THE SULFONAMIDE-REQUIRING MUTANT OF NEUROSPORA: THREONINE-METHIONINE ANTAGONISM[1]

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It has previously been reported (Emerson and Cushing, 1946; Emerson, 1947) that the so-called sulfonamide-requiring mutant strain of *Neurospora* (strain E-15172, hereafter designated by the symbol "sfo") does not grow on minimal medium at 35 C, that the addition of sulfanilamide (SA) permits maximum growth at that temperature and augments the growth occurring at lower temperatures, and that the SA-dependent growth is competitively inhibited by *p*-aminobenzoic acid (PABA).

It was further shown (Zalokar, 1948) that the failure of this strain to grow at higher temperatures is due to inhibition by the amounts of PABA normally produced and that the growth-stimulating action of SA results from an antagonism of this inhibition. The sfo mutant was combined with a mutant (1633, designated "pab") that is unable to synthesize PABA, so that the amount of this vitamin available to the mycelium could be controlled by adding known amounts to the medium. The double mutant (sfo, pab) grows maximally on low concentrations of PABA and is inhibited strongly by slightly higher concentrations, which are still 100,000-fold less than are required to inhibit the wild type. Growth inhibition by the higher concentrations of PABA is competitively antagonized by SA.

The present paper reports experiments that partially identify the reaction responsible for the sulfonamide-requiring character. From studies previously reported it has been supposed that there is a gene-controlled deleterious reaction (in sfo strains) that requires relatively higher levels of PABA than are needed for other PABA-dependent metabolic reactions. The present studies show that the deleterious reaction also requires somewhat more methionine (or homocysteine) than the minimum required for normal growth and that the inhibition resulting from excess methionine is competitively antagonized by threonine (or homoserine).

NOTE ON METHOD OF PRESENTING DATA

Best results with the sfo character have been obtained by the growth-tube method (Ryan, Beadle, and Tatum, 1943). Growth tubes 220 mm in length,

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containing 10 ml of 1.5 per cent agar medium were inoculated at one end with conidia, and the position of the mycelial frontier was recorded at daily intervals. Growth curves were plotted from such data. Growth curves obtained under varying conditions differ from one another not only in the final growth rate but especially in the time required to attain that rate. No single measurement accurately represents both of these characteristics of the growth curves, but a fairly good approximation is obtained by using the amount of growth in 3 days, as this is a function of both the length of the lag phase and the rate of growth thereafter.

With the exception of growth of the double mutant (sfo, me-2) on varying concentrations of methionine, the entire series of experiments has been repeated with a freshly isolated ascospore culture of the sfo strain. Both series of experiments agree closely, but only the more recent is summarized in the figures

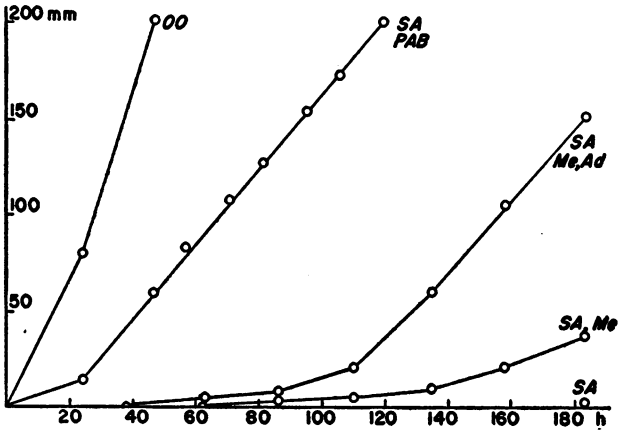

Figure 1. Action of sulfonamide and some of its antagonists on growth of *Neurospora*. Growth curves of wild type (E-5256): *00*—on minimal medium; *SA, PAB* = on 2×10^{-3}M sulfanilamide plus 10^{-5}M *p*-aminobenzoic acid; *SA, Me, Ad* = on sulfanilamide plus 10^{-3}M methionine and 10^{-4}M adenine sulfate; *SA, Me* = on sulfanilamide and methionine; *SA* = on 2×10^{-3}M sulfanilamide.

