THE NUTRITIONAL EQUIVALENCE OF PANTOTHENATE AND *p*-AMINO BENZOATE FOR THE GROWTH OF *BACTERIUM LINENS*¹

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Received for publication May 28, 1953

Knowledge concerning the function of *p*-aminobenzoate in biological systems has been derived largely by the use of specific competitive inhibitors. Thus, the reversal of either sulfanilamide or 2-chloro-4-aminobenzoate inhibition by methionine and other amino acids (Lampen *et al.*, 1946; King *et al.*, 1948; Strandkov, 1947; Winkler and DeHaan, 1948), by pteroylglutamic acid (Nimmo-Smith *et al.*, 1948; Jukes and Stokstad, 1948), and by certain purines and pyrimidines (Cutts and Rainbow, 1949; Lampen and Jones, 1947) has been considered evidence that *p*-aminobenzoate functions in the synthesis of these metabolites. Similarly, the data of King *et al.* (1948) imply that *p*-aminobenzoate also functions in the synthesis of pantothenate since pantothenate effectively antagonized the inhibition of 2-chloro-4-aminobenzoate in a mutant of *Escherichia coli*. High levels of pantothenate also partially satisfied the *p*-aminobenzoate requirements of a mutant of *Saccharomyces cerevisiae* (Pomper, 1952).

The function of *p*-aminobenzoate in the synthesis of folic acid or pteroylglutamic acid has been attributed to its incorporation into the pteroylglutamic acid molecule (Angier *et al.*, 1946). Whether *p*-aminobenzoate is involved indirectly through folic acid or a like metabolite in the synthesis of amino acids, purines, etc., or whether it has a separate catalytic role in the synthesis of metabolites other than pteroylglutamate has not been decided conclusively.

From a nutritional study of 25 strains of *Bacterium linens*, it was observed (Purko *et al.*, 1951) that one strain (no. 456) is able to grow when either pantothenate or *p*-aminobenzoate is present. While an interaction between *p*-aminobenzoate and pantothenate may be indicated from the use of 2-chloro-4-aminobenzoate by

King *et al.* (1948), this substitution of pantothenate for *p*-aminobenzoate and *vice versa* for the growth of *B. linens* provides more direct evidence for an interrelationship between these vitamins. In addition, it offers an opportunity to study a function of *p*-aminobenzoate without the obligatory use of competitive inhibitors.

Data presented in this report, showing the equivalence of pantothenate and *p*-aminobenzoate for the growth of *B. linens*, indicate that *p*-aminobenzoate functions in the synthesis of pantothenate and also suggest that pantothenate may be involved in the synthesis of *p*-aminobenzoate.

MATERIALS AND METHODS

Bacteriological. *Bacterium linens*, strain 456, a salt tolerant, obligately aerobic, gram positive, short rod (Albert *et al.*, 1944), one of 25 strains, was isolated from limburger cheese and identified in this laboratory by Meyer (1949). The inoculum was prepared by growing cultures with aeration at room temperature ($27 \pm 3^\circ\text{C}$) for 24 hours in a medium composed of yeast extract, 1 per cent; tryptone, 1 per cent; dipotassium phosphate, 0.5 per cent; and glucose, 0.5 per cent (Wood and Gunsalus, 1942). The cells were washed three times with sterile distilled water, resuspended, and diluted to an optical density of 0.1. One drop of this suspension was used to inoculate each tube.

The composition of the semi-synthetic basal medium and supplements is shown in table 1. The constituents for 100 ml of medium were dissolved in 71.5 ml of distilled water, 5 ml aliquots of which were dispensed in 18 by 150 mm test tubes. Supplements and water were added to each tube to a total volume of 7 ml. The tubes were plugged with aluminum foil-coated rubber stoppers equipped with cotton plugged, glass-venting tubes and sterilized by autoclaving. After inoculation, the stoppers

¹ A preliminary report was presented at the 51st SAB Meeting, Boston, Massachusetts, May, 1952.

were sealed to the glass with collodion, the tubes inclined at an angle of approximately 37 degrees from horizontal and aerated by shaking [120 strokes (1.3 in) per min] at room temperature or in later experiments in an incubator at 25 C.

The growth was estimated turbidimetrically at desired intervals in an Evelyn colorimeter equipped with a no. 660 filter and is expressed as optical density $\times 10$.

