GENETIC CONTROL OF BIOCHEMICAL REACTIONS IN NEUROSPORA: AN "AMINOBENZOICLESS" MUTANT*

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Para-aminobenzoic acid has recently been recognized as a factor required for the growth of a number of microörganisms[1] and as a member of the vitamin B group.[2] One of the number of x-ray induced mutants of *Neurospora crassa*, obtained as described elsewhere,[3] is characterized by the loss of ability to synthesize *p*-aminobenzoic acid. This "aminobenzoicless" mutant is differentiated from normal by a single gene, is unable to grow on unsupplemented synthetic medium, but its growth is indistinguishable from normal when *p*-aminobenzoic acid is supplied.

The mutant culture was maintained on "complete" medium containing yeast extract, malt extract and sucrose. Conidia from this stock culture were used for inoculating test cultures. The test medium was the "synthetic" or "minimal" medium previously described[3] containing inorganic salts and nitrogen, sucrose and 4 gammas of biotin[4] per 1000 ml. Agar used in this synthetic medium was freed of *p*-aminobenzoic acid by repeated washing with distilled water. Cultures were incubated at 25°C.

Crosses were made of the *p*-aminobenzoicless mutant (sex *A*, the equivalent of + in Lindegren's terminology[5]) with the normal strain (sex *a*); ascospores were dissected in order from asci and grown on complete medium. These cultures were tested by transferring them to minimal medium and observing for growth. In all, 142 single spore cultures were grown and tested. Of these, 72 proved to be able to synthesize *p*-aminobenzoic acid and 70 were unable to accomplish this. Of 11 asci from each of which all eight ascospore cultures were obtained, all had four normal and four mutant-type spores, and in no case were more than four spores of a kind obtained from any one ascus. The results in the 17 asci in which segregations for both sex and aminobenzoicless could be deduced are summarized in table 1. It is evident from these results that the aminobenzoicless character is inherited as though it were differentiated from normal by a single gene. There is no evidence of linkage of aminobenzoicless and sex. The fact that in 10 out of 17 asci aminobenzoicless segregated from its normal allele in the second division indicates that the aminobenzoicless locus is an appreciable distance from the centromere.

In addition to the original aminobenzoicless mutant (ascospore isolate 1633), which was used for all physiological investigations described except those otherwise noted, an apparently independent occurrence of this mutant was recorded (isolate 5359). A cross between 5359 and 1633-63-6

TABLE 1

SEGREGATION FOR SEX AND ABILITY TO SYNTHESIZE *p*-AMINOBENZOIC ACID, IN ASCI FROM A CROSS OF NORMAL (SEX *a*) AND AMINOBENZOICLESS (SEX *A*)

The base and apex of the ascus are regarded as equivalent and the orientation of second division spindles is disregarded unless this has significance with regard to recombination. Since the third division is equational, each pair of products of this division is reported as a unit.

TYPE OF SPORE ARRANGEMENT				NUMBER OF ASCI OBSERVED
A +	A +	a *pab*	a *pab*	3
A *pab*	A *pab*	a +	a +	4
A +	A *pab*	a +	a *pab*	9*
A +	a +	A *pab*	a *pab*	0
A +	a *pab*	A +	a *pab*	0
A *pab*	a +	A *pab*	a +	0
A +	a *pab*	A *pab*	a +	1

* In two asci the results were such that it was presumed that two spores had been transposed in dissection.

