AN IMPROVED MICROBIOLOGICAL METHOD FOR THE DETERMINATION OF NICOTINIC ACID BASED ON THE USE OF PROTEUS HX19

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(Received for publication, August 27, 1946)

The biological importance of nicotinic acid and its derivatives has stimulated the development of various microbiological methods of determining this substance. The most widely used is that of Snell and Wright (1). This method is, however, relatively insensitive, 0.1 γ being the smallest amount of nicotinic acid which can be estimated by it.

The aim of the present investigation was to improve the method of Lwoff and Querido (2). This procedure is based on the use of the non-pathogenic organism Proteus HX19, which is available in every bacteriological laboratory. The main modification introduced concerns the composition of the basal medium. The sensitivity of the assay is increased 10-fold by this modification. The method has been found to be applicable to the determination of nicotinic acid in a variety of biological materials.

EXPERIMENTAL

Organism—The test organism is Proteus HX19, the antigen of the Weil-Felix reaction for typhus. The cultures are carried on standard agar slants. Transfers are made at monthly intervals, and incubation is at 30° for 24 hours. Between transfers the cultures are stored in the refrigerator.

Basal Medium—The medium is a modification of that of Lwoff and Querido (2). The response of Proteus to nicotinic acid in the original medium is feeble and sluggish. Good growth appears in it only after 4 days and only if large inocula are used. As a result perceptible growth also takes place in controls. The sensitivity of the original test is rather low. Considerably better results are obtained by the use of a medium having the following composition: (NH₄)₂SO₄ 0.75 gm., KH₂PO₄ 4.50 gm., KCl 0.50 gm., NaNO₃ 1.00 gm., distilled water 750 cc.

This medium was brought to pH 7.4 by adding 2 to 2.5 cc. of 10 N NaOH; it was then transferred in 7.5 cc. amounts into 16 × 150 mm. Pyrex test-tubes, plugged with cotton, and autoclaved at 15 pounds pressure for 30 minutes.

To sterile mineral base sterile quantities of the following are added
as aseptically: (a) 30 per cent glucose 0.2 cc., (b) 0.1 per cent ferric citrate 0.1 cc., (c) 0.5 per cent magnesium sulfate 0.1 cc., (d) 10 per cent acid-hydrolyzed casein 0.1 cc.

An alternative method is to prepare a mixture of (a), (b), (c), and (d) in the proportions specified and to sterilize it by passage through a Seitz filter, 0.5 cc. of the filtrate being added per test-tube containing the mineral base. Graded amounts of nicotinic acid or of the sample to be tested are then introduced and the volume is made up to 10 cc. with distilled water. The tubes are then ready for inoculation.

If there is reason to suspect the presence of sulfonamides in the test sample, addition of 0.1 cc. of a 0.1 per cent solution of p-aminobenzoic acid per test-tube of the above medium is recommended.

Acid-Hydrolyzed Casein—This is the only constituent of the culture medium which requires special preparation. Both technical and “vitamin-free” casein can be used. The method of preparation is the following. 50 gm. are hydrolyzed with 250 cc. of 25 per cent sulfuric acid. The mixture is autoclaved for 10 hours at 15 pounds pressure, and the sulfuric acid is then removed with baryta. Excess of barium is carefully removed with sulfuric acid (pH 4 to 5). Nicotinic acid which may be present in the hydrolysate must be completely removed. This is accomplished by stirring the hydrolysate with 2 per cent norit for 30 minutes and filtering. If the casein is of a technical grade, repetition of this procedure is generally necessary. Adsorption may be considered to be complete if the Proteus shows scanty growth in the medium in the absence of nicotinic acid, and full growth on the same medium after addition of 0.15 γ of nicotinic acid per 10 cc. When this is the case, the hydrolysate is brought to pH 7.0 with concentrated sodium hydroxide solution and is diluted or concentrated to give a final concentration of 10 per cent.

Procedure

Inoculum—A small number of Proteus organisms are removed from the surface of a 24 hour agar slant culture with a wire loop, care being taken to avoid direct contact with the agar. The loopful of Proteus is suspended in enough sterile 0.9 per cent sodium chloride solution to yield a just barely turbid suspension; 1 cc. of the latter is added to 100 cc. of saline. 2 drops (about 0.1 cc.) of a suspension obtained in this way are added to each assay tube. The inoculated tubes are incubated in a slanted position (angle, 10° to 15°) for 40 hours at 30°.

Standard Curve—Each set of assays is run together with a set of tubes containing per 10 cc. of medium 0.0, 0.005, 0.01, 0.02, 0.03, 0.04, 0.06, 0.08, and 0.1 γ of nicotinic acid. From the growth data a standard curve is constructed. The growth is followed turbidimetrically by means of a photo-electric colorimeter of the Evelyn type (Fig. 1).
Specificity—Nicotinamide and nicotinic acid are equally effective. Recovery experiments gave satisfactory results, the maximal error not exceeding ±10 per cent. Other nicotinic acid analogues were unavailable to this laboratory and were, therefore, not assayed. Pelczar and Porter (3), however, found that ethynicotinamide, diethylnicotinamide, ethyl nicotinate, and nicotinylglycine were all effective for many strains of Proteus X19, whereas picolinic acid, quinolinic acid, trigonelline, pyridine betaine-3-carboxylic acid, and α-aminopyridine-3-carboxylic acid were inactive.

