.M. plus F.A. Part IV, C1), recombine freely with each other.

In scoring progeny of crosses of the type *fy* x *fz* only two types of progeny could be distinguished - whether the two mutants were in the same locus or in two different loci - one having an f^+ and the other having an *f* phenotype. Hence, if the double recombinant *fy fz* arose it was probably indistinguishable from the parental *f* type. As colonies having the *f* phenotype were not isolated from such crosses and back crossed to both parents, it is impossible to decide this point.

To find out whether or not the system is suitable for fine genetic analysis, the cross paba1 y; ad23; f3 x bi1; w3; f4 (f3 x f4) was analysed by plating a heavy suspension of ascospores on A.M. plus para-aminobenzoic acid, and proper dilutions on M.M. plus para-aminobenzoic acid. The frequency of f^+ progeny among the total recombinant progeny of this cross was 0.1% which is much higher than the reversion frequency of f3; f4 does not revert at all (Table 21). Thus the system shows itself to be suitable for fine genetic analysis.

The recessivity of the f mutants to their wild type allele was established by synthesizing diploids (Roper, 1952) heterozygous for f mutants (Table 27). In all cases tested recessivity was confirmed for all the examined characteristics of the f mutants. By synthesizing diploids between various pairs of f mutants (Table 27) it was found that when the f mutants are at two different loci they do complement, but when they are at the same locus they do not complement. This confirms their recessivity and might suggest that the three loci correspond to three cistrons.

b) Location

i) Mitotic analysis

By haploidization of diploids between f mutant strains and tester strain MSD all the f mutants tested were located in linkage groups

27. Growth of diploids heterozygous and homozygous for f mutants

Table 27. Growth of diploids heterozygous and homozygous for f mutants on three media

Combination			Type of diploid	Media		
				A.M.	F.A.M.	S.M. + S.F.A.M.
ad23; f3; cha	/	paba1; w3	f3 / f^+	+	-	-
bi1; w2; f8	/	MSD	f8 / f^+	+	-	-
paba1; w3; f101 ribo2	/	ad23; cha	f101 / f^+	+	-	-
paba1; f102	/	MSD	f102 / f^+	+	-	-
bi1; f301	/	MSD	f301 / f^+	+	-	-
bi1; f302	/	MSD	f302 / f^+	+	-	-
bi1; f303	/	MSD	f303 / f^+	+	-	-
bi1; f305	/	MSD	f305 / f^+	+	-	-
bi1; f306	/	MSD	f306 / f^+	+	-	-
bi1; f307	/	MSD	f307 / f^+	+	-	-
bi1; f308	/	MSD	f308 / f^+	+	-	-
bi1; f309	/	MSD	f309 / f^+	+	-	-

Table 27 continued

paba1 y; ad23; f2	/ w3; pyro4; f3	f2 / f3	-	+	+
paba1 y; ad23; f2	/ bi1; w3; f4	f2 / f4	-	+	+
paba1 y; ad23; f2	/ w3; pyro4; f101	f2 / f101	+	-	-
paba1 y; ad23; f2	/ w3; pyro4; f102	f2 / f102	+	-	-
paba1; y; f3	/ w3; pyro4; f101	f3 / f101	+	-	-
ad23; f3	/ w3; pyro4; f102	f3 / f102	+	-	-
y; pyro4; nic2 f3 ribo5	/ bi1; f303	f3 / f303	-	+	+
y; pyro4; nic2 f3 ribo5	/ bi1; f305	f3 / f305	-	+	+
y; pyro4; nic2 f3 ribo5	/ bi1; f306	f3 / f306	-	+	+
y; s1; f101	/ w3; pyro4; f102	f101 / f102	+	-	-
paba1; w3; f101 ribo2	/ bi1; f301	f101 / f301	+	-	-
paba1; w3; f101 ribo2	/ bi1; f302	f101 / f302	-	+	+
paba1; w3; f101 ribo2	/ bi1; f308	f101 / f308	-	+	+
paba1; w3; f101 ribo2	/ bi1; f309	f101 / f309	-	+	+
w3; pyro4; f102	/ bi1; f301	f102 / f301	-	+	+
w3; pyro4; f102	/ bi1; f307	f102 / f307	-	+	+
bi1; f301	/ y; ad1; s12; f307	f301 / f307	-	+	+

V or VIII, (Tables 28-30). The following diploids were synthesized:

1) MSD / paba1; ad23; f3
2) MSD / paba1; w3; f101
3) MSD / paba1; f102
4) MSD / bi1; f301
5) MSD / bi1; f302
6) MSD / bi1; f303
7) MSD / bi1; f305
8) MSD / bi1; f306
9) MSD / bi1; f307
10) MSD / bi1; f308
11) MSD / bi1; f309

Haploidization of diploid 1 (Table 28) failed to locate f3 to a particular linkage group as all the haploids obtained had the f phenotype. However, as they were also phe$^+$; lys$^+$ location in the third or the fifth linkage group is suggested (phe2 is selected against when haploidization is made with P.F.P.A. and lys5 is rarely recovered under the conditions of the test). Therefore the diploid w3 pyro4; f3 / pro1; paba1 y; pal A1 was synthesized (pal A1 being located in the third linkage group, Dorn, 1963). The thirty-three haploids isolated segregated in the following way:

	f	pal		
parental	+	-	3	18
	-	+	15	
recombinant	+	+	11	15
	-	-	4	

Table 28. Location of f3 in linkage groups III or V by haploidization, after treatment with P.F.P.A., of diploid MSD/paba1; ad23; f3

