89. ESTIMATION OF NICOTINIC ACID IN ANIMAL TISSUES, BLOOD AND CERTAIN FOODSTUFFS

2. APPLICATIONS[1]

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In the previous paper [Kodicek, 1940] the cyanogen-*p*-aminoacetophenone method for the estimation of nicotinic acid [Harris & Raymond, 1939, 1, 2] was tested on certain biological materials, and the individual steps of the reaction were examined in detail in order to ascertain the most accurate conditions for the application of the method. The procedure finally adopted has been used for determinations on animal tissues, blood and a number of foodstuffs and medicinal preparations. So far the only known biochemical action of nicotinic acid consists in hydrogen transfer in the phosphopyridine-nucleotide coenzyme systems. Therefore, for control tests the nicotinic acid content of preparations of cozymase was estimated and the concentration of cozymase was calculated from it and compared with the concentration found by the spectrographic method.[2] As yet it has not been found possible to distinguish between the coenzymes and free nicotinic acid, but an attempt was made to differentiate three separate fractions.

Working details

The method is in most respects almost identical with that described by Harris & Raymond [1939, 2] for urine, but, as success depends on close adherence to the minutiae of procedure, they are given here in full.

Reagents required. (1) CNBr solution: prepared fresh daily by adding ice-cold 10% aqueous solution of KCN drop by drop to ice-cold saturated Br water until it is just decolorized.

(2) Amino reagent: 5 g. *p*-aminoacetophenone and 30 ml. 32% HCl (sp.gr. 1·16), made up to 100 ml. with distilled water.

(3) 96% ethyl alcohol.

(4) 40% NaOH.

(5) 5% $NaHCO_3$.

(6) Standard solution of nicotinic acid: 100 μg. per ml. This solution is prepared weekly from a solution of 10 times the strength.

Process for animal tissues

(1) *Extraction, hydrolysis, alcohol precipitation and neutralization.* 0·5–2·0 g. of tissue are cut into small slices (only rarely was it necessary to grind the material) and placed with 5 ml. of distilled water plus 1 ml. of 40% NaOH in a small conical flask. The mixture is heated on a steam bath for 1 hr., a glass funnel

[1] Communicated to the Biochemical Society, 9 December 1939 [Kodicek, 1939].

[2] The spectrographic estimation was carried out by Dr D. E. Green, to whom I wish here to express my thanks.

being used to prevent excessive evaporation. The amide and other substances yielding nicotinic acid, such as phosphopyridine nucleotides, are converted by this hydrolysis into nicotinic acid. The mixture is cooled, and 40 ml. of 96% ethyl alcohol are added. The precipitate is centrifuged. The clear solution is neutralized accurately to *p*H 6·5 with conc. HCl in presence of 1–2 ml. of 5% $NaHCO_3$ to adjust the *p*H precisely, bromothymol blue being used as external indicator. The solution is made up with ethyl alcohol to a volume of 50 ml.

Notes. (*a*) The solution is sometimes slightly turbid, but clears when water or CNBr is added. If the natural colour of the material is too intense, a dilution to 100 ml. or more with ethyl alcohol is recommended.

(*b*) The amount of tissue taken for estimation should contain about 50 μg. of nicotinic acid.

(*c*) After neutralization the solution can be left overnight in the refrigerator without loss of nicotinic acid.

Example: Liver, estimated immediately ... 126 μg./g.
Liver, after 24 hr. ... ... 129 μg./g.

(2) *Opening of the pyridine ring and development of colour.* Four graduated 15 ml. stoppered flasks are taken (*X*, *A*, *B* and *C*). To flasks *B* and *C*, 0·2 and 0·4 ml. respectively are added of the standard solution of 10 mg./100 ml. of nicotinic acid (=20 and 40 μg. of nicotinic acid). 10 ml. of the prepared extract are run into each of the 4 flasks. Sample *X* is kept as the blank. All 4 flasks are placed in a water bath at 70–80° for *ca.* 10 min. Then 2 ml. of freshly prepared CNBr reagent are added to flasks *A*, *B* and *C*, which are mixed well and left for 5 min. in the water bath. To the blank, *X*, 2 ml. of distilled water are added instead. Then the 4 flasks are cooled for 5 min. in cold water in a dark room. 0·4 ml. of the amino reagent (see above) is added to all 4 flasks which are then filled to the 15 ml. mark with 96% ethyl alcohol. The contents are mixed and allowed to stand for 5 min. in a dark cupboard. Immediately afterwards, colorimetric measurements are taken with the Pulfrich photometer, using 3 cm. cells and filter S 47. Flask *X* is used as the blank, and samples *A*, *B* and *C* are examined for their contents of nicotinic acid.

