CCL. ASSESSMENT OF THE LEVEL OF NUTRITION. A METHOD FOR THE ESTIMATION OF NICOTINIC ACID IN URINE[1]

By LESLIE J. HARRIS and
W. D. RAYMOND[2]

*From the Nutritional Laboratory, University of Cambridge
and Medical Research Council*

(*Received 31 October 1939*)

WHEN in 1937 the pellagra-preventing factor was identified as nicotinic acid it became desirable to try and devise a method to estimate it in urine, with the object of working out a test for determining the "level of nutrition" similar to that already used for vitamins C [Harris & Ray, 1935] and B_1 [Harris & Leong, 1936].

After consultation with Dr S. Greenburgh, Public Analyst at Cambridge, it was decided to investigate the possibilities of three alternative methods of estimation.

Methods for estimating nicotinic acid

(1) The first of these to be considered, decarboxylation of nicotinic acid yielding pyridine and recovery of the latter after distillation, was judged to be unsatisfactory and inconvenient as compared with the procedure finally adopted.

(2) A second method, namely a colour reaction given by pyridine derivatives with 2:4-dinitrochlorobenzene [Vongerichten, 1899; Zincke, 1904], was also examined but was abandoned after a time as we reached the conclusion that it was more suited for a rough qualitative test than for a specific quantitative determination. This reaction has already been recommended by Vilter *et al.* [1938] for estimating nicotinic acid in urine, but it must be mentioned that the values which they cite as obtained by its use are widely divergent from those to be recorded in this paper.

(3) The method which we finally chose was based on the colour test for pyridine derivatives introduced by König in 1904, namely the reaction with cyanogen bromide plus an aromatic amine. A variation of this reaction was used by Strafford & Parry Jones [1933] and by Barta [1935] for detecting pyridine present as an impurity in nicotine. Swaminathan [1938] and Bandier & Hald [1939] and others have recently described a test for nicotinic acid based upon this reaction, but in the form set out by these workers the test does not, as we find, actually permit of the quantitative determination of nicotinic acid in urine. As Euler *et al.* [1938] have pointed out, the depth of the coloration given by pyridine derivatives in a König test depends on the *p*H, on the concentration of salts, and on other variable factors. By the procedure described in the present paper we have however been able to circumvent these disturbing influences.

[1] Communicated to the Biochemical Society 1 July 1939 [Harris & Raymond, 1939].

[2] Of the Medical Laboratory, Dar-es-Salaam, Tanganyika.

Chemistry of the König reaction

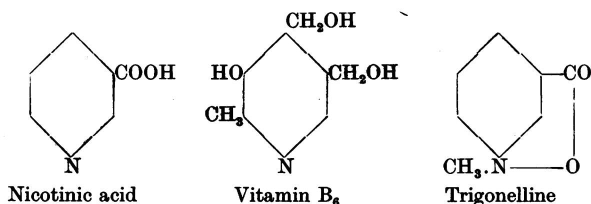
According to König [1904] the general type of the interaction of pyridine derivatives with CNBr and aromatic amines follows a course of which the special features are the formation of an addition compound with CNBr, the substitution of two or more aromatic nuclei into the resulting compound, followed by the possible migration of one H atom and accompanied by an opening of the ring structure. König considered that with increasing concentrations of the amine an increasing number of aromatic groups might be forced into combination.

EXPERIMENTAL

The work now to be described falls roughly under three headings: first a series of preliminary control tests to examine the specificity of the reaction; secondly a study of the more exact quantitative relations of the reaction, with the object of devising an accurate analytical procedure; and thirdly, the application of this method to controlled biological material.

Preliminary control tests

(*a*) *Specificity.* Since it has recently been shown that vitamin B_6 is, like nicotinic acid, a pyridine derivative it was thought advisable to examine its behaviour in the colour reaction. Fortunately it was found not to interfere under



Nicotinic acid Vitamin B_6 Trigonelline

the conditions of our test, carried out as specified below. In confirmation of the observation of Swaminathan [1938] trigonelline, a closely related compound which may also occur in urine, was likewise found not to yield a König reaction.

(*b*) *Varying intensity of colour.* Preliminary experiments at once made it obvious that the colour developed was very sensitive and changed in intensity as the *p*H was altered, or as salts were added to the medium, and with other variables, as found by Euler *et al.* [1938]. Later work, described in the quantitative section, was therefore designed to try and overcome these difficulties.

(*c*) *Necessity for blank.* Substances presumably of an aldehydic nature were found to be present in urine and to give a colour with the aromatic amine even in absence of CNBr. Accordingly it was concluded that a "blank" test without CNBr must form part of the procedure in order to allow for such "non-specific" coloration.

(*d*) *Choice of aromatic amine.* A large number of miscellaneous aromatic bases, including those listed in Table I, were examined in turn with the object of discovering which seemed most suitable for the test. It will be noticed that among the substances tried was *p*-aminoacetophenone which was thought of because of its previous use by Prebluda & McCollum [1937] as a colour reagent for vitamin B_1. This substance was finally chosen as having the following advantages among others:

(1) The colour is relatively stable if kept protected from light.

