Vitamin Methods

7. A NEW MODIFICATION OF THE *p*-AMINOACETOPHENONE METHOD FOR ESTIMATING NICOTINIC ACID IN URINE

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Harris & Raymond [1939] developed a method for estimating nicotinic acid in urine with the object of assessing the nutritional status in this vitamin, along the same lines as those employed in the saturation tests previously devised for vitamin C [Harris & Ray, 1935] and vitamin B_1 [Harris & Leong, 1936]. The method was based on the cyanogen bromide reaction of König [1904], a novel feature being the reagent used as the aromatic amine, namely, *p*-aminoacetophenone. Although other aromatic amines have also been used for determining nicotinic acid, e.g. aniline and *p*-aminophenol, several investigators have concluded that Harris & Raymond's reagent is the most advantageous [Naganna, Giri & Venkatesam, 1941; Stotz, 1941; Arnold, Schreiffler & Lipsius, 1941].

With urines and highly coloured extracts the colour reaction is always less easy to perform than with colourless material. To overcome this difficulty, several alternative procedures have been suggested, but some of these are too tedious, while others run the risk of loss of nicotinic acid. For this reason we have undertaken an extensive study with special reference to the following: the removal of interfering coloured and non-coloured substances; the problem of the 'blank' correction; the conditions of the preliminary conversion of related substances to nicotinic acid. The optimal conditions for the reaction of CNBr with nicotinic acid and the subsequent development of colour [Harris & Raymond, 1939; Kodicek, 1940 *a*, *b*] have been reinvestigated with regard to the complex composition of urinary extracts.

As a result, an improved modification of the *p*-aminoacetophenone method has been devised, which has the advantage of being simple and reliable. The method, which is based on the original procedure of Harris & Raymond [1939], has been used in this laboratory for over two years with good results.

GENERAL CONSIDERATIONS

(1) *Removal of interfering colour*

There are, in general, three methods of estimating a substance in a coloured solution: (*a*) by removing the substance from the solution, thus freeing it from the interfering colour; (*b*) by eliminating the colour from the solution without losing the compound to be determined; and (*c*) by estimating it in the coloured solution and allowing for the colour by a blank correction.

Separation of nicotinic acid from urine by removing it on adsorbents or by extracting it with an immiscible solvent is not very practicable owing to the difficulty of effecting a complete separation, and furthermore, some pigment is removed with the vitamin. A further disadvantage is the difficulty of eluting the vitamin quantitatively after adsorption. Perlzweig, Levy & Sarret [1940] claimed to have obtained good results by using Lloyd's reagent after preliminary oxidation. Dann & Handler [1941], using Perlzweig's decolorization procedure, confirmed this finding. The decolorization procedure, however, is more tedious than the oxidation method described in this paper and is much dependent upon various physical properties of the adsorbing agent. The adsorption of nicotinic acid by medicinal charcoal, as suggested by Giri & Naganna [1941], failed to give, in our hands, reproducible results. Swaminathan [1939] used charcoal in a different manner—to remove pigments from the urine, leaving nicotinic acid in the solution. He thereby eliminated the use of a blank correction except in cases in which the test solutions were still coloured. The adsorption was carried out in an alkaline medium, and it was claimed that no nicotinic acid was lost by this treatment. Melnick & Field [1940 *a*, *b*], like Swaminathan, used charcoal to remove interfering pigments, but performed the adsorption in acid alcoholic solutions. They admitted that their method was liable to give results with urine which are too high.

The method to be described combines principles (*b*) and (*c*) mentioned above, without recourse to adsorption. It consists of a preliminary washing of the hydrolyzed urinary solution with isobutanol, which removes part of the interfering coloured substances, and a subsequent oxidation of the remaining colour with potassium permanganate. The treatment usually produces a solution which is almost colourless. Any residual coloured or colourless interfering substances are allowed for by a special blank correction.

(2) *The blank correction*

Several ways of allowing for the blank correction have been suggested by various workers, but none of them seems to be entirely satisfactory. Bandier [1939*b*] used as a blank a sample of the urine treated as the 'unknown' but without the addition of the amine. The inadequacy of this blank, at least for *p*-aminoacetophenone, will be shown later when the different types of blank are compared. Harris & Raymond [1939] omitted CNBr from their blank, which had otherwise been treated exactly like the 'unknown'. This has been

