Spectrum of Forward Mutants in the *pyr-3* Region of *Neurospora*

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SUMMARY

About 200 forward mutants of *Neurospora* obtained as suppressors of *arg* were studied. All fell in the *pyr* region, and all appeared deficient in aspartic transcarbamylase (ATC) to various degrees: extreme deficiencies in ATC (*pyr-N* alleles) resulted in pyrimidine-dependence as well as suppression of *arg*, while partial ATC deficiencies (*pyr^{su-arg}* alleles) led only to suppression. Five different complementation groups were represented among the *pyr-N* alleles studied. Evidence is presented which suggests that *pyr-N* and *pyr^{su-arg}* alleles affect ATC via different mechanisms.

INTRODUCTION

The genetics and the complementation pattern of mutants at the *pyr-3* (*pyr*) region of *Neurospora* was studied by Mitchell & Mitchell (1956), by Suyama, Munkres & Woodward (1959) and by Woodward (1962). Davis (1960), investigating the enzymic defects in the same mutants, showed that the *pyr* region is functionally heterogeneous: it comprises mutants which lack aspartic transcarbamylase (ATC) activity, as well as mutants with normal values of the same activity and presumably deficient in a previous step (Davis, 1961, 1962). The former or ATC-less mutants will be designated *pyr-3N* (*pyr-N*), and the latter *pyr-3M* (*pyr-M*). The results just outlined were obtained with mutants isolated by the usual methods for the selection of auxotrophs. A special screening method, which will be referred to as the suppressor method, is also available for the isolation of *pyr* mutants (Reissig, 1963). This method takes advantage of the fact that *pyr* mutants (*pyr-N* mutants, as will be seen) suppress the arginine requirement of a strain carrying the *arg-2* (*arg*) mutant gene. Even partial decreases in the activity of the normal *pyr⁺* allele result in suppression of *arg* (Reissig, 1960). Consequently the suppressor method screens for a whole spectrum of mutant alleles, ranging from the subthreshold *pyr^{su-arg}* (no pyrimidine requirement) to *pyr* alleles allowing no trace of growth in the absence of pyrimidines. With two basically different methods available for the selection of mutants at *pyr*, it seemed profitable to attempt a more thorough assessment of the genetic information contained in this region.

The following abbreviations are used: *arg* for *arg-2*; *pyr* for *pyr-3*; ATC for aspartic transcarbamylase; Rg_{arg} for growth in the absence of arginine relative to growth in its presence; Rg_{ud} for growth in the absence of uridine relative to growth in its presence.

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METHODS

General methods were described in the preceding paper (Reissig, 1962). Additional details are given in this section.

Strains. Several *pyr* mutants obtained by auxotroph selection following treatment with u.v.-radiation, were kindly provided by K. D. Munkres (strains prefixed KS; Suyama *et al.* 1959) and by M. B. Mitchell; representing types N and M (Davis, 1960; and personal communication): KS 36a, KS 36A, 70007-45502-32723a (*pyr-3d co*) and 45502-1507-1A (*pyr-3d T*) all type N; and KS 138a, KS 138A and ED 354-1a (a re-isolate of 37301) all type M. The St Lawrence wild types 74A and 73a were also used.

Measurement of growth responses. Growth was measured as in the usual tube method (Ryan, Beadle & Tatum, 1943); but by using 15 × 150 mm. test tubes with a notch near the open end, laid horizontally so that the notch retained the medium (5 ml.). Each strain was tested on four different media: unsupplemented, supplemented with arginine (0.1 g./l.), supplemented with uridine (0.05 g./l.), and supplemented with arginine + uridine.

Strain ED 416-1a, the source of all mutants screened, is arginine-dependent, but can grow slowly on arginine-free medium. This slow growth is characterized by a long lag phase (5-6 days instead of the usual 1 or 2) followed by growth at a constant and normal rate. Therefore, linear growth rate is useless as a measure of growth response to arginine: a meaningful growth index must take into account the duration of the lag phase. The growth index used was defined as the reciprocal of the time taken by the mycelial front to travel 11 cm. from the point of inoculation. Measurements were discontinued on the 14th day, and extrapolated whenever necessary.