Notes. (*a*) It is important not to expose the flasks to light after addition of the amino reagent, as the colour is sensitive to light.

(*b*) The dilution of the alcohol with water may cause a slight turbidity, measurable only by the sensitive photometer. The addition of 2 ml. of distilled water to the blank satisfactorily eliminates the possibility of error on this account. As the blank also contains the same amount of the amino reagent, another potential source of error is likewise eliminated. Similarly the colour of the extract itself does not interfere, as it is present also in the blank. The solution must be practically clear as the photometer acts also as a nephelometer.

(*c*) The amount of the amino reagent added must be accurately measured. During the course of the work it became clear, especially after the experiment illustrated in Fig. 1 of the previous paper, that a larger amount of amino reagent would be better, but during these series of estimations the original smaller amount of amino reagent was maintained. It is advisable to renew the *p*-aminoacetophenone weekly.

(*d*) The preparation of CNBr in ice-cold solutions was found to be important, as otherwise some formation of complex compounds probably takes place which may interfere [Kulikow & Krestowosdwigenskaja, 1930].

Calculations. From the 3 readings (say *A*, *B*, *C*) a graph can be constructed, which follows Beer's law and is a straight line—the colorimetric values being

taken as ordinates and the amounts of nicotinic acid added (viz. zero, 20, 40 μg.) as abscissae. This line when produced backwards cuts the horizontal axis at a point whose distance from the zero point represents the amount of nicotinic acid present in the "unknown" (sample A). By this procedure the amount of nicotinic acid can be estimated graphically, being dependent on the readings on the abscissae corresponding with the additions of 20 and 40 μg. of nicotinic acid. Fuller instructions will be found in the paper of Harris & Raymond [1939, 2].

It has proved of use to apply the following mathematical formula for calculating the content of nicotinic acid, thus eliminating the possibility of a small personal error in the graphical calculations.

$$\mu\text{g. of nicotinic acid per g. tissue} = \frac{A}{B+C-2A} \times \frac{6V}{n},$$

where A, B and C are the corresponding extinctions for the unknown (sample A), and for the unknown plus 20 and 40 μg. of nicotinic acid, respectively (samples B and C); V = the final total volume to which the extract was made up after neutralization, and n the number of grams of tissue taken for estimation.

Example: Extinction found for $A = 0{\cdot}21$, for $B = 0{\cdot}63$, for $C = 1{\cdot}05$; $n = 1$ (since 1 g. of fresh weight of tissue, pancreas, was taken for the estimation); $V = 50$ (neutralized extract made up to 50 ml.).

$$\text{Nicotinic acid} = \frac{0{\cdot}21}{0{\cdot}63+1{\cdot}05-0{\cdot}42} \times \frac{6 \times 50}{1} = 50\ \mu\text{g. per g. fresh tissue.}$$

The formula was calculated upon the basis of the observation that the higher colour values have a greater relative error than the lower, so that the mean of the extinction values B and C gives a fairer assessment of the true result. This mean value represents an extinction equal to 30 μg. of nicotinic acid.

Note. For routine work only 2 readings need be used, since the comparison with the standard reference curve gives another valuable check on the result. As a general rule, however, when working with unknown materials, it is advisable to adhere to the original method (i.e. 3 readings).

Results

(*a*) *Animal tissues and some medicinal preparations.* The results for various animal tissues are given in Table 1. The figures seem to run parallel with the reputed biological values. Liver and adrenals have the highest amount, then follow kidney, heart, muscle and other organs. It may be noted that the eye-lens has a fair amount of nicotinic acid and in human cataract no drop to zero was found as may happen with ascorbic acid [Müller, 1935; Hradecka *et al.* 1937; and others]. Salmon was very rich in nicotinic acid, which accords with previous biological findings [Goldberger & Wheeler, 1928].

