A METHOD FOR THE DETERMINATION OF NICOTINIC ACID, NICOTINAMIDE, AND POSSIBLY OTHER PYRIDINE-LIKE SUBSTANCES IN HUMAN URINE*

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The discovery of nicotinic acid in rice bran (1) and in yeast (2, 3) directed attention of biochemists to the natural occurrence of this pyridinecarboxylic acid. Funk suggested that it had some relation to vitamin B1, but subsequent investigation showed that it made no part of vitamin B1 or B2. The discovery by Warburg and associates (4) that it was present as the amide in coenzyme and by von Euler et al. (5) that it was a part of cozymase revived interest in it and indicated that it was playing an important part in the respiratory metabolism of both animal and plant cells. Following these clues, Elvehjem and his associates (6) discovered that its administration would cure the disease of black tongue in dogs, and Spies, Cooper, and Blankenhorn (7) reported its favorable action in the treatment of human pellagra, an action confirmed by others (8).

Since there is no evidence that pyridine can be formed in the human body, it is evident that nicotinic acid or its amide must be an important article of human diet and that it is apparently necessary for the normal health of the mucosa of the mouth and intestine, nervous system, and skin, since these all are impaired in pellagra and improved by the ingestion of nicotinic acid or the amide. It must be necessary for the muscles also, and probably for the liver and some other organs, since it is an essential

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part of cozymase and of the coenzyme (Warburg et al.) of these tissues and organs.

These facts made it desirable to discover a method by which its presence in animal tissues, fluids, and secretions could be detected and the amount present determined. The pressing need for the discovery of a method making it possible to detect a prepellagrous condition, before the well marked skin and other changes were so developed as to make diagnosis easy, led to the first efforts being directed toward a method for measuring the amount in the urine both in normals and in subjects with dietary deficiency diseases, such as pellagra. Such a method was found, as announced in a preliminary communication, and showed at once that pellagrins when untreated, and normal people, when on a diet deficient in nicotinic acid, had no substances in the urine which would develop the color reaction of the method. They excreted no nicotinic acid conjugates, at least in a form which would give the reaction described herein.¹

An investigation of the literature revealed a characteristic color reaction for pyridine and certain of its homologues. It was first described by Vongerichten (10), and was developed by Zincke (11, 12), König (13), Reitzenstein (14), Baumgarten (15), and others. The reaction involved the addition of 2,4-dinitrochlorobenzene to the tertiary nitrogen of the pyridine ring. This addition product was decomposed by amines, such as aniline, or by alkali hydroxides, to yield a deeply colored product. Further study by König showed that cyanogen bromide gave a similar addition product with pyridine and that decomposition by aniline or by alkalies yielded similar soluble colored products. The opinions in regard to the identity of these colored substances have been controversial, but the most reliable evidence indicates that the derivatives are open chain compounds with conjugated double bonds, and that they are related to glutaric dialdehyde (15). It was decided to try this reaction upon nicotinic acid, the β-mono-carboxylic acid of pyridine, and upon certain other related compounds such as the true salts and conjugated derivatives, and nicotine itself, since a study of the literature did not reveal any

¹ After this paper had been submitted for publication, practically the same method was published by Karrer and Keller for the determination of nicotinamide in cozymase (9).
application of this reaction to substances other than the pyridine-like bases.

It was soon observed that free nicotinic acid, the sodium salt, and the amide would not give a color with 2,4-dinitrochlorobenzene and alkali at the temperatures 60-70° and in conditions used in previous work with pyridine and other like bases as described by the authors cited. However, it was noticed that in the absence of a solvent, nicotinic acid and the above reagent fused at a low temperature, between 85-100°, depending upon the proportion of each. After this fusion, the addition of an alcoholic solution of alkali to the residue gave a brilliant purple color. Since this color was similar to the purple pyridine derivative, it was thought at first that the color was due to some pyridine formed by the fusion. Subsequent work, however, showed this to be improbable, since cyanogen bromide, which reacted with the pyridine, did not yield a colored derivative of nicotinic acid under any of the conditions which have been tried. Furthermore, in an attempt to extract the colored compounds with organic solvents, it was observed that although the colored pyridine derivative was easily extracted with isopropyl and other higher alcohols from the alkaline liquid, the purple derivative of nicotinic acid had a greater affinity for the aqueous alkali layer and would exhibit a partition in the two solvents dependent upon the concentration of the alkali present. The greater the concentration of the alkali, the less went into the alcohol. This gave evidence that the colored substance was acidic and that the carboxyl group remained intact during the reaction with 2,4-dinitrochlorobenzene. The two derivatives, i.e. from pyridine and from nicotinic acid, were evidently not identical. Hence the color was not due to pyridine formed from nicotinic acid as had at first been supposed.