TABLE 1
Composition of the medium

BASAL MEDIUM		VITAMIN SUPPLEMENTS	
	mg/ml		$\mu\text{g/ml}$
Casein hydrolyzate, enzymatic	10.0	Thiamin-HCl..	0.3
Glucose....	40.0	Riboflavin.....	0.3
L-Tryptophan.....	0.2	Pyridoxine-HCl.....	0.3
L-Cystine....	0.4	Calcium pantothenate....	0.3
Adenine sulfate...	0.02	Niacin.....	0.3
Guanine-HCl.....	0.02	Biotin.....	0.002
Uracil.....	0.02	Folic acid.....	0.07
Salts A.....	0.01 ml	Inositol.....	0.3
Salts B.....	0.01 ml	Choline-HCl..	0.07
pH.....	6.8	p-Aminobenzoate.....	0.014
		Vitamin B ₁₂ ...	0.0014

Salts A: Dissolve 25 g K_2HPO_4 and 25 g KH_2PO_4 in 250 ml of H_2O .

Salts B: Dissolve 10 g $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.5 g NaCl , 0.5 g $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, and 0.5 g $\text{MnSO}_4 \cdot 3\text{H}_2\text{O}$ in 250 ml of H_2O .

Microbiological assay—pantothenate. Cells (500 mg dry weight) of *B. linens* were suspended in 50 ml of distilled water. The suspension was adjusted to pH 6.8 and autoclaved for 15 minutes. After cooling, 50 mg of "mylase P" (Wallerstein Laboratories) and 2 ml of 2 M sodium acetate were added. The sample then was adjusted to pH 4.5 to 4.8 with 1 N HCl, layered with toluene and incubated for 24 hours at 50 C. After dilution to 100 ml and filtration through Whatman no. 40 filter paper, aliquots (0.1, 0.5, 1, 2, 3, 4, and 5 ml) were assayed for pantothenate with *Lactobacillus arabinosus*, ATCC no. 8014, according to the procedure of Skeggs and Wright (1944). The values obtained may be low since Neilands and Strong (1948) have shown that

the bound pantothenate is not released completely by this procedure.

p-Aminobenzoate. Bacterial cells (500 mg dry weight) were suspended in 5 ml of 6 N H_2SO_4 , autoclaved for one hour, and then neutralized with saturated barium hydroxide. The barium sulfate was washed thoroughly with distilled water, the combined filtrates adjusted to pH 6.0 with 1 N HCl, and diluted to 100 ml. Aliquots (0.5, 1, 2, 3, 4, and 5 ml) of the filtrate were added to the basal medium (medium A) described by Davis and Mingioli (1950) and assayed for p-aminobenzoate^{*} with an *Escherichia coli* mutant (Davis and Mingioli, 1950).

Although the assay organism grows only in the presence of p-aminobenzoate, the possibility exists that cellular materials other than p-aminobenzoate stimulate the growth of this organism. Consequently, the values obtained for the p-aminobenzoate content of cells may be slightly high. However, the presence of the compound was substantiated further using a p-aminobenzoateless mutant of *Neurospora crassa* (Agarwala and Peterson, 1950) and *Rhodotorula aurantiaca* (Robbins and Ma, 1944).

Materials. DL-Pantoyl-tauryl-p-sulfamylamide and D(+)-pantoyl-tauryl-p-aminoanilide were furnished by Dr. Robert O. Roblin, Jr., of the American Cyanamid Company. The folinic acid (50 per cent pure, 257 units per mg), α -ketoisovalerate, and coenzyme A (350 units per mg) were obtained from Dr. I. C. Gunsalus of the University of Illinois. Pantethine (*Lactobacillus bulgaricus* factor) was supplied by Dr. O. D. Bird of Parke, Davis and Company. The other materials and reagents used in these experiments were commercial preparations.

RESULTS

The vitamin requirements of *B. linens* were tested by adding single vitamins to the basal medium in concentrations shown in table 1. The results shown in table 2 clearly indicate that growth occurred when either pantothenate or p-aminobenzoate was present and that other vitamins including pteroylglutamic acid were ineffective. The growth with pantethine containing the optimal level of pantothenate (3 μg per ml, see below) was similar to that

^{*} Personal communication. Dr. B. L. Davis, Cornell University Medical College, New York, New York.

obtained in the presence of pantothenate. Coenzyme A (13 units per mg), containing 3 μ g of pantothenate per ml, gave erratic results, but rapid growth was observed with a highly purified preparation (350 units per mg) (figure 1). As shown in figure 1, both folic acid and folinic acid (70 μ g per ml) were inactive. When the concentration of folic acid and folinic acid was increased 40-fold, growth occurred after 70 hours. However, the response was equivalent to that evoked by 1/200th the concentration of *p*-aminobenzoate.

