*AN ENZYMATIC DIFFERENCE AMONG PYR-3 MUTANTS OF NEUROSPORA CRASSA**

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The *pyrimidine-3* mutants of *Neurospora crassa* have received considerable attention in the past. Mitchell and Mitchell[1] described experiments, using *pyr-3a* (37301), *3b* (37815; temperature sensitive), and *3d* (45502), which indicated that the *pyr-3a* and *3b* mutations represent a genetic unit distinct from *3d*. The evidence for this view was that pyrimidine-independent heterocaryons could be formed by the combination of *pyr-3a* or *3b* with *3d;* that *3d*, unlike *3a* or *3b*, was unaffected by a suppressor, *s;* and that crosses between *3a* or *3b* and *3d* gave rise to a few (less than 0.01 per cent) wild type progeny. The segregation of a closely linked colonial marker (*co*), however, suggested that the wild type progeny arose through a mechanism different from simple reciprocal chromosome exchange during meiosis.

Suyama *et al.*[2] have confirmed and extended these data with a large series of independently isolated *pyr-3* mutants. Their work indicates that the *pyr-3* locus consists, on the basis of heterocaryon complementation, of two functional units, and that a large portion of the wild type progeny arising from intercrosses of mutants can be ascribed to a mechanism ("gene conversion") which does not involve the recombination of closely linked genetic markers. All of the *pyr-3* mutants, including both functional units revealed by heterocaryon complementation, appear to be arranged in a continuous, linear order, and reasonable additivity of distances prevails in the genetic map. The maximum percentage of prototrophs from crosses of mutants was approximately 0.02 per cent, which indicated a very short length of the *pyr-3* complex. (The *pyr-3d* mutant is reported to be identical with a mutant, KS36, in one of the functional units described by Suyama *et al.*;[2] *pyr-3a* and *3b* presumably represent the other unit.)

Because the patterns of heterocaryon complementation or suppressor relationship observed among *pyr-3* mutants has been observed in other mutant series concerned with one[3–6] or with more than one enzyme, it cannot be decided with the evidence available whether or not all *pyr-3* mutations affect a single metabolic function.

This investigation was designed to relate enzymatic features of the *pyr-3* mutants to the implied physiological differences between them and to the genetic structure of the *pyr-3* locus. Preliminary data have indicated that *pyr-3a*, *3b*, and *3d*, like the wild type strain, all contain the series of enzymes required for the transformation of ureido-succinic acid (US; = carbamyl aspartic acid) to uridylic acid via the orotic acid pathway, and that deficiencies in the activity of certain of these enzymes are responsible for the pyrimidine requirement of other *pyr* mutants.[7] US does not satisfy the nutritional requirement of any of the *pyr-3* mutants. The preliminary enzymatic studies, however, were considered compelling evidence that the metabolic block in *pyr-3* mutants concerned the synthesis of US or earlier steps of pyrimidine metabolism.[8] Attention was therefore directed to the synthesis of

US in *pyr-3* mutants; this study deals with the enzyme, aspartic carbamyl transferase, which catalyzes the following reaction:

L-aspartic acid + carbamyl phosphate → L-US + inorganic phosphate.

This enzyme has previously been demonstrated in *E. coli*[9, 10] and higher organisms.[11] The data presented here indicate a fundamental difference between *pyr-3d* on the one hand, and *pyr-3a, 3b,* and wild type on the other.

Materials and Methods.—Cultures of *pyr-3a, 3b, 3d,* and of wild type 5297 were obtained from the culture collection of the California Institute of Technology. *Pyr-3b* requires a pyrimidine as a nutritional supplement only at temperatures higher than 32°C.[1]

Mycelia were grown for 18 to 24 hr, with shaking, in 2,500 ml culture flasks containing 700 ml minimal medium. Uridine (50 μg/ml) was used as a nutritional supplement for the pyrimidine mutants. The mycelia were harvested, washed, dried in a Buchner funnel, and dry powders were prepared after the pads were ground in cold acetone.

The extracts for enzyme work were prepared by extracting three grams of the powder twice with 20 ml 0.02 *M* tris(hydroxymethyl)-aminomethane-acetic acid (Tris-acetate) buffer, pH 8.0. The extracts were combined and acetone fractionation was applied. A precipitate which formed between 33 and 60 per cent acetone was collected by centrifugation, redissolved in 0.005 *M* buffer, pH 8.0, and dialyzed against the same buffer in small diameter tubing for 6 hr with stirring. Up to 15 per cent of the activity was lost on dialysis. A sevenfold purification could be achieved by adding aged calcium phosphate gel until the transferase activity began to decline. For most experiments, the dialyzed acetone fraction, which contained from 10 to 15 mg protein per ml, was used.