(2) The "blank" for the reagents, i.e. all reagents, mixed together in absence only of nicotinic acid, is quite colourless.

Table I. *Aromatic amines tested*

Metol
Aniline
Benzidine
o-, *m*- and *p*-Toluidine
α- and β-Naphthylamine
Dianisidine
α-Aminoanthroquinone
2:4-Dichloraniline
p-Dimethylaminoaniline
1-Amino-2-naphthol-4-sulphonic acid
p-Aminoacetophenone

(3) The intensity of the colour is unusually high, e.g. about five times that given by metol or aniline.

(4) The colour can be extracted by ethyl acetate, and this property may prove, in certain modifications of the test, suitable for use for other biological assays.

Aniline, used by Swaminathan, and metol used by Bandier & Hald seemed less satisfactory in our hands for these and other reasons.

Quantitative relationships

In the more detailed quantitative investigation of the reaction, undertaken with the object of elaborating a reliable analytical procedure, attention had to be paid to all of the following seven factors: (*a*) influence of light, (*b*) influence of *p*H and salts, (*c*) effect of variations in concentration of the amine reagent used, (*d*) effect of variations in concentration of CNBr, (*e*) action of excess acid, (*f*) applicability of reaction to nicotinamide, (*g*) limits of sensitivity.

Unless otherwise stated values for colour intensity given below were those obtained by the standardized procedure set out later in this paper.

(*a*) *Effect of exposure to light.* Early in the work it was found that the fully developed colour, given at the last stage of the reaction, faded fairly quickly on exposure to light. Control tests (e.g. Table II) indicated that, provided this

Table II. *Effect of light*

Time min.	*E*: Solution kept in dark	*E*: Solution exposed to light
0	1·47	1·47
15	1·47	1·2
30	1·47	<1·2
60	1·47	—

coloured solution were kept in the dark, no appreciable fading occurred over a reasonably long time. For this reason we have stipulated in the working directions that certain of the operations must be carried out in such a way as to prevent exposure to bright light.

We are indebted to Dr E. Kodicek for the observation that when more dilute solutions are employed than that referred to in Table II, some measurable fading will occur within a period of say about 1 hr. even in the dark. Nevertheless, under the actual working conditions of our test, the loss in an experimental period of about 15–30 min. is still so small as to be of no practical significance, provided that the solution is kept in the dark.

(*b*) *Change of* p*H by buffer.* The striking effect of the addition of a buffer mixture on the intensity of the colour is sufficiently illustrated by the one example given in Fig. 1. It will be noticed that under the particular conditions of the experiment in question the presence of an acetate buffer at *p*H *c.* 3 sufficed to reduce the colour to about one-third of its intensity for any particular concentration of nicotinic acid. But it is important to note that for any given set of conditions, either for the pure unbuffered solution of nicotinic acid (upper curve) or for the solution with the addition of a fixed amount of buffer (lower curve), the relation between colour intensity and concentration of the unknown (nicotinic acid) remains a linear one—i.e. the system within these limits obeys Beer's law. Use is made of this fact in the method of test to be described.

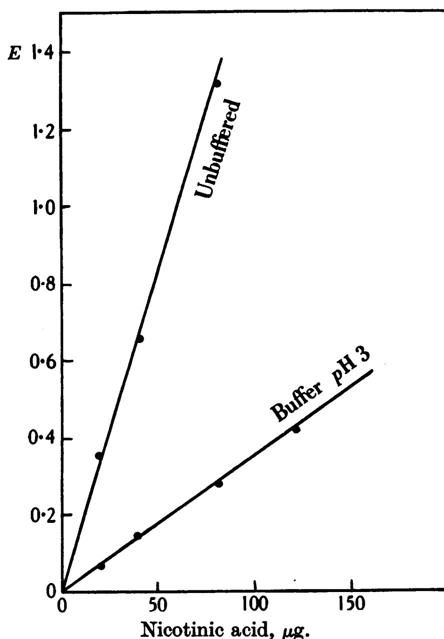


Fig. 1.

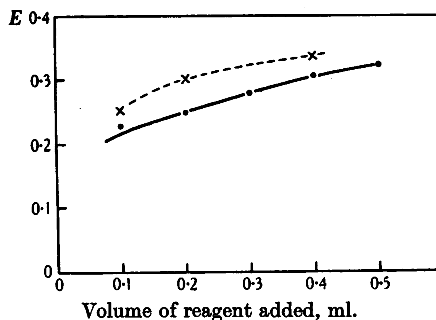


Fig. 2.

Fig. 1. Influence of buffer on slope of curve. Above, unbuffered solution. Below, in presence of acetate buffer at *p*H *c.* 3.