TABLE 2

Vitamin requirement of Bacterium linens, strain 456

Composition of the basal medium and vitamin concentrations are as shown in table 1. Cultures were incubated with aeration for 90 hours at room temperature ($27\text{ C} \pm 3\text{ C}$).

ADDITION	OPTICAL DENSITY $\times 10$
None.....	0.132
Ca pantothenate.....	4.32
<i>p</i> -Aminobenzoate.....	2.84
Thiamin-HCl.....	0.088
Riboflavin.....	0.132
Pyridoxine-HCl.....	0.88
Biotin.....	0.223
Folic acid.....	0.177
Choline-HCl.....	0.132
Inositol.....	0.132
Niacin.....	0.132
Vitamin B ₁₂	0.223
All vitamins.....	7.46

In order to determine the optimum vitamin concentration, media containing graded levels of either pantothenate or *p*-aminobenzoate were inoculated and the course of growth followed turbidimetrically. The rate of growth was approximately proportional to vitamin concentration at low levels. The amount of growth after 115 hours as a function of vitamin concentration is shown in figure 2. An increase in growth was observed until the concentration of pantothenate approached approximately 0.7 μ g per ml. Beyond this point (0.7 to 4.6 μ g per ml), only a slight increase resulted. Similarly, as the concentration of *p*-aminobenzoate was increased from 1.43 to 57 μ g per ml, a marked increase in growth was observed; concentrations

in excess of 57 μ g per ml, however, did not produce a greater effect.

While rapid growth was obtained in the presence of optimum pantothenate (3 μ g per ml) or *p*-aminobenzoate (57 μ g per ml), the rate and amount of growth were different in each case (figure 3). With pantothenate, the culture grew rapidly after about 20 hours, and the amount of growth was approximately one-half that obtained with a mixture of pantothenate and *p*-aminobenzoate. As already shown in figure 2, this amount of growth could not be increased by adding more pantothenate. With *p*-amino-

Figure 1. Growth on pantothenate (PA) or *p*-aminobenzoate (PABA) derivatives. Conditions are as in table 2 except that pantothenate (3 μ g per ml), pantethine (LBF) (95 units per ml $\approx$ 3 μ g of pantothenate per ml), and coenzyme A (CoA) (4.3 units per ml $\approx$ 3 μ g of pantothenate per ml) were added as indicated.

benzoate the growth was somewhat slower but eventually exceeded that on pantothenate and leveled off at about two-thirds that obtained with both vitamins.

That the observed substitution of pantothenate for *p*-aminobenzoate and *vice versa* was not due to a carryover of vitamins in the inoculum was shown by the fact that each of four successive subcultures in media containing pantothenate, *p*-aminobenzoate, and pantothenate plus *p*-aminobenzoate gave the results shown in figure 3. Furthermore, several sources of pantothenate and *p*-aminobenzoate were used, thus lessening the possibility that the vitamins were contaminated.

The addition of niacin, thiamin, pyridoxine, pteroylglutamic acid, and riboflavin, singly or in combination in the amounts shown in table 1, did not alter appreciably the results

Figure 2. Effect of *p*-aminobenzoate and pantothenate concentration on the growth. Conditions are as in figure 1 except that the vitamin concentrations were changed as indicated. The incubation time was 115 hours.

Figure 3. Growth with *p*-aminobenzoate or pantothenate. Conditions are as in figure 2 except that 3 μ g of pantothenate and 57 μ g of *p*-aminobenzoate per ml were added.

shown in figure 3. Biotin (1.4 μ g per ml), on the other hand, in the presence of pantothenate or *p*-aminobenzoate increased the rate and amount of growth. No effect was observed when biotin was added to a medium containing optimum levels of both pantothenate and *p*-aminobenzoate. Biotin appears to be stimulatory rather than required since it alone did not

support growth, and, as already noted, several subcultures in biotin-free media containing either pantothenate or *p*-aminobenzoate or both did not result in a progressive decrease in growth rate or amount in each subculture as would be expected if biotin were required. In addition, an amino acid mixture was substituted successfully for casein hydrolyzate in an otherwise biotin-free medium, thereby eliminating that source of biotin (and other growth factors) as a contaminant. In such a medium, no biotin dependence developed, and the response to pantothenate and *p*-aminobenzoate was similar to that recorded in figure 3.