Linkage group	Tester marker	f^+	f
I	paba$^+$	0	7
	paba	0	15
II	Acr$^+$	0	8
	Acr	0	14
II	ad$^+$	0	14
	ad	0	8
III	phe$^+$	0	22
	phe	0	0
IV	pyro$^+$	0	6
	pyro	0	16
V	lys$^+$	0	22
	lys	0	0
VI	s$^+$	0	17
	s	0	5
VII	nic$^+$	0	13
	nic	0	9
VIII	ribo$^+$	0	12
	ribo	0	10

Table 29. Location of f101 in linkage group VIII by haploidization, after treatment with P.F.P.A., of diploid MSD/paba1; w3; f101

Linkage group	Tester marker	f+	f
I	paba+	7	11
	paba	4	4
II	Acr+	5	8
	Acr	6	7
II	w+	6	7
	w	5	8
III	phe+	11	15
	phe	0	0
IV	pyro+	6	7
	pyro	5	8
V	lys+	9	11
	lys	2*	4
VI	s+	3	8
	s	8	7
VII	nic+	4	11
	nic	7	4
VIII	ribo+	0	15
	ribo	11	0

* See next page

* These two haploids did not grow on A.M. like a usual f^+ strain because they carry the mutant lys5. However, due to the morphology of these colonies on acetate, they could be distinguished from f colonies, and their pattern of growth was completely identical to the pattern of growth of strains carrying the mutant lys5 on A.M.

Table 30. Location of f102 in linkage group VIII by haploidization, after treatment with P.F.P.A., of diploid MSD/paba1; f102

Linkage group	Tester marker	f^+	f
I	paba$^+$	3	10
	paba	3	7
II	Acr$^+$	4	8
	Acr	2	9
III	phe$^+$	6	17
	phe	0	0
IV	pyro$^+$	3	5
	pyro	3	12
V	lys$^+$	6	15
	lys	0	2
VI	s$^+$	2	11
	s	4	6
VII	nic$^+$	2	11
	nic	4	6
VIII	ribo$^+$	0	17
	ribo	6	0

i.e. f3 is not located in linkage group III.

The diploid w3; pyro4; f3 / y; nic2 ribo5 (nic2 and ribo5 are in the fifth linkage group) was then synthesized and fifty-four haploids were analysed. They segregated as follows:

	f	nic	ribo		
parental	+	-	-	14	54
	-	+	+	40	
recombinant	+	+	+	0	0
	-	-	-	0	

which indicates location of f3 in the fifth linkage group. Also all the haploids isolated from diploids 6, 7 and 8 were f^- and pho^+ and lys^+, which indicates location in either the third or the fifth linkage group.

Haploidization of diploids 2 (Table 29) and 3 (Table 30) located both f101 and f102 in linkage group VIII and haploidization of diploids 4, 5, 9, 10 and 11 confirmed this location for the other mutants known to map at these loci (Table 26).

All this evidence leads to the conclusion that locus f3 is located in the third linkage group, while loci f101 and f102 are unlinked (Table 26) and located in the eighth linkage group.

By haploidization one can detect translocations by finding complete linkage between two markers which are normally located in two different linkage groups. During the course of this work translocations were found in the strains paba1, between linkage groups VI & VII, bi1; w2, between linkage groups III & VIII, and bi1; w3, between linkage groups III & VIII and VI & VII. Therefore for further isolation of mutants the strain bi1 (which was found to be without translocations) was used.

ii) Meiotic analysis

f3

By analysing 300 progeny of the cross y; nic2; ribo5 x w3; pyro4; f3 (Table 31), f3 was located between nic2 and ribo5 very loosely linked to both of them.

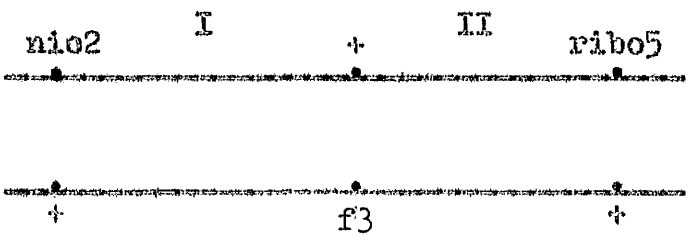
f101

Crosses between strains carrying the mutant f101 and strains carrying other markers of the eighth linkage group were analysed. Linkage was detected only between f101, ribo2 and arg3 (Table 32), showing that

Table 31. Location of f3 by meiotic analysis

Cross - y; nic2 ribo5 x w3; pyro4; f3

The data are tabulated only in respect of the markers nic2, ribo5, f3 as in a three point cross.



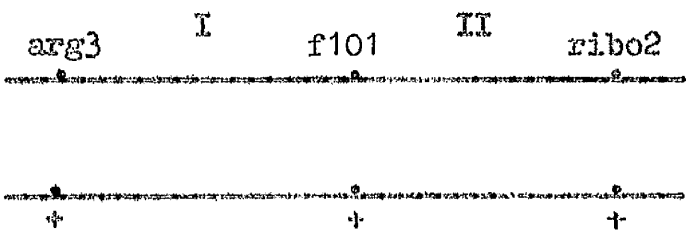
Crossover regions	Genotypes				
none	nic	+	ribo	54)	114
	+	f	+	60)	
I	nic	+	+	41)	81
	+	+	ribo	40)	
II	nic	+	+	39)	67
	+	f	ribo	28)	
I and II	nic	f	ribo	19)	38
	+	+	+	19)	

Linkage map. nic2 40 ± 5.6 f3 35 ± 5.4 ribo5

Table 32. Location of f101 by meiotic analysis

Cross - bi1; orn9 cha x bi1; arg3 f101 ribo2

Data are tabulated only in respect of the markers arg3, f101, ribo2 as in a three point cross.