Fig. 2. Effect of varying the amount of aromatic amine added. Other conditions, e.g. concentration of nicotinic acid, kept constant. • = results with aqueous solutions. × = results with urine.

(*c*) *Effect of variation in concentration of amine reagent.* As will be seen from Fig. 2 the intensity of the coloration increases only relatively slowly as the concentration of the amine reagent is increased, provided that the latter exceeds values of about 0·1 or 0·2 ml. While therefore it is important that in carrying out an estimation the amount of reagent added should be kept reasonably constant on all occasions, nevertheless any small variation in volume or concentration due to unavoidable experimental error will have no serious effect on the accuracy of the result.

(*d*) *Influence of variations in amount of CNBr used.* Again with the CNBr, the intensity of colour is increased as its concentration rises. However, provided that a reasonable excess is used, the effect is not significant.

(e) *Effect of acid.* Table III illustrates how, under a given set of circumstances, the addition of acid causes the intensity of colour gradually to rise and then as more acid is added it once more begins to diminish. It is essential therefore that the amount of acid should be kept constant for any particular test.

Table III. *Illustrative experiment showing effect of addition of acid on intensity of colour produced in a buffered solution of nicotinic acid*

Vol. of conc. HCl added to 1 vol. of urine	*E*
0·0	0·182
0·1	0·234
0·2	0·202
0·4	0·186

(f) *Conversion of nicotinamide into nicotinic acid.* As the pellagra-preventing factor in urine occurs partly as free nicotinic acid and partly as nicotinamide, the procedure used must be capable of estimating both forms. The results given in Table IV indicate that the preliminary hydrolysis which we employ is adequate to convert the amide into the acid and give quantitative recovery. The question of the possible occurrence and estimation in urine of other related substances, e.g. nicotinuric acid [Ackermann, 1912], will be discussed in a later paper.

Table IV. *Recovery of added nicotinic acid*

Description	Amount taken μg.	Amount found μg.	Recovery %
Aqueous solution	98	103	105
,,	39·3	41	104
Added to urine	98	96	98

(g) *Limit of sensitivity.* With the use of the standard procedure described below, it has been found possible to detect as little as 1 μg. of nicotinic acid. Below this amount, detection would be difficult.

Principle of method recommended

The special feature of the method to be described is that the urine, after preliminary heating with NaOH to convert any amide into the acid, is divided into four equal portions: one is kept as blank and to the other three nicotinic acid is added in known graded amounts, viz. 0, 20 and 40 μg. These three solutions, which are kept protected from the light, are all treated in the same way, being in turn warmed with CNBr, cooled, treated with *p*-aminoacetophenone allowed to stand, acidified, and then their colour-intensities measured in a Pulfrich step photometer. From what has already been said in the preceding sections, it will be clear that for any given specimen the depth of colour will vary with *p*H and the concentration of salts (and with other factors); but these three readings are obtained under conditions which are identical except for the concentration of nicotinic acid added, and it is found therefore that if we plot the colour intensities (as ordinates) against the amounts of nicotinic acid added (as abscissae) the readings will always lie on a straight line. If this line is now produced backwards it will cut the X-axis at a distance from the origin which represents the amount of nicotinic acid which would have to be removed from the urine to give a zero content, that is the nicotinic acid content of the original urine itself.

Working details

Since success in the use of the method depends on close attention to details, it is advisable to describe the procedure in full.

Reagents. (1) *CNBr solution.* Prepared fresh daily by adding a 10% aqueous solution of KCN drop by drop to saturated Br water until it is just decolorized. (2) *Amine reagent.* *p*-Aminoacetophenone 5 g., plus HCl (10 g. HCl per 100 ml.) 14 ml., plus distilled water to 50 ml.

Apparatus. Pulfrich step photometer, 15 ml. graduated stoppered flasks, pipettes, burettes, water bath with false bottom.

Process for urine. (1) *Hydrolysis of amide and esters of nicotinic acid.* A 25 ml. specimen of the urine is measured out, 5 ml. of 20% NaOH are added and the mixture heated for 30 min. on a steam bath to convert any nicotinamide into free nicotinic acid. The solution is next accurately neutralized, for which purpose 2 ml. of 4% $NaHCO_3$ is first added to help stabilize the end-point; and then concentrated HCl (usually about 1·8 ml.) is run in very cautiously drop by drop from a microburette reading to 0·01 ml. until the solution is precisely at *p*H 6 (bromothymol blue as external indicator). The contents of the flask and washings are then transferred to a 50 ml. graduated flask and made up to the mark.

Notes. (*a*) Excess of acid or alkali should be avoided as it adversely affects subsequent reactions.

(*b*) With urines rich in nicotinic acid dilution to 100, 200 or even 500 ml. may be necessary.