criticized by Melnick & Field [1940*b*] on the ground that the amine reacts with the interfering constituents of urine to form a colour which is not produced in the presence of CNBr. We could not observe, however, any bleaching effect of CNBr under our experimental conditions, nor could we find in our new 'acid blank' any unspecific side reaction which would not be present also in the 'unknown' [cf. Melnick, Oser & Siegel, 1941]. Dann & Handler [1941] state that the cyanogen bromide procedure worked well only in a colourless solution. They adopt the procedure of Perlzweig *et al.* [1940] to produce an almost colourless extract, and introduce a blank correction which consists of the residual colour of the extract and the colour of the reagents measured separately. Although we agree with Dann & Handler that a colourless extract is to be preferred to a coloured one, we feel that their blank correction may show, with certain extracts, too low a value. Their blank is based on the assumption that a colourless extract contains no interfering substances which may react with the reagents to give a colour. We find that occasionally an apparently colourless solution may still react with the reagents to produce a slight colour. In the procedure to be described we have included all the reagents and the test solution in one blank carried out in such a way that the nicotinic acid in the test solution does not react.

(3) *Preliminary conversion to nicotinic acid of related substances*

Another point which has been the subject of much controversy among different workers is the question whether the conversion to nicotinic acid of related substances should be carried out in acid or in alkaline solution. Swaminathan digested the urine in 2 *N* NaOH for a period of 3 hr. Under such conditions, a considerable portion of trigonelline may be changed to nicotinic acid, which may account for his high values for nicotinic acid output. Melnick & Field, on the other hand, subjected the urine to acid hydrolysis. As pointed out by Perlzweig *et al.* [1940], this has the danger of preventing the complete conversion of nicotinuric acid to nicotinic acid. In the method of Harris & Raymond the digestion was carried out in *N* NaOH for 30 min. It will be shown later that this short digestion with the relatively dilute alkali is preferable, as it does not cause any appreciable conversion of trigonelline to nicotinic acid even in the presence of large quantities of the former compound. Moreover, it is much more convenient, and yields a lighter coloured solution than the method with acid digestion.

(4) *Optimal conditions for the König reaction and subsequent development of colour*

The various factors which influence the König reaction have been extensively investigated by Harris & Raymond [1939] and Kodicek [1940*a*]. There were, however, a few more points which needed further study, especially the conditions for the reaction of CNBr with nicotinic acid. All these factors have been investigated in the present work, and are discussed below in §§ IV and V.

EXPERIMENTAL

(I) *Comparison of preliminary acid and alkaline digestion*

Three 40 ml. portions of a sample of urine were digested with 20 ml. of 12 *N* HCl on a boiling water-bath for 5 hr., neutralized, and decolorized as described in § II(*a*) below. Further batches of eight 50 ml. portions of the same urine, four with added trigonelline and four without, were digested with *N* NaOH for 45 min. on the water-bath, neutralized and decolorized as before. They were then analyzed for nicotinic acid. It will be seen from Table 1 that the same values were obtained whether the digestion was carried out in acid or alkali, and, in the latter case, with or without the addition of trigonelline. The slight variations in the individual values are due to the experimental error, since, with the low values of extinction, the personal error in reading is from 5 to 10%.

Table 1. *Comparison of results with preliminary alkali and acid digestions*

Estimations of nicotinic acid in the presence and absence of trigonelline

	Digestion in alkaline solution				Digestion in acid solution.
	Extinction with				
Material	Acid blank	Urine	Urine +10μg. of nicotinic acid	Nicotinic acid found in urine (μg./ml.)	Nicotinic acid found in urine (μg./ml.)
Urine alone: Test 1	0·049	0·135	0·352	0·79	0·74
Test 2	0·052	0·141	0·355	0·83	0·73
Test 3	0·049	0·125	0·321	0·78	0·77
Test 4	0·049	0·146	0·350	0·95	—
Average	0·050	0·137	0·341	0·84	0·75
Urine, same specimen +0·5 mg. of trigonelline/10 ml.	0·048	0·142	0·346	0·92	—
Urine, same specimen +0·5 mg. of trigonelline/10 ml.	0·054	0·132	0·336	0·77	—
Average	0·051	0·137	0·341	0·84	—
Urine, same specimen +1·0 mg. of trigonelline/10 ml.	0·053	0·157	0·381	0·93	—
Urine, same specimen +1·0 mg. of trigonelline/10 ml.	0·055	0·146	0·360	0·85	—
Average	0·054	0·152	0·371	0·89	—

(II) *Decolorization of urinary digests*

After the preliminary digestion, which serves to convert related substances to nicotinic acid, the urine usually becomes quite dark in colour. It is necessary to remove the colour; otherwise the blank correction will be too high and the accuracy of the method much decreased. Since digestion with alkali always yields a lighter coloured solution than digestion with acid, and as shown above, trigonelline does not interfere, it was decided to use the former method of digestion and to carry out the decolorization of this solution. Two methods of decolorization were tried:

(*a*) *Charcoal adsorption.* The procedure used for decolorization with charcoal as recommended by Swaminathan [1938] was used with some modifications. The method is as follows:

After the preliminary digestion with alkali (unlike Swaminathan, we digested with *N* NaOH, i.e. 5 ml. of 40% (w/v) NaOH added to 50 ml. of urine, and the time of digestion restricted to 45 min.), the urine was neutralized with conc. HCl, and then adjusted to a less strongly alkaline reaction by the addition of 0·5 ml. of 40% NaOH. The precipitate was centrifuged off. The centrifugate was made up to 50 ml., and boiled with 1 g. of norite charcoal. The solution was filtered hot with suction and the charcoal on the filter paper boiled in about 30 ml. of dilute alkali (0·3 ml. of 40% NaOH to 100 ml. of water) for 1–2 min., filtered again and the residue washed with 20 ml. of the hot dilute alkali. The combined filtrates were brought to pH 6·5–6·8 with HCl and made up to 100 ml. The solution was then analyzed for nicotinic acid according to the procedure described below.

This procedure for decolorization gave good recoveries (Table 2). Larger amounts of charcoal (2 g. as used by Swaminathan), however, caused 20–40% loss of added nicotinic acid. The slight yellow colour that remained after adsorption was allowed for by a blank correction. However, owing to the difficulty of obtaining charcoal of uniform quality, this procedure was given up in favour of the second method to be described, which not only gives a more colourless extract but also has other advantages and gives more reliable results.

Table 2. *Recovery of added nicotinic acid after decolorization of urine with charcoal*

Exp. no.	Nicotinic acid in original urine (μg./ml.)	Nicotinic acid added (μg./ml.)	Additional nicotinic acid found (μg./ml.)	Recovery (%)
1	5·1	10·0	9·8	98
2	1·7	10·0	11·2	112
3	4·0	10·0	10·3	103
4	1·24	1·00	1·16	116
5	1·05	2·00	2·15	108
6	0·58	2·00	2·32	116
7	0·48	2·00	1·98	99
8	1·0	0·93	0·74	80
			Average	104

Standard deviation of single determination ±12.

(*b*) *Washing with isobutanol and oxidation with* $KMnO_4$. Urinary pigments may be effectively removed from solution by extracting them with immiscible solvents, such as phenol or benzylalcohol, but unfortunately a great proportion of nicotinic acid is removed as well. Isobutanol extracts, albeit incompletely, a considerable part of the urinary pigments, while it leaves practically all the nicotinic acid behind. The distribution coefficient of nicotinic acid between water-saturated isobutanol and isobutanol-saturated water below pH 4·5 is about 1 : 19. By using 1 vol. of isobutanol to every 2 vol. of urinary digest we have found it possible to remove a large part of the pigments without losing any significant amount of the nicotinic acid (Table 3), but for very accurate work, a correction factor could be introduced to account for the amount extracted by the isobutanol. The residual colour is then removed by oxidation with $KMnO_4$. The oxidation is carried out in acid solution at about 50°. A 4% solution of $KMnO_4$ is added drop by drop with continuous stirring until the colour of the permanganate stays for about 30 sec. The colour is allowed to disappear completely. This usually takes 5–10 min.* The solution is then neutralized carefully to pH 6·5–

Table 3. *Recovery of nicotinic acid after a solution in M/45 phosphate buffer* (pH 4·5) *had been washed with isobutanol*

	Extinction	Amount found (μg.)	Recovery (%)
(*a*) 10 μg. of nicotinic acid present:			
After washing with isobutanol	0·200	10·5	105
Control without treatment	0·251	10·4	104
(*b*) 25 μg. of nicotinic acid present:			
After washing with isobutanol	0·786	23·8	95
After washing with isobutanol	0·809	24·2	97
Control without treatment	0·852	25·7	103

Table 4. *Recovery of added nicotinic acid with the new method*

	Nicotinic acid			
Exp. no.	In original urine (μg./ml.)	Added (μg./ml.)	Additional found (μg./ml.)	Recovery (%)
1	1·5	2·5	2·34	94
2	0·82	2·5	2·7	108
3	0·89	2·5	2·3	92
4	0·87	2·5	2·48	99
5	0·93	2·5	2·44	98
6	0·61	2·5	2·59	104
7	0·82	2·5	2·3	92
8	0·96	2·5	2·59	104
9	1·15	2·5	2·55	102
10	0·63	2·5	2·39	96
11	0·83	2·5	2·32	93
12	1·17	2·0	2·09	105
13	0·26	2·0	1·85	93
			Average	98·5

Standard deviation of single determination ±5·6.

