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Studies on the Biosynthesis of *p*-Aminobenzoic Acid by Symbiosis Experiments

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In a previous paper [1] it has been reported that two different strains of lactic acid bacteria can be grown together in symbiosis in a synthetic medium, each producing growth factors needed by the other. These investigations have been continued in this laboratory with the special aim of obtaining information concerning the relationships between these growth factors, especially vitamins and amino acids.

It was first shown by Woods [2] in 1940 that *p*-aminobenzoic acid was an essential metabolite in living organisms, and that it inhibited the bacteriostatic action of sulfonamide drugs. It has since been shown to be a growth factor for certain micro-organisms (reviewed by Knight [3] and Peterson and Peterson[4]). However, almost nothing is known of the mechanism by which it is synthesized in nature. The data recently presented by Davis [5–7] on aromatic biosynthesis indicate that shikimic acid serves as a precursor of *p*-aminobenzoic acid in the mutant strains of *E. coli*.

In the present work using the technique of symbiosis it was found that L-phenylalanine can replace *p*-aminobenzoic acid as growth factor for the phenylalanine-requiring strain *Lactobacillus arabinosus* 17—5. This finding suggests that L-phenylalanine functions as a precursor for the synthesis of *p*-aminobenzoic acid in this organism. In an attempt to obtain some information on possible intermediates in this biosynthesis, tests were made on whether certain compounds related to *p*-aminobenzoic acid and phenylalanine can replace *p*-aminobenzoic acid. In these experiments some observations have been made indicating a possible mechanism that may be involved in the conversion of L-phenylalanine to *p*-aminobenzoic acid.

EXPERIMENTAL

Cultures and method. The organisms used were *Lactobacillus arabinosus* 17—5 and *Streptococcus faecalis* R (obtained from Professor E. E. Snell, University of Wisconsin, U.S.A.). The stock cultures were maintained by stab inoculation in the glucose citrate tryptone yeast extract agar as described previously [1]. The inocula were prepared by transferring the organism from stab culture to 7 ml of this medium, without agar.

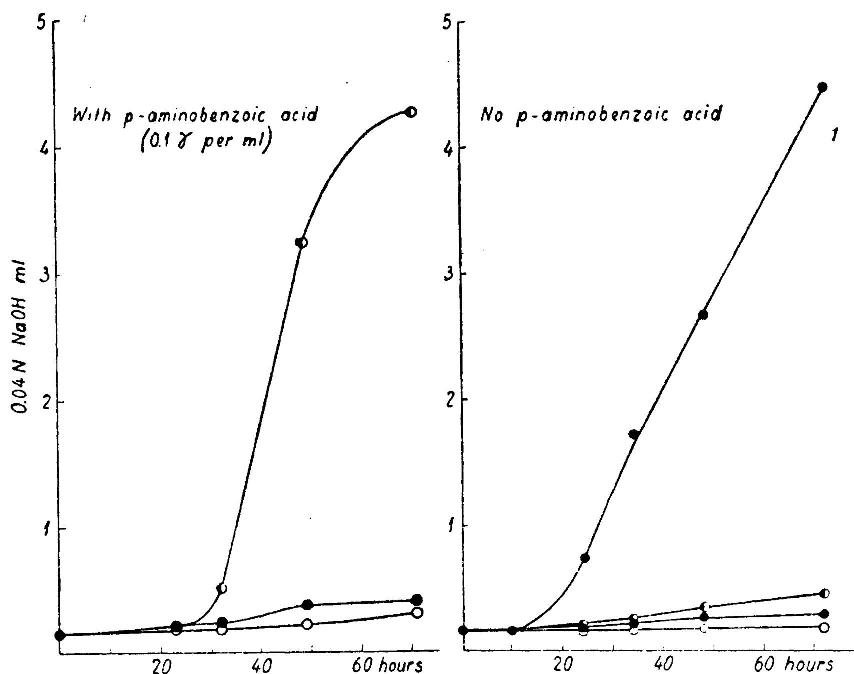


Fig. 1. The replacement of p-*aminobenzoic acid by* L-*phenylalanine. Basal medium without folic acid, phenylalanine, adenine, guanine, xanthine, uracil, and* p-*aminobenzoic acid (Medium B). Curve 1. Medium B supplemented with 10 γ of* L-*phenylalanine per ml.*
● *L. arabinosus 17—5 (phenylalanine-requiring strain)*
○ *Str. faecalis R (folic acid-requiring strain)*
◐ *L. arabinosus 17—5 and Str. faecalis R together.*

After incubating for 16—18 hours at 37° C the cells were centrifuged out and washed with 10 ml of 0.9 % sterile saline. This process was repeated, the cells being washed 2—3 times. The cells were finally suspended in saline and the suspension diluted to contain approximately half a million organisms per ml. One drop of this suspension, containing about 2×10^4 bacteria, was used as inoculum for 2 ml of the final medium.