(2) *Reaction with CNBr and aromatic amine.* Four 15 ml. standard flasks are taken, labelled X, A, B and C. (X is the blank, A the unknown and B and C controls to which known amounts of nicotinic acid are added.) In B is placed 0·2 ml. and in C 0·4 ml. of a standard solution of nicotinic acid (100 μg. = 1 ml.). By means of a pipette 10 ml. of the prepared urine are run into each of the flasks A, B, C and X, care being taken to wash down any of the strong solution of standard nicotinic acid adhering to the sides of the flask. All flasks are placed in an opaque-sided water bath and kept at 80° for *c.* 10 min. or until they acquire the temperature of the bath. CNBr reagent, 2 ml., is added to each of A, B and C but not X, the contents of the flasks being mixed by rotating them. At the end of a further 4 min. the flasks are removed to a bath of cold water (not exposed to strong light). After 4 min. cooling, 0·2 ml. of the aromatic amine reagent (solution no. 2) is added to each of the flasks A, B, C and X. The contents are mixed and the flasks are placed in a dark cupboard for 15 min.

(3) *Development of latent colour.* To each of flasks X, A, B and C 0·4 ml. of a 10% solution of HCl is added, the contents are diluted to the mark with distilled water and well mixed, and the flasks replaced in the dark cupboard. Approximately 15 min. after the addition of the acid, colorimetric measurements are made with the Pulfrich photometer. Solution X is placed in one 3 cm. cell and A, B and C introduced successively in the other 3 cm. cell, an S 47 filter being used. An exact match with solution C is sometimes difficult, on account of slight differences in the shade of the blue observed.

Notes. (*a*) It is important that all the flasks be similarly treated with respect to the addition of acid. Similarity of treatment is more important than the exact quantity or strength of the acid. A 2 ml. micro-burette divided into 0·01 ml. divisions is satisfactory for measuring the acid.

(*b*) Although it is not necessary to work in absolute darkness, the colours are extremely sensitive to light, especially so when in acid reaction. The solutions should be exposed as little as possible to strong daylight. The colours are sufficiently stable to allow accurate measurement in the photometer. The stages from the cooling onwards are best carried out in a darkened room.

(*c*) The CNBr is poisonous and should be added from a burette.

(*d*) Any turbidity in the treated urine may be a source of serious error. No difficulty is usually experienced with human urine, but urine from experimental animals often requires to be centrifuged before hydrolysis and recentrifuged after hydrolysis and then diluted to a known volume. If there is any marked difference in turbidity in the final coloured solutions X and A a third centrifuging may be necessary before the colour measurement. A preferable procedure [Kodicek, 1939] is to clarify by addition of 60 ml. of ethanol after the preliminary hydrolysis; this addition does not change the intensity or stability of the colour, or the recovery of nicotinic acid.

(*e*) *Preservation of urine.* Toluene used as preservative must be repurified ("sulphur-free"); otherwise the impurities present in it will interfere in the colour reaction. Urine preserved with such purified toluene retains the nicotinic acid well, for some days at least.

Calculations. From the three photometer readings (say k_A, k_B, k_C) a graph is constructed, taking these values as ordinates and the corresponding known amounts of nicotinic acid added (0, 20 and 40 μg.) as abscissae. A straight line is so obtained, which when produced backwards cuts the axis at a distance from the origin representing the amount of nicotinic acid in the specimen examined.

For routine work it is sufficient to prepare three aliquots (A, B and X) instead of four (A, B, C and X) and to calculate the result thus:

Nicotinic acid in μg. per ml. $=\frac{k_A}{(k_B-k_A)}\times\frac{20}{n}$ where n equals the number of ml. of urine taken in one aliquot of the diluted urine.

Examples illustrating principle of method. In Table V are entered the records of an experiment in which three separate estimates were made on one and the same specimen of urine, but under three different sets of experimental conditions. The data are treated graphically in Fig. 3, and it will be noted that

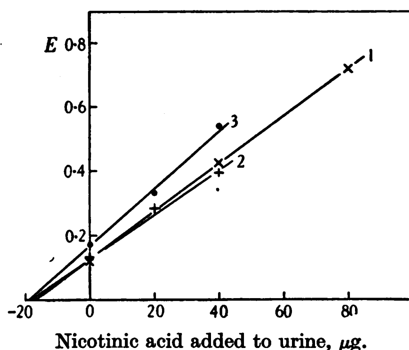


Fig. 3. Triplicate determinations on same specimen of urine under varying conditions, showing the agreement in the final results:

Curve 1, 40 and 80 μg. added:	N.A. found = 17 μg.
2, 20 and 40 μg. added:	N.A. found = 18 μg.
3, 20 and 40 μg. added, less acid:	N.A. found = 19 μg.

notwithstanding the experimental variations in question, e.g. in the pH or in the amounts of nicotinic acid added to the control tubes, the results obtained on the three occasions were yet in fairly good agreement, viz. 17, 18 and 19 μg. of nicotinic acid in the 5 ml. of urine taken for examination. This example has been specially chosen to illustrate the underlying principle of the method, viz. that although the slope of the graph and the values of the initial readings on the urine itself may vary in determinations done under different conditions, nevertheless the results as obtained by extrapolation are always in good accord.

