AN EVALUATION OF MICROBIOLOGICAL ASSAYS FOR p-AMINOBENZOIC ACID

by

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AN EVALUATION OF MICROBIOLOGICAL ASSAYS FOR p-AMINOBENZOIC ACID

INTRODUCTION

The discovery by Woods (31) of the antisulfonamide activity of p-aminobenzoic acid*, coupled with the later work of Kohn and Harris (5,10,11), Shive and his co-workers (23,25), and Lampen and Jones (12,13), has established the role of PABA in the biological synthesis of methionine, serine, purines, folic acid and perhaps other unidentified growth factors. With these developments in the chemistry of the vitamin, a need has arisen for its quantitative determination in natural materials.

Since PABA had been found only in extremely minute quantities (of the order of 0.1 to 1 microgram per gram in most materials), it appeared at the outset that PABA could not be assayed by chemical methods because of their lack of sensitivity. This has been borne out by subsequent investigations (2,9). Animal assays are inaccurate since much of the animal requirement is satisfied because of PABA synthesis by the bacterial flora of the large intestine (1). There remains the possibility of assay through microbiological means.

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*p-aminobenzoic acid will be referred to in the remainder of this thesis as PABA.

Shortly after the discovery of the vitamin, Underkofler, and his coworkers (15, 30) found that PABA functioned as a growth factor for one of the acetic acid bacteria, Acetobacter suboxydans, an organism which has been often utilized industrially in the oxidation of polyhydric alcohols. Landy and Dicken (16) used Underkofler's work as a basis for the first published account of a microbiological technique for the estimation of PABA.

Lactic acid bacteria had at that time become popular assay organisms for several of the B vitamins. After Isbell (6) noted that a strain of Lactobacillus arabinosus 17-5 required PABA for growth, Lewis (18) developed an assay method with this organism. Unfortunately, it was later found that the organism could adapt to media containing suboptimal quantities of PABA, thus eliminating this vitamin as a growth requirement, and necessarily eliminating the method for quantitative assay.

Following these discoveries, Thompson, Isbell and Mitchell (19,29) introduced a method using Neurospora crassa "p-aminobenzoicless". This organism, which is one of the many mutant molds produced by Beadle, et al. (28), was stated to be more sensitive to the vitamin than was Acetobacter.

Meanwhile, Lampen and Peterson (14), both of whom had been involved with the original work on the Acetobacter growth factors, were investigating a method using an anerobic organism, Clostridium acetobutylicum. Their results were later published and this assay has been used in a few laboratories for PABA determinations.

The use of Leuconostoc mesenteroides P-60 for PABA assay was described briefly by Pennington (20), who indicated that the method was reliable and that it gave reproducible values with many materials. Supporting data were lacking, however, and the composition of the growth medium was not given.

Some important features of the above described methods are listed in Table I. In addition, a recent note by Rainbow (21) suggests that several strains of brewer's yeast, particularly "Yeast 45" and "Yeast 47" could well be adapted to microbiological assay procedures, since the organisms required PABA for growth.

It is not possible to assume that each of these assay methods is as desirable as any other. In order to evaluate them, the methods have been studied with regard to their general utility, and the growth media have been examined for extraneous stimulatory materials. Where feasible, improvement of the assays has been undertaken.

From the results shown in this thesis, and in the literature, it seems evident that the assay method introduced by Pennington (20) using Leuconostoc mesenteroides P-60 is superior to the other microbiological assays for this vitamin. It is extremely sensitive, the growth period is short, and it gives reproducible results over a relatively broad range of concentrations of the vitamin.

TABLE I

PABA ASSAY METHODS

ORGANISM	INCUBATION TIME IN HOURS	RANGE IN MICROGRAMS	GROWTH MEASUREMENT	REFERENCE	REMARKS
Acetobacter suboxydans	48	0.005-0.05	turbidity	Landy & Dicken (16)	Not sensitive enough; incubation time too long.
Lactobacillus arabinosus 17-5	72	0.00015-0.0005	acid production	Lewis (18)	Incubation time is long. Organism has dispensed with PABA requirement.
Neurospora crassa "p-aminobenzoicless"	20	0.005-0.03	diameter of mold colony	Thompson Isbell & Mitchell (29)	Mold is more difficult to handle and more difficult to measure growth response; not sensitive enough.

