THE REPLACEMENT OF PARA-AMINOBENZOIC ACID BY METHIONINE IN THE GROWTH OF A NEUROSPORA MUTANT

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Present knowledge of the function of *para*-aminobenzoic acid (PABA) has been derived almost exclusively from sulfanilamide (SAN) inhibition studies. Shive and Roberts (1946) have advanced the hypothesis that PABA is catalytically active in the synthesis of a number of metabolic products in *Escherichia coli* and that the enzyme systems concerned in their production are differentially sensitive to SAN inhibition. They present evidence that the most sensitive reaction to SAN is that concerned with methionine synthesis, and adenine synthesis is the next most sensitive step. However, since methionine and adenine are incapable of reversing SAN inhibition in all concentrations at which PABA was effective, it was concluded that PABA is concerned in other unknown synthetic reactions. Later, Winkler and de Haan (1948) reported that, in the presence of methionine and adenine, serine and pteroylglutamic acid (or thymine) would reverse the inhibitory effects of higher concentrations of SAN. The results indicate that the latter compounds are also products of reactions in which PABA is concerned.

Strandskov (1947), in a further study of PABA antagonists, found that the inhibition of growth of *E. coli* by 2-chloro-PABA could be reversed by methionine at any concentration of the inhibitor. Strains that became resistant to 2-Cl-PABA were shown, however, not to be resistant to SAN, and from these data Strandskov concluded that SAN inhibits a step in methionine synthesis in addition to that inhibited by 2-Cl-PABA.

The present report is concerned with the characteristics of a biochemical mutant of *Neurospora* whose growth requirements can be satisfied by either methionine or PABA. The response of this mutant to various growth factors in the presence and absence of SAN has been studied. The results indicate that one of the rate-limiting steps in the growth of this organism is the methylation of homocysteine to form methionine.

MATERIALS AND METHODS

The biochemical mutant of *Neurospora* (8043-M) was obtained in this laboratory from the wild type after treatment with nitrogen mustard (McElroy, Cushing, and Miller, 1947). In growth studies the mutant strain was grown in 125-ml Erlenmeyer flasks containing 20 ml of Fries basal medium to which

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the various inhibitors and growth-supporting compounds were added prior to autoclaving (Beadle and Tatum, 1941). Inoculations were made from sterile conidial suspensions of the mutant, and the flasks were then incubated at 29 C for 2 to 8 days, depending on the experiment. At the end of the incubation period the mycelium was harvested, dried at 80 C overnight, and weighed on a torsion microbalance. All determinations were made in duplicate.

EXPERIMENTAL RESULTS

Growth requirements. The response of mutant 8043-M to varying concentrations of methionine or PABA is shown in figure 1. The growth on methionine is characterized by a relatively long lag period in contrast to the growth on PABA (figure 2). Since it was possible that some factor was accumulating in the medium

Figure 1. Growth of mutant 8043-M on varying concentrations of PABA and DL-methionine at 29 C. Growth on methionine for 7 days. Growth on PABA for 3 days.

or in the mycelium that was necessary for rapid growth, inoculations were made from rapidly growing cultures into methionine-containing media and also into sterile media in which the mutant had grown for 3 to 4 days on methionine. No significant removal of the lag was obtained in either test. The growth lag could not be removed by growing the mutant in the presence of methionine at various temperatures (23, 29, and 35 C). Likewise, variations in pH had no significant effect and, in addition, the growth lag could not be removed by the addition of vitamins (except PABA), amino acids, purines, and pyrimidines, singly or in combination.

In order to test the possibility that this mutant is capable of synthesizing small amounts of PABA, or some compound with PABA activity, in the presence of methionine, the mutant was grown in 2-liter Fernbach flasks containing 400 ml of Fries basal medium and 20 mg of DL-methionine. For comparison, a wild

type strain was grown in the basal medium. After 3 and 8 days, the mycelia were harvested, dried, ground, and then hydrolyzed for 30 minutes in N HCl at 120 C. The hydrolyzates were filtered, neutralized, and brought to a total volume of 25 ml per g dry weight of mycelium. Another PABA mutant that is incapable of responding to methionine or factors other than PABA was used for assay. Table 1 shows the amount of PABA in the mycelium of the mutant and the wild type grown under these conditions. It is clear from these data that mutant 8043-M does produce some PABA, although in very small amounts—10 to 15 per cent of the normal wild type.

