GENETICS OF AROMATIC AMINO ACID BIOSYNTHESIS

IN

ASPERGILLUS NIDULANS.

by

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A THESIS SUBMITTED TO THE UNIVERSITY OF GLASGOW

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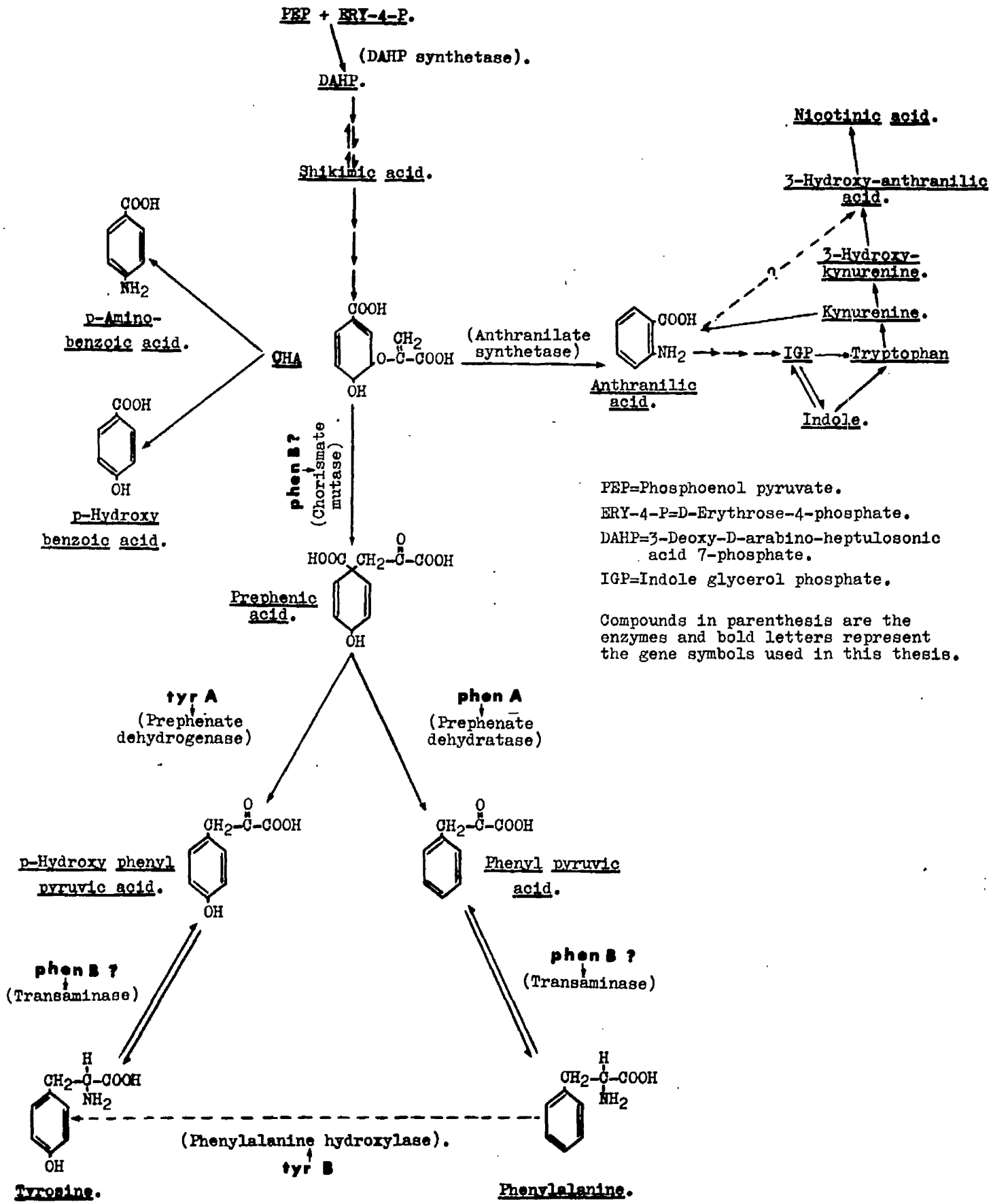
CONTENTS.

ABBREVIATIONS USED

C.M.	Complete medium.
FPA	DL-p-Fluorophenylalanine.
LEU	L-leucine.
METH	DL-methionine.
M.M.	Minimal medium.
NTG	N'-Methyl-N-nitro-N-nitrosoguanidine.
PABA	p-aminobenzoic acid.
PHE	L-b-Phenyl-a-alanine.
TRY	DL-tryptophan.
TYR	DL-tyrosine.
VAL	L-valine.



PEP=Phosphoenol pyruvate.

ERY-4-P=D-Erythrose-4-phosphate.

DAHP=3-Deoxy-D-arabino-heptulosonic acid 7-phosphate.

IGP=Indole glycerol phosphate.

Compounds in parenthesis are the enzymes and bold letters represent the gene symbols used in this thesis.

BIOSYNTHETIC PATHWAY OF AROMATIC AMINO ACIDS.

Fig.-1.

I GENERAL INTRODUCTION

The work described in this thesis is mainly an investigation of the genetics of aromatic amino acid biosynthesis in Aspergillus nidulans. The genetic control of aromatic amino acid biosynthesis is of interest because as compared to bacteria, yeast and Neurospora, very little is known about it in Aspergillus nidulans and there are no pointers towards the possible significance of metabolic blocks in different (phen 3 and its alleles and phen 6) phenylalanine (PHE) - requiring mutants recovered so far in this mould. Besides, what is already known, indicates towards a possible difference in the genetic control of aromatic amino acid biosynthesis between other micro-organisms on the one hand and A. nidulans on the other.

The scheme of aromatic amino acid biosynthesis (Fig. 1) is a summary of information from the study of mutants and from enzymatic and isotopic labelling techniques in Escherichia coli (Davis, 1951, 1952, 1955; Davis and Mingioli, 1953; Weiss and Mingioli, 1956; Rudman and Meister, 1953; Miller et al., 1957; Schwinck and Adams, 1959 and Morgan et al., 1963), Aerobacter aerogenes (Davis, 1951 and Morgan

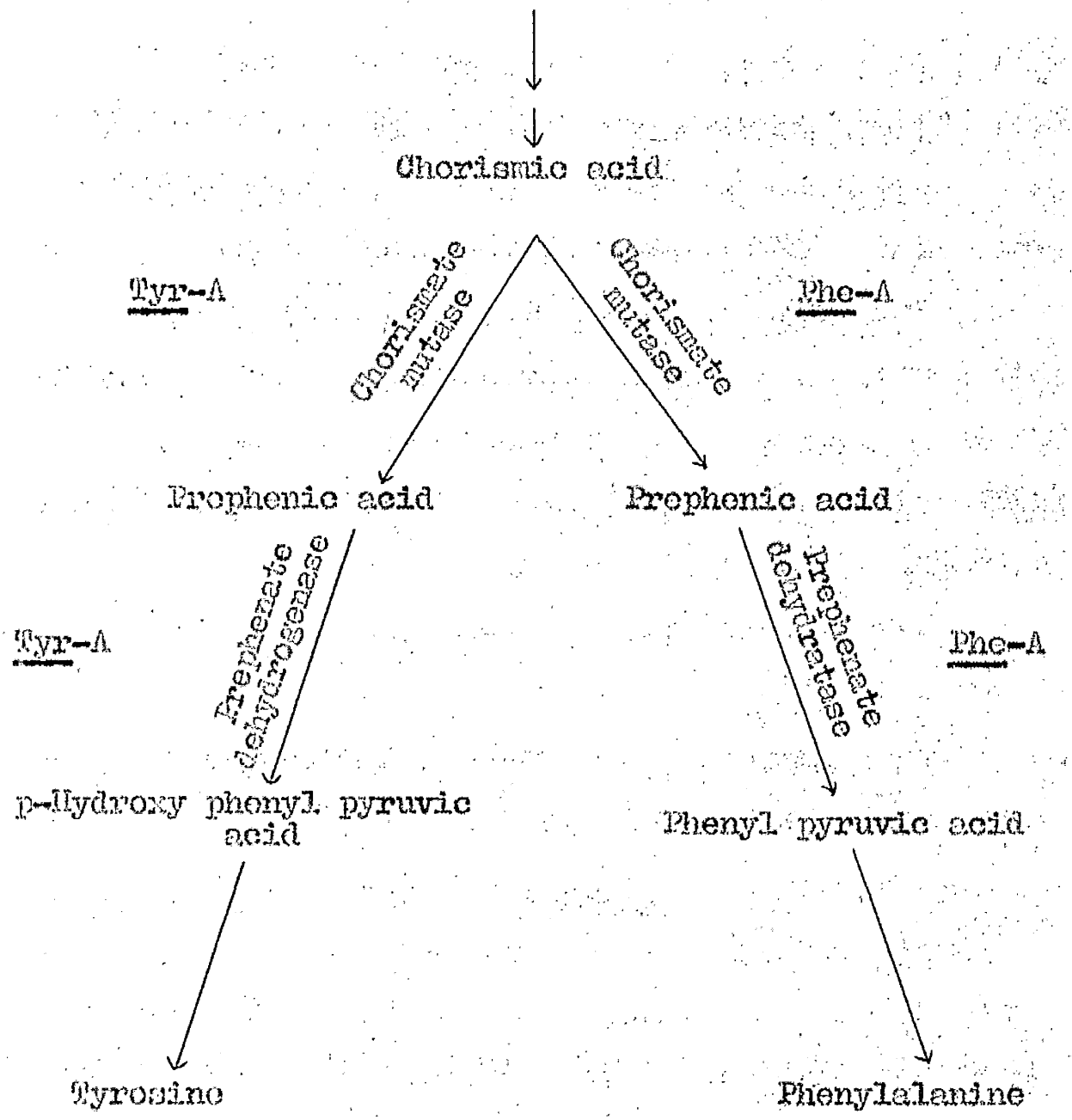
et al., 1963), Neurospora crassa (Davis, 1951; Barratt et al., 1956; Gross, 1958; Gross and Fein, 1960; Bonner et al., 1960 and 1965), Aspergillus nidulans (Pontecorvo, 1952a; Pontecorvo et al., 1953; Roberts, 1967 and Hutter and DeMoss, 1967), rat, man (Garrod, 1909, 1923; Mitchell in Beadle, 1945; Woolf, 1963) and a variety of other micro-organisms, higher plants and animals. In Aspergillus nidulans only tryptophan (Roberts, 1967 and Hutter and DeMoss, 1967), nicotinic acid (Pontecorvo, 1952a and Pontecorvo et al., 1953) and p-aminobenzoic acid (Pontecorvo et al., 1953) - requiring mutants have been studied extensively both genetically and physiologically.

There is no report of any study concerned with PHE and TYR biosynthesis in A. nidulans except of the existence of two loci (phen 3 and its alleles and phen 6), mutants at which require PHE for their optimum growth (Kafer, 1958 and McCully, 1964). During routine procedures for the isolation of auxotrophic mutants in the Department of Genetics, Glasgow, no TYR-requiring mutants have been recovered in about 20 years of work. Whereas, in E. coli, N. crassa, A. aerogenes and many other micro-organisms, tyrosine-requiring mutants blocked between prephenic acid and p-hydroxyphenylpyruvic acid have been frequently isolated in various laboratories (Meister, 1965).

In all organisms studied so far (Meister, 1965 and Broquist and Trupin, 1966) the pathway for PHE, TYR, TRY, nicotinic acid, p-aminobenzoic acid and p-hydroxybenzoic acid biosynthesis is common up to chorismic acid, from where it branches off in different directions. The aromatic amino acids PHE and TYR are synthesised from chorismic acid through prophenic and phenylpyruvic acids and prephenic and p-hydroxyphenylpyruvic acids respectively.

The intermediate - prophenic acid - is extremely acid labile and decays spontaneously to phenylpyruvic acid (Gilvarg, 1955). Therefore, E. coli mutants, unable to catalyse the reaction which converts prophenic acid to phenylpyruvic acid can, nevertheless, show delayed growth without phenylalanine. If the culture medium is kept alkaline they have an absolute requirement for phenylalanine (Hayes, 1963).

In A. aerogenes and E. coli W there are two chorismate mutase isoenzymes that are separable as two distinct peaks when cell free extracts are chromatographed on a DEAE cellulose column. Prophenate dehydrogenase activity is associated with one of these peaks and prephenate dehydratase activity with the other (Cotton and Gibson, 1965). PHE-requiring and TYR-requiring mutants of E. coli K-12 are

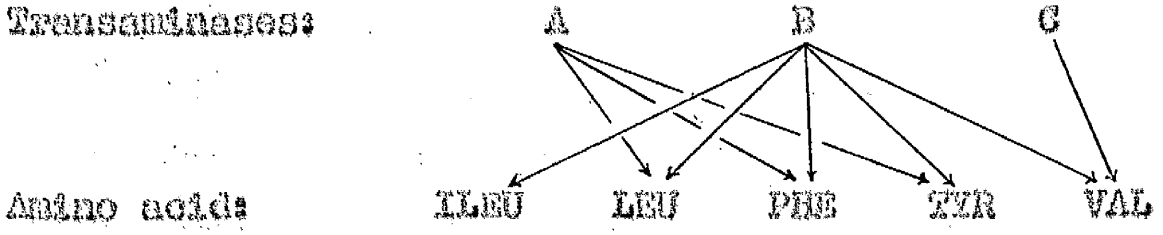


Possible metabolic blocks in Tyr-A and Phe-A mutants of E.coli K-12.(After Pittard and Wallace,1966).

Fig.-2.

deficient in prephenate dehydratase and prephenate dehydrogenase activities respectively and these mutants simultaneously lose their respective chorismate mutase activities (Pittard and Wallace, 1966) (Fig. 2). In other words, two steps are catalysed by a single enzyme or a single mutation results into a deficiency of two enzyme-activities. But in N. crassa, mutants requiring PHE + TYR are known, which are presumably blocked between chorismic acid and prephenic acid (Davis, 1955).

Biochemical evidence indicates that transamination is the last step involved in the biosynthesis of amino acids by micro-organisms. The broad specificity of transaminases is the reason why little genetic work has been done on these terminal steps. In E. coli W (a strain not easily amenable to genetic analysis) there appear to be at least three transaminases (A, B and C) involved in such reactions in respect of five amino acids (Rudman and Meister, 1953).



Transaminases A and B aminate the keto-analogues of TYR, PHE and LEU; although A is more active in TYR and PHE synthesis

than in LEU synthesis. Both B and C are active towards VAL whereas ILEU is formed only by the enzyme B. There seem to be at least two transaminases for each conversion, with the exception of that leading to ILEU. With the usual techniques of selection, therefore, it is unlikely to select mutants that are specifically blocked at a transamination step.

One of the reasons for the failure to recover TYR-requiring mutants during routine searches for auxotrophs, could be that there are two pathways for TYR synthesis in A. nidulans.

Another pathway for TYR synthesis (different from that present in micro-organisms and plants and described above) is known in man (Garrod, 1909, 1923; Beadle, 1945; Harris, 1959) and many animals including rat, sheep, dog and some invertebrates (Kaufman, 1957, 1963; Zannoni et al., 1966; Woolf, 1963). In contrast to micro-organisms and plants which are able to synthesise aromatic compounds, all animals, studied so far, require dietary PHE and TRY and TYR is produced by the hydroxylation of PHE. This hydroxylation is a quantitatively important one for animals and was first postulated as early as 1909 by Neubauer (Meister, 1965). More recently, the enzyme PHE-hydroxylase

has been purified from rat and sheep liver and shown to act only on PHE and not on p-fluorophenylalanine (FPA) or any other amino acid (Kaufman, 1963).

Reports about the occurrence of PHE-hydroxylase in micro-organisms are meagre and conflicting. Earlier reports about the possible conversion of PHE to TYR in E. coli (Doudoroff and Shive, 1946, 1947, and Borgman et al., 1953) have been contradicted (Simmonds, 1954; Davis, 1955 and Miller and Simmonds, 1957). In Bacillus subtilis Nester et al. (1963) could not detect any conversion of PHE to TYR. Mitoma and Cooper (1954) too could not detect any PHE-hydroxylase activity in either E. coli or B. subtilis. In yeast, E. coli and Streptococcus faecalis Udenfriend and Cooper (1952) tried in vain to detect an enzyme with such activity.

Substantial amount of PHE-hydroxylase activity has been detected in some strains of Pseudomonas but only when the organism is grown in the presence of PHE or TYR, indicating thereby that it is an inducible enzyme (Mitoma and Cooper, 1954 and Guroff and Ito, 1963). Certain aromatic mutants of N. crassa too show the ability to convert PHE to TYR (Barratt et al., 1956). This suggests that some micro-organisms may be able to use the "animal pathway" for TYR synthesis in addition to (at least under certain circumstances)

the shikimic acid pathway. If both plant (shikimic acid) and animal (hydroxylation of PHE) pathways for TYR synthesis existed in A. nidulans, it could account for the non-recovery of TYR-requiring mutants during routine searches for auxotroph because only mutants blocked in both pathways would show such a requirement.

Of interest is the discovery of Morpurgo (1962) that some of the FPA resistant mutants of A. nidulans have a partial requirement for TYR or PHE and all of them are selectively inhibited by indole or aminotyrosine + phenyl-anthranilic acid. But the possible causes of resistance, requirement and inhibition of these mutants is not understood. Warr and Roper (1965) found that FPA resistance suppresses nicotinic acid requirement in certain mutants of A. nidulans. These findings indicate that studies of FPA resistant mutants can throw some light on the aromatic amino acid biosynthesis.

In fact a novel approach to the study of metabolite synthesis and utilisation was opened up as early as 1940, by the suggestion of Woods that sulphonamides exert their inhibitory actions because of their structural similarities to p-aminobenzoic acid and that the drug competes with the vitamin for combination with an enzyme, the action of which is essential for growth. Since then, the effects of many

structural analogues of essential metabolites on different organisms have been studied both *in vivo* and *in vitro* (Work and Work, 1948; Hochster and Quastel, 1963 and Meister, 1965). Amongst many analogues of aromatic amino acids, the effect of PFA on a variety of organisms has been most extensively studied and it has been found to compete with its natural counterpart (PHE) at more than one stage of metabolism.

Selection and study of PFA resistant mutants in a variety of micro-organisms has thus provided a new tool for the study of different cell-processes that are under genetic control. In many micro-organisms PFA resistant mutants have been found to lack the uptake system for aromatic amino acids and some other compounds - depending on the specificity (Surdin *et al.*, 1965; Kappy and Metzenberg, 1965; Gronson, 1966; Gronson *et al.*, 1966; Gibs and Gronson, 1966; Stadler, 1966 and Gronson, 1967) of the uptake system. PFA-resistance due to a mutation in the PHE regulatory mechanism (resulting into derepression of PHE pathway) has been found to result in PHE-overproduction in *E. coli* (Adelberg, 1958) and possibly also in *S. typhimurium* (Ames, 1964). Another mutant strain of *E. coli*, resistant to PFA, synthesises an altered phenyl-alanyl-s-RNA synthetase which activates PHE but not PFA (Fangman and Neidhardt, 1964a, 1964b).

It has been proved beyond doubt that PFA is incorporated randomly into the proteins of *B. coli* and *B. cereus* entirely at the expense of Phe and not at all at the expense of any other amino acid and the extent of replacement is about the same (up to 75 %) throughout different proteins of the cells (Munier and Cohen, 1959; Yoshida, 1960; Richmond, 1960 and 1963).

Most of the work concerning PFA resistance has been carried out with bacteria and little is known about it in fungi in general and *Aspergillus* in particular. Morpurgo was the first (1962) to isolate PFA resistant mutants in *A. nidulans*. All his mutants map (DePalma and Morpurgo, 1963) at one locus - designated *fpA* in the symbolism of the present work. Warr and Roper (1965) too have isolated one mutant, possibly at the same locus. McCully (1964) has isolated 5 more PFA-resistant mutants in *A. nidulans* which map at three loci (2 at *fpA*, 1 at *fpB* and 2 at *fpD*) - one of them perhaps identical to that previously identified by Morpurgo (1962).

Based on the experiences of previous workers with *A. nidulans* and other organisms, the work presented in this thesis was carried out in an attempt to understand the mechanism of aromatic amino acid biosynthesis in *A. nidulans*.

In Chapter III of this thesis, characterisation of newly and previously isolated PHE-requiring mutants has been described. Isolation of partial and exacting TYR-requiring mutants - based on the hypothesis of the existence of two pathways for TYR synthesis in A. nidulans - and their formal and physiological genetic analysis is the subject matter of Chapters IV and V. In Chapter VI, experiments have been described leading to the suggestion that partial TYR-requirers are FPA-resistant, possibly due to an oversynthesis of PHE. In the last section (Chapter VII), genetic evidences have been presented to suggest that there are other mechanisms too (such as a mutation in the aromatic amino acid uptake system) for FPA resistance in A. nidulans.

II MATERIALS AND METHODS

1) Life cycle of Aspergillus nidulans.

A. nidulans (Eidam) Winter, is a homothallic ascomycete belonging to the family Aspergillaceae of the order Plectascineae. Detailed descriptions of its mycology, genetics and cytology are available elsewhere (Thom and Raper, 1945; Pontecorvo et al., 1953; Pontecorvo, 1959 and Elliott, 1960) and only the salient features will be summarised here.

The vegetative mycelium is branched, septate, coenocytic and haploid. Anastomosis between adjacent hyphae followed by nuclear migration is of frequent occurrence. The uninucleate asexual spores (conidia) are produced in chains from bottle shaped sterigmata arranged on top half of globose vesicles. Conidia of a single chain have genetically identical nuclei but different chains may have genetically different nuclei.

Binucleate sexual spores (ascospores) are produced in peritheeia (more exactly cleistotheeia). A young peritheeium contains a mass of "ascogenous" hyphae on which many small asceus primordia develop. Each primordium contains two nuclei which fuse to form a zygote of the asceus. The

zygotic nucleus immediately undergoes two meiotic divisions which are followed by a mitosis of the four products. Each of the eight resulting nuclei is included in an ascospore inside which they undergo a further mitosis. The eight binucleate ascospores are contained in each of the tens of thousands of fragile asci of a tough spherical pertithecium.

Genetic analysis has shown that a pertithecium, borne by a heterokaryotic mycelium, usually but not invariably, contains asci of exclusively crossed or exclusively selfed origin (Pontecorvo et al., 1953). Most probably all the asci in a single pertithecium originate from a pair of nuclei which enter into conjugate divisions to give rise to dikaryotic ascogenous hyphae from which the asci originate (Pontecorvo et al., 1953; Apirion, 1963).

2) Media and stock solutions.

Unless otherwise stated, all chemicals used are of analytical reagent grade.

(a) Minimal medium (M.M.) (Pontecorvo et al., 1953).

Ingredients per litre:-

D - glucose	10 g.
$NaNO_3$	6 g.
KCl	0.52 g.
$MgSO_4$	0.52 g.

KH_2PO_4	1.52 g.
$FeSO_4.7H_2O$	traces
$ZnSO_4.7H_2O$	traces
Agar	10 g.

adjusted to pH 6.5 with NaOH or HCl.

(b) Base medium (B.M.)

As M.M. but without glucose.

(c) Complete medium (C.M.)

It is similar to that given in Pontecorvo et al. (1953) with slight modifications. It consists of M.M. supplemented with the following ingredients per litre:-

Difco bacto peptone . .	2 g.
Yeastrol (Brewers' Food Supply Company Ltd., Edinburgh)	1 g.
Difco bacto casamino acids technical	1.5 g.
Riboflavin	1 mg.
Nicotinamide	1 mg.
Para-aminobenzoic acid.	0.5 mg.
Pyridoxin.HCl	0.5 mg.
Aneurin.HCl	0.5 mg.
Biotin	0.02 mg.

(d) Acetate medium (A.M.)

B.M. with 10 g. of ammonium acetate and adjusted to pH 6.1 with NaOH or HCl.

(o) Liquid media (L.M.)

Any of the various media in a liquid form i.e. without agar.

(f) Stock solutions

These solutions were added to media deficient in them for the growth or inhibition of growth of particular genotypes.

	Stock solution	Amount per 180 ml. of medium
Acriflavine	0.5 % (w/v)	1 ml.
Adenine	0.05 M	2 ml.
L-arginine	0.2 M	1 ml.
Aneurin.HCl	20 γ/ml.	1 ml.
p-Aminobenzoic acid	0.001 M	1 ml.
DL-Aminobutyric acid	1 % (w/v)	1 ml.
Biotin	4 γ/ml.	1 ml.
DL-p-Fluorophenyl-alanine	1 % (w/v)	2.5 ml.
Galactose	20 % (w/v)	1 ml.
L-Leucine	0.5 % (w/v)	1 ml.
L-Lysine mono - HCl	0.2 M	1 ml.
L-Methionine	30,000 γ/ml.	1 ml.
L-Nicotinamide	2,000 γ/ml.	1 ml.
L-Phenylalanine	0.05 M	1 ml.
L-Proline	2 % (w/v)	1 ml.

	Stock solution	Amount per 180 ml. of medium
Pyridoxine - HCl	10 γ/ml.	1 ml.
Riboflavine	20 γ/ml.	1 ml.
Sodium nitrite	1 M	1 ml.
Sodium thiosulphate	0.2 M	1 ml.
DL-Tryptophan	0.05 M	1 ml.
DL-Tyrosine	0.05 M	2 ml.

3) Methods of culture and plating

Cultures were normally incubated at 35 - 37°C. Strains were maintained on slopes of suitable media. Suspensions of conidia for plating were made in sterile saline containing about 0.2 % Tween 80 as a wetting agent. The chains of conidia were broken by vigorous shaking on a "Vortex mixer". The density of the suspension was estimated from a haemocytometer count. After a suitable dilution, 0.1 ml. of conidial suspension was spread over the surface of solid media with a sterile glass rod. Ascospore plating was done in the same way as conidia. Before breaking open to liberate the ascospores, the perithecia were rolled on hard agar (2.5 % -w/v) to get rid of sticking conidia and Hülle cells.

Dilutions were made to give less than 100 colonies

per petri-dish. When it was only necessary to make a colony count, up to 500 conidia were plated per dish on a medium containing 0.1 % sodium deoxycholate (w/v) (Mackintosh and Pritchard, 1963).

4) Auxanographic tests

The general principles and techniques have been described by Pontecorvo (1949).

5) Crossing and analysis of crosses

The conidia of two strains to be crossed were streaked together on thick layers of suitable media. The dishes were then sealed with sellotape and incubated for a further period of 6 - 10 days. This method is in common use and has resulted from the cumulative experience of various workers over 20 years.

Two methods are available (Pontecorvo et al., 1953) for the analysis of crosses and have been employed in this work:-

(a) Recombinant selection:- Ascospores from several perithecia were collected and plated on a selective medium on which only ascospores recombinant in respect of two or more markers could grow. This method requires that the two parental strains carry complementary genes determining nutritional requirements

When morphological markers (e.g. colour of the conidia) were used, selection was made by visual inspection.

(b) Perithecium analysis:- This analysis is based on the fact (Hemmons, Pontecorvo and Eufon; 1952, 1953) that asci from a single perithecium tend to be exclusively of either selfed or of crossed origin. Ascospores of a perithecium were stored at 4°C and a sample plated on C.M. If the sample revealed the perithecium to be crossed, the next sample was plated and the colonies, thus obtained, were transferred to master plates of C.M., 26 to a plate; and replicated, using a multiple wire replicator, to various media to reveal their genotypes.

6) Synthesis of heterokaryons:-

Heterokaryons were generally synthesised between strains of different nutritional requirements and of different conidial colour so that they could be recognised by the mixed spore colours as well as by the ability to grow on M.M. A mixture of conidia from the two strains was allowed to germinat and grow on L.C.M. for about 12 hours to give a hyphal mat. This mat was teased out on M.M. or M.M. supplemented only with growth factors that both component strains required. Heterokaryotic mycelia usually grew from most of the teased pieces.

7) Synthesis of diploids

Roper's technique (1952) of selecting prototrophic conidia from those of a balanced heterokaryon of the relevant strains was used. Approximately 10^7 conidia from the heterokaryon were mixed with a cooled (40-45°C) M.M. supplemented with necessary growth factors and poured into petri-dishes. Diploid colonies which grew, were isolated and purified by replating.

8) Mitotic analysis

(a) For assigning genes to linkage groups:- A diploid between a master strain and the strain carrying a marker not yet located was synthesised and haploidised with FPA (Lhoas, 1961; McCully and Forbes, 1965). The genotypes of haploid segregants were examined. Since in haploidisation there is recombination between markers of different linkage groups but not within linkage groups (Pontecorvo et al., 1953; Pontecorvo et al., 1954 and Pontecorvo and Kafer, 1958) the unlocated marker will recombine with the master strain markers of all other seven linkage groups but not with that of its own linkage group.

(b) For determining the sequence of linked markers:- Heterozygous diploids were allowed to grow on C.M. and diploid segregants arising via mitotic crossing-

over were selected and their phenotypes determined. Since a somatic crossing-over proximal to a marker will lead to its homozygosis whereas a somatic crossing-over distal to it will have no effect on its heterozygous arrangement, the sequence of different markers with respect to the centromere and each other can be determined (Pontecorvo and Kafer, 1958).

9) Replica - plating for isolation of mutants

The method has been described by Mackintosh and Pritchard (1963).

MUTANTS USED AS GENETIC MARKERS.