The most potent source of nicotinic acid, however, was the proprietary liver extract Eli Lilly "343", which contained 1090 μg. per g. of powder, equivalent to 52 μg. of nicotinic acid per g. fresh weight of liver. Yeastrel and marmite are very rich. Baker's and brewer's yeasts have high concentrations of nicotinic acid allowing for the high content of water.

(*b*) *Dairy products* (*milk and eggs*) *and human milk.* From the values given in Table 2 it will be seen that a surprising finding is the very low content of nicotinic acid in milk, which varied from less than 1 to 5 μg. per ml. The estimations were made during the months of November and December. Since, owing to the sensitivity of the method, even less than 1 μg. can be detected,

Table 1. *Concentrations of nicotinic acid in animal tissues and medicinal preparations*

Material	Nicotinic acid, μg. per g. fresh wt. of tissue*	Reputed biological (pellagra-preventing) value
Liver (ox)	170	Good
Liver (sheep)	200	Good
Adrenals (sheep)	135	—
Heart (sheep)	60	—
Muscle, skeletal (ox)	43	Good
Kidney (sheep)	75	—
Pancreas (sheep)	40	—
Spleen (ox)	53	—
Lung (ox)	43	—
Brain (ox)	30	—
Eye-lens (ox)	50	—
Eye-lens, human, cataract	100	—
Fish:		
Salmon	84	Good
Herring	40	—
Cod	30	—
Roe, herring	21	—
Roe, turbot	23	—
Medicinal preparations:		
Liver extract, Eli Lilly "343"	1090	Very good
Yeastrel	400	—
Marmite	640	—
Yeast, baker's (moisture 69%)	74	Good
Yeast, brewer's (moisture 78%)	91	Good

* Results have been calculated as mean values, from at least three estimations.

Table 2. *Nicotinic acid in dairy products, milk and eggs, and human milk*

Material	Nicotinic acid μg. per ml. or g. fresh wt. respectively	Biological (pellagra-preventing value)
Egg-white	<0·5	None*
Egg-yolk	10	
Milk,† fresh, Nov.–Dec.	<1–5	Fair [Sebrell, 1934; Wheeler & Sebrell, 1933]
8 samples, av.	3	None*
Milk, dried	25	
Human milk:†		
Mrs E.	<1	
Mrs R.	<1	
Mrs M.	<1	

* Harris & Kodicek [1940] found egg-white and fresh milk inactive in blacktongue when given in curative tests in doses as high as 90 g. and 120 ml. per kg. wt. of dog, respectively.

† 2, 4 and 8 ml. of milk were used for the estimations; hydrolysis with 4, 8 and 10% NaOH yielded the same low results.

there seems no doubt that the amount of nicotinic acid in milk is indeed negligible, and especially is this so for human milk. The higher concentration of nicotinic acid in dried milk corresponds well with the amount found in fresh milk calculating on the wet basis. Recovery of added nicotinic acid was 105% [cf. previous paper: Kodicek, 1940].

Egg-white was found to be negative, whereas the yolk contained 10 μg. per g. fresh weight. The biological values for milk given by Wheeler & Sebrell [1933] and Sebrell [1934][1] do not agree with these chemical findings, but in our own

[1] See Discussion, below.

experiments [Harris & Kodicek, 1940] we were able to confirm the negative result. Even 120 ml. of fresh milk per kg. of body weight daily failed to cure blacktongue in dogs. Egg-white likewise proved to be deficient when tested on dogs in curative or preventive doses of 100 g. daily [Harris & Kodicek, 1940].

(*c*) *Blood.* A special modification was worked out as follows: 5 ml. of oxalated blood are hydrolysed with 0·5 ml. of 40 % NaOH for 1 hr. on a boiling water bath. After cooling, 50 ml. of acetone are added, the mixture is centrifuged and the clear solution neutralized. The contents are made up to the 50 ml. mark with

Table 3. *Estimation of nicotinic acid in blood*

Description	A Estimated in acetone solution.* Nicotinic acid μg. per ml.	B Acetone evaporated off, estimation carried out in ethyl alcohol.† Nicotinic acid μg. per ml.
Horse blood:		
Whole blood, oxalated	4·7	4·8
Red cells	14·0‡	—
Serum	0·0	0·0
Plasma	0·0	0·0
Sheep blood:		
Whole blood, oxalated	4·7	4·5

* A = 5 ml. of oxalated blood hydrolysed with aqueous NaOH and estimated in acetone solution in usual manner.