The growth of each mutant in the absence of arginine, relative to its growth in the presence of arginine, was called $R_{g_{arg}}$ and calculated from equation (1):

$$R_{g_{arg}} = \frac{\text{index on minimal} + \text{index on uridine}}{\text{index on arginine} + \text{index on arginine} + \text{uridine}}. \quad (1)$$

Similarly, relative growth without uridine ($R_{g_{ud}}$) is given by equation (2):

$$R_{g_{ud}} = \frac{\text{index on minimal} + \text{index on arginine}}{\text{index on uridine} + \text{index on arginine} + \text{uridine}}. \quad (2)$$

The measurement of response to uridine does not present complications of the type discussed in connexion with arginine response: uridine-stimulated mutants respond to this metabolite by shortening the lag phase and increasing the linear growth rate. However, for the sake of uniformity, the growth index was defined throughout as previously indicated. It should be noted that substitution of rates of linear growth for the growth indexes in equation (2), leaves $R_{g_{ud}}$ values practically unchanged. Therefore, the choice of growth index is not imposing any arbitrary distortion upon the description of the experimental data. A difference in $R_{g_{arg}}$ or in $R_{g_{ud}}$ larger than 0.2 is probably significant. However, statistical controls were not included in the experiments to be reported: therefore only the major features of these results can be discussed.

Complementation tests. Unless otherwise specified, mutants were tested in pair-

wise combinations for their ability to complement (i.e. to form a prototrophic heterokaryon) on plates of minimal medium + arginine in the manner described by de Serres (1956). Unsupplemented plates yielded similar results. Two weeks were allowed whenever possible before classing a test as negative, but positive reactions were in evidence between the 2nd and 4th day except when unrelated strains were used. Complementation tests between *arg* and *pyr* (or *arg pyr*) strains were made on plates supplemented with orotic acid (1 g./l.). This supplement inhibits the residual growth of *arg* and stimulates only slightly *pyr* strains.

RESULTS

The frequency distribution of Rg_{arg} values among 190 mutants obtained with the suppressor method, is shown in Fig. 1. Clearly, partially arginine-dependent mutants (Rg_{arg} in the 0.4–0.8 range) were also obtained. This is so with the usual scoring schedule; but by picking 2 days earlier, only fully arginine-independent mutants are recovered.

The distribution of Rg_{ud} values among the same 190 mutants is strikingly bimodal (Fig. 2). Before attempting to interpret these results we must ascertain the genetic basis of the mutations examined. This is particularly important because it is known from previous work that the screening method used yields back-mutants at *arg* as well as mutants at *pyr*. The genotype of the prototrophs was ascertained by recombination analysis, as reported in the preceding paper of this series (Reissig, 1962); the results are indicated in Fig. 2: *pyr^{su-arg}* strains by vertical hatching; and back mutants by dotted areas. Pyrimidine-dependent mutants were tested for allelism by complementation criteria (see section on allelism), and all found to be allelic as indicated by horizontal hatching in Fig. 2.

Mutants with low Rg_{arg} values (< 0.85) could not easily be tested by the recombination method, because the segregation of partial arginine-dependence interferes. Unshaded areas in Fig. 2 correspond to mutants of this type. Preliminary tests performed with some of them favour the hypothesis that they are predominantly *pyr^{su-arg}*.

It is clear that the distribution of mutants at *pyr* is bimodal. There is no reason to suspect that this may be due to an artefact of the selection method. Bimodality points, rather, to a duality in function, as will be considered in the discussion.

Complementation pattern

In a previous study (Reissig, 1959) 9 *pyr* mutants obtained by the suppressor method were tested for complementation in all possible pairwise combinations. This disclosed two complementation groups or cistrons: alpha, represented by 5 mutants, and beta, represented by 4 mutants. Complementation testing was now extended to 204 new mutants of independent origin, induced by u.v.-radiation, ethylmethane sulphonate or nitrous acid (Reissig, 1963). Testing in all possible combinations was not attempted; instead, 1 alpha tester and 3 beta testers were chosen at random, and their complementation reactions with all other strains were determined. The results of these and some additional tests are shown in Table 1. Four complementation groups (alpha, beta, gamma, delta) were revealed, such that any member of one group complemented with any member of another, but did not complement with members of the same group. A fifth group (non-complementing group) comprises

mutants unable to complement with representatives of any of the other 4 groups.