Locus symbol (allelic mutants are placed in one line).	Phenotype
abl	aminobutyric acid requiring
Acr1	acriflavine resistant
ad3	adenine requiring
ad14	adenine requiring
ad17	adenine requiring
ad20	adenine requiring
an1	aneurine requiring
arg1	arginine requiring
arg2	arginine requiring
arg3	arginine requiring
bi1	biotin requiring
br42	morphological mutent
cha	chartreuse conidia
cnxd14	unable to utilise nitrate and xanthine
facA303	unable to utilise ammonium acetate
fpA1, fpA12	p-fluorophenylalanine resistant
fpB37	p-fluorophenylalanine resistant
fpD11, fpD43	p-fluorophenylalanine resistant
fw	fawn conidia
gal1	unable to utilise galactose
gal5	unable to utilise galactose
lu1	leucine requiring
med15	morphological mutent

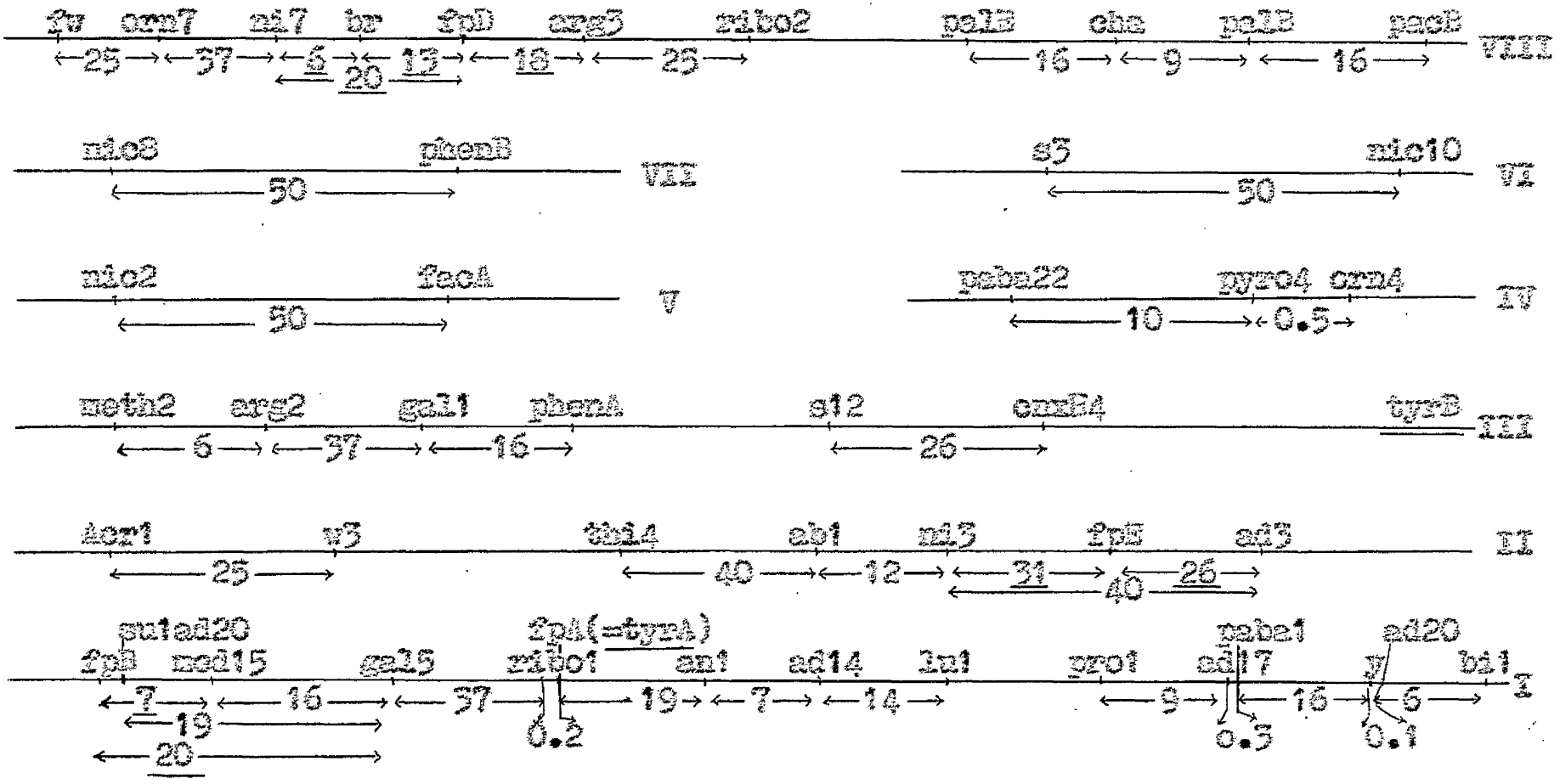
MUTANTS USED AS GENETIC MARKERS(Contd.)

Locus symbol (allelic mutants are placed in one line)	Phenotype
meth2	methionine requiring
ni3	unable to utilise nitrate
ni7	unable to utilise nitrate
nic2	nicotinic acid requiring
nic8	nicotinic acid requiring
orn4	ornithine requiring
orn7	ornithine requiring
paba1	p-aminobenzoic acid requiring
peba22	p-aminobenzoic acid requiring
pal3	alkaline phosphataseless
pal5	alkaline phosphataseless
phenA1, phenA2, phenA3, phenA4 and phenA5	phenylalanine requiring
phenB6	phenylalanine requiring
pro1	proline requiring
pyro4	pyridoxine requiring
ribo1	riboflavine requiring
ribo2	riboflavine requiring
s12	unable to utilise sulphate
sulad20	suppressor of ad20
thi4	thiazole requiring
w2, w3, w6	white conidia
y	yellow conidia

N.B.-Strains carrying br4 2 and med15 were kindly supplied by Dr.A.J.Clutterbuck of this laboratory. All other strains were from the Glasgow stock of strains.

Linkage relationships of loci referred to in this thesis.

Throughout the thesis map distances are given in percent recombination(own data underlined).



Distribution of markers on eight linkage groups.

III PHENYLALANINE REQUIRING MUTANTS

The isolation and physiological and genetic studies of mutants requiring phenylalanine are reported in this section.

Choice of the mutagen

Nutritionally deficient mutants of *A. nidulans* have been isolated following exposure of conidia to X-rays or U.V. light (Pontecorvo *et al.*, 1953) or treatment of conidia with mustard gas (Hockenhull, 1949) or nitrous acid (Siddiqi, 1962) or N-methyl-N'-nitro-N-nitrosoguanidine (NTG) (Clutterbuck and Sinha, 1966). In the present work the last mutagen has been often used in preference to others.

X-ray and U.V. irradiation are known to cause gross chromosomal aberrations among the survivors of a wide variety of organisms (Miller and Mackenzie, 1939; Stadler, 1941; Swanson and Stadler, 1955; Lindegren and Lindegren, 1941 and Hollaender *et al.*, 1945). Cumulative experience of several workers in this laboratory with *A. nidulans* has shown that chromosomal aberrations, particularly reciprocal translocations and inversions lead to abnormal linkage

relationships, both in mitotic (Pontecorvo, Tarr-gloor and Forbes, 1954; Pontecorvo and Kafer, 1958) and meiotic analysis. Haploidisation of diploid strains synthesised with special multiply marked "tester strains" (Forbes, 1959) has been used for assigning markers of unknown location to one of the eight chromosomes of A. nidulans (McCully and Forbes, 1965). Obviously, translocations come in the way of unambiguous assignments. Six PABA-requiring mutants were available in the Glasgow-stock of strains. At least two of them carry a translocation (Table 1).

Table 1

'phen' mutants already available in the Glasgow-stock

Mutant	Mutagen	Linkage group	Translocation
phen 1	X-ray	III	not tested
phen 2	U.V.	III	I-VIII translocation (Kafer,1965)
phen 3	U.V.	III	I-IV translocation
phen 4	U.V.	III	not tested
phen 5	U.V.	III	not tested
phen 6	U.V.	VII	free of translocation

On the other hand NTG appears to be the most potent

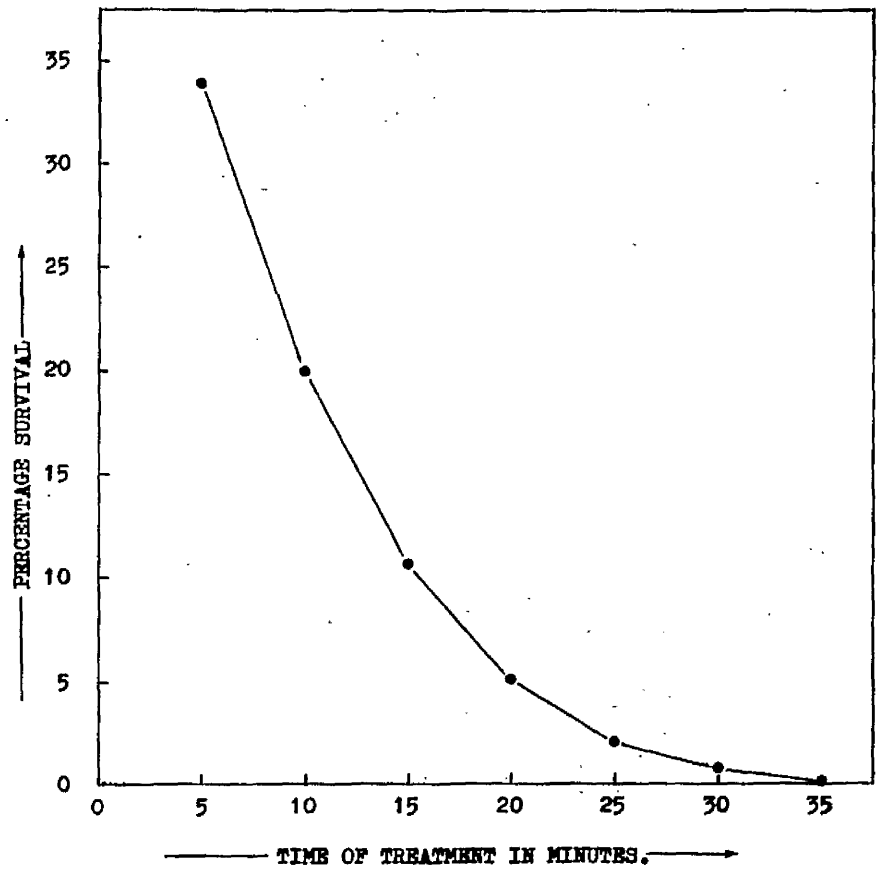


Fig.-3 : Survival of bi1 (green) conidia after treatment with NTG.

chemical mutagen yet discovered. Its mutagenicity was first reported by Mandell and Greenberg (1960) and since then it has been widely used by microbial geneticists. In E. coli K-12, it induces at least one mutation per treated cell under conditions permitting over 50 per cent survival and a mutant type such as valine-resistance, which can result from a mutation in any of several loci, is produced at a frequency of over 0.2 % of the cells treated (Adelberg et al., 1965). There has been no report as to the exact type of mutations produced by NTG. It is a well known alkylating agent, and alkylating agents are known to cause a variety of transitions and transversions. NTG induced mutants in the aminotransferase gene (G gene) of the histidine operon in Salmonella typhimurium suggest that it causes only transitions and transversions and no additions or deletions (Whitfield et al., 1966).

Method of treatment with NTG

The method of Adelberg et al. (1965) for mutagenesis has been successfully applied to A. nidulans (Clutterbuck and Sinha, 1966). Conidia harvested from fresh slopes are suspended in 2 ml. (about 10^9 conidia/ml.) of Tris and Maleic buffer (each at a final concentration of M/20) adjusted to pH 6.0 with NaOH. Six mg. NTG are dissolved in 10 ml. of

Tris buffer. The two solutions (2 + 10 ml.) are mixed and incubated at 37°C with occasional agitation. Thus the concentration of NTG in the treatment mixture is 0.5 mg/ml and the concentration of conidia about 10^8/ml. Treatment is stopped by centrifuging down the conidia and washing them at least twice in sterile distilled water. The percentage survival of a bil strain goes down with the increase in the time of treatment (Fig. 3). Even with viabilities as high as 50 %, a high yield of mutants is obtained. In this work a viability of about 25 % has been aimed at.

Isolation and characterisation of phenylalanine-requiring mutants.

As shown in Table 2, no PHE-requiring mutant was recovered after U.V. or HNO_2 treatment of bil conidia. However, five PHE-requiring mutants were recovered after NTG treatment of bil; w6 and bil strains - two from the former and three from the latter. Treated conidia were plated on MM + biotin + phenylalanine and replicated on MM + biotin, following replica plating technique (Mackintosh and Pritchard, 1963). Colonies growing on the former but not on the latter plate were isolated, purified by single colony isolation or micromanipulation and retested for their requirements.

Table 2

Isolation of phenylalanine-requiring mutants by replica plating

Experiment number	Strain treated	Mutagen	Percentage survival	Number of colonies tested	PHE-auxotrophs recovered
A	bil	HNO_2	2.73	9,638	none
B	bil	HNO_2	8.0	18,000	none
C	bil	HNO_2	2.0	12,800	none
D	bil	HNO_2	1.6	6,500	none
E	bil	HNO_2	4.5	12,000	none
F	bil	U.V.	15.0	7,500	none
G	bil	U.V.	8.5	1,250	none
H	bil	U.V.	2.5	12,500	none
I	bil	U.V.	10.0	12,000	none
J	bil;w6	NTG	25.0	15,000	2-phen 7 and phen 8
K	bil	NTG	25.0	12,000	none
L	bil	NTG	10.0	12,000	2-phen 9 and phen 10
M	bil	NTG	10.0	15,000	1-phen 11

Complementation tests

Complementation tests (in *trans* arrangement) between different PHE-requiring mutants in diploids and heterokaryons gave identical results. All mutants were found to be recessive and fell in two complementation groups. *phen 2, 3, 4, 5* (old strains), *7, 8, 9, 10* and *11* did not complement in any combination while *phen 6* (old strain) complemented with all the others. Previous workers had already located *phen 2, 3, 4* and *5* in linkage group III and *phen 6* in linkage group VII and the additional mutants did, therefore, not add to the number of cistrons already identified. In this particular instance, the number of cistrons corresponds with the number of loci which are hereafter called *phen A* (*phen 2, 3, 4, 5, 7, 8, 9, 10* and *11*) and *phen B* (*phen 6*).

Tests for translocation and location of mutants in linkage groups by mitotic haploidisation.

(a) Principle:- Haploidisation of diploid strains (Pontecorvo *et al.*, 1953; Pontecorvo, 1956 and Pontecorvo and Kafer, 1956, 1958) has been used to assign markers of unknown location to one of the eight linkage groups of *A. nidulans*. This work was enormously facilitated by the introduction of "tester strains" (Forbes, 1963) in the technique developed by Forbes (1959) and improved by McCully and

Forbes (1965).

Master-strains allow a rapid detection of reciprocal translocations (McCully and Forbes, 1965) which are known to be common in A. nidulans (Kafer, 1962;1965). If a reciprocal translocation is present in the strain to be tested, after haploidisation of a heterozygous diploid, two markers of the master-strain, known to be on different linkage groups, show complete linkage in elg. This is because both the recombinant classes have a deficiency and a duplication of different chromosomal segments and are, therefore, usually non-viable. But in the case of an insertional translocation, one recombinant class is deficient for a chromosomal segment and the other has it duplicated. The latter class may be partially viable.

(b) Results:-

By haploidisation with master-strain F (McCully and Forbes, 1965) (genotype = sulad20, y, ad20; AcrI; galI; pyro4; facA303; s3; nicB; ribo2), phenB6 (Table 3) and phenA7 (Table 4) were found to be free of reciprocal translocations whereas phenA3 (Table 5) was found to harbour a I - IV translocation.

In conformity with previous results of complementation tests, these experiments indicated that phenB6 is in linkage group VII and phenA3 and phenA7 are in linkage group III.

Table 3

Location of phen B6 by mitotic haploidisation on PPA and test of translocation

Segregation of markers in 27 haploids isolated from the diploid:-

I	II	III	IV	V	VI	VII	VIII
ad20, y, ad20, +, bil	Acrl;	gal1;	pyro4;	facA303;	s3;	nic8, +, (phonB6?)	; ribo2

	Parentals		Recombinants	
	++	--	+-	-+
y;Acrl	6	8	12	1
y;gal1	7	5	11	4
y;pyro4	8	8	10	1
y;facA303	3	5	15	4
y;s3	1	0	17	9
y;nic8	0	9	18	0
y;ribo2	6	7	12	2
Acrl;gal1	2	11	5	9
Acrl;pyro4	3	13	5	7
Acrl;facA303	2	15	5	5
Acrl;s3	2	12	5	8
Acrl;nic8	0	20	5	0
Acrl;ribo2	2	14	5	6
gal1;pyro4	4	11	7	5
gal1;facA303	2	7	9	9
gal1;s3	5	11	6	5
gal1;nic8	0	16	11	0
gal1;ribo2	7	15	4	1
pyro4;facA303	4	15	5	3
pyro4;s3	1	9	8	9
pyro4;nic8	0	18	9	0
pyro4;ribo2	4	14	5	4

Table 3 (continued)

	Parentals		Recombinants	
	++	--	+-	-+
facA303;s3	4	14	3	6
facA303;nic8	0	20	7	0
facA303;rib02	1	13	6	7
s3;nic8	0	17	10	0
s3;rib02	3	12	7	5
nic8;rib02	0	19	0	8

	Parentals		Recombinants	
	-+	+-	--	++
phenB6;y	0	9	0	18
phenB6;Acxl	0	20	0	7
phenB6;gal1	0	16	0	11
phenB6;pyro4	0	18	0	9
phenB6;facA303	0	20	0	7
phenB6;s3	0	17	0	10
phenB6,nic8	0	27	0	0
phenB6;rib02	0	19	0	8
y,bil	2	18	0	0

Conclusion: phenB locus is in linkage group VII and the strain bil;phenB6 is free of translocations. In this case and in others, in which haploids are selected on FPA, the phen parental or recombinant types are not recovered because of the higher toxicity of FPA for phen strains.

Table 4

Location of phen A7 by mitotic haploidisation on FPA and test of translocation

Segregation of markers in 37 haploids isolated from the diploid:-

I II III IV V VI VII VIII

$$\frac{sulad20,y,ad20,+}{+}\,,+,\,\frac{+}{+}\,,bil;\ \frac{Acrl,+}{+}\,,u6;\ \frac{gall,+}{+}\,,(phenA7?);\ \frac{pyro^{4},facA303,s3,nic8,ribo2}{+}$$

	Parentals ++	Parentals --	Recombinants +-	Recombinants -+
y;Acrl	4	10	19	4
y;gall	0	14	23	0
y;pyro^{4}	11	9	12	5
y;facA303	15	6	8	8
y;s3	14	5	9	9
y;nic8	10	5	12	9
y;ribo2	7	9	16	5
Acrl;gall	0	29	8	0
Acrl;pyro^{4}	3	16	5	13
Acrl;facA303	4	10	4	19
Acrl;s3	5	11	3	18
Acrl;nic8	7	17	1	12
Acrl;ribo2	8	25	0	4
gall;pyro^{4}	0	21	0	16
gall;facA303	0	14	0	23
gall;s3	0	14	0	23
gall;nic8	0	18	0	19
gall;ribo2	0	25	0	12

Table 4 (continued)

	Parentals		Recombinants	
	++	--	+-	-+
pyro^{+};facA303	10	8	6	13
pyro^{+};s3	11	9	5	12
pyro^{+};nic8	8	10	8	11
pyro^{+};ribo2	5	14	11	7
facA303;s3	14	5	9	9
facA303;nic8	11	7	12	7
facA303;ribo2	5	7	18	7
s3;nic8	13	8	10	6
s3;ribo2	9	11	14	3
nic8;ribo2	8	14	11	4

	Parentals		Recombinants	
	+-	-+	++	--
phonA7;y	14	0	23	0
phonA7;Acr1	29	0	8	0
phonA7,gal1	37	0	0	0
phonA7;pyro^{+}	21	0	16	0
phonA7;facA303	14	0	23	0
phonA7;s3	14	0	23	0
phonA7;nic8	18	0	19	0
phonA7;ribo2	25	0	12	0
bil,y	14	23	0	0

Conclusion: 4 out of the 8 white (=Acr1^{+}) segregants must be of the genotype y;w6 because they carry the bil^{+} allele. The strain bil;w6;phonA7 is free of translocations and the locus phonA is in linkage group III.

Table 5

Location of phenA3 by mitotic haploidisation on FPA and test of translocation

Segregation of markers in 34 haploids isolated from the diploid:-

I II III IV V VI VII VIII.

$$\frac{\text{oulod20}}{+},\frac{y}{+},\frac{\text{ad20}}{+},\frac{+}{\text{bil}};\frac{\text{Acrl}}{+};\frac{\text{gall}}{+},\frac{+}{(\text{phenA3?})};\frac{\text{pyro}^{4}}{+};\frac{\text{facA303}}{+};\frac{\text{s3}}{+};\frac{\text{nic8}}{+};\frac{\text{ribo2}}{+}$$

	Parentals		Recombinants	
	++	--	+-	-+
y;Acrl	3	15	2	14
y;gall	0	29	5	0
y;pyro4	5	29	0	0
y;facA303	0	22	5	7
y;s3	5	14	0	15
y;nic8	2	16	3	13
y;ribo2	5	11	0	18
Acrl;gall	0	17	17	0
Acrl;pyro4	3	15	14	2
Acrl;facA303	3	15	12	2
Acrl;s3	12	9	5	8
Acrl;nic8	8	10	9	7
Acrl;ribo2	15	9	2	8
gall;pyro4	0	29	0	5
gall;facA303	0	27	0	7
gall;s3	0	14	0	20
gall;nic8	0	19	0	15
gall;ribo2	0	11	0	23
pyro4;facA303	0	22	5	7
pyro4;s3	5	14	0	15
pyro4;nic8	2	16	3	13
pyro4;ribo2	5	11	0	18

Table 5 (continued)

	Parentals		Recombinants	
	++	--	+-	-+
facA303;s3	4	11	3	16
facA303;nic8	7	19	0	8
facA303;rib2	7	11	0	16
s3;nic8	9	8	11	6
s3;rib2	16	7	2	7
nic8;rib2	13	9	2	10

	Parentals		Recombinants	
	-+	+-	--	++
phenA3;y	0	29	0	15
phenA3;Acr1	0	17	0	17
phenA3,gal1	0	34	0	0
phenA3;pyro4	0	29	0	5
phenA3;facA303	0	27	0	7
phenA3;s3	0	14	0	20
phenA3;nic8	0	19	0	15
phenA3;rib2	0	11	0	23
y,bi1	29	5	0	0

Conclusion: No recombinants were recovered between the markers of linkage group I ($\frac{su1;ad20,y,ad20,+}{+\ ,+,\ +\ ,bi1}$) and IV ($\frac{+}{pyro4}$) indicating the presence of a translocation involving linkage groups I and IV. Other linkage groups, including linkage group III which carries the phenA locus, were found to be free of translocations.

A translocation-free bil; phenA3 strain

In order to eliminate the translocation present in the bil; phenA3 strain, a cross was made between bil; phen3 and bil; lul expecting that a quarter of bil,lul; phenA3 recombinants (those which carry linkage groups I and IV from the parent bil, lul) should be free of any translocation. bil, lul was chosen as the other parent because a bil, lul; phenA3 recombinant was required for some experiments to be described in the last Section and the strain bil, lul was found to be free of translocations (Table 6). Seven bil, lul; phenA3 recombinants were picked up and the first one tested turned out to be free of translocations (Table 7). This translocation-free bil, lul; phenA3 strain was then crossed with (1) MSF, (2) y;w3;arel and (3) ad3,y to get various recombinants as well as a translocation-free bil; phenA3 strain.

Table 6

Location of lul by mitotic haploidisation on FPA and test of translocation

Segregation of markers in 24 haploids isolated from the diploid:-

I II III IV V VI VII VIII

$$\frac{sul\,ad20,\ +}{+}\ ,\frac{y,\ ad20,\ +}{+}\ ;\frac{Acrl}{+};\frac{gall}{+};\frac{pyro4}{+};\frac{facA303}{+};\frac{s3}{+};\frac{nic8}{+};\frac{rib02}{+}.$$

(lul?), + , + , bil

	Parentals		Recombinants	
	++	--	+-	-+
lul, bil	24	0	0	0
ad20, y	0	9	15	0
Acrl; gall	6	7	3	8
Acrl; pyro4	5	6	4	9
Acrl; facA303	4	11	5	4
Acrl; s3	6	4	3	11
Acrl; nic8	5	4	4	11
Acrl; rib02	9	0	0	15
gall; pyro4	6	2	8	8
gall; facA303	6	8	8	2
gall; s3	11	4	3	6
gall; nic8	10	4	4	6
gall; rib02	14	0	0	10
pyro4; facA303	5	7	2	3
pyro4; s3	9	2	5	8
pyro4; nic8	10	4	4	6
pyro4; rib02	14	0	0	10
facA303; s3	5	4	3	12
facA303; nic8	5	5	3	11
facA303; rib02	8	0	0	16

Table 6 (continued)

	Parentals		Recombinants	
	++	--	+-	-+
s3;nic8	14	5	3	2
s3;ribo2	17	0	0	7
nic8;ribo2	16	0	0	8

	Parentals		Recombinants	
	+-	-+	--	++
ad20,lul	0	9	0	15
ad20,bil	0	9	0	15
lul;Acrl	15	0	0	9
lul;gall	10	0	0	14
lul;pyro4	10	0	0	14
lul;facA303	16	0	0	8
lul;s3	7	0	0	17
lul;nic8	8	0	0	16
lul;;ribo2	0	0	0	24
lul,y	24	0	0	0

Conclusion: The strain bil,lul is free of translocations and a somatic crossing-over between sulad20 and lul has taken place.

Table 7

Test of translocation in the strain bil,lul;phonA3

Segregation of markers in 37 haploids isolated from the diploid:-

I	II	III	IV	V	VI	VII	VIII
sulad20, + ,y,ad20, + ,Acrl, gall, + ; pyro4; facA303; s3; nic8; ribo2							
+ ,lul,+, + ,bil + + ,phonA3 + + + + + +							

Table 7 (continued)

	Parentals		Recombinants	
	++	--	+-	-+
y;Acrl	0	14	0	23
y;gal1	0	37	0	0
y;pyro4	0	21	0	16
y;facA303	0	25	0	12
y;s3	0	12	0	25
y;nic8	0	19	0	18
y;rlbo2	0	17	0	20
Acrl;gal1	0	14	23	0
Acrl;pyro4	11	9	12	5
Acrl;facA303	7	9	16	5
Acrl;s3	16	5	7	9
Acrl;nic8	14	10	9	4
Acrl;rlbo2	13	7	10	7
gal1;pyro4	0	21	0	16
gal1;facA303	0	25	0	12
gal1;s3	0	12	0	25
gal1;nic8	0	19	0	18
gal1;rlbo2	0	17	0	20
pyro4;facA303	8	17	8	4
pyro4;s3	12	8	4	17
pyro4;nic8	7	10	9	11
pyro4;rlbo2	10	11	6	10
facA303;s3	9	9	3	16
facA303;nic8	7	14	5	11
facA303;rlbo2	10	15	2	10
s3;nic8	12	6	13	6
s3;rlbo2	15	7	10	5
nic8;rlbo2	10	9	8	10
bil,lul	32	0	0	0
bil;phonA3	37	0	0	0

Table 7 (continued)

	Parentals		Recombinants	
	+-	-+	++	--
y,bil	0	37	0	0
y,lul	0	37	0	0
y;phenA3	0	37	0	0

Conclusion: The strain bil,lul;phenA3 is free of translocations Only yellow haploids were visually selected. nal+ segregants were not recovered because nal1 and phenA are in the same linkage group.

Auxanographic tests and leakiness of phenylalanine-requiring strains.

Auxanographic tests of strains phen 2-11 showed that they all grow on both PHE and phenylpyruvic acid (ketoacid analogue of PHE). But the response to phenylpyruvic acid does not mean much because a chromatographic analysis revealed that the sample used was contaminated with PHE.

These tests further showed that all PHE-requiring strains (isolated so far) can grow to a limited extent even without added phenylalanine. Even on plating (about 50 conidia per dish) the conidia formed colonies of the size of pin-heads in the absence of added PHE. When about 50 conidia of bil;phenA3 strain were washed and suspended in 20 ml. of liquid MM + biotin and the mixture was poured into a sterile

plastic petri-dish, taking care to use minimum of washed glassware, after about 48 hours of incubation small ball-like colonies of the size of pin-heads were obtained. This to a large extent, excluded the possibility of contamination with PHE and indicated that the PHE-requiring strains analysed are leaky. It was noticed that the *phenP6* strain is more leaky than the mutants at the *phenA* locus (*phenA2, 3, 4, 5, 7, 8, 9, 10* and *11*).

There was no effect of change of temperature of incubation (25, 30, 37, 42°C) or pH (6.6-7.8) of the medium on the leakiness of PHE-requiring mutants.

DISCUSSION

If the general scheme for aromatic amino acid biosynthesis in *A. nidulans* is the same as in other micro-organisms (Fig. 1), *phenA* and *phenB* mutants should be blocked somewhere after chorismic acid, because a block before that would result into a requirement for TYR + PHE + TRY + PABA + p-hydroxybenzoic acid (if the block is between shikimic acid and chorismic acid) or for shikimic acid (if the block is before that).

Ten mutants at the *phenA* locus (*phen1-11* except *phen6*) and only one at the *phenB* locus (*phen6*) have been isolated so far and *phenB6* has been found to be more leaky than mutants at *phenA* locus. This may be a reason why mutants at *phenB* locus are not likely to be frequently identified and isolated. It is not known whether the blocks in *phenA* and *phenB* mutants are at different points or at the same point for different reasons. It is not possible to conclude anything from the positive growth of both these mutants on phenylpyruvic acid because the samples used were found to contain PHE as an impurity and *phenB6* was more leaky than *phenA* mutants.

Mutants at the *phenA* locus in *A. nidulans* are perhaps blocked between prophenic acid and phenylpyruvic acid.

Mutations affecting this step are the only ones so far recovered as PHE-requirers in *E. coli* (Davis, 1955; Pittard and Wallace, 1966), *Salmonella* (Demerec *et al.*, 1956 and Sanderson and Demerec, 1965), *A. aerogenes* (Davis, 1951), *N. crassa* (Barratt and Ogata, 1966) and many other microorganisms (Meister, 1965).

At the branching of the pathways, conceivably there are two enzymes - prephenate dehydrogenase and prephenate dehydratase - operating on the synthesis of TYR and PHE respectively. The former makes p-hydroxyphenylpyruvic acid and the latter phenylpyruvic acid from prephenic acid. It could well be that *phenA* mutants lack prephenate dehydratase activity and they are leaky because some PHE can be made by prephenate dehydrogenase which is present in a *phenA;tyrA*$^+$ mutant. In other words, perhaps the specificities of prephenate dehydrogenase and prephenate dehydratase slightly overlap. If this were true, a *phenA;tyrA* double mutant should not be leaky and this is what has been found and reported in the next Section.

If the block in *phenB* mutant is at different point as compared to the block in *phenA* mutants (for which there is no evidence), it could be either at the chorismate mutase step or at the transamination step. In either case the mutant should require PHE + TYR but both these requirements

are probably satisfied by exogenous PMS in *A. nidulans* because as indicated in the next two Sections of this thesis, PMS can be converted to TMR in this organism. If the block were at the chorismate mutase step, leakiness could be explained by the lability (Jensen and Nester, 1965) of chorismic acid and if the block were at the transaminationstep, leakiness could be explained on the basis of non-specificities of transaminases (Rudman and Meister, 1953).

SUMMARY

1. Five additional PHE-requiring mutants have been isolated after NTG treatment of bil and bil;v6 conidia.

2. phen 2,3,4,5(old strains),7,8,9,10 and 11 have been found not to complement in any combination and to be in linkage group III whereas phen6 (old strain) has been found to complement with all others and its location in linkage group VII, determined by McCully, has been confirmed.