Crossover regions	Genotypes					
none	arg	f	ribo	71	158	
	+	+	+	87		
I	arg	+	+	12	35	
	+	+	ribo	23		
II	arg	f	+	6	8	
	+	f	ribo	2		
I and II	arg	+	ribo	0	1	
	+	+	+	1		

Linkage map. arg3 — 17.8 ± 5.3 — f101 — 4.45 ± 2.9 — ribo2

f101 is closely linked to ribo2 and suggesting that it lies between arg3 and ribo2. To confirm this order selective platings were made by sandwiching between S.M. and S.F.A.M. ascospores from hybrid peritheeia of the following crosses:

1) bi1; orn9 cha x bi1; arg3 f101 ribo2
2) paba1 x bi1; arg3 f101 ribo2
3) bi1; arg3 x bi1; f101 ribo2.

In each case selection was made for the ribo^{+} f recombinants which were analysed for the segregation of arg3 (Table 33). The data confirmed the order (arg3 f101 ribo2).

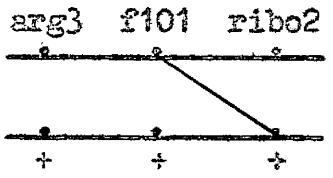
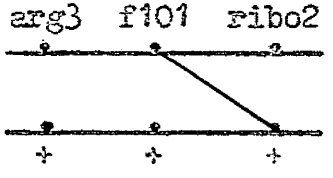
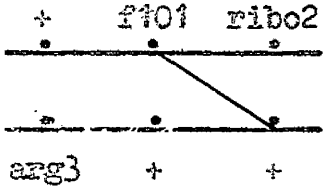
f102

The allele f307 represents locus f102 as this allele is more easily distinguished on the various media from its wild type allele than f102. Three crosses involving markers of the eighth linkage group were made, but no linkage between f307 and any other marker was detected, about 200 progeny from each cross being analysed. The crosses were as follows:

1) y; ad1; s12; f307 x bi1; arg3 ribo2
2) y; ad1; s12; f307 x bi1; w3; crn7 ribo2 gal7
3) y; ad1; s12; f307 x bi1; ad23 cha pal B7.

Table 33. Ordering the mutants arg3 f101 ribo2

By selecting ribo$^+$ f recombinants from three crosses; in each of which these three mutants were involved. Selection - by sandwiching ascospores between S.M. and S.F.A.M.; only ascospores from hybrid perithecia were plated.

Cross	Selection	No. of ribo$^+$ f recombinants analysed	No. of ribo$^+$ f recombinants carrying the mutant arg3	Order of markers	Distance arg3-f101*
bi1;orn9eha x bi1;arg3f101ribo2		156	103	arg3 f101ribo2	27.6±7.2
paba1 x bi1;arg3f101ribo2		103	69	arg3 f101 ribo2	33.0±9.8
bi1;arg3 x bi1; f101 ribo2		103	21	arg3 f101 ribo2	20.4±7.9

* The differences in the distance arg3 - f101 are due to the poor viability of the strains carrying mutant arg3.

2) 'Back'-mutants

'Back'-mutants were subjected to further analysis with a view to deciding whether they were due to extra-cistron or intra-cistron suppressors. Hence crosses between the revertant strains and strains carrying the wild type alleles were made. The analysis of those crosses (Table 34) showed that those suppressors of alleles f3, f8 and f102 which were tested were extra-cistronic and unlinked to the allele they suppressed, while the suppressors of the allele f101 might be intra-cistronic. These results are in accord with a hypothesis put forward in Part IV, D, as all the revertants analysed except for revertants of the allele f101 are 'small' on A.M.

A thick suspension of ascospores from the cross w3; pyro4; f101 b2-f101 x paba1 y; ad23 was plated on F.A.M. plus para-aminobenzoic acid. From an estimated 0.8 x 10^6 viable recombinant ascospores plated, two f colonies were recovered. One of them recombined freely with f101, which indicated a mutational origin; the other did not recombine with f101 (no recombinant was found among 2,500 viable progeny of a cross of this f mutant to f101), which indicates that this f mutant was either f101 itself, or the suppressor, b2-f101 on its own having a mutant phenotype (Crick et al., 1961). This example demonstrates the adequacy of the system for mapping of extremely closely linked suppressors. Dominance of two suppressor mutants of f101 which

Table 34. Crosses between f revertant strains and wild type strains

Crosses analysed by 'selective plating', selection for *pyro*$^+$ *ad*$^+$ recombinants.