Table V. *Determination of nicotinic acid in urine under varying experimental conditions*

Nicotinic acid added, μg.	Exp. 1 (Determination no. 1) Photometer reading	Exp. 2 (Duplicate under somewhat different conditions) Photometer reading	Exp. 3 (Triplicate, with 0·1 ml. HCl instead of 0·2 ml.) Photometer reading
0	0·12	0·13	0·18
20	—	0·28	0·33
40	0·43	0·39	0·54
80	0·72	—	—
Nicotinic acid found, μg.	17	18	19

A second example is given in Table VI; and Fig. 4 illustrates how, when the corresponding values are plotted out, the same final result is reached from either of the two duplicate determinations.

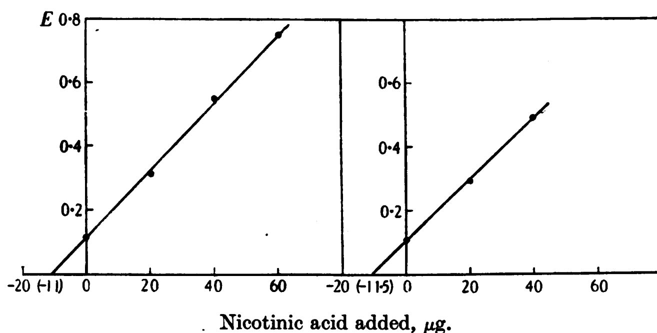


Fig. 4. Duplicate determinations, case of anorexia nervosa.

Value found, left = 11 μg.
Value found, right = 11·5 μg.

Table VI. *Duplicate determinations of nicotinic acid on single specimen of urine: case of anorexia nervosa*

Amount of nicotinic acid added, μg.	Photometer readings: First experiment	Photometer readings: Second experiment
0	0·12	0·11
20	0·31	0·29
40	0·55	0·48
Nicotinic acid found	11 μg.	11·5 μg.

Degree of accuracy attainable. The figures given in the above tables for the photometer readings always represent the average of three readings. They are to be regarded as accurate to about $\pm$0·01–0·02 unit, and the points plotted from them are generally found to be on the straight line, within this same limit. From the results already cited, and from other determinations, it can be stated that duplicates agree well, within an experimental error of about $\pm$10%, and that added nicotinic acid or amide is quantitatively recovered.

TESTS ON NORMAL AND DEFICIENT EXPERIMENTAL ANIMALS

Since cases of pellagra are not easily available in this country we decided that control tests should be begun in the first place with experimental animals kept on various levels of adequacy or deficiency of nicotinic acid.

(a) *Experiments on guinea-pigs*

Gradual loss of nicotinic acid from urine during development of deficiency disease. As has been recorded elsewhere [Harris, 1939], guinea-pigs are unable to survive on a diet devoid of nicotinic acid. In the representative experiment illustrated in Fig. 5, one guinea-pig was kept on a regimen deficient in nicotinic acid (a modified Goldberger diet) while a second guinea-pig, serving as positive control, received daily supplements of the substance. The animal having no nicotinic acid began to show the first evidence of deficiency with a corresponding loss of weight after about 3 weeks, and his condition progressively deteriorated thereafter until he finally succumbed after 4–5 weeks; the positive control on the other hand continued to thrive. It will be noticed that as the deficiency disease advanced the amount of nicotinic acid in the urine fell steadily until at the last stages it had become virtually zero. It is important to recognize however that at the onset of the disease nicotinic acid was still being excreted in significant amounts, and that it was not until the animal was already severely ill that the excretion entirely ceased.

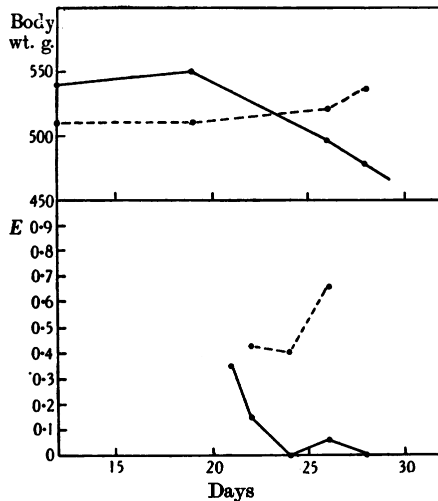


Fig. 5. Loss of nicotinic acid from urine of guinea-pig during development of deficiency. Above, weight curves. Below, colour value of urines.

——— Guinea-pig on Goldberger diet.
- - - - - Guinea-pig on Goldberger diet + nicotinic acid.

