CCXXI. QUANTITATIVE ESTIMATION OF NICOTINIC ACID IN URINE

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SINCE Warburg & Christian [1934] and von Euler *et al.* [1935] have shown that coferment and cozymase contain nicotinamide, and particularly since the effect of nicotinic acid in the treatment of the disease of blacktongue in dogs [Elvehjem *et al.* 1937] and pellagra in man [Fouts *et al.* 1938] became established, the question of the excretion in the urine of nicotinic acid or its derivatives has attracted considerable attention.

Vilter *et al.* [1938] have devised a colorimetric reaction "for the determination of nicotinic acid, nicotinamide and possibly other pyridine-like substances in human urine" and state: "by the application of this method to human urine it has been found that individuals on a normal diversified diet excrete daily colour-producing substances equivalent to 20 to 50 mg. of nicotinic acid or its conjugates. We believe these substances to be nicotinic acid conjugates."

This method which is based on the colour reaction of pyridine with 2:4-dinitrochlorobenzene and alkali hydroxide [Vongerichten, 1899], is, however, rather intricate and tedious. Moreover, decoloration is performed with charcoal, which seems rather ill-suited for the purpose, since the usual kind of charcoal at any rate adsorbs nicotinic acid [Shaw & Macdonald, 1938; Bandier & Hald, 1939; Porjé, 1939]. Covello [1938] has also employed Vongerichten's method with the modification that he uses 3:4-dinitrochlorobenzene instead of 2:4-dinitrochlorobenzene.

Quite recently, Porjé [1939] has described another colorimetric method for the estimation of nicotinic acid (pyridine derivatives) in urine based on König's [1904] observation that a colour reaction ensues when pyridine reacts with CNBr and a primary or secondary aromatic amine (e.g. aniline). With this method it has been found that normal subjects excrete 1.2 to 20 mg. nicotinic acid in 24 hr. Porjé makes the reservation that the colour reaction developed in analysis of the urine may be due to some other derivative of pyridine, though he does not subject the matter to closer investigation.

In both methods the colour produced by nicotinic acid is very unstable; this makes them unsuitable, at least for serial work. Moreover, Vilter *et al.*, Covello and Porjé perform the analysis without subjecting the urine to hydrolysis, which, as will be shown below, is not without importance.

In this paper, a description will be given of a method for the quantitative estimation of nicotinic acid (—derivative) in urine, involving the colorimetric reaction with CNBr and metol described in a previous article by Bandier & Hald [1939].

As attempts at decoloration (of the urine) with charcoal, fuller's earth and heavy metal salts have been unsuccessful, it has been necessary to introduce a blank value for the original colour of the urine.

If the reaction is performed on untreated urine, added nicotinic acid is not recovered quantitatively. This means that urine contains substances that mask

the colour reaction. Better, although not entirely satisfactory, results are obtained if the reaction is performed in the presence of KH_2PO_4 . However, added nicotinic acid is recovered quantitatively if the urine is first saturated with NaCl and the nicotinic acid extracted with a large excess of acetone; the extract is transferred to water containing KH_2PO_4 , and the colorimetric reaction carried out on this solution.

Saturation with NaCl causes flocculation of the precipitate formed on adding acetone; without NaCl, the precipitate adheres in a slimy layer to the sides of the flask. The necessity of using KH_2PO_4 has been explained in a previous publication [Bandier, 1939]. As some urinary pigment also is extracted by acetone, it is essential to include a blank value for the original colour.

The pH decreases to 2–3 after metol is added. The same value also is obtained by adding about 0.18 ml. 2N H_2SO_4 . On examining a series of urines, it has been found that addition of this amount of H_2SO_4 after treatment with CNBr, has very little influence on the original colour (blank value).

Technique

5 ml. urine and 2 g. NaCl are mixed in a 50 ml. graduated flask with a glass stopper and 45 ml. acetone are added from a burette. The mixture is shaken thoroughly and centrifuged for about 2 min. in a 50 ml. tube capped with thin parchment paper. 20 ml. of the acetone extract (representing 2 ml. urine) are then pipetted off, and placed in a 100 ml. round-bottomed boiling flask together with about 3 ml. distilled water. The acetone extract is evaporated *in vacuo* with continual shaking (bath temperature 40–50°).