It has been proposed to designate these loci as phenA and phenB respectively.

3. The strains bil;phenB6 and bil;v6;phenA7 have been found to be free of translocations and a I - IV translocation present in the strain bil;phenA3 has been eliminated by outcrossing.

IV PARTIAL TYROSINE-REQUIRING MUTANTS

General considerations

A number of TYR requiring mutants have been isolated and studied in E. coli (Davis, 1955; Smith and Yanofsky, 1960), S. typhimurium (Demerec et al., 1956), Streptomyces coelicolor (Hopwood- personal communication) and Neurospora (Tatum et al., 1954; Perkins and DeBusk in Barratt and Ogata, 1966). Perhaps the best known and well studied case of genotically determined TYR deficiency is phenylketonuria in man (Garrod, 1923; Harris, 1959 and Woolf, 1963) and similar situations in rats and other animals (Kaufman, 1963 and Zannoni et al., 1966).

Before the present work there was no report of the isolation of a complete TYR-requiring auxotroph in A. nidulans. Morpurgo (1962) isolated a number of PPA-resistant mutants in A. nidulans and found that some had a partial requirement for TYR or PHE. But the nature of metabolic block in these mutants was not known.

A fresh search was, therefore, made for TYR-requiring auxotrophs in A. nidulans.

Failure to isolate a tyrosine-requiring auxotroph in a bil strain.

U.V. or NTG treatment of bil conidia did not yield any

TYR auxotrophs although 108,500 colonies, grown on MM + biotin + TYR, were replicated on MM + biotin (Table 8).

Table 8

Failure to isolate tyrosine-requiring mutants in a bil strain by replica plating

Experiment number	Mutagen used	Percentage survival	No. of viable colonies tested	Number of tyrosine auxotrophs recovered
A	U.V.	12.0	16,500	none
B	U.V.	8.5	13,000	none
C	U.V.	6.2.	12,500	none
D	NTG	20.0	12,000	none
E	NTG	20.0	13,500	none
F	NTG	18.0	15,000	none
G	NTG	18.0	12,000	none
H	NTG	25.0	14,000	none

The hypothesis of two pathways for tyrosine synthesis in Aspergillus nidulans.

From what is known of the conversion of PHE to TYR in man, it is conceivable that there are two pathways for TYR synthesis in A. nidulans: one is the general one, known in

micro-organisms and is in part common to all aromatic compounds. The other is, like in man, by PHE hydroxylation. A block in one pathway would still allow for substantial growth, even in the absence of exogenous TYR, because of the still functioning other pathway.

It would be difficult thus to identify and isolate a TYR auxotroph. Even if one were a major pathway and the other only a minor one, a metabolic block in the major pathwey might call for an increased synthesis or activity of the enzyme or enzymes concerned in the minor pathway -thus leading to too much growth even in the absence of TYR.

The shikimic acid pathway (Fig. 1) as known in E. coli and other organisms, consists of a series of anaerobic reactions utilising glucose as a carbon source for the synthesis of aromatic compounds. The phenolic hydroxyl group of TYR originates from a hydroxyl group of glucose (Davis, 1955). The pathways for TYR and PHE synthesis branch from prophenic acid; going through phenylpyruvic acid to PHE and through p-hydroxy-phenylpyruvic acid to TYR. If this were the only way of synthesising TYR a genetic block between prophenic acid and TYR should lead to a TYR-requirement.

In contrast to micro-organisms and plants which are able to synthesise aromatic amino acids from simpler compounds,

animals require dietary PHE and TYR. The former is converted by hydroxylation to TYR (Kaufman, 1963). Animal tissues lack the ability to synthesise the benzene ring and they use molecular oxygen as the source of phenolic hydroxyl group during the conversion of PHE to TYR. Perhaps, in animals, TYR can also arise as an intermediate during the catabolic disposal of the aromatic compounds (Block, 1962).

The shikimic acid pathway is the general one known in micro-organisms and higher plants for the joint synthesis of all aromatic compounds. Before the work to be reported here, tyrosine formation by hydroxylation of PHE was known in animal tissues and in some specialised micro-organisms like PHE adapted pseudomonads (Mitoma and Leeper, 1954) and certain aromatic mutants of N. crassa (Barratt et al., 1956). In both these micro-organisms TYR-requiring mutants, blocked in the shikimic acid pathway are known, which are not leaky and in each of these organisms absolute TYR requirement results due to a mutation at only one locus, so far known (Davis and Mingioli, 1953; Bonner et al., 1965). This suggests that under ordinary conditions, TYR is not formed from PHE either in Pseudomonas or in N. crassa or at least this conversion is not detectable and the aerobic formation of TYR from PHE in the specialised strains of these micro-

organisms is an adaptation under unfavourable circumstances. In other words, except in one strain of N. crassa and one strain of Pseudomonas, in all micro-organisms and plants, so far investigated, there is only one pathway for TYR synthesis and that is the shikimic acid pathway. Besides, the genetical studies of PHE-hydroxylation step have not been carried out in any organism in which the shikimic acid pathway is known or likely to occur.

Perhaps both these pathways for TYR synthesis: shikimic acid pathway and hydroxylation of PHE, are operative in A. nidulans and are quantitatively important. Their genetical and preliminary biochemical investigations are reported in the following pages.

Isolation of a tyrosine-requirer in a phenylalanine-requiring strain.

(a) Principle:-

If two pathways existed for the synthesis of TYR in A. nidulans -one by the shikimic acid pathway and the other by hydroxylation of PHE, it would be difficult to detect a TYR-requiring mutant in a phen$^+$ strain, because this would require two blocks i.e. a simultaneous occurrence of two mutations in a single nucleus or nuclear lineage. On the other hand it might be possible

to recognise a TYR-requiring mutant in a *phen* strain, by limiting PHE in the medium so as just to satisfy the PHE requirement without having enough of it to be converted to TYR. Under these conditions, a *phen;tyr* double mutant should show a growth response related to the level of TYR supplied.

In addition to this approach, there is another one suggested by the fact (discussed in the previous section) that all the available PHE-requiring mutants, at the two loci (*phenA* and *phenB*) so far known, are leaky. On a medium devoid of PHE, *phen* strains show this leakiness by slight growth. This leakiness of *phenA* mutants could well be due to slight non-specificity of prephenate dehydrogenase (Fig. 1) which may lead to slight growth in the presumed absence of prephenate dehydratase activity. If this assumption is correct, mutants blocked in the shikimic acid pathway both in the PHE branch and in the homologous step in the TYR branch, should be non-leaky. In other words, one might expect two general types of blocks in the synthesis of TYR: one in the two or more steps between prephenic acid via p-hydroxy-phenylpyruvic acid to TYR (and these may involve enzymes with specificities similar to that involved in the homologous steps in the synthesis of PHE)

and the other in the hydroxylation of PHE.

(b) Experimental:-

bil;phenA3 conidia were treated with NTG, grown on MM + biotin + TYR + optimum PHE (1 ml. of M/20 per 180 ml. of medium, i.e. final concentration M/3600) and the well isolated colonies from such platings were replicated on MM + bi + limiting PHE (0.1 ml. of M/20 per 180 ml. of medium i.e. M/36000). The colonies which grew on the former medium but failed to grow on the latter were isolated, purified and retested for their requirements. Two TYR-requiring mutants were thus recovered.

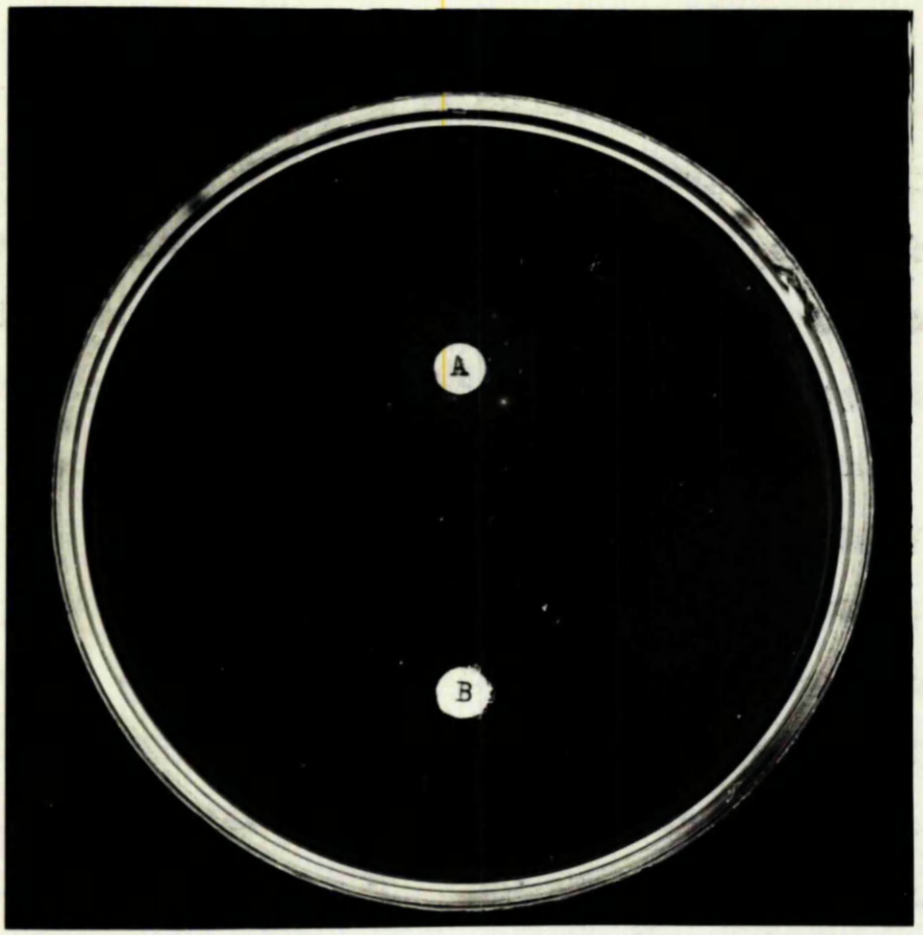
Following a similar procedure but by replicating the growing colonies on MM + biotin only (taking advantage of the fact that phenA strains are leaky) and by comparing the master plates and replicas, 72 hours after replication (allowing the time for the leaky strain to grow), eight more TYR-requiring auxotrophs were recovered in a bil; phenA3 strain (Table 9).

Table 9

Isolation of tyrosine-requiring mutants by replica plating after NTG treatment of bil;phonA3 conidia

Experiment number	Percentage survival	No. of viable colonies tested	Grown on	Replicated on	Tyrosine auxotrophs Total	Tyrosine auxotrophs Isolation number
A	22.0	14,000	MM +	MM+biotin + limiting phonyl-alanine	1	tyr1
B	22.0	12,500	biotin+		1	tyr2
C	20.0	15,000	tyrosine + optimum phonyl-alanine	MM+biotin	1	tyr3
D	30.0	9,000			2	tyr4 tyr5
E	30.0	6,000			1	tyr6
F	40.0	8,500			2	tyr7 tyr8
G	40.0	4,000			5*	tyr9
H	40.0	3,000			1	tyr10

*) In view of the relatively high number of auxotrophs recovered in this experiment, which could well have have arisen as a clone, only one isolate was taken for further work.



bi_1, $tyrA_7$; $phen_3$ conidia pregerminated 6 hrs at 37°C. in Minimal medium + biotin. Supplements were added at marked positions.

A. L-phenylalanine. B. L-tyrosine.

Plate.- 1

Auxanographic test of a phenA;tyr double mutant.

Auxanography of a bil,tyr1;phenA3 strain revealed that TYR-requirement is satisfied by either TYR or p-hydroxyphenyl-pyruvic acid (ketonoid analogue of TYR) and not by PHE, phenyl-pyruvic acid, shikimic acid, TRY or p-aminobenzoic acid. The double mutant is inhibited by excess of either TYR or PHE and grows only at a certain ratio of the two metabolites, as is shown by the central arc of growth in Plate 1. The narrowness of the arc indicates that only a narrow ratio of concentrations of the two metabolites, within a wider range of absolute concentrations, is suitable for growth. The background growth around PHE as well as around TYR indicates that none of these requirements is absolute i.e. both phenA and tyr mutants are leaky. The arc of growth is bent towards TYR perhaps because it becomes limiting and is much less soluble than PHE. All TYR-requiring mutants (tyr1-10) behaved in the same way.

In auxanographic tests TRY inhibited both the requirements (PHE and TYR) competitively, as was evident by the competitive inhibition of the leaky growth around PHE (due to the leakiness of a tyr strain) and around TYR (due to the leakiness of a phenA strain).

Assay of relative requirements for TYR and PHE of a phenA;tyr double mutant.

The auxanographic test suggested that a phenA;tyr double mutant grows optimally at a narrow range of ratios of the concentration of PHE to that of TYR. In order to get an idea of those concentrations, about 20 conidia per dish of a bil,tyr1;phenA3 strain were plated on media with different relative concentrations of PHE and TYR and the growth of colonies on different dishes was compared after 48 hrs. of incubation.

Table 10

Growth of a bil,tyr1;phenA3 strain at different relative concentrations of tyrosine and phenylalanine

Final concentration in the medium: PHE \ TYR	M/400	M/800	M/1600	M/3200
M/400	✣ ✣ ✣ ✣	✣ ✣ ✣	✣ ✣	-
M/800	✣ ✣ ✣ ✣	✣ ✣ ✣ ✣	✣ ✣	-
M/1600	✣ ✣ ✣ ✣	✣ ✣ ✣ ✣	✣ ✣ ✣	✣
M/3200	✣ ✣ ✣ ✣	✣ ✣ ✣ ✣	✣ ✣ ✣	✣ ✣

N.B. Number of ✣s represent the degree of growth and sporulation. (✣✣✣✣ = wild type growth).

As shown in Table 10, optimal growth of a tyrI;phonA double mutant was obtained with the concentration of TYR between M/400 and M/800 and of PHE between M/800 and M/3200. M/3600 of PHE is the minimum concentration to support optimal growth of a phon single mutant.

Complementation between different tyrosine-requiring mutants.

All ten tyrosine-requirers isolated in the bil;phonA3 strain were phenotypically similar. Heterokaryons and diploids were synthesised between the strains tyr8,nabal;v3; phonA3 (obtained from a cross bil,tyr8;phonA3 x pibol,prol, nabal,ad20,bil;v3) and bil,tyrn;phonA3 (where n is the isolation number 1 to 10) and were found to grow like a (tyr$^+$) wild type on MM + TYR + limiting PHE but like a mutant (tyr) on MM + limiting PHE. tyr mutants 1-10, therefore, are allelic to one another and define a single locus which is hereafter called the tyrA locus. Most of the isolates were further confirmed to be mutants at the same locus, by analysing appropriate crosses.

Location of tyrosine-requiring mutants (tyrA) in a linkage group by mitotic haploidisation and test of translocation.

A heterozygous diploid was synthesised between 'Master strain F' and bil,tyrA8;phonA3. It was haploidised on FPA,

the segregants were classified and tabulated (Table 11). The results show that the locus *tyrA* is in linkage group I because *tyrA8* did not recombine with markers on that "chromosome" (*bil* and *y*). It also shows that the strain *bil,tyrA8;phenA3* is free of reciprocal translocations.

Table 11

Location of tyrA8 by mitotic haploidisation on FPA and test of translocation

Segregation of markers in 85 haploids isolated from the diploid:-

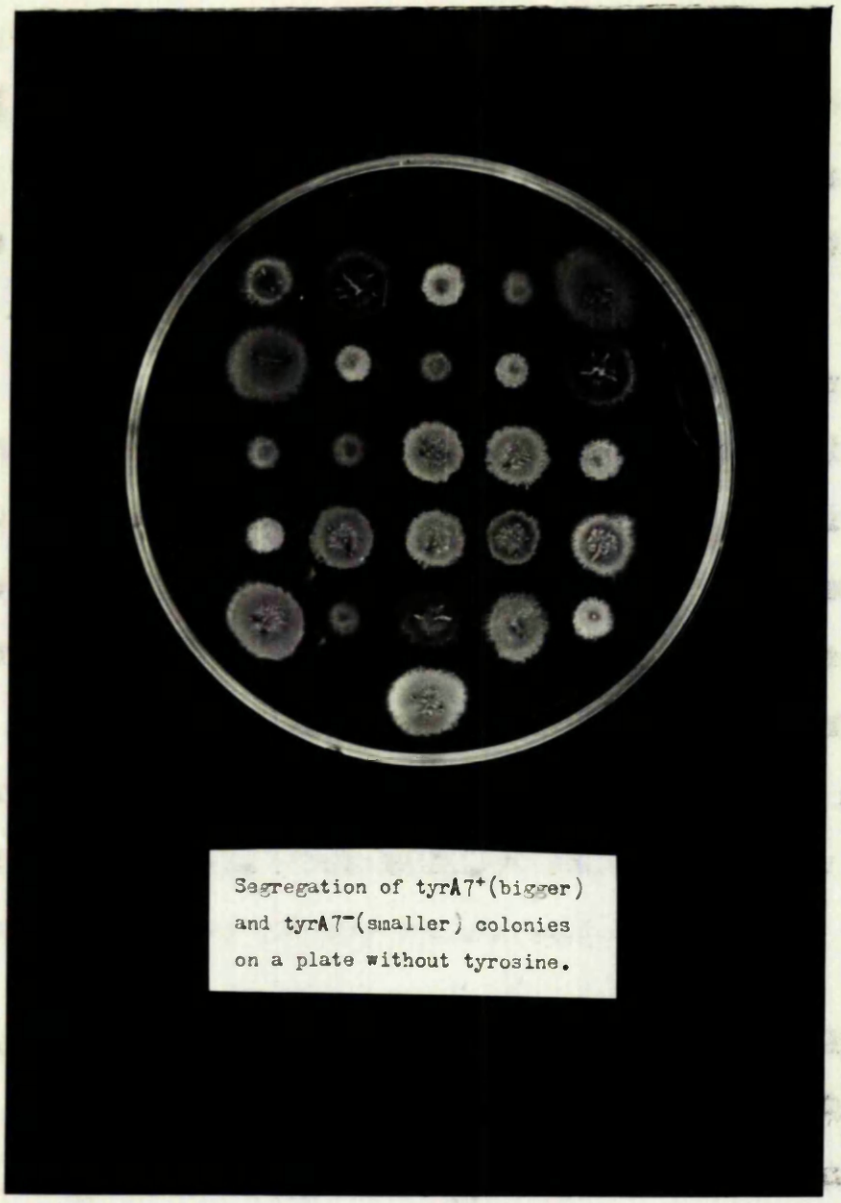
I	II	III	IV	V	VI	VII	VIII	?
sul, y, ad20, ✣, ✣, ✣, bil	Acr1	gal1	✣, phenA3	pyro4	facA303	s3	nic8	rib02	✣, tyrA8

	Parentals		Recombinants	
	✣✣	▬▬	✣▬	▬✣
y;Acr1	25	18	27	15
y;gal1	0	33	52	0
y;pyro4	25	14	27	19
y;facA303	32	17	20	16
y;s3	25	13	27	20
y;nic8	36	20	14	13
y;rib02	24	25	28	8
Acr1;gal1	0	14	40	0
Acr1;pyro4	18	19	22	26
Acr1;facA303	21	18	19	27
Acr1;s3	20	20	20	25
Acr1;nic8	26	20	14	25
Acr1;rib02	14	27	26	18

Table 11 (continued)

	Parentals		Recombinants	
	++	--	+-	-+
gal1;pyro4	0	41	0	44
gal1;facA303	0	37	0	48
gal1;s3	0	40	0	45
gal1;nic8	0	34	0	51
gal1;ribo2	0	53	0	32
pyro4;facA303	26	19	18	22
pyro4;s3	23	19	21	22
pyro4;nic8	26	16	18	25
pyro4;ribo2	17	27	27	15
facA303;s3	24	16	24	21
facA303;nic8	29	15	19	22
facA303;ribo2	22	27	26	10
s3;nic8	26	15	19	25
s3;ribo2	20	28	25	12
nic8;ribo2	19	21	32	13
bil;phonA3	33	0	0	52
bil;tyrA8	33	52	0	0
tyrA8;phonA3	33	0	0	52

	Parentals		Recombinants	
	+-	-+	++	--
tyrA8;y	33	52	0	0
tyrA8;Acz1	18	25	15	27
tyrA8;gal1	33	0	0	52
tyrA8;pyro4	14	25	19	27
tyrA8;facA303	17	32	16	20
tyrA8;s3	13	25	20	27
tyrA8;nic8	20	38	13	14
tyrA8;ribo2	25	24	8	28
phonA3;y	33	0	52	0
phonA3;Acz1	45	0	40	0
phonA3;gal1	85	0	0	0
phonA3;pyro4	41	0	44	0
phonA3;facA303	37	0	48	0
phonA3;s3	40	0	45	0
phonA3;nic8	34	0	51	0
phonA3;ribo2	53	0	32	0



Segregation of tyrA7^{+}(bigger) and tyrA7^{-}(smaller) colonies on a plate without tyrosine.

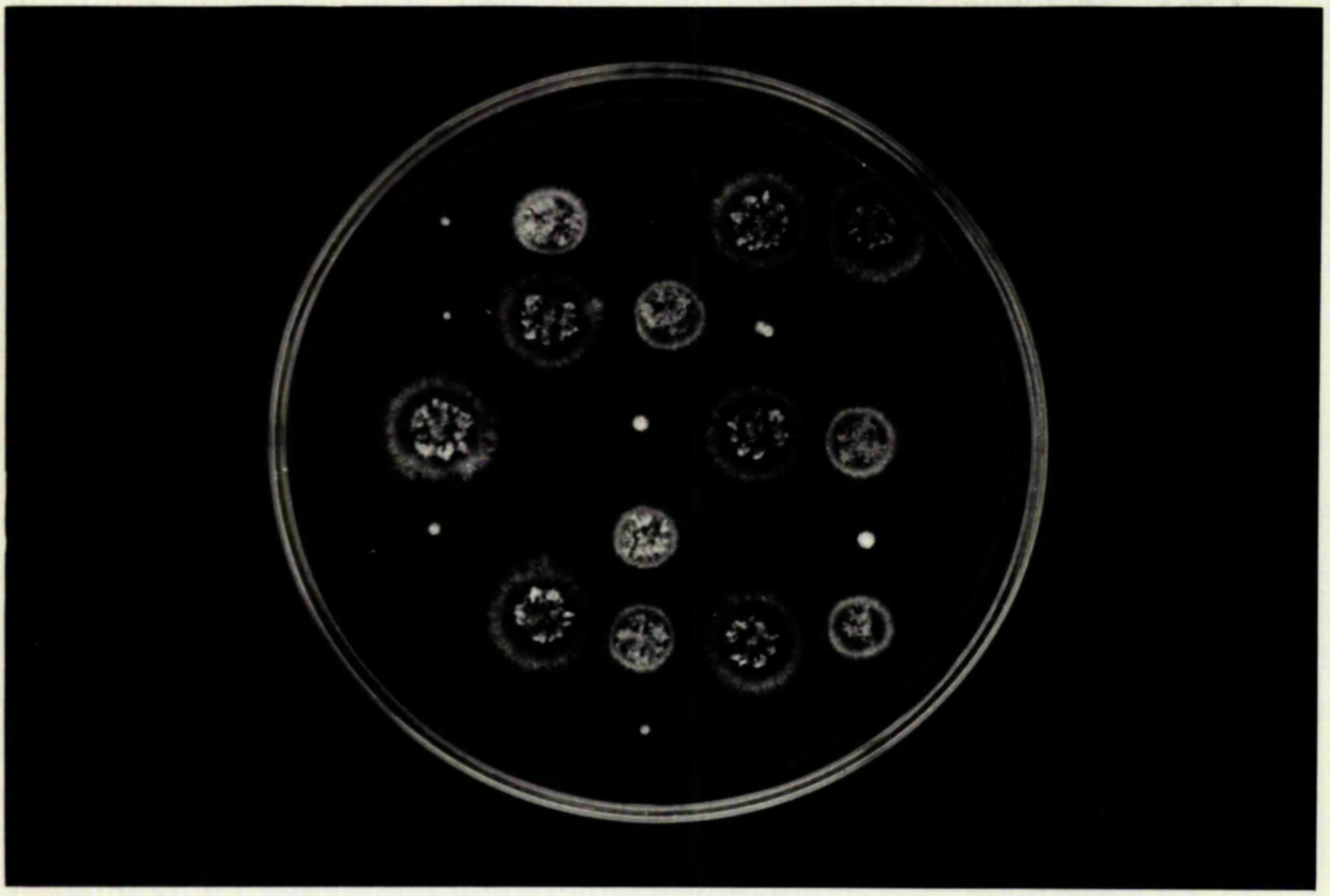
Plate - 2.

Haploid sectors were selected on CM + TYR + FPA. As both TYR and FPA are inhibitory to phenA strains, only phenA3^{+} segregants were recovered. Since phenA and gal1 loci are in the same linkage group (III), a selection for phenA^{+} segregants would automatically select only gal1 segregants (and not gal1^{+}), because the two markers were in trans in the heterozygous diploid.

Distinction between TYR-requirers and non-requirers was difficult in the presence of PHE in the medium. Scoring for segregants at tyrA locus was, therefore, done both in the presence and absence of added PHE. As shown in Plate 2, in the absence of PHE-requirement, TYR-requirers form colonies smaller than those formed by non-requirers. Besides, the TYR-requirers look whitish from the back of the plate and have a button-like, sharp edged, compact colony habit.

Growth patterns of tyrA;phenA , tyrA;+ , +;phenA and +;+ strains on different supplements.

In order to separate the phenA3 and tyrA7 mutations, a bil,tyrA7;phenA3 strain was crossed to y;w2;arg1. Colonies from single ascospores from a hybrid perithecium were replicated on media with different supplements. As it was difficult to distinguish tyrA and tyrA^{+} colonies on a medium with PHE, the master plates were replicated with a multiple



Segregants from a cross

bi1,tyrA7;phenA3 X y;w2;arg1

on a medium without tyrosine and phenylalanine.

phenA3;tyrA7 = Not growing (↗)
phenA3; + = Very small colonies
+ ;tyrA7 = Colonies of intermediate size
+ ; + = Large colonies

Plate.- 3

(26) wire replicator on a medium devoid of TYR and PHE. On such a plate 4 types of colonies corresponding to the 4 types of segregants: phenA3;tyrAZ , tyrAZ;+ , +;phenA3 and +;+ were distinguishable (Plate 3).

Comparison of the growth rates and patterns of four strains: (1) bil, (2) y;phenA3, (3) y;tyrAZ (recovered from the cross bil,tyrAZ;phenA3 x y,w2;arg1) and (4) bil, tyrAZ; phenA3 when plated (Table 12) or mass-inoculated (Table 13) on MM + different supplements, revealed that (1) phen A3 cannot grow on TYR but it becomes slightly more leaky in the presence of TYR, (2) phenA3 is inhibited by excess of TYR and tyrAZ is inhibited by excess of PHE, and (3) tyrAZ is very leaky.

Table 12

Relative growth and sporulation about 72 hours after plating about 30 conidia per plate

MM + Supplements \ Strains	bil	y;phenA3	y,tyrAZ	bil,tyrAZ; phenA3
nil	-	-	+	-
Bl	+ + + +	-	+ +	-
PHE	-	+ + + +	+	-
TYR	-	-	+ + + +	-
Bl + PHE	+ + + +	+ + + +	+ +	-
Bl + TYR	+ + + +	-	+ + + +	-
Bl + PHE + TYR	+ + +	+	+ + + +	+ + + +
Bl+limiting PHE + TYR	+ + +	-	+ + + +	-

No. of +s represent the degree of growth and sporulation.

Table 13

Colony diameter in mm. - 72 hours after mass inoculation

(an average of 4 colonies)

MM + Supplements \ Strains	bi1	y;pheA3	y;tyrA7	bi,tyrA7; pheA3
nil	11	07	35	00
B1	38	08	34	00
PHE	14	40	35	02
TYR	08	07	41	02
B1 + PHE	40	42	38	08
B1 + TYR	40	15	42	09
B1+PHE+TYR	34	28	40	34
B1+limiting PHE + TYR	35	15	42	11

Mapping tyrA mutants by meiotic analysis.

By appropriate crosses tyrA8 (cross 1), tyrA7 (cross 2), tyrA9 (cross 3) and tyrA10 (cross 4) were located very close to ribol on the left arm of linkage group X -the recombination fraction for ribol-tyrA being 0.0-1.5 %. This confirmed the results of complementation that all tyrA mutants (1 to 10) are allelic to each other and represent mutations at a single locus.

CROSS -1

$$\frac{+ \; , tyrA8, \; + \; , \; + \; , \; + \; , \; + \; , \; + \; , \; + \; , phenA3}{ribol, \; + \; , anl, prol, ad20, bil, Acrl, v2, \; +}$$

Only phen$^+$ segregants were selected.

Distribution of markers of linkage group I.

		tyrA8				+				
		ribol		+		ribol		+		
		anl	+	anl	+	anl	+	anl	+	
prol	ad20	0	0	3	11	32	2	0	0	48
	+	0	0	15	15	16	1	0	0	47
+	ad20	0	0	1	4	2	1	0	0	8
	+	0	0	7	55	20	6	0	0	88
		0	0	26	85	70	10	0	0	191

Distribution of markers of linkage group II.

	Acrl	+	
v2	79	20	99
+	23	69	92
	102	89	

Allele ratios:-

	tyrA8	ribol	anl	prol	ad20	Acrl	v2
+ allele	80	111	95	96	137	89	92
- allele	111	80	96	95	56	102	99

The allele ratio of ad20 is disturbed.

Recombination fractions:-

tyrA8	- ribol	=	0.0 %
tyrA8	- anl	=	18.84 ± 2.82 %
tyrA8	- prol	=	38.21 ± 3.51 %
tyrA8	- ad20	=	32.46 ± 3.38 %
anl	- prol	=	30.89 ± 3.33 %
anl	- ad20	=	39.79 ± 3.53 %
prol	- ad20	=	28.79 ± 3.27 %
Aeyl	- v2	=	22.51 ± 3.02 %

Conclusion: tyrA8 is very closely linked to ribol.

cross -2

$$\frac{+\ ,\ tyrA7,\ +\ ,\ +\ ,\ +\ ,\ bil,\ phenA3}{ribol,\ +\ ,\ adl^{+},\ prol,\ y,\ +\ ,\ +}$$

Distribution of segregants:-

				✣				bll				
				✣		y		✣		y		
				✣	phon A3	✣	phon A3	✣	phon A3	✣	phon A3	
		adlb	prol									
			✣									
	zibol		prol									
		✣	✣									
tyrA7			prol			12	4	4				20
		adlb	✣					6	1			7
	✣		prol	2		13	4	1				20
		✣	✣		1	9	2	38	11			61
		adlb	prol			35	7	5	1	4	2	54
			✣	2		4		9	1			16
	zibol		prol			6	1	1			1	9
		✣	✣			1		9	4			14
✣			prol									
		adlb	✣									
	✣		prol				1					1
		✣	✣			1			1			2
				4	1	81	19	73	19	4	3	204

Allele ratios

	tyrA7	ribol	ad14	pro1	bil	y	phenA3
+ allele	96	111	107	100	105	97	162
- allele	108	93	97	104	99	107	42

The allele ratio of phenA3 is disturbed.