Excretion at various levels of intake. In continuation of these observations, groups of guinea-pigs were dosed with varying amounts of nicotinic acid. The graphs corresponding with two typical urinary analyses are illustrated in Figs. 6 and 7, the first indicating that there was a zero excretion by two guinea-pigs on a deficient diet and the second that there was an excretion of 400 μg. daily by the two guinea-pigs receiving 2 mg. each daily. These charts are included

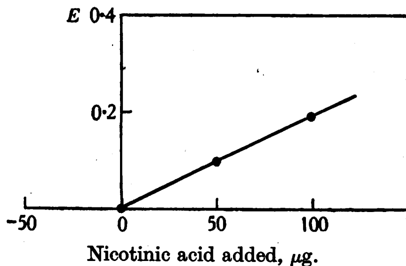


Fig. 6.

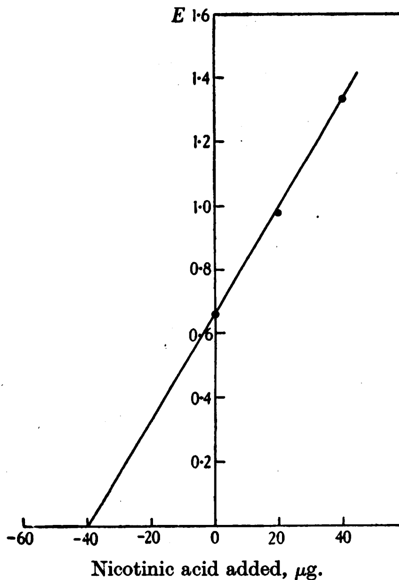


Fig. 7.

Fig. 6. Test on urine from two deficient guinea-pigs showing the absence of nicotinic acid (cf. Fig. 7).

Fig. 7. Test on urine of normal guinea-pig receiving 2 mg. nicotinic acid daily. Nicotinic acid found = 40 μg. (≡400 μg. in 24 hr. specimen).

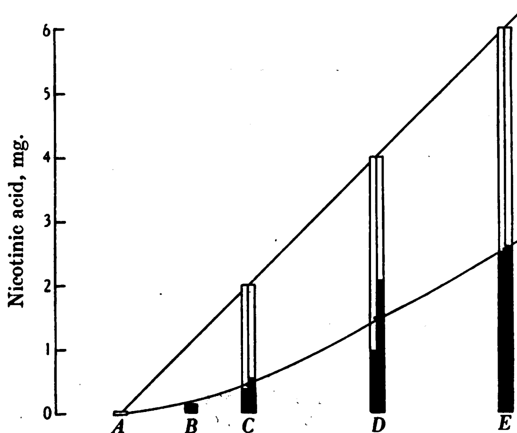


Fig. 8. Excretion of nicotinic acid with increasing level of intake.

Open columns = intake; black columns = excretion.

A = deficient diet.
B = stock diet of bran and oats, etc.
C = deficient diet + 2 mg. nicotinic acid.
D = deficient diet + 4 mg. nicotinic acid.
E = deficient diet + 6 mg. nicotinic acid.

The proportion excreted rises with increasing intake.

because they show that the method of calculation used with human urine is equally applicable for work with guinea-pigs (as we have found it also to be for dogs and other species); i.e. the experimental points always lie on a straight line, so that the amount of nicotinic acid originally present can be determined by extrapolation. The results of a series of such experiments are collected in Table VII. It is apparent that the percentage of nicotinic acid excreted rises continuously as the intake increases. The relation is more clearly seen from the graphical summary of results in Fig. 8.

Table VII. *Excretion of nicotinic acid by guinea-pigs*

Diet	mg. Individual animals	mg. Average	% excreted
Bran, oats, etc.	0·14; 0·14	0·14	—
Deficient	0·0; 0·0	0·0	0
Deficient + 2 mg. nicotinic acid	0·38; 0·54	0·46	23
Deficient + 4 mg. nicotinic acid	0·96; 2·06	1·51	38
Deficient + 6 mg. nicotinic acid	2·5; 2·6	2·55	44

(*b*) *Experiments on dogs*

Tests leading to similar results were also carried out with dogs. The animals were kept on a Goldberger blacktongue diet, with or without addition of yeast, liver extract (or other source of nicotinic acid) or nicotinic acid itself. Those dogs having no supplement duly developed blacktongue, and the amount of nicotinic acid in the urine was found to fall gradually as the disease progressed. Controls receiving the nicotinic acid, or the various addenda containing it, excreted nicotinic acid in corresponding amounts. These confirmatory experiments were carried out in collaboration with Dr E. Kodicek to whom we are indebted for the analyses in Table VIII.