TABLE 2

RESPONSE OF *p*-AMINOBENZOICLESS MUTANT 1633 TO COMPOUNDS RELATED TO *p*-AMINOBENZOIC ACID

Inactive Compounds

Benzoic acid	Sulfanilic acid
o-Aminobenzoic acid	Tyrosine
m-Aminobenzoic acid	2, 4-Diaminotoluene
p-Hydroxybenzoic acid	Acetanilide
p-Chlorobenzoic acid	*p*-Methylcyclohexanol

Active Compounds

SUBSTANCE	ACTIVITY COMPARED WITH THAT OF *p*-AMINOBENZOIC ACID* BY WEIGHT IN LIQUID MEDIUM	BY GROWTH RATE ON SOLID MEDIUM
Acetyl-*p*-aminobenzoic acid	1/50†	
p-Nitrobenzoic acid	1/100	1/120
p-Dimethylaminobenzaldehyde	1/1150‡	1/170‡
p-Toluidine	1/1400	1/1600
Aniline	1/13,000	
p-Nitrotoluene§	1/14,000	1/5000
p-Aminoacetophenone§	1/100,000	

* Activity values based on several series of varied concentrations, and when possible calculated from the concentrations giving $^1/_2$ maximum growth.

† Value obtained by visual comparison with control *p*-aminobenzoic dilutions.

‡ The difference between these two values seems to be correlated with the use of liquid and solid media.

§ Only very slight growth possible with any concentration, figures for activity therefore not too significant.

(derived mutant of sex *a*) gave 68 offspring from single ascospores taken at random, and all of these failed to grow in minimal medium. Presumably

these two aminobenzoicless strains represent independent occurrences of the same gene change. It is improbable that the two strains are the result of mutations of closely linked genes or that the second represents merely a tube contaminated with a spore of the first, although these two possibilities cannot be rigorously excluded.

Physiologically the two aminobenzoic strains are similar if not identical. Although exhaustive measurements of the *p*-aminobenzoic acid require-

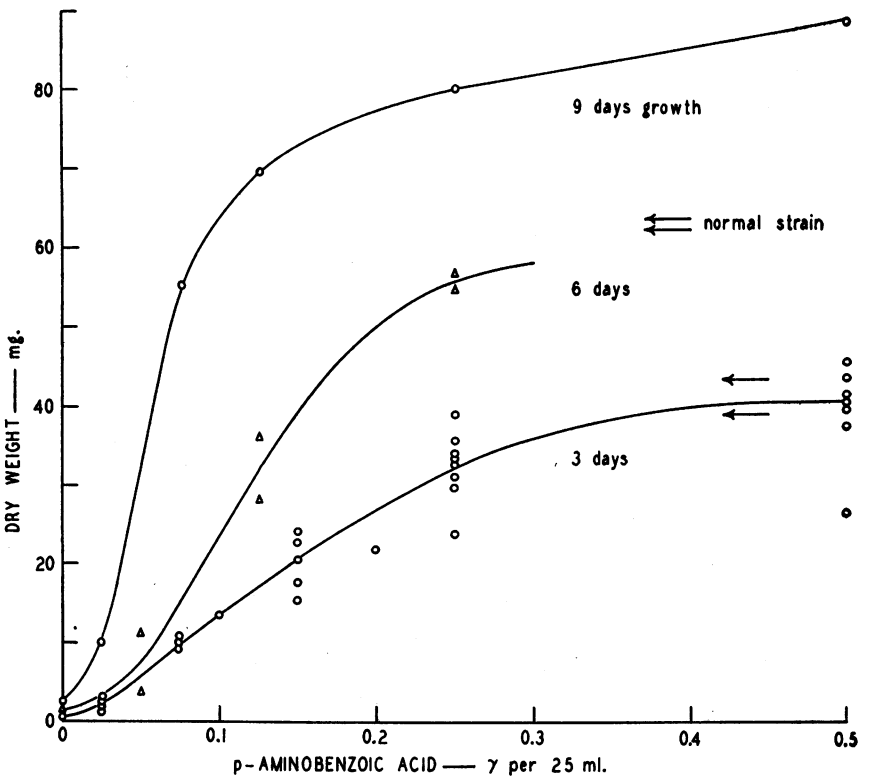

FIGURE 1

Dry weight attained after three, six and nine days as a function of *p*-aminobenzoic acid concentration. Weights of normal strain indicated by arrows.

ment of strain 5359 have not been made, it can be said that this is of the same order of magnitude as that of strain 1633. The quantitative growth responses to the compounds related to *p*-aminobenzoic acid which are listed in table 2 are similar in the two strains. Furthermore they are similarly inhibited by sulfanilamide as indicated in figure 4.