† B = 5 ml. of oxalated blood hydrolysed with aqueous NaOH, 50 ml., of acetone added, solution centrifuged till clear, acetone evaporated off *in vacuo*, residue dissolved in 96 % ethyl alcohol, made up to 50 ml. and estimated.

‡ The value for whole blood calculated from the haematocrit reading gives 4·5 μg. per ml. of whole blood, in good agreement with the direct experimental estimation.

Table 4. *Different fractions of nicotinic acid in blood (horse)*

Description	Nicotinic acid μg. per ml. whole blood
"Fraction 1". Unhydrolysed alcohol-soluble fraction (free nicotinic acid plus one-fifth of amide)	0·0
"Fraction 2". Hydrolysed alcohol-soluble fraction (nicotinic derivatives soluble in alcohol, which are converted into nicotinic acid upon hydrolysis)	0·0
"Fraction 3". Alcohol-insoluble fraction hydrolysed (residuum consisting of derivatives insoluble in ethyl alcohol, 96 %)	5·0
"Fraction 4". Unhydrolysed acetone-soluble fraction	0·0
"Fraction 5". Hydrolysed acetone-soluble fraction	0·5
"Fraction 6". Acetone-insoluble fraction hydrolysed	4·0

"Fraction 1" was obtained by the following treatment: 5 ml. of whole oxalated horse blood were extracted with 40 ml. of 96 % ethyl alcohol for 30 min. at 30° and vigorously stirred. The mixture was centrifuged, the clear solution made up with ethyl alcohol to 100 ml. and estimated without hydrolysis.

"Fraction 2". The same as "Fraction 1", but 50 ml. of the final solution taken and hydrolysed with 5 ml. of 40 % NaOH using a reflux cooler, then the nicotinic acid estimated as usual.

"Fraction 3". The residuum obtained after the centrifuging of "Fraction 1" was hydrolysed with 5 ml. of distilled water and 0·5 ml. of 40 % NaOH.

"Fractions 4, 5 and 6" are similar to "Fractions 1, 2 and 3", respectively, but acetone was used for extraction instead of ethyl alcohol.

96% ethyl alcohol and the estimation is carried out as described for animal tissues. Table 3 shows the results and the effect of different modifications in procedure.

No nicotinic acid was found in serum and plasma, the whole amount being in the red blood cells. As the simpler method with acetone described under A gives the same results as estimations in ethyl alcohol, the former was adopted as a routine.[1] Only a few examples of the estimations on blood are given in this paper, and more extensive results will be presented later.

In the work summarized in Table 4 an attempt was made to differentiate various forms of nicotinic acid derivatives present in blood. The methods used were the same as those adopted for animal tissues (see Table 8 in this paper, where more details will be given). From these findings it may be concluded that the whole of the nicotinic acid, which is present only in the red blood cells, is bound to substances which are insoluble in 96% ethyl alcohol and probably also in acetone. At present it is not certain whether the whole of the nicotinic acid can be attributed to phosphopyridine nucleotides or whether there are also other substances containing the nicotinic acid grouping.

The results in Table 4 are in good agreement with values obtained by quite a different procedure and recorded in Table 3, the total nicotinic acid being in the former 5·0 and 4·5 μg. of nicotinic acid per ml. of whole blood, respectively, and in the latter 4·7 and 4·8 μg. per ml., respectively.