Various compounds related to methionine and PABA were tested for their growth-stimulatory activity. The results of this study are summarized in table 2.

Figure 2. Growth of mutant 8043-M on 1 mg methionine per 20 ml basal medium and on 1×10^{-4} mg of PABA per 20 ml basal medium for 1 to 7 days at 29 C.

It will be noted, as observed earlier by Tatum and Beadle (1942), that growth is obtained on benzene rings with attached nitrogen in either the amino form or in nitro groups. If, however, other substituents are present on the ring in the *ortho* or *meta* position (except for *m*-toluidine), or if the amino group itself is substituted, as in the case of *p*-dimethylaminobenzoic acid, growth-stimulatory activity is destroyed. In contrast to the mutant studied by Tatum and Beadle (1942), pimelic acid produced no appreciable growth. It is interesting to note that 2-chloro-PABA supports growth. The homocysteine thiolactone-HCl was synthesized from homocysteine by the method of Riegel and du Vigneaud (1935). The fact that homocysteine is incapable of supporting the growth of this mutant is of some interest, especially when compared to the fact that homocysteine is not capable of antagonizing sulfanilamide inhibition (Winkler and de Haan, 1948).

Sulfanilamide inhibition. Since the most sensitive reaction to sulfanilamide appears to be that concerned with methionine synthesis, it was of some interest to study the effect of SAN on this mutant. The concentration of SAN to give 50 per cent inhibition of growth in the presence of different concentrations of

TABLE 1
PABA synthesis by mutant 8043-M

ORGANISM	PABA $\mu\text{g}/\text{mg}$ DRY MYCELIUM $\times 10^{-4}$	GROWTH PERIOD (DAYS)
Wild type (5256-A)	41.0	3
8043-M	6.5	3
8043-M	4.5	8

PABA synthesized by mutant 8043-M and wild type in 3 and 8 days as determined by bioassay with mutant 3450-M (McElroy, Cushing, and Miller, 1947). Details in text.

TABLE 2
Growth of mutant 8043-M on various compounds

COMPOUND TESTED	RESPONSE	COMPOUND TESTED	RESPONSE
Benzoic acid (BA)	—	<i>m</i> -Toluidine	+
<i>p</i> -OH-BA*	—	Aniline	+
<i>o</i> -OH-BA*	—	Folic acid	$\pm$
<i>o</i> -Cl-BA*	—	PABA	+
<i>o</i> -NO ₂ -BA*	—	DL-Methionine	+
<i>o</i> -NH ₂ -BA*	—	Pimelic acid	—
<i>p</i> -Cl-benzaldehyde*	—	L-Cystathionine†	—
<i>m</i> -NO ₂ -benzaldehyde*	—	DL-Homocysteine thiolactone·HCl	—
<i>p</i> -Dimethyl-amino-BA*	—	DL-Homocystine	—
<i>p</i> -NO ₂ -benzamide*	+	DL-Cysteine	—
2-Cl-PABA‡	$\pm$	Choline	—
<i>p</i> -NH ₂ -acetophenone	$\pm$		
<i>p</i> -Toluidine	+		

Compounds to be tested for growth-promoting activity were added to 20 ml Fries basal medium, inoculated, and incubated for 7 days at 29 C. Duplicate determinations were made at 2 different concentrations. —, no response; $\pm$, slight response; +, good response. Concentrations added: PABA, 0.1 and 1 μg per 20 ml; methionine, homocysteine, homocystine, and cysteine, 1 and 5 mg per 20 ml; pimelic acid and cystathionine, 1 and 2 mg per 20 ml; choline, 50 μg per 20 ml; all others, 1 and 100 μg per 20 ml.

* Courtesy of Dr. A. H. Corwin.

† Courtesy of Dr. H. J. Teas.