The experimental procedure was essentially the same as that described in an earlier paper [1]. The basal medium of Henderson and Snell [8] in slightly modified form and with appropriate omission was also used in this study. The basal medium was added to test tubes in 1 ml portions, diluted with water or supplements to 2 ml, and autoclaved at 112° C for 5 minutes. In addition, filter-sterilized solutions of the test compounds were prepared (excluding sulfanilamide, benzoic acid, and *p*-aminohippuric acid) and added, with sterile technique, to the autoclaved basal medium in order to avoid possible decomposition of the compounds due to autoclaving. The incubation temperature was 37° C. The growth response was followed titrimetrically. The acid produced was titrated electrometrically directly in the test tubes with 0.04 *N* sodium hydroxyde, using a Cannon automatic titrator.

RESULTS

In an earlier study [1] it was demonstrated that *Str. faecalis* R and *L. arabinosus* 17—5 are able to grow together, but not alone, in a medium from which folic acid and phenylalanine have been omitted, although folic acid is required

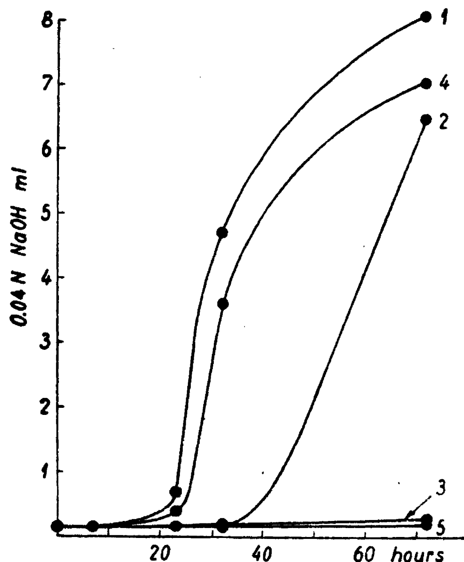


Fig. 2. The replacement of p-*aminobenzoic acid by* L-*phenylalanine is inhibited by sulfanilamide. Test organism L. arabinosus 17—5. Medium B added with sulfanilamide 0.1 γ per ml.*

Curve 1. 1 000 γ of L-*phenylalanine per ml.*
Curve 2. 100 γ » » » »
Curve 3. 10 γ » » » »
Curve 4. 10 γ » » » », but without sulfanilamide.
Curve 5. No L-*phenylalanine and sulfanilamide.*

by *Str. faecalis* R and phenylalanine by *L. arabinosus* 17—5. It was suggested that good growth results because each organism produces a growth factor needed by the other, phenylalanine and folic acid respectively. If, besides these compounds, all purine and pyrimidine bases (adenine, guanine, xanthine and uracil) were also omitted, both bacteria could still grow together, as can be seen from Fig. 1. However, if *p*-aminobenzoic acid, in addition to folic acid, phenylalanine, purines, and uracil, was omitted from the basal medium, the two organisms could not be grown together at all (Fig. 1). It appears, therefore, that the omission of *p*-aminobenzoic acid was the limiting factor for the growth of the two strains in association.

Since *Str. faecalis* R does not require *p*-aminobenzoic acid and since, moreover, this compound cannot replace the folic acid requirement of this organism, it was obvious that the inhibition of the symbiotic growth was due to the inability of *L. arabinosus* 17—5 to synthesize *p*-aminobenzoic acid and folic acid in the absence of phenylalanine. The fact that L-phenylalanine can substitute for *p*-aminobenzoic acid as a growth factor for *L. arabinosus* 17—5 is illustrated in Fig. 1.

As can be seen from Fig. 2 and Fig. 3 the replacement of *p*-aminobenzoic acid by phenylalanine was inhibited by sulfanilamide and also by benzoic acid.