Table VIII. *Gradual fall in excretion of nicotinic acid by dogs on diets deficient in P.P. factor*

Dog no.	Day of exp.	Diet	Nicotinic acid excreted µg. per day	Notes
1	5	Deficient	335	Goldberger diet (plus riboflavin and vitamin B_1) to 30th day
	11	Deficient	250	
	18	Deficient	73	
	28	Deficient	0	
	33	Supplemented with liver extract	108	Eli Lilly "343" liver powder, 4 g. daily, given from 30th to 45th day
	45	Supplemented with liver extract	420	
	57	Deficient	0	Supplement discontinued from 45th day to end
2	5	Deficient	155	Goldberger diet to 30th day
	28	Deficient	0	
	33	Supplemented with nicotinic acid	336	Nicotinic acid, 10 mg. daily, given from 30th to 36th day
	45	Deficient	120	Supplement discontinued after 36th day
	57	Deficient	0	
3	11	Positive control	347	Goldberger diet supplemented with Eli Lilly "343" liver powder, 4 g. daily
	18	Positive control	660	
4	28	Positive control	820	Goldberger diet supplemented with nicotinic acid, 2 mg. per kg. body weight daily

(c) *Excretion of nicotinic acid by rats*

Rats differ from the species hitherto discussed—man, guinea-pig and dog—in that they are able to remain free from symptoms of deficiency disease when kept on a Goldberger diet [Birch *et al.* 1935]. It was of interest therefore to examine the urine of such rats. We found that appreciable amounts of nicotinic acid were still excreted in the urine, even after the rats had been kept for long periods on the deficient diet (Table IX). The most likely explanation seems to be that the rat can synthesize nicotinic acid under certain conditions, either in its tissues or by a symbiotic process analogous with refection. This would account for the relative non-susceptibility of the rat as contrasted for example with man, monkey, pig or guinea-pig. The alternative view is that rats do need nicotinic acid but that a comparatively minute amount suffices in comparison with the larger requirements of other species. These problems can best be settled by balance experiments on rats kept on "synthetic" as well as on maize diets, an account of which will be published separately. In the meantime it may be noted that with large additions of nicotinic acid to the diet the excretion is further raised (Table IX).

Table IX. *Excretion of nicotinic acid by rats*

Diet	Daily excretion in urine (μg.)
Basal diet	25, 25, 23, 12·5
Basal diet + nicotinic acid (10 mg. test dose)	100

Preliminary surveys on human subjects

Effect of test doses. Since the methods previously introduced for assessing the level of nutrition for vitamins C [Harris & Ray, 1935] and B_1 [Harris & Leong, 1936] involved the administration of test doses, to determine the "degree of

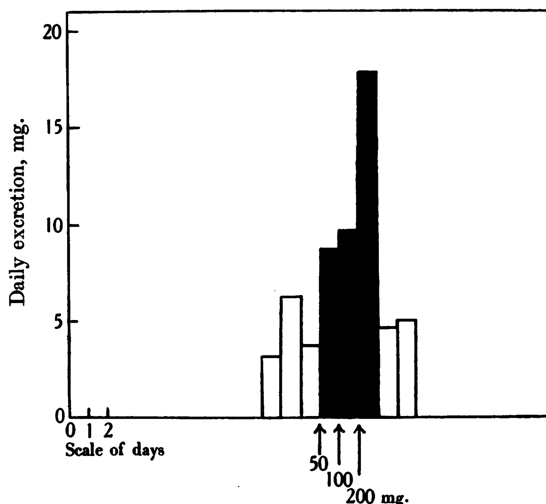


Fig. 9. Increased excretion of nicotinic acid by human subject after test doses (amounts given as shown at arrow).

saturation" of the tissues, it was decided to try out a similar procedure for nicotinic acid. From the tests on experimental animals already recorded it is

clear that with other species at any rate the excretion does in fact increase with the intake. A number of experiments upon ourselves, one of which is shown graphically in Fig. 9, indicate that a similar relation holds for human beings.

Range of resting values and dependence upon intake. Determinations on a group of presumed normal subjects (research workers and laboratory assistants) showed that the usual rate of excretion of nicotinic acid in the urine as determined by this method was about 4–5 mg. per day, the minimum value noted being 3·1 and the maximum 6·2; higher values were however seen when the diet had been enriched with foods especially active in the P.P. factor, e.g. marmite. These values, together with the results of test-dosing, are all collected together for easy reference in Table X.

Table X. *Excretion of nicotinic acid by human subjects, mg. per day*

Description	Resting levels	After test doses, mg.		
		50	100	200
Normal:				
L. J. H.	3·1, 6·2, 3·6, 4·6, 5·0	8·6	9·6	17·9
W. D. R.	3·5, 5·0	14·1	12·9	14·9
W. D. R. smoking heavily	7·3	—	—	—
W. D. R. smoking heavily + marmite	9·8, 12·6, 10·0	—	—	—
Y. L. W.	3·9	—	—	—
G. G. G.	4·9	—	—	—
Deficient:				
Anorexia nervosa	2·5, 2	—	—	—
Pellagra	1·8, 2·9	—	—	—
Pellagra during treatment	4·6, 4·8, 10	—	—	—
? Pellagra spinal-cord condition	2·76	—	—	—

An indication of increased values in subjects who are smoking heavily is worthy of further study.