‡ Courtesy of Dr. F. B. Strandkov.

methionine, methionine plus adenine, or PABA is shown in table 3. Since the reaction in the synthesis of methionine is already limiting, it is not surprising that the concentration of SAN to give 50 per cent growth inhibition remains essentially constant irrespective of the methionine concentration. The results show that the inhibition of growth on methionine by a given concentration of

SAN can be reversed, however, by the addition of adenine (purine) and that the reversal of this inhibition is of a noncompetitive nature. The mutant will not grow on adenine, other purines, or their derivatives, although all are effective in combination with methionine. Pyrimidines were without effect. PABA exhibits an approximately constant ratio to SAN for 50 per cent inhibition over a 100-fold concentration range.

In addition to the action of the purines in combination with methionine in reversing SAN inhibition, a mixture of ten amino acids produced a slight growth in the presence of concentrations of SAN that cannot be reversed by methionine plus adenine. The effect of the mixture, which contains aspartic acid, nor-leucine, alanine, glutamic acid, glycine, tryptophan, cystine, cysteine, serine,

TABLE 3

Inhibition of growth of mutant 8043-M on methionine, methionine + adenine, and para-aminobenzoic acid by added sulfanilamide

CONCENTRATION OF GROWTH-PROMOTING SUBSTANCE μg/20 ml (A)	CONCENTRATION OF SAN TO GIVE 50% INHIBITION μg/20 ml (B)	RATIO B/A
1,000 methionine (DL)	54.5	0.054
2,000 methionine	58.0	0.028
5,000 methionine	64.5	0.012
100 adenine + 1,000 methionine	76.0	0.76
500 adenine + 1,000 methionine	176.0	0.35
1,000 adenine + 1,000 methionine	140.0	0.14
0.15 para-aminobenzoic acid	400.0	2,600
1.5 para-aminobenzoic acid	5,000.0	3,300
15.0 para-aminobenzoic acid	50,100.0	3,340

Appropriate amounts of methionine, adenine, PABA, and SAN were added to 20 ml of basal medium in 125-ml Erlenmeyer flasks, inoculated, and incubated (29 C) for 7 days. SAN concentrations to give 50 per cent inhibition were extrapolated from inhibition curves and this value is recorded in column B. The ratio of B to A was calculated using the concentration of the first compound listed in column A.

and proline, is greater than the effect of any single amino acid. Glycine and serine, singly or together, however, seem to be the most effective in reversing the inhibition.

DISCUSSION

The interpretation of the growth requirements of this mutant most consistent with the experimental results is as follows: The activity of a gene or genes controlling PABA synthesis has been modified in such a way that the amount of PABA produced is decreased to about one-tenth of the amount synthesized by the wild type. This amount of PABA is not sufficient to meet requirements for methionine synthesis but is sufficient to permit enzyme systems involved in the synthesis of purines, serine, etc., to function at a reduced rate. That the immediate genetic block is in methionine synthesis was thought improbable, since growth on methionine is not so rapid as on PABA and since other methionine

mutants will not grow on PABA. Since it was possible to inhibit the growth of this mutant on a methionine-containing medium by adding sulfanilamide, it is unlikely that methionine synthesis is the only function of PABA. Moreover, there are other PABA mutants that will not respond to methionine. The inability of homocysteine to replace methionine in supporting the growth of this mutant indicates that the step in methionine synthesis that is blocked is that concerned with the methylation of homocysteine. This had previously been suggested from inhibitor studies by Winkler and de Haan.²

It is impossible to determine at present the nature of the genetic change that results in a lowered production of PABA. The production of an inhibitor of a normal gene (suppressor) or gene product, the production of a less efficient or more unstable gene product (hypomorph), or the removal of a precursor by an accelerated side reaction are all possibilities that might explain the results observed. This mutant should afford good material for the study of possible mechanisms of genetic hypomorphs.

The most likely explanation of the long lag phase in the growth of this mutant on methionine is as follows: Owing to the low rate of production of PABA, functions other than the production of methionine in which PABA is concerned proceed at a reduced rate and thus prevent cell division at a normal (wild type) rate. After a certain cell density has been obtained, however, other factors become rate-limiting in the growth of the mutant both on PABA and methionine. Since the same factors are limiting in both cases, the slopes of the curves approximate each other in this phase of growth.