At present, after a study of the factors necessary to produce this color on a quantitative basis, it is believed that an addition compound similar to that described by Zincke (11) for pyridine is formed when an alcoholic (95 per cent) solution of nicotinic acid and 2,4-dinitrochlorobenzene is evaporated to dryness at 95-105°. The fusion and reaction are complete within 10 minutes.

Since the paper was submitted, the successful use of cyanogen bromide to produce a colored derivative of nicotinic acid has been described by Swaminathan (16).
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at 100–105° after the solvent is evaporated. The addition product is yellowish but develops red color on the addition of alkali. When only a slight excess of the reagent, 2,4-dinitrochlorobenzene, is used, and the colored solutions are diluted to a fixed volume with more of the cold alcoholic sodium hydroxide, the depth of color produced is proportional to the quantity of nicotinic acid or amide taken. The amount of light absorption of the color can then be successfully reproduced for different concentrations of nicotinic acid in the range of 0.1 to 0.5 mg. on the Sheard-Sanford photelometer, and the amount of nicotinic acid or the amide thus determined. In this photelometer colored filters, a photoelectric cell, and a sensitive microammeter are used. Once calibrated for the colored substance under investigation this photelometer is very convenient. Still smaller amounts could no doubt be determined, but with less accuracy with this instrument.

Nicotinic acid itself is not the only substance giving the color. Other compounds related to it which have been tested are nicotinamide, sodium nicotinate, coramine (diethyl nicotinamide), trigonellin, picolinic acid, α-picoline, and nicotine. The sodium salt of nicotinic acid gives a purple color as the free acid does. The betaine, trigonellin, gives no color; i.e., a negative test. The amide gives a Burgundy red, a yellow-red in dilute solutions; but the substituted amide, coramine, again gives a purple color. Why the substitutions of ethyl groups for the hydrogen atoms of the amide nitrogen should shift the absorption toward the red is not yet understood. Picolinic acid, the α-monocarboxylic acid of pyridine (m.p. 136°, uncorrected), does not give a colored product under the same conditions. However, a color does develop after the acid is heated with the reagent over a free flame. The odor of pyridine is then apparent. The closely related base, α-picoline, i.e. α-methylpyridine, gives a purple color under the same conditions as pyridine (4) or nicotinic acid, so the “hindrance” to the reaction in picolinic acid due to the carboxyl group in the α position is of interest. The picolinic acid-glycine conjugate, kindly supplied us by Mr. Birnbaum, also failed to develop color under the conditions of the nicotinic acid reaction. Nicotine

Obtained from the Central Scientific Company of Chicago.

This fact was communicated to us by Mr. S. M. Birnbaum who is working on the excretion of picolinic acid.
itself, however, gives a purple color. This fact should be useful, since tests for this alkaloid are not numerous.

The reaction is, then, sufficiently selective and delicate to enable one to make a quantitative estimation of very small amounts of nicotinic acid or amide in the absence of other pyridine bases.

The method developed was the following: The photelometer is first calibrated by the use of solutions of the acid or amide of known strength, amounts of solution being taken containing
between 0.1 and 0.5 mg. The calibration curves thus obtained with the green filter of the photometer are given in Fig. 1.

It will be seen from Fig. 1 that for quantities between 0.1 and 0.5 mg. the points when plotted on logarithmic paper with the per cent of transmission of the light, as read from the photometer, as ordinates, fall along straight lines, but that the line of the amide differs from that of the acid.

The standard solutions used to determine the points in Fig. 1 were treated in the following way: 1, 2, 3, and 5 cc. samples of aqueous solutions containing 10 mg. of nicotinic acid in 100 cc., and others containing 10 mg. of nicotinamide in 100 cc., were pipetted into 30 cc. beakers; the solutions were evaporated cautiously at 80-100° or on the water bath and were removed as soon as the solvent had evaporated. To each beaker was then added 1.0 cc. of a 1 per cent solution of 2,4-dinitrochlorobenzene in 95 per cent alcohol. The beakers were allowed to stand for 1 hour or more at room temperature and then evaporated to dryness at 100-105° and heated 10 minutes between these limits of temperature in order to fuse the reagent with the substances to be determined. An excess of the dinitrochlorobenzene must be avoided, as it develops a yellow or orange color by itself when made alkaline. A small excess in the presence of 0.1 to 0.5 mg. of nicotinic acid or nicotinamide is permitted as the color is then too faint to affect the result significantly, but a blank must be run in order to make sure no significant color is produced by the reagent itself in the amounts added. The 1 cc. of the reagent used by us is sufficient for developing maximum color in 0.1 to 0.5 mg. If more than this be taken, more reagent is needed. The heating should not be higher than 105°, as a brown color may develop at higher temperatures. The amide requires a longer heating to develop maximum color than does the acid. The amide should be heated for 10 minutes between 100-105°, while 5 minutes are enough for the acid. After this fusion, the fusion mixture is cooled to 25°, dissolved in 10 cc. of a clear solution of 0.1 per cent NaOH in 95 per cent alcohol which has been cooled to 10°, and filtered at once.