Growth of the aminobenzoicless mutant is normal only in the presence of *p*-aminobenzoic acid. In tests of other known growth factors a slight

but significant response to pimelic acid has been noted. This response is apparent only after a period of five or six days, whereas that to *p*-aminobenzoic acid is evident after one day. The pimelic acid effect has been observed with both liquid and solid media and with carefully recrystallized pimelic acid. This phenomenon, which obviously needs further investigation, suggests either some sort of adaptation or a possible conversion of pimelic acid to *p*-aminobenzoic acid.[6] Whatever its nature may be,

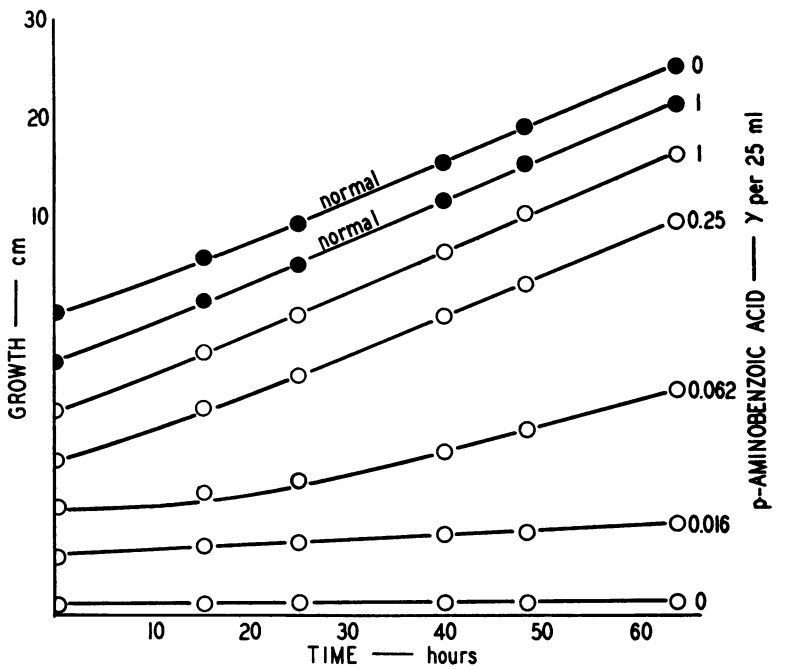

FIGURE 2

Position of mycelial frontiers in growth tubes as a function of time. Results with normal strains indicated by solid circles and those with *p*-aminobenzoicless strain (1633) indicated with open circles. Vertical scale offset for each successive curve in the series.

recovering and retesting strains grown in the presence of pimelic acid shows that the effect does not involve a genetic change in the organism.

The growth of the aminobenzoicless mutant is a function of the amount of *p*-aminobenzoic acid supplied to it. This quantitative relation can be determined either by the increase in dry weight of mycelium in liquid medium, or by rate of progression of the mycelium front through a tube partly filled with agar medium.

For the dry weight determinations, cultures were grown in 250-ml.

Erlenmeyer flasks containing 25 ml. of synthetic medium supplemented with *p*-aminobenzoic acid. Each flask was inoculated with a drop of a water suspension of conidia. At the end of the incubation period, generally 3 days, the mycelium was removed, washed and dried at 105°C. on a weighed watch glass. Figure 1 gives the results of a number of series. The relation between weight and *p*-aminobenzoic acid is almost linear up to a concentration of 0.25 gamma per 25 ml. The average weight reached