The present work does not present any new concepts of PABA function; however, the results are of interest since they support previous suggestions concerned with the function of PABA which were based almost entirely on inhibitor studies. Thus, this mutant duplicates the growth requirements of the wild type grown in the presence of a concentration of SAN just sufficient to prevent methionine synthesis. In one case the PABA deficiency is genetically determined; in the other it is determined by the added antimetabolite. The exact role of PABA in metabolism is still incompletely known; however, from this and inhibitor studies (Shive and Roberts, 1946; Shive *et al.*, 1947; Winkler and de Haan, 1948) as well as the implication of both PABA and choline methyl groups in serine synthesis (Sakami, 1949), it would appear that PABA is concerned in the transfer of one-carbon units.

SUMMARY

The growth requirements of *Neurospora crassa* mutant 8043-M have been determined both in the presence and absence of sulfanilamide. This mutant will

² After this manuscript was completed the recent report of the isolation of PABA mutants in *E. coli* by Lampen, Jones, and Roepke (1949) appeared. It would appear that either the metabolism of PABA with respect to methionine synthesis is different in these two organisms or that different reactions become rate-limiting in the synthesis of methionine when PABA function is interfered with in the two organisms. Some, but not all, of the variants of their mutant would seem to fall into categories somewhat similar to those of the *Neurospora* mutant reported here.

grow on a basal medium supplemented with either DL-methionine or *para*-aminobenzoic acid (PABA) as well as certain compounds closely related to the latter. Growth on methionine is characterized by a pronounced lag phase of 2 to 3 days.

The amount of PABA synthesized by this mutant when grown on methionine is 10 to 15 per cent of the amount synthesized by the wild type.

Sulfanilamide inhibition studies on this mutant agree with the results of earlier workers.

From the fact that this mutant will not grow on DL-homocysteine thiolactone-HCl, it is indicated that PABA is concerned in the methylation of this compound to form methionine, and from this and previous work a general metabolic function of PABA is suggested.

REFERENCES

- BEADLE, G. W., AND TATUM, E. L. 1941 Genetic control of biochemical reactions in *Neurospora*. Proc. Natl. Acad. Sci. U. S., **27**, 499-506.
- LAMPEN, J. O., JONES, M. J., AND ROEPKE, R. R. 1949 Mutant strains of *Escherichia coli* unable to synthesize *p*-aminobenzoic acid. J. Biol. Chem., **180**, 423-434.
- McELROY, W. D., CUSHING, J. E., AND MILLER, H. 1947 Induction of biochemical mutations in *Neurospora crassa* by nitrogen mustard. J. Cellular Comp. Physiol., **30**, 331-346.
- RIEGEL, B., AND DU VIGNEAUD, V. 1935 Isolation of homocysteine and its conversion to a thiolactone. J. Biol. Chem., **112**, 149-154.
- SAKAMI, W. 1949 The formation of the β -carbon of serine from choline methyl groups. J. Biol. Chem., **179**, 495-496.
- SHIVE, W., ACKERMANN, W. W., GORDON, M., GELZENDAUER, M. E., AND EAKIN, R. E. 1947 5(4)-Amino-4(5)-imidazolecarboxamide, a precursor of purines. J. Am. Chem. Soc., **69**, 725-726.
- SHIVE, W., AND ROBERTS, E. C. 1946 Biochemical transformations as determined by competitive analogue-metabolite growth inhibition. II. Some transformations involving *p*-aminobenzoic acid. J. Biol. Chem., **162**, 463-471.
- STRANDSKOV, F. B. 1947 Inhibition of methionine synthesis in *Escherichia coli* by 2-chloro-4-aminobenzoic acid and sulfanilamide. J. Bact., **53**, 555-559.
- TATUM, E. L., AND BEADLE, G. W. 1942 Genetic control of biochemical reactions in *Neurospora*: an "amino-benzoicless" mutant. Proc. Natl. Acad. Sci. U. S., **28**, 234-243.
- WINKLER, K. C., AND DE HAAN, P. G. 1948 On the action of sulfanilamide. XII. A set of non-competitive sulfanilamide antagonists for *E. coli*. Arch. Bioch., **18**, 97-107.