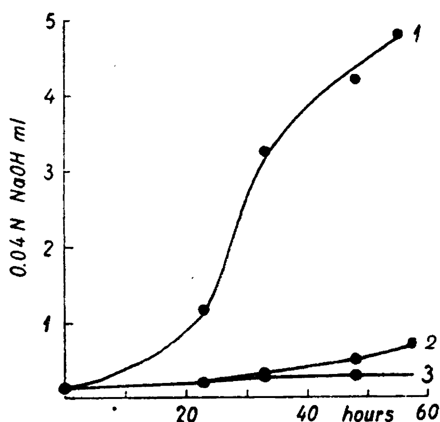


Fig. 3. *The replacement of* p-*aminobenzoic acid by* L-*phenylalanine is inhibited by benzoic acid. Test organism L. arabinosus 17—5. Medium B. Curve 1. 10 γ of* L-*phenylalanine per ml. Curve 2. 10 γ of* L-*phenylalanine and 10 γ benzoic acid per ml. Curve 3. No* L-*phenylalanine and benzoic acid.*

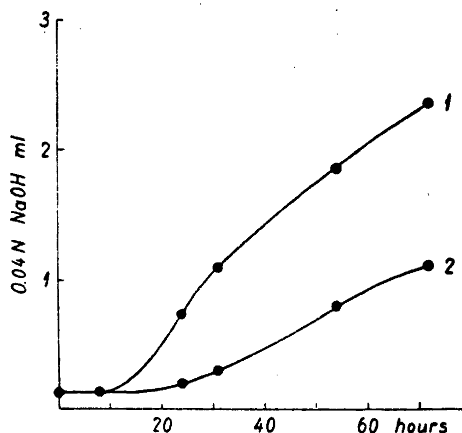


Fig. 4. *The promoting effect of* p-*nitrobenzoic acid on the utilization of* L-*phenylalanine with L. arabinosus 17—5. Medium B. Curve 1. 1 γ of* L-*phenylalanine and 1 γ of* p-*nitrobenzoic acid per ml. Curve 2. 1 γ of* L-*phenylalanine per ml.*

However, benzoic acid was a distinctly weaker inhibitor than sulfanilamide. The inhibitory effect of both compounds disappeared as soon as minute amounts (0.1 γ per 2 ml) of *p*-aminobenzoic acid were added to the medium.

Since it was found that *p*-nitrobenzoic acid had a promoting effect on the utilization of L-phenylalanine by *L. arabinosus* 17—5, but only in the absence of *p*-aminobenzoic acid (Fig. 4), certain compounds were tested for their ability to replace *p*-aminobenzoic acid in associations of these bacteria. The results of these experiments are summarized in Table 1 and in Figs. 5 and 6. In addition, the ability of these same compounds to replace phenylalanine was tested on *L. arabinosus* 17—5 alone. The results of these experiments are given in Table 1 and in Fig. 7.

As shown in Figs. 5 and 6 and in Table 1, *p*-nitrobenzoic acid, *p*-iodobenzoic acid, *p*-aminophenylacetic acid, *p*-nitrophenylacetic acid and *p*-aminohippuric acid could replace *p*-aminobenzoic acid as growth factor in symbiosis experiments. In the presence of *p*-nitrophenylacetic acid or *p*-aminophenylacetic acid it was shown microscopically that the *L. arabinosus* 17—5 symbiont grew relatively more rapidly than *Str. faecalis* R as compared with the experiments in which these substances were replaced with *p*-aminobenzoic acid [1]. Only one compound, shikimic acid, could replace phenylalanine for *L. arabinosus* 17—5 in the complete basal medium, as can be seen from Table 1 and Fig. 7.

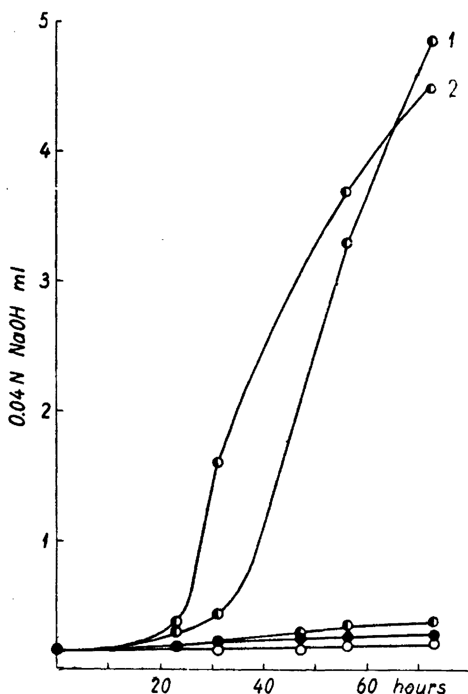


Fig. 5. The replacement of p-*aminobenzoic acid by* p-*iodobenzoic acid and* p-*nitrobenzoic acid. Medium B. Curve 1. 1 γ of* p-*iodobenzoic acid per ml. Curve 2. 10 γ of* p-*nitrobenzoic acid per ml.*
● *L. arabinosus 17—5*
○ *Str. faecalis R*
◐ *L. arabinosus 17—5 and Str. faecalis R together.*

DISCUSSION

The data obtained from the present symbiosis experiments can readily be explained by assuming the participation of phenylalanine as a precursor in *p*-aminobenzoic acid formation in *L. arabinosus* 17—5. In the medium used, this organism requires *p*-aminobenzoic acid only in the absence of L-phenylalanine, which is an essential amino acid for it (for the synthesis of the cell proteins). When L-phenylalanine is available it can rapidly synthesize *p*-aminobenzoic acid, which will then be a non-essential vitamin for this organism. On the basis of this fact it is also very understandable why these two bacteria cannot be grown in symbiosis in the absence of *p*-aminobenzoic acid, folic acid, and phenylalanine.