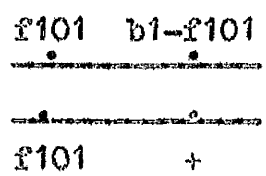
Cross	Type of cross	No. of progeny with the phenotype f^+	No. of progeny with the phenotype f	Total	Linkage between suppressor and suppresse*	Test for 3:1 ratio χ^2_1	Test for 3:1 ratio P
w3; pyro4; f3; (b1-f3) x paba1 y; ad23	f3; b1-f3 x + +	54	14	68	41	0.70	0.41
bi1; w2; f8; (b1-f8)** x "	f8; b1-f8 x + +	33	18	51	>50	2.88	0.09
w3; pyro4; f101 b1-f101 x "	f101b1-f101 x + +	104	0	104	<2	-	
w3; pyro4; f101 b2-f101 x "	f101b2-f101 x + +	108	0	108	<2	-	
w3; pyro4; f101 b3-f101 x "	f101b3-f101 x + +	106	0	106	<2	-	
w3; pyro4; f102; (b1-f102) x "	f102b1-f102 x + +	36	10	46	44	0.26	0.61
w3; pyro4; f102; (b2-f102) x "	f102b2-f102 x + +	58	26	84	>50	1.59	0.20

* These calculations are based on the assumption that the progeny having the *f* phenotype represent half of the recombinants.

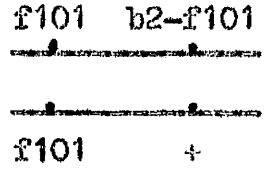
** In this cross selection was made for *bi*$^+$ *ad*$^+$ recombinants.

is expected from intra-cistronic suppressors, was demonstrated by synthesizing diploids homozygous for f101 and heterozygous for the suppressors. The diploids were as follows:

1) w3; pyro4; f101 b1-f101 / y; s1; f101



2) w3; pyro4; f101 b2-f101 / y; s1; f101

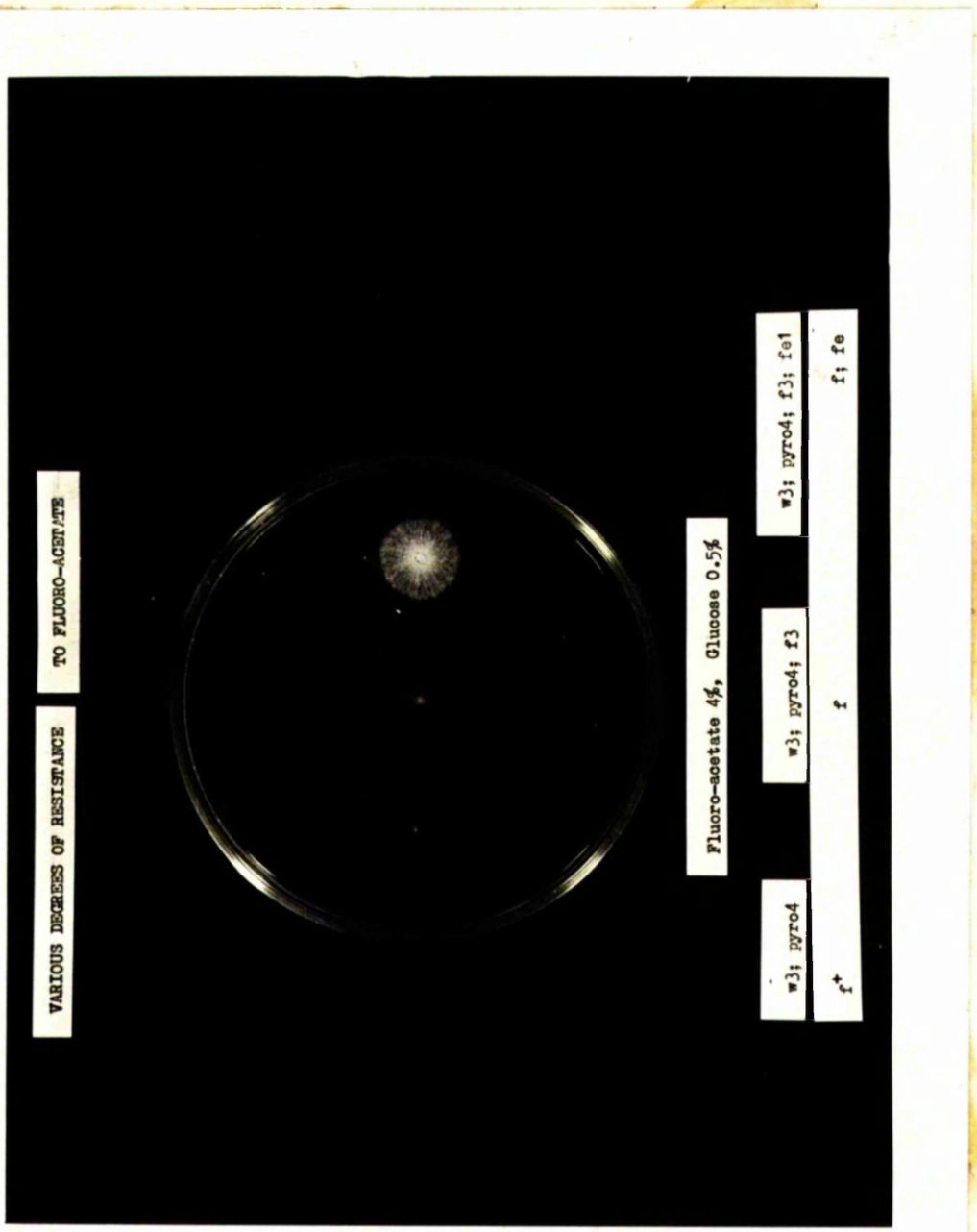


Both diploids were found to have an f^+ phenotype on A.M. and F.A.M.