ROLE OF METHIONINE IN PABA CONSERVATION AND IN INHIBITION BY SA

It has been shown that PABA deficiency in bacteria, whether a result of mutation or of SA inhibition, can be relieved by supplementing the medium with several unrelated substances (cf. Kohn, 1943). Methionine suppresses the greater part of SA inhibition (Harris and Kohn, 1941, *a*, *b*; Lampen, Roepke, and Jones, 1946). Purines, when added with methionine, alleviate inhibition by SA even more (Harris and Kohn, 1941 *a*,*b*; Shive and Roberts, 1946). In some bacteria, thymine can replace the requirement for folic acid, a derivative of PABA (Snell and Mitchell, 1941; Stokes, 1944). In a recent paper Strehler (1950) describes a mutant of *Neurospora* that requires either PABA or methionine for growth. Our results, reported here, are consistent with his observations.

The inhibition of wild type *Neurospora* by SA is partially counteracted by methionine (figure 1). The further addition of adenine or guanine results in a

still greater reduction in the inhibition caused by SA, but these supplements never completely overcome the effect of SA. Similarly, when grown on suboptimal amounts of PABA, the pab strain responds much better if methionine and adenine (or guanine) are added to the medium (figure 2), but these substances do not completely substitute for PABA. Thymine is without effect in this relationship.

METHIONINE AS INHIBITOR OF SFO STRAIN

The sfo mutant, on the other hand, is slightly inhibited by methionine even in the presence of SA. As in the earlier studies in which the double mutant (sfo, pab) was used in showing a PABA requirement of the sfo strain (Zalokar, 1948), it is possible in this case to study the effect of methionine on the sfo character by introducing into the sfo strain mutant genes that block the synthesis of methionine. In this instance the responses of double mutants (carrying sfo

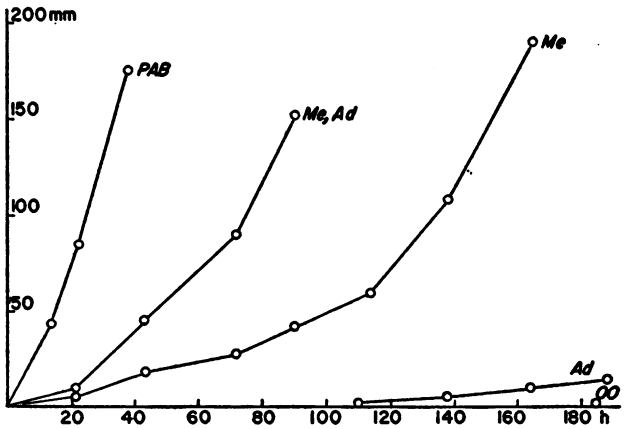

Figure 2. Growth curves of *p*-aminobenzoic-acid-less strain of *Neurospora* (1633): *PAB* = on 10^{-6}M *p*-aminobenzoic acid; *Me, Ad* = on 10^{-3}M methionine and 10^{-4}M adenine sulfate; *Me* = on methionine alone; *Ad* = on adenine sulfate alone; *00* = on minimal medium.

and a genetic block to methionine synthesis) differed depending upon the particular step in the synthesis that was blocked (see summary in table 1).