* If the liquid is not sufficiently reducing, as with a solution of the pure vitamin, the excess of permanganate has to be reduced by the addition of a few drops of 0·5% H_2O_2. The excess of H_2O_2 must be removed (e.g. by catalase), as otherwise it may interfere with the subsequent development of colour.

6·8. Any manganous hydroxide separating out is centrifuged off. The solution at this stage should be practically colourless or of light straw colour and is ready for treatment with CNBr. Recovery of added nicotinic acid in solutions so treated is complete (Table 4). The advantage of this oxidation method is not limited to the production of an almost colourless extract. It gives also a solution free from reducing substances, such as ascorbic acid or sulphites, which when present in large amounts may prevent the proper development of colour. Other reducing substances so far tried (sodium thiosulphate, uric acid) had the same effect. The presence of such substances decreases the sensitivity of the method, and the advantage of the oxidation procedure is that it eliminates this interference. Hence the new modification seems to be a further improvement in comparison with other methods.

(III) *Comparison of different blanks*

Three different blanks were tried, (*a*) 'CNBr blank', i.e. a sample of urine treated in the same manner as the 'unknown' but without the amine; (*b*) 'amine blank', i.e. without CNBr; and (*c*) 'acid blank', see below.

Theoretically no blank can be considered perfect which omits any of the reactants. The 'CNBr blank' advocated by Bandier and described above, errs probably on the low side. The 'amine blank' of Harris & Raymond gives a higher correction, but the fact that CNBr is omitted might be criticized. It was found that when nicotinic acid was treated with CNBr in an acid solution, no reaction took place. This observation makes it possible to include all the reactants in the blank by simply adding acid to the test solution in the reversed order, namely, *before* treatment with CNBr. This blank is referred to in future as the 'acid blank'. It will be seen from Table 5 that the value of this blank is

Table 5. *Nicotinic acid estimated in urines with use of different blanks and charcoal decolorization*

	Nicotinic acid found (mg./day) with				
Sample	Urine blank*	CNBr blank	Amine blank	Amine blank plus CNBr blank	Acid blank
1	4·40	3·88	2·88	2·37	2·24
2	3·38	2·83	2·65	2·06	2·21
3	3·01	2·62	1·98	1·51	1·56
4	6·04	6·04	4·78	4·78	4·68
5	4·90	4·20	2·66	1·95	1·85
6	2·69	2·27	1·87	1·46	1·37
7	2·92	2·50	1·54	1·13	1·12
8	3·58	3·34	1·98	1·74	1·77
9	3·30	2·85	2·02	1·55	1·55
10	1·80	1·54	0·99	0·73	0·71

* Urine treated exactly in the usual manner, but without the addition of CNBr and amine reagent.

approximately equal to the 'CNBr blank' and the 'amine blank'. When the washing with isobutanol and the oxidation by $KMnO_4$ were used, the 'acid blank' showed even lower values, corresponding approximately to the sum of the original colour of the CNBr and amine. It is interesting to note that the values of normal human excretion of nicotinic acid obtained by means of this new blank tally well with those of Perlzweig *et al.* [1940] and of Snell & Wright [1941] determined by a different technique.

(IV) *Optimum conditions for the reaction of* CNBr *with nicotinic acid*

In their original method, Harris & Raymond used a temperature of 80° and a duration of 10 min. for the reaction. Kodicek [1940*b*] allowed 5 min. for the reaction to take place. The effect of both temperature and time was reinvestigated by varying these two factors.

(*a*) *Temperature.* A solution of pure nicotinic acid containing 2 μg./ml. was used. The CNBr was added and the reaction mixture placed for 5 min. on a water-bath maintained at constant temperature. The results are given in Table 6. It will be seen that the optimum temperature for maximal colour production is between 50 and 60°.

Table 6. *Effect of temperature on reaction of* CNBr *with nicotinic acid*

Temp. (° C)	Extinction
20	0·148
40	0·305
50	0·388
60	0·418
70	0·329
80	0·236

(*b*) *Time.* The same solution of nicotinic acid was used, and the temperature was kept at 56–58°. The reaction mixture was placed in the water-bath for varying lengths of time. As illustrated in Table 7, the highest extinction was obtained within 2–5 min. It was, therefore, decided to use 4 min. for the reaction in our procedure.

