CXXXVII. QUANTITATIVE ESTIMATION OF NICOTINIC ACID IN BIOLOGICAL MATERIAL

By ERIK BANDIER

From the Biochemical Laboratory, Medicinalco, Ltd., Copenhagen S.

(*Received 11 May 1939*)

A COLORIMETRIC method involving the use of cyanogen bromide and metol for the quantitative estimation of nicotinic acid was described in a previous paper [Bandier & Hald, 1939]. The practical value of the method was shown by a series of analyses on yeast. The method is very accurate and the colour has the merit of remaining constant for several days. The intensity of the colour is directly proportional to the concentration of nicotinic acid.

I. *The specificity of the method*

The method is based on the observation of König [1904] that a colour reaction ensues when pyridine reacts with CNBr and a primary or secondary aromatic amine.

If the analysis is carried out in the presence of KH_2PO_4—as described below—no coloration ensues with small amounts of pyridine, nor will picolinic acid, α-picoline, trigonelline or methylpyridinium chloride cause coloration, even if heating with strong acid or alkali on a boiling water bath has first taken place. Larger amounts of pyridine (about 1 mg.) yield an inconstant and unstable pale yellow-red colour. Such a concentration of pyridine causes the solution to smell strongly of pyridine.

Like nicotinic acid, nicotinamide yields a clear yellow colour. By the present method of analysis, however, nicotinamide is hydrolysed to nicotinic acid; this happens in a few moments when it is heated with strong alkali or acid.

If KH_2PO_4 is not used, pyridine yields a deeper yellow-red colour which is also inconstant and unstable. Picoline gives a slight, almost negligible, coloration; the other compounds mentioned give none. The method thus appears to be fairly specific.

II. *Estimation of nicotinic acid in biological material*

The technique described in the above-mentioned paper for analysing dry yeast, is unsuitable for the determination of nicotinic acid in animal tissue (liver, muscle etc.). A method will now be described which on repeated trials has proved perfectly satisfactory for the estimation of nicotinic acid in animal tissues.

(1) *Technique.* The sample of tissue is minced, dried (in a dry current of air heated to 40°) and coarsely ground. The loss of weight is calculated. 5 ml. 4*N*·NaOH are run into a 10 ml. graduated flask with a glass stopper and 2·5 g. dried material are placed in the liquid with a glass spatula. The flask is closed with a wad of non-absorbent cotton-wool and placed in a boiling water bath for about 30 min., during which time it is shaken once or twice. This causes complete solution of the organic material. The mixture is cooled a little (not too much, as gelatinization then takes place) and 1 ml. conc. 36% HCl is added;

the stopper is tightly fitted and the flask turned upside down twice. After cooling for a moment, a further 0·8 ml. conc. 36 % HCl is added and the contents are shaken as before. As there is considerable formation of gas precautions must be taken to prevent the stopper from being blown out. After cooling to 20° distilled water is added to make the volume up to 10 ml. After thorough shaking the mixture is centrifuged for about 10 min. in an ordinary centrifuge tube. An almost clear brownish fluid and a supernatant compact layer are obtained. 1 ml. of the liquid, representing 250 mg. dried organic material, is transferred to another tube and 9 ml. acetone are added. The tube is carefully closed with a rubber stopper; the stopper must be of a material that is not attacked by acetone, otherwise a milky turbidity appears on transference of the acetone extract to water. After vigorous shaking the mixture is centrifuged for 3–4 min. Two layers are formed: a small (about 0·3 ml.) highly coloured very viscid aqueous phase and a clear, slightly coloured, layer of acetone. 4 ml. of the acetone layer (corresponding to 100 mg. dried material) and about 3 ml. distilled water are run into a round-bottomed flask; the acetone is evaporated with a water vacuum pump without other heating than the warmth of the hand. The contents are then quantitatively transferred with the aid of 5 ml. 2 % KH_2PO_4 to a graduated flask of 20 ml. capacity and heated for 5 min. in a water bath at 75–80°. 1 ml. 4 % freshly prepared aqueous CNBr is added and after standing 5 min. in the water bath, the flask is cooled under the tap to about room temperature. 10 ml. saturated (about 5 %) fresh aqueous metol are added and the volume is made up to 20 ml. with distilled water. After standing 1 hr. or more at room temperature protected from light, the colour developed is read off in a Pulfrich photometer (filter S. 43) with a blank (prepared simultaneously) containing the same amounts of CNBr, KH_2PO_4, metol and distilled water to make the volume up to 20 ml.

The solution obtained by transference of the acetone extract to water is not quite colourless; the degree of coloration depends on the organ examined. Therefore, a second portion of 4 ml. of the acetone layer used for the analysis is taken and treated as above (transference to water + 5 ml. 2 % KH_2PO_4—heating with CNBr), but instead of metol, distilled water is added to make up the volume to 20 ml. The colour is measured in the Pulfrich photometer (filter S. 43) with distilled water in the other cell and the value found is subtracted from the first reading. From this result and the coefficient of extinction determined in a standard solution (0·1 mg. nicotinic acid), the amount of the nicotinic acid in the dried or fresh organic material can be calculated.