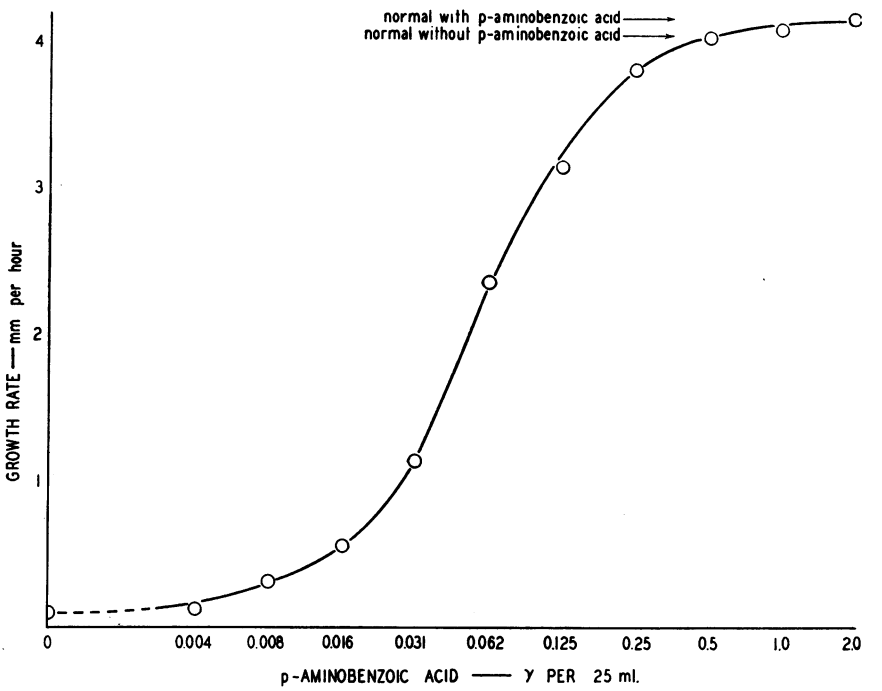

FIGURE 3

Rate of growth in tubes of aminobenzoicless strain (1633) as a function of *p*-aminobenzoic acid content of medium. Rates of normal strains indicated by arrows. Individual points obtained by averaging four independently obtained values.

in 3 days with 0.5 gamma per 25 ml. is about 40 mg., the weight reached by the normal, unsupplemented culture in the same time. The variability is rather great, due to the fact that only one determination can be made for each culture and due to variations in inoculum, length of initial lag period and probably other factors. Because of these difficulties and because significant weights cannot be reached in much less than 3 days, this method is only fairly satisfactory for assay purposes.

A more satisfactory method of determining the relation between *p*-aminobenzoic acid concentration and growth is to measure the rate of

progression of the mycelium front along the surface of agar medium in a horizontal tube as previously described.[3] This method has the advantage that variations inherent in the liquid culture method are not included in the measurements, since the final rate of progression is determined after equilibrium is reached and several measurements may be made in a given culture tube. This method has the further advantage that significant rate measurements may be made over a relatively short period of time, after the rate has become constant. The results of a series of *p*-aminobenzoic acid concentrations are shown in figure 2. As more *p*-aminoben-