(*d*) *Cereals and vegetables.* Table 5 shows the results of tests on cereals and vegetables. As has already been stated in the previous paper, certain difficulties appeared in the interpretation of the results for these substances. No such trouble was encountered with animal tissues, from which it was found that all the nicotinic acid could be readily extracted with boiling water. This suggests that in animal tissues all the nicotinic acid is present in the free state, or bound to substances soluble in water (such as phosphopyridine nucleotides) or very easily split off by boiling water. This question will be dealt with later. But in the case of some cereals it was found that only a small part of the chromogen ("apparent" nicotinic acid) passed into solution upon extraction with water at 100°. As it seemed certain that this behaviour was not due to imperfect extraction, it was necessary to assume that some unknown substance giving the cyanogen-*p*-aminoacetophenone reaction was more strongly bound and liberated only after hydrolysis with NaOH. This difference between cereals and animal tissues is of some importance, and it was not at first certain which of the two methods of extraction—by hydrolysis (extractions *a* and *b*) or by boiling water (extraction *c*)—gave the true content of nicotinic acid which would correspond with the biological test. Such discrepancies between the reputed biological values of these cereal foodstuffs and the chemical values as obtained after the more drastic method of extraction supported the view that some unspecific chromogen was being set free which was not in fact active as a pellagra-preventing substance.[2] During the course of the present investigations Harris & Kodicek [1940] were able to test this theory. In experiments on dogs with blacktongue, a specimen of yellow maize flour (sample *C*), which showed chemically an unusually high content of the "apparent" nicotinic acid, 27 μg. per g. material, was tested. It was found in fact that this sample was just as deficient as the others examined at the same time, 4 dogs developing blacktongue in the usual period of about

[1] With blood, acetone was found to be preferable to alcohol since a clear solution free from pigments was more readily obtained.

[2] The possibility of such an unspecific reaction was discussed in greater detail in the previous paper.

Table 5. *Estimation of chromogens ("pyridine-like substances") in cereals*

Method of extraction ... (see below)	Chromogens calculated as μg. of nicotinic acid per g. of material				Biological pellagra-preventing values [Sebrell, 1934]
	a	*b*	*c*	*d*	
Cereals:					
White flour	5	8	—	—	—
	12	—	—	—	—
Hovis flour	26	—	—	—	—
Whole wheat	33	—	—	—	Slight
Wheat germ	39	38	27	17	Good
White bread (moisture, 31%)	<5	—	—	—	—
Brown bread (moisture, 34%)	25	28	12	13	—
	33	—	—	—	—
Rice powder	16	—	—	12	—
	27	26	17	5	—
White maize meal	13	—	—	—	Deficient*
Yellow maize meal:					
Sample A	10	—	—	—	Deficient*
Sample B	18	33	—	—	—
Sample C	12	27	6	—	Deficient†
Sample D	41	45	8	37	—
Pea meal	49	—	—	—	—
	25	20	18	5	—
Caseinogen ("Glaxo", A/E)	<5	—	—	—	—
Sugar	<2	—	—	—	—
Starch, rice, raw	<5	—	—	—	—
Goldberger diet:					
I	10	—	—	—	Deficient*
II	17	26	6	20	Deficient†
Vegetables:					
Potatoes	20	—	—	—	None
Spinach	17	—	—	—	Fair
Cabbage	3	—	—	—	Fair
Carrots	<5	—	—	—	Slight
Tomatoes	<5	—	—	—	Good
Apples	<5	—	—	—	None

* Harris & Kodicek [1940] have used these cereals successfully in the production of black-tongue. Goldberger diet I contained: white maize meal 4000, pea meal 500, extracted casein A/E (Glaxo) 600, salts 130, cottonseed oil 300. If the content of nicotinic acid is calculated for this diet from the individual constituents, it is found to be 13 μg. per g., which is in good agreement with the figure obtained by actual determinations on the whole diet itself.

† Goldberger diet II contained the same amount of yellow maize meal (sample C) instead of white maize meal.

Extractions. The different methods of extraction have been more fully discussed in the previous paper, Table 1 [Kodicek, 1940].

a = Extracted during hydrolysis with aqueous NaOH = total chromogens, active plus inactive.

b = Extracted during hydrolysis with alcoholic NaOH = total chromogens, active and inactive.

c = Extracted with water at 100° for 1 hr., centrifuged and solution hydrolysed = active nicotinic acid (?).

d = Residuum left after water extraction *c*, extracted during hydrolysis with alcoholic NaOH.

10–30 days. The water extract of this sample yielded very low chemical values—6 μg. per g.—which would agree with the negative biological result. Moreover, the solubility in boiling water runs parallel with the behaviour of the active nicotinic acid present in animal tissues from which it appears to be completely extracted by water. It is therefore probable that extraction with boiling water is the process which shows the true content of "active" nicotinic acid.

In Table 5 some results are given in column *c* obtained with this procedure. There is, however, no need to assume that the substance so extracted from

cereals by boiling water is necessarily identical with the active pellagra-preventing substance until experiments, which are now in progress, have proved this suggestion to be true or otherwise.