TABLE 3

Pantothenate and p-aminobenzoate synthesis by Bacterium linens

VITAMIN ADDED	VITAMIN CONTENT— μ g/MG DRIED CELLS	
	Pantothenate	<i>p</i> -Amino-benzoate
Pantothenate.....	4.12 $\pm$ 0.37*	2.4 $\pm$ 0.12
Pantothenate, biotin..	1.62 $\pm$ 0.12	2.90 $\pm$ 0.16
<i>p</i> -Aminobenzoate....	4.18 $\pm$ 0.19	6.80 $\pm$ 0.14
<i>p</i> -Aminobenzoate, biotin.....	4.49 $\pm$ 0.15	3.66 $\pm$ 0.15
Pantothenate, <i>p</i> -aminobenzoate..	10.04 $\pm$ 0.16	8.77 $\pm$ 0.30
Pantothenate, <i>p</i> -aminobenzoate, biotin.....	7.86 $\pm$ 0.29	6.64 $\pm$ 1.19

$$* \text{Standard deviation} = \sqrt{\frac{\sum d^2}{n-1}} \quad (n = 5).$$

Pantothenate and p-aminobenzoate synthesis. Two possible explanations for the observed pantothenate-*p*-aminobenzoate interaction may be: (1) In an otherwise adequate medium one vitamin, say pantothenate, is required for growth; *p*-aminobenzoate is required (in the absence of pantothenate) only for pantothenate synthesis. Thus, when *p*-aminobenzoate is added, pantothenate is formed and growth occurs; or (2) Two vitamins, pantothenate and *p*-aminobenzoate, are required for growth, and each is required for the synthesis of the other. In this case growth occurs when either pantothenate or *p*-aminobenzoate is added because the omitted vitamin is synthesized. In the first possibility, one vitamin (pantothenate) would be found in

the cells when pantothenate was added, and both vitamins (pantothenate and *p*-aminobenzoate) would be found in the cells grown on *p*-aminobenzoate. In the second possibility, both vitamins would be found in the cells regardless of which vitamin was present in the medium.

To test which of these possibilities is involved, dried cells obtained from 5 day cultures grown in 1 L of medium (table 1) containing pantothenate (3 μ g per ml) and/or *p*-aminobenzoate (0.06 μ g

and *p*-aminobenzoate + biotin, table 3). Each of these values represents the mean of five determinations. The consistency of these values was satisfactory since the variations (expressed as standard deviation, Snedecor, 1946) were due to variations in different lots of cells as well as to variations among assays.

Sulfanilamide and salicylate inhibition. As shown in figure 4, sulfanilamide (3 μ g per ml) completely inhibited growth in a medium containing *p*-aminobenzoate (57 μ g per ml).

Figure 4. Reversal of sulfanilamide and salicylate inhibition. Conditions: The medium contained *p*-aminobenzoate (57 μ g per ml) and either sulfanilamide (3 μ g per ml) or salicylate (57 μ g per ml) (labeled "no additions"). Pantothenate (3 μ g per ml), pantoate (2 μ g per ml), or biotin (1.4 μ g per ml) was added as indicated.

per ml) were assayed for each of these vitamins. It is evident from the results shown in table 3 that in each instance the omitted vitamin was synthesized during growth. Thus, 2 μ g of *p*-aminobenzoate per mg dry weight of cells were synthesized during growth in a medium containing pantothenate. Four μ g of pantothenate per mg dry weight were found in the cells grown in the presence of *p*-aminobenzoate. These values are in essential agreement with those of Woods *et al.* (1942) for pantothenate in *Pseudomonas fluorescens* and Strehler (1950) for *p*-aminobenzoate in *Neurospora crassa*, strain 8043-M. More of the omitted vitamin and less of the added vitamin were found in cells grown in the presence of biotin (pantothenate + biotin

The inhibition was reversed by the addition of either pantothenate (3 μ g per ml), pantoate (2 μ g per ml), or biotin (1.4 μ g per ml). Additional attempts to demonstrate the function of *p*-aminobenzoate in pantothenate synthesis, using 2-chloro-4-aminobenzoic acid (15 μ g per ml) as the inhibitor, failed because it alone supported rapid growth.