TABLE I (CONTINUED)

PABA ASSAY METHODS

ORGANISM	INCUBATION TIME IN HOURS	RANGE IN MICROGRAMS	GROWTH MEASUREMENT	REFERENCE	REMARKS
Clostridium acetobutylicum	20-24	0.0005-0.0020	turbidity	Lampen & Peterson (14)	Anerobe; difficult to prepare oxygen-free medium.
Leuconostoc mesenteroides P-60	15	---	turbidity	Pennington (20)	None

ASSAY USING *ACETOBACTER SUBOXYDANS* (16)

EXPERIMENTAL

In view of the discovery of a panthothenic acid conjugate*, a new growth factor active for *Acetobacter suboxydans* by King, Locher and Cheldelin (8) in this laboratory, it appeared impossible for existing assay methods using this organism (3,16,17) to be accurate, since no suitable source of PAC had been included in the modified medium. Although Landy and Dicken (16) claimed that a growth period of 48 hours produced sufficient growth for assay purposes, the strain used herein required approximately 72 hours for full growth when the free vitamin was employed. However, King *et al.* (8) have observed in their pantoic acid assay that in the presence of PAC, growth reaches a maximum in 40 hours. It was therefore felt that the PABA assay might be improved by adding PAC to the medium in place of panthothenic acid.

Organism. — The test organism employed was a strain of *Acetobacter suboxydans* which was listed as No. 621 by the American Type Culture Collection. This organism was carried on yeast extract-glycerol-agar slants (2% agar, 5% glycerol, 0.5% Difco yeast extract,

*Panthothenic acid conjugate will be referred to in the remainder of this thesis as PAC.

pH 6). The stock slants were transferred bimonthly. Slants for use in daily inoculations were renewed twice weekly. For daily inoculum, a loop of organisms was transferred from the semi-weekly slant to a broth medium (glycerol 5%, Difco yeast extract 0.5%), which was incubated for 24 hours at 30° C prior to use in assays.

Basal Medium. — The basal medium is shown in Table II. It is a double concentration medium, being diluted 100% in each assay flask.

Several precautions should be taken in preparing the various parts of the medium. It is suggested that Difco Vitamin-free Casamino acids be used as the nitrogen source at the level suggested in the table. In testing various U. S. P. vitamin-free casein sources, it was found that these casein sources were contaminated with PABA to a higher degree than the Difco product.

Tryptophane is most conveniently weighed out when the medium is mixed together. Solution in hot water can be accomplished at a concentration of 10-20 mg. per ml. Tryptophane solutions tend to decompose when stored even for short lengths of time.

Cystine is easily dissolved in 6N HCl with heating, at concentrations of 15-20 mg. per ml. This solution should be stored under toluene at room temperature, since

much of the amino acid precipitates in the cold. No decomposition of standard cystine solutions has been observed.

Adenine hydrochloride, guanine sulfate and uracil may be dissolved in 6N HCl with heat at concentrations of 2-3 mg. of each per ml. and stored under toluene for several weeks.

Inorganic salts are commonly grouped under headings of Salts A and B (26). These solutions supply all the necessary inorganic materials for growth.

Vitamins may be kept in stable solutions of 1 mg. per ml. for several weeks if stored under toluene and refrigerated.

TABLE II

*BASAL MEDIUM FOR PABA ASSAY
WITH ACETOBACTER SUBOXYDANS

Difco Casamino Acids	10 g. ***
Glycerol	10 g.
Glucose ***	5 g.
Liver Extract *** (24)	2 g.
Peptone *** (24)	5 g.
Tryptophane	200 mg.
Cystine	150 mg.
Adenine, guanine and uracil **	5 mg. each
Salts A and B	10 ml. each
Nicotinic Acid	200 γ
Folic Acid	100 γ
Calcium Pantothenate	200 γ
Distilled water to 1 liter	
pH 6.0	

* Pantothenic acid conjugate (PAC) equivalent to 3.0 γ pantothenic acid per mg. was added individually to each flask at a level of 3 mg. per flask.

** Purines were added to this modified medium at the level suggested by Landy and Streightoff (17).

*** Glucose, additional casein, and peptone, liver mixture were added following the suggestion of Cheldelin and Bennett (3).

Pantothenic acid conjugate (PAC) (8) supported maximum growth when supplied at levels of 3 mg. per tube. The extract, containing the equivalent of 3.0 γ pantothenic acid per mg., was known as Preparation No. 801.

