A MICROBIOLOGICAL ASSAY METHOD FOR *p*-AMINOBENZOIC ACID

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Since the establishment of *p*-aminobenzoic acid as a member of the B vitamin group, a considerable interest has been shown in methods of determination in natural materials. Since known chemical methods are not sufficiently sensitive, it became evident that microbiological tests should be the most practicable. The organism *Clostridium acetobutylicum* has been used (1) but no general assay procedure has been presented. Several bacterial strains which respond to *p*-aminobenzoic acid have been investigated in this laboratory, but satisfactory assay procedures with these organisms have not yet been devised.

For the discovery of the test organism used in the procedure described in this paper, we are indebted to Dr. Beadle and Dr. Tatum who kindly furnished us with a culture of their *p*-aminobenzoic acid requiring a mutant strain of *Neurospora crassa*, designated by them as *Neurospora crassa p-aminobenzoicless* No. 1633 (2). This mold will grow optimally on a medium consisting of inorganic salts, ammonium tartrate, sucrose, biotin, and *p*-aminobenzoic acid. For purposes of assay, however, it has proved advantageous to supplement this basal medium with natural extracts which are either naturally low in *p*-aminobenzoic acid or have been treated to remove it. With such a complex medium, the possibility of interference by toxic substances or stimulatory substances other than *p*-aminobenzoic acid which might be present in samples to be assayed is reduced to a minimum.

Since the completion of a considerable part of the experimental work described in this paper, microbiological assay methods for *p*-aminobenzoic acid have been published by Landy and Dicken (3) utilizing the organism *Acetobacter suboxydans* and by Lewis (4) using *Lactobacillus arabinosus* 17-5.

EXPERIMENTAL

Organism—Stock cultures of the organism are carried on slants of the same composition as the assay medium except for the addition of 0.05 γ of *p*-aminobenzoic acid per tube. In preparing the inoculum for an assay, a large loopful of spores is thoroughly dispersed in 1 or 2 ml. of sterile liquid agar medium identical in composition with the assay medium except for the addition of 5 millimicrograms of *p*-aminobenzoic acid. This agar medium

with suspended spores is poured into a sterile Petri plate and incubated 16 hours at 30° and placed in the refrigerator until used. In this length of time the spores will have germinated and the incipient mycelia, distributed uniformly throughout the agar, will be just visible to the unaided eye. Blocks of agar cut from this plate are used for inoculating the plates containing the sample to be assayed. These inoculum blocks may best be removed with the aid of a sterile 8 mm. cork borer fitted with a plunger for extruding the block of agar. Care must be taken to secure circular blocks of uniform thickness, as any irregularity in the shape of the inoculum block will be reflected in the subsequent growth of the mold.

Medium—The medium employed has the following composition: basal, ammonium tartrate 5.0 gm., KH_2PO_4 1.0 gm., NH_4NO_3 1.0 gm., $MgSO_4 \cdot 7H_2O$ 0.5 gm., NaCl 0.1 gm., $CaCl_2$ 0.1 gm., $FeCl_3$ 5.0 mg., sucrose 15 gm., biotin 4 γ, agar (washed) 20 gm.; supplements, vitamin-free, acid-hydrolyzed, charcoal-treated casein 1.0 gm., enzymatically digested beef liver extract 0.5 gm., enzymatically digested beef muscle extract 0.1 gm., charcoal-treated yeast extract 0.1 gm.; water to make 1 liter. The basal medium is that employed by Tatum and Beadle (2). The preparation of the supplements is described below.

Acid-Hydrolyzed, Charcoal-Treated Casein—50 gm. of vitamin-free casein (Labco) are mixed with 500 ml. of 6 N HCl, and the mixture refluxed for 10 hours. The HCl is then removed by repeated vacuum distillation, the pH adjusted to 3.0, and the volume to 500 ml. 5 gm. of Darco G-60 decolorizing charcoal are added; the mixture is shaken 15 minutes, and then filtered to remove charcoal. The pH is adjusted to 7.0 and the solution preserved under toluene.

Enzymatically Hydrolyzed Beef Liver and Muscle—10 gm. of finely ground fresh beef liver or muscle are suspended in 50 ml. of acetic acid-sodium acetate buffer, pH 4.5, and to this mixture is added 0.1 gm. each of the enzyme preparations clarase[1] and caroid.[2] The mixture is allowed to autolyze under benzene for 24 hours, after which time the benzene is removed by steaming and the solids by filtration.

Charcoal-Treated Yeast Extract—10 gm. of yeast extract (Difco) are dissolved in 100 ml. of water and the pH adjusted to 3.0. 2 gm. of Darco G-60 decolorizing charcoal are added and the mixture shaken 15 minutes. The charcoal is then removed by filtration and the pH adjusted to 7.0.

Agar—Commercial grades of agar often contain appreciable amounts of *p*-aminobenzoic acid and must therefore be thoroughly washed to remove these traces. This may be accomplished by dispersing the agar in a large volume of distilled water, allowing it to settle, and decanting the water.