The steps through which the biosynthesis of methionine takes place in *Neurospora* have been worked out by Horowitz (1947*a*,*b*), and mutant strains that block each step are known (figure 3). Mutant 39816 is blocked before the synthesis of cysteine (step me-4); it will grow if supplied with cysteine or any product appearing later in the reactions leading to methionine. Strains 9666, 36104, and 39103 (which are genetically distinct; Buss, 1944) block the synthesis of cystathionine (step me-3); their growth is not supported by cysteine, but they do grow if supplied cystathionine or any later product. Mutant H-98 cannot split cystathionine to give homocysteine (me-2) and accumulates cystathionine in the medium; its growth is supported by either homocysteine or methionine, but not by products earlier in the series. Finally strains 32213, 35809, 37603, 38706, 44704, 47806, and 48003 are unable to methylate homocysteine to pro-

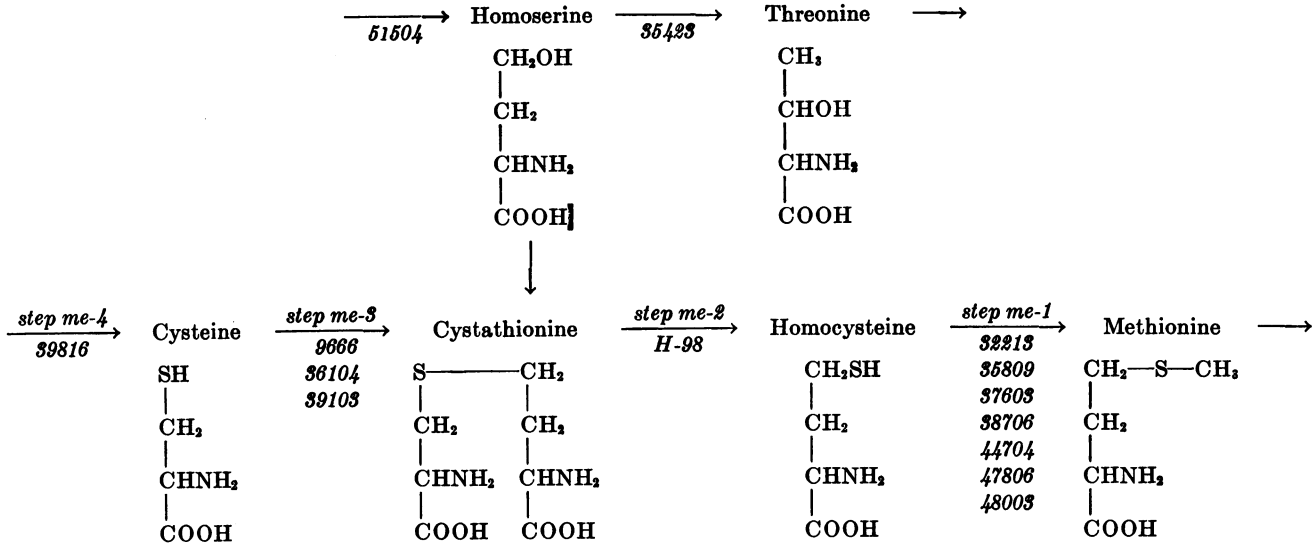

Figure 3. Methionine and threonine synthesis in Neurospora. (After Horowitz, 1947 *a*, *b*; Teas, Fling, and Horowitz, 1948.)

duce methionine (me-1); these have specific requirements for methionine, with the exception of 37603, which requires either methionine or choline (the genetic relationships of these strains are not completely known).

Double mutants (sfo, me-3 and sfo, me-2), in which the block to methionine synthesis precedes homocysteine, show increased growth with increasing DL-methionine concentration up to about 2×10^{-5}M (3 μg per ml). Higher concentrations of methionine are inhibitory (figure 4). The growth of the double mutants at any given concentration is always less than that of the corresponding simple methionineless mutant.