After adjustment of pH to 6.0, to remove turbidity, the basal medium may be autoclaved at 15 lbs. pressure for five minutes and then filtered. This eliminates cloudiness in the assay flasks due to heat precipitable materials in the basal medium.

Assay Procedure. — Standard flasks were set up in the following manner to obtain a reference curve. PABA solutions were added to each of the 50 ml. Erlenmeyer flasks at successive concentrations from 0.001 to 0.030 micrograms per flask. Natural materials to be assayed may be diluted to the same range and substituted for the PABA solutions, usually at four levels (1 to 4 ml.). Basal medium at a level of 5 ml. was added, followed by dilution to 10 ml. with distilled water.

Erlenmeyer flasks allow more profuse growth than 6 inch bacteriological test tubes since the organism is an obligate acrobe and grows only on the surface of the medium.

After additions to the flasks were concluded, they were plugged with cotton and autoclaved at 15 lbs.

pressure for 15 minutes. Following cooling, the tubes were inoculated with a suspension of the test organism.

To prepare inoculum suspensions, the contents of the inoculum flask were centrifuged, the supernatant medium was decanted and the organisms were resuspended in 10 ml. of sterile physiological saline. This process was repeated twice. One drop of the final suspension (in 15 ml. sterile saline, optical density approximately 0.6) was added aseptically to each flask.

After incubation at 30° C. for 40 to 48 hours, the resultant turbidity of the cultures was measured in a Pfaltz and Bauer fluorophotometer. Readings were plotted on cross hatched paper vs. PABA contents of the tubes. When natural materials are assayed, the turbidimetric values at different levels are taken and the PABA content is read directly from the graph.

RESULTS

With Acetobacter, as with each organism tested, growth has been observed not only as a function of PABA, but also as produced by the other constituents of the various media, in the presence of optimal and suboptimal concentrations of the vitamin.

As suggested above, improvements in the Acetobacter assay can be realized. The growth response (shown in Table III) is obtained in 40 hours, rather than in 60 to 72 hours when pantothenic acid was employed. Tryptophane was omitted, since it is not needed either in a medium containing hydrolyzed casein or in a synthetic one containing isoleucine, alanine, histidine, cystine, valine, and proline, as suggested by Stokes and Larsen (27).

The addition of five mg. each of adenine, guanine and uracil per liter of medium has been recommended (17). The stimulatory nature of these substances has been confirmed in the present work, but it has also been found that increasing the concentration of these substances produces inhibition, beginning at about 10 mg. of each base per liter. This inhibition has been demonstrated both at suboptimal (0 and 0.004 microgram per flask) and

optimal (0.01 microgram) levels of PABA. It appears then that the assay cannot easily be used for natural materials which are rich in purines, since the inhibitory action will give low apparent values for the PABA assayed.

TABLE III

GROWTH RESPONSE OF *ACETOBACTER SUBOXYDANS* TO ADDED PABA

Micrograms PABA per culture	Optical Density (2-log transmission)
0	.240
0.001	.310
0.002	.340
0.004	.435
0.006	.510
0.008	.525
0.01	.595
0.03	.700

EXPERIMENTS USING "YEAST 45"

EXPERIMENTAL

Following the suggestion of Rainbow (21), an attempt was made to utilize "Yeast 45" in an assay procedure for determining PABA. King*, has noted that PABA requirements of this organism are dispensed with, if adequate purines, folic acid, and methionine are added to the medium. These results have been confirmed in the present study.

Organism. — The organism tested was "Yeast 45", a single cell strain of top fermentation yeast, isolated in an English brewery.

The yeast was grown on molasses agar slants (4.0% molasses, 0.12% $(NH_4)H_2PO_4$, 2.0% agar) for 24 hours at 30° C and then refrigerated. For daily inoculum, the yeasts were transferred from this stock slant to a similar agar slant which was incubated 24 hours prior to use.

Basal Medium. — The basal medium was similar to that suggested by King and Cheldelin (7). It is shown in detail in Table IV. Each of the constituents was handled as suggested in the Acetobacter assay method.

*Private communication.