3) Enhancers

Often, when f strains were inoculated into F.A.M., super-resistant sectors arose. Therefore, the strain paba1; w3; f101 ribo2 was inoculated into a petri dish containing F.A.M. plus the necessary growth factors. Super-resistant sectors arose and were isolated. The new strains were designated fe1, fe2 etc. (fe = fluoroacetate resistance enhancer, Fig. 6). The strains paba1; w3; f101 ribo2 fe1, paba1; w3; f101 ribo2 fe2, paba1; w3; f101 ribo2 fe3 have the f phenotype on acetate and succinate. By synthesizing the diploid

Figure 6


VARIOUS DEGREES OF RESISTANCE
TO FLUORO-ACETATE
Fluoro-acetate 4%, Glucose 0.5%
w3; pyro4
w3; pyro4; f3
w3; pyro4; f3; fe1
f+
f
f; fe

y; s1; f101 / paba1; w3; fe1; f101 ribo2

f101	+
f101	fe1

fe1 was found to be recessive as this diploid has the phenotype of an f101 strain on F.A.M. As expected, this diploid strain failed to grow on A.M. while the diploid

bi1; arg3 / paba1; w3; fe1; f101 ribo2

f101	fe1
+	+

grew on A.M. and was sensitive to F.A.

The cross paba1; w3; fe1; f101 ribo2; x y; ad1; s12 (fe1; f101 x +;+) was analysed. Three types of progeny were distinguished:

1) progeny which do not grow on A.M. and are super-resistant to F.A. (assumed genotype fe1; f101),
2) progeny which grow on A.M. and are sensitive to F.A. (assumed genotypes +; +; and fe1; +),
3) progeny which do not grow on A.M. and are resistant to F.A. (assumed genotype f101; +).

The number of progeny in class 2 was roughly equal to the number of progeny in classes 1 and 3. These results suggest that the enhancer

mutation (fe) is separable from the f101 locus, and that the enhancer mutant by itself (fe) is indistinguishable (by the above criteria) from an fe^+ allele. To confirm this, several recombinants from this cross (class 2) were isolated. One of them y; ad1; s12, assumed to carry in addition the mutant fe1, was crossed with three strains representing the three f loci. Analysis of these crosses (Table 35) confirmed that the genotype of this strain is y; ad1; s12; fe1 and that by itself fe1 is phenotypically indistinguishable from the wild type allele fe^+ (on the tested criteria), that fe1 is unlinked to any of the known f loci, and that fe1 is probably a general enhancer for any f mutant irrespective of its location. This lends further support to the idea that all the three f loci are engaged in the same primary function (Part IV, E2e).

Table 35. Crosses between y; ad1; s12; fe1 and three strains each carrying an f mutant representing one of the three f loci

Crosses analysed by 'perithecium analysis', with selection for ad^+ segregants.

Cross	Type of cross	No. of progeny super-resistant to F.A.	Total No. of progeny analysed	Test for 1:3 ratio χ^2_1	P
w3; pyro4; f3 x y; ad1; s12; fe1	f3 x fe1	23	90	0.015	0.90
paba1; w3; f101 ribo2 x "	f101 x fe1	29	102	0.94	0.34
bi1; f307 x "	f307 x fe1	32	104	1.84	0.17

Segregation for growth and non-growth on A.M. among the progeny of all these crosses did not deviate significantly from 1:1 ratio.

Summary

1) The mutants isolated have been shown to behave like a single Mendelian factor.

2) They have been shown to be recessive.

3) By genetical (crosses) and functional (complementation) tests, all *f* mutants examined fall into three loci which correspond to three cistrons.

4) One of the loci is located in linkage group III; the other two are located in linkage group VIII, and are unlinked.

5) Some revertants of various *f* 'forward'-mutants were analysed and have been found to result from unlinked suppressors.

6) One of the *f* 'forward'-mutants *f101*, reverts by extremely closely linked or intra cistronic suppressors.

7) An enhancer mutation to fluoroacetate resistance has been shown to be indistinguishable by itself from the corresponding wild type allele, to be unlinked to any of the three *f* loci and to enhance any of three *f* mutants, each representing one of the three *f* loci.

(G) Complementation in heterokaryons and diploids

Recessive mutants from different loci usually complement when tested either in the heterokaryon or in the heterozygous diploid. So far, differences in complementation between diploids and heterokaryons, each having the same genotype, have been found in a few cases (Pontecorvo, 1952; Roberts, 1961; for more details see Pontecorvo, 1963). The most striking of these is probably the case of mutants in three methionine suppressor loci in Coprinus lagopus (Lewis, 1961, and unpublished results) where most of the combinations between mutants from different loci do not complement in the heterokaryon in the trans configuration.

The f mutants of Aspergillus nidulans represent a somewhat similar situation with the difference that so far none of the combinations tested (between mutants from different loci) do complement in the heterokaryon, which renders the possibility of exceptions unlikely. Moreover, while complementation between suppressors is expressed as failure to grow, complementation between auxotrophic mutants is expressed by growth. Complementation between f mutants was verified by testing growth of the relevant heterokaryon and diploid on A.M.

Three of the f mutants (f3, f101 and f102) were tested for

recessivity in the heterokaryon and in the heterozygous diploid and found to be recessive; i.e. they grew on acetate medium. The following combinations were tried:

1) w3; pyro4; f3 / paba1 y (f3 / f$^+$)

2) w3; pyro4; f101 / paba1 y (f101 / f$^+$)

3) w3; pyro4; f102 / paba1 y (f102 / f$^+$)

(see also Table 27).