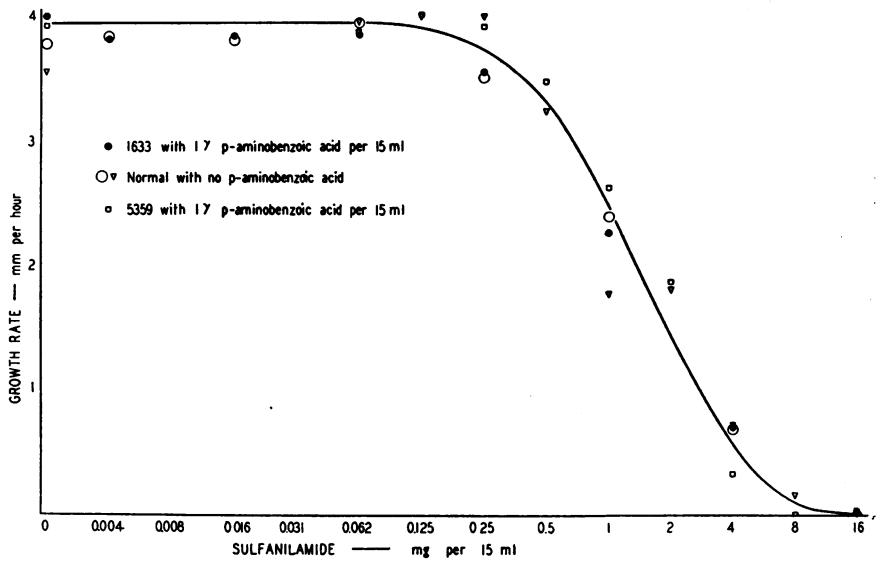

FIGURE 4

Inhibition of normal and aminobenzoicless strains (1633 and 5359) with sulfanilamide. Mutant strains supplied with one gamma *p*-aminobenzoic acid per 15 ml. medium.

zoic acid is supplied the rate of progression increases until with 0.5 gamma per 25 ml. it is the same as that of the normal strain (approximately 4 mm. per hour at 25°C. Figure 3 gives the final relation between the rate and vitamin concentration.

The results of both methods show that when enough *p*-aminobenzoic acid is supplied to the mutant it is indistinguishable from the normal strain. The inability to synthesize this vitamin seems therefore to be the only differentiating factor.

The biological significance of *p*-aminobenzoic acid was first recognized as a result of its action in overcoming the bacteriostatic or inhibitory

effects of sulfanilamide.[7] This antagonism has been interpreted as a competitive reaction, with an excess of sulfanilamide displacing the normally present *p*-aminobenzoic acid from its functional rôle, and an excess of *p*-aminobenzoic acid overcoming the resulting inhibition. The widespread existence of this *p*-aminobenzoic acid—sulfanilamide antagonism has been taken as indicating the essential nature of *p*-aminobenzoic acid for many diverse organisms including diatoms[8] and fungi (trichophyton).[9] Sulfanilamide also completely inhibits the growth of normal and mutant strains of *Neurospora*, and in both cases this inhibition is completely overcome by an excess of *p*-aminobenzoic acid. Figure 4 shows that growth on solid medium is completely inhibited by a sulfanilamide concentration of 16 mg. per 15 ml. In these tests enough *p*-aminobenzoic acid was supplied to the mutant to permit normal growth in the absence of sulfanilamide. The quantitative effect of sulfanilamide was the same on the normal and the mutant strains, demonstrating that the utilization of *p*-aminobenzoic acid rather than its synthesis is blocked by sulfanilamide, and that the amount of *p*-aminobenzoic acid synthesized by the normal strain is not appreciably greater than that required by the mutant for normal growth. Similar results were obtained by measurements of dry weight increase. The effectiveness of added *p*-aminobenzoic acid in overcoming the inhibition by 8 mg. sulfanilamide per 25 ml. in liquid medium was also investigated. The vitamin had the same anti-sulfanilamide activity for the normal and mutant strains, but its activity under these conditions was only about $^1/_{100}$ of its vitamin activity for the mutant strain in the absence of sulfanilamide.

As a working hypothesis, a single gene may be considered to be concerned with the primary control of a single specific chemical reaction.[10] If this premise is accepted, the biosynthesis of *p*-aminobenzoic acid in the mutant strain, which differs from normal by a single gene, should be blocked in only one specific step in the entire complex of reactions. An attempt was made to trace the course of *p*-aminobenzoic acid synthesis and to determine the step that is blocked by the mutant gene. This involved testing a number of substances for their ability to replace *p*-aminobenzoic acid in the growth of the mutant strain.[11] The results obtained by the dry weight and growth rate methods as summarized in table 2 were reasonably consistent with each other. *Ortho-* and *m*-aminobenzoic acids were inactive, as were all the compounds tested which did not have an aromatic N. With the amino group replaced by Cl or OH as in *p*-Cl or *p*-OH-benzoic acids, or as in tyrosine, no activity could be detected. These facts indicate that an aromatic substituted N is essential for activity and that if a second substituent group is present it must be in para position to the N. These results and the inactivity of tyrosine, which is presumably synthesized by the mutant strain, suggest the possibility that the reaction blocked by the

mutant gene may be concerned with the introduction of an amino N into the benzene ring.