TABLE IV

BASAL MEDIUM FOR YEAST GROWTH (7)

Glucose	20 g.
$(NH_4)_2SO_4$	3 g.
KH_2PO_4	2 g.
Salt Solution 1 and 2	1 ml. each
Inositol	15 mg.
Riboflavin	200 γ
Nicotinic Acid	200 γ
Pyridoxine	200 γ
Calcium Pantothenate	200 γ
Thiamin Hydrochloride	100 γ
Biotin	1 γ
Water to	1 liter
pH	4.8-5.0

Testing Procedure. — Folic acid, methionine and a solution of adenine, guanine and uracil were tested for stimulatory effect. A standard curve for PABA was obtained at successive concentrations between 0.001 and 0.01 microgram of PABA. Each of these solutions was tested at three levels of PABA (0,0.002 and 0.006 microgram). Finally another growth response curve was obtained with the medium, including in each tube 0.1 microgram of folic acid, 1 mg. methionine and 0.1 mg. each of the listed purines and pyrimidines. In this manner, it was possible to show the effect of each of these additions.

The test substances were measured into 20 x 150 mm. lipless pyrex test tubes, diluted to a total volume of 2 ml., and 5 ml. of the medium shown in Table IV was added to each tube. The tubes were plugged with cotton and autoclaved at 15 lbs. pressure for 15 minutes. Following cooling they were inoculated.

For an inoculum, a loopful of yeast from the freshly grown slant was suspended in a tube of sterile medium and the yeast concentration determined turbidimetrically with the aid of a standard calibration curve. A measured amount of this suspension was transferred to a flask containing a known volume of sterile medium so that the concentration of moist yeast was 2.4 mg.

per 100 ml. The test tubes were then seeded with 1 ml. of this suspension added to each tube to give a final volume of 8 ml. and a yeast concentration of 0.3 mg. per 100 ml. The tubes were incubated at 30° C (for 40-48 hours) and the growth measured turbidmetrically on a Pfaltz and Bauer fluorophotometer.

RESULTS

The results are shown in Table V. "Yeast 47" has been shown to give similar results.*

*Unpublished communication from T. E. King.

TABLE V

GROWTH RESPONSE OF "YEAST 45"

PABA γ per tube	PURINES* mg. per tube	FOLIC ACID γ per tube	METHIONINE mg. per tube	OPTICAL DENSITY
0				.010
0.002				.010
0.006				.015
0.010				.040
0	0.05			.015
0	0.2			.015
0.002	0.05			.020
0.002	0.2			.025
0.006	0.05			.075
0.006	0.2			.070
0		0.05		.010
0		0.2		.025
0.002		0.05		.015
0.002		0.2		.015
0.006		0.05		.020
0.006		0.2		.030
0			0.5	.015
0			3	.015
0.002			0.5	.020
0.002			3	.035
0.006			0.5	.145
0.006			3	.275
0	0.1	0.1	1.0	.470
0.002	0.1	0.1	1.0	.435
0.006	0.1	0.1	1.0	.405
0.01	0.1	0.1	1.0	.575

*Adenine, guanine and uracil.

ASSAY USING *LEUCONOSTOC MESENTEROIDES* P-60

In view of the lack of sufficient information on the growth medium and experimental results in Pennington's note (20), it seemed desirable to investigate the constituents of the proposed medium for stimulatory effect and also to determine the optimal levels for all the added materials. In addition, more concrete evidence of accuracy was needed in the form of recoveries of known amounts of the added vitamin and in the degree of agreement at all assay levels.

EXPERIMENTAL

Organism. — The organism used in this assay is a strain of *Leuconostoc mesenteroides* P-60. This organism, which was received from Dunn (4) of the University of California at Los Angeles, who in turn received his organism from Snell at the University of Texas, has different growth requirements than an organism which was received at this laboratory from Snell. Since these variations have been noted, and since the organism carried by the American Type Culture Collection is

different from the above two*, care should be exercised in the choice of the assay organism.

The organism was carried on a glycerol-yeast extract-agar medium (glycerol 5.0%, yeast extract 0.5%, agar 2%) in the form of a stab culture and was transferred bimonthly to renew its activity. Subcultures (stabs) were transferred weekly from the stock culture for daily use. For daily inoculum, the organism was transferred to a nutrient broth (Table VI) and incubated at 37°C for 24 hours prior to use.

*Unpublished observations of the author.