[1] Takamine Laboratories, Inc., Clifton, New Jersey.
[2] American Ferment Company, Buffalo, New York.

Such a procedure repeated fifteen or twenty times over a period of from 24 to 48 hours is satisfactory.

The washed agar and supplements are added to the basal medium just prior to use.

Procedure—The standard *p*-aminobenzoic acid solution and solutions for analysis are pipetted into test-tubes and diluted when necessary to 1 ml. 15 ml. of the hot agar medium are then added to each tube and the tubes autoclaved for 15 minutes. Directly after removal from the autoclave, the contents of the tubes are transferred to sterile Petri plates. For this purpose molded soft glass plates have proved most satisfactory, since the bottoms are uniform, giving a layer of agar of uniform thickness. Most Pyrex plates are unsatisfactory, since they are deeper at the edges than in the center. When the agar has cooled, an inoculum block is placed upon the surface in the center of each plate. The plates are incubated right side up (to prevent mold from growing downward) at 30° for approximately 20 hours. Standard plates containing amounts of *p*-aminobenzoic acid varying from 4 to 40 millimicrograms (10^{-9} gm.) are satisfactory for establishing a standard curve. Amounts of the extracts to be assayed must be selected by preliminary experiment (or estimate) to contain an amount of *p*-aminobenzoic acid within the range of the standard curve.

Measurement of Response to p-Aminobenzoic Acid—The diameter of the mold growth surrounding the inoculum block is measured with calipers and is dependent on the amount of *p*-aminobenzoic acid in the culture plate. This growth is quite uniformly circular if care is taken to cut perfect inoculum blocks from a culture containing a heavy, uniform distribution of spores. This method of measuring the growth response has been found to be superior to measuring, by means of a planimeter, the area of the colony, and is in general more convenient than measuring the rate of growth along a horizontal tube as was done by Beadle and Tatum (5). A typical growth response curve is shown in Fig. 1.

Preparation of Materials for Assay—Materials to be assayed must be extracted in such a manner as to make available to the mold all of the *p*-aminobenzoic acid present in the material. As we have indicated in a previous note (6), simple hot water extraction does not release all of the *p*-aminobenzoic acid from liver. The amount of *p*-aminobenzoic acid obtained from a sample of beef liver and from beef kidney by various extraction procedures is shown in Table I. The partial destruction of pure *p*-aminobenzoic acid by the hydrolysis procedures is also demonstrated. In experiments not described it is indicated that this destruction also occurs in the presence of tissue samples to about the same extent as in the pure solution. Since hydrolysis with 6 N H_2SO_4 produces a maximum yield of *p*-aminobenzoic acid in spite of this destruction, averaging about 15 per cent,

the following procedure for the preparation of samples was adopted. The sample[3] to be hydrolyzed is finely ground and 5 ml. of 6 N sulfuric acid added per gm. of material. This mixture is autoclaved for 1 hour at 15 pounds pressure and the sulfuric acid then nearly neutralized with barium carbonate. The precipitated barium sulfate and the undigested material are removed by filtration and the pH adjusted to neutrality with ammonium

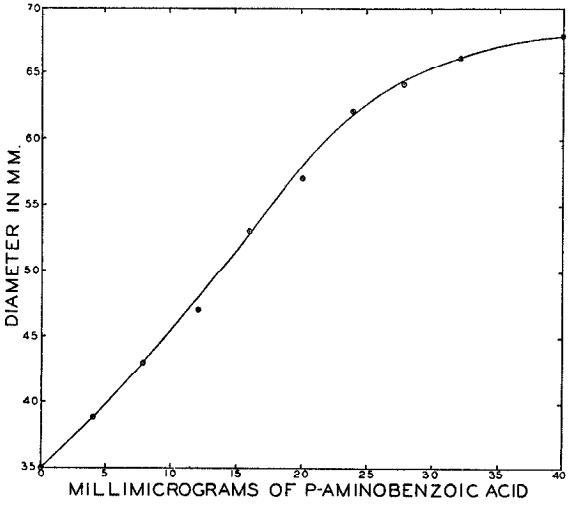

FIG. 1. A typical mold growth curve

TABLE I

Effect of Acid and Alkali on p-Aminobenzoic Acid

All samples were autoclaved under 15 pounds pressure for 1 hour.

	6 N H_2SO_4	5 N NaOH	1 N NaOH	0.1 N NaOH
Beef kidney, γ *per gm.*	2.4	0.75	0.60	0.50
" liver, γ " "	2.1	0.80		
p-Aminobenzoic acid (0.4 γ per ml.), % *recovered*	83		88	85

hydroxide. Neutralization of the sulfuric acid with sodium hydroxide or ammonium hydroxide is not permissible, as excessive amounts of salt are toxic.