No explanation has yet been found why, after hydrolysis in water, lower figures are sometimes found than after hydrolysis in ethyl alcohol.

Stability of nicotinic acid

It is known that nicotinic acid is very stable to heat and many kinds of chemical treatment. Table 6 shows that the storage of foodstuffs for as long as 3 years did not lower their content of nicotinic acid. Also urine stored for 1 month showed no appreciable loss of nicotinic acid.

Table 6. *Stability of nicotinic acid*

Material	Nicotinic acid μg. per g.
Yeastrel stored:	
(*a*) Since Nov. 1936	380
(*b*) Since Sept. 1937	440
(*c*) Since June 1938	360
(*d*) Since Aug. 1939	320
Urine, human, preserved with sulphur-free toluene in refrigerator:	
1st day	12
8th day	12
18th day	10
25th day	14

Table 7. *Recovery of nicotinic acid from cozymase*

Description	Amount of cozymase taken, expressed as nicotinic acid* μg.	Amount of nicotinic acid found μg.	Recovery of nicotinic acid %
Cozymase (Euler), 90% purity:			
(*a*) Without hydrolysing, "free nicotinic acid"	17	0	0
(*b*) Hydrolysed in 5 ml. with 2 ml. NaOH, 20%	17	15	88
Cozymase (Green), sample A, 11·3% purity:			
(*a*) Without hydrolysing, "free nicotinic acid"	45	0	0
(*b*) Hydrolysed in 5 ml.			
(1) With 1 ml. NaOH, 20%	45	37	82
(2) With 2 ml. NaOH, 20%	45	40	89
Cozymase (Green), sample B, 52% purity:			
(*a*) Without hydrolysing, "free nicotinic acid"	58	0	0
(*b*) Hydrolysed in 5 ml. with 2 ml. NaOH, 20%	58	56	97

* The amount of nicotinic acid was calculated from the concentration of pure cozymase determined spectrographically by Dr D. E. Green after enzymic reduction to dihydrocozymase. Molecular weight of cozymase was taken as 681; that of nicotinamide is 122 [Lutwak-Mann, 1939].

Recovery of nicotinic acid from cozymase

Since the main if not the only source of nicotinic acid in animal tissues is probably the phosphopyridine nucleotides, the amount of nicotinic acid in different preparations of cozymase was estimated (Table 7) and compared with the calculated amount, the degree of purity of these different enzyme specimens

being determined spectrographically by Dr D. E. Green. No so-called "free nicotinic acid" was found in untreated preparations of cozymase. The recovery of nicotinic acid from preparations of cozymase was calculated to be from 88 to 97%. The linkage of nicotinic acid in cozymase is very weak, and after heating for 60 min. on a steam bath nicotinic acid already appeared in the free state in a concentration of 13%. As it is probable that it was the amide in solution which was being estimated, a five-times higher value ought to be calculated, the nicotinamide when unhydrolysed giving only 20% of the colour of nicotinic acid itself (see section on the recovery of nicotinamide in previous paper). It may therefore be concluded that as much as *ca.* 65% of nicotinamide would be liberated simply by boiling for 60 min.

Differentiation of various fractions

Using three different methods of treatment three different sets of results have been obtained for animal tissues, and this may indicate that nicotinic acid in animal tissues can be divided into three or more different fractions. This suggestion is, however, at present nothing more than provisional. Table 8 shows

Table 8. *Different fractions of nicotinic acid in animal tissues and some other substances*

	"Fraction 1" Unhydrolysed alcohol-soluble fraction "Free nicotinic acid", μg. per g. fresh wt.	"Fraction 2" Hydrolysed alcohol-soluble fraction Nicotinic acid μg. per g. fresh wt.	"Fraction 3" Total nicotinic acid μg. per g. fresh wt.
Animal tissues:			
Liver (ox)	20	90	170
Liver (sheep)	25	120	200
Adrenals (sheep)	24	66	135
Heart (sheep)	15	20	60
Muscle skeletal (ox)	7	11	43
Kidney (sheep)	25	80	75
Pancreas (sheep)	10	30	40
Brain (ox)	8	10	30
Spleen (ox)	0	63	53
Blood (horse)	0	0	4·7
Other materials:			
Liver powder, Eli Lilly "343"	250	1030	1090
Do.: calculated per g. fresh wt. of liver	12	49	52
Yeastrel	380	380	400
Marmite	600	580	640
Baker's yeast (moisture 69%)	10	25	74
Brewer's yeast (moisture 78%)	14	60	91

"Fraction 1." Material first extracted with 96% ethyl alcohol by grinding with sand or stirred for 2 hr., and without subsequent hydrolysis = "unhydrolysed alcohol-soluble fraction", representing free nicotinic acid plus 20% of free amide.