TABLE VI

INOCULUM BROTH FOR *LEUCONOSTOC MESENTEROIDES* P-60

Alkali treated peptone	1.0 g.
Glucose	4.0 g.
Sodium acetate	3.0 g.
Casein	0.4 g.
Cystine	20 mg.
Salts A and B	1 ml. each
Yeast extract	100 mg.
Liver extract	100 mg.
Water to	200 ml.
pH	6.7

Basal Medium. — The basal medium for the Leuconostoc Mesenteroides P-60 assay is shown in Table VII. All constituents of the basal medium are best handled as previously suggested in the Acetobacter studies, with the following exceptions:

Difco Vitamin-free Casamino acids should not be used as a nitrogen source for this assay. Several different commercial samples of U. S. P. Vitamin-free casein allowed more rapid and luxuriant growth than the Difco product. Each of the casein samples was HCl hydrolyzed, the HCl being removed by distillation. Noriting the resulting solution 2-3 times at pH 3.0 to remove residual vitamins, seemed to give best growth in addition to lower values in the blank (no PABA added) tubes.

Assay Procedure. — Standard tubes were set up in the following manner to obtain a reference curve. PABA solutions were added to each of the bacteriological test-tubes (20 x 150 mm. lipless pyrex test-tubes) at successive concentrations from 0.1 to 1.5 milli-micrograms. Natural materials were diluted to the same range and substituted for the PABA at three or four levels (1 to 4 ml.). Basal medium at a level of 5 ml. was added, followed by dilution to 10 ml. with distilled water.

TABLE VII

BASAL MEDIUM FOR PABA ASSAY
WITH *LEUCONOSTOC MESENTEROIDES* P-60

Casein	10 g.
Sodium Acetate	12 g.
Glucose	20 g.
Cystine	200 mg.
Tryptophane	200 mg.
Adenine, guanine, uracil	20 mg. each
Salts A and B	10 ml. each
Thiamin	600 γ
Nicotinic Acid	600 γ
Calcium pantothenate	600 γ
Pyridoxine	600 γ
Biotin	2 γ
Pyridoxal	1 γ
Water to	1 liter
pH	6.8

After additions to the tubes were completed, the entire wire rack of tubes was covered with a clean towel and autoclaved for 15 minutes at 15 lbs. pressure. After cooling, the tubes were inoculated with a suspension of the test organism.

To prepare inoculum suspensions, the inoculum tubes were centrifuged, the supernatant medium was discarded and the organisms were resuspended in 10 ml. of sterile physiological saline. This process was repeated twice. One ml. of the final suspension was diluted to 15 ml., with one drop of this suspension (optical density approximately 0.25) serving to inoculate each tube.

After incubation at 37°C for 15 hours, the resultant turbidity of the cultures was measured turbidimetrically. If desired, the cultures may be incubated at 37°C for 72 hours, with acid production being used as the growth determinant. Optical density readings (or ml. of 0.1 N acid produced) were plotted on cross hatched paper vs. PABA contents of the tubes (Figures I and II). When natural materials were assayed, the turbidimetric values (or ml. of acid produced) at different levels were taken and the PABA content was read directly from the graph.

Preparation of Samples for Assay. — Assay samples were prepared by two methods: water extraction, and acid hydrolysis. One gram of the solid, natural material was suspended in 50 ml. of water or 6 N HCl, using Erlenmeyer flasks. The flasks were plugged with cotton and autoclaved at 15 lbs. pressure for 30 minutes. The water extracted samples were then diluted to provide a PABA concentration within the operating range of the assay and then filtered. The acid hydrolyzed samples were diluted to a satisfactory concentration, neutralized with NaOH and then filtered. In this manner, the resultant test solutions were clear and usually colorless.