L. arabinosus 17—5 cannot synthesize folic acid for *Str. faecalis* R in a medium lacking these three growth factors, and the latter strain is therefore unable to produce phenylalanine, with the result that the growth of both bacteria is prevented. In addition, it is of interest to note that folic acid contains *p*-aminobenzoic acid as a structural unit and therefore it must be assumed that *p*-aminobenzoic acid is one essential component for the biosynthesis of folic acid in *L. arabinosus* 17—5. It also seems possible that the function of *p*-aminobenzoic acid is inhibited by sulfanilamide and benzoic acid, these compounds preventing the participation of *p*-aminobenzoic acid in the synthesis of folic acid *(Cf. e. g.* [9-11]*)*.

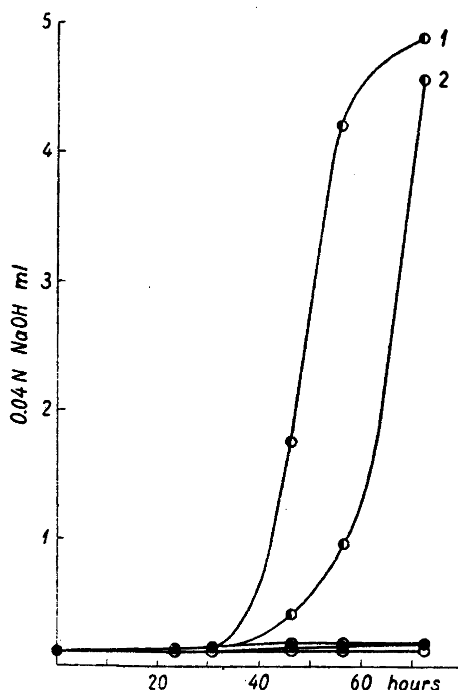


Fig. 6. The replacement of p-*aminobenzoic acid by* p-*aminophenylacetic acid and* p-*nitrophenylacetic acid. Medium B. Curve 1.* p-*Aminophenylacetic acid 10 γ per ml. Curve 2.* p-*Nitrophenylacetic acid 10 γ per ml.*
● *L. arabinosus 17—5*
○ *Str. faecalis R*
◐ *L. arabinosus 17—5 and Str. faecalis R together.*

Table 1. Activity of certain compounds to substitute for p-*aminobenzoic acid and phenylalanine.*

Compound	Activity to substitute for *p*-aminobenzoic acid *	Activity to substitute for phenylalanine *
p-Nitrobenzoic acid	active	inactive
m- » »	inactive	
o- » »	»	
p-Iodobenzoic acid	active	i nactive
m- » »	inactive	
o- » »	»	
p-Aminophenylacetic acid	active	inactive
p-Nitrophenylacetic acid	»	»
Phenylacetic acid	inactive	»
Phenylpropionic acid	»	»
Phenylpyruvic acid	»	»
p-Hydroxybenzoic acid	»	»
p-Aminohippuric acid	active	»
Shikimic acid	inactive	active
Quinic acid	»	inactive
Benzoic acid, Sulfanilamide		inhibitory effect on the utilization of phenylalanine in the absence of *p*-aminobenzoic acid

* By the technique of symbiosis (with *L. arabinosus* 17—5 and *Str. faecalis* R).
** With *L. arabinosus* 17—5.