"Fraction 2." Same as "Fraction 1" but subsequently hydrolysed = "hydrolysed alcohol-soluble fraction".

"Fraction 3." Original material or aqueous extract first hydrolysed as a whole = "total nicotinic acid".

the results expressed as different fractions. "Fraction 3" is the total nicotinic acid present in animal tissues; "Fraction 2" is the hydrolysed alcohol-soluble fraction, which consists of substances soluble in 96% ethyl alcohol. This fraction is partly the nicotinic acid and amide in the free state and partly alcohol-soluble

'bound nicotinic acid" which only gives the cyanogen-*p*-aminoacetophenone reaction after hydrolysis. The latter portion can easily be calculated, knowing the concentration of free nicotinic acid and roughly that of the amide, estimated in "Fraction 1". In "Fraction 1" the unhydrolysed alcohol-soluble part is estimated, which seems to consist of free nicotinic acid plus one-fifth of the free amide, since unhydrolysed amide yields only 20% of the maximum colour.

The differences between the total nicotinic acid and the fraction obtained on extraction with ethyl alcohol before hydrolysis (hydrolysed alcohol-soluble fraction) are not due to imperfect extraction of the latter. No difference was found whether the alcohol extraction was performed simply by grinding the tissue with sand in ethyl alcohol or by stirring for 2 hr. at 50°. Nor is the variation due to the difference in *p*H. It is true that the "alcoholic fraction" is extracted at a neutral reaction and that the "total nicotinic acid" fraction is treated with ethyl alcohol at a very strong alkaline reaction after hydrolysis: control tests showed, however, that neutralization of the hydrolysed total nicotinic acid extract "Fraction 3" before addition of ethyl alcohol did not alter the results.[1] The different figures for the various fractions must therefore be due either to incomplete precipitation of certain nicotinic acid derivatives or else to selective precipitation of certain fractions. How the phosphopyridine nucleotides will behave in this direction has not yet been examined. Liver, adrenals, heart, brain and muscle showed notable differences between the total amount of nicotinic acid and the hydrolysed alcohol-soluble fraction, indicating that in these tissues a considerable portion of the nicotinic acid was estimated in a form which is insoluble in ethyl alcohol. The possible effect of autolysis in causing such difference has still to be examined.

Yeastrel and marmite showed no differences as between the 3 fractions, indicating no doubt that during their preparation the nicotinic acid had been liberated, probably by autolysis. In Eli Lilly "343" liver powder only the total and hydrolysed alcohol-soluble fractions gave similar figures. The unhydrolysed fraction was only 25% of the total nicotinic acid. This may mean that in this preparation this small part only is present as free nicotinic acid, or that it is present entirely as amide, which gives a less intense colour if unhydrolysed. It is interesting to observe that this product, which is presumably prepared from alcoholic extracts of liver, contains nearly the same amount of nicotinic acid, calculated for fresh weight of liver, as the hydrolysed alcohol-soluble fraction of liver described in this paper (52 and 90 μg. per g. fresh liver, respectively).

The unhydrolysed alcohol-soluble fraction, the so-called "free nicotinic acid", is present only in traces in most tissues, and it is possible that it appears only on autolysis and that no free nicotinic acid is present as such in animal tissues. In horse blood all the nicotinic acid is present in substances which are precipitated by ethyl alcohol, so that both the hydrolysed and unhydrolysed alcohol-soluble fractions yield no nicotinic acid.

Discussion

The concentrations of nicotinic acid found in animal tissues agree fairly well with the reputed biological values. The high concentration in the eye-lens is interesting as nicotinic acid, being concerned with hydrogen transfer in phosphopyridine nucleotides, may play an important role in the respiration of the eye-

[1] Added nicotinic acid (100 μg.) could be recovered to the extent of 110 and 90% in "Fraction 2" of liver; in unhydrolysed alcohol-soluble fraction added nicotinic acid could be recovered to the extent of 100%.

lens as possibly also does ascorbic acid. The latter, unlike nicotinic acid, appears however to be diminished in cataract.