The failure of this fifth group to complement could be due to relationships intrinsic to the *pyr* region; or to extrinsic factors, such as additional mutations at loci which determine barriers to the formation of heterokaryons. To determine whether the ability to form heterokaryons was impaired in the 7 non-complementing mutants, heterokaryons were attempted between them and the *arg* strain

Fig. 1

Fig. 1. Distribution of $R_{g_{arg}}$ values among *Neurospora* mutants obtained by the suppressor method. ■, Original *arg* strain. For definition of $R_{g_{arg}}$, see Methods.

Fig. 2

Fig. 2. Distribution of $R_{g_{ud}}$ values among *Neurospora* mutants obtained by the suppressor method. ■, Original *arg* strain; ▨, back mutants (*arg*⁺); ▤, not tested. Forward mutations at *pyr*: ▤, as established by complementation; ▨, as established by recombination; ▤, as established by complementation and recombination. For definition of $R_{g_{ud}}$, see Methods.

from which all *pyr* mutants derived. Positive responses were obtained in every case, and the true heterokaryotic nature of the resulting prototrophic mycelium was confirmed by the isolation of hyphal tips, subcultivation, and plating of microconidia

on appropriately supplemented media. Therefore failure to complement was not due to incompatibility barriers, and was most likely related to the type of functional alteration at *pyr*. A clear correlation was noted between failure to complement, and failure to grow at all on minimal medium (Table 2).

Table 1. *Complementation among pyrimidine-dependent mutants of Neurospora obtained by the suppressor method*

Mutants of *Neurospora* were classified in 5 complementation groups. The complementation reactions of all members of each group with representative strains from the same and from other groups are indicated in the table: +, complementation; 0, no complementation. The figures in parentheses indicate the number of different pairs tested in each case.

No. of mutants	Complementation group	Alpha	Beta	Gamma	Delta	Non-complementing
155	Alpha	0 (444)	.	.	.	.
38	Beta	+	0 (41)	.	.	.
3	Gamma	+	+	0 (3)	.	.
1	Delta	+	+	+	0 (1)	.
7	Non-complementing	0 (28)	0 (28)	0 (21)	0 (7)	0 (1)
204						

Table 2. *Correlation between failure to grow on pyrimidine free medium, and failure to complement*

Growth on pyrimidine free medium*	No. of <i>pyr</i> mutants	
	Complementing†	Non-complementing
Yes	101	0
No	39	10

* Visible growth by the 14th day on arginine supplemented medium.

† Either in alpha, beta, gamma or delta complementing groups.

Comparison with mutants obtained by auxotroph selection

Two types of mutants are known to occur at the *pyr* region (Davis, 1960): *pyr-N* mutants lacking ATC; and *pyr-M* mutants with normal levels of ATC. In first analysis, *pyr-N* and *pyr-M* mutants can be identified with two complementation groups, which will be called, respectively, *n* and *m*. New complementation groups have appeared in more recent work (Woodward, 1962; see also Mitchell & Mitchell, 1956), but the earlier identification of *pyr-N* and *pyr-M* with two complementation groups is still useful as a preliminary criterion for comparative studies. The results just described refer to mutants obtained by auxotroph selection. It is pertinent to ask whether the same picture applies to mutants obtained by the suppressor method. The data in Table 3 show that this is not so: suppressor selection appears to yield only mutants of the *n* complementation type. But before attempting an interpretation of the data in Table 3, it was essential to find out whether the negative complementation tests might result from incompatibility factors extraneous to the *pyr* locus. Incompatibility factors were ruled out by three different criteria.