Table 7. *Effect of time on reaction of* CNBr *with nicotinic acid*

Time (min.)	Extinction	
	Exp. 1	Exp. 2
2·0	—	0·408
2·5	0·408	—
3·5	—	0·408
5·0	0·410	0·410
10·0	0·335	—
20·0	0·267	—

(V) *Optimum conditions for the development of colour*

(*a*) *Amount of amine.* It was felt that the amount of amine used previously by Harris & Raymond [1939] and Kodicek [1940 *a*, *b*] might not be the optimum amount for

Table 8. *Effect of variation in the amount of amine used*

10% ethanolic *p*-aminoacetophenone added (ml.)	Extinction for solution of nicotinic acid containing (μg.)	
	10	20
0·2	0·218	0·469
0·3	0·246	0·503
0·6	0·292	0·585
0·9	0·314	0·635
1·2	0·311	0·633
1·5	0·320	0·648

the reaction, as has already been pointed out [Kodicek, 1940*a*]. The fact that the urinary extract was different in composition from other extracts used by one of us (E.K.) made it necessary to reinvestigate this point. It is clear from Table 8 that a nearly optimum development of colour is reached with the addition of about 1·0 ml. of a 10% solution of *p*-aminoacetophenone in ethanol.

(*b*) *Effect of acid upon the final development of colour.* The intensity of colour produced by the reaction between the amine and the CNBr-treated-nicotinic acid depends on the acidity of the solution. The optimal acidity corresponds to a pH of about 1·5, as illustrated by Table 9. It will be seen that the intensity of colour varies only slightly with changes of acidity below a pH value of 2. It was found that 0·6 ml. of a 10% HCl was sufficient to bring the pH of the urines so far examined to a value between 1 and 2 under the conditions of our procedure.

Table 9. *Effect of amount of acid*

10% HCl (ml.)	Extinction for 10 μg. nicotinic acid in *M*/45 phosphate buffer of pH 6·6	pH (quinhydrone electrode)
0·1	0·177	2·51
0·2	0·232	2·14
0·3	0·252	1·87
0·4	0·257	1·68
0·6	0·275	1·45
1·0	0·243	1·13

(*c*) *Stability of the colour produced.* It will be seen from Table 10 that the colour is fairly stable and there is very little fading after standing in the dark for periods up to 30 min. This agrees with the findings of Harris & Raymond [1939].

Table 10. *Fading of developed colour*

Time (min.)	Extinction for solution of nicotinic acid containing (μg.) 12·5	25	50
5	0·255	0·500	1·010
10	0·263	0·519	1·030
15	0·264	0·517	1·017
20	0·259	0·514	1·022
25	0·253	0·514	1·010
30	0·253	0·518	1·008
60	0·239	0·480	0·948

METHOD ADOPTED

Reagents

CNBr *solution.* Prepared fresh daily by titrating a saturated solution of ice-cold bromine water with 10% ice-cold NaCN solution until the colour of bromine disappears. (NaCN was found to be better than KCN.)

Alcoholic amine reagent. 10 g. of recrystallized *p*-aminoacetophenone were dissolved in 96% ethanol and made up to 100 ml. The solution should be prepared fresh every week as older solutions tend to deepen in colour. (The *p*-aminoacetophenone was recrystallized from a warm saturated solution in 96% ethanol by adding about 3 vol. of distilled water. After several hours at 0° the precipitate was filtered off. The process of crystallization may be repeated a second time if necessary. The purified product was dried *in vacuo* over $CaCl_2$. It should be of a light creamy colour.)

HCl *solution* 10% w/w.

Concentrated HCl *solution* 36% w/w.

NaOH *solution* 40% w/v.

$KMnO_4$ *solution* 4% w/v.

Isobutanol. Redistilled and saturated with water.

Standard solution of nicotinic acid. 20 μg./ml. in 25% ethanol. Prepared weekly from a stronger solution containing 1 mg./ml. in 25% ethanol.

Procedure

(1) *Preliminary digestion.* If the total amount of urine excreted each day is less than 1500 ml., dilute the urine to this volume. Take 20 ml. of urine, add 2 ml. of 40% NaOH and heat on a boiling water-bath for 45 min. Cool, adjust pH to 2–3 with conc. HCl, with thymol blue as external indicator. Make up the volume to 25 ml. with distilled water. Pour the treated urine into a 50 ml. separating funnel; add isobutanol corresponding to one-half the final volume of digested urine and shake for 2 min. Allow to separate, then run out the aqueous layer. Take 20 ml. of this water layer and evaporate the isobutanol present in the solution on a boiling water-bath for about 15 min. in an open beaker. Some isobutanol is still left in the solution, but as it does not interfere with the final estimation, its complete removal is not necessary. Add to the hot solution 15 ml. of distilled water, and cool it down to about 40–50°. Acidify further with 6 drops of concentrated HCl and oxidize by adding a 4% solution of $KMnO_4$ drop by drop with continuous stirring, until the urine is completely decolorized and the pink colour of the permanganate stays for more than 30 sec. Allow the solution to cool. Stand for about 15 min. to ensure that any excess of $KMnO_4$ is completely reduced, then neutralize cautiously with 40% NaOH to pH 6·5–6·8 with bromothymol blue as external indicator. It is essential that the pH 6·8 should not be overstepped, as an alkaline reaction causes a brownish coloration. Make up to 50 ml. with distilled water and centrifuge off any precipitate that is formed.