Salicylic acid has been reported by Maas (1952) to inhibit growth by interfering with pantoate synthesis. This finding is substantiated by studies with *B. linens*. As shown in figure 4, growth on *p*-aminobenzoate is completely inhibited by 57 μ g per ml of salicylic acid. When pantothenate or pantoate is added, unimpaired growth results even at very high levels of salicylate (1.145 mg

per ml). These data are consistent with the results of the previous experiments which indicated that *p*-aminobenzoate participates in the synthesis of pantothenate.

The use of pantothenate antagonists, such as pantoyltaurine, D(+)-pantoyl-tauryl-*p*-aminanilide, and DL-pantoyl-tauryl-*p*-sulfamylanilide, for a similar purpose resulted in rapid growth in the absence of vitamins, perhaps due to the liberation of the pantoyl moiety by peptidase action. Thus, it was not possible to obtain evidence for pantothenate functioning in *p*-aminobenzoate synthesis by observing the antagonistic effect of *p*-aminobenzoate upon these pantothenate competitive inhibitors.

DISCUSSION

The nutritional equivalence of pantothenate and *p*-aminobenzoate for the growth of *B. linens* represents the first direct evidence of an interaction between pantothenate and *p*-aminobenzoate. Microbiological assays of cells show that the omitted vitamin is synthesized in either case. Thus, this situation is not analogous to that reported by Holden *et al.* (1949) in which either D-alanine or vitamin B₆ was required for the growth of *Streptococcus faecalis*. In that case D-alanine was required for growth and B₆ was required in the absence of D-alanine only to mediate the synthesis of D-alanine. Hence, vitamin B₆ grown cells contained both B₆ and D-alanine, but D-alanine grown cells did not contain vitamin B₆. Since *B. linens* contains both pantothenate and *p*-aminobenzoate when grown on either pantothenate or *p*-aminobenzoate it is apparent that the omitted vitamin of the pair was synthesized. Growth did not occur in the absence of both vitamins. Thus, it is plausible that the synthesis of pantothenate requires added *p*-aminobenzoate. The reversal of sulfanilamide and salicylate inhibition by pantothenate may be taken as further evidence that *p*-aminobenzoate is involved in pantothenate synthesis. Similar data concerning a function of pantothenate in *p*-aminobenzoate synthesis have not been obtained due to the ability of the organism to grow on all of the pantothenate inhibitors tested. It has been postulated by Sevag and Green (1944), however, that pantothenate is involved in aromatic ring formation.

The inability of folic acid and folinic acid to support growth except at very high concentra-

tions might be taken as evidence that *p*-aminobenzoate has a function other than to become a part of a folic acid-like molecule. However, if the *p*-aminobenzoate-folic acid interaction can be represented schematically as follows:

it is conceivable that *B. linens* can synthesize a coenzyme containing a pteroylglutamic acid moiety from *p*-aminobenzoate through an intermediate X, whereas the enzymes or enzyme system for conversion of folic acid and folinic acid to the coenzyme through X is lacking or functions very slowly.

Although biotin is not required for growth, its ability to stimulate growth, its depressing effect upon the pantothenate and *p*-aminobenzoate content of cells, as well as its ability to reverse sulfanilamide inhibition, indicate a role in pantothenate or *p*-aminobenzoate metabolism. Further experiments are necessary, however, in order to elucidate further its function.

ACKNOWLEDGMENT

The authors wish to express their appreciation to Dr. F. M. Clark, Bacteriology Department, University of Illinois, for the *p*-aminobenzoate determinations with *Rhodotorula aurantiaca*.

SUMMARY

Bacterium linens, strain 456, requires either pantothenate or *p*-aminobenzoate for growth. Folic acid or folinic acid did not substitute for *p*-aminobenzoate except at very high concentrations. Biotin was not required but stimulated growth when either pantothenate or *p*-aminobenzoate was present.

Cells grown in the presence of pantothenate contained *p*-aminobenzoate and those grown on *p*-aminobenzoate contained pantothenate. The addition of biotin reduced the amount of the added vitamin (either pantothenate or *p*-aminobenzoate) found in the cells.

Growth in media containing *p*-aminobenzoate was inhibited by salicylate and sulfanilamide and reversed by pantothenate, pantoate, and biotin.

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