Carbamyl phosphate (CAP) was prepared by the method of Jones *et al.*[12] and was 96 per cent pure.

Aspartic carbamyl transferase activity was measured at 25° or 35° by the appearance of US after 15 min in the following reaction mixture: 10 μmoles CAP, 40 μmoles K^+ L-aspartate, 250 μmoles glycine-NaOH buffer, pH 9.1, and 0.2 to 0.4 ml of the dialyzed acetone fraction; final volume = 3.25 ml. The reaction was stopped by the addition of 0.5 ml of 2 *M* perchloric acid. After centrifuging the mixtures, 3 ml were passed quickly through a Dowex-50W column (1 × 1 cm, H^+ form), the column was rinsed with an equal volume of water,[10] and the eluate was tested by the procedure of Koritz and Cohen.[13] Inorganic phosphate was measured directly on cold perchloric acid-precipitated reaction mixtures by the method of Lowry and Lopez.[14]

Aspartic Carbamyl Transferase from Wild Type.—Wild type extracts displayed aspartic carbamyl transferase activity, and the activity was directly proportional to the amount of extract in the reaction mixtures. US was identified as a product of the reaction by chromatography[15] and by the method of Reichard and Hanshoff,[9] which involves isotope dilution and recrystallization of US to constant specific activity. D-aspartate would not serve as a substrate for the reaction. The appearance of phosphate is stoichiometrically equivalent to the US formed if the aspartate-independent breakdown of CAP is taken into account: in two experi-

ments, the ratio of μmoles phosphate to μmoles US was 1.17 to 1.09 and 0.85 to 0.84.

No cofactor requirement for the reaction could be detected. The activity was not inhibited by cyanide (0.01 *M*), arsenate (0.06 *M*), fluoride (0.01 *M*), EDTA (0.05 *M*), phosphate (0.2 *M*) or D-aspartate (0.01 *M*), but it was inhibited by iodoacetate and *p*-chloromercuribenzoate (90 per cent inhibition with 10^{-4} *M* PCMB). The inhibition brought about by PCMB was reversible to a great extent by glutathione. These results suggest that the activity of the enzyme is dependent upon sulfhydril groups; the enzyme thus resembles the equivalent transferase isolated from *E. coli*.[9] Other data pertaining to the wild type *Neurospora* enzyme are presented in the following section.

Comparison of Mutant and Wild Type Extracts.—Extracts of *pyr-3a* mycelia contained greater aspartic carbamyl transferase activity than wild type extracts.

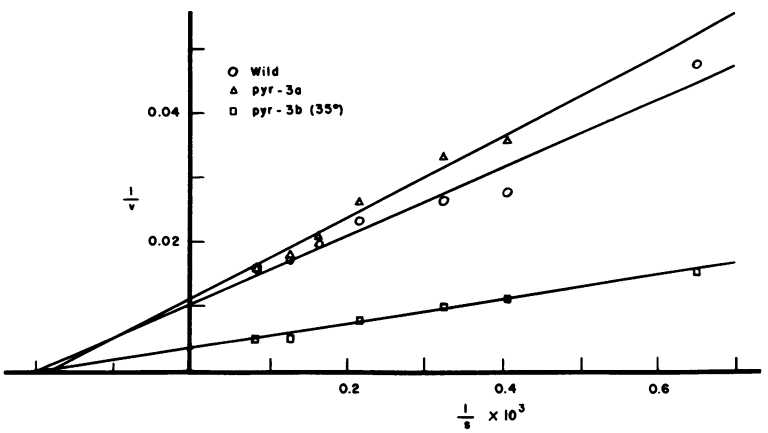

FIG. 1.—Lineweaver–Burk plot of aspartic carbamyl transferase reaction velocity, measured by the appearance of US, as a function of aspartate concentration. The standard conditions were used, except that the aspartate concentration (molar) was varied. The reactions were carried out at 25°C in the case of wild type and *pyr-3a*, and at 35°C in the case of *pyr-3b*. The lines were fitted by the method of least squares, and K_m values (see text) were derived from the X-intercepts of the least squares regressions.

Similar extracts of *pyr-3b*, grown either at 25°C or at 35°C, were active when the reaction was carried out at 25°C or at 35°C, and the relationship of the activities at the two reaction temperatures was much the same as that observed in wild type extracts (Table 1).