If this is the reaction blocked in the aminobenzoicless mutant, normal strains should be able to introduce an amino N into a benzene ring. It is known that normal strains synthesize *p*-aminobenzoic acid or some other active substance, and practically all of the excess of this is contained in the culture medium. It is therefore possible to test such strains for their ability to introduce an aromatic amino group by growing them in the presence of compounds containing a nitrogen-free benzene ring and determining whether the *p*-aminobenzoic acid content of the medium is thereby increased. When grown for 3 days in the absence of such compounds, *N. crassa* produced about 0.1 gamma of *p*-aminobenzoic acid per 25 ml. of medium, indicating that the synthesis of this growth factor is normally not greatly in excess of its needs. This is likewise indicated by the sulfanilamide inhibition data. In the presence of benzoic acid, *p*-hydroxybenzoic acid, or tyrosine, in concentrations up to 1 mg. per 25 ml., the amount of *p*-aminobenzoic acid produced was not detectably increased. In fact, benzoic acid appreciably decreased the amount of *p*-aminobenzoic acid recovered. The conclusion that these compounds are not converted into *p*-aminobenzoic acid is confirmed by the fact that they do not have the anti-sulfanilamide activity to be expected with an increased yield of *p*-aminobenzoic acid.

The failure to detect any conversion of the tested nitrogen-free compounds to *p*-aminobenzoic acid, tends to discredit the suggestion that the mutant gene blocks the introduction of an aromatic amino group. It indicates, rather, that the normal synthesis of *p*-aminobenzoic acid does not involve the introduction of an aromatic N or amino group into a preformed benzene ring. It should be pointed out, however, that the interpretation of all such experiments may be complicated by factors such as penetration into the cells, secondary chemical reactions or the inability to add the possible intermediates in the biological or "active" condition.

A normal precursor of *p*-aminobenzoic acid which is located in the synthetic sequence leading up to the break due to the mutant gene should theoretically be completely inactive for the mutant strain. Similarly, a precursor coming after this break should be comparable in activity to *p*-aminobenzoic acid itself. If all the effective substances listed in table 2 owe their activity to their conversion to *p*-aminobenzoic acid, *Neurospora* must be able to reduce a nitro group,[12] introduce a carboxyl group para to an amino group, oxidize a methyl or aldehyde group and de-acetylate or de-methylate an amino group. The activities of the effective compounds are so much lower than that of *p*-aminobenzoic acid itself, however, that it seems doubtful that any is its normal precursor, or that any of these reactions is involved in the normal synthesis of *p*-aminobenzoic acid.

These compounds may be somewhat active *per se*, or may owe their activity to a conversion to a *p*-aminobenzoic acid by a relatively inefficient mechanism not concerned in the normal biosynthesis of *p*-aminobenzoic acid. This accessory conversion mechanism should be equally efficient in the normal and mutant strains. In either case the compounds should have the same anti-sulfanilamide activity for both strains if they are not normal precursors of *p*-aminobenzoic acid. Of all the active compounds listed (table 2), only *p*-nitrobenzoic acid and aniline had any anti-sulfanilamide activity,[13] and no quantitative difference in their effect on the normal and mutant strains was observed.

It may be concluded that the active compounds listed in table 2 are probably not concerned in the normal synthesis of *p*-aminobenzoic acid. In this biosynthesis the N or amino group is probably introduced before the formation of the benzene ring.

Summary.—An x-ray induced mutant strain of *Neurospora crassa* has been obtained which requires *p*-aminobenzoic acid for growth. Its growth is a function of the amount of *p*-aminobenzoic acid supplied, and it is indistinguishable from normal when adequate amounts of *p*-aminobenzoic acid are available.

The mutant differs from normal by a single gene, which must therefore control an essential step in the synthesis of *p*-aminobenzoic acid, and which is presumably primarily concerned only with the synthesis of *p*-aminobenzoic acid.