(2) *Reaction with* CNBr. Four graduated 15 ml. flasks are labelled A, B, C and D. To flasks C and D add 0·5 ml. of diluted standard nicotinic acid solution. To flask A add 0·6 ml. of 10% HCl; this flask is to be used as a blank. Measure 10 ml. of the urine solution into each of the four flasks. Mix well, especially flask A, then add to all four flasks 2 ml. of CNBr solution. After the mixing, place the flasks for 4 min. in a water-bath kept at 56–58°. Cool the flasks for 5 min. in tap water (temperature 18–20°) in a darkened place.

(3) *Development of colour.* To each flask add 1 ml. of the alcoholic amine reagent and shake. Then to flask B, C and D add 0·6 ml. of 10% HCl. Fill the flasks up to the 15 ml. mark with distilled water. Mix the contents and allow to stand for 5 min. in a darkened place. Immediately afterwards take measurements with the Pulfrich photometer, using 3 cm. cells and filter S 47 [Kodicek, 1940*a*, *b*]. Flask A is used as the blank, and the flasks B, C and D are examined for their contents of nicotinic acid.

(4) *Calculation of result.* The relation between intensity of colour (extinction) and nicotinic acid present is linear [Harris & Raymond, 1939]. Hence, nicotinic acid content, μg./ml. of urine, equals

$$\frac{B-A}{(C-A)-(B-A)} \times S \times \frac{F}{10} \times 1{\cdot}1 \times \frac{25}{20} \times \frac{1}{V_0}$$

$$= \frac{B-A}{C-B} \times S \times \frac{F}{V_0} \times 1{\cdot}375.$$

When, according to the procedure, $S = 10$ μg., $F = 50$ ml. and $V_0 = 20$ ml., the formula is simplified to $\frac{B-A}{C-B} \times 3{\cdot}44$ μg./ml. of urine. A = extinction of solution in flask A (blank), B = extinction of solution in flask B (urine), C = mean of extinctions of solution in flasks C and D (urine + added nicotinic acid), S = μg. of nicotinic acid added to flasks C and D, F = final volume of urine after washing with isobutanol, oxidation and neutralization, V_0 = original volume of urine taken for analysis, 1·1 = factor allowing for the increase in volume of washed urine due to isobutanol.

Reproducibility of method

To test the reproducibility of the method separate determinations were done on one specimen of urine. The results are shown in Table 11. The standard error of a single determination is about ±2·4%. This degree of precision is highly satisfactory.

Table 11. *Data testing the reproducibility of method*

Exp. no.	Nicotinic acid in same specimen of urine (μg./ml.)
1	0·81
2	0·86
3	0·85
4	0·82
5	0·81
Average	0·83

Standard deviation of single determination ±0·02 (2·4%).

RESULTS

Daily excretion of nicotinic acid by normal individuals determined by the new procedure

Ten normal subjects were examined for their daily excretion of nicotinic acid under standard conditions (see below). The final modification described above was used. The resting levels of adults varied from 0·85 to 2·33 mg. of nicotinic acid/day, with an average of 1·15 mg. (Table 12). These figures agree with the values found by the earlier procedure of charcoal decolorization.

Table 12. *Daily excretion of nicotinic acid by normal individuals as determined by the final modification*

Subject	Age	Sex	Daily volume of urine (ml.)	Nicotinic acid excreted (mg./day)
J.E.P.	17	F	880	0·85
E.M.C.	41	F	1450	1·03
J.Y.	30	M	1360	1·14
L.W.M.	33	M	1400	1·16
A.G.D.	17	M	1040	1·43
E.K.	33	M	1100	1·53
S.G.I.	30	M	2160	1·77
T.M.	41	M	2350	1·92
D.W.	19	M	1630	2·33
J.K.	4	F	650	0·46

*Excretion after experimental dosing of healthy subjects with nicotinic acid**

Four normal subjects living on their usual diets were examined for their urinary excretion of nicotinic acid. The daily output of urine was collected in two portions, a morning 3 hr. sample and the remaining 21 hr. portion; each portion was analyzed separately. Test doses were taken with or after breakfast. Table 13 gives the results of the experiment.