A further comparison of wild type and mutants was made in regard to the effects of changes in substrate concentration and the pH of the reaction mixture. By means of a Lineweaver-Burk analysis[16] (Fig. 1), the derived K_m values for aspartate for the enzymes of wild type, *pyr-3a*, and *pyr-3b* (the reaction was carried out at 35°C in the case of *pyr-3b*) were 5.0, 5.75, and 4.9 × 10^{-3} *M*, respectively. Moreover, no great differences in the dependence of the reaction on CAP concentration or pH could be observed in extracts of wild type and *pyr-3a* (Fig. 2).

In contrast to extracts of *pyr-3a* and *3b*, those of the mutant *pyr-3d* were consistently inactive (Table 1); an activity of one per cent of the wild type extracts would have been detected. When an extract of *pyr-3d* was added to wild type

or *pyr-3a* extracts, the activities of the latter two were not significantly altered. This rules out, to a great extent, (*a*) a free inhibitor, (*b*) an unsatisfied cofactor requirement, and (*c*) a reaction which competes with the synthesis of US, as causal factors in the inability of *pyr-3d* to yield aspartic carbamyl transferase activity.

The relationship of the activity, or lack of it, in *pyr-3a*, *3b*, *3d* and wild type was the same if untreated extracts of fresh homogenized mycelia were used instead of acetone powder extracts.

Discussion.—The foregoing data clearly indicate that the *pyr-3a* and *pyr-3b* mutations are not correlated with significant changes in the enzyme aspartic carbamyl transferase with regard to differences in its activity at different temperatures, pH's, or substrate concentrations. The enzyme activity of *pyr-3d* extracts, on the other hand, has been found to be less than one per cent that of similar extracts of wild type mycelia. The enzymatic difference between the *pyr-3* mutants corresponds to that observed in studies of their genetics,[1, 2] heterocaryon comple-

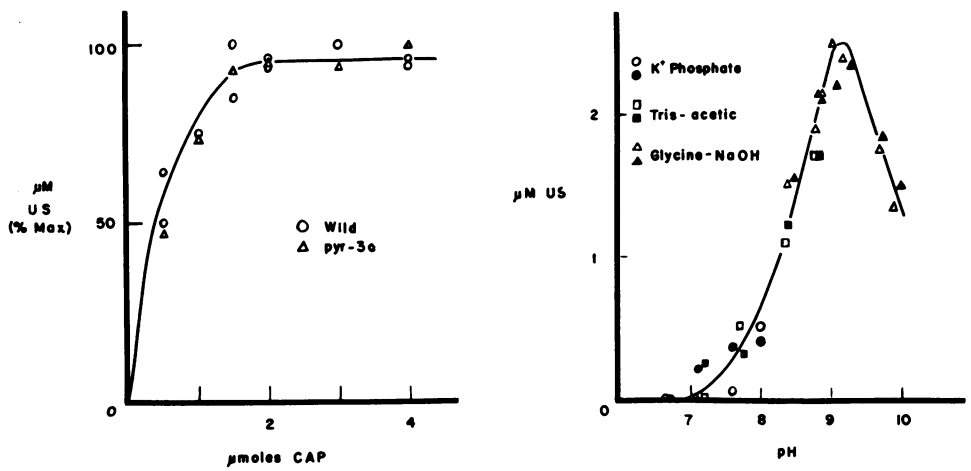

FIG. 2.—The effect of CAP concentration (μmoles per reaction mixture) (left) and *pH* (right) on aspartic carbamyl transferase activity of wild type and *pyr-3a* extracts. In the right-hand figure, solid symbols represent wild type, open symbols *pyr-3a*.

mentation,[1, 2] and suppressor response.[1] Four other pyrimidine-requiring strains presumed to represent the *pyr-3* locus, 49001, 63902, 68902, and 47102, have also been found to lack aspartic carbamyl transferase activity. (Mutant 49001 has been tested and found to resemble *pyr-3d* in its lack of response to the suppressor.[1]) It may be concluded that the lack of aspartic carbamyl transferase activity in these strains and in *pyr-3d* is directly related to the pyrimidine requirement of the mutants themselves. In light of the findings reported here, it is improbable that the series of *pyr-3* mutations directly affects only one enzyme. What has been designated as the "*pyr-3*" locus, a genetic complex covering less than (0.05) map units, is undoubtedly concerned with more than a single function.