Sulfanilamide inhibits the growth of both the normal and mutant strains, and in both cases the inhibition is overcome by an excess of *p*-aminobenzoic acid.

A number of substances related to *p*-aminobenzoic acid are able to replace it, but their activities are much less than that of *p*-aminobenzoic acid itself.

The addition of benzoic or *p*-OH-benzoic acids or tyrosine did not increase the amount of *p*-aminobenzoic acid produced by the normal strain.

It is concluded that none of the compounds tested is concerned with the normal synthesis of *p*-aminobenzoic acid, and that this biosynthesis probably does not involve the introduction of an amino group into a preformed benzene ring.

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[1] See Fildes, P., *Lancet*, **238**, 955 (1940); Rubbo, S. D., and Gillespie, J. M., *Nature*, **146**, 838 (1940); and Lampen, J. O., and Peterson, W. H., *Jour. Am. Chem. Soc.*, **63**, 2283 (1941).

[2] Ansbacher, S., *Science*, **93**, 164 (1941); and Sure, B., *Ibid.*, **94**, 167 (1941).

[3] Beadle, G. W., and Tatum, E. L., *Proc. Nat. Acad. Sci.*, **27**, 499 (1941).

[4] S. M. A. Corporation, Chagrin Falls, Ohio. Biotin concentrate 1000.

[5] Dodge originally used "*A*" and "*a*" to designate the two sex types in *Neurospora crassa*. Lindegren later substituted the symbols "+" and "−" for these. Because of the desirability of using the symbol + for the normal allele of a gene and also to designate a positive experimental result, we prefer Dodge's original designations.

[6] Neither conversion of pimelic acid to *p*-aminobenzoic acid by the normal strain nor anti-sulfanilamide activity of pimelic acid could be detected.

[7] See Woods, D., and Fildes, P., *Chem. Ind.*, **59**, 133 (1940); Woods, D., *Brit. Jour. Exp. Path.*, **21**, 74 (1940); and Landy, M., and Wyeno, J., *Proc. Soc. Exp. Biol. Med.*, **46**, 59 (1941).

[8] Wiedling, S., *Science*, **94**, 389 (1941).

[9] Dimond, N. S., *Ibid.*, **94**, 420 (1941).

[10] See Beadle, G. W., and Tatum, E. L., *Amer. Nat.*, **75**, 107 (1941), and loc. cit., footnote 3.

[11] All the compounds tested were carefully purified to remove any possible traces of *p*-aminobenzoic acid. The aniline was prepared by hydrolysis of recrystallized acetanilide and subsequent distillation.

[12] A bacteriostatic action of *p*-nitrobenzoic acid has been reported. See King, J. T., and Henschel, A. F., *Proc. Soc. Exp. Biol. Med.*, **47**, 400 (1941).

[13] The other active compounds were toxic in concentrations theoretically high enough to overcome the sulfanilamide inhibition.

THE DISSOCIATION CONSTANT IN NITROGEN FIXATION BY AZOTOBACTER

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The dissociation constant of nitrogen fixation by *Azotobacter* was first estimated by Lineweaver, Burk and Deming[1] from their data on the influence of the pN_2 on rate of fixation by this organism. These workers measured fixation in the Warburg respirometer by observing the increase in the rate of oxygen uptake with time. The initial steps of nitrogen fixation by this organism were formulated as:

$$E + N_2 \rightleftarrows N_2E \text{ (rapid)} \quad (1)$$

$$N_2E \rightarrow E + P \text{ (slow)} \quad (2)$$

where E = Enzyme (nitrogenase) concerned with first step of fixation,

P = Products (increase in *Azotobacter* cells).

Using the method of Lineweaver and Burk,[2] they calculated a Michaelis constant (K_{N_2}) for reaction 1 of 0.21 atm. In their experiments the pO_2 was kept constant at 0.2 atm. and hydrogen added to bring the total pressure to one atmosphere whenever the pN_2 was less than 0.8 atm. Since