To test the accuracy of the method when the acid and amide

5 Karrer and Keller avoided this difficulty by extracting the excess reagent from the melt with ether. This is an improvement on our method.
are mixed with the organic and inorganic matter of urine, urine was decolored by filtering through charcoal and then treated with Lloyd's reagent and filtered. Known quantities of the amide or acid were then added to this urine and the amounts determined from the calibration curves made with the pure substances in water. Table I gives the results.

From these results, it is seen that when the procedure is carefully performed to prevent loss of material, and the reaction completed at temperatures consistent with minimum loss and maximum color development, the determination is accurate within 5.0 to 10 per cent. Errors in the determination of aqueous solutions of the pure substances may be caused by incomplete reaction and possibly slight losses by vaporization. These, however, can be reduced to a minimum by care.

The next step was to add the known quantities of nicotinic acid or amide to fresh urine, before its decoloration, to see how accurately the amounts could be determined. Table II, containing the results, shows that both the acid and amide may be accurately determined within about 10 per cent when thus added. Furthermore, neither the acid nor the amide was absorbed by the charcoal used for decoloration of the urine. The per cent of error of Table II is calculated from the average of two or more determinations in each case.

### Table I

<table>
<thead>
<tr>
<th>Substance</th>
<th>Amounts determined (average of three or more readings)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1 cc.</td>
</tr>
<tr>
<td>Nicotinic acid</td>
<td>mg.</td>
</tr>
<tr>
<td>Solution in water, 5.5 mg. per 100 cc.</td>
<td>0.053</td>
</tr>
<tr>
<td>Standard solution of acid in treated urine, 10 mg. per 100 cc.</td>
<td>0.109</td>
</tr>
<tr>
<td>Nicotinamide</td>
<td>mg.</td>
</tr>
<tr>
<td>Solution in water, 3.6 mg. per 100 cc.</td>
<td>0.038</td>
</tr>
<tr>
<td>Standard solution of nicotinamide in treated urine, 10 mg. per 100 cc.</td>
<td>0.094</td>
</tr>
</tbody>
</table>
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Since the color is not permanent but slowly fades after filtration, the influence of the time factor was studied. The results (Table III) show that the maximum color slowly develops in the first 3 to 5 minutes and then fades, but in from 5 to 15 minutes the

**Table II**

*Determination of Nicotinic Acid and Nicotinamide Added to Untreated Urine*

<table>
<thead>
<tr>
<th>Untreated urine + nicotinic acid</th>
<th>Conjugate present normally</th>
<th>Nicotinic acid added</th>
<th>Total present</th>
<th>Total determined</th>
<th>Error</th>
<th>per cent</th>
</tr>
</thead>
<tbody>
<tr>
<td>cc.</td>
<td>mg.</td>
<td>mg.</td>
<td>mg.</td>
<td>mg.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>0.016</td>
<td>0.106</td>
<td>0.122</td>
<td>0.12</td>
<td>-1.6</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>0.032</td>
<td>0.212</td>
<td>0.244</td>
<td>0.244</td>
<td>0.0</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>0.048</td>
<td>0.318</td>
<td>0.366</td>
<td>0.326</td>
<td>-10.9</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Untreated urine + nicotinamide</th>
<th>Nicotinamide added</th>
<th>mg.</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.016</td>
<td>0.057</td>
</tr>
<tr>
<td>2</td>
<td>0.032</td>
<td>0.114</td>
</tr>
<tr>
<td>3</td>
<td>0.048</td>
<td>0.171</td>
</tr>
</tbody>
</table>

**Table III**

*Stability of Color at Successive Time Intervals*

<table>
<thead>
<tr>
<th>Substance added</th>
<th>Per cent light transmission</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>30 sec.</td>
</tr>
<tr>
<td>1 cc. nicotinic acid, 0.1 mg. per cc. water</td>
<td>86</td>
</tr>
<tr>
<td>1 cc. nicotinamide, 0.1 mg. per cc. water</td>
<td>76</td>
</tr>
<tr>
<td>3 cc. nicotinic acid, 0.106 mg. per cc. urine</td>
<td>51</td>
</tr>
<tr>
<td>3 cc. nicotinamide, 0.057 mg. per cc. urine</td>
<td>53</td>
</tr>
</tbody>
</table>

per cent of transmission as read with the filter remains very constant.