FIGURE I

TURBIDIMETRIC GROWTH RESPONSE OF LEUC. MESENTEROIDES TO PABA

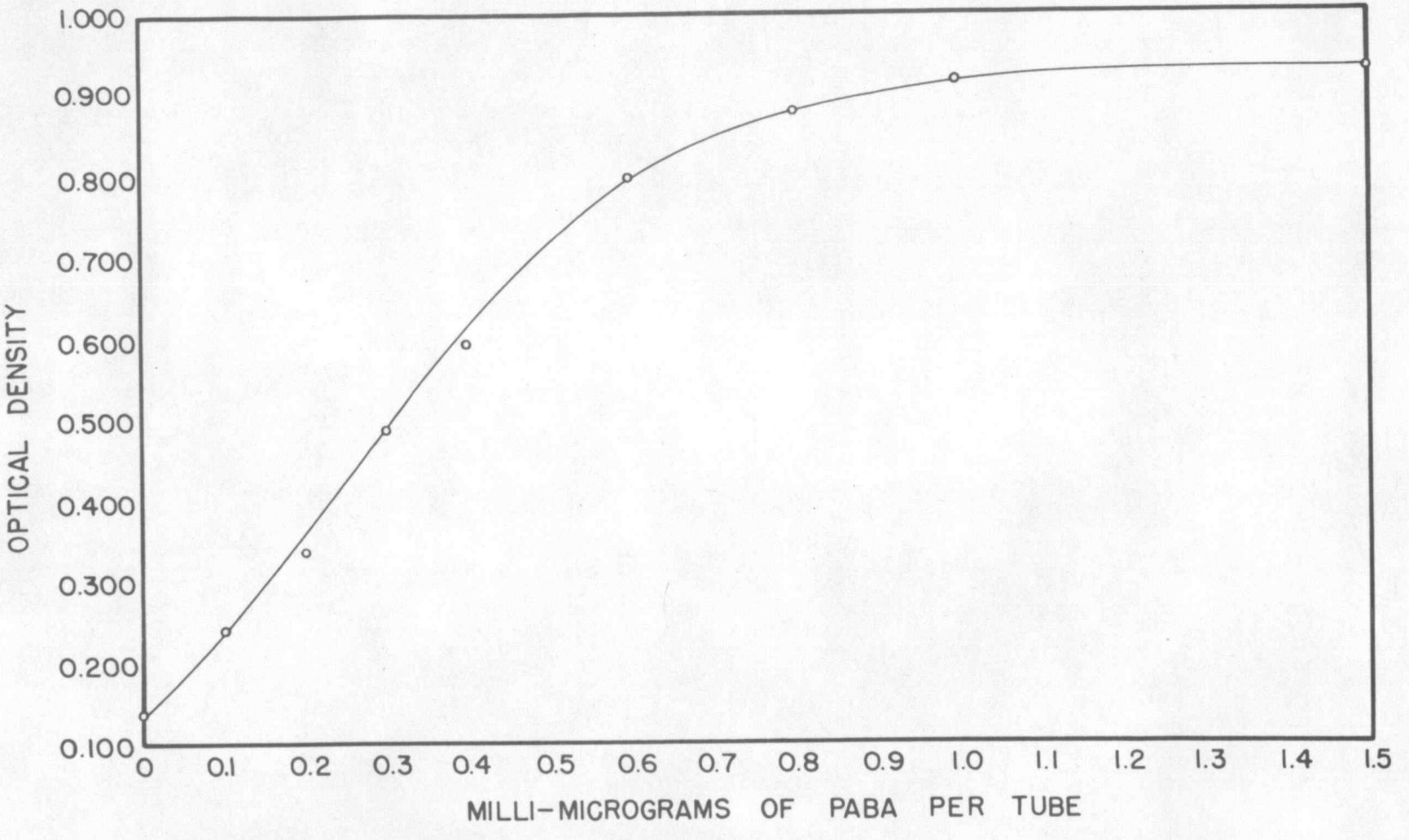
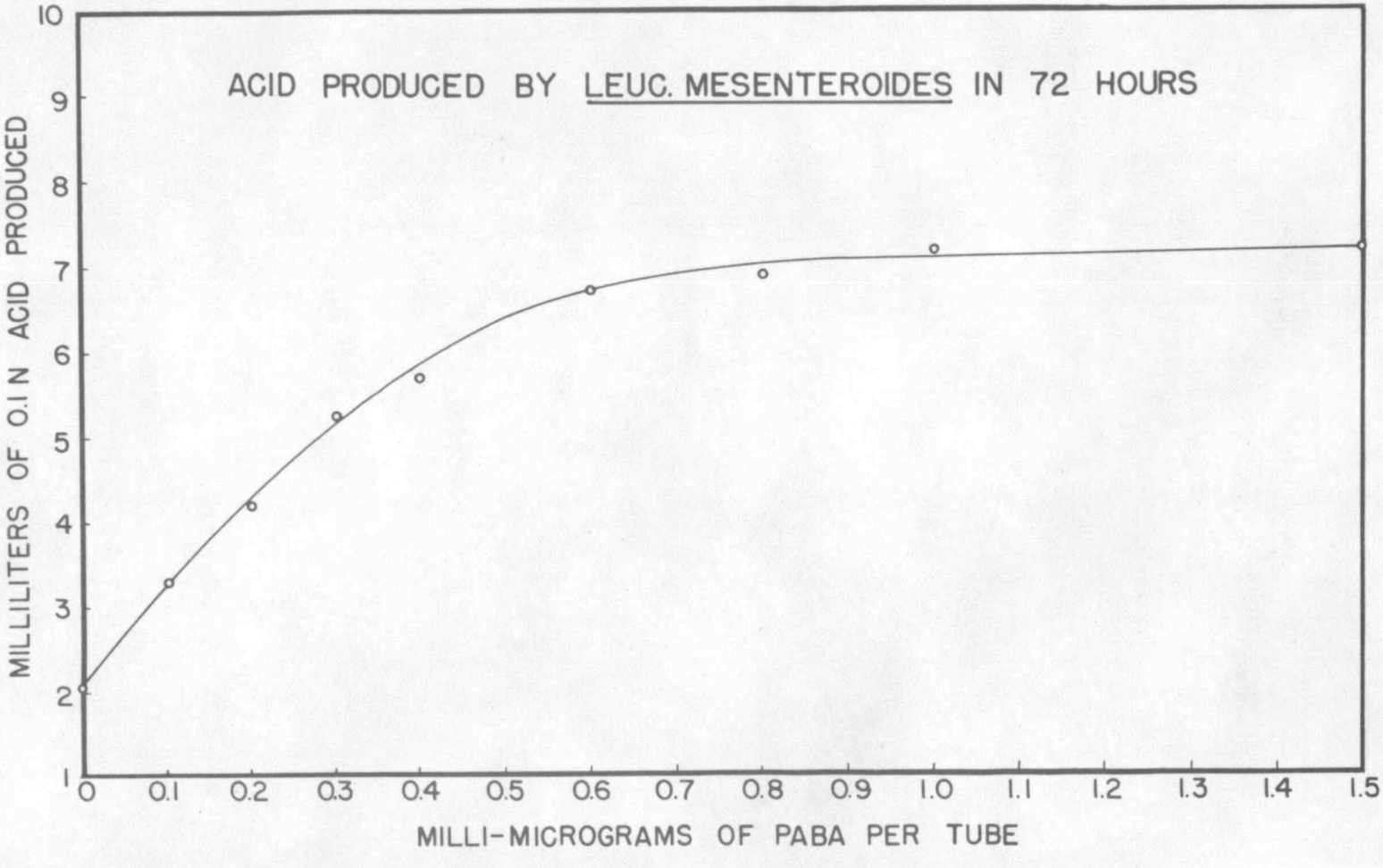