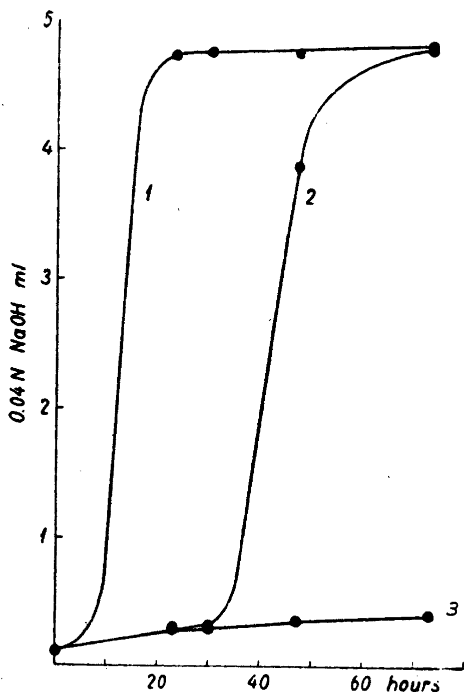
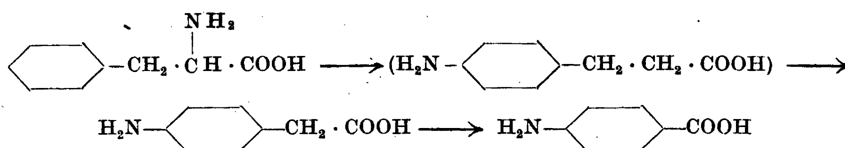


Fig. 7. The replacement of phenylalanine by shikimic acid in L. arabinosus 17—5. From the original basal medium is lacking phenylalanine. Curve 1. L-*phenylalanine 10 γ per ml. Curve 2. Shikimic acid 20 γ per ml. Curve 3. Medium without phenylalanine and shikimic acid.*

Evidence indicates that one of the cellular functions of *p*-aminobenzoic acid is to catalyze the synthesis of purine bases in certain micro-organisms[9,12,13]. In view of this fact it seems possible that the omission of purines has increased the requirement of *L. arabinosus* 17—5 for *p*-aminobenzoic acid in these symbiosis experiments. Neither of the strains required purines and uracil in the original basal medium.

The ability of certain compounds related to *p*-aminobenzoic acid to replace this growth factor in the symbiosis experiments indicates a possible mechanism allowing the conversion of phenylalanine to *p*-aminobenzoic acid. Because *p*-aminophenylacetic acid and *p*-nitrophenylacetic acid could replace *p*-aminobenzoic acid, it seems probable that the carbon chain of phenylalanine undergoes oxidation (presumably after deamination) and gives rise to a carboxyl group. However, since phenylpropionic acid and phenylacetic acid were inactive, before oxidation the hydrogen atom in the *para*-position of the benzene ring is presumably replaced by the amino group (or, by some other reactive group, which again would be replaced by an amino group). The activity of *p*-nitrobenzoic acid and *p*-iodobenzoic acid indicates that the organism concerned is able to replace the nitro group and iodine by the amino group. It should be noticed that only *para*-compounds of nitrobenzoic acid and iodobenzoic acid were active. It seems, therefore, reasonable to postulate the following mechanism:



The activity of *p*-aminohippuric acid must be due to the hydrolysis of this compound, presumably by *L. arabinosus* 17—5, by which *p*-aminobenzoic acid will be liberated.

The inactivity of *p*-hydroxybenzoic acid indicates that this compound cannot be converted into *p*-aminobenzoic acid by the lactic acid bacteria used. Similar observations have also been made with *E. coli* mutants, in which, however, the conversion of *p*-aminobenzoic acid to *p*-hydroxybenzoic acid seems to be possible [6].

According to Davis [5–7] shikimic acid (and certain related compounds) function as a common precursor in the synthesis of aromatic amino acids, *p*-aminobenzoic acid, and *p*-hydroxybenzoic acid in *E. coli* mutants. As shown in Table 1 and in Fig. 7, shikimic acid could not replace *p*-aminobenzoic acid, but instead phenylalanine in *L. arabinosus* 17—5. It must therefore be concluded that shikimic acid can serve as a precursor of phenylalanine in this organism, but not directly as a precursor of *p*-aminobenzoic acid, as is evidently the case in *E. coli* mutants.

SUMMARY

Using the technique of symbiosis it was found that L-phenylalanine may be substituted for *p*-aminobenzoic acid as a growth factor for *L. arabinosus* 17—5, which cannot synthesize phenylalanine at all in the basal medium used. The replacement of *p*-aminobenzoic acid by L-phenylalanine is very susceptile to sulfanilamide inhibition. It is concluded that L-phenylalanine functions as a precursor for the synthesis of *p*-aminobenzoic acid in this organism and tests have therefore been made on the ability of various compounds to replace *p*-aminobenzoic acid. *p*-Aminophenylacetic acid, *p*-nitrophenylacetic acid, *p*-nitrobenzoic acid, *p*-iodobenzoic acid, and *p*-aminohippuric acid were active. In addition shikimic acid could substitute for L-phenylalanine. The mechanism that may be involved in the conversion of phenylalanine to *p*-aminobenzoic acid has been discussed.

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