*Method and Results When Applied to Urine*—In the urine of people who have ingested nicotinic acid or amide there might be trigonellin, nicotinic acid, nicotinic acid-glycine conjugate, and nicotinamide. It has been shown that in dog urine the acid,
the glycine conjugate, and trigonellin are present after ingestion of considerable quantities of nicotinic acid. In rabbit urine only the glycine conjugate and free acid are present (17, 18). Of these substances trigonellin, which is known to occur sometimes in human urine, gives no color reaction by our procedure. The acid, if present, gives a purple, the amide a red, and the color of the glycine conjugate has not been determined but probably would be a red. Actually we have found that if not more than 100 mg. of nicotinic acid are ingested, the color given by the urinary constituent is red as with the amide; but if large single doses of 100 to 500 mg. are ingested repeatedly, the color of the urinary constituent for the next few hours after each such dose is the purple color of nicotinic acid or its sodium salts. We interpret this to mean that in the first case either the amide or a conjugate is present. And only when large doses are given does the free acid or a salt appear in the urine.

The method worked out for the urine was as follows: 15 cc. of urine are decolored by boiling with 0.1 to 0.3 gm. of Darco charcoal (Coleman and Bell). Crude vegetable charcoals do not absorb nicotinic acid (1). The solution when filtered should be clear and colorless. 3 cc. samples are then measured into 30 cc. beakers and are evaporated just to dryness in an oven at 80-100°. When dry, the beakers are removed and 1 cc. of an alcoholic solution of 2,4-dinitrochlorobenzene (1 gm. in 100 cc. of alcohol) is added. 10 mg. of 2,4-dinitrochlorobenzene are sufficient theoretically for development of maximum color with about 5 mg. of both acid and amide. After the samples have stood at room temperature with the reagent for 1 to 3 hours to allow for an intimate mixture, they are again evaporated to dryness and heated 10 minutes at 105°. The temperature must not exceed 105°. 10 minutes of heating at 105° gives the maximum color. Samples which are browned by overheating should be discarded.

The beakers are then cooled to 25°, or lower, and 10 cc. of a clear, cold (10°) solution of 0.1 per cent sodium hydroxide in 95 per cent alcohol are added and the residue stirred from the bottom. The colored solution is then made up just to 15 cc. by addition of about 5 cc. more of cold alcoholic sodium hydroxide. Since these alcoholic solutions are not clear they must be quickly filtered while cold. If for any reason the filtration is slow, it may be
facilitated by suction or centrifugation, but we have employed simply gravity, the solution running readily through the filter paper.

The quantitative reading must be made quickly (within 15 minutes) after the color has developed, although the chilled alcoholate retards the fading.

The colored but clear solution is transferred as soon as filtered to the photelometer cell, and with a green filter the per cent of transmission of green light by the solution is read from the photelometer scale.

A blank determination of the same quantities of the reagent with alkali alone should be run when fresh solutions are prepared, as too concentrated alkali produces a decided yellow color with any excess of the 2,4-dinitrochlorobenzene.

If the urinary color is a red like that caused by the amide, as it usually is, the amount is read from the amide curve and recorded as an equivalent amount of nicotinamide.

If it be a clear purple like that of nicotinic acid, the amount is read from the nicotinic acid curve.

If, however, as may happen after large doses of nicotinic acid have been ingested, the color is a mixture of red and purple, only an approximation or a semiquantitative estimation can at present be obtained with the photelometer. This happens, however, only in specially treated patients or when nicotinic acid has been ingested for experimental purposes. Ordinarily the reading is from the amide curve. With the use of special filters the amount of each reacting substance might be estimated separately but with the three filters of the photelometer at present available that is not possible.

**Results**

No red, or purple, color-producing substances have been found to be present in the urine in all the cases of pellagrins in relapse or in the acute disease so far examined. None of these pellagrins has had enough nicotinic acid or its conjugates in the urine to develop color. We also found little or no color to develop in urine tests from normal persons who were maintained for some time on a strictly controlled "pellagra-producing" diet, a diet such as is usually eaten by pellagrins. The constituents of such a diet are
described later. However, the urine from a person suffering from inanition and dehydration, which came to our attention, showed a weak but positive reaction for nicotinamide, although her mouth was sore, like that of a pellagrin. The patient died before an accurate diagnosis could be made.