Recombination fractions:

tyrA7 - ribol = 1.47 ± 0.84 %

tyrA7 - ad14 = 25.98 ± 3.07 %

tyrA7 - pro1 = 35.3 ± 3.34 %

tyrA7 - bil = 41.67 ± 3.45 %

tyrA7 - y = 37.75 ± 3.4 %

bil - y = 5.9 ± 2.72 %

Conclusion: tyrA7 is very closely linked to ribol.

CROSS -3

$$\frac{+}{ribol,} \; , \frac{tyrA9,}{+} \; , \frac{+}{ad14,} \; , \frac{+}{lul,} \; , \frac{+}{y,} \; , \frac{bil,}{+} \; ; \frac{phenA3}{+}$$

only ad14$^+$, lul$^+$; phenA3$^+$ recombinants were selected by selective plating.

Segregation of ribol, tyrA9, bil and y:-

		+		tyrA9		
		+	ribol	+	ribol	
+	+	0	3	0	0	3
	y	0	31	18	0	49
bil	+	0	17	31	0	48
	y	0	1	2	0	3
		0	52	51	0	103

Allele ratios:

	tyrA9	ribol	bil	y
+ allele	52	51	52	51
- allele	51	52	51	52

Recombination fractions:

tyrA9 - ribol = 0.0 ± 0.0 %

tyrA9 - bil = 34.95 ± 4.7 %

tyrA9 - y = 38.83 ± 4.8 %

bil - y = 5.82 ± 2.3 %

Conclusion: tyrA9 is very closely linked to ribol.

Cross -4

$$\frac{+\ ,\ tyrA10,\ +\ ,\ +\ ,\ +,bil;phonA3}{ribol,\ +\ ,adl4,lul,\ y,\ +\ \ +}$$

Only adk$^+$,lul$^+$;phonA3 recombinants were recovered by selective plating.

Segregation of ribol,tyrA10,bil and y:

		+		tyrA10		
		+	ribol	+	ribol	
+	+	0	1	3	0	4
	y	0	22	19	0	41
bil	+	0	20	28	0	48
	y	0	0	2	0	2
		0	43	52	0	95

Allele ratios:

	tyrA10	ribol	bil	y
+ allele	43	52	45	52
- allele	52	43	50	43

Recombination fractions:

tyrA10 - ribol = 0.0 ± 0.0 %

bil - y = 6.31 ± 2.49 %

Conclusion: tyrA10 is very closely linked to ribol.

DISCUSSION

The results support the idea that there are two pathways for TYR synthesis in *A. nidulans*; one through the shikimic acid pathway and the other by the hydroxylation of PHE; and that *tyrA* mutants are blocked in the shikimic acid pathway and not in the PHE-hydroxylation step. *tyrA* mutants are leaky because of the still functioning other pathway which allows almost 50 % of normal growth. The fact that it is difficult to distinguish between *tyrA* and *tyrA*$^+$ colonies on a PHE-containing medium, supports the suggestion that *tyrA* mutants are blocked in the shikimic acid pathway. The fact that a *phenA;tyrA* double mutant does not grow at all on a medium devoid of PHE and TYR, strengthens this contention and further suggests that in both *phenA* and *tyrA* mutants the metabolic blocks are at homologous positions in the shikimic acid pathway. Thus, *phenA* mutants are leaky probably because the corresponding enzyme in the TYR pathway is slightly non-specific. *phenA;tyrA* double mutants do not grow at all on MM because there is no endogenous synthesis of either PHE or TYR. They are able to grow on PHE because it can be converted (hypothetically by hydroxylation) to TYR

and thus both PHE and TYR requirements can be satisfied. On the other hand they do not grow on TYR alone, indicating thereby that TYR cannot be converted to PHE in A. nidulans. tyrA mutants grow on either TYR or p-hydroxyphenylpyruvic acid. Therefore, the metabolic block appears to be between prephenic acid and p-hydroxyphenylpyruvic acid. A block before prephenic acid would result in a multiple requirement or in a requirement for PHE only.

In A. nidulans phenA mutants are competitively inhibited by leucine at high concentrations (Pontecorvo -personal communication) or TYR or TRY and tyrA mutants are competitively inhibited by PHE or TRY. Similarly, arginine-requiring mutants are competitively inhibited by lysine and viceversa. In the case of LYS - ARG, possibly the inhibition operates against uptake or utilisation of the growth factors (Pontecorvo, 1952). Various naturally occurring amino acids have been found to inhibit the growth of certain amino acid - requiring mutants of N. crassa (Brockman, 1964). Amino acids of a particular 'family' are more inhibitory to mutants requiring the member or members of their own group than to mutants requiring members of another group. On the basis of studies with a variety of micro-organisms, it is now commonly believed that such a type of inhibition is due to a competition for a common site -mainly the site of entry

into the cell. In most organisms there is a specific transport system for transferring aromatic amino acids from the medium into the cell (Britten and McClure, 1962; Ames, 1964; Brockman, 1964; Stadler, 1966; Wiley and Matchett, 1966). Studies with amino acid analogue resistant mutants throw some light on such phenomena and are the subject of Section VII of this thesis.

SUMMARY

1. Two possible pathways for TYR synthesis in *A. nidulans* have been proposed.

2. Ten allelic *tyrA* mutants, blocked in the shikimic acid pathway (probably lacking prephenate dehydrogenase activity) have been isolated after NTG treatment of *bil;phenA3* conidia.

3. *tyrA* locus has been found to be very closely linked (0.0 - 1.5 % recombination fraction) to *ribol* on the left arm of linkage group I.

4. *tyrA* mutants have been found to be competitively inhibited by PHE or TRY, and *phenA3* mutant has been found to be competitively inhibited by TYR or TRY.

V NON LEAKY TYROSINE-REQUIRING STRAINS

General Considerations:

All tyrA (1-10) mutants are only partial TYR requirers, as one would expect if only one of the two pathways for TYR synthesis were blocked in them. In fact they were obtained by the device of limiting the presumed substrate or precursor (PHE) of the alternative pathway. This in itself could be taken to indicate that tyrA mutants are blocked in the shikimic acid pathway and a part of the TYR requirement of the organism is met by the conversion of PHE to TYR. Starting with a tyrA mutant, therefore, it should be possible to obtain, by a further mutation, an absolute TYR-requirer (non-leaky) blocked in both the pathways for TYR synthesis. The second metabolic block, in such an absolute TYR requirer, would be in the conversion of PHE to TYR. So far genetic studies of this hydroxylation step have been carried out only in mice and man (Garrod, 1923; La Du et al., 1958; Woolf, 1963) and not in micro-organisms which are much more suitable for various types of genetic and biochemical analysis.

Isolation of an absolute tyrosine-requiror from a partial tyrosine-requiring strain.

NTG treated bil,tyrA7 (a recombinant obtained from the cross bil,tyrA7;phenA3 x ribol,adl4,prol,v)conidia were grown on MM + biotin + TYR and replicated on MM + biotin + TYR and MM + biotin, using the velvet replica plating technique. The colonies that grew on the former medium but not on the latter were isolated, purified and retested for their requirements. Thus, four non-leaky TYR-requirers were isolated out of 64,000 colonies tested (Table 14)

Table 14

Isolation of absolute tyrosine-requirers by replica plating after NTG treatment of bil,tyrA7 conidia

Experiment number	Percentage survival	Total No. of colonies tested	Absolute tyrosine-requirers recovered	
			Total	Isolation no. ø
A	33.0	12,000	2	tyr(1) and tyr(2)
B	31.0	8,000	none	-
C	27.0	8,500	1	tyr(3)
D	25.0	15,000	none	-
E	25.0	12,500	1	tyr(4)
F	24.0	8,000	none	-

ø Thus the presumed genotype of these isolates will be bil,tyrA7;tyr(n) where n=isolation number of new mutant.

Phenotypes of the segregants recovered after outcrossing a bil,tyrA7;tyr(n) strain (with respect to tyrosine requirement)

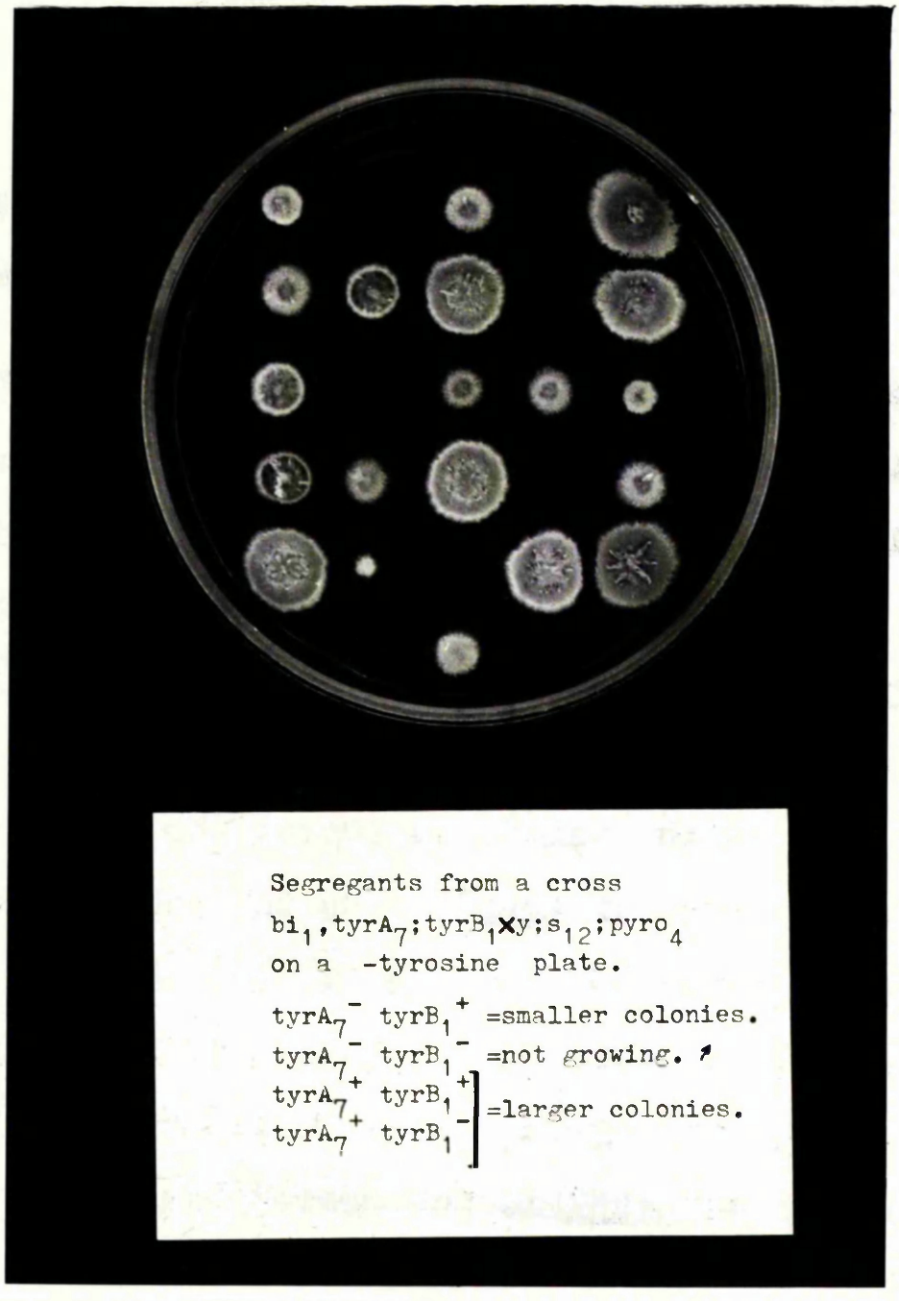
A cross was made between strains bil,tyrA7;tyr(2) and ribol,nd14,lul,y to separate the mutants responsible for TYR-requirement, expecting that absolute requirement is due to two mutations at different loci, each showing a partial TYR-requirement, when separated from the other. Colonies from single ascospores from a hybrid perithecium were replicated on media supplemented with different supplements. Segregation of TYR-requirement with respect to ribol was found to be as given below-

Total number of colonies analysed = 205

ribo 1 = 103 — growing like a wild type even without TYR.

ribol^{+} = 102 — 49 = absolute TYR-requiring; 53 = partial TYR-requiring

As ribol and tyrA7 were in trans in the cross and the two loci are very tightly linked, the great majority (say 99 %) of the ribo^{+} colonies must carry the tyrA7 allele and the great majority of the ribol colonies must carry the tyrA7^{+} allele. Thus, one can deduce the genotypes of the above segregants to be as follows -



Segregants from a cross

$bi_1, tyrA_7; tyrB_1 \times y; s_{12}; pyro_4$

on a -tyrosine plate.

$tyrA_7^-$ $tyrB_1^+$ =smaller colonies.

$tyrA_7^-$ $tyrB_1^-$ =not growing.

$tyrA_7^+$ $tyrB_1^+$

$tyrA_7^+$ $tyrB_1^-$ =larger colonies.

Plate - 4.

<u>ribol</u> = 103	<u>tyrA7</u>$^+$; <u>tyr(2)</u> <u>tyrA7</u>$^+$; <u>tyr(2)</u>$^+$	Two indistinguishable classes if <u>tyr(2)</u> by itself is so leaky as to overlap the wild type.

<u>ribol</u>$^+$ = 102	49 = <u>tyrA7</u>; <u>tyr(2)</u> = absolute TYR requirers. 53 = <u>tyrA7</u>; <u>tyr(2)</u>$^+$ = partial TYR requirers.

The proportions of the two phenotypes in the <u>ribol</u>$^+$ class indicates free recombination between mutants at two distinct loci, responsible when together for the absolute TYR requirement of the original <u>tyrA7</u>; <u>tyr(2)</u> strain. One locus is <u>tyrA</u> and the other one is hereafter designated '<u>tyrB</u>', and the individual mutants at it with the added numerals. It is impossible to follow the segregation of <u>tyrB</u> in the presence of <u>tyrA</u>$^+$. On the other hand, out of 102 <u>tyrA</u> segregants, 49 (about 50 %) are absolute TYR-requirers (carry the <u>tyrB2</u> allele) and 53 (about 50 %) are partial TYR-requirers (carry <u>tyrB2</u>$^+$) i.e. it is possible to follow the segregation of <u>tyrB2</u> in the presence of mutants at the <u>tyrA</u> locus.

Plate 4 shows the relative degrees of growth of segregants from a cross <u>bil,tyrA7;tyrB1</u> x <u>y;pl2;pyro4</u> on a medium devoid of tyrosine. Only three phenotypes (with respect to <u>tyrA</u> and <u>tyrB</u>) are distinguishable.

Auxanographic and complementation tests of absolute tyrosine-requirers.

All four tyrA7; tyr(n) strains respond to TYR and to p-hydroxyphenylpyruvic acid but not to PHE, TRY, PABA, phenylpyruvic acid or shikimic acid.

Complementation tests showed that all four mutants are allelic, thus representing a single locus (tyrB).

Results of complementation tests

Component strains of the heterokaryon	Growing on MM	Growing on MM + TYR
y,tyrA7;arg2,tyr(1)$^{\emptyset}$ + bil,tyrA7;tyr(1)	not growing	growing
y,tyrA7;arg2,tyr(1) + bil,tyrA7;tyrB(2)	not growing	growing
y,tyrA7;arg2,tyr(1) + bil,tyrA7;tyr(3)	not growing	growing
y,tyrA7;arg2,tyr(1) + bil,tyrA7;tyr(4)	not growing	growing

$\emptyset$ This was a recombinant from a cross bil,tyrA7;tyr(1) x y;arg2,meth2.

Allocation of the tyrB locus to its linkage group by mitotic haploidisation.

A heterozygous diploid was synthesised between Master Strain F and bil,tyrA7;tyrB1. It was haploidised with FPA and the haploid segregants were classified. The results

(Table 15) show that bil,tyrA7;tyrB1 is free of reciprocal translocations and that the locus tyrB is in linkage group III.

tyrA7;tyrB1 segregants were not recovered because they are sensitive to FPA. tyrA;tyrB$^+$ segregants were readily recognisable by their growth pattern.

The results show that tyrB locus is in linkage group III because by selecting tyrB$^+$ segregants, only gall segregants were recovered. tyrB is not in linkage group I because tyrB and tyrA were separable by mitotic haploidisation even though they were in cis arrangement in the heterozygous diploid.

Table 15

Location of tyrB by mitotic haploidisation

Segregation of markers in 19 haploids isolated from the diploid:

$$\frac{\text{I}}{\text{sn1ad20,y,ad20}} \quad \frac{\text{II}}{\text{+}} \quad \frac{\text{III}}{\text{+}} \quad \frac{\text{IV}}{\text{+}} \quad \frac{\text{V}}{\text{+}} \quad \frac{\text{VI}}{\text{+}} \quad \frac{\text{VII}}{\text{+}} \quad \frac{\text{VIII}}{\text{+}} \quad \frac{?}{\text{+}}$$

$$\frac{+}{+,+,} \quad \frac{+}{+} \quad \frac{+}{\text{bil,tyrA7}}; \quad \frac{\text{Acr1}}{+}; \quad \frac{\text{gal1}}{+}; \quad \frac{\text{pyro4}}{+}; \quad \frac{\text{facA303}}{+}; \quad \frac{\text{s3}}{+}; \quad \frac{\text{nic8}}{+}; \quad \frac{\text{rib2}}{+}; \quad \frac{+}{\text{tyrB1}}$$

	Parentals		Recombinants	
	++	--	+-	-+
y;Acr1	8	0	11	0
y;gal1	0	0	19	0
y;pyro4	12	0	7	0
y;facA303	13	0	6	0
y;s3	5	0	14	0

Table 15 (continued)

	Parentals		Recombinants	
	++	--	+-	-+
y;nic8	12	0	7	0
y;ribo2	11	0	8	0
Acrl;gal1	0	11	8	0
Acrl;pyro4	5	4	3	7
Acrl;facA303	4	2	4	9
Acrl;s3	2	8	6	3
Acrl;nic8	5	4	3	7
Acrl;ribo2	6	6	2	5
gal1;pyro4	0	7	0	12
gal1;facA303	0	6	0	13
gal1;s3	0	14	0	5
gal1;nic8	0	7	0	12
gal1;ribo2	0	8	0	11
pyro4;facA303	10	4	2	3
pyro4;s3	3	5	9	2
pyro4;nic8	8	3	4	4
pyro4;ribo2	6	2	6	5
facA303;s3	3	4	10	2
facA303;nic8	8	2	5	4
facA303;ribo2	6	1	7	5
s3;nic8	4	6	1	8
s3;ribo2	1	4	4	10
nic8;ribo2	6	2	6	5
bi,tyrA7	0	19	0	0
bi;tyrB1	0	0	0	19
tyrA7;tyrB1	0	0	0	19

	Parentals		Recombinants	
	+-	-+	++	--
tyrA7,y	0	19	0	0
tyrA7;Acrl	0	8	0	11

Table 15 (continued)

	Parentals		Recombinants	
	+-	-+	++	--
tyrA7;gal1	0	0	0	19
tyrA7;pyro4	0	12	0	7
tyrA7;facA303	0	13	0	6
tyrA7;s3	0	5	0	14
tyrA7;nic8	0	12	0	7
tyrA7;ribo2	0	11	0	8
tyrB1;y	0	0	19	0
tyrB1;Acr1	11	0	8	0
tyrB1;gal1	19	0	0	0
tyrB1;pyro4	7	0	12	0
tyrB1;facA303	6	0	13	0
tyrB1;s3	14	0	5	0
tyrB1;nic8	7	0	12	0
tyrB1;ribo2	8	0	11	0

Mapping the tyrB locus by meiotic analysis

Lack of suitable markers on linkage group III and dearth of suitable recombinants stand in the way of locating tyrB locus by meiotic analysis. Another difficulty in meiotic analysis is the scarcity of hybrid perithecia which has turned out to be a feature of crosses involving the bil,tyrA7;tyrB1-4 strain as one parent. A cross with a phenA strain poses another problem i.e. classification of tyrA7,tyrB and phenA recombinants. However, tyrB and phenA mutants are not allelic because the strains bil,tyrA7;tyrB1

and tyrA7,adl4,lul,y;phonA3 (isolated from the cross bil,tyrA7; phonA3 x ribol,adl4,lul,y) complement in diploids as well as in heterokaryons. In addition, the growth responses of tyrA;tyrB and tyrA;phonA strains are also different - the former requires only TYR and the latter both TYR and PHE for its optimal growth.

However, tyrB is not linked to arg2 as is shown from the following results:-

cross:-

$$\frac{\text{ribol}^{+}}{+}\ ,\ \frac{+}{\text{tyrA7}}\ ,\ \frac{\text{adl4}^{+}}{+}\ ,\ \frac{\text{lul}^{+}}{+}\ ,\ \frac{\text{y}^{+}}{+}\ ,\ \frac{+}{\text{bil}}\ ;\ \frac{\text{arg2}^{+}}{+}\ ,\ \frac{+}{\text{tyrB1}}$$

312 ribol^{+}, adl4^{+}, lul^{+},bil^{+} colonies were recovered by selective plating and the following segregation of tyrA7,tyrB1 and arg2 was observed.

	tyrA7		
	tyrB1	+	
arg2	46	101	147
+	57	108	165
			312

Conclusions: Viability of tyrB is poor and tyrB and arg2 loci are unlinked (recombination fraction = 44.37 ± 4.4 %).

Sparing effect of tyrosine on phenylalanine requirement.

If a part of PHE synthesised by *A. nidulans* is converted to TYR, PHE-requiring mutants should grow like a wild type on suboptimal concentrations of PHE if supplemented by TYR. In order to test this, conidia of *biI;phenA1* strain were plated on MM + suboptimal concentrations of PHE (minimal optimal concentration = M/3600) + varying concentrations of TYR. Growth and sporulation of colonies on different dishes was compared after 48 hours of incubation (Table 16).

Table 16

Growth and sporulation of a *biI;phenA*$_2$ strain at different relative concentrations of phenylalanine and tyrosine

Concentration of supplements in the medium		Growth and sporulation
PHE	TYR	
	nil	+ + + + + +
	M/3600	+ + + + +
	M/1800	+ + + +
M/18000	M/1200	+ + +
	M/900	+ +
	M/750	-
	M/480	-

Table 16 (continued)

M/9,000	nil	+ + + + + + + + + + +
	M/3600	+ + + + + + + + + + + + +
	M/1,800	+ + + + + + + + + + + + + +
	M/1,200	+ + + + + + + +
	M/900	+ + + + + + +
	M/750	+ + + + + +
	M/480	+ + + + +

Number of +s represent the degree of growth and sporulation.

As shown in the lower half of the Table, at lower concentration TYR spares a part of PHE requirement but at higher concentrations it is inhibitory. This sparing effect of TYR is not evident in an auxanographic test.

Biochemical studies with tyrosine-requiring mutants.

(a) General considerations:- As pointed out earlier, mutants with the characteristics of tyrB were expected on the assumption that they should be unable to convert PHE to TYR, presumably because of an absence of PHE-hydroxylase activity. The assumption was that this enzyme is present in tyrA mutants and is the cause of their leakiness. On the other hand the absence of this enzyme

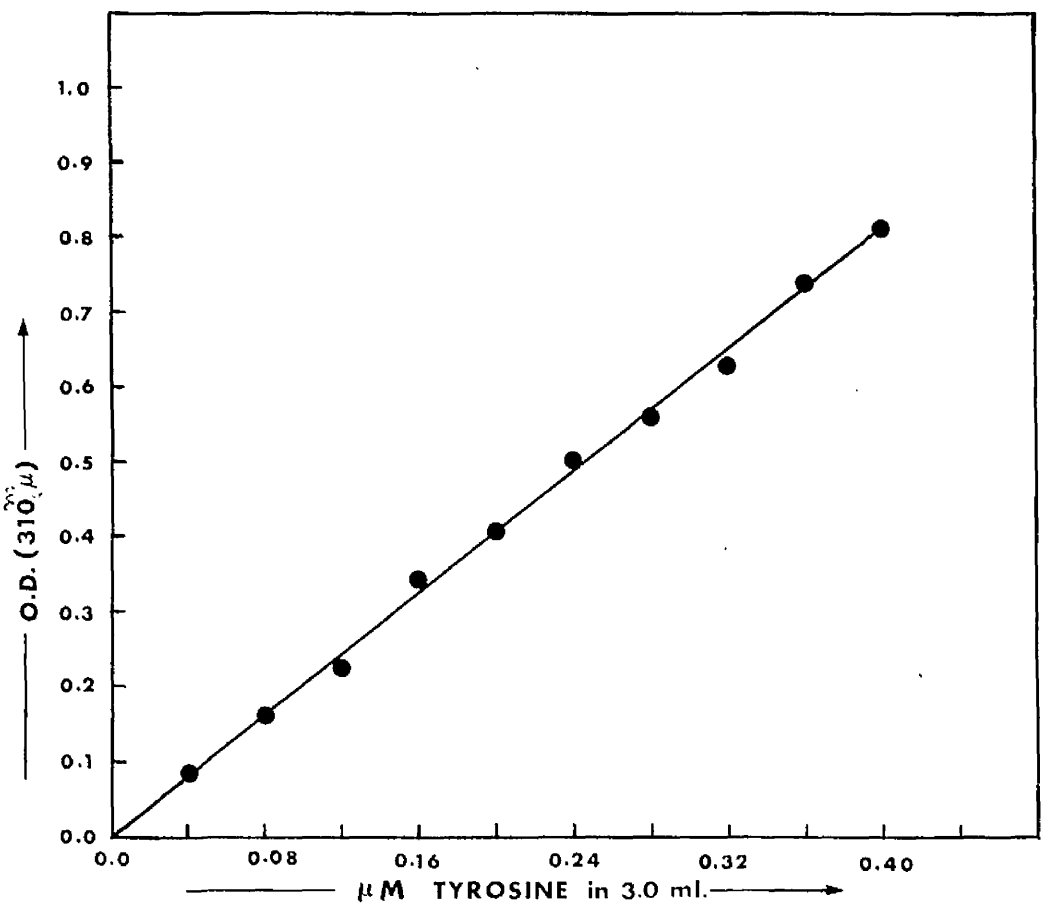

1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
O.D. (310 mμ)
0.0
0.08
0.16
0.24
0.32
0.40
μM TYROSINE in 3.0 ml.

FIG.-4

alone (as, e.g. in tyrA^{+};tyrB strains) seems to have so little effect that the two alternative phenotypes are not distinguishable. In order to test the various possibilities one can follow the conversion of different presumed precursors of TYR (p-hydroxyphonylpyruvic acid and PHE) to TYR in cell-suspensions and cell-free crude enzyme extracts. For this one needs a reasonably sensitive method to assay TYR.

(b) Assay of tyrosine:- Kaufman's (1957) method of TYR estimation by iodination in alkaline solution has been followed. In this method a suitable amount of TYR solution is pipetted in a Beckman cuvette containing 0.4 ml. of un-neutralised 2M Tris and 0.05 ml. of 0.1 M iodine solution containing 2.5 gms. of Potassium iodide per 100 ml. The mixture is allowed to stand for 2 min. at room temperature after which 0.2 ml. of 0.1M sodium thiosulphate is added and the final volume is made up to 3 ml. with distilled water. The solution is stirred and optical density measured at 310 mμ (U.V. range) in a D.B. Spectrophotometer (Beckman). A water-blank or an untreated -TYR solution- blank or a treated -water- blank makes no noticeable difference. As shown in Fig. 4, O.D. is proportional to the tyrosine concentration. This method is sensitive enough to measure as little as 0.04 mμ

of TYR in 3 ml. of final reaction mixture. Even 100 times as much PHE does not interfere with the assay of TYR but an equal or even lesser amount of p-hydroxyphenylpyruvic acid strongly interferes with the assay. This means that by this method one can detect the conversion of PHE to TYR but not of p-hydroxyphenylpyruvic acid to TYR.

(c) Preparation of crude extracts:- Freshly harvested bil and bil,tyrA7 conidia were grown in liquid MM + biotin for about 20 hours. 750 ml. of the medium was seeded with 5-8 x 10^8 conidia and flasks were kept shaking in a Gyrotory shaker at 37°C. Hyphae were filtered on a Buchner's funnel, washed thoroughly with distilled water and homogenised (4.5 g. wet weight from one flask) in 20 ml. of 0.1M phosphate buffer pH 7.0± at half speed in a MSE homogeniser for ½ hour with almost equal weight of ballotini (Vir Tis # 16-220, from Scientific Glass) at about 4°C. The homogenate was centriguged at 500 r.p.m. to get rid of ballotini. The supernatant was then spun at 30,000 x G in a MSE 18 centrifuge at 4°C to separate the cell debris from the sap. The supernatant was then desalted by passing through a Sephadex (G - 25 coarse) column. The crude enzyme extract was collected in different fractions and usually

the fractions after the first one were tested for enzyme activities. All manipulations were done in the cold room at about 4°C.

(d) Measurement of enzyme activity:- The activity of the enzyme PHE-hydroxylase was measured by assaying the amount of TYR formed from a given amount of PHE. The reaction mixture contained the following components:

2.0 ml. of soluble system containing the enzyme.

0.4 ml. of M/20 phenylalanine = 20 μ moles.

0.4 ml. of M phosphate buffer pH 6.8 = 400 μ moles.

0.5 ml. of M/1,000 TPN NaH_2 = 0.5 μ moles.

0.3 ml. of M glucose = 300 μ moles.

0.01 ml. of glucose-6-phosphate dehydrogenase.

(In some experiments instead of the last three substances, a boiled extract of bll strain was added as a source of possible co-factors).

After incubation at 37°C with occasional stirring in open beakers for 30 minutes, the reaction was stopped by adding 3 ml. of 12 % trichloro-acetic acid to each of the reaction mixtures. In the control sets TCA was added to the reaction mixtures before adding the soluble system. The denatured protein was removed by centrifugation at about 1,000 G for 10 minutes and the amount of

tyrosine in the supernatant was assayed. The relative amounts of proteins in different preparations was determined spectrophotometrically by measuring the O.D. at 280 mμ.