The average daily excretions of nicotinic acid by the four individuals were 1·09, 1·42, 1·47 and 2·10 mg., and the corresponding morning 3-hourly outputs averaged 0·23, 0·09, 0·13 and 0·16 mg. respectively. Smoking did not appreciably change the values. After daily ingestion of 100 mg. of nicotinic acid for several days, the average daily excretions of the last three individuals increased to 7·77, 3·98 and 9·51 mg., and the corresponding 3-hourly excretions to 5·34, 2·02 and 6·38 mg. respectively.

It will be seen from Table 13 that while the excretions of nicotinic acid by the different subjects on their usual diets changed very little from day to day and varied little from one to another, the outputs after ingestion of test doses fluctuated considerably,

* Decolorization of urine in these experiments had been performed by charcoal adsorption as described in § II(*a*). Urine collected after dosing had, however, such a high concentration of nicotinic acid that the charcoal adsorption could be replaced by a simple dilution of the urine.

Table 13. *Response of normal individuals to test doses of nicotinic acid*

Subject	Day	Test dose (mg.)		Nicotinic acid excreted (mg.) in 3 hr.	Nicotinic acid excreted (mg.) in 24 hr.	Notes
Y.L.W.	1	0		—	2·24	No smoking
Body weight	2	0		—	2·21	,,
65 kg.	3	0		—	1·56	,,
	4	0		—	1·26	Smoking
	5	0		0·05	1·21	,,
	6	0		0·12	0·71	,,
	7	0		0·10	1·40	,,
	8	0		0·08	0·77	,,
			Average	0·09	1·42	
	9	100		2·91	4·68	
	10	100		5·16	8·38	
	11	100		2·34	3·74	
	12	100		2·98	4·92	
	13	100		16·30	21·20	
	14	100		2·32	3·70	
			Average	5·34	7·77	
L.J.H.	1	0		—	0·63	No smoking
Body weight	2	0		—	2·21	,,
72 kg.	3	0		—	2·16	,,
	4	0		—	2·56	Smoking
	5	0		—	1·90	,,
	6	0		0·14	2·06	,,
	7	0		0·12	1·25	,,
	8	0		0·17	1·09	,,
	9	0		0·20	1·91	,,
			Average	0·16	2·10	
	10	100		1·33	3·07	
	11	100		5·16	7·82	
	12	100		4·48	7·60	
	13	100		17·50	23·98	Dose taken on empty stomach
	14	100		2·00	4·28	
	15	100		7·82	10·30	
			Average	6·38	9·51	
	16	0		0·53	2·30	
	17	0		0·17	1·27	
	18	0		0·59	4·39	
E.K.	1	0		—	2·50	No smoking
Body weight	2	0		—	1·85	,,
87 kg.	3	0		—	1·37	,,
	4	0		—	1·12	Smoking
	5	0		—	1·77	,,
	6	0		0·10	0·82	,,
	7	0		0·15	1·00	,,
	8	0		0·14	1·48	,,
	9	0		0·14	1·28	,,
			Average	0·13	1·47	
	10	100		1·13	2·58	
	11	100		1·75	2·98	
	12	100		1·49	2·47	
	13	100		0·61	2·32	
	14	100		4·60	9·20	
	15	100		2·24	3·81	
	16	100		2·35	4·47	
			Average	2·02	3·98	
	17	0		1·22	3·08	
	18	0		0·11	2·53	
G.G.	1	0		—	1·55	
Body weight	2	0		—	0·71	
83 kg.	3	0		—	0·92	
	4	0		—	0·91	
	5	0		—	0·77	
	6	0		0·16	0·71	
	7	0		0·10	0·86	
	8	0		0·42	1·55	
	9	0		0·24	1·85	
			Average	0·23	1·09	

making it difficult to draw any definite conclusion from the experiment. It will be noted also that the different individuals varied a great deal in their response to dosing. Immediately after the cessation of dosing, the excretion of nicotinic acid returned to the previous level.

In view of the big fluctuations in excretion after the intake of test doses, it was thought advisable to standardize the conditions under which the doses were administered. Three subjects were given a special breakfast which contained little nicotinic acid or trigonelline, and consisted of two cups of tea, two slices of white bread, jam, butter and about 50 ml. of milk, and the dose given with the breakfast after one cup of tea and one slice of bread. The urines were collected and analyzed as before, and the results are given in Table 14. It will be seen that the 3-hourly excretions after dosing were fairly constant from day to day, and also the 24 hr. outputs did not fluctuate as before when the conditions of dosing were not standardized. It is obvious that if test doses of nicotinic acid are to be used for assessing the body reserves of the vitamin, they must be administered under a standardized condition, otherwise little information can be gained from the results.