Low values in disease. We have not yet examined any significant number of deficient subjects but, for the sake of comparison, included in Table X are references to three miscellaneous cases which we were able to investigate through the kindness of Dr Yudkin, Prof. Drummond and Dr Ungley, respectively. It is noteworthy that in these cases (in all of which a defective intake of nicotinic acid may reasonably be presumed to have occurred) the excretions, although certainly subnormal and lower than any of those seen in the normal series, were yet *not vastly* below the normal range and in no single instance did they approach a zero value. It would be unprofitable to enter on any detailed discussion of this finding pending the completion of a more comprehensive survey of deficient cases and the establishment of standards of normality and deficiency, both for resting levels and for responses to test doses. In the meantime it is worth remarking that the conditions for the human may very well be found to resemble those already established in the above pages for the experimental animal: that is, that appreciable amounts of nicotinic acid may still continue to be excreted as the deficiency disease is developing, and therefore that it is not until a late stage of depletion has been reached that the excretion may be expected to fall to near zero. Advanced cases of pellagra are uncommon in this country and we have not yet had an opportunity of examining one. The alternative explanation would be that the test is not completely specific and that a residue of traces of other unidentified pyridine derivatives distinct from nicotinic acid are being measured in the deficient human subject by our test. The animal experiments lend no support to this view, however, since the ultimate value in the late avitaminosis is in fact zero.

Much laborious quantitative work will necessarily be required before standards of normality can be set up with any finality. The object of this paper is rather to describe the working details of the method recommended; and it can be claimed that the method in question does give reproducible results, that it permits of quantitative recovery of added nicotinic acid, that it differentiates between nicotinic acid and certain other pyridine derivatives (such as vitamin B_6 or trigonelline), that we have shown that the titre does in fact vary in accordance with the past intake and is responsive to test doses, and that deficiency in experimental animals can be satisfactorily diagnosed by its use.

Summary

The process, which is based on the König colour reaction, is so planned as to circumvent the operation of various disturbing factors in urine referred to by Euler and by others.

The urine is heated with NaOH, to convert any amide into the acid, and neutralized. The specimen is divided into four portions; one is kept as blank, and to the other three are added 0, 20 and 40 μg. of nicotinic acid. The solutions, which must be protected throughout from the light, are warmed with CNBr, cooled, treated with *p*-aminoacetophenone, allowed to stand and acidified, and the three colour intensities measured in a Pulfrich photometer with S 47 filter, comparing with the blank to which no CNBr has been added. For any given specimen the depth of colour varies with the *p*H, with the concentration of salts, and possibly with other factors, but the three readings always lie on a straight line and by extrapolation to zero the content of nicotinic acid can be accurately determined.

Duplicates agree well, within an error of about $\pm 10\%$, and added nicotinic acid or amide is quantitatively recovered.

In man the output varied according to the intake, and rose after test doses; lowered values were found in pellagra, and with anorexia. The normal range of values was usually from 3 to 5 mg. per day; but further detailed work is still needed to establish exact quantitative standards of normality.

Guinea-pigs or dogs deprived of nicotinic acid showed a progressive fall in the excretion as the symptoms of deficiency developed and, ultimately, a zero value. In rats the output was increased with a high intake of the vitamin, but the continued excretion on a deficient diet suggests some power of synthesis by the rat.

Vitamin B_6 does not interfere.

References

Ackermann (1912). *Z. Biol.* **59**, 17.
Bandier & Hald (1939). *Biochem. J.* **33**, 264.
Barta (1935). *Biochem. Z.* **277**, 412.
Birch, György & Harris (1935). *Biochem. J.* **29**, 2830.
Euler, Schlenk, Heiwinkel & Högberg (1938). *Hoppe-Seyl. Z.* **256**, 208.
Harris (1939). *Chem. Ind.* **58**, 471.
—— & Leong (1936). *Lancet*, **1**, 886.
—— & Ray (1935). *Lancet*, **1**, 71.
—— & Raymond (1939). *Chem. Ind.* **58**, 652.
Kodicek (1939). In the Press.
König (1904). *J. Prakt. Chem.* **69**, 105; **70**, 19.

Prebluda & McCollum (1937). *J. biol. Chem.* **119**, Proc. lxxix.
Strafford & Parry Jones (1933). *Analyst*, **58**, 380.
Swaminathan (1938). *Nature, Lond.*, **141**, 830; *Ind. J. med. Res.* **26**, 427.
Vilter, Spies & Matthews (1938). *J. Amer. chem. Soc.* **60**, 731.
Vongerichten (1899). *Ber. dtsch. chem. Ges.* **32**, 2571.
Zincke (1904). *Liebigs Ann.* **330**, 361.

Note added 13 December 1939. Following the preliminary publication of these results [Harris & Raymond, 1939] we have learned privately from Dr W. R. Aykroyd that very similar values for the excretion of nicotinic acid in urine have been found by Dr M. Swaminathan [paper in Press, *Indian Journal of Medical Research*].