(e) Results:- Table 17 gives the abilities of cell extracts of bil and bil,tyrA7 strains to convert PHE to TYR.

Table 17

Test for PHE-hydroxylase activity in crude extracts of bil and bil,tyrA7 strains

Without boiled extract of bil,	Experiment	Control	Difference (Exp-control)	
	Extract from bil	Extract from bil + TCA at 0 time		
O.D.	0.32	0.32	0.0	No PHE-hydroxylase activity in tyrA⁻ strain
Amount of TYR per 2 ml. of extract	0.16 μmole	0.16 μmole	0.0	Amount of TYR formed=nil/ml. of crude extract.
	Extract from bil,tyrA7	Extract from bil,tyrA7 + TCA at 0 time		
O.D.	0.31	0.215	0.0955	very little activity in tyrA mutant
Amount of TYR per 2 ml. of extract	0.15 μmole	0.10 μmole	0.05 μmole	Amount of TYR formed= 0.025/ml. of crude extract

*. tyrA has, if any, more hydroxylase activity than tyrA⁺.

Table 17 (continued)

With a boiled extract of bil (0.5 ml. of the extract boiled for one minute),

	Experiment	Control	Difference (Exp.-control)	
	Extract from bil	Extract from bil + TCA at 0 time		
O.D.	0.423	0.335	0.09	very little PHE-hydroxylase activity, if any
Amount of TYR per 2 ml. of extract	0.21 µmole	0.16 µmole	0.05 µmole	the amount of TYR formed= 0.025 µmole per ml. of cell extract
	Extract from bil, tyr47	Extract from bil, tyr47 + TCA at 0 time		
O.D.	0.455	0.313	0.142	more PHE-hydroxylase activity
Amount of TYR per 2 ml. of extract	0.22 µmole	0.15 µmole	0.07 µmole	amount of TYR formed= 0.035 µmole per ml. of cell extract

Table 17 (continued)

Estimation of protein in the crude extract (without any correction)

1 ml. of the crude extract was diluted to 45 ml. and 3 ml. of it was put in a cuvette.

Strain	O.D.280
bil	0.42
bil, tyrA7	0.325

∴ Ratio of bil : bil, tyrA7 = 1 : 0.774

(f) Observations:- In both experiments (whether boiled extracts were added as a source of co-factors or not) the amounts of TYR formed by extracts of a tyrA7 strain were greater than the amounts of TYR formed by the extracts of a tyrA^{+} (bil) strain; although the latter strain's extract had 1.292 times as much protein (without any correction for nucleic acid contents).

The boiled extract of a bil strain was not desalted and therefore contained TYR and its precursors. This might have resulted into the detection of some more TYR in the experiments in which boiled extracts were added to the reaction mixtures.

Preliminary experiments to test induction or repression of PHE-hydroxylase by exogenous TYR or PHE were inconclusive and need further investigation.

DISCUSSION

The experimental results presented in this section suggest that if there are two pathways for TYR synthesis in A.nidulans , tyrA mutants are not blocked in the PHE-hydroxylation pathway because they have either equal or negligibly greater PHE hydroxylase activity as compared to a wild type. This lends support to the conclusion , drawn in the previous Section , that tyrA mutants are blocked in the shikimic acid pathway. The The fact that tyrA;tyrB double mutants are exacting TYR-requirers , suggests that the block in tyrB mutants is in the alternative (other then the shikimic acid) pathway for TYR synthesis i.e. in the hydroxylation of PHE. The critical experiment would had been to show that tyrB has much less or no PHE-hydroxylase activity as compared to the wild type. But with the techniques described in this Section,no or very little PHE-hydroxylase activity was detected even in the wild type. This means that more refined and precise biochemical techniques are necessary to detect the differences

in PHE hydroxylase activities of different strains. Another difficulty is in the separation of tyrA and tyrB mutations from the strain bil, tyrA7; tyrB1-1.

The fact that tyrB mutants are not distinguishable from $tyrB^+$ in the presence of $tyrA^+$, suggests that if tyrB mutants are blocked in the hydroxylation of PHE, this is not a quantitatively important pathway in a $tyrA^+$ strain though it may become decisive in a tyrA strain. Thus it appears that shikimic acid pathway is the major pathway for TYR synthesis and when the mutants are blocked in this pathway, more PHE is converted to TYR by the hydroxylation pathway. This may be due to an increase in the activity or amount of PHE-hydroxylase or it may be simply due to an increased availability of its substrate. Mitchell has suggested that in man TYR can also be formed by the hydroxylation of phenylpyruvic acid followed by a transamination (Beadle, 1945). It is conceivable that tyrA mutants -blocked in the shikimic acid pathway- after prephenic acid- accumulate some precursor (perhaps prephenic acid) which can be metabolised to over-synthesise phenylpyruvic acid or PHE or both, which could be converted to TYR.

As expected on the basis of PHE hydroxylase activity, although TYR has no growth promoting activity for phenA

mutants, at lower concentrations it (TYR) spares a part of the PHE requirement of a *pheA* mutant. It is assumed that generally a metabolite which has no growth promoting activity for a given mutant, but which spares that mutant's requirement, does so because it is an essential metabolic product of the compound which the mutant requires (Brockman, 1964).

Thus, although there is no direct evidence, all facts suggest that *tyrB* mutants lack PHE-hydroxylase activity.

SUMMARY

1. Four absolute TYR-requiring strains have been isolated by further mutations in a bil.tyrA7 strain.

2. These four mutants have been found to be allelic to each other and to represent an independent locus, designated tyrB.

3. The locus tyrB has been assigned to the linkage group III and has been found to be meiotically unlinked to asn2.

4. It has been suggested that tyrB mutants perhaps lack the PHE-hydroxylase activity.

5. Preliminary biochemical experiments have suggested that in a tyrA strain (as compared to tyrA^{+}) more PHE is converted to TYR.

VI para-FLUOROPHENYLALANINE RESISTANCE OF PARTIAL TYROSINE-REQUIRERS (*tyrA*)

General considerations:

Morpurgo (1962) has reported that some of the eleven (exact number not mentioned) allelic FPA resistant mutants of *A. nidulans*, isolated by him, have a partial requirement for TYR or PHE and all of them are selectively inhibited by indole. Later on it has been found that they are also inhibited by aminotyrosine + phenylanthranilic acid (Morpurgo and Volterra, 1966). De Palma and Morpurgo (1963) mapped *pfp-1** only 0.2 unit proximal to *riboI* on the left arm of the first linkage group. Warr and Roper (1965) isolated *pf-21** and located it 0.3 units from *riboI* but this mutant has no requirement. McCully (1964) has isolated a number of FPA resistant mutants, two of which (designated here as *fpA1* and *fpA12*) map near *riboI* and others (one mutant at *fpB* and two at *fpD* locus) are unlinked to *riboI*.

Since *tyrA* mutants map at about the same location as FPA resistant mutants isolated and mapped by earlier workers near *riboI* and some of the FPA-resistant mutants turn out to be partial TYR or PHE requirers (Morpurgo, 1962), it seemed

* p-fluorophenylalanine resistant mutants are designated as *pfp* mutants in Morpurgo's lab., as *pf* mutants in Roper's lab. and as *fp* mutants in this lab.

likely that both of these phenotypic expressions are determined by the same locus. In order to explore this possibility, tyrA mutants were tested for PPA-resistance and some more PPA-resistant mutants were isolted and the requirements of those that were linked to ribol were determined. Furthermore tyrA , fpA1, fpA12 and other newly isolted PPA resistant mutants linked to ribol were tested for allelism against each other. The results of these and some other relevant experiments are presented in the following pages.

tyrA mutants are PPA resistant.

As shown in Table 16, the bil strain is sensitive whereas tyrA1 , tyrA7 , tyrA6, fpA1 and fpA12 mutants are resistant to four different concentrations of PPA to almost the same extent. A bil,tyrA7;phenA3 strain was found to be PPA sensitive and none of the 103 ribol segregants from the cross ribol,adl4,lul,y x bil,tyrA7;tyrB2 (described on page 71) were found to be PPA resistant. As explained on page 72 of this thesis, half of those segregants must carry tyrB mutation. This indicates that tyrB mutants are not PPA resistant.

Table 18

Diameter of colonies (in mms.) 72 hours after mass inoculation of conidia of different strains on MM + different concentrations of FPA + required supplements (a mean of four measurements).

Strains tested	concentration of FPA in the medium (v/v)			
	0.00625%	0.0125%	0.025%	0.05%
bil	4.0	3.5	2.0	2.0
tyrA1,y	24.0	23.5	23.5	23.0
tyrA7,bil	23.5	24.0	23.0	22.5
tyrA8,bil	24.0	23.5	23.0	23.0
fpA1,bil	24.5	24.5	23.5	24.0
ribo1,fpA12, bil	24.5	24.0	24.0	23.0

Isolation of FPA resistant mutants closely linked to ribo1 and their growth requirements.

FPA resistant strains of A. nidulans were isolated by point inoculating bil (green) conidia on MM + biotin + 0.05 - 0.1 % FPA (final concentration of FPA in the medium -

At those concentrations of FPA wild type colonies grew spidery and very slowly. In order to avoid starting from a clone each inoculum was derived from a different wild type (bil) colony. After 10-12 days of incubation, fast growing and well sporulating sectors developed from some of the inoculated points. Conidia from one such sector from each such original inoculum were touched by a needle and subcultured on MM + biotin. Each of 12 such isolates were purified by a single colony isolation and tested for FPA resistance.

Each of the 12 FPA resistant isolates (designated fp46-57) were tested for their TYR requirements and were crossed to a ribol,ad14,lu1,y strain to find out any linkage of FPA resistance to ribol. As shown in Table 19, for 10 strains the mutant determining FPA-resistance was found to be closely linked to ribol and 9 out of these 10 strains turned out to be partial TYR-requirers. Further investigations with other two strains (fp48 and fp56) are reported in the next Section.

Table 19

Linkage of fp46-57 to ribol

(Only ribol$^+$,ad14$^+$,lu1$^+$,bil$^+$ recombinants were selected by

Table 19 (continued)

plating the ascospores from hybrid perithecia on MM and the segregation of FPA resistance was followed)

Isolate	Growth response to FUR	Results of a cross *ribo1,ad14,lu1,y* × *bi1,fpX*$^+$		
		Number of segregants analysed	Number of *ribo*$^+$,*fpX*$^+$ recombinants obtained	% age recombination between *ribo1* and *fpX*
bi1,fp46	+	206	0	0
bi1,fp47	+	208	0	0
bi1,fp48	-	208	95	45.7 ± 3.45
bi1,fp49	+	198	1	0.5 ± 0.5
bi1,fp50	-	187	0	0
bi1,fp51	+	200	3	1.5 ± 0.86
bi1,fp52	+	203	0	0
bi1,fp53	+	204	0	0
bi1,fp54	+	208	0	0
bi1,fp55	+	206	0	0
bi1,fp56	-	201	103	51.2 ± 3.52
bi1,fp57	+	205	0	0

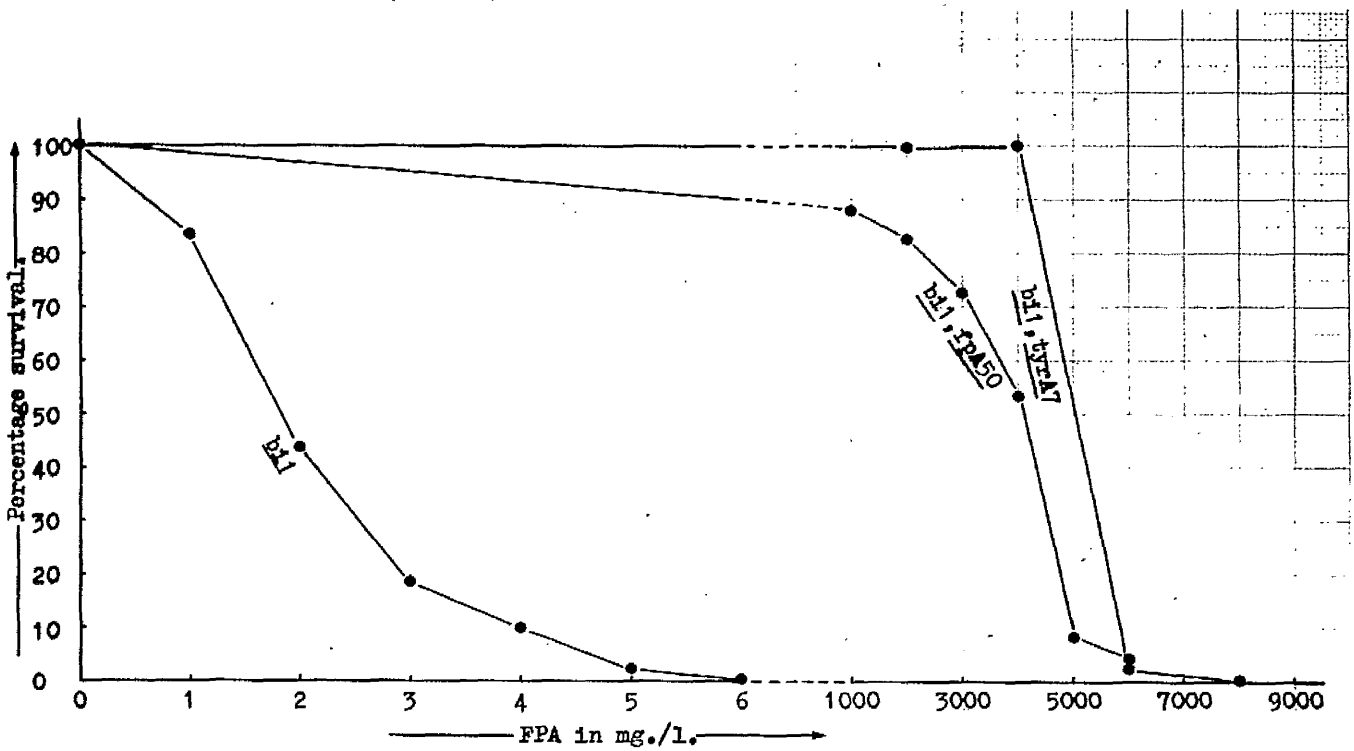
+) X = isolation number 46 to 57.

Tests for allelism between tyrA, fpA and freshly isolated FPA resistant mutants closely linked to ribol.

Diploids were synthesised between the strain y,fpA1; pabal and each of the following 13 strains:- (1) bil,tyrA7; phenA3, (2) bil,ribol,fpA1, (3) bil,ribol,fpA12, (4) bil, fp46, (5) bil,fp47, (6) bil,fp49, (7) bil,fp50, (8) bil, fp51, (9) bil,fp52, (10) bil,fp53, (11) bil,fp54, (12) bil,fp55 and (13) bil,fp57. All were found to be resistant to FPA. On the other hand diploids (bil,tyrA7; phenA3 + MSF) and (bil,tyrA7;phenA3 + y,fpB37,pabal,ad14) were found to be sensitive to FPA.

The results show that tyrA7 and fpB37 are recessive in heterozygous diploids and they are not allelic to each other On the other hand tyrA7 is allelic to fpA1 (fpA mutants are recessive -McCully, 1964). It further showed that FPA resistant mutants that are closely linked to ribol (isolated so far) are allelic to fpA1, provided they are recessive.

Thus, all tyrA mutants tested so far, are FPA resistant but the converse is not always true; there are fpA mutants which are not partial TYR requirers e.g. strains bil,fpA50 , bil,fpA12 , pf -21 (Warr and Roper, 1965) and some of the 11 allelic mutants of Morpurgo (1962).



Percentage survival of conidia on MM+biotin+different concentrations of FPA after 48 hrs. of incubation at 37°C.

Degrees of FPA resistance of tyrA7 and fpA50 as compared to a wild type strain (bil).

In order to compare the degrees of FPA resistance of tyrA7 (partial TYR-requiring mutant at fpA locus) and fpA50 (a mutant at fpA locus which has no requirement) mutants as compared to a FPA sensitive bil strain, freshly harvested conidia of (1) bil, (2) bil,tyrA7 and (3) bil,fpA50 strains were plated on MM + biotin + different concentrations of FPA and at each concentration of FPA the perdentage survival (as revealed by the number of colonies visible after 48 hours of incubation) was calculated.

As shown in Table 20 and 21 and Fig. 5, 5-6 mg./l of FPA is inhibitory to a bil (FPA-sensitive) strain whereas mutants at fpA locus are inhibited to the same extent only by about 1000 times as much FPA. And a partial TYR-requiring strain is more resistant to FPA than a non-requiring strain.

Table 20

Percentage survival of bil conidia on different concentrations of FPA

No. of conidia plated per dish, estimated from haemocytometer counts = 120 ± 11. 0.1 ml. of conidial suspension was plated per dish.

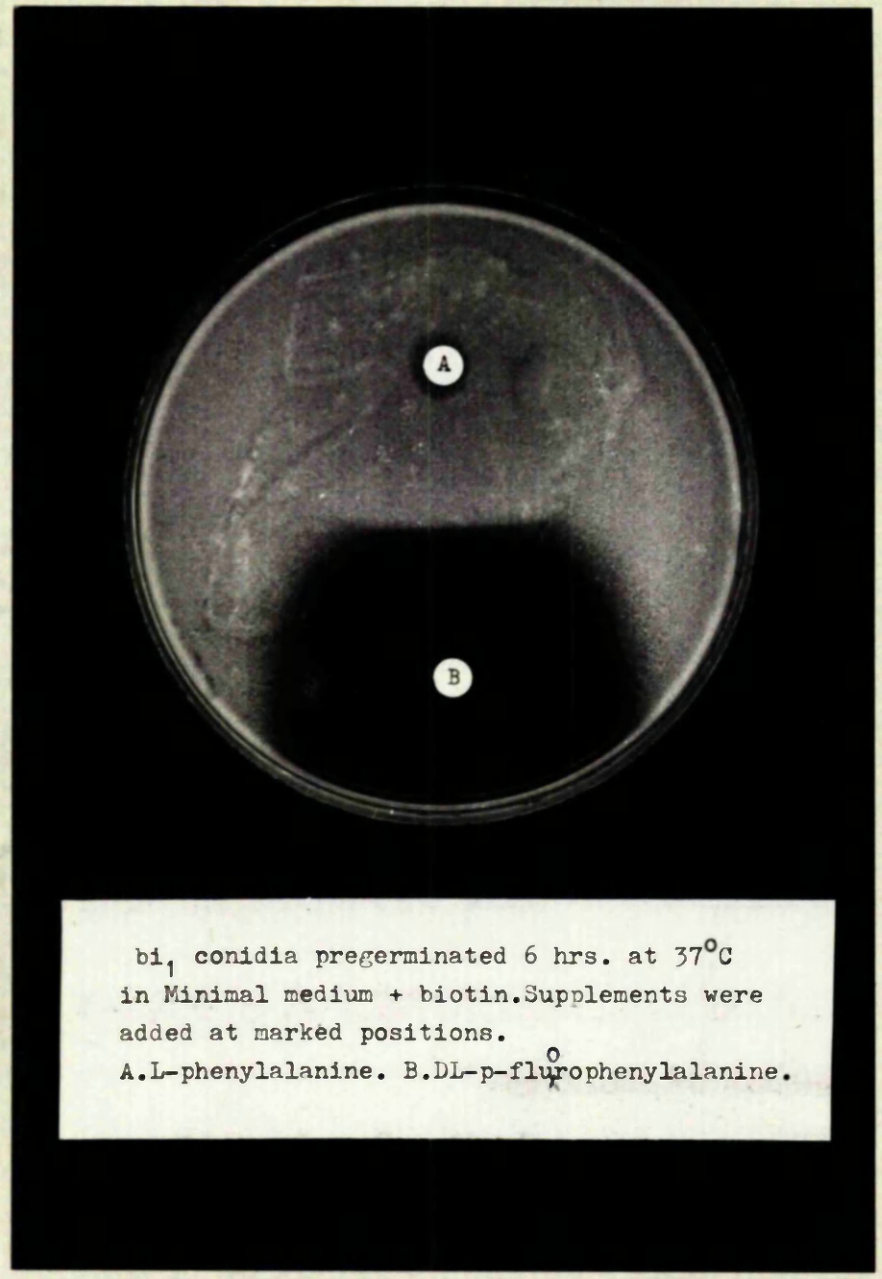
Table 20 (continued)

Concentration of FPA in mg/l	Total no. of colonies on four dishes	Percentage survival
0.0	418	100.00
1.0	340	83.68
2.0	183	43.78
3.0	78	18.66
4.0	42	10.05
5.0	10	2.39
6.0	1	0.24
7.0	0	0
8.0	0	0
9.0	0	0
10.0	0	0

Table 21

Percentage survival of bil.tyrA7 and bil.fnA50 conidia on different concentrations of FPA when 151 ± 12 and 100 ± 10 conidia were plated respectively.

0.1 ml. of conidial suspension was plated per dish and density of the suspension was estimated from haemocytometer counts.



bi_1 conidia pregerminated 6 hrs. at 37°C in Minimal medium + biotin. Supplements were added at marked positions.
A. L-phenylalanine. B. DL-p-fluorophenylalanine.

Plate - 5.

Table 21 (continued)

Concentration of FPA in mg/l	bil,tyrA7		bil,fpA50	
	Total number of colonies on four dishes	Percentage survival	Total number of colonies on four dishes	Percentage survival
nil	559	100.0	361	100.0
1,000	-	-	317	88.0
2,000	556	99.46	298	82.5
3,000	-	-	263	72.5
4,000	558	99.82	194	53.7
5,000	-	-	30	8.3
6,000	13	2.33	15	4.16
7,000	1	0.18	-	-
8,000	-	-	-	-
9,000	-	-	-	-
10,000	0	0	-	-

Efficiency of phenylalanine and other compounds in reversing the inhibitory effects of FPA.

An auxanographic test of a bil strain indicated that the inhibition of growth of A. nidulans by FPA is competitively reversed by PHE (Plate 5). Phenylpyruvic acid also was found to be quite effective in reversing the inhibitory effect of FPA but its action was not competitive. TYR, TRY, p-hydroxyphenylpyruvic acid and anthranilic acid also were slightly effective only when the relative concentration of FPA was very low.

DISCUSSION

Partial TYR requiring mutants at tyrA locus are resistant to FPA. In fact they are allelic to fpA mutants isolated by McCully (1964) and during the present investigation. They are probably also allelic to pfp-r mutants of Morpurgo (1962) and pf-21 mutant of Marr and Roper (1965) because they all map at about the same position in linkage group I; very closely linked to ribol. However, only direct crosses and tests of allelism between the mutants obtained in this laboratory and the others could give a definite answer.

Antimetabolites have been very often used for the isolation of mutants producing increased amounts of metabolites(Adelberg,1958; Scherr and Rafelson,1962). Studies on enzyme regulation in branched biosynthetic pathways in bacteria have shown that overproduction of one end product metabolite might lead to a reduction in the rate of production of the common intermediate to a level below that needed for

the optimal biosynthesis of another end product metabolite - especially in the biosynthesis of amino acids of Aspartic acid 'family' and of aromatic amino acids (Cohen, 1965). On this basis, FPA resistant mutants at the fpA (= tyrA) locus in A. nidulans could well be resistant because of an accumulation or overproduction of PHE due to a derepression (or loss of feedback inhibition) of some enzyme specific for PHE synthesis. The partial TYR requirement could then be a consequence of too much diversion of common precursors towards PHE synthesis. This hypothesis however makes it difficult to explain the growth responses of phenA;tyrA and tyrA;tyrB double mutants. On the other hand, all the observations are well explained on the basis of the hypothesis that tyrA (= fpA) mutants are blocked in the shikimic acid pathway, and that this is one of the two alternative pathways for the biosynthesis of TYR in A. nidulans.

TYR-requiring mutants of E. coli (Huang, 1964) and Mc. glutamicus (Nakayama et al., 1961), blocked in the shikimic acid pathway, are known to accumulate up to 2 g/l of l-PHE when growing under optimal conditions. In fact these mutants are used for industrial production of PHE. Conceivably, tyrA mutants of A. nidulans too, accumulate or overproduce PHE due to a block in the shikimic acid pathway

of TYR synthesis, as a consequence of which they are simultaneously FPA resistant. The observation, that a *fpA; phenA* double mutant is perfectly viable but not resistant to FPA, supports this possibility.

If *tyrB* mutants are deficient in PHE hydroxylase activity, they also might accumulate or overproduce PHE, thus making these strains FPA resistant. But they are FPA sensitiv perhaps because the hydroxylation of PHE is a minor pathway in TYR synthesis and is not very active in a *tyrA*$^{+}$ strain.

All *tyrA* mutants (tested so far) are FPA resistant but all *fpA* mutants are not partial TYR requirers. Most of the *fpA* mutants (9 out of 10 isolated during the present investigation) are partial TYR requirers and one which is partial TYR requirer is more resistant to FPA than the one which does not require TYR for its optimal growth. Thus, it appears that partial TYR requirers have presumably lost the ability to synthesise an active enzyme for which *fpA* (= *tyrA*) locus is responsible whereas little or non-requirers (not detectable) (but still FPA resistant and, by hypothesis, overproducing PHE) have only a partial defect thus producing an enzyme which has reduced efficiency. In other words, in *fpA* mutants which apparently do not require TYR for their optimum growth, the requirement for TYR is below the level of detection.

The observation that if the medium is supplemented with aminotyrosine (an analogue of TYR) and phenylanthranilic acid (an analogue of anthranilic acid) at a concentration of 0.01 and 0.047 % (w/v) respectively, all the fpA mutants (whether they require TYR or not) are totally incapable of growth (Morpurgo, Sermonti, Petrelli and Ricci, in Calvori and Morpurgo, 1966) again suggests that all fpA mutants represent metabolic blocks in TYR synthesis -whether partial or complete. Marr and Roper's (1965) observation of suppression of nic8 by pf-21 can also be explained if we assume that pf-21 is a mutant in the shikimic acid pathway of TYR synthesis and thus results in an accumulation of some common precursor -perhaps chorismic acid- from where the pathways of PHE, TYR, TRY, PABA, p-hydroxybenzoic acid and nicotinic acid synthesis branch in different directions.

Thus, all the circumstantial evidences support the hypothesis that in A. nidulans FPA resistance (due to a mutation of fpA locus) is due to a metabolic block in the shikimic acid pathway of TYR synthesis which results in an accumulation or overproduction of PHE which, in its own turn, competes against the antimetabolite.

SUMMARY

1. Mutants at the *tyrA* locus have been found to be allelic to mutants at the *fpA* locus.

2. Ten more mutants at the *fpA* locus have been isolated and nine of them have been found to be partial TYR-requirers.

3. *fpA* mutants have been found to be resistant to about 1000 times as much FPA as is inhibitory (5.6 mg/l) to a *bil* strain.

4. Two FPA resistant mutants unlinked to *ribol* have been isolated. Their further investigations along with the investigations of three other mutants (*fpB37*, *fpD11* and *fpD43* -isolated by McCully, 1964) are reported in the next Section.

VII RESISTANCE TO FPA DUE TO MUTATIONS AT LOCI

VII RESISTANCE TO FPA DUE TO MUTATIONS AT LOCI

OTHER THAN fpA (= tyrA)

At the time the investigation reported in this thesis was undertaken, three loci were known in *A. nidulans*, mutations at which resulted into FPA resistance (Table 22).

Table 22

FPA resistant mutants isolated by previous workers

Isolates	Reference	Linkage group	Location and other features	
11 allelic pfp-r mutants	Morpurgo, 1961; DePalma and Morpurgo, 1963	I	0.2 units proximal to *riboI* and recessive in a heterozygote	Linkage relationships indicate that they may be allelic to each other. Considered in the previous Section
pf-21	Warr and Roper 1965	I	0.3 units from *riboI* and recessive in a heterozygote	
fpA1 and fpA12	McCully, 1964 -unpublished-	I	Closely linked to *riboI* and recessive in a heterozygote	
fpB3Y	available in Glasgow stock	I	Unlinked to *biI*, *pabaI*, *riboI* and *adY*, and recessive in a heterozygote	
fpA3		VIII	Unlinked to *pacB*, *pelD*, *fhe*, *paIE* or *ribo2* -semidominant in a heterozygote	*
fpI1		III ?*	Unlinked to *phenA3*- dominant in a hetero zygote	*

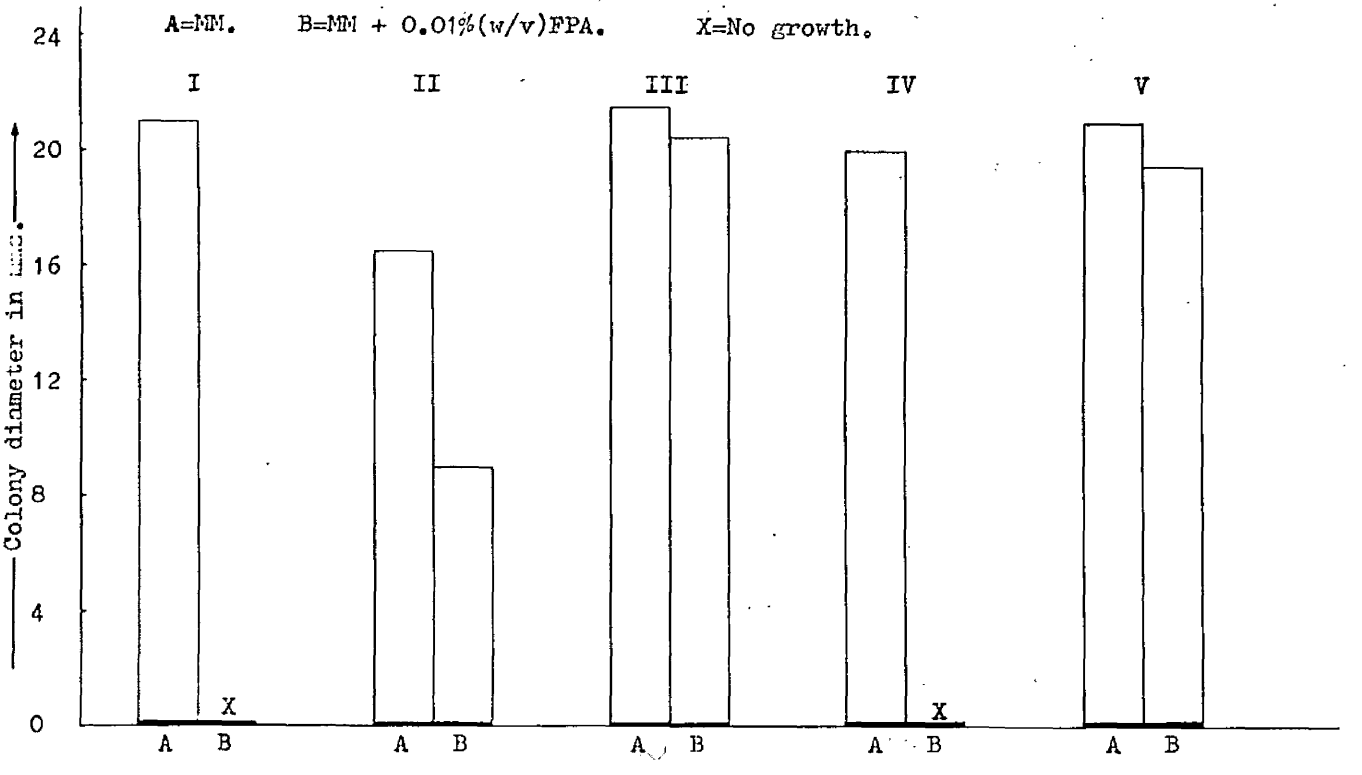
*) McCully (1964) assigned *fp43* to linkage group VIII and *fp11* to linkage group III and gave them the locus symbols C and D respectively. During the present investigation both these mutants were found to map at about the same position on linkage group VIII and provisionally the locus symbol D has been used for both of them.