(2) *Comments.* Added nicotinic acid is recovered quantitatively, which proves the reliability of the method. The time during which the material is subjected to hydrolysis is ample; periods varying from $\frac{1}{4}$ to 2 hr. have yielded identical results. If the hydrolysis is carried out first in alkali and then in acid, the result is the same as when alkaline hydrolysis only is used.

After heating the tissue with 5 ml. 4*N* NaOH and adding 1·8 ml. 36 % HCl the reaction is distinctly acid (pH = 3–4). Closer study has shown that a quantity of HCl equivalent to the amount of NaOH used is insufficient, i.e. a certain excess of HCl must be added. The presence of greater quantities of HCl (as much as 2 ml. 36 % HCl) has no influence on the result of the analysis. If KH_2PO_4 is omitted the analysis will give lower values. The amount used (5 ml. 2 % KH_2PO_4) has proved adequate. To obtain a correct reading in the Pulfrich photometer the extinction must not exceed 0·50; the size of the cell must, therefore, be chosen with this in view. The samples of tissue from various organs give blanks representing 5–25 % of the values of the actual analytical results.

The organic material is dried in order to minimize the volume of the reagents; the method may of course be adapted to direct analysis of fresh material.

(3) *Analysis of small amounts of material.* When only a very small amount is available, the analysis may be carried out as follows: 0·5 ml. 4*N* NaOH and 250 mg. dried organic material are placed in a graduated centrifuge tube. After closing the tube with a wad of non-absorbent cotton-wool the contents are heated on a boiling water bath for about ½ hr. (the tube is shaken once or twice to ensure complete solution of the material). 0·18 ml. 36% HCl is added; after shaking and cooling distilled water is run in to make up the volume to 1 ml.; 9 ml. acetone are added and the analysis proceeds as before.

On transference of the acetone extract to water, a rather marked turbidity appears which disturbs the photometric reading; the former method involving the use of 2·5 g. dried substance has therefore been adopted as the normal procedure. Identical results can be obtained by the two methods.

(4) *Examples.* Pig kidney, wt. 189 g., dry wt. 37 g., i.e. dry wt. = 19·6% of wet wt. 2·5 g. dry wt., 5 ml. 4*N* NaOH and 1·8 ml. 36% HCl are used. 5 ml. 2% KH_2PO_4 are employed for the colour reaction.

	mg./100 g.
Analysis: the extinction is 0·363 in 1 cm. layer.	
Blank: extinction 0·215 in 5 cm. layer.	
0·1 mg. nicotinic acid: extinction 0·444 in 0·5 cm. layer.	
The extinction of nicotinic acid in 100 mg. dry renal tissue is therefore $0{\cdot}363 - (0{\cdot}215 \div 5) = 0{\cdot}320$.	
This corresponds to a nicotinic acid concentration of	36·0
Calculated for fresh material this is	7·09
If 1·58 ml. 36% HCl (which are equivalent to the amount of NaOH) are used, the result is	29·5
With 2 ml. 36% HCl the result is	35·5
i.e. the same as when 1·8 ml. are used.	
If KH_2PO_4 is omitted the result is	31·4
With 1 ml. 2% KH_2PO_4 the result is	35·7
With 2 ml. 2% KH_2PO_4 the result is	35·9
With 5 ml. 2% KH_2PO_4 the result is	36·0
If the same sample is subjected first to alkaline and then to acid hydrolysis the result is ... ... (1 g. dried organic material is heated on a boiling water bath with 2 ml. 4*N* NaOH and distilled water to make the volume up to 6 ml., then 1·35 ml. 36% HCl are added. After reheating for 20 min. on the boiling water bath, 2 ml. 4*N* NaOH are added, the mixture is cooled and the volume made up to 10 ml. by adding distilled water. The rest of the analysis proceeds as above.)	36·3
With the technique used for very small amounts of tissue (250 mg.) the result is ...	36·0

Dried pig kidney. Found nicotinic acid mg./100 g.	Dried pig kidney + 40 mg./100 g. nicotinic acid. Total nicotinic acid found mg./100 g.	% added nicotinic acid recovered
36·0	77·0	102·5

(5) *Other results.* The results of analyses made on various organs and tissues are given in Table I. The organic material was obtained from recently slaughtered healthy animals; drying was effected within a few hours of the death of the animal. The figures give the mean values of duplicate analyses made on samples of two organs.