After evaporation of the acetone, the contents are transferred quantitatively with the aid of 5 ml. 2% KH_2PO_4 to a graduated 20 ml. flask and heated for 5 min. on a water bath at 75–80°. Then 1 ml. 4% freshly prepared aqueous CNBr is added, and after 5 min. on the water bath the flask is cooled under the tap to about room temperature. 10 ml. of a fresh aqueous saturated solution of metol (about 5%) are added and the total volume made up to 20 ml. with distilled water. After 1 hr. at room temperature, protected from light, the colour developed is read off in a Pulfrich photometer (filter S 43) against a solution prepared simultaneously, containing the same amounts of CNBr, metol, KH_2PO_4 and distilled water to make up the volume to 20 ml.

For the blank, a second portion of 20 ml. of the acetone extract is treated as above, 0.18 ml. 2N H_2SO_4 being added instead of metol. The colour is measured in the Pulfrich photometer (filter S 43) with distilled water in the other cell. The value found is reduced to correspond to the same thickness of cell as used in the first reading and subtracted from this. From the result and the coefficient of extinction determined on a standard solution (0.1 mg. nicotinic acid) the amount of nicotinic acid corresponding to the colour developed can be calculated. The blank value for untreated normal urine may amount to 6 times the value of the colour reaction proper; for urine subjected to alkaline hydrolysis, the blank value is about twice the value of the colour reaction.

As the method is based on a reaction that is characteristic of pyridine, the intensity of the colour cannot be accepted directly as a quantitative measure of nicotinic acid. Thus besides free nicotinic acid, the following pyridine derivatives at least are liable to be encountered: nicotinamide—free and in combination in cozymase and coenzyme [Warburg & Christian, 1936], nicotine (demonstrated in the urine of tobacco smokers by Helmer *et al.* [1939], methylpyridinium hydroxide [Kutscher & Lohmann, 1906] and trigonelline [Linneweh & Reinwein, 1932]. In this connexion it should be mentioned that Ackermann

[1912] found nicotinuric acid in the urine of a dog that had received nicotinic acid by mouth.

Methylpyridinium hydroxide and trigonelline give no coloration, nor presumably do coenzyme and cozymase, as the pyridine N atom is pentavalent in the two latter [von Euler & Schlenk, 1937] as well as in the two former substances. Moreover, it is stated by Warburg & Christian [1935] that active coferment yields no colour with CNBr and sulphanilic acid. Nicotine, nicotinamide and nicotinuric acid give the same yellow colour as nicotinic acid. In equimolar concentrations, the colour produced by nicotinamide is considerably stronger than that yielded by nicotinic acid, while nicotinuric acid, and particularly nicotine, cause feebler colorations. The relations are the following: nicotinic acid: 100; nicotinamide: 142; nicotinuric acid: 42; nicotine: 8.

A desirable first step in the analysis would be, therefore, to transform the derivatives into nicotinic acid. Coenzyme, cozymase and nicotinamide are easily hydrolysed by heating with strong (about *N*) acid or alkali. Nicotinuric acid, however, is uninfluenced by heating with about *N* HCl up to 1 hr. on a boiling water bath, but is easily decomposed in alkaline solution. Thus heating on a boiling water bath for 15–30 min. with *N* NaOH causes complete hydrolysis and liberation of nicotinic acid. (The investigations have been carried out on pure nicotinuric acid, synthetically prepared in this laboratory by Jens Hald.)

Trigonelline and methylpyridinium hydroxide are not affected by heating on a water bath with strong acid or alkali.

As a result of these findings, the analysis has been performed as follows: 15 ml. urine and 2 ml. 10*N* NaOH are mixed in a 20 ml. graduated flask which is closed with a wad of non-absorbent cotton wool and heated for 30 min. on a boiling water bath (which is sufficient to produce a maximal colour reaction subsequently). After cooling the solution, the *pH* is adjusted to about 5 by adding 36 % HCl (usually 1.6 ml. HCl), and the volume made up to 20 ml. with distilled water (the *pH* is determined with narrow strips of "Universalindicator-papier Merck"). After centrifuging, the analysis proceeds as described above. It follows that in calculating the amount of nicotinic acid, the degree of dilution of the urine must be taken into account.