Sulfanilamide antagonizes the inhibition of the double mutants in a noncompetitive manner. In the presence of enough SA to permit growth of the sfo strain, the double mutants (sfo, me) respond to increased methionine concentra-

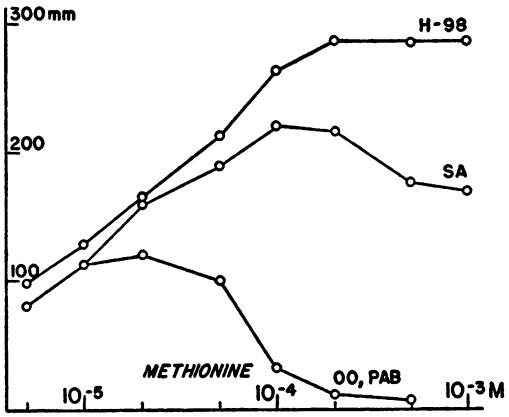

Figure 4. Growth rates expressed as mm grown in 72 hours of the double mutant, sulfonamide-requiring, methionineless (sfo, me-2) on varying concentrations of methionine *OO, PAB* = identical curves on minimal medium with or without addition of 10^{-4}M *p*-aminobenzoic acid; *SA* = growth on minimal medium supplemented with 10^{-4}M sulfanilamide; *H-98* = growth of simple me-2 mutant.

tion as the simple methionineless mutants, except for the slight inhibition at high concentrations characteristic of the simple sfo strain (figure 4).

When the double mutant (sfo, me) is grown on the concentration of methionine permitting greatest growth (about 2×10^{-5}M), added PABA is without effect as a growth inhibitor (figure 4).

Homocysteine, the precursor of methionine, has an inhibitory action similar to, but less pronounced than, that of methionine. In practice, homocysteine was added to the medium in the more stable form of its thiolactone hydrochloride,[4] which has to be reconverted in the organism to homocysteine. As the thiolactone, homocysteine has been found to be less effective than methionine in supporting growth of mutants blocked before homocysteine (Horowitz, 1947*b*). It is therefore not surprising that it is also less effective in inhibiting growth of the sfo strains.

[4] I am indebted to Dr. Marguerite Fling who kindly supplied me with homocysteine thiolactone hydrochloride that she had synthesized.

The behavior of mutant strains blocked between homocysteine and methionine (step me-1) is not completely self-consistent (table 1). Double mutants derived from crosses between sfo and strains 38706, 44704, and 47806 all behave in the same way. They do not grow at any concentration of methionine whatsoever. In the presence of SA these double mutants respond to methionine exactly like the simple methionineless strains.

Mutant 37603, which can grow on either methionine or choline but not on homocysteine, differs from other mutants that can utilize methionine but not homocysteine. When this mutant gene is combined with sfo, the resulting double mutant responds differently depending upon whether choline or methionine is supplied to the medium. With increasing methionine concentration growth rises to a peak at 2×10^{-5} M, above which inhibition sets in. In this respect

TABLE 1

Growth of different sulfonamide-requiring, methionineless double mutants on methionine

DOUBLE MUTANT		METHIONINE ADDED				
Type	Methionineless parent	0	2×10^{-5} M	10^{-3} M	10^{-3} M 10^{-4} M SA	10^{-3} M 10^{-3} M threonine
sfo, me-1	38706	−	−	−	++	++
	44704	−	−	−	++	++
	47806	−	−	−	++	++
sfo, me-1	37603	−	+	−		+
sfo, me-1	32213	−	±*	±*	++	+
	35809	−	±*	+*		++
	48003	−	±*	+*		++
sfo, me-2	H-98	−	+	−	++	++
sfo, me-3	36104	−	+	−	++	++

* Initial growth, but stopping after a short interval.

the mutant resembles those that are blocked before homocysteine rather than after. When choline is used to support growth, on the other hand, growth of the double mutant increases with increasing concentration, with no inhibition resulting from excess choline.

Another group of mutants (32213, 35809, and 48003) that also utilize methionine but not homocysteine have given still different results. Double mutants in which these mutants are combined with sfo make an initial growth when methionine alone is supplied to the medium, and the higher the methionine concentration, the greater the initial growth rate, but growth is not maintained on any concentration of methionine.