The amount of nicotinic acid found in pig and ox muscle is more than 200 times as great as the amount found by Karrer & Keller [1938]. This discrepancy is undoubtedly due to the procedure adopted by these investigators, i.e. evaporation to dryness of the tissue extract and extraction of the nicotinic

Table I. *The amount of nicotinic acid in some organs and tissues expressed in mg./100 g. of fresh material*

	Nicotinic acid, mg./100 g.		
	Pig	Ox	Cod
Liver	11·8	12·2	—
Kidney	6·83	Cortex 6·56 Medulla 5·17	—
Spleen	4·04	4·42	—
Heart muscle	5·34	5·93	—
Striped muscle	4·73	4·90	1·95
Testis	4·42	—	—
Ovary	3·84	—	—
Adrenal cortex	—	6·54	—
Adrenal medulla	—	4·90	—
Thyroid	1·59	3·00	—
Pancreas	5·00	—	—
Thymus	3·25	—	—
Roe	—	—	1·52

acid with warm benzene; this extraction is not quantitative—at any rate the method has failed in my hands.

The amount of nicotinic acid in pig and ox liver agrees well with the results of Swaminathan [1938] for sheep's liver.

III. *Estimation of nicotinic acid in some medicinal preparations*

The amount of nicotinic acid in some medicinal preparations (yeast, liver and stomach preparations) used in the treatment of pellagra is shown in Table II.

Table II. *The amount of nicotinic acid expressed in mg./100 g. or 100 ml. of some medicinal preparations*

Yeast preparations		Stomach preparations	
Name	Nicotinic acid mg./100 g.	Name	Nicotinic acid mg./100 g.
Levurine (A.B.)	47·8	Ventriculin (MCO)	16·9
Leogaer (Leo)	49·7	Ventriculin (P.D. and Co.)	13·2
Bevital (Leo)	47·7	Gastrosic (Leo)	15·4
		Ventriculus siccatus (Orthana)	15·0
		Intricula (Ido)	12·8

Liver preparations			
For oral administration		For injection	
Name	Nicotinic acid mg./100 g.	Name	Nicotinic acid mg./100 g.
Exhepa (Ido)	122	Campolon (Bayer)	41·3
Mecolever (MCO)	119	Exhepa fort. p. inj. (Ido)	1·6
Exhepa liquid. (Ido)	43·7	Hepsol (MCO)	2·9
Extractum hepatis fluidum (A.B.)	6·0	Hepsol fortior (MCO)	116·0
Extractum hepatis fluidum (Exoglen)	32·3	Extract. hepatis p. inj. (Gea)	39·2
		Perhepar (Gedeon Richter A.G.)	49·4

The method described above has been used in analysing the dry preparations. As the fluid preparations contain fewer buffer substances, less HCl is needed after the alkaline hydrolysis to make the reaction slightly acid (*p*H about 4–5).

The preparations also have been analysed after the addition of a known amount of nicotinic acid; this has been recovered quantitatively in every instance.

Investigations by Spies *et al.* [1938], Vilter *et al.* [1939], Sebrell & Butler [1938], Chick *et al.* [1938], Day *et al.* [1938] and Langston *et al.* [1938] have shown, however, that nicotinic acid is not the only factor which plays a role in the therapy of pellagra. At present, therefore, it cannot be decided if the amount of nicotinic acid in organic preparations is the sole indicator of their therapeutic value in pellagra. There is no relation between the concentration of nicotinic acid in liver preparations and their therapeutic effect in pernicious anaemia.

With regard to considerations concerning the identity of the vitamin B_2 complex with the "cyanide-insensitive enzyme complex" composed of the "yellow respiration ferment" [Warburg & Christian, 1932], the coferment [Warburg & Christian, 1934] or cozymase [v. Euler *et al.* 1935] and the "Zwischenferment", the reader is referred to an article by the author which will shortly appear elsewhere.

SUMMARY

1. The colorimetric method for the quantitative estimation of nicotinic acid described in a previous paper is fairly specific, as small amounts of pyridine, picolinic acid, α-picoline, trigonelline or methylpyridinium chloride give no colour when the analysis is carried out in the presence of a certain amount of KH_2PO_4.

2. The technique for analysing biological material and medicinal organic preparations is described.

3. The result is given of a series of analyses of pig and ox organs and tissues, as well as some organic preparations used in the treatment of pellagra.

REFERENCES

Bandier & Hald (1939). *Biochem. J.* **33**, 264.
Chick, Macrae, Martin & Martin (1938). *Biochem. J.* **32**, 2207.
Day, Langston & Darby (1938). *Proc. Soc. Exp. Biol., N.Y.*, **38**, 860.
v. Euler, Albers & Schlenk (1935). *Hoppe-Seyl. Z.* **237**, 180, I.
Karrer & Keller (1938). *Helv. Chim. Acta*, **21**, 1170.
König (1904). *J. prakt. Chem.* **69**, 105.
Langston, Darby, Shukers & Day (1938). *J. exp. Med.* **68**, 923.
Sebrell & Butler (1938). *U.S. Publ. Hlth Rep.* **53**, 2282.
Spies, Grant, Stone & McLester (1938). *South M. J.* **31**, 1231.
Swaminathan (1938). *Ind. J. med. Res.* **26**, 427.
Vilter, Vilter & Spies (1939). *J. Amer. med. Ass.* **112**, 420.
Warburg & Christian (1932). *Biochem. Z.* **254**, 438.
—— —— (1934). *Biochem. Z.* **275**, 112.