First criterion. Two mutants, respectively alpha and beta type, were crossed to

74A, a strain highly compatible with KS strains. Eighteen segregants (11 alpha, and 7 beta) were tested for complementation with *m* (KS138A, KS138a, and ED354-1a) and with *n* (KS36a, KS36A, *pyr-3d co*, *pyr-3d T*) strains. Although the speed and intensity of the positive reactions were much improved in most cases, all reactions reported negative in Table 3 were again negative.

Table 3. *Complementation between pyrimidine-dependent mutants of Neurospora obtained by the suppressor method and by auxotroph selection*

Suppressor mutants		Complementation reaction against auxotroph selection mutants			
		Group <i>m</i> mutants		Group <i>n</i> mutants	
		KS138a	ED 354-1a	KS36a	<i>pyr-3d co</i>
No. tested	Group				
5	Alpha	+	+	0	0
6	Beta	+	+	0	0
8	Gamma	+	+	0	0
1	Delta	+	+	0	0
10	Non-complementing*	+	+	0	0

* That is, non-complementing when tested against suppressor mutants (Table 1).

Second criterion. An alternative method for testing complementation involves crossing both auxotrophic strains and determining whether prototrophic pseudo-wild type ascospores appear. This test is more sensitive than the usual heterokaryon test (de Serres, 1960) and eliminates incompatibility barriers (Mitchell & Mitchell, 1956). A total of 10,300 viable ascospores was examined in crosses of $n \times \alpha$; 7700 ascospores in crosses of $n \times \beta$; and 1030 ascospores in crosses of $n \times$ non-complementers. In every case the results were negative. Control crosses of the same to *m* strains gave an average of 0.2 % prototrophs.

Third criterion. The strain from which all suppressor mutants derive is arginine-dependent. It was a simple matter to test whether this strain (ED416-1a) is heterokaryon-compatible with KS36a, one of the strains used as *n* tester in the experiment of Table 3. Mixed inoculation allowed growth on orotic acid supplemented plates. Isolation of hyphal tips, cultivation on minimal medium slopes, and plating of microconidia on appropriately supplemented media, proved that a true heterokaryon had formed. Therefore, no barriers existed which prevented heterokaryon formation.

Conclusion. The complementation results shown in Table 3 reflect functional relations at the *pyr* locus, and are not distorted by extrinsic factors. Pyrimidine-dependent mutants representative of the different types obtained as suppressors of *arg*, are all of the *n* complementing type, and thus presumably deficient in ATC.

Allelism of suppressor mutants

In this section we shall consider all mutants obtained by the suppressor method, with the exception of back mutants at *arg*. Allelism between *pyr^{su-arg}* and *pyr* was demonstrated to within ± 0.1 map units in two instances in a previous publication (Reissig, 1960). Recently 89 new *pyr^{su-arg}* mutations were identified by genetic tests (Reissig, 1962). These tests involved crossing *arg pyr^{su-arg} × arg pyr*. If *pyr^{su-arg}* and

pyr were not allelic, recombination would yield *arg* + + strains, arginine-dependent but pyrimidine-independent. Yet no arginine-dependent strain was found among 748 pyrimidine-independent segregants isolated from crosses involving the 89 new strains. Thus most if not all mutants described as *pyr*^{su-arg} are alleles of *pyr*, or closely linked to this locus.

Allelism of all pyrimidine-dependent mutants is most simply demonstrated by a functional criterion: they all can be classified in five complementation groups, such that one of the groups fails to complement with the other four (α , β , γ , δ). Genetic evidence for allelism between an alpha mutant and a beta mutant was presented earlier (Reissig, 1959). Five additional mutants representing the various complementation groups were crossed to a sixth *pyr* mutant; again no pyrimidine-independent recombinant was detected among several thousand ascospores from each cross.