There is other evidence indicating that mutant strains that utilize methionine but not homocysteine do not constitute a homogeneous group. They can be separated into three groups on the basis of their ability to reduce selenite under

different conditions (Zalokar, 1950), and there is recent evidence that members of the three groups so defined are genetically distinct (M. Fling, personal communication). Those mutants which, when combined with sfo, do not grow on methionine belong to the group that reduces selenite only in the presence of methionine. Those which as double mutants produce some growth on methionine belong to the group that fails to reduce selenite under any condition. The third group contains mutants that are always able to reduce selenite.

THREONINE ANTAGONISM OF METHIONINE INHIBITION

In the search for methods of identifying the reaction involved in the inhibition of the sfo strain by methionine a number of postulates were considered. One of these presupposed that homocysteine (the precursor of methionine and perhaps the real inhibitor) might combine with homoserine to give a thioether (homo-

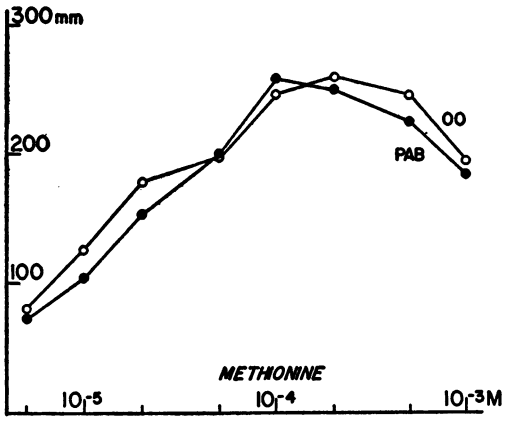

Figure 5. Growth rates of the double mutant (sfo, me-2) on different concentrations of methionine: *OO* = minimal medium with 10^{-3}M threonine; *PAB* = minimal medium with threonine and 10^{-5}M *p*-aminobenzoic acid.

lanthionine; Stekol, 1948) resembling cystathionine, which is similarly formed from cysteine and homoserine (cf. Binkley and Du Vigneaud, 1942). One result of such a reaction might be a deficiency of homoserine for other essential reactions, and since threonine is derived from homoserine (Teas, Horowitz, and Fling, 1948), a deficiency for threonine might result. No direct evidence for the production of the postulated thioether has been obtained, but the sfo strain does respond as if it had a deficiency of threonine or homoserine.

Either homoserine or threonine can support growth of the sfo strain at 35 C in the complete absence of SA. At concentrations of 60 μg per ml or more of either growth of the sfo strain equals that of the wild type. Furthermore, at relatively high concentrations of threonine, PABA has no inhibitory effect (figure 5).

In the double mutants (sfo, me-2 and sfo, me-3), in which the available methionine can be controlled, there is a competitive antagonism between threonine and methionine. Methionine inhibition is reduced about 50 per cent by

equimolar proportions of threonine and is completely overcome at molar concentrations two or three times greater than that of methionine (figure 6). At high concentrations of both substances, threonine is relatively more efficient as an antagonist to methionine.

The antagonistic action of threonine and homoserine toward methionine is fairly specific. Many other amino acids were tested by two methods. In one

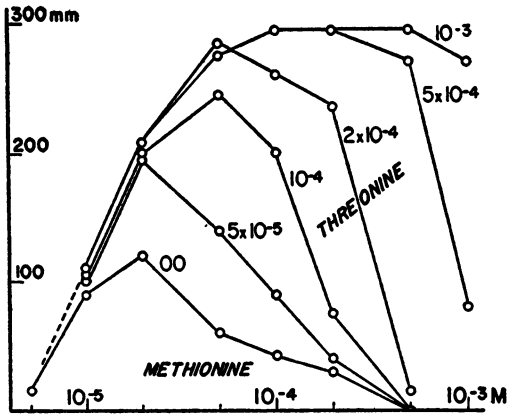

Figure 6. Growth rates of the double mutant (sfo, me-2) on varying concentrations of methionine and threonine.