By this procedure, the total amount of free and conjugated nicotinic acid is determined. Nicotine is also included, while methylpyridinium hydroxide and trigonelline give no colour. Similarly, investigations of the specificity of colorimetric reaction have shown that no colour is yielded by picoline or picolinic acid. Thus it seems that pyridine derivatives in which the pyridine nitrogen is pentavalent, and α -substituted derivatives are inactive in the colour reaction. When the analysis is performed with KH_2PO_4 , small amounts of pyridine yield no colour; large quantities (about 1 mg.), which cause the solution to smell strongly of pyridine, give a pale yellow colour which is inconstant and unstable. If KH_2PO_4 is not used, the colour developed is stronger, but it is still unstable and inconstant.

The colour developed is stronger when the urine is hydrolysed with HCl than it is after alkaline treatment; this enhanced colour reaction is not however due to nicotinic acid.

15 ml. urine are heated with 1.6 ml. conc. HCl on a boiling water bath for about 30 min. and the *pH* adjusted to about 5 with 2 ml. 10*N* NaOH. The analysis then proceeds as described above, partly with KH_2PO_4 and partly with 1 ml. 36 % HCl in the main analyses as well as in the blanks. The addition of HCl eliminates nicotinic acid from the colour reaction, as this substance yields no colour with CNBr and metol in the presence of free strong acid. Concurrently,

a second sample of urine is subjected to alkaline hydrolysis and processed partly with KH_2PO_4 and partly with HCl .

On carrying out the colour reaction after HCl has been added, it is advisable to heat and cool the main analysis and the blank together, as the remainder of the original colour of the urine turns darker on being heated with HCl and CNBr .

The results are shown in Table I.

Table I

	Colour-yielding substances equivalent to nicotinic acid, mg. per 100 ml.
1. Analysis of urine after acid hydrolysis and with the use of 5 ml. 2% KH_2PO_4	0.58
2. After acid hydrolysis and with the use of 1 ml. conc. 36% HCl	0.35
Difference between 1 and 2	0.23
3. Analysis of urine after alkaline hydrolysis and with the use of 5 ml. 2% KH_2PO_4	0.24
4. After alkaline hydrolysis and with the use of 1 ml. conc. HCl	0.00

It will be noticed that the increased power to produce colour induced by subjecting the urine to acid hydrolysis, is maintained when the analysis is performed in the presence of HCl (where nicotinic acid, as already mentioned, yields no colour).

The difference between the colour reactions of urine subjected to acid hydrolysis at the usual reaction and in a strongly acid solution, corresponds fairly well to the strength of the colour yielded by the sample of urine subjected to alkaline hydrolysis. In this sample, as in the case of pure nicotinic acid, the colour fails to appear in the presence of HCl .

RESULTS

Analyses of a series of daily urines from healthy adults on a normal mixed diet containing an adequate supply of meat have given the results contained in Table II.

Table II. *Nicotinic acid excretion by normal subjects on mixed diet*

No.	Diuresis ml.	Colour-yielding matter equivalent to nicotinic acid, mg. per 100 ml.		Total excretion of colour-yielding substances equivalent to nicotinic acid (after alkaline hydrolysis), mg.	Urine + 0.67 mg. nicotinic acid per 100 ml. Total nicotinic acid found (after alkaline hydrolysis) mg. per 100 ml.	Nicotinic acid recovered %
		Urine not subjected to hydrolysis	Urine subjected to alkaline hydrolysis			
1	850	0.14	0.23	2.0	0.89	98
2	830	0.09	0.29	2.4	0.93	96
3	1565	0.15	0.32	5.0	1.01	103
4	725	0.07	0.21	1.5	0.88	100
5	1430	—	0.27	3.9	0.92	97
6	1100	—	0.30	3.3	0.98	101
7	1550	—	0.23	3.6	0.91	101
8	1150	—	0.20	2.3	0.88	101
9	780	0.09	0.30	2.3	0.96	98
10	1070	—	0.37	4.0	1.05	102

In order to investigate the mode of excretion in healthy adults of pure nicotinic acid ingested by mouth, the following experiment was performed.