Whereas the results with animal tissues seem unequivocal, it was found that cereals contain some interfering substance or substances, possibly pyridine derivatives, which, though giving a positive colour reaction, are inactive in black-tongue. It seems likely that extraction with boiling water will prove to be more specific, but this is a matter for further experiments which are in progress. This procedure certainly gives significantly lower results for cereals and is a reliable procedure for animal tissues. At present no final conclusions can be given about cereals.

A surprising finding is the very low concentration of nicotinic acid in milk. Wheeler & Sebrell [1933] and Sebrell [1934] estimate the pellagra- and black-tongue-preventing value of milk as "fair". Amounts of 30 ml. per kg. weight of dog daily delayed the onset of blacktongue in dogs for a considerable length of time in their experiments. Milk is also recommended in nearly all text-books as being a good source of the pellagra-preventing factor for patients suffering from pellagra. However, dogs which we have kept on a blacktongue-producing diet [Harris & Kodicek, 1940] showed no improvement even after doses of 120 ml. of fresh milk per kg. body weight, nor in prophylactic tests did doses of about 30 ml./kg. delay the appearance of symptoms of blacktongue beyond the time observed for comparable deficient control animals (e.g. in *ca.* 2–4 weeks). This biological result therefore confirms the chemical determination. Again, human milk was found chemically to be deficient. This result raises questions about the metabolism of the growing organism. As infants need nicotinic acid for their coenzyme systems, it would be interesting to ascertain whether infants are born with a large store of nicotinic acid or have some means of synthesizing it.

In blood nicotinic acid was found only in the red cells, and in a form which is totally precipitated by ethyl alcohol and acetone. Whether the total nicotinic acid in blood is derived only from phosphopyridine nucleotides cannot at present be ascertained, but it seems highly probable.

The differentiation of various fractions is so far nothing more than provisional. It seems that in animal tissues free nicotinic acid appears very rapidly on autolysis, but that in living tissues it is not present, or else is present in only minute amounts. The rate of appearance of free nicotinic acid (or amide) would thus be an expression of the concentration of enzymes which are able to split the phosphopyridine nucleotides and other nicotinic derivatives. According to this point of view kidney, pancreas and spleen would be rich in such enzymes. More work is needed to differentiate the coenzymes from other possible sources of nicotinic acid in tissues, and investigations are in progress.

Summary

1. The cyanogen-*p*-aminoacetophenone method of Harris & Raymond [1939, 1, 2] was applied to the estimation of nicotinic acid in numerous animal tissues, medicinal preparations, blood, dairy products and cereals. The results were compared with the reputed biological pellagra-preventing values, as given by other authors or as found in new experiments in this institute.

2. Results for animal tissues run parallel with the biological values. The highest values were found for liver and adrenals. Salmon is rich in nicotinic acid. Eye-lens, normal and with cataract, contains a fair amount.

3. Egg-white was found to be deficient, both in chemical and biological tests. Milk was also found to be surprisingly low, and this was confirmed in experiments on canine blacktongue.

4. In horse and sheep bloods about 4·7 μg. of nicotinic acid per ml. were found, all of it in the red cells. The whole of the nicotinic acid seems to be bound to substances which are insoluble in excess of ethyl alcohol and in acetone.

5. Substances are present in cereals which give a non-specific colour reaction and complicate the estimation of the active nicotinic acid. It seems probable that extraction with water may be used to separate the active antiblacktongue principle from interfering substances.

6. Storage of certain food extracts over a period of years did not diminish their contents of nicotinic acid.

7. Cozymase was found to be quantitatively hydrolysed and recovered (viz. 88–97 %).

8. Nicotinic acid is present in various forms of combination and an attempt was made at a provisional differentiation of the different fractions. Certain tissues, namely liver, adrenals, heart and skeletal muscle, appeared to contain various fractions, differentiated by their insolubility in ethyl alcohol.

9. It appears that little or no free nicotinic acid is present in living animal tissues but that on autolysis it is rapidly set free from coenzymes or other combined forms.

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