Vitamins other than nicotinic acid were found not to affect the growth curve when added in various concentrations either separately or in combinations. The following substances were tested: riboflavin, thiamine, biotin, pyridoxine, glutamine, pantothenic acid, and p-aminobenzoic acid.

Reliability of Method—The method presented is at least 10 times as sensitive as those of other workers (Lwoff and Querido (2), Isbell, Woolcy, Butler, and Sebrell (4), and Snell and Wright (1)). The question arises whether it is as reliable as the most sensitive of these methods, that of Snell and Wright (1). Unfortunately, direct comparison of these methods was not feasible; however, we applied our procedure to standard materials and compared the results with findings of other authors as reported in the

Fig. 1. Response of Proteus HX19 to nicotinic acid. The ordinate scale represents photometer readings; the abscissa, micrograms of nicotinic acid per 10 cc. of medium.
literature. Recently Stokes, Gunness, and Foster (5) reported vitamin values for various ingredients of microbiological culture media prepared by Difco. They determined nicotinic acid by the method of Snell and Wright. Table I compares their results with those obtained on the same materials with our own method. It can be seen that the results are almost identical.

**Table I**

Comparison of Results of Nicotinic Acid Assays of Ingredients of Bacteriological Media, by Different Methods

<table>
<thead>
<tr>
<th>Material (Difco)</th>
<th>Snell and Wright method*</th>
<th>Authors' method</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>γ per gm.</td>
<td>γ per gm.</td>
</tr>
<tr>
<td>Beef extract</td>
<td>993</td>
<td>927</td>
</tr>
<tr>
<td>Peptone</td>
<td>29.4</td>
<td>30</td>
</tr>
<tr>
<td>Neopeptone</td>
<td>152</td>
<td>150</td>
</tr>
<tr>
<td>Proteose peptone</td>
<td>149</td>
<td>120</td>
</tr>
<tr>
<td>Tryptone</td>
<td>26.3</td>
<td>25.5</td>
</tr>
</tbody>
</table>

* From Stokes et al. (5).

**Table II**

Nicotinic Acid Content of Various Materials

The results are expressed in micrograms per 100 gm. or cc.

<table>
<thead>
<tr>
<th>Vegetables</th>
<th>Fruits</th>
<th>Miscellaneous</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Beet</td>
<td>135</td>
<td>Soy bean, flour</td>
</tr>
<tr>
<td>Radish</td>
<td>170</td>
<td>Standard &quot;</td>
</tr>
<tr>
<td>Cucumber</td>
<td>210</td>
<td>Beans, green</td>
</tr>
<tr>
<td>Pepper, green</td>
<td>315</td>
<td>Rice flour</td>
</tr>
<tr>
<td>Carrot</td>
<td>320</td>
<td>Bakers' yeast</td>
</tr>
<tr>
<td>Marrow</td>
<td>320</td>
<td>Blood 1 (human)</td>
</tr>
<tr>
<td>Cabbage</td>
<td>370</td>
<td>&quot; 2 &quot;</td>
</tr>
<tr>
<td>Eggplant</td>
<td>520</td>
<td>Urine 1 &quot;</td>
</tr>
<tr>
<td>Potato</td>
<td>735</td>
<td>&quot; 2 &quot;</td>
</tr>
<tr>
<td>Sweet potato</td>
<td>375</td>
<td>&quot; 2 &quot;</td>
</tr>
</tbody>
</table>

Determinations on Natural Products—The usual methods of extraction were employed. The test materials for the most part were extracted by being autoclaved at 15 pounds pressure for 30 minutes in a large excess of water (100:1). In certain cases boiling alkali or acid was tried, but yielded the same results as did extraction by water, an observation also made by Snell and Wright (1). For estimation of nicotinic acid in blood the samples
were treated according to Isbell et al. (4); 0.1 cc. of blood sufficed for a determination.

In order to obtain reliable results the samples are assayed simultaneously at three levels within the useful range and the mean of readings is taken.

Table II reports findings of nicotinic acid in various foodstuffs (local fruits and vegetables), blood, and urine.

**DISCUSSION**

The method described above is a modification of that of Lwoff and Querido (2). The principal changes introduced by us are the inclusion of casein hydrolysate and nitrate in the culture medium, and incubation of the assay tubes in a slanted position. By this means the range of the response is shifted and extended from 0.07 to 1.0 \( \gamma \) to 0.005 to 0.1 \( \gamma \) per 10 cc. of medium. The sensitivity is therefore 10-fold. The improved procedure permits estimation of the response of the cells in terms of turbidity without interference from the color or turbidity of the test material. Determinations carried out on standard materials have shown that our method yields the same results as that of Snell and Wright (1). The new procedure is, however, faster, and involves a test organism available in every bacteriological laboratory.

**SUMMARY**

1. A new method of determining nicotinic acid based on the use of *Proteus* HX19 is described.

2. The range of response of the new method is 0.005 to 0.1 \( \gamma \) per 10 cc. of culture medium.

3. Determinations of nicotinic acid in foodstuffs (local vegetables and fruits), blood, and urine are reported.

4. Merits of the new method, *viz*., sensitivity, simplicity, and speed relative to older methods, are discussed.

**BIBLIOGRAPHY**

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