As described and discussed in the previous Section, the locus *fpA* could be concerned with TYR synthesis and mutations at this locus could well lead to FPA resistance due to an accumulation or overproduction of PHE. But at the time the present investigation was started, there was no clue as to the mechanism of FPA resistance determined by mutants at *fpB* and *fpD* loci and their genetical studies too were incomplete. Besides, two more (*fp48* and *fp56*) FPA resistant mutants, unlinked to *ribol*, were isolated which needed characterisation.

Further studies with two newly isolated (*fp48* and *fp56*) and three already available (*fpB7*, *fpD43* and *fpD11*) FPA resistant mutants are reported in the following pages.

Requirements of FPA resistant mutants.

The mutant *fp48* grows very slowly either on MM or CM but grows well on both if supplemented with TRY. Nicotinic acid has no such effect. (In the CM, as prepared routinely



Colony diameter(average of four colonies) of heterozygous diploids (I-V) on MM and MM+FPA after 48 hours of incubation at 37°C.(Dominance test).

most of the MM is destroyed.

The other mutants (*fp56*, *fpB37*, *fpD43* and *fpD11*) grow optimally on MM.

Tests for allelism and dominance

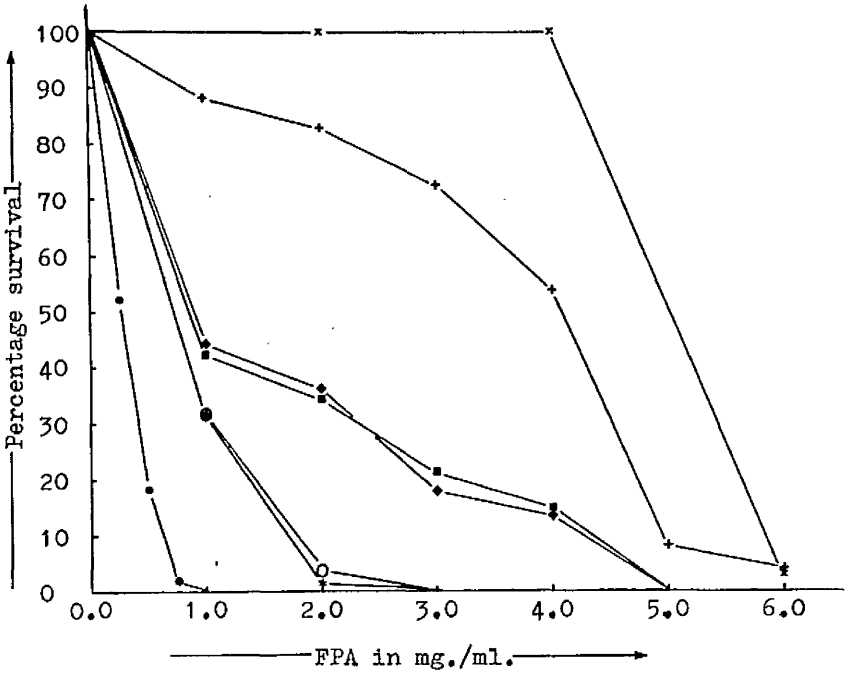
As FPA resistant mutants can be recessive, semidominant or dominant in a heterozygous diploid (McCully, 1964), tests for allelism by complementation will not give any conclusive result when one or both of the alleles in question are dominant or semidominant. In order to find out which mutations are dominant and which are recessive, heterozygous diploids (Table 23 and Fig. 6) were synthesised with each of the FPA-resistant isolates and were inoculated on MM and MM + FPA (0.01 % w/v). The colony diameters were measured after 48 hours of incubation. Fig. 6 summarises the results and indicates that *fpB37* and *fpk8* are recessive whereas *fpD43* is semidominant and *fpD11* and *fp56* are dominant.

Table 23

Genotypes of heterozygous diploids (Fig. 6)

I $\frac{fpB37,\ +\ ,y,\ +\ ,nabol,adl4;\ +}{+\ ,tyrA7,+,bil,\ +\ ,\ +\ ;phenA3}$

II $\frac{nbol,\ +\ ,+,bil,\ +\ ,\ +\ ,fpC43}{+\ ,fpA1,y,\ +\ ,pabol,adl4;\ +}$



Percentage survival of conidia of (•) bi1,ribo1,fpB37, (*)bi1;fpE48,(o)bi1,ribo1;fpD43,(♦)bi1;fpD56,(▪)bi1,ribo1;fpD11, (+)bi1,fpA50 and (×)bi1,tyrA7 on different concentrations of FPA after 48 hours of incubation at 37°C.

Fig.-7

III $\frac{ribo1,\ +\ ,+\ ,\ +\ ,b11,+,fpD11}{+\ ,fpA1,pabal,ad14,\ +\ ,y,+}$

IV $\frac{+\ ,+,b11,\ +\ ,fp48,+}{sulad20,y,\ +\ ,ad20,+,Acrl,+}; \frac{+\ }{gall}; \frac{+\ }{pyro4}; \frac{+\ }{facA303}; \frac{+\ }{s3}; \frac{+\ }{nic8}; \frac{+\ }{ribo2}$

V $\frac{+\ ,+,b11,\ +\ }{sulad20,y,\ +\ ,ad20}; \frac{+\ }{Acrl}; \frac{+\ }{gall}; \frac{+\ }{pyro4}; \frac{+\ }{facA303}; \frac{+\ }{s3}; \frac{+\ }{nic8}; \frac{fp56,\ +\ }{+\ ,ribo2}$

As the mutant fp48 was found to be recessive in a heterozygous diploid, a diploid was synthesised between the strains b11,fp48 and y,fpB37,pabal,ad14 and was found to be FPA sensitive. This suggested that fp48 is not allelic to fpB37 and therefore it was given the locus symbol "E".

The relative degrees of FPA resistance of mutants fpB37, fpD11, fpF43, fp56 and fpE48 as compared to fpA1 and fpA50 are given in Tables 21 and 24 and Fig. 7.

Table - 24.

Percentage survival of conidia of different strains on different concentrations of FPA after 48 hours of growth.

Figures in parenthesis represent number of conidia plated as estimated from haemocytometer counts.

Concentration of FPA in mg/l.	bi1,ribo1,fpB37 (600±25)		bi1;fpB48 (510±22)		bi1,ribo1;fpD11 (630±25)		bi1,ribo1;fp43 (450±21)		bi1;fp56 (580±24)	
	Total no. of colonies.	Percentage survival	Total no of colonies	Percentage survival	Total no of colonies	Percentage survival	Total no of colonies	Percentage survival	Total no of colonies	Percentage survival
nil	570	100	486	100	609	100	431	100	544	100
250	298	52.3								
500	103	18.1								
750	11	1.99								
1,000	nil	0.0	153	31.5	259	47.5	136	31.56	241	43.3
2,000			8	1.6	211	34.6	16	3.71	196	36.0
3,000			nil	0.0	131	21.5	nil	0.0	98	18.0
4,000			nil	0.0	93	15.25	nil	0.0	76	13.98
5,000					nil	0.0	nil	0.0	nil	0.0
6,000					nil	0.0	nil	0.0	nil	0.0

STUDIES WITH THE LOCUS fpE

Requirements of the mutant fpE48 for optimal growth.

In auxenographic tests the mutant fpE48 was found to respond to anthranilic acid, indole, tryptophan or kynurenine and not to shikimic acid, PABA, PHE, TYR, nicotinic acid or 3-hydroxy-anthranilic acid. It is slightly leaky so that it can grow slowly even without any supplement. In its leakiness and response to various supplements, fpE48 was found to be quite different from other mutants that are available in the Glasgow-stock and that respond to tryptophan, anthranilic acid or nicotinic acid (Table 25)

Table 25

Growth responses of nicotinic acid requiring mutants

	Strain	Response to: Anth. acid	Indole	Trypt-ophan	Kynure-nine	3-OH Anth. acid	Nicot-inic acid	Lin-kage group	Other information
Strains already available in the Glasgow Stock.	nic1	+	+	+	+	+	+		nic1, 3, 8 and 9 have the same response
	nic2	+	±	-	-	+	+	V	nic2, 4, 5, 6 and 7 have the same response
	nic8	+	+	+	+	+	+	VII	
	nic10	-	-	-			+	VI	
	nic11	+	+	+					
	nic12								
	nic13	+		+					not allelic to nic2 or nic8
	nic14	+	+	+	-	-	+		
	fpE48	+	+	+	+	-	-		isolated as a FPA resistant mutant

The mutant *fpE43* does not grow well on C.M. and produces non-sporulating cottony sectors whereas on M.M. it sporulates uniformly well and grows very slowly. 0.01% (w/v) of tryptophan either in MM or CM promotes a healthy growth (like a wild type). Auxanographic tests revealed that the growth of a *fpE* mutant on tryptophan is competitively inhibited by MMB or 7MR.

Location of *fpE*.

The chromosomal location of *fpE* was determined mitotically by the standard procedure (Forbes, 1959 and Forbes and McCully, 1965). A heterozygous diploid between *bil;fpE43* and the 'mater strain P' was synthesised. It was haploidised with PPA, the segregants were classified and tabulated (Table 26). The results show that the strain *bil;fpE43* is free of translocations and the locus *fpE* is in linkage group II.

The meiotic location of *fpE43* was done by crossing the strain *bil;fpE43* to each of the strains *bil;Acr1,w3,ab1,nil,ad3* (cross 1) and *nic1,bil;Acr1,w3,thi4,nil,ad3* (cross 2). The results of these crosses, as shown are not very conclusive because of the distortions of allele ratios and recovery of too many double cross-overs, but suggest that *fpE* locus could be located in the *nil* - *ad3* interval on the right arm of linkage group II.

Table 26

Location of ΣpE+8 by mitotic haploidisation on FPA

Segregation of markers in 37 haploids isolated from the diploid

I: $\frac{an1\,ad20}{+}$, $\frac{y}{+}$, $\frac{ad20}{+}$, $\frac{+}{bi1}$; II: $\frac{Acr1}{+}$; III: $\frac{gal1}{+}$; IV: $\frac{pyro4}{+}$; V: $\frac{facA303}{+}$; VI: $\frac{s3}{+}$; VII: $\frac{nic8}{+}$; VIII: $\frac{rib2}{+}$; ?: $\frac{+}{\Sigma pE+8}$

	Parentals		Recombinants	
	++	--	+-	-+
y;Acr1	2	23	9	3
y;gal1	10	10	1	16
y;pyro4	5	15	6	11
y;facA303	7	16	4	10
y;s3	8	12	3	14
y;nic8	2	17	9	9
y;rib2	7	10	4	16
Acr1;gal1	3	9	2	23
Acr1;pyro4	1	19	2	13
Acr1;facA303	1	16	2	16
Acr1;s3	2	12	3	20
Acr1;nic8	0	21	5	11
Acr1;rib2	3	12	2	20
gal1;pyro4	11	6	15	5
gal1;facA303	14	8	12	3
gal1;s3	18	7	8	4
gal1;nic8	9	9	17	2
gal1;rib2	20	8	6	3
pyro4;facA303	11	16	5	5
pyro4;s3	13	13	3	8
pyro4;nic8	4	15	12	6
pyro4;rib2	9	8	7	13

Table 26 (continued)

	Parentals		Recombinants	
	++	--	+-	-+
facA303;s3	12	10	5	10
facA303;nic8	7	16	10	4
facA303;ribo2	12	9	5	11
s3;nic8	7	11	15	4
s3;ribo2	14	6	8	9
nic8;ribo2	8	11	3	15
bil fpE48	23	2	3	9

	Parentals		Recombinants	
	+-	-+	++	--
fpE48;y	23	2	9	3
fpE48,Acx1	32	5	0	0
fpE48,gal1	9	3	23	2
fpE48;pyro4	19	3	13	2
fpE48;facA303	16	1	16	4
fpE48;s3	12	2	20	3
fpE48;nic8	21	0	11	5
fpE48;ribo2	12	3	20	2

Some out of 5 fpE48;nic8 colonies may be of the genotype fpE48;nic8^{+}. It is difficult to distinguish between these two types of segregants because both will grow on MM + tryptophan but none of them will grow on MM + nicotinic acid. Other segregants for nicotinic acid were classified on tryptophan and nicotinic acid -less medium. But this does

not affect unambiguous assignment of fpE locus to linkage group II.

CROSS -1

$$\frac{bil}{bil} \times \frac{Acrl.w3.abl.nl3.ad3}{+ \quad + \quad + \quad + \quad +} \; ? \quad \frac{+}{(fpE48)}$$

Segregation of markers:-

			fpE48				+				
			Acrl		+		Acrl		+		
			w3	+	w3	+	w3	+	w3	+	
abl.	nl3	ad3	1		2		15	6	11	26	61
		+	1			2	2		4	4	13
	+	ad3			1		2				3
		+	4		1	4					9
+	nl3	ad3					6	2	2	7	17
		+		2			1	2	2	2	9
	+	ad3	1		1	1	13	2	3	11	32
		+	14	2	4	12	9	4	1	8	54
			21	4	9	19	48	16	23	58	198

Allele ratios:

	fpE48	Acrl	w3	abl	nl3	ad3
+ allele	145	109	97	112	98	85
- allele	53	89	101	86	100	113

Segregation of fpE48 is disturbed.

Recombination fractions:-

Acrl	-	v3	= 26.26	± 3.13
2pl+3	-	v3	= 52.52	± 3.56
2pl+3	-	Acrl	= 53.53	± 3.54
2pl+3	-	abl	= 46.66	± 3.54
2pl+3	-	nl3	= 30.81	± 3.28
2pl+3	-	ad3	= 23.23	± 3.09
abl	-	nl3	= 19.2	± 2.71
nl3	-	ad3	= 28.8	± 3.21
abl	-	ad3	= 35.9	± 3.41

These results indicate that 2pl is on the right arm of linkage group II in the nl3 - ad3 interval.

Cross -2

bil, ribol, Acrl, v3, thl+, nl3, ✧ , ad3
bil, ✧ , ✧ , ✧ , ✧ , ✧ , 2pl+3, ✧

Distribution of markers of linkage group II.

			2pl+3				✧				
			Acrl		✧		Acrl		✧		
			v3	✧	v3	✧	v3	✧	v3	✧	
	nl3	ad3					13	10	3	11	37
thl+		✧	3	1			6	1		3	13
	✧	ad3					3	3	1	1	8
		✧	4	1	6	9	2			3	21
	nl3	ad3					13	3	1	7	24
✧		✧				1	8	3		2	14
	✧	ad3	2				8	1	2	3	16
		✧	10	3	1	11	16	7		6	54
			18	5	7	17	69	28	7	36	187

Allele ratios:

	spiA3	AcrI	w3	thi4	nl3	ad3
+ allele	140	67	86	108	99	102
- allele	47	120	101	79	88	85

Recombination fractions:

AcrI	- w3	= 25.1	± 3.17 %
spiA3	-thi4	= 52.4	± 3.65 %
spiA3	- nl3	= 39.0	± 3.35 %
spiA3	- ad3	= 31.0	± 3.38 %
thi4	- nl3	= 35.8	± 3.50 %
thi4	- ad3	= 45.5	± 3.64 %
nl3	- ad3	= 27.3	± 3.25 %

The results of this cross also indicate that the locus spiA is perhaps in the nl3 - ad3 interval on the right arm of linkage group II.

PABA and nicotinic acid requiring mutants are not resistant to PPA

In all the organisms investigated so far, the pathway for PABA, p-hydroxybenzoic acid, PHB, HMH and PMP is common up to chorismic acid (Fig. 1), from whose it branches off. It has been suggested that in A. nidulans nicotinic acid can also be synthesised from anthranilic acid via an alternative short cut as well as via indole, tryptophan, kynurenine and 3-OH-anthranilic acid (Pontecorvo, 1950; Pontecorvo et al., 1953).

A genetical block in the synthesis of PABA or p-hydroxy-benzoic acid or nicotinic acid, just after chorismic acid, may, therefore, lead to the accumulation or overproduction of PHB and thus to FPA-resistance in these mutants.

(1) bil;nic2, (2) nabn1 and (3) bil;paba22 strains were, therefore, tested for their growth (as compared to a wild type, FPA-sensitive, bil strain) on MM + 0.01% (v/v) FPA. None of these strains grew even on mass inoculation.

It was concluded that none of these strains are FPA-resistant.

Studies with the mutants fpDll, fp43 and fp56.

Relative location of fpDll, fp43 and fp56 with respect to each other:

The results of three crosses (Table 27) suggest that the three mutants fpDll, fp43 and fp56 are either allelic to each other or are located very close to one another in the same linkage group.

Table 27

Non-recovery of FPA sensitive recombinants from crosses (1) fp56 x fpDll, (2) fp56 x fp43 and (3) fpDll x fp43.

Strains involved in the crosses	Type of cross	Total number of segregants analysed	Segregation of FPA resistance and colour	
			FPA resistant: FPA sensitive	Green: yellow
bil;fp56 x pabal,ad17,y;fpDll	fp56 x fpDll	203	203 : 0	92 : 111
bil;fp56 x pabal,ad17,y; fp43	fp56 x fp43	194	194 : 0	93 : 101
bil,albol;fpDll x pabal,ad17,y; fp43	fpDll x fp43	208	208 : 0	113 : 95

Chromosomal location of fpDll.

Chromosomal location of fpDll was determined mitotically

by the standard procedure (Forbes, 1959; Forbes and McCully, 1965). A heterozygous diploid between bil,ribo1;fnDll and the tester strain 'MSF' was synthesised. Haploid segregants from this diploid were selected on CM + FPA, classified and tabulated (Table 28) to show that the strain bil,ribo1;fnDll is free of reciprocal translocations and the locus fnD is in linkage group VIII.

Table 28

Location of fnDll by mitotic haploidisation on FPA

Segregation of markers in 25 haploids isolated from the diploids:-

I II III IV V VI VII VIII ?

$$\frac{su1\,ad20,y,ad20,+,+}{+,+,+,bil,rib1} ; \frac{Acr1}{+} ; \frac{gal1}{+} ; \frac{pyro4}{+} ; \frac{facA303}{+} ; \frac{s3}{+} ; \frac{nic8}{+} ; \frac{rib2}{+} ; \frac{+}{fnDll}$$

	Parentals		Recombinants	
	++	--	+-	-+
y;Acr1	-	17	-	8
y;gal1	-	13	-	12
y;pyro4	-	13	-	12
y;facA303	-	7	-	18
y;s3	-	9	-	16
y;nic8	-	11	-	14
y;rib2	-	-	-	25
Acr1;gal1	5	10	3	7
Acr1;pyro4	6	11	2	6
Acr1;facA303	7	6	1	11
Acr1;s3	6	7	2	10
Acr1;nic8	6	9	2	8
Acr1;rib2	8	-	-	17

Table 28 (continued)

	Parentals		Recombinants	
	++	--	+-	-+
gal1;pyro4	6	7	6	6
gal1;facA303	9	4	3	9
gal1;s3	8	5	4	8
gal1;mic8	10	9	2	4
gal1;rib02	12	+	+	13
pyro4;facA303	9	4	3	9
pyro4;s3	9	6	4	7
pyro4;mic8	7	6	4	7
pyro4;rib02	12	+	+	13
facA303;s3	12	3	6	4
facA303;mic8	9	2	9	5
facA303;rib02	18	+	+	7
s3;mic8	7	2	9	7
s3;rib02	16	+	+	9
mic8;rib02	14	-	-	11
bil;rib02	25	-	-	-
bil;fpDll	-	-	25	-
fpDll;rib02	-	-	-	25

	Parentals		Recombinants	
	+-	-+	++	--
fpDll;y	-	-	-	25
fpDll;Ace1	-	8	-	17
fpDll;gal1	-	12	-	13
fpDll;pyro4	-	12	-	13
fpDll;facA303	-	18	-	7
fpDll;s3	-	16	-	9
fpDll;mic8	-	14	-	11
fpDll;rib01	-	25	-	-

Since, from the green heterozygous diploid, only yellow haploid segregants were selected, all of them carried the *ylb1*$^{+}$ allele. As the selection of haploids was made on 6M + PPA, only *fpl1* recombinants were recovered as fast growing sectors and thus the *ylb2* segregants were selected against, because both *fpl* and *ylb2* loci belong to the same linkage group (VIII) and were in *trans* in the heterozygous diploid. *fpl1* recombined with all other markers.

Mapping *fpl1*, *fp56* and *fp43* mutants by meiotic analysis.

By appropriate crosses *fpl1*, *fp56* and *fp43* were located in the *ad7 - arg3* interval in linkage group VIII. This again confirmed the earlier conclusion that these mutants are either allelic or are located very close to each other. Results of various crosses are presented herewith and summarised in Table 29.

Meiotic location of *fp43*:

Cross -1

$$\frac{bil,+,+}{+,y,pabal,ad17} \times \frac{arg3,+}{+,fp43}$$

Markers *bil*, *y*, *pabal* and *ad17* being in linkage group I, were irrelevant for the purpose of this cross, and were, therefore, not classified. The results from two hybrid

perithecia, pooled together, show the following segregation of markers of linkage group VIII.

Segregation of markers and allele ratios

	fp+3	+	
arg3	30	113	143
+	136	20	156
	166	133	299

Recombination fraction

fp+3 - arg3 = 16.7 ± 2.16 %

CROSS -2

$$\frac{bi1,+,+,+,nd17,+}{+,y,pabal,+,+,fp+3}$$

Only pabal+, nd17+ recombinants were selected.

Segregation of markers

		fp+3		+		
		nd17	+	nd17	+	
bi1	y		1			1
	+	9	71	54	21	155
+	y	6	17	11	6	40
	+	2	5	1	1	9
		17	94	66	28	205

Allele ratios:

	fp^{+}3	n17	bil	y
+ allele	94	122	49	104
- allele	111	83	156	41

Allele ratios of bil and y are disturbed because both are linked to pabal and adl7 and only pabal^{+}, adl7^{+} progeny were selected.

Recombination fractions:

fp^{+}3 - n17 = 21.95 ± 2.89 %
bil - y = 4.9 ± 1.52 %
pabal - y = 20.0 ± 2.79 %
pabal - bil = 23.9 ± 2.98 %

$$\frac{\text{bil}, +}{+, y} \times \frac{\text{CROSS -3}}{\text{I} \quad \text{II}} \times \frac{+, \text{br}^{+}2, +}{\text{n17}, +, \text{fp}^{+}3}$$

Segregation of markers of linkage group VIII.

+	br^{+}2	+	= 88	174	-Parentals
n17	+	fp^{+}3	= 86		
+	+	fp^{+}3	= 2	6	-Cross-over in interval I
n17	br^{+}2	+	= 4		
+	br^{+}2	fp^{+}3	= 9	22	-Cross-over in interval II
n17	+	+	= 13		

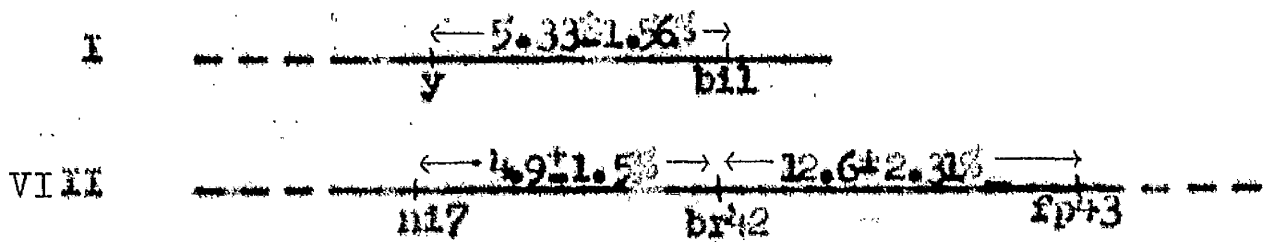
+	+	+	= 3	4	- Cross-overs in intervals I and II
ni7	br42	fp43	= 1		

Segregation of markers of linkage group I.

bil + = 90 , + y = 105 .... 195 = Parentals

bil y = 6 , + + = 5 .... 11 = Recombinants

Linkage map



Meiotic location of fp56

Cross -4

$$\frac{bil.+}{+\ ,y} \times \frac{fp56.+}{+\ ,ni7}$$

The recombinant y;ni7 was obtained from the cross

bil;ni7 x y,pabal,ad17;fp43.

Segregation of markers:-

		sp56		+		
		ni7	+	ni7	+	
bi1	y	1		5	2	7
	+	3	42	41	4	91
+	y	13	41	35	10	99
	+	2	1	2	1	6
		20	84	83	16	203

Allele ratios:

	sp56	ni7	bi1	y
+ allele	99	100	105	97
- allele	104	103	98	106

Recombination fractions

bi1 - y = 6.4 ± 1.71 %
sp56 - ni7 =17.7 ± 2.68 %

Meiotic location of fpb11

CROSS -5

bi1, +, ribol, fpb11, +
+ ,y, + + ,ni7

Segregation of markers:

			spl1		+ +		
			n17	+	n17	+	
+	bil	y	1		1		2
		+	4	14	11	3	32
	+	y	2	15	15	2	34
		+	1			1	2
+	bil	y	1	1	2		4
		+	5	20	22	6	53
	+	y	2	20	20	5	55
		+		1	2	1	4
			16	79	73	18	186

Allele ratios:

	spl1	n17	+	bil	y
+ allele	91	97	116	95	91
- allele	95	89	70	91	95

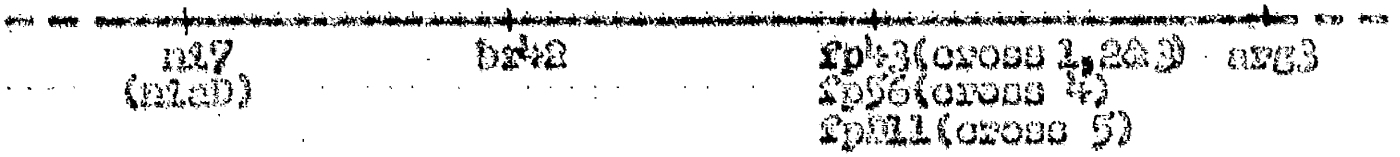
Recombination fractions:

bil - y = 6.5 ± 1.81 %
spl1-n17 = 18.3 ± 2.83 %

Table 29

Probable location of fp43, fp56 and fp111 in linkage group VIII

Only the relevant part of the chromosome has been represented here.



Cross 1 ← 16.7±2.16 % →

Cross 2 ← 21.95±2.89% →

Cross 3 ← 4.9±1.5 % → ← 12.6±2.31% →

Cross 4 ← 17.7±2.68% →

Cross 5 ← 10.3±2.03 % →

In view of the mapping results the three mutants can be provisionally assigned to a single locus fp.

If these three mutants are allelic to each other, dominance of fp56 and fpD11 in a heterozygous diploid and semidominance of fp43 under the same conditions can be explained on the basis of the incompleteness of the metabolic block in the latter case (fp43) and its completeness in fpD11 and fp56.

Interaction between nutrition and FPA-resistance determined by fpD locus.

The results of five crosses presented in Table 30, show that there is some interaction between FPA resistance (due to a mutation at fpD locus) and METH or PHE requirement in A. nidulans.

Table 30

Segregation of nutritional markers with respect to fpD locus in 5 crosses

(Figures in parenthesis are for recombinants)

Strains involved in the cross	No. of colonies from a single perithecium analysed	Pairs of markers considered	Segregants ++	+-	-+	--
bil;cnxB4 x pabaA, adA7,y;fpD11	208	fpD11-cnxB4	(63)	51	50	(44)

Table 39 (continued)

y;ad2 x bi1,ribo1;spi1	201	spi1-ad2	(61)	29	67	(4)
		spi1-ribo1	52	(39)	(66)	45
		spi1-bi1	48	(42)	(44)	67
y;arg3,meth2 x bi1,ribo1;spi1	199	spi1-arg3	(75)	47	71	(5)
		spi1-meth2	(72)	51	72	(4)
		spi1-ribo1	61	(61)	(43)	28
		spi1-bi1	51	(71)	(36)	40
y;meth2,phen42 x bi1,ribo1;spi1	208	spi1-meth2	(59)	80	51	(28)
		spi1-phen42	(60)	71	69	(0)
bi1,arg3 x pabe1,ade7, y;spi3	299	spi3-arg3	(20)	136	213	(30)

There is no interaction between PPA resistance (due to a mutation at spi locus) and nitrite, thiosulphate, riboflavine, biotin or arginine requirement. But very few spi1;meth2 and no spi1;phen42 recombinants were obtained.