Having demonstrated that the major mutations screened with the suppressor method are allelic, the possibility should be considered that concomitant mutations at modifier genes might be responsible for the minor phenotypic differences observed. This seemed unlikely, since it would require simultaneous mutation of two genes (*pyr* and modifier) affecting the same character, but nevertheless was tested. Four *pyr* mutants with $Rg_{ud} = 0.6-0.7$ (*pyr*_{0.6-0.7}) were crossed to a *pyr* strain with $Rg_{ud} = 0.0$ (*pyr*_{0.0}) marked with *co* (colonial), about 8 units away from *pyr*. Both parents carried also the *cot* (colonial at 32°) allele. When ascospores from such a cross are incubated at 32°, all growing clones make small colonies. The morphological difference between *co* and *co*⁺ is masked at this temperature, and size differences are related to growth requirements only. Cooling then to 25° the difference between *co* and *co*⁺ can be clearly appreciated in a few hours. Ascospores from crosses of *co*⁺*arg pyr*_{0.6-0.7}*cot* × *co arg pyr*_{0.0}*cot* were plated on minimal medium with and without pyrimidines. On the former medium the colonies formed were of normal size, half *co* and half *co*⁺. On medium lacking pyrimidines, the colonies which appeared were small, and most (695 out of 702) were *co*⁺. This result shows that the factor allowing residual growth is closely linked to *co*.

In another experiment, ascospores from the cross *co arg pyr*_{0.2}*cot* × *co*⁺*arg pyr*_{0.0}*cot* were plated on minimal medium. Seven days later, microcolonies could be distinguished and transferred to pyrimidine supplemented medium. On cooling, all 174 colonies were found to be *co*. Control platings on pyrimidine medium yielded half *co* and half *co*⁺. Control platings on minimal of crosses involving *pyr*_{0.0} × *pyr*_{0.0} gave no microcolonies. Thus, linkage between the residual growth factor and *co* was again demonstrated. In all likelihood this means that residual growth is an attribute of the *pyr* alleles characteristic of each mutant, and that additional modifier mutations are not involved.

ATC activities

Dr R. H. Davis, at the University of Michigan, has assayed some of our suppressor mutants for ATC activity and very kindly made available his results for insertion in the present publication (Table 4). It is clear that *pyr* mutants belonging to the five complementing groups are all deficient in ATC. Mutants with low Rg_{ud} have no demonstrable ATC activity, where 5% of the normal value would have been detectable; and mutant KØ493-50a, capable of appreciable growth in the absence

Fig. 8. Summary of comparisons between pyrimidine-dependent mutants of *Neurospora* obtained by auxotroph selection and by the suppressor method. The numbers indicate numbers of mutants found in each class. Failure to complement is signified when bars overlap. The scheme is based on the complementation map of Suyama *et al.* (1959). The more recent results of Woodward (1962) differ by the finding of complementation among certain '*m*' mutants, and in some combinations of '*m-n*' with '*n*' or '*m*'. *ATC = aspartic transcarbamylase.

Table 4. *Aspartic transcarbamylase activities in Neurospora mutants isolated by the suppressor method, and in controls*

Enzyme assays performed by Dr R. H. Davis (personal communication). Methods are described elsewhere (Davis & Woodward, 1962). Cultures in liquid minimal medium, supplemented with arginine and uridine (each 50 mg./l.). All strains derived from ED416-1a by one-step mutation, except 73a.

Source of extract				Specific ATC activity in extract (μ mole ureido-succinate/mg. protein/hr.
Strain number	Genotype	Complementation type	R _{Gad}	
Mutants:				
K0493-14a	<i>arg pyr</i>	Alpha	0.18	0.0
K0493-54a	<i>arg pyr</i>	Beta	0.02	0.0
K0556-185a	<i>arg pyr</i>	Gamma	0.00	0.0
K0545-122a	<i>arg pyr</i>	Delta	0.00	0.0
K0545-31a	<i>arg pyr</i>	Non-complementing	0.00	0.0
K0493-50a	<i>arg pyr</i>	Alpha	0.60	0.55
K0493-40a	<i>arg pyr^{su-arg}</i>	.	1.05	0.48
Controls:				
ED416-1a	<i>arg pyr⁺</i>	.	1.17	1.66
K0493-56a	<i>arg⁺ pyr⁺</i>	.	1.01	1.89
73a	<i>arg⁺ pyr⁺</i>	.	ca. 1	1.72

of uridine (Rg_{ad} = 0.6), had about one-third of the activity in the controls. Mutant K0493-40a, a typical *pyr^{su-arg}* strain, also displayed decreased ATC activity; while K0493-56a, a back-mutant of ED416-1a, had the same activity as ED416-1a or an unrelated wild strain.