Several hundred urine specimens have been examined by this method both in normal persons after oral and intravenous administration of various amounts of nicotinic acid and its salts, and in disease. The urines from three pellagrins in relapse gave negative tests before treatment, but both they and persons on a pellagra-producing diet (i.e., a limited diet without nicotinic acid-containing food) showed a quick response to nicotinic acid therapy by increased excretion of color-producing substances in the urine; but the excretion reverted to the low level normal for the individual on such a diet within 24 hours of ceasing ingestion of nicotinic acid.

The amount of nicotinic acid or its amide excreted daily by so-called normal persons on usual diets has varied considerably, but the value as determined by the method on the urines of students, internes, and patients not ill of pellagra lay between 20 and 50 mg. daily.

After nicotinic acid was given by mouth, the quantity in the urine was much increased, but never was equal to the amount ingested. How much was converted into trigonellin has not been determined.

The attempt is being made to apply the reaction to blood filtrate, spinal fluid, gastric juice, and tissue hydrolysates. The results are not yet satisfactory, for although it is possible to see a deepening in the color developed in samples of the blood protein-free filtrate taken after nicotinic acid therapy as contrasted with the pretreatment blood, the color is masked by an intense yellow and is different from that of any of the pure substances so far examined.

We have also tested in a preliminary way the alcohol-water (70 per cent) extracts of foodstuffs, but only successfully when the extract was sufficiently light in color. A qualitative test on active liver concentrates, i.e. liver concentrates curative for pellagra and pernicious anemia, was positive, but not quantitatively satisfactory because of the natural color of the material.
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In the case of dried brewers' yeast, hydrolyzed and unhydrolyzed samples gave small positive qualitative tests. Similar extractives of hydrolyzed and unhydrolyzed foodstuffs used as a pellagra-producing diet gave negative tests. Extractives of hominy grits, corn-meal, white flour, sweet potato, cabbage, spinach, pork fat, and sugar, foods constituting such a diet, all were negative. While these results are interesting as showing an important difference between the above foodstuffs and certain natural foods which contain pellagra-preventive substances, such as liver and yeast, the work is incomplete and the methods must be improved. Some of the disturbing pigments can no doubt be removed by proper solvents.

DISCUSSION

Until the present work was carried out there have been only two pyridine compounds known to occur in normal urine, namely, trigonellin and methyl pyridinium hydroxide. These substances already have the valences of the nitrogen saturated and this seems to prevent any addition product, such as is necessary to the first step of this reaction. For this reason, and because trigonellin has been found on trial not to give the reaction, we believe that neither of these substances will develop color under the conditions of this reaction, although we have not tested the N-methyl pyridinium hydroxide. Hence, a positive test, such as we have obtained, is fairly certain evidence of the presence of nicotinic acid, nicotinamide, certain of its salts, or, possibly, of its conjugated derivative, in normal human urine. Thus a third pyridine compound, nicotinic acid, is indicated as a constituent of normal human urine.

We conclude, after the examination of many specimens, that a failure of the urine to yield a positive reaction by this test indicates a serious deficiency of nicotinic acid or amide in the individual's diet. Hence this may prove to be a diagnostic sign of a prepellagrous condition, of value in those early cases in which the skin and mouth lesions are not yet well developed.

SUMMARY

1. A color reaction has been described for the detection of nicotinic acid, nicotinamide, substituted amides of nicotinic acid, and certain salts of the acid in urine and other fluids.
2. Colors are developed with nicotinic acid, nicotinamide, sodium nicotinate, and diethyl nicotinamide (coramine); no color is developed by trigonellin and picolinic acid.

3. By the application of this method to human urine it has been found that individuals on a normal diversified diet excrete daily color-producing substances equivalent to 20 to 50 mg. of nicotinic acid or its conjugates. We believe these substances to be nicotinic acid conjugates.

4. Pellagrins in relapse, or in the first acute disease, or normal individuals on a diet such as pellagrins usually consume, excrete little if any color-producing nicotinic acid derivatives.

5. Extracts of various foods, such as liver, yeast, and some others known to be pellagra-preventive foods, are shown to contain substances giving this nicotinic acid reaction, but the method does not yet permit a quantitative determination owing to the presence of other disturbing pigments.

6. The findings suggest that this method is useful as a confirmatory test of the pellagrous and prepellagrous states and for studying the excretion of nicotinic acid and other closely related substances which give colored products in vitro with 2,4-dinitrochlorobenzene, when alkalinized.

7. Since nicotinic acid or the amide appears to be one of the vitamins, the method enables one to estimate the amount of this vitamin in urine.

8. Work is being continued on the estimation of the quantity of nicotinic acid and amide in different tissues and foods.

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