To test for complementation in heterokaryons, conidia of both strains were mixed in liquid A.M. and after 4-5 days incubation the mycelium was transferred to A.M.

These three mutants, representing three unlinked loci (f3, f101, f102), were tested in all possible combinations in heterokaryons and in heterozygous diploids. All of the heterokaryons failed to grow on A.M. while the diploids did grow (Tables 27 and 36, Fig. 7). The pattern of complementation did not change when the complementing nutritional markers of the combination f3 / f101 were changed (Table 36). Furthermore, as the f mutants grew slightly on A.M. it was possible to transfer hyphal tips from this slight growth to M.M. where, after a day or two, a vigourously growing heterokaryon was obtained. On two occasions diploid sectors arose from the slight growth on A.M. of heterokaryons between two unlinked f mutants (Fig. 8). This indicates that although the heterokaryon is formed it cannot grow i.e. a genuine failure of growth and not of forming a heterokaryon.

Table 36. Tests for complementation of unlinked f mutants in heterokaryons and in heterozygous diploids

Combination	Type of combination	Heterokaryons			Heterozygous diploids	
		A.M.	A.M. & requirements	M.M.	A.M.	M.M.
paba1 y; f3 / w3; pyro4; f101	f3 / f101	-	-	+	+	+
ad23; f3 / w3; pyro4; f102	f3 / f102	-	-	+	+	+
y; s1; f101 / w3; pyro4; f102	f101/ f102	-	-	+	+	+
ad23; f3 / y; s1; f101	f3 / f101	-	-	+	+	+

+ indicates wild type growth

- indicates mutant growth

Figure 7

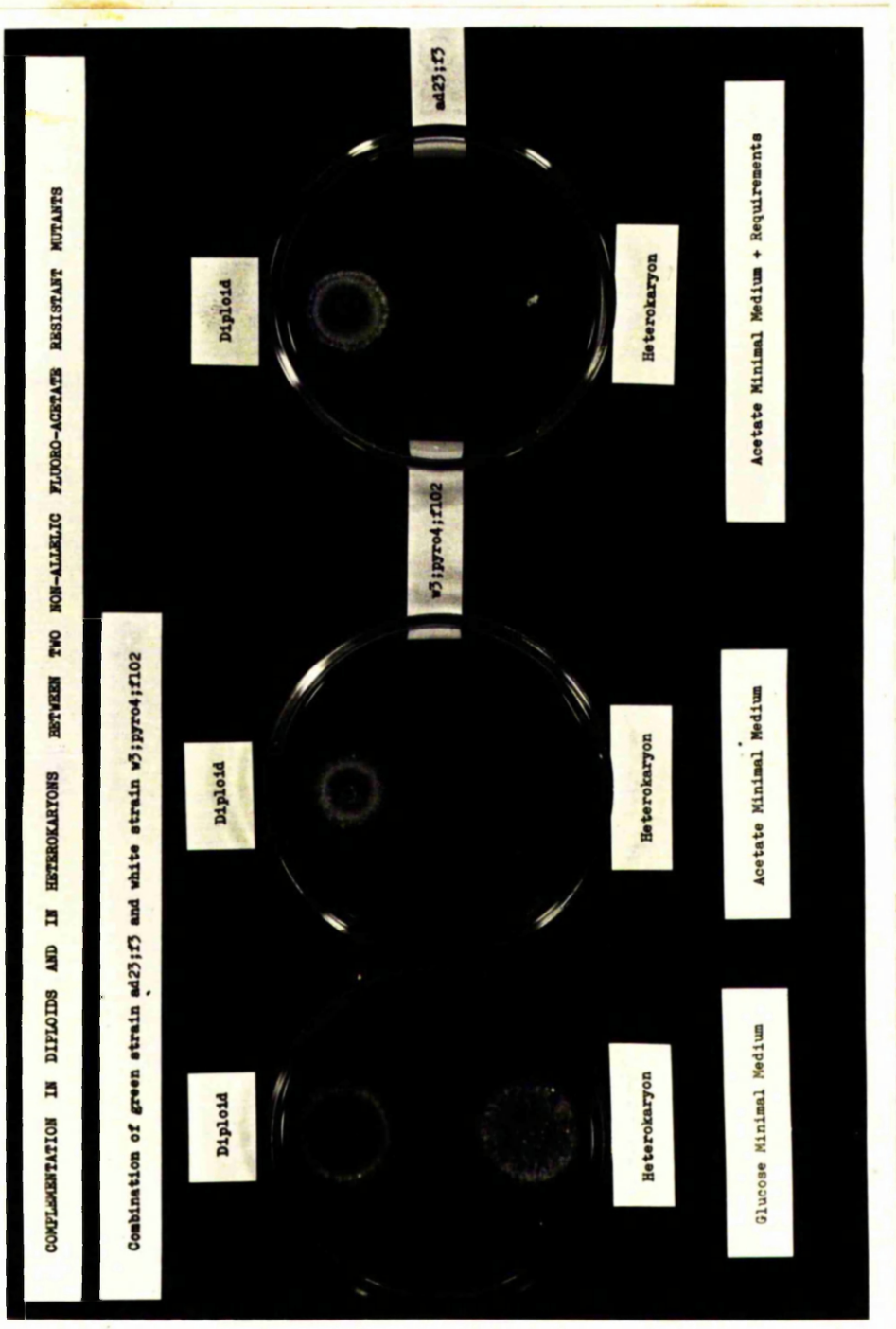

COMPLEMENTATION IN DIPLOIDS AND IN HETEROKARYONS BETWEEN TWO NON-ALLELIC FLUORO-ACETATE RESISTANT MUTANTS
Combination of green strain a23;f3 and white strain w3;pyro4;f102
Diploid
Diploid
Diploid
w3;pyro4;f102
a23;f3
Heterokaryon
Heterokaryon
Heterokaryon
Glucose Minimal Medium
Acetate Minimal Medium
Acetate Minimal Medium + Requirements