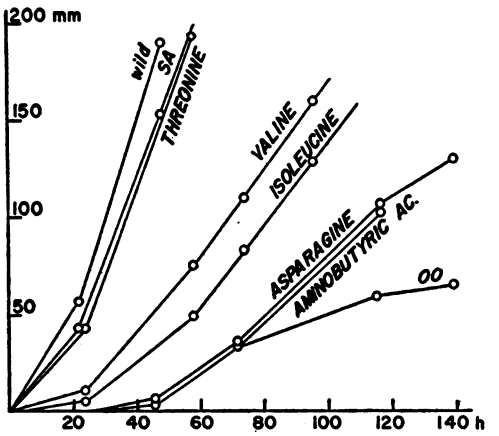

Figure 7. Growth curves of a sulfonamide-requiring strain on different amino acids, added in 10^{-3}M to the medium. Curve of wild type and of sfo type on 10^{-4}M sulfonamide (*sa*) and on minimal medium (*00*) for comparison.

procedure, the amino acids were tested (at 10^{-3} M concentrations) for a threonine-like stimulation of the sfo mutant. In the other, tests were made with the double mutant (sfo, me-2) in the presence of 2×10^{-4} M threonine and 2×10^{-4} M methionine, a mixture resulting in about 50 per cent inhibition of growth. In this situation, if the test substance (again added at 10^{-3} M) was antagonistic to methionine, growth stimulation should result; if it was indifferent there should be no

change; and if the substance itself was inhibitory there should be reduced growth. The following substances proved to be indifferent by both tests: DL-alanine, L-arginine, DL-citrulline, L-cystine, L-glutamic acid, glycine, L-histidine, L-leucine, L-lysine, DL-norleucine, DL-ornithine, L-proline, DL-serine, L-tryptophan, and L-tyrosine.

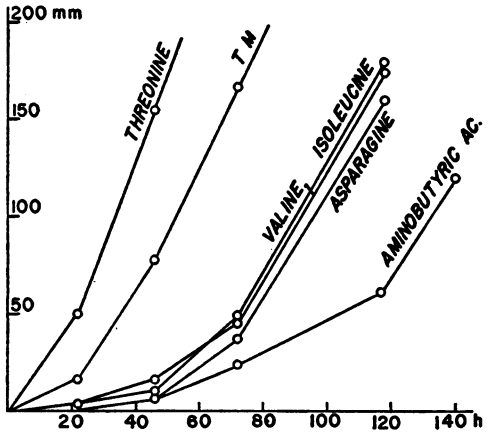

Figure 8. Growth curves of double mutant, sfo, me-2, on different amino acids. All media except the threonine control were supplemented with 2×10^{-4}M methionine and 2×10^{-4}M threonine; other amino acids indicated were added in 10^{-3}M concentration.

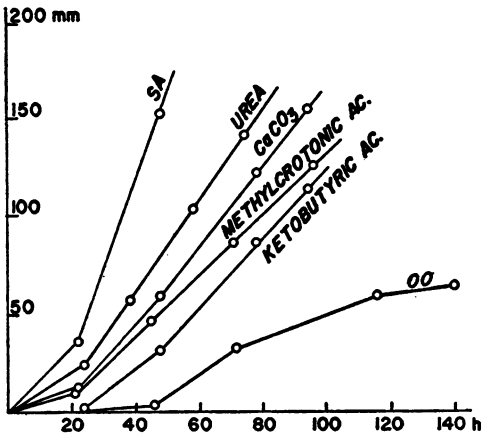

Figure 9. Growth curves of a sulfonamide-requiring strain on pH-raising substances and two C_4-chain growth-promoting substances.

A paradoxical behavior was found in tests with DL-isoleucine, DL-valine, and, in lesser degree, α-aminobutyric acid and asparagine. They each stimulate growth of the sfo strain (figure 7). They each reduce the inhibition of the sfo me-2 double mutant resulting from 30 μg per ml of methionine. In the presence of equal amounts of threonine and methionine, however, these substances are rather inhibitory (figure 8). No substance tested (except SA) is equal to threonine or homoserine in stimulatory action, nor to methionine as an inhibitor.