Going back to the colonies recovered by plating the ascospores of a hybrid pentaploidum from the cross y;meth2,phen42 x bi1,ribo1;spi1 on M + all required growth factors, it was noticed that even after 72 hours of incubation, spi1;meth2 recombinants formed very small, badly sporulating and fluffy

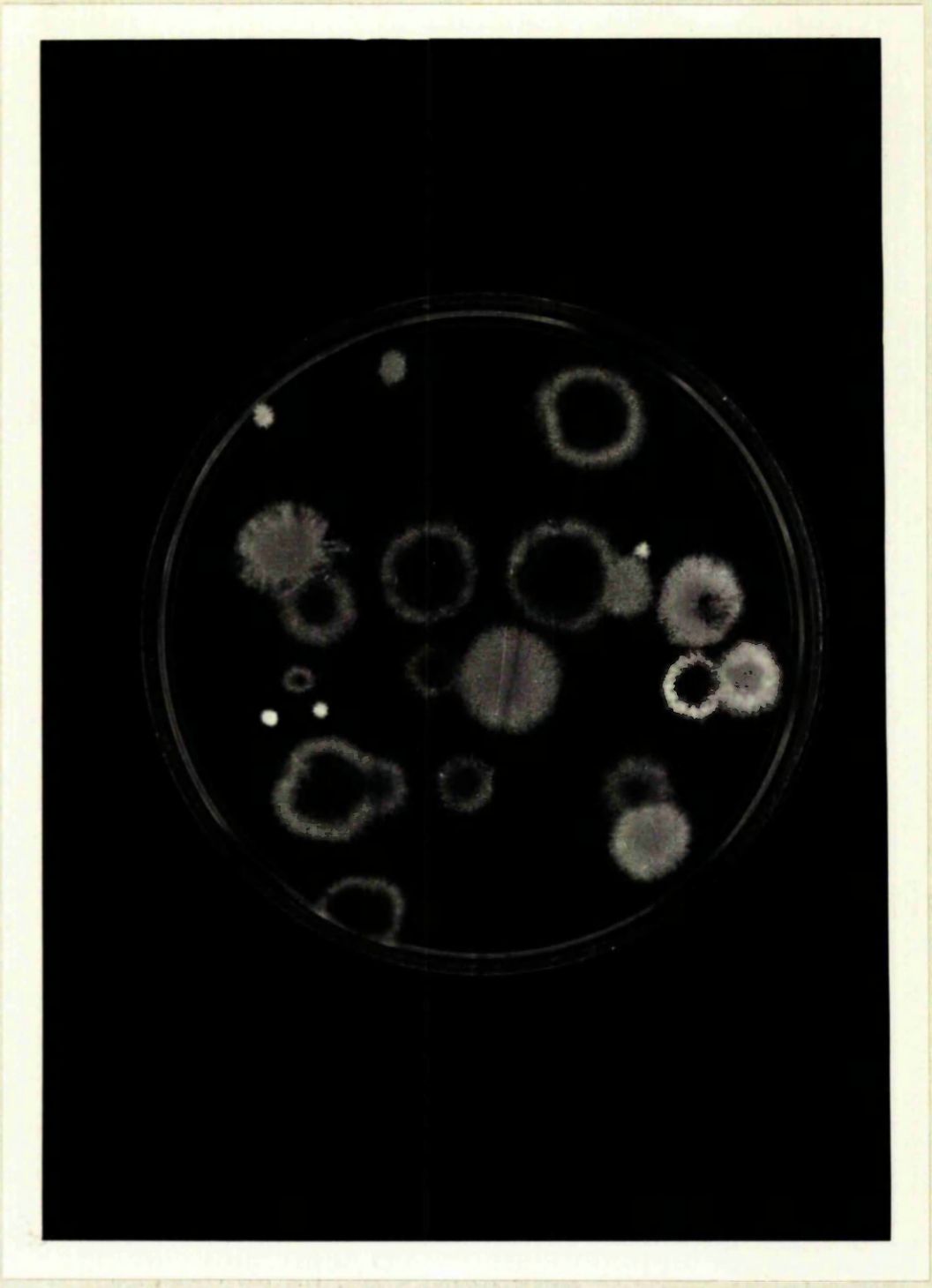


Plate-6 : Segregants from a cross y;meth2,phenA2 × bi1,ribo1;fpD11.The colonies indicated by arrows are fpD11;meth2 recombinants.

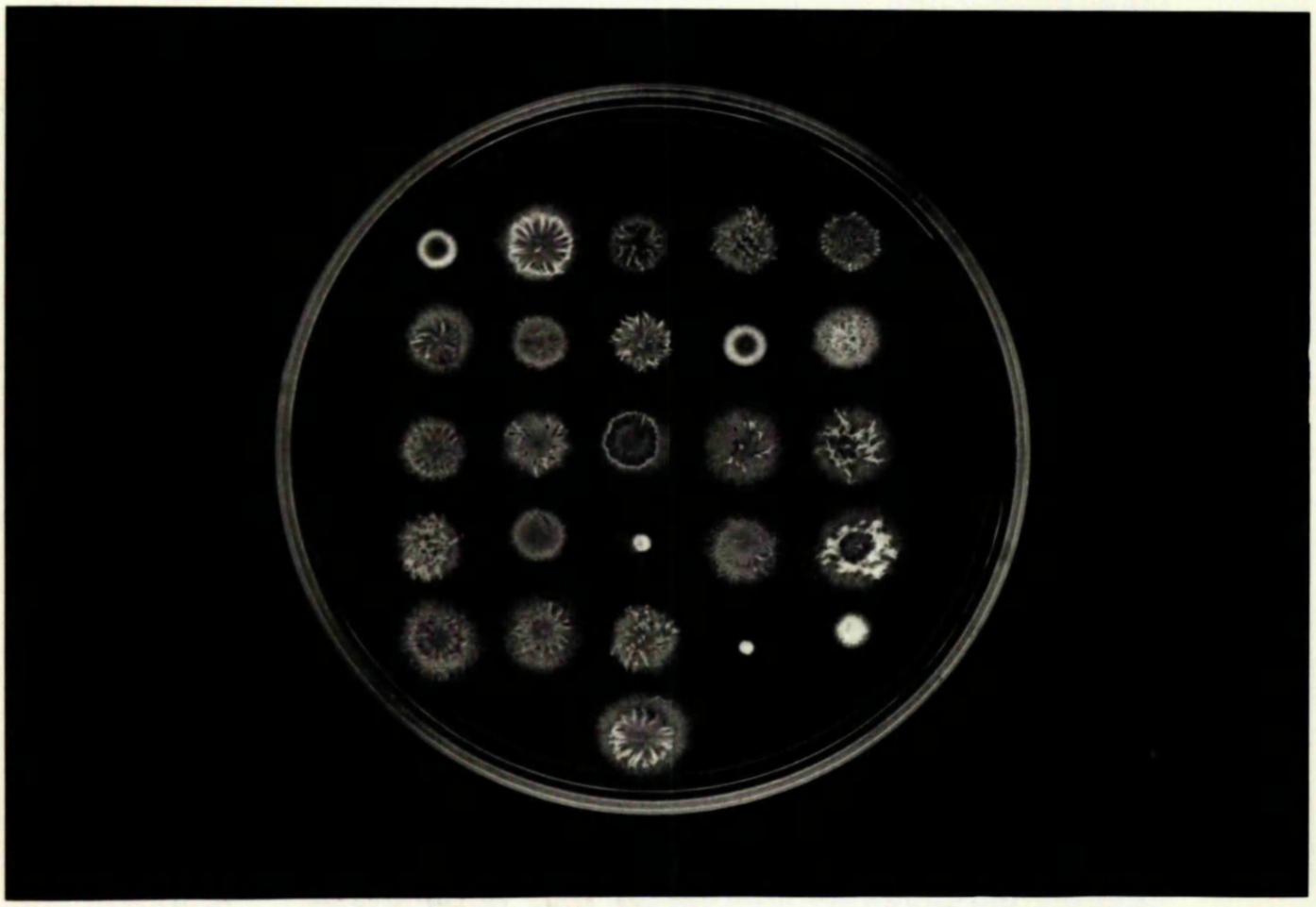


Plate-7 : A fpD11;meth2 recombinant,indicated by an arrow,producing a methionine-independant sector.

colonies (Plate 6). Even on master plates (Plate 7) such colonies were morphologically distinguishable and very often produced well-sporulating and well growing sectors (Plate 7) which turned out to be methionine independent and PPA resistant. Perhaps poor growth, poor sporulation and difficulty in transferring to the testor medium of *thr1;metH2* recombinants, tend to eliminate them at each of the successive steps of master plate preparation, transfer to the testor medium and scoring.

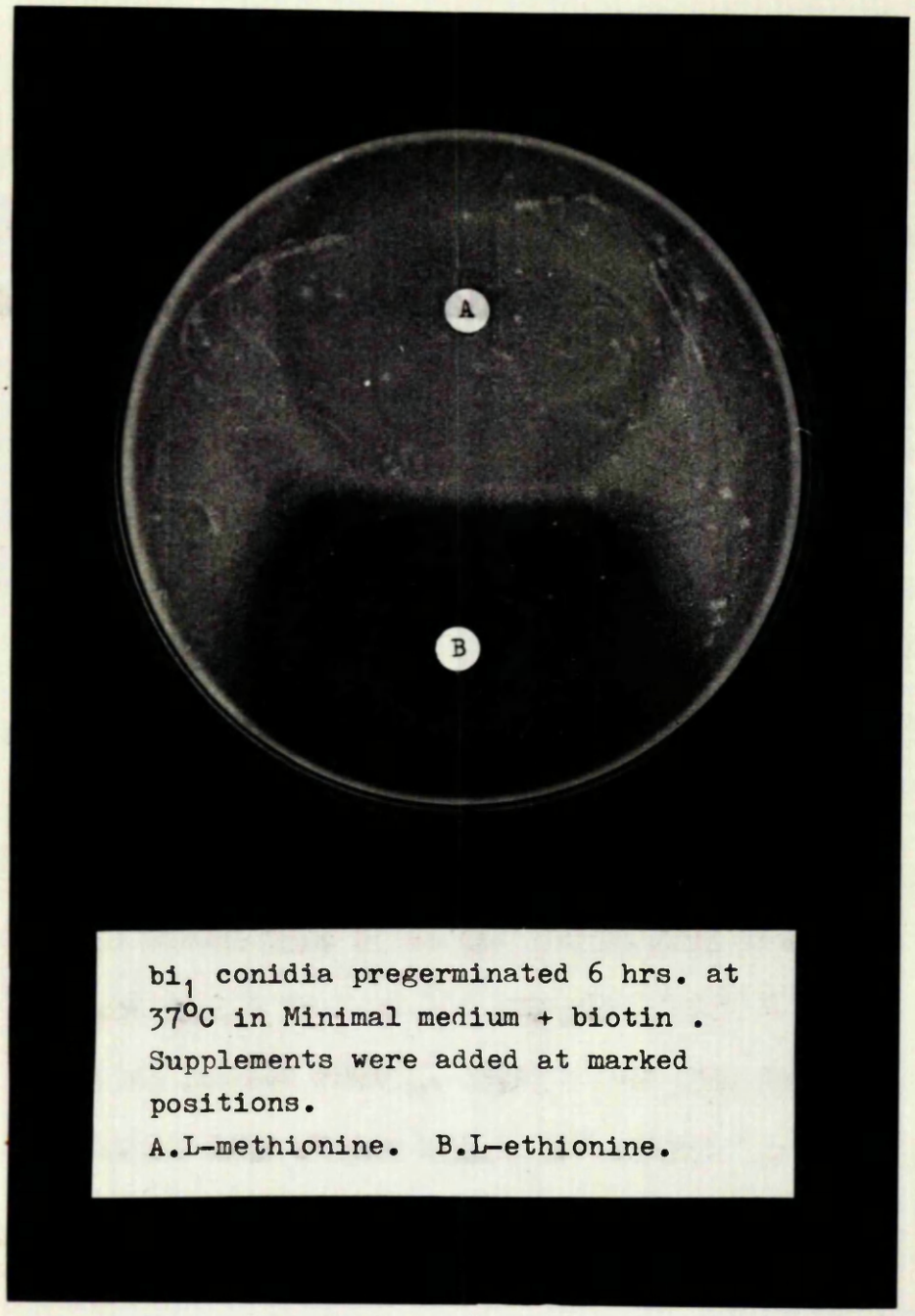
But the recombinant *thr1,phen2* was not recovered at all perhaps because it is not viable.

Thus it appears that mutants at *two* loci have reduced or no uptake of PHE, PPA and MET.

Resistance to other amino acid analogues.

Principle:- Analogues of some essential metabolites are inhibitory to a variety of micro-organisms and these analogues are taken up into the cell by the same route and mechanism as their natural competitors (Richmond, 1962). Therefore, the mutants that have reduced uptake of metabolites are resistant to the corresponding analogues.

Experimental:- Sensitivity of wild type (*thr* - green) *A. nidulans* to a variety of amino acid analogues was tested auxanographically. The following amino acid analogues showed



bi_1 conidia pregerminated 6 hrs. at 37°C in Minimal medium + biotin . Supplements were added at marked positions.
A.L-methionine. B.L-ethionine.

Plate.- 8

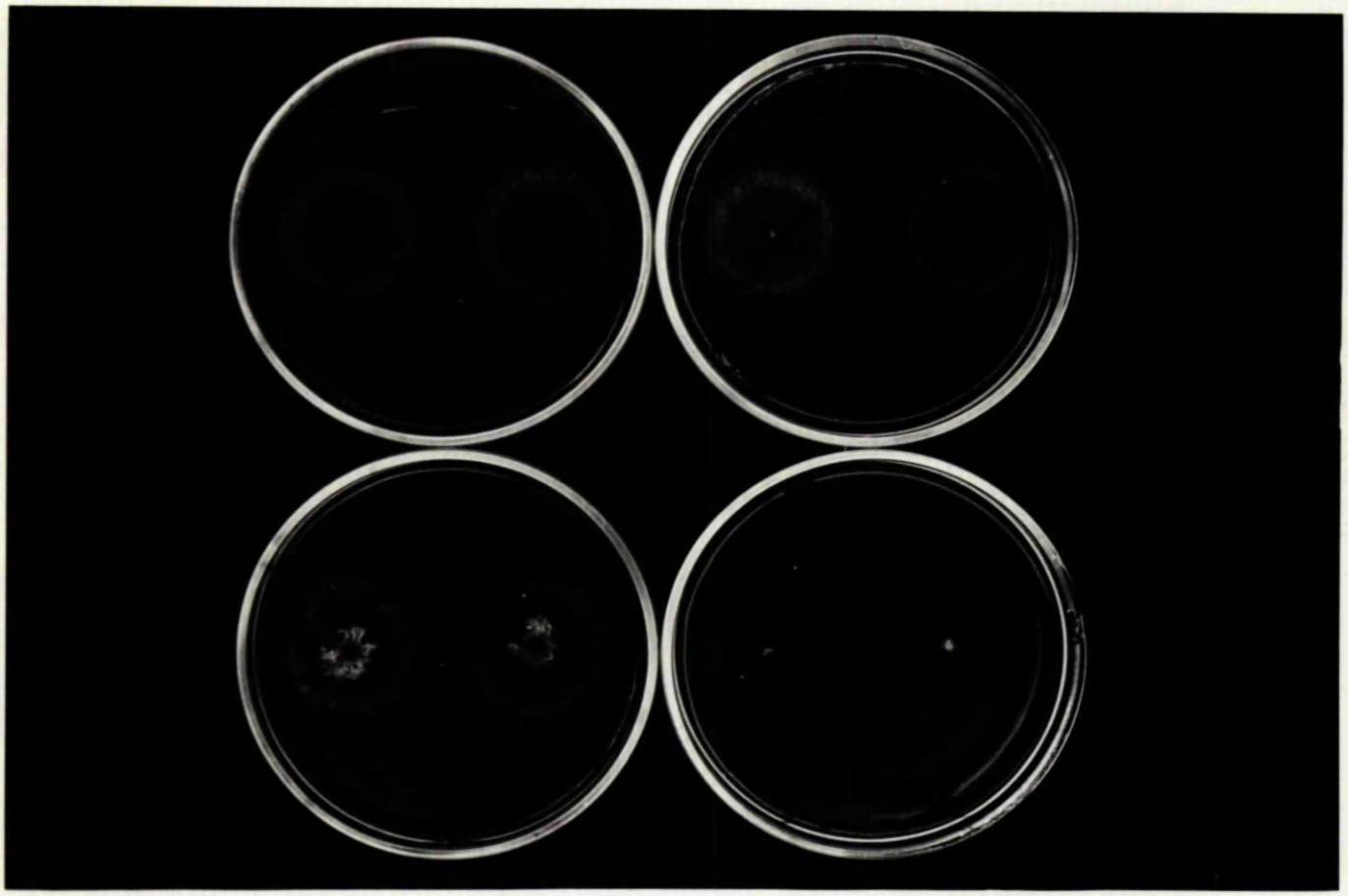
no significant inhibition of growth (compounds in parenthesis are the corresponding natural metabolites): b-2-thienylalanine (phenylalanine), dl-5-methyltryptophan (tryptophan), norvaline (valine), dl-homoserine (serine) and l-canavanine (arginine).

By contrast, l-ethionine (methionine) and l-3-amino-tyrosine-2HCl (tyrosine) were found to inhibit the fungal growth. Inhibition by l-ethionine was competitively reversed by methionine (Plate 8) and not by any other amino acid. Excess of methionine too was found to be inhibitory. Inhibition by l-3-aminotyrosine-2HCl was competitively reversed by either PHE or TYR but not by any other amino acid.

Arginography of (1) bil,mtol;SpDl, (2) bil,mtol; SpDh3 and (3) bil;SpD56 strains revealed that unlike the wild type they are resistant to both ethionine and l-3-aminotyrosine-2HCl. This again supported the earlier indication that SpD mutants lack the transport system which is responsible for the uptake of aromatic amino acids and methionine.

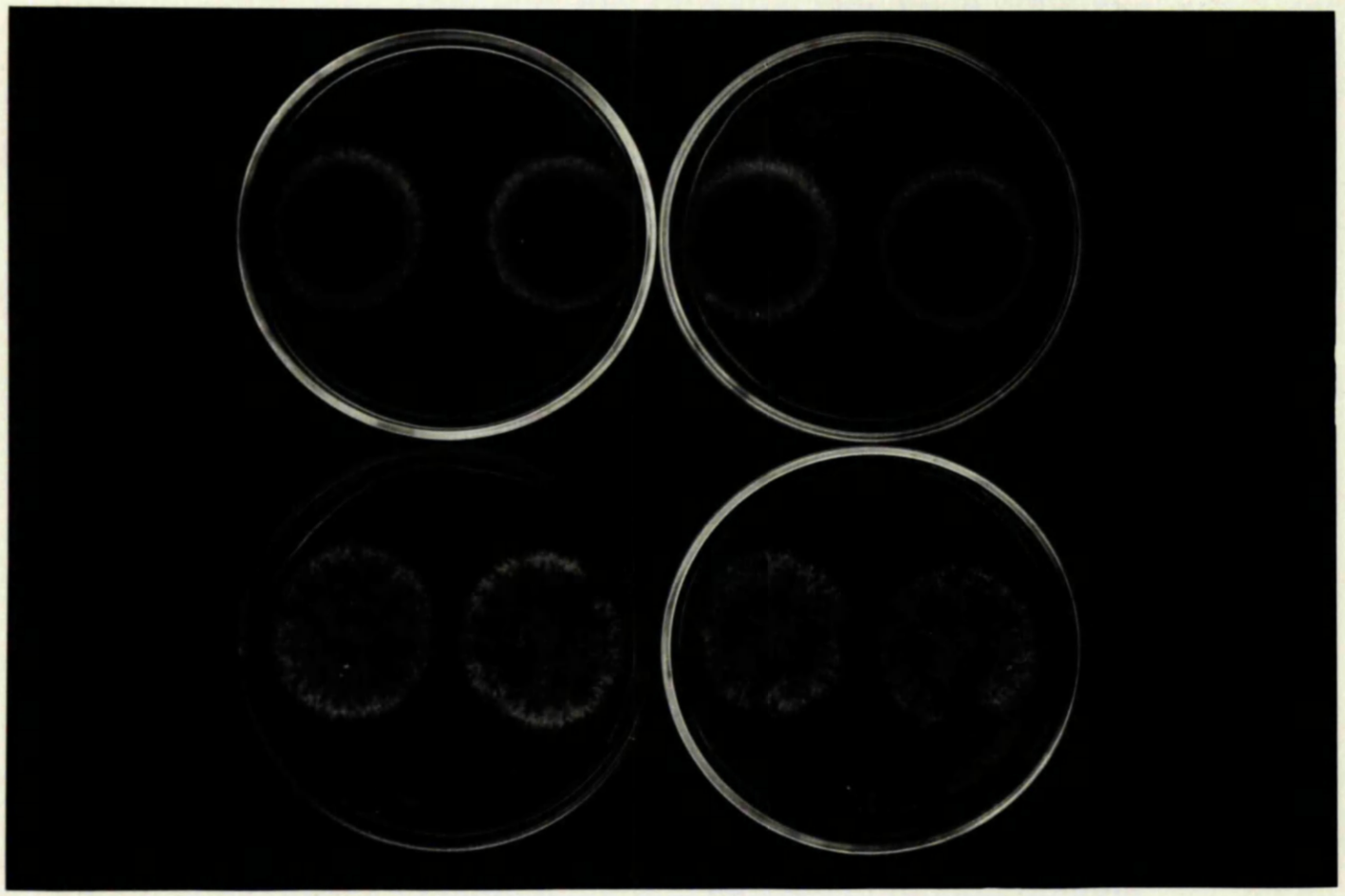
Dominance of SpDl in a heterozygote but its recessivity in a heterokaryon.

General considerations: The analytical potentialities of comparing the effects of same combinations of alleles in heterozygotes (within the same nucleus) and heterokaryons



Homozygous (fpD11/fpD11) (left) and heterozygous (fpD11/ +) (right) diploids (top row) and corresponding heterokaryons (bottom row) on M.M.+0.05% FPA (w/v).

Plate.- 10



Homozygous (fpD11/fpD11) (left) and heterozygous (fpD11/+) (right) diploids (top row) and corresponding heterokaryons (bottom)on M.M.

Plate.- 9.

(in different nuclei but in the same cytoplasm)have been considered by Pontecorvo (1950, 1952, 1963). Complementation tests with both heterokaryotic and heterozygotic association of genetic elements have been extensively done in A. nidulans and comparatively very few differences have been found between the heterozygotes and the corresponding heterokaryons. The differences have been interpreted to indicate an effective localization of the reactants in the process (Pontecorvo, 1963; Gonsalon and Lewis, 1967).

The mutant fmP11 in A. nidulans presents yet another example of a difference in the phenotype of a heterozygote and the corresponding heterokaryon.

Experimental: In order to test whether the mutant fmP11 is dominant over its wild type allele only in a heterozygous diploid or also in the corresponding heterokaryon, homozygous and heterozygous diploids and corresponding heterokaryons were synthesized and their ability to grow on MM and MM + 0.05 % FPA (v/v) was compared. As shown in Plates 9 and 10 and Table 32, both the homozygous diploid and the corresponding heterokaryon grow on MM as well as on MM + FPA whereas the heterozygous diploid grows on MM and MM + FPA but the corresponding heterokaryon grows on MM only and fails to grow on MM and FPA.

Table 31

Dominance of fpD11 in a heterozygote but its recessivity in a heterokaryon

Component strains	Type of combination	Growth of Diploid		Growth of Heterokaryon	
		on MM	on MM + FPA	on MM	on MM + FPA
bil,ribol;fpD11 and y,pabal,ad17;fpD11	fpD11 and fpD11	+	+	+	+
bil,ribol;fpD11 and y;meth2,phenA2	fpD11 and +	+	+	+	-

+ = growth; - = no growth.

When the heterokaryon, which failed to grow on MM + FPA, was rescued on to MM, a vigorously growing heterokaryon was obtained. This indicated that it was a genuine failure of growth rather than a failure of the formation of a heterokaryon.

If one of the component strains of the heterokaryon did not have a requirement for PHE (e.g. a heterokaryon between bil;pyro4,orn4 and y,pabal,ad17;fpD11), it could grow very slowly even on MM + 0.05 % FPA (v/v). This was perhaps because both the component strains could grow a bit, cross feed each

other and then grow again. This is indicated by the fact that even a FPA-sensitive strain grows slowly on FPA concentrations as high as 0.1 % (w/v) until it sends out FPA-resistant sectors. When a slow growing (on FPA) heterokaryon, of the above mentioned constitution, was examined, the proportion of green heads (strain carrying *fpD*$^{+}$ allele) was found to be very low and the conidia from these heads were found still to be FPA sensitive.

When conidia from a slow growing (on FPA) heterokaryon (*y,pabal,adl2;fpD11*) + (*bil;pyro*^{4}*,orn*^{4}) were collected by means of a loop and plated on different media, it was found that only about 10 % of the spores belonged to the strain carrying the *fpD*$^{+}$ allele (Table 32).

Table 32

Medium	No. of yellow colonies	No. of green colonies	Total	%age of yellow colonies	%age of green colonies
MM+supplements	586	71	657	89.2	10.8
MM + 0.01% FPA (w/v)+ supplements	171	21*	192	89.06	10.94

* these colonies were brown, poorly conidiating and very slow growing - typical of FPA-sensitive colonies on a medium with FPA.

Thus it appears that the gene-product which is responsible (either directly or indirectly) for the uptake of amino acids (PHE, MEPH) and their analogues from the medium is not synthesized in the heterozygous diploid (*pgl1*/+) whereas it is fully or partially synthesized in the corresponding heterokaryon. It remains to be determined whether this gene-product is an enzyme concerned with the uptake of PHE and MEPH or is a repressor which regulates the activity of a structural gene (Jacob and Campbell, 1959; Jacob and Monod, 1961; Koonin and Maganault, 1967) or of another regulator (Pontecorvo, 1963).

Studies with the locus fpB.

One PPA resistant mutant at the fpB locus was isolated by McCully and was assigned to linkage group I. He also found fpB37 to be unlinked to y,bil,pabaI,adE7 and riboI (1964). Further genetic analysis of this mutant has been carried out which is presented in the following paragraphs.

Genetic analysis of fpB37 based on mitotic recombination.

There is no known marker distal to bil with respect to which fpB37 can be meiotically tested for linkage. In order to know whether fpB37 is located distal to bil, a genetic analysis based on mitotic recombination (Pontecorvo and Kafer, 1958) was attempted.

A heterozygous diploid was synthesized in the usual way (Roper, 1952) between y,trpC6;phenA3 and y,pabaI,adE7, fpB37. Ten conidia were plated on C.M. and one yellow segregant (appearing as a yellow head) was isolated from each colony. The segregants were classified as to their phenotypes and haploids were discarded. Segregants for the phenA locus were not considered because it is on a different linkage group (III). The results (Table 33) show that the fpB locus is not distal to yellow i.e. it is either in the bil-pabaI interval or is located distal to galU on the left arm.

Table 33

Phenotypes of yellow diploid segregants from the diploid:

$$\frac{(spB37)\ ?,\ \diamond\ ,\ ad17,pabal,y,\ \diamond\ \diamond}{\diamond\ ?,lys10,\ \diamond\ ,\ \diamond\ ,\diamond,bil,phenA3}$$

Phenotype	Number
Prototrophs	6
pabal,ad17,y	14
spB37,pabal,ad17,y	5

Meiotic location of spB37

The locus spB was located distal to med15 and gal5 on linkage group I.

CROSS -1

$$\frac{(spB37)\ ?,\ \diamond\ ,\ \diamond\ ,ad17,pabal,y,\ \diamond\ \diamond\ ,\ \diamond}{\diamond\ ?,med15,gal5,\ \diamond\ ,\ \diamond\ ,\diamond,bil,lys,\ \diamond\ ,orn7}$$

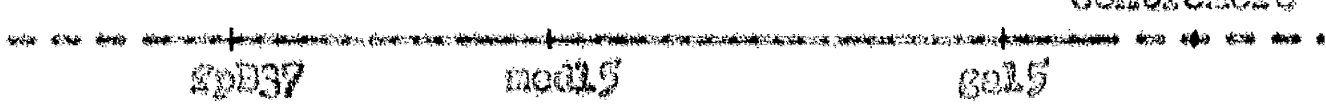
151 spB37+, ad17+, pabal+, bil+, orn7+ segregants were selected and the following segregation of other markers was obtained:-

spB37	◇	◇	= (selected against)	Parentals
◇	med15	gal5	= 120	

CROSS #2 (continued)

spB37	mod15	gal5	= (selected against)		Cross-overs in interval I
+	+	+	=	8	
spB37	+	gal5	= (selected against)		Cross-overs in interval II
+	mod15	+	=	21	
spB37	mod15	+	= (selected against)		Cross-overs in intervals I and II
+	+	gal5	=	2	

Linkage map:-



Interaction between nutrition and resistance determined by Sp3 locus.

In a cross lu1,lu1 x +,spB37,mod1,ad17, no spB37,lu1 recombinant was recovered out of 20+ segregants from ascospores of a hybrid perithecium, although the other recombinant - spB37+,lu1+- was quite frequent. Segregation of spB37 with

respect to the nutritional markers involved in this cross is shown in Table 34.

Table 34

Segregation of nutritional markers with respect to SpB37

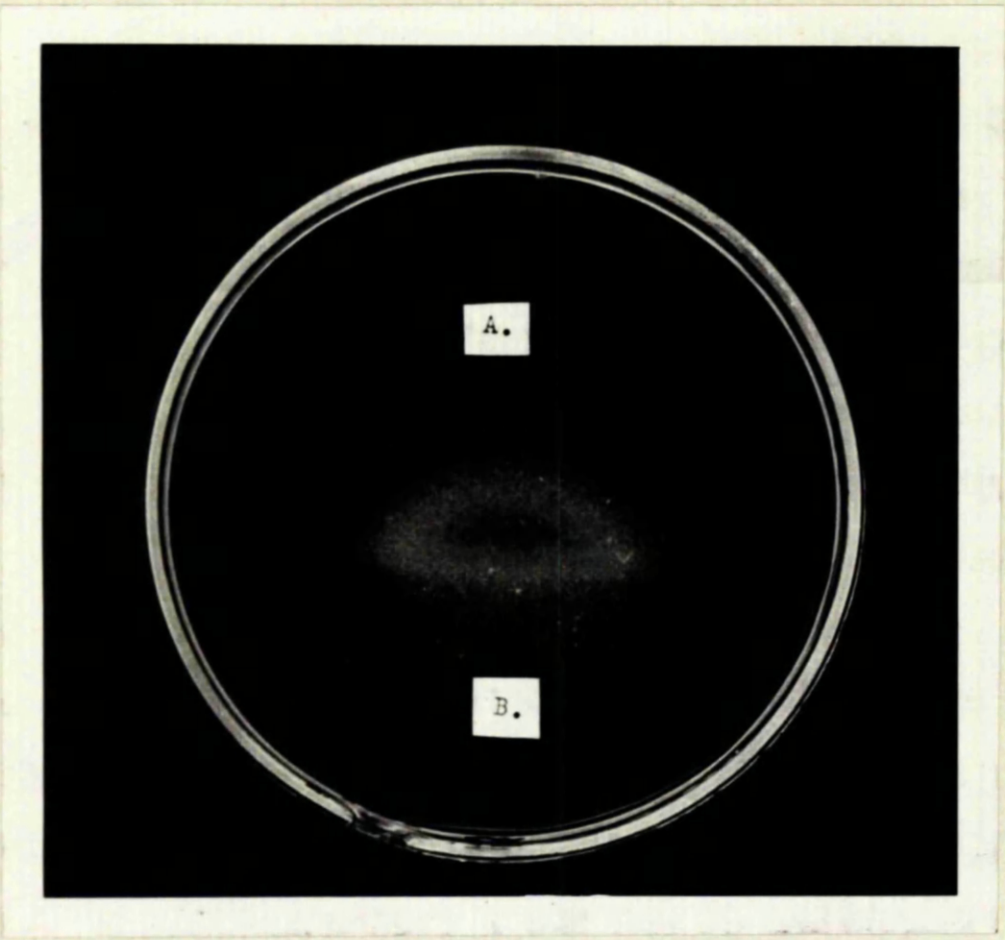
	Segregants			
Pairs of loci	+/+	+/-	-/+	-/-
SpB37 - lul	(69)	53	62	(0)
SpB37 - pabal	64	(58)	(23)	59
SpB37 - ad17	65	(57)	(22)	60
SpB37 - bil	61	(62)	(55)	27

Figures in parenthesis are those for recombinants.