These data further show that the inability of *pyr-3d* to grow on medium supplemented with US does not reflect the site of the metabolic block in this mutant.[17] There is, therefore, no reason to think that a similar lack of response to US by *pyr-3a* and *3b* is significant either. This suggestion, together with the preliminary

enzymatic data on *pyr-3* mutants referred to above, allow the assumption that *pyr-3a* and *3b* are blocked before the synthesis of US.

The nature of the biochemical lesion in *pyr-3a* and *3b*, however, has not yet been elucidated. In a series of experiments, Mitchell and Mitchell[18] made observations of the nutritional requirements of strains carrying any one of several arginine mutations and *pyr-3a* or *s*. Their data imply that there is a significant connection between the metabolic block in *pyr-3a* and the synthesis of arginine, and that the synthesis of arginine and pyrimidines is to some extent competitive. Such a competition may operate in the utilization of carbamyl phosphate or aspartic acid, both of which are normally required in pyrimidine and arginine synthesis. A

TABLE 1

ASPARTIC CARBAMYL TRANSFERASE ACTIVITIES OF WILD TYPE AND MUTANT EXTRACTS*

Source of Extract	μM US formed at 25°	μM US formed at 35°	Ratio: Act. 35° / Act. 25°
wild type (5297) grown at 25°	1.43	2.58	1.8
pyr-3a (37301) grown at 25°	6.20	..	..
pyr-3b (37815) grown at 25°	2.33	3.99	1.7
pyr-3b (37815) grown at 35°	1.11	2.11	1.9
pyr-3d (45502) grown at 25°	0.00	..	..

* Approximately 3 mg protein of the dialyzed acetone fraction was used in assays of all strains except *pyr-3d*, where 20 mg was used.

study of the relationship of arginine and pyrimidine synthesis in wild type and *pyr-3a* has been initiated. It is hoped that these further investigations can be profitably related to the extensive genetic analysis of Suyama *et al.*[2]

Summary.—Several pyrimidine-requiring mutants of *Neurospora crassa*, presumed to represent a single locus (*pyr-3*), have been studied in regard to the activity of aspartic carbamyl transferase of mycelia. It has been found that *pyr-3d* and certain other mutants yield no activity, while *pyr-3a* and *3b* appear to contain an enzyme indistinguishable from that demonstrated in the wild type. The results suggest that the *pyr-3* "locus" is concerned with more than a single function.

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*ANTAGONISM BETWEEN SUBSTRATE AND REPRESSOR IN CONTROLLING THE FORMATION OF A BIOSYNTHETIC ENZYME**

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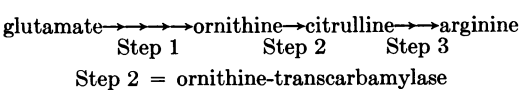
Since the formation of many degradative enzymes is induced by adding to the culture medium the corresponding substrate, it has often been suggested that the "constitutive" nature of biosynthetic enzymes depends on the endogenous formation of their substrates. It would appear possible to test this hypothesis by using a genetic block to prevent this endogenous formation. However, previous attempts to study the problem in this way[1, 2] have led to inconclusive results, since growth on the substrate was compared with growth on the end product of the biosynthetic sequence, and this end product has since been found to repress the formation of the enzyme. Furthermore, the added substrate was also serving as a source of the repressor. An additional possible complication of such experiments is formation of the substrate from the added end product, in those cases where the reactions between the two compounds are reversible.

In undertaking a reinvestigation of this problem with ornithine transcarbamylase of *Escherichia coli*, an enzyme of arginine biosynthesis which catalyzes the conversion of ornithine to citrulline, we have controlled these interfering factors by two devices: the use of a chemostat and the use of double mutants blocked both before and after the reaction under study. The chemostat made it possible to reduce repression by supplying mutants with the required arginine at low, controlled concentrations.[3] In the double mutants (Fig. 1), the earlier block



Step 2 = ornithine-transcarbamylase

FIG. 1.—Schematic pathway of arginine biosynthesis.

prevents endogenous formation of the substrate (ornithine), while the later block prevents both formation of the end product of the seqence from added substrate and formation of the substrate from added end product. In *E. coli* there appears to be no additional pathway to ornithine.[4] The study has been extended to include not only arginine-repressible strains but also strains in which arginine does not have this effect.

Bacterial Strains.—In the present experiments the wild type and mutants of different strains of *E. coli* have been studied: the W strain, in which added arginine