Specificity of Assay Method—That the mold responds specifically to the *p*-aminobenzoic acid content of the samples being assayed is indicated by the general parallelism of response to natural materials and to pure *p*-aminobenzoic acid. Beadle and Tatum have tested a large number of

[3] Usually 1 to 5 gm.

compounds structurally related to *p*-aminobenzoic acid, and in no cases have these compounds shown more than a fraction of a per cent of the activity of *p*-aminobenzoic acid (5). We have tested the acetyl derivative and the amide of *p*-aminobenzoic acid, and also *p*-nitrobenzoic acid. Of these compounds, only the amide possessed appreciable activity, being three-hundredths as active as *p*-aminobenzoic acid. This activity is probably best interpreted as being due to hydrolysis effected by the mold.

p-Aminobenzoic acid added to natural materials after acid hydrolysis is accounted for in the assay with an average error of ±10 per cent.

Application of Assay Method to Natural Materials—A variety of natural materials has been assayed and the results are shown in Table II. The samples were extracted by acid hydrolysis in the manner described above, and also by steaming for 15 minutes, in an effort to differentiate between the

TABLE II

p-Aminobenzoic Acid Content of Natural Materials

The results are given in micrograms per gm. of moist tissue.

Sample	Acid hydrolysis	Hot H_2O extraction	Per cent "bound"	Sample	Acid hydrolysis	Hot H_2O extraction	Per cent "bound"
Carrots	0.22	0.08	64	Pork	0.8	0.3	63
Potatoes	0.36	0.34	6	Beef liver	2.5	0.2	92
Spinach	0.6	0.12	80	" muscle	0.64	0.3	53
Yeast cake	4.0	3.6	10	Rat brain	0.7	0.14	80
Milk	0.1	0.08	20	" kidney	1.8	0.13	93
Sweet potato	0.12	0.11	8	" heart	1.35	0.15	89
Egg (whole)	0.4	0.07	83	" blood	0.27	0.06	78
Molasses	0.32	0.2	38	" muscle	1.7	0.15	91
Wheat germ	1.8	0.5	72	Urine	0.5	<0.02	
Whole wheat	0.6	0.25	58				

"bound" and "unbound" *p*-aminobenzoic acid. As will be observed, the various tissues differ widely in the fraction of *p*-aminobenzoic acid which is water-extractable.

DISCUSSION

The assay method here presented is applicable to a wide variety of materials with a reproducibility of results of ±15 per cent. The method of measurement of response to *p*-aminobenzoic acid is probably not capable of the precision obtainable in the turbidimetric or titrimetric determination of bacterial growth, since the diameter and regularity of the mold growth are affected by such factors as irregularity in the shape of the inoculum block and irregularity in the surface of the Petri plate. The method has, however, some unique features which more than compensate for the sources of deviation in assay values mentioned above. It is more rapid than any

test for *p*-aminobenzoic acid yet published, since it requires only a 20 hour growth period. As the growth period is short and the medium relatively acidic, difficulties due to contamination are not encountered. The time required for setting up an assay is somewhat longer than for a bacterial test but the determination of the amount of growth is much more rapid.

The use of this completely different type of organism is a distinct advantage for the purpose of comparison of assay methods.

An unusually high degree of specificity has been obtained in this method by supplementation of the medium with enzyme hydrolysates of liver and muscle tissues. Such extracts contain considerable quantities of known and undoubtedly of unknown growth-promoting substances but can be used directly in this test because the *p*-aminobenzoic acid present is bound in such a form that it is not utilized by the organism or released by enzymatic hydrolysis.

The question of extraction of *p*-aminobenzoic acid from natural materials involves certain difficulties which have yet to be eliminated. We have found conditions which give a maximum yield with respect to acidic hydrolysis and to clarase and caroid hydrolysis. There is, however, some destruction of the released *p*-aminobenzoic acid during this process amounting to about 15 per cent of the total present. Compared to the increase in yield of hydrolysis with 6 N acid over other treatments, this loss in activity by destruction is not particularly significant.

The last column in Table II lists the approximate per cent of the *p*-aminobenzoic acid content of various substances which is bound. It is perhaps significant that this percentage is high for most animal tissues, the lowest being beef muscle, 53 per cent, and the average on eight tissues being 79.9 per cent. On the other hand ten miscellaneous substances mostly of plant origin averaged 44 per cent bound.

At the present it is not possible to compare the results obtained by this method with those of Landy and Dicken (3) and Lewis (4), since samples for assay have not been prepared in the same way. Such a comparison of all three methods on the same samples is now in progress in this laboratory.

SUMMARY

1. A microbiological test of high specificity for the determination of *p*-aminobenzoic acid in biological materials is described. The organism used is a mutant strain of the mold *Neurospora crassa* produced by Tatum and Beadle (2). The method is rapid and the results reproducible.

2. A procedure for the hydrolysis of "bound" *p*-aminobenzoic acid is described and its limitations discussed.

3. The *p*-aminobenzoic acid content of a number of natural materials has been determined and values on the amounts bound and unbound are included.

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