Figure 8

A diploid growing out of a heterokaryon

Note: the diploid sector is on the right-hand side of the plate.
The heterokaryon is between the strains pabaA y; f3 and w3; pyro4; f101 (f3 and f101 are unlinked).
The plate contains acetate medium.

The other combinations between alleles from different loci which were compared in the heterokaryon and in the heterozygous diploid (in trans) were the following:

f2 / f101, f102 f3 / f8, f301 f8 / f101 f102 / f303.

All these combinations fell into the same pattern i.e. heterokaryons did not grow on acetate while heterozygous diploids did. Also, 45 heterokaryons were synthesized between fifteen f mutants and each of the mutants f3, f101 and f102. All these 45 heterokaryons failed to grow on A.M. although at least 30 of these combinations were between non-allelic mutants. The following strains were used:

w3; pyro4; f3, w3; pyro4; f101, w3; pyro4; f102

and strains carrying the following f mutants:

f1, f6, f9, f10, f13, f18, f20, f21, f22, f24, f26, f28, f30, f32, f34.

All these strains, in addition to the f mutant carried the mutant bi1.

To explain this phenomenon one can postulate several models:

1) The protein involved in growth on acetate does not migrate outside the nucleus, and therefore the mutants fail to complement in the heterokaryon (Pontecorvo, 1963).

2) Assuming one protein made up of two or three different polypeptide chains, local concentrations of them in the heterokaryon are not sufficient to allow assembly of the polypeptide chains (Pontecorvo, 1963). Assembly of polypeptide chains is known to occur for haemoglobin (Itano and Singer, 1958).

3) The information of each nucleus for synthesis of the enzyme or enzymes concerned moves en masse to particles of the cytoplasm where these enzymes are normally situated, in this case probably mitochondria; or, if the information does not move en masse each of the particles receives information from one particular nucleus only. This model assumes further that there are no interactions among the particles, and predicts the occurrence of one kind of particle in the cytoplasm of the diploids, and of two kinds of particles in the heterokaryotic cytoplasm. A model of this kind can be postulated for every case in which the proteins are not freely soluble in the cytoplasm, but are localized.

4) Assuming that of the three genes involved, one is structural and two are regulatory, and that the product of the regulatory gene is restricted to the nucleus, either because there is no possibility of migrating out, or because it exists in very few copies; one would expect in all cases in which one regulatory gene and one structural gene, or two regulatory genes, are involved, to find differences in the complementation pattern between the heterokaryon and the heterozygous diploid.

It would seem that in dealing with regulatory genes comparisons between heterokaryons and heterozygous diploids might decide whether or not the products of regulatory genes (repressors) are effectively restricted to the nucleus i.e. whether or not they are cytoplasmic

(Jacob and Monod, 1963). For an elaborate model of interactions between structural and regulatory genes which accounts for the differences in the complementation pattern of the same combination in the heterokaryon and the heterozygous diploid see Pontecorvo (1963).

At least one point may be drawn from this: if in Aspergillus nidulans the diploid condition did not exist and complementation was tested only in the heterokaryon (as in Neurospora crassa), mutants which are in different linkage groups may have been taken to be allelic, i.e. ectopic allelism (Pontecorvo, 1958).

Summary

1) All the combinations, in trans configuration, of two f mutants (pairs consisting of mutants from different cistrons) do complement in the heterozygous diploids but not in the corresponding heterokaryon.

2) Certain possibilities which could explain this phenomenon have been suggested.

(H) Discussion

Systems of the type described in this work are primarily suited to use in micro-organisms but could be applied to cells in tissue cultures and might be adapted for use in other organisms such as Drosophila, as selection for resistance is relatively simple. A 'two-way selection' would also facilitate procurement of auxotrophic mutants which in certain organisms are difficult to obtain (Chlamydomonas reinhardi, Sager, personal communication).

The assumption that mutants of the same cistron always have a similar phenotype presumably arises in most cases from the availability of only one phenotypic characteristic by which mutant strains may be distinguished from corresponding wild type strains. In the f system, however, mutants are distinguished from corresponding wild types on at least three criteria, and mutants can be obtained which do not exhibit all of these characteristics, for example, mutants which are resistant to F.A. but can utilize acetate. Such mutants were isolated and designated fa but were not further analysed.

A mechanism which could explain occurrence of fa mutants in one of the f cistrons is the following: consider that the protein coded (or regulated) by the f cistron(s) does not distinguish between the

metabolite (acetate or a derivative of it) and its analogue (fluoroacetate or a derivative of it), but distinguishes between them after a change arising from a mutation. The protein will then reject the analogue thereby causing resistance, but still deal with the metabolite, thereby causing prototrophy. Thus, mutants resistant to fluoroacetate which still utilize acetate might map in an f cistron, occupying only a minor fraction of its mutable sites, and mapping at particular places in it, as presumably only very definite and restricted changes in the protein would enable it to distinguish between a metabolite and its analogue.