Dr M. Nazario (personal communication; Instituto Nacional de Microbiología, Buenos Aires) has confirmed the absence of ATC activity in strains KØ493-14, KØ493-54, KØ545-122 and KØ545-81, by using a method capable of detecting 0.3 % of the normal activity. He measured incorporation of ^{14}C carbamyl phosphate in the acid-stable fraction, using extracts of strains cultured under conditions of de-repression, i.e. with limiting uridine.

The biochemical data reported confirm the conclusions arrived at in previous sections on the basis of genetic and complementation data alone, namely that suppressor mutants are deficient in ATC (Fig. 3); and that *pyr^{su-arg}* is an allele of *pyr*.

DISCUSSION

The mutants screened by the suppressor method fall into two groups: back mutations at *arg* and forward mutations at *pyr*. The latter involve drastic (*pyr-N*) or partial (*pyr^{su-arg}*) deficiencies in ATC activity. Since selection is based on the suppression of the *arg* gene, it could be concluded that this suppression entails in every case a total or partial block in reaction (1):

The available evidence (Reissig, 1960; Davis, 1962) indicates that *arg* has a partial (cf. Fig. 1) block in the synthesis of carbamyl phosphate, a common precursor of arginine and pyrimidines. From carbamyl phosphate onwards, the arginine and pyrimidine biosynthetic pathways diverge via reactions catalysed, respectively, by ornithine transcarbamylase (OTC) and by ATC (reaction 1). Thus, OTC and ATC compete for carbamyl phosphate. Why a block in carbamyl phosphate synthesis should result in a mutant responding to arginine, but not to pyrimidines, has not been explained with certainty; but this might easily result from irreversibility of certain reactions, channelling, or prevailing regulatory systems. At any rate, suppression of *arg* by a second mutation creating a deficiency for ATC, is readily understood in terms of a diminished competition for carbamyl phosphate. Diminution of ATC activity to about one-third its normal value suffices for full suppression of *arg* (Table 4). Other mutations in the *pyr* region cause only partial suppression of *arg* (Reissig, 1960, and present paper), presumably as a result of smaller decreases in ATC activity.

There are two types of genes which control protein synthesis: regulatory and structural (Jacob & Monod, 1959). In the present study, the frequency distribution of ATC-deficient mutants is clearly bimodal when they are classified according to the extent of their pyrimidine dependence (Fig. 2), and presumably also when classified by ATC activity (Table 4). This can be most simply explained on the hypothesis that most pyrimidine-dependent types (*pyr-N*) are due to mutations at a structural gene, and most prototrophs (*pyr^{su-arg}*) to mutations at a regulatory gene. Of course, the evidence for the existence of both regulatory and structural genes in this system is not compelling, and is indirect. Alternative but less plausible explanations are possible. For instance, the structural relations in the ATC molecule may be such that amino acid substitution would most often lead to two discontinuous activity states. Or discontinuity may follow from the peculiar effects of chromosomal sites of high mutability ('hot-spots', in the terminology of Benzer & Freese, 1958). The

latter alternative is rendered improbable by the fact that the same distribution of mutant types can be obtained with different mutagens (Reissig, 1963).

An interesting feature of the mutants discussed here is the fact that mutants defective in the same enzymic activity are able to complement in heterokaryons. It was noted (Table 2) that there is a positive correlation between residual growth of the various mutants and their ability to complement. This correlation should be kept in mind when comparing mutants obtained by the suppressor method with mutants obtained by the usual methods for the selection of auxotrophs, since the latter methods discriminate against mutants which exhibit residual growth, while the suppressor method does not. A possible theoretical basis for the correlation between residual growth and complementation is provided by the observation (Yanofsky & St Laurence, 1960; Lacy & Bonner, 1961) that tryptophan synthetase mutants which possess CRM (an immunologically related protein) complement more frequently than those which lack it. In turn, possession of CRM might be a condition for residual growth.

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This is paper 3 in the series on forward and back mutation in the *pyr-3* region of *Neurospora*.

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