A common characteristic of all amino acids that either inhibit or antagonize the inhibition of the sfo mutant is a 4-carbon chain. Some other C_4 substances were tested. Acetoacetic, fumaric, malic, and succinic acids are without activity. Crotonic acid is inhibitory, but it is also inhibitory to the wild type. Of the substances tested, α-ketobutyric acid and β-methyl crotonic acid (synthesized by Dr. B. Arreguin-Lozano) have a very obvious stimulatory action. The growth of the sfo strain promoted by those substances, however, was not equal to that promoted by threonine (figure 9). β-Methyl crotonic acid is inhibitory to both the wild type and the mutant. A one-thousandth molar solution distinctly inhibits the wild type, reducing the growth to less than half. One quarter of this amount is still inhibitory to the wild type, but at the same time stimulatory for the mutant.

EFFECT OF HYDROGEN ION CONCENTRATION

The expression of the sfo character was found to depend on hydrogen ion concentration. The sfo strain fails to grow on minimal medium at 35 C in the range pH 4 to pH 6, which is generally realized in the culture medium. If the medium is kept about pH 7 by addition of $CaCO_3$, growth of the mutant is nearly normal (figure 9). A high pH can also be obtained by the addition of urea to the medium. Urea is decomposed by mold enzymes and ammonia liberated, keeping the pH high. Urea supports very good growth of the sfo strain in the absence of sulfanilamide or threonine. In this case, however, the response may be due to free ammonia as well as to high pH.

A medium containing no ammonia, one in which all nitrogen is in the form of nitrate, does not stimulate growth of the sfo strain even at high pH. It may be that the only effect of high pH is to keep free ammonia in the medium and make it available to the organism.

DISCUSSION

The reported results show that methionine or its precursor inhibits threonine utilization under the influence of the sfo gene, thus inhibiting growth. A similar inhibition was reported by Teas, Horowitz, and Fling (1948) in the homoserine-less mutant 51504. This mutant requires homoserine, or both threonine and methionine for growth, but too much methionine inhibits growth in competition with threonine. This inhibition cannot be of the same nature as that reported here. It is not influenced by temperature and sulfanilamide cannot antagonize it. By contrast, α-aminobutyric acid and isoleucine are better antagonists than they are in our case. Only L-methionine inhibits the mutant 51504; D-methionine is without action, although it can support growth of methionine-deficient mutants. Tests of the inhibitory action of both methionine isomers on the sfo mutant show that both inhibit but that L-methionine is several times as active as D-methionine, DL-methionine being intermediate in effect.

The failure of the sfo strain to grow on unsupplemented media can best be accounted for by the supposition that there is a reaction in this strain that is catalyzed by PABA, that uses methionine (or homocysteine) as substrate, and

that leads to a deficiency of threonine (or homoserine). Homocysteine shows some of the inhibitory action characteristic of methionine. Mutants blocked after homocysteine but before methionine are able to produce the first substance (and possibly accumulate it) but not the second substance. Double mutants between certain of these and the sfo mutant (sfo, me-l) behave as though they are completely inhibited by methionine no matter how little methionine is added to the medium (cf. table 1). In such double mutants the inhibition must be caused by a precursor of methionine. The precursor of methionine that is directly involved is not necessarily homocysteine. There must be two or three "steps" to the methylation of homocysteine, since the over-all reaction is blocked by at least three genetically independent mutations which differ from one another in relation to methionine inhibition of the sfo character and selenite reduction. The steps controlled by the particular mutants under discussion are not known.