Table 14. *Response of normal individuals under standardized conditions of dosing*

Subject	Day	Test dose of nicotinic acid (mg.)		Nicotinic acid excreted (mg.) in 3 hr.	Nicotinic acid excreted (mg.) in 24 hr.
Y.L.W.	1	0		0·12	1·20
Body weight	2	0		0·16	1·21
65 kg.			Average	0·14	1·21
	3	100		9·70	12·28
	4	100		11·20	13·80
	5	100		8·40	—
			Average	9·97	13·04
	6	0		0·21	6·61
	7	0		0·18	1·68
	8	0		0·15	0·90
E.K.	1	0		0·41	1·64
Body weight	2	0		0·11	1·40
87 kg.			Average	0·26	1·52
	3	100		0·85	2·36
	4	100		1·27	3·31
	5	100		1·03	11·03
			Average	1·05	5·57
	6	0		0·25	1·90
	7	0		0·25	1·99
	8	0		0·22	1·05
G.G.	1	0		0·16	0·71
Body weight	2	0		0·10	0·86
83 kg.	3	0		0·42	1·55
	4	0		0·24	1·85
			Average	0·23	1·26
	5	100		10·89	13·69
	6	100		9·85	11·17
	7	100		11·20	13·93
			Average	10·65	12·93
	8	0		0·20	1·40
	9	0		0·10	1·23

Time curve of excretion of ingested nicotinic acid under uniform conditions

In one normal subject the hourly excretion of nicotinic acid after ingestion of 100 mg. of nicotinic acid was studied under standardized conditions. The excretion was followed at hourly intervals for 5 hr. (Table 15). Our findings, similar to those of Bandier [1939*a*], were that 91 % of the administered nicotinic acid was excreted in the first 5 hr. after the dose, and that the maximum excretion occurred in the first hour and amounted to about 63 %. This rapid elimination suggests that the organism is not able to utilize efficiently all the ingested nicotinic acid and therefore excretes the greater part of it promptly. This phenomenon does not occur when our subjects are dosed with nicotinic amide. Both the acid and the amide, however, produce a large

Table 15. *Rate of excretion of ingested nicotinic acid*

Time	Nicotinic acid excreted (mg./hr.)
3 hr. period before dosing	0·23
Total 24 hr. period before dosing	1·26
100 mg. nicotinic acid given by mouth	
0 hr.	—
1	7·77
2	2·46
3	0·66
4	0·37
5	0·25
5 hr. total excretion	11·51
5–24 hr.	2·18
24 hr. total excretion	13·69

increase in the excretion of trigonelline and related N-methyl pyridinium compounds which can be regarded as normal metabolites. The fate of ingested nicotinic acid or amide in the body has been briefly discussed in our previous report [Kodicek & Wang, 1941]. The detailed results will be published in a subsequent paper. On the basis of these findings, we have recommended dosing with nicotinic amide, followed by the estimation of the urinary excretion both of trigonelline and nicotinic acid, as a method of assessing the level of nutrition with regard to the anti-pellagra vitamin.

SUMMARY

1. The cyanogen bromide-*p*-aminoacetophenone method of Harris & Raymond for the estimation of nicotinic acid in urine has been further studied, and several additional improvements have been introduced, viz. (*a*) decolorization of the digested urine by washing with isobutanol and oxidation with $KMnO_4$; (*b*) the use of a lower temperature, a shorter time for the reaction of CNBr with nicotinic acid, and the addition of larger amounts of *p*-aminoacetophenone to the reaction mixture; and (*c*) the introduction of a modified blank.

2. The normal excretion of nicotinic acid by ten healthy subjects and the response to test doses of nicotinic acid by four of them has been studied.

3. The maximum excretion of ingested nicotinic acid occurs in the first three hours, the first hour showing the highest output.

4. It is emphasized that when test doses of nicotinic acid are used for assessing the body reserves of the vitamin, they should be administered under standardized conditions.

5. For obtaining reliable information of the nutritional status in regard to the anti-pellagra vitamin, dosing with nicotinic amide is to be preferred to dosing with nicotinic acid, and estimation should then be made of the excretion of trigonelline, as well as that of other substances related to nicotinic acid.

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The Etherification of Hydroxyamino-acid Residues in Silk Fibroin by Dimethyl Sulphate

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In recent years it has been recognized that in addition to tyrosine the hydroxyamino-acids serine and threonine are major protein constituents. The mode of linkage of the —OH groups of these latter in the protein is of interest, since such groups might play a part in non-peptide linkage of amino-acid residues