Perhaps a SpB37,lul recombinant is not viable.

Competitive inhibition of PHE-requiring strains by LEU.

The observations that PHE-requiring strains are competitively inhibited by higher concentrations of leucine (Ponteocrvo -personal communication) or PPA and that SpB37; phenA2 as well as SpB37,lul recombinants are not recovered whereas comparatively fewer SpB37;meth2 recombinants are recovered, suggest that there is a common site of interaction for PHE, PPA, LEU and perhaps METH.



bi1,lu1;phenA3 conidia pregerminated 6 hours at 37°C in Minimal medium+biotin.Supplements were added at marked positions.

A.L-phenylalanine. B.L-leucine.

Plate.- 11.

Autoradiography of a bil,lul;phenA3 strain (Plate 11) revealed that a PHE-requiring strain is competitively inhibited by LEU but a LEU-requiring strain is not inhibited by PHE.

Further analysis of LEU-PHE inhibition was carried out by measuring the growth rate of a bil,lul;phenA3 strain at different relative concentrations of LEU and PHE. Growth of a colony was measured by measuring the colony diameter at regular intervals of time, along a marked line on the back of a petri-dish.

First of all, a crude assay was done to assess the range of relative concentrations (in terms of molar ratios) of LEU and PHE at which there was marked inhibition of growth of a bil,lul;phenA3 strain. Rate of growth was measured at 4 concentrations of LEU and 7 concentrations of PHE = 28 combinations in all. Growth measurements were started after a lag period of 24 hours. Table 35 gives the results and Table 36 emphasises the salient points.

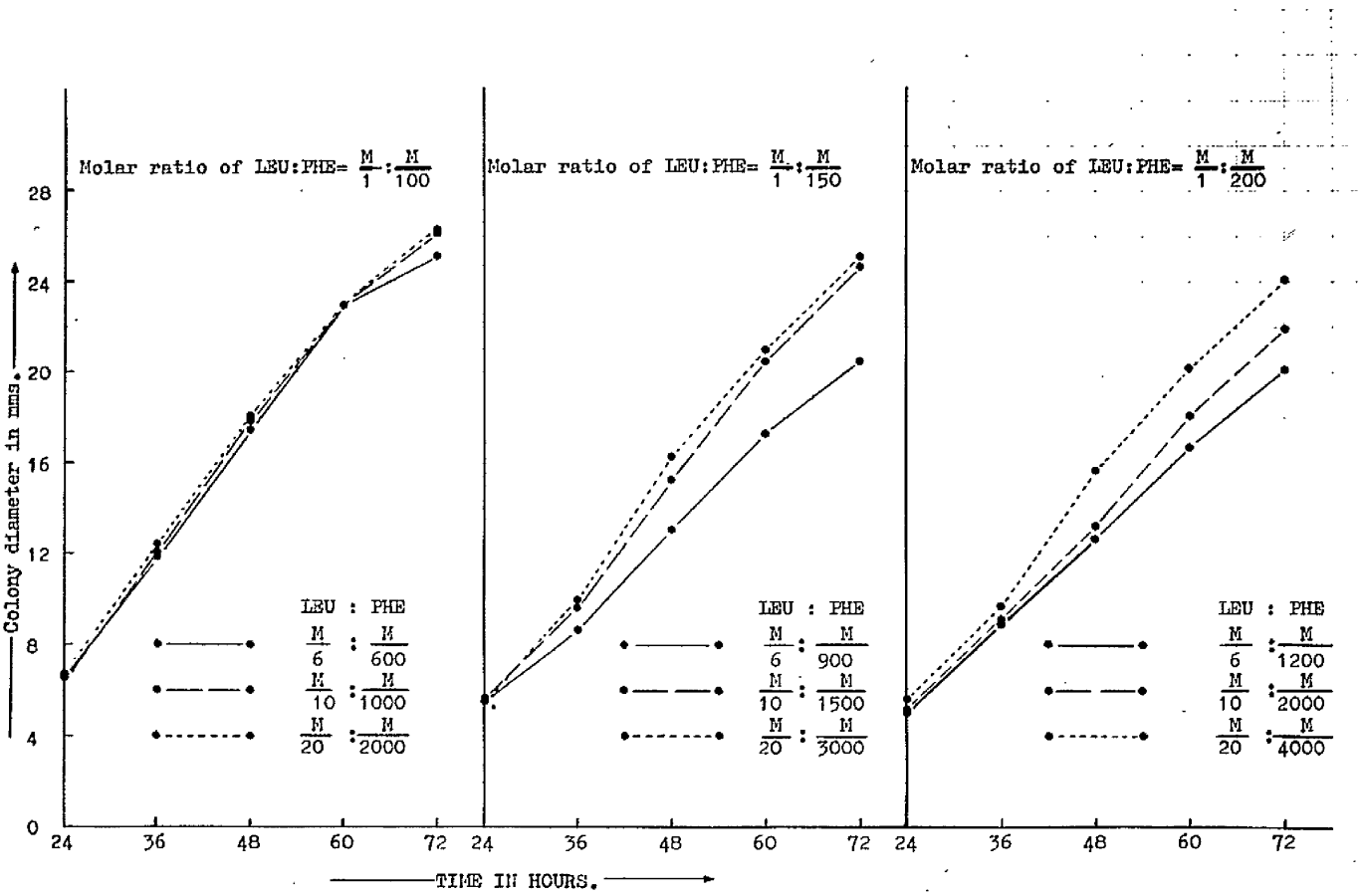
Table 35

Effects of varying relative concentrations of leucine and phenylalanine on the growth of bil,lul;phenA3

Table 35 (continued)

Molar concentrations in the medium		Growth in mm. during successive intervals* of 8 hrs. each (increase in colony diameter					
LiCl	PMS	1	2	3	4	5	6
M/6	M/3200	0.125	0.0	0.0	0.5	1.75	2.625
	M/1600	1.5	1.625	2.125	2.75	2.50	3.25
	M/700	1.35	3.875	3.50	3.00	2.75	2.625
	M/320	4.00	4.00	4.125	3.125	2.50	2.50
	M/192	4.00	4.50	4.125	3.125	2.25	2.375
	M/140	4.00	4.875	4.625	2.875	2.875	3.125
	M/70	4.125	4.75	4.625	3.375	3.625	4.00
M/8	M/3200	0.50	0.0	1.0	2.00	2.375	3.125
	M/1600	1.875	2.0	2.75	3.125	3.0	3.225
	M/700	3.75	3.75	4.00	3.125	2.625	2.50
	M/320	4.00	4.50	4.00	3.25	2.375	2.50
	M/192	4.00	4.875	4.00	3.25	2.50	3.00
	M/140	3.875	4.50	4.50	3.25	2.625	3.00
	M/70	4.125	4.00	4.75	3.50	3.75	4.25
M/12	M/3200	1.125	1.5	2.5	2.75	2.75	3.375
	M/1600	3.25	3.625	3.25	3.50	2.50	2.75
	M/700	4.00	3.75	3.875	3.375	2.625	2.625
	M/320	3.875	4.375	4.50	3.375	2.25	2.75
	M/192	4.125	4.125	4.625	3.625	2.875	2.375
	M/140	4.00	4.125	4.75	3.50	3.25	3.50
	M/70	4.25	4.25	4.50	4.00	4.375	4.125
M/24	M/3200	2.75	3.00	3.75	3.50	2.00	3.375
	M/1600	3.625	3.75	3.875	3.00	2.75	3.25
	M/700	3.75	3.625	4.25	3.375	3.00	2.875
	M/320	3.75	4.125	4.25	3.50	3.375	3.50
	M/192	3.875	3.75	4.00	3.75	3.375	4.075
	M/140	4.125	3.75	3.75	3.75	3.875	4.25
	M/70	3.875	4.25	4.125	4.125	3.625	4.375

* An average of four readings.



Rate of growth of bi1,lu1;phenA3 at the same molar ratios but different relative concentrations of LEU and PHE.

Fig.- 8

Table 36

Rate of growth	Molar concentration in the medium	
	LEU	PHE
Slowest	M/6	M/3200
Slower	M/6	M/1400
Slow	M/6	M/700
Normal	M/6	M/320
Normal	M/6	M/192
Normal	M/6	M/140
Normal	M/6	M/70

N.B. Molar concentration of PHE for optimum growth = M/3600.

These results indicated that LEU is inhibitory only when the LEU:PHE ratio was 100:1.

Measurement of the rate of growth of *bil, lul, pro A1* at the same molar ratios but different relative concentrations of LEU and PHE showed that it is the molar ratio that matters for inhibition and not the concentration of either of the two metabolites, independent of the other. The experiment was carried out at three molar ratios and at each molar ratio, five different concentrations of the metabolites were used. The results are given in Table 37 and Fig. 8.

Table 37

Increase in colony diameter of a Mi.Mu.phoA3 strain at the same molar ratios but different relative concentrations of LEU and PHE

Molar ratio of LEU:PHE	Concentration in the medium LEU	PHE	Colony diameter (mean of four readings) in mm. after 24 hrs.	36 hrs.	48 hrs.	60 hrs.	72 hrs.
$\frac{M}{1}:\frac{M}{100}$	M/6	M/600	6.750	11.875	17.500	23.125	25.250
	M/8	M/800	6.750	12.250	18.250	23.125	26.250
	M/10	M/1000	6.625	12.125	17.875	23.000	26.125
	M/15	M/1500	6.625	12.125	17.875	22.750	26.250
	M/20	M/2000	6.875	12.500	18.000	23.000	26.250
$\frac{M}{1}:\frac{M}{150}$	M/6	M/900	5.500	8.750	13.125	17.375	20.750
	M/8	M/1200	5.750	9.625	14.375	19.500	23.125
	M/10	M/1500	5.750	9.875	15.375	20.500	24.625
	M/15	M/2250	5.500	9.500	15.000	20.375	24.500
	M/20	M/3000	5.500	10.000	16.375	21.000	25.125
$\frac{M}{1}:\frac{M}{200}$	M/6	M/1200	5.125	8.875	12.375	16.750	20.250
	M/8	M/1600	5.250	8.875	13.375	18.000	22.000
	M/10	M/2000	5.250	9.125	13.375	18.125	21.875
	M/15	M/3000	5.500	9.250	14.750	19.250	23.625
	M/20	M/4000	5.750	9.875	15.375	20.250	24.125

Lastly, an attempt was made to find out whether LEU is inhibitory only within a narrow range of PHE concentrations and it was found that toxic LEU (M/6) is inhibitory to a

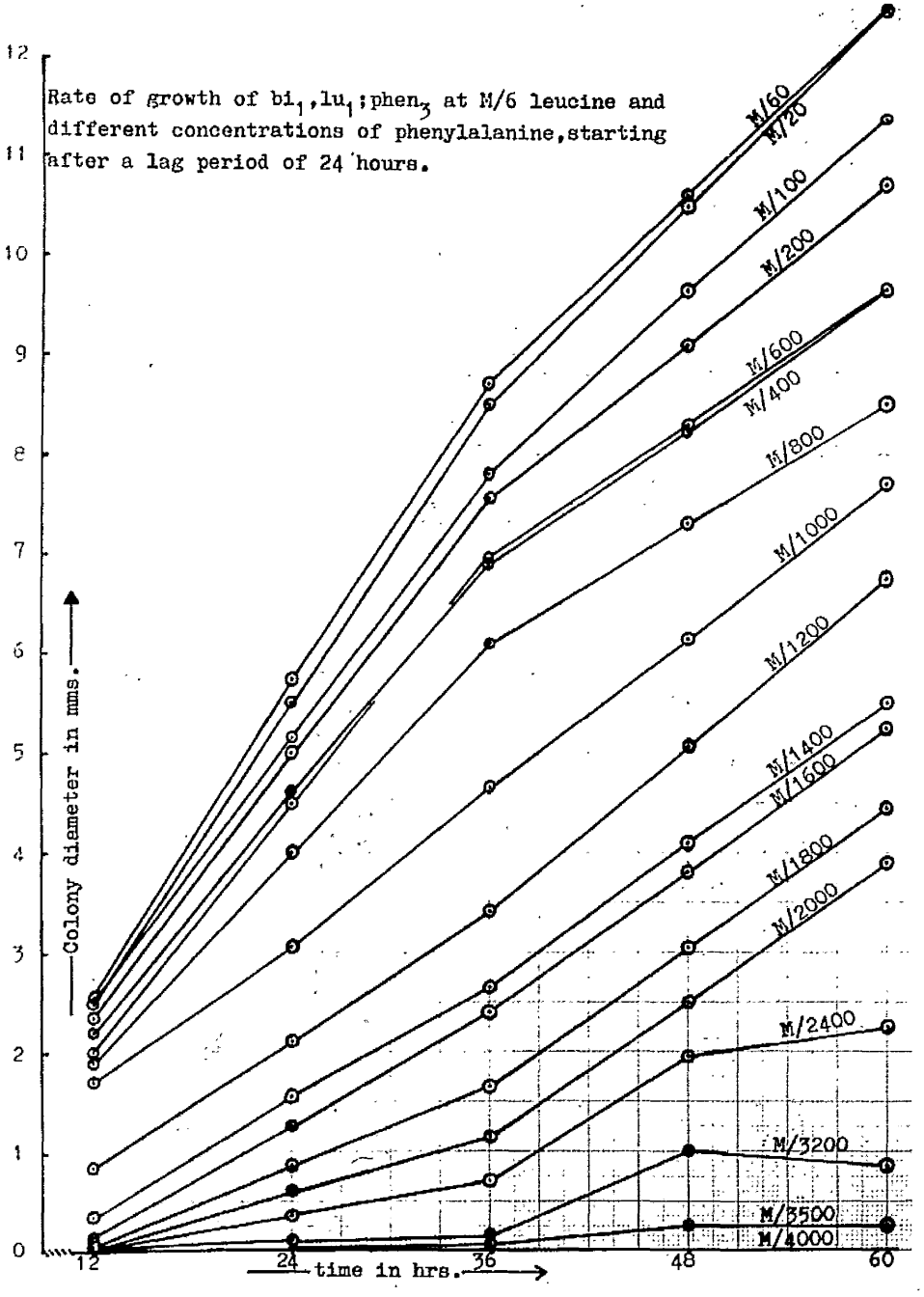

Rate of growth of bi1,lu1;phen3 at M/6 leucine and different concentrations of phenylalanine,starting after a lag period of 24 hours.
M/60
M/20
M/100
M/200
M/600
M/400
M/800
M/1000
M/1200
M/1400
M/1600
M/1800
M/2000
M/2400
M/3200
M/3500
M/4000
Colony diameter in mms.
time in hrs.
0
1
2
3
4
5
6
7
8
9
10
11
12
24
36
48
60

Fig.-9

bil,lul;phonA3 at all concentrations of PEM and with the increase in PEM-concentration, the rate of growth increased (Table 38 and Fig. 9).

Table 38.

Rate of growth of a bil,lul;phonA3 strain at a fixed concentration (M/6) of LiCl and varying concentrations of PEM, counting after a lag period of 24 hours.

Conc^n. of LiCl	Conc^n. of PEM	Increase in colony diameter (in mm) during (mean of four readings) 12 hrs.	24 hrs.	36 hrs.	48 hrs.	60 hrs
	M/20	2.50	5.50	8.50	10.50	12.50
	M/60	2.55	5.75	8.70	10.60	-
	M/100	2.55	4.00	7.80	9.65	-
	M/200	2.40	5.00	7.55	9.20	10.70
	M/400	2.20	4.60	6.90	8.25	9.65
	M/600	2.00	4.50	6.95	8.30	9.65
	M/800	1.90	4.00	6.10	7.30	8.50
	M/1000	1.40	3.05	4.65	6.15	7.70
M/6	M/1200	0.85	2.10	3.40	5.05	6.25
	M/1400	0.35	1.55	2.65	4.05	5.50
	M/1600	0.15	1.25	2.50	3.80	5.25
	M/1800	0.05	0.85	1.65	3.05	4.45
	M/2000	0.00	0.60	1.15	2.50	3.90
	M/2400	0.00	0.35	0.70	1.95	2.25
	M/2800	0.05	0.35	0.65	2.10	2.55
	M/3200	0.10	0.10	0.35	1.00	0.85
	M/3500	0.00	0.00	0.05	0.25	0.25
	M/4000	0.00	0.00	0.00	0.00	0.00

DISCUSSION

Because of its growth response to anthranilic acid, indole or tryptophan, the biochemical block in the mutant *trpE9* seems to be between chorismic acid and anthranilic acid in the pathway for tryptophan biosynthesis. *A. nidulans* mutants, which lack anthranilate synthetase (the enzyme which catalyses the conversion of chorismic acid to anthranilic acid) do not accumulate any intermediate of tryptophan biosynthesis (Roberts, 1967; Rutter and DeMoss, 1967). This may result into non-utilization of chorismic acid which can be metabolised to accumulate or overproduce all or some of the products of the common pathway e.g. PABA, p-hydroxybenzoic acid, PHB and PYR. An accumulation or overproduction of PHB by *trpE9* can account for its resistance to FPA. A similar situation has been discussed in the previous section where a partial TRP requiring (*trpA* or *trpA*) mutant is believed to be resistant to FPA due to an overproduction or accumulation of PHB and consequent competition against the antimetabolite.

It has been shown that *trpE9* mutant is not as resistant to FPA as the mutants at the *trpA* (= *trpA*) locus. As *trpA* and

pheA mutants are PPA-resistant because they either accumulate or overproduce PHE, it would appear that *pheH* is not accumulating or overproducing as much PHE as are the *pheA* mutants. This may be due to either or both of the following two reasons: (1) A partial TYR-requirement, resulting from a block between prephenic acid and p-hydroxyphenylpyruvic acid, leads to a diversion of the utilization of a common (to PHE and TYR) precursor (probably prophenic acid) entirely towards the synthesis of PHE, whereas an anthranilic acid requirement (as in *pheH*) results into a lowered demand for chorismic acid (a common precursor of PABA, p-hydroxybenzoic acid, nicotinic acid, TRY, TYR and PHE), only a fraction of which is converted to PHE (other fractions are converted to other end-products). (2) In a wild type, a major part of chorismic acid is utilized to produce PHE and TYR and only a minor part is utilized to produce TRY. Thus, a metabolic block in TYR-synthesis will result into more overproduction or accumulation of PHE, than a metabolic block in TRY synthesis (lack of anthranilate synthetase). This idea is supported by the fact that in *E. coli*, as compared to PHE or TYR, lesser amount of TRY is present in the overall protein. The amino acid sequences of *E. coli* proteins reveal that the frequencies of PHE, TYR and TRY residues are approximately in the ratio of 3:2:1 respectively (Braggio, 1964). In other words, for every five molecules of chorismic acid

converted to PHE and TYR, only one is converted to PHE.

A similar explanation can be given for the observation that nic2 and PABA-requiring mutants in A. nidulans are not resistant to FPA. Both these vitamins are required in extremely small quantities as compared to the amino acids. Therefore, only a trace of chorismic acid will be utilised in their synthesis and a metabolic block between chorismic acid and PABA or nicotinic acid may not result in an appreciable non-utilisation of chorismic acid so as to overproduce or accumulate other end products (TRY, TYR or PHE).

FPA resistant mutants fpA1, fpA3 and fpA6 map at about the same place in linkage group VIII. fpA1 and fpA6 are dominant and fpA3 is semidominant in a heterozygous diploid. Tests of allelism by complementation, therefore, are not possible. In other respects (cross resistance to ethionine and 1-3-andnetyrosine-2HCl) their behaviour is similar. The three mutants, therefore, are perhaps allelic to each other, representing mutations at a locus which can be designated fpA. Semi-dominance of fpA3 in a heterozygous diploid and its lesser degree of FPA resistance (as compared to fpA1 and fpA6) can be attributed to an incompleteness of the metabolic block.

Incorporation of FPA resistance (due to a mutation at the fpA locus) and nutrition can be taken to suggest that the fpA

locus is somehow responsible for the transport of PIH and compounds which are supposedly taken up by the same uptake process (like PPA, MEPH, ethionine, aminotyrosine) from the medium into the cell. Cross resistance of these mutants to ethionine and aminotyrosine, strengthens this contention. There is substantial evidence in other organisms that uptake or transport mechanisms are under genetic control (Holston, 1965; Stadler, 1966 and Gronson, 1967). Circumstantial evidences have led to the suggestion that the transport of amino acids is mediated by enzyme-like mechanisms (Cohn and Monod, 1957). Widespread use of the term "permease" reflects the popularity of this idea, although there is no direct evidence to support this.

On the basis of present investigation it appears that in A. nidulans, there is yet another (independent uptake system for hypoxanthine, uric acid and allantoin have been proposed by Arst and Cove, 1966) transport system with restricted specificity. It has more affinity for PIH and its analogues than for NEPH and ethionine and it has no affinity for arginine, riboflavin, thiosulphate, sodium nitrate and biotin. Moreover, perhaps there is only one uptake system responsible for the uptake of PIH because the mutant spi1, which perhaps lacks this transport system, does not

grow at all on PHE. It also follows from this that PHE is not handled by any non-specific or general transport system, even if it existed in *A. nidulans*. On the other hand, perhaps METH is only partly transported by PHE-specific uptake system and there could be a METH-specific uptake system or another transport system with broader specificity for efficient or complete uptake of METH.

Calvori and Morpurgo (1966) have reported that their *pfn-r* mutants (*fpA* or *tyrA* of this thesis) fail to grow on MM + 0.01 % (w/v) l-3-aminotyrosine + 0.047 % (w/v) phenylanthranilic acid whereas wild type colonies can grow. Using this medium and starting with a *pfn-r* mutant, they have isolated strains of *A. nidulans* which grow on this medium like a wild type and grow faster than a wild type on MM +PPA. They have concluded that the genotypes of such strains is *su-pfn-r,pfn-r*. They assume that by their method of selection, only true back mutants and suppressors can be selected. Perhaps they fail to realise that strains of the genotype *fnD;fpA* (with an additional mutation at *fnD* locus) can also be recovered by their methods of selection and in fact their results can be better explained by assuming that their "*suppressors of pfn-r*) are actually mutants with properties like *fnD* mutants. The mutant *fnD11* has been found to be dominant or semidominant in heterozygous diploids and recessive in heterokaryons. Instances of such differences

in the phenotypes of heterokaryons and corresponding heterozygotes have been interpreted to indicate an effective localisation of gene products (Pontecorvo, 1952b, 1963; Casselton and Lewis, 1967).

There is very little known about the *fpl* locus. However, the failure to recover *thiV.lul* double mutants suggests that this locus also is in some way concerned with the uptake of amino acids. On the basis of inhibition analysis in *N. crassa*, leucine has been put in the same family of amino acids as PHE and TYR (Haddox, 1952; Brockman, 1964). In *A. nidulans*, PHE-requiring mutants are competitively inhibited by higher concentrations of leucine (Pontecorvo - personal communication). It will not be, therefore, surprising if it is proved that the uptake of both PHE and LEU are under the control of the same transport system. In the meantime, it appears most likely that a mutation at *fpl* locus prevents or decreases the entry of PFA into the cell - thus making it resistant. There could well be other sites of PHE-LEU-PFA interaction such as the sites of attachment with activating enzymes or t-RNA.

SUMMARY

1. The loci *fpD*, *fpB* and *fpE* have been assigned to linkage groups I, VIII and XI respectively.

2. The mutant *fpB48* has been found to require tryptophan, indole or anthranilic acid for its optimal growth and it has been suggested that this mutant is PPA resistant because it accumulates or overproduces PEP.

3. The fact that mutants *fpB1*, *fp56* and *fpb3* map at about the same place, are resistant to PPA, ochlorine and 3-3-anthotyrosine-2HCl, and behave as dominants (*fpB1* and *fp56*) or semidominant (*fpb3*) in a heterozygote, have been considered to suggest that these three isolates are mutant at the same locus - *fpB*.

4. Interactions of PPA resistance (due to mutations at *fpD* and *fpE* locus) and nutrition have been interpreted to suggest that these two loci are somehow concerned with amino acid uptake.

5. Mutants at *fpD*, *fpD* and *fpE* loci have been found to differ from each other in degrees of resistance to PPA and have been found to be much less resistant than the mutants at *fpA* (= *tyrA*) locus.

VIII GENERAL DISCUSSION AND SUMMARY

DISCUSSION.

The experimental results presented in this thesis allow one to draw some tentative conclusions as to the metabolic pathways of aromatic amino acid biosynthesis and PPA regulation in *Aspergillus nidulans*.

It appears that in this ascomycete there are two routes for tyrosine synthesis: one through the shikimic acid pathway and the other by the hydroxylation of phenylalanine; the former being the major pathway. Otherwise, the general scheme appears to be the same as in other micro-organisms and higher plants (Fig. 2).

Hydroxylation of phenylalanine is the only known mechanism for the biosynthesis of tyrosine in animals (Kaufman 1963). There are some biochemical evidences for the presence of PHE-hydroxylase activity in phenylalanine-adapted pseudomonads (Mitoma and Keeper, 1954) and an aromatic mutant of *N. crassa* (Barratt *et al.*, 1956), although genetic studies do not suggest anything like this because in both these micro-organisms single step mutants, blocked in the shikimic acid pathway, are known which result into absolute tyrosine-requirements.

Thus, A. nidulans is the only organism, investigated so far, in which the genetic studies (as reported in this thesis) suggest that both the "animal" (PHE-hydroxylation) and "plant" (shikimic acid) pathways are operative for the biosynthesis of tyrosine. Preliminary biochemical studies support the conclusions drawn from the genetical studies.

p-Fluorophenylalanine resistance of partial tyrosine-requiring mutants (tyrA or tyrA) and of an anthranilic acid requiring mutant (trpB) of A. nidulans suggests that these mutants either accumulate or overproduce phenylalanine which competes against the antimetabolite. Tyrosine-requiring mutants in E. coli (Ahtang, 1964) and Mc. glutamicus (Nakayama et al., 1961) are known to accumulate up to 2 g/l of phenylalanine but it is not known whether these mutants are PFA-resistant; perhaps it is difficult to test them because they are absolute tyrosine-requirers and tyrosine is active (although not as active as PHE) in reversing the inhibitory effects of PFA. On the other hand, phenylalanine overeating mutants of E. coli, derepressed in the PHE-pathway, are known to be PFA resistant (Adelberg, 1958) and it has been suggested that PFA is inhibitory to E. coli primarily because it feed back inhibits the activity of PHE-sensitive enzymes (Previc Pinkey, 1964a, 1964b, and Egerlol, 1965). But it appears that A. nidulans has a perhaps comparatively simple mechanism

of overpynthesising one metabolite (PAB) by cutting down the synthesis of the other (PAR).

Interactions of PPA resistance (due to mutations at either the *fol* or the *fol* locus) and amino acid requirements as well as cross resistance of *fol* mutants to ethionine and methotrexate, suggest that uptake of amino acids and their analogues is under the genetic control of *fol* and *fol* loci in *A. nidulans*. Precise biochemical studies of different mutants are desirable to establish the existence and specificities of different uptake systems in this mould.

Thus it appears that in *A. nidulans* there are at least two mechanisms of PPA resistance: one by the overpynthesis of PAB which competes against the antimetabolite and the other by the loss of or defect in the uptake system which normally transports amino acids and their analogues from the medium into the cell. The fact that *pyrA* (= *folA*) mutants are much more resistant to PPA than mutants at *fol* or *fol* loci, suggest that the first mechanism of PPA-resistance is much more effic than the second one.

The observation that *folB1* mutant is dominant in a heterozygous diploid but recessive in a heterokaryon, suggests the possibility of an effective localization (Pontecorvo, 1963; Apinis, 1966, and Gamulton and Lowis, 1967) of the product of this gene in *A. nidulans*. It is not known that

the gene product is?.

It is not clear as yet whether fpH and fpH loci have no functional relationship or they have a regulator gene - structural gene relationship (Jacob and Campbell, 1959; Jacob and Monod, 1961; Pontecorvo, 1963, and Loomis and Magasanik, 1967) or they control the synthesis of different polypeptides of heteropolymeric uptake systems which could have overlapping specificities. Isolation and study of some more FPA-resistant mutants can help in settling the matter one way or the other and in bringing to light some more mechanisms of FPA resistance in A. nidulans, if they exist at all.

SUMMARY

1. Five additional PHE-requiring mutants have been isolated but they do not add to the number of loci already known (*phenA* and *phenB*).

2. The strains *bil;phenB6* and *bil;w6;phenA7* have been found to be free of translocation and a I - IV translocation present in the strain *bil;phenA3* has been eliminated by outcrossing.

3. Two pathways for tyrosine synthesis in *A. nidulans* have been proposed: the well-known one via shikimic acid and an alternative one, like in animals, by the hydroxylation of phenylalanine.

4. Ten allelic partial tyrosine-requiring mutants (*tyrA*), presumably blocked in the shikimic acid pathway have been isolated after NTG treatment of *bil;phenA3* conidia.

5. Four partial tyrosine-requirers at another locus, designated *tyrB*, have been isolated after NTG treatment of *bil;tyrA7* conidia. They are presumably blocked in an alternative pathway of tyrosine synthesis i.e. in the

phenylalanine hydroxylation pathway.

6. *tyrA* mutants have been found to be PPA resistant and allelic to mutants at *pA* locus. *tyrB* mutants have been found to be very leaky and PPA sensitive and a *tyrA;tyrB* double mutant has been found to be an aromatic tyrosine requirer.

7. Mutants at locus *pA* (= *tyrA*) and *pE* (anthranilic acid requiring) have been interpreted to be PPA resistant due to an overysynthesis of PHE.

8. Mutants at locus *pI* and *pH* have been interpreted to be uptake-deficient mutants.

9. Loci *tyrA*, *tyrB*, *fdI*, *fdI* and *pE* have been located both mitotically (to linkage groups) and meiotically on linkage groups I, XII (only mitotically), I, VIII and II respectively.

10. *pI* mutants have been found to be dominant over their wild type alleles in heterozygotes but recessive in corresponding heterokaryons.

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