FIGURE II


ACID PRODUCED BY LEUC. MESENTEROIDES IN 72 HOURS
MILLILITERS OF 0.1 N ACID PRODUCED
10
9
8
7
6
5
4
3
2
1
0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
0.9
1.0
1.1
1.2
1.3
1.4
1.5
MILLI-MICROGRAMS OF PABA PER TUBE

RESULTS

The PABA content of several processed natural materials is shown in Table VIII. The values for these materials usually showed less than a 6% average deviation in each assay when calculated from three different assay levels. Good agreement among values was obtained for a given material tested on successive days.

Listed in Table IX are the recoveries of added PABA to samples of natural materials. The recoveries vary from 84% to 105% which is within the accepted range for microbiological determinations.

All constituents of the medium were checked and found to be non-stimulatory at higher levels. This in itself is partial assurance of the accuracy of the method. Although the vitamin requirements of the organism are much less than the quantities found in the medium, the larger amounts have been added routinely to prevent stimulation by the materials being assayed. Pyridoxal, though not needed for growth, is slightly stimulatory at the level incorporated in the medium.

The natural substances assayed were chosen because of their general stimulatory effect upon micro-organisms. It was felt that if the assay would give good results on these materials, it should be satisfactory for most foodstuffs.

TABLE VIII

PABA CONTENT OF PROCESSED NATURAL MATERIALS

Material	PABA Content (γ per gram)	
	Water Extraction	Acid Hydrolysis
Difco Yeast Extract	57.	77.
Difco Autolyzed Yeast	6.4	17.
Difco Tryptone	0.14	0.38
Difco Proteose-Peptone	0.27	0.30
Difco Peptonized Milk	0.11	0.22
Difco Peptone	0.10	0.13
Difco Malt Extract	0.43	1.1
Wilson Liver Fraction "L"	0.80	9.0
Wilson Liver Concentrate	2.9	22.
Baker Peptone	0.10	0.12

TABLE IX

PABA ANALYSES AND RECOVERIES

Material	Sample			PABA Found	PABA per gram	Recovery
	Added PABA milli-micrograms			milli-micrograms	micrograms	per cent
Difco Yeast Extract	0.02 mg.			.100	50.0	
	0.03 mg.			.185	60.5	
	0.04 mg.			.235	59.0	
	0.02 mg.	PABA	0.1	.205		105
	0.03 mg.	PABA	0.15	.332		99
	0.04 mg.	PABA	0.2	.425		95
Difco Malt Extract	0.6 mg.			.275	0.46	
	0.8 mg.			.330	0.42	
	1.0 mg.			.405	0.41	
	0.6 mg.	PABA	0.3	.555		93
	0.8 mg.	PABA	0.4	.665		84
Wilson Liver Fraction "L"	0.3 mg.			.220	0.75	
	0.3 mg.	PABA	0.3	.505		95
Difco Peptone	4.0 mg.			.505	0.12	

DISCUSSION

The microbiological assay for PABA using Leuc. mesenteroides P-60, has been shown to be the best of the methods examined for determination of the vitamin. It meets all the requirements of a satisfactory assay method. It is more sensitive (sensitivity 0.0001 micrograms per culture) than most of the microbiological assays for any of the vitamins, and the growth period is short (16 hours). Growth is slight without PABA, and abundant when adequate PABA is added. There are no extraneous stimulatory materials incorporated in the basal medium. Excellent agreement at different levels of the assayed material have been observed and recoveries of added vitamin are within the range of a satisfactory microbiological technique. In addition, the assay procedure is not entirely rigid, since it has been shown that the vitamin may also be determined by titration of the acid produced by the organism in 72 hours.

It is essential to obtain standard curves with steep initial slopes as shown in Figure I. If the lower portions of the standard curve are appreciably sigmoidal, the assay results will be anomalous. The cause of this "lag" is not completely understood, but it is accentuated by the use of a dilute inoculum. Variations in the

shape of the curve have been observed with different lots of hydrolyzed casein and with different treatment of the casein. Excessive noriting (4-5 times) at pH 3 also reduces the initial slope suggesting that the removal of other growth factors may be the cause of this phenomenon.

It has not been possible to reduce the growth in the absence of added PABA. Continued treatment of the casein with Norite as just described produces optical densities in the "blank" tubes as low as 0.090, but the assay results are unsatisfactory as mentioned, and maximum growth readings are less than 0.7. It seems likely that the present basal medium contains traces of PABA, but until a synthetic medium can be developed for this organism which will permit maximum growth to be reached rapidly (see also Dunn et al.) (4), it is felt that the present method should suffice.

The generally higher yields of PABA after acid hydrolysis, shown in Table VIII, have also been observed by previous workers. It is possible that water extraction may be inadequate, but it seems more likely that the higher values represent peptides (22) and other conjugates of the vitamin, including folic acid. Since the extent and nature of all naturally occurring forms is not known, a relatively drastic hydrolysis procedure

seems desirable to release the vitamin from its conjugates. This is best effected by acid hydrolysis, according to Pennington (20) or by alkaline treatment, in the opinion of Lampen and Peterson (14).

SUMMARY

1. PABA assay time using *Acetobacter suboxydans* has been reduced to 40-44 hours by additions of pantothenic acid conjugate.
2. The medium for this organism has been revised, omitting tryptophane, since it is not needed for growth.
3. Purines, at levels above 10 mg. per liter have been found to be inhibitory. This limits the use of the assay to materials containing small concentrations of purines.
4. The PABA requirement for "Yeast 45" has been shown to be completely fulfilled by adding purines, folic acid and methionine, therefore it is impossible to use this organism for assay purposes.
5. A growth promoting medium for use with *Leuconostoc mesenteroides* P-60 in PABA assays has been formulated.
6. Experiments showing the accuracy and superiority of this assay method have been noted. It fulfills the requirements of a good PABA assay.

7. Water extraction was unsatisfactory to remove PABA from solid materials. Acid hydrolysis has been shown to give higher apparent PABA values.

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