Since methionine is the best inhibitor although the evidence just mentioned suggests its precursor as inhibitor, it must be supposed that methionine is readily converted into that precursor. In animal experiments there is evidence that methionine can give up the methyl group and be converted to homocysteine (cf. Du Vigneaud 1942–1943; Virtue and Lewis, 1934; Brand, Cahill, and Block, 1935). There is no direct proof of a similar reaction in *Neurospora*. Indirectly, the supposition finds support in the observation that methionine can substitute for cysteine.

Methionine could be transformed to cysteine either in the reverse of the pathway of synthesis (figure 3) or by some other route resulting in a cyclic reaction. The second supposition is more likely since methionine can be used as a cysteine substituent even when the synthetic steps between the latter and the former are blocked by mutation. A double mutant, cysteineless (39816) and methionineless (H-98), should require both cysteine and methionine for growth if methionine could not be converted into cysteine. Actually growth is supported by the addition of methionine alone. Similar results have been obtained by S. C. Shen (unpublished) using double mutants between cysteineless (36106) and methionineless (29627 and 44704) strains in which the block is between homocysteine and methionine. Both double mutants utilize methionine as the sole source of sulfur.

Since both threonine and homoserine are completely effective in overcoming the inhibition caused by methionine (or homocysteine) in the sfo strain, and since such diverse substances as valine, isoleucine, β-methyl crotonic acid, urea, and ammonia at high pH are all partially effective, it is difficult to guess the nature of the normal process that is interfered with in this inhibition. On the other hand, homoserine has been shown to be a precursor of both threonine and methionine (Teas, Horowitz, and Fling, 1948), isoleucine can partially substitute for threonine in one of the threonineless mutants (Teas, 1948), and isoleucine and valine are closely interrelated (Bonner, 1946). It may be that one of the normally occurring interactions between these substances is the one that is inhibited by methionine in the sfo strain. *Beta*-methyl crotonic acid may owe its effectiveness to its structural similarity to valine or to some intermediate in

threonine synthesis. The effectiveness of urea and of ammonia at high pH suggests that the inhibited reaction may be one involving amination. Since the antagonistic reactions discussed are competitive, it would seem that inhibition by methionine must involve a reaction in which threonine is utilized; a precursor of threonine could be utilized directly in the inhibited reaction only if the steps between the precursor and threonine were reversible.

SUMMARY

If the synthesis of *p*-aminobenzoic acid (PABA) by the sulfonamide-requiring (sfo) strain of *Neurospora* is prevented by the introduction of a genetic block (Zalokar, 1948), growth is supported by low concentrations of PABA but is inhibited by higher concentrations. Inhibition by PABA is competitively antagonized by sulfanilamide (SA).

Experiments are reported which show that if the synthesis of methionine by the sfo strain is similarly prevented by the introduction of a genetic block preceding the synthesis of homocysteine, growth is supported by low concentrations (2×10^{-5} M) of methionine (or homocysteine) but is inhibited by higher concentrations. Inhibition by methionine is competitively antagonized by threonine (or homoserine).

Four antagonistic reactions have now been observed in the sfo strain. The inhibition of growth by excess PABA is competitively antagonized by SA and noncompetitively by threonine. Inhibition by excess methionine is competitively antagonized by threonine and noncompetitively by SA.

Five methods are now known by which growth of the sfo strain can be supported. These are (1) by the addition of SA, (2) by reducing available PABA by genetic means, (3) by reducing available methionine by genetic means, (4) by the addition of threonine, and (5) by making free ammonia available.

The present interpretation of these results is that the sfo gene controls a deleterious reaction with the following characteristics: (1) it requires a precursor of methionine as substrate, and the amount required is greater than that needed for essential reactions normally using the same substrate; (2) it requires PABA as a catalyst, and in much larger amounts than are required by other reactions using the same catalyst; (3) at the same time SA is a much stronger competitor of PABA in this reaction than in other reactions requiring PABA as catalyst; and (4) the reaction, or a product of the deleterious reaction, interferes with the normal utilization of threonine. That the step in threonine utilization interfered with may involve amination is suggested by the growth-promoting activity of free ammonia.

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