After securing a specimen of urine from the preceding 24 hr., 90 mg. nicotinic acid (3 tablets of 30 mg. taken with a large glass of water) were given on an empty stomach, and the urine collected first at hourly intervals and subsequently at intervals of several hours. An example is shown in Fig. 1.

Fig. 1. Diagram showing the excretion in the urine of nicotinic acid given by mouth. Analysis carried out on alkaline-hydrolysed urine.

The calculated amounts of nicotinic acid were recovered from urine subjected to alkaline hydrolysis. The excretion is largest during the first hour, but rapidly diminishes to the normal level in about 6 hr. In the case illustrated, 13.75 mg. nicotinic acid were excreted during the first 6 hr.; subtraction of the normal amount contained in the urine (determined by analysis of the urine passed before the ingestion of nicotinic acid), i.e. 1.25 mg., gives 12.5 mg.; this is 13.9% of the ingested amount.

On considering the results given in Table III of a series of analyses of urine passed by a normal person during the first hour after ingestion of pure nicotinic

Table III. *Analyses of urine passed by a normal subject after ingestion of nicotinic acid*

	Colour-producing substances equivalent to nicotinic acid in mg. per 100 ml.
Analysis of urine not subjected to hydrolysis	3.30
Analysis of urine subjected to acid hydrolysis	3.58
Analysis of urine heated with NaOH on a boiling water bath for 15 min.	6.02
Analysis of urine heated with NaOH on a boiling water bath for 30 min.	6.11
Analysis of urine heated with NaOH on a boiling water bath for 60 min.	6.09

acid by mouth, it seems probable that most of the nicotinic acid recovered from the urine was excreted as nicotinuric acid. It has been mentioned already, that this substance promptly liberates nicotinic acid on being subjected to alkaline hydrolysis, while it is not attacked by heating in the presence of acid under the given conditions. Computed for equivalent amounts, the coefficient of extinction of nicotinuric acid is $2/5$ of the coefficient of extinction of nicotinic acid.

Urine contains substances which mask the colour reaction; the masking is most conspicuous when a large amount of nicotinic acid is present, but is relatively insignificant for amounts approaching values in normal urines. This drawback has been obviated in the present method of analysis by extracting with acetone.

It is imperative, therefore, to perform the extraction with acetone in analysis of urine containing a large amount of nicotinic acid (e.g. in investigations on the excretion of nicotinic acid after ingestion of a certain amount). However, it is possible that the extraction may be omitted in analyses of urines from ordinary patients; the colour reaction could then be performed in the presence of KH_2PO_4 directly on the urine after alkaline hydrolysis. Nevertheless, the simplified method should not be used before reliable data on the variations in urines from normal persons are available.

SUMMARY

1. An account is given of a technique for the quantitative estimation in urine of nicotinic acid and nicotinic acid conjugates by the colorimetric method involving the use of CNBr and metol described in a previous paper.

2. Part of the nicotinic acid or its conjugates must be present in the urine in combined form, as the reaction increases two- or three-fold if the urine is first subjected to alkaline hydrolysis.

3. Acid hydrolysis of the urine liberates substances that yield a similar colour reaction which cannot however be due to nicotinic acid.

4. The urines of 10 normal persons have been analysed after alkaline hydrolysis; it was found that the colour-producing substances excreted in 24 hr. were equivalent to 1.5–5 mg. nicotinic acid.

5. After oral ingestion of 90 mg. nicotinic acid, 14 % is excreted, chiefly during the first hour.

6. After oral ingestion, part of the nicotinic acid is excreted in a combined form which is hydrolysed under the same conditions as is synthetic nicotinuric acid (nicotinyl glycine).

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Note added 1 November 1939. Further study has shown that in investigations on urines from ordinary patients, i.e. *without previous ingestion of free nicotinic acid or nicotinamide*, the colour reaction can be performed on 2 ml. of alkaline-hydrolysed urine after the addition of 5 ml. 2% KH_2PO_4 .

For the blank another portion of 2 ml. of the alkaline-hydrolysed urine is treated in the same way, 0.18 ml. 2*N* H_2SO_4 being added instead of metol.

Care must be taken that the patients do not smoke tobacco, as nicotine, like coramine (diethylnicotinamide) will give a slight colour reaction with CNBr and metol.