It is not known if the various techniques used for selection of 'forward'-mutants select mutants mainly of a particular locus, as only mutants selected by the S.M. + S.F.A.M. technique were mapped in numbers sufficient for consideration (these are the eight mutants, f301 - f303, f305 - f309). These mutants do not represent a random sample, having been selected by reason of their showing slight variations in growth on succinate medium, and in residual growth on acetate medium; they cannot, therefore, provide any satisfactory answer. If, however, the difference in growth of f mutants on succinate medium (Part IV, E2ci) is accepted as a basis for distinguishing mutants of the f3 locus from mutants of the f101 and f102 loci, it is possible to ascertain which technique will select preferentially mutants of

the f3 locus. This being so would emphasize the suitability of the f system for the purpose of 'two-way selection' within one locus.

With the above considerations in mind, the problem of which mutants revert and which do not (Table 21) may also be re-examined. The vast majority of mutants which do not revert are of the type which grows more rapidly on succinate medium. If it is accepted that these mutants map at locus f3, then unless they are gross aberrations in the genetic material this phenomenon is difficult to understand.

Relevant to the effect of selection conditions on mutants selected is the frequency of occurrence of enhancer mutants when f colonies are grown on F.A.M. containing 4% fluoroacetate and 0.5% glucose, as compared to the rarity of their occurrence when the medium contains only 2% fluoroacetate and 0.5% glucose. This is probably due to the increase in size of f colonies grown on medium containing the latter concentrations in comparison to colonies grown on medium containing the first concentrations.

It was suggested previously (Part IV, E2a) that the slight basic growth of f mutants on A.M. might be due to an alternative and inefficient pathway for acetate utilization which is not blocked by the f mutants. It was also considered that the 'proline effect' might be due to activation of this pathway (Part IV, E2b). Mutations

blocking this pathway may in fact be the enhancer mutants (fe, Part IV, F3), which by themselves cannot be distinguished from the corresponding wild types. This is supported by the fact that certain strains carrying an f mutant together with an fe mutant are completely inert on acetate medium and do not demonstrate the 'proline effect'. Alternatively, fe mutants may result from a block in a permeability system, but this explanation is weakened by the fact that strains carrying an fe mutant (but not an f mutant) grow on acetate medium as well as the corresponding wild type strains.

V GENERAL SUMMARY

I The importance of selective techniques in genetical studies has been discussed and two types of selective systems have been emphasized - 'selective tetrad analysis' and 'two-way selection' systems.

II Materials and methods used in this work were described, those which are in common use in the study of Aspergillus nidulans and those which were particularly designed in the course of this work.

III In order to obtain a system for 'selective tetrad analysis', blue and colourless ascospore mutants have been isolated. Four blue mutants have been located in one locus (Linkage group II), and, of five colourless mutants, four have been located in one locus (Linkage group I), and one in Linkage group IV. These mutants were found to be 'non-autonomous, but analysis of crosses between them led to the conclusion that in Aspergillus nidulans the phenotype of the perithecium and its ascospores - in respect of the characters examined - is determined by the nuclear constitution of the protoperithecium which gave origin to it.

IV

A) The intended work with acetate non-utilizing mutants and a brief account of findings concerning them is given.

B) The principles for establishing 'two-way selection' systems based on correlating resistance and auxotrophy are outlined, and some results - confirming these principles - obtained by using a metabolite (acetate) and its analogue (fluoroacetate) - are represented. Mutants obtained (resistant to fluoroacetate and unable to utilize acetate) have been designated f.

C) Various techniques for the selection of mutants resistant to fluoroacetate have been used, and the most suitable for a 'two-way selection' system has been shown to be the sandwiching of spores between succinate medium and succinate fluoroacetate medium.

D) Revertants able to utilize acetate have been selected from f 'forward'-mutants. Most of the f mutants do not revert and this failure is thought to be genuine. There is an inverse correlation between the frequency of reversion of f mutants and the size of the revertant colonies on acetate medium.

E) It has been shown that fluoroacetate inhibits competitively growth of Aspergillus nidulans on various carbon sources.

A residual growth of f mutants on acetate medium has been observed and attributed to a second pathway for acetate utilization

unaffected by an f mutation. Proline has been shown to have a 'sparing effect' in f mutant strains grown on acetate medium, and this has been attributed to the activation of the second pathway.

f mutant strains have been shown to grow more rapidly on intermediates of the Krebs' cycle (succinate, fumerate and malate) and to be more sensitive to malonate than corresponding wild type strains.

It was concluded that all f mutants are impaired in the same primary function, (in the sense that only one protein is involved).

F) f mutants have been shown to be recessive, to map in three meiotically unlinked loci corresponding to three cistrons, and the loci to map in two linkage groups, (V and VIII).

Revertants, prototrophic on A.M., have been shown to result from extra-cistronic suppressors. One mutant, f101, reverts by extremely closely linked or intra-cistronic suppressors.

An enhancer mutation to fluoroacetate resistance has been shown to be recessive, to be indistinguishable by itself from the corresponding wild type allele, to be unlinked to any of the three f loci, and to enhance f mutants of all three loci.

G) It has been shown that two f mutants, in two different loci, in the trans configuration do not complement in heterokaryons but do complement in the corresponding heterozygous diploids when tested on acetate medium.

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