[CONTRIBUTION FROM THE DIVISION OF ANIMAL NUTRITION, UNIVERSITY OF ILLINOIS]

Nicotinic Acid Metabolism. II. The Metabolism of Radioactive Nicotinic Acid and Nicotinamide in the Rat[1,2]

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Following the injection of C^{14}-carboxyl-labeled nicotinic acid and nicotinamide into rats, the radioactive metabolites excreted in the urine were separated by paper chromatography and detected by radioautographs. Nicotinuric acid accounted for 60% of the C^{14} excreted following large doses of radionicotinic acid, while an N^1-methylnicotinamide-like compound or compounds accounted for 65% of the C^{14} excreted following large doses of nicotinamide. At least 7 radioactive metabolites resulted from the administration of nicotinic acid and 9 from nicotinamide. Of the unidentified metabolites, the chief one, designated compound 8, follows N^1-methyl-2-pyridone-5-carboxylamide closely on paper chromatograms and results from the metabolism of both nicotinic acid and nicotinamide.

The metabolism of nicotinic acid has been studied and many of its metabolic excretion products identified in numerous experiments reported in recent years.[3-12]

We have shown that most of these known metabolites of nicotinic acid can be separated and identified on paper chromatograms.[13]

The over-all metabolism of radioactive nicotinic acid and nicotinamide in mice was studied by Roth, *et al.*,[14] who showed that 45% of the radioactivity administered was excreted in the urine while only 4.2% appeared in the expired CO_2 in 100 hours.

We have reported the application of paper strip chromatographic methods to the study of the metabolism of radioactive nicotinic acid and nicotinamide in the rat.[15] Leifer, *et al.*,[16] have since reported the application of similar methods to the study of nicotinic acid and nicotinamide metabolism in the dog, mouse and hamster, as well as in the rat. The paper strip chromatographic method reported in the previous paper[17] has been used to separate the metabolic products excreted in the urine of the rat following the intraperitoneal injection of radioactive nicotinic acid and nicotinamide.

Experimental

Glass metabolism cages modified from those of Roth, *et al.*,[14] were used. The rats were given the radionicotinic acid or amide by intraperitoneal injection and immediately placed in the metabolism cages. A water cup and a food cup (in those cases in which the animal was fed) were fastened to the screen floor of the cage and the food was slightly moistened with water to minimize scattering. Dry CO_2-free air was drawn through the system at the rate of 1.5 to 2 liters per minute measured by a wet test-meter. Urine, feces and expired CO_2 were collected over various periods depending on level of radionicotinic acid or amide intake.

The radiocarbon content of the carcass, feces, expired CO_2, and urine (by difference) of rats injected with each compound is given in Table I. All measurements of radioactivity were done with the vibrating-reed electrometer following combustion or (in the case of expired air) liberation of the CO_2 from $BaCO_3$.

TABLE I

DISTRIBUTION OF CARBON-14 IN CO_2, FECES, CARCASS AND URINE OF RATS AFTER A 48-HOUR COLLECTION PERIOD FOLLOWING THE ADMINISTRATION OF RADIONICOTINIC ACID OR NICOTINAMIDE

Rat no.	Radioactive comp. injected	Administered radioactivity (μc.)	Carcass radioactivity in μc./g. live wt.	Total radioactivity, % Expired CO_2	Feces[a]	Carcass[b]	Urine (by diff.)
1	Nicotinic acid	93.5	0.088	2.26	2.79	30.5	64.7
3	Nicotinic acid	67.3	.060	1.41	0.873	23.1	75.8
2	Nicotinamide	94.5	.092	5.58	0.975	23.3	69.1
4	Nicotinamide	58.1	.072	24.3	2.26	28.6	43.4

[a] Each sample was extracted by steaming 3 times with water and washing with alcohol; the extract was made up to 100 ml. An aliquot of 100 μl., was measured into a combustion boat and dried before placing on the combustion line. [b] Each carcass was digested by the procedure of Weinman.[22] The digest was made to volume of one liter with water and aliquot of 100 μl., was measured into the combustion boat and dried before being placed in the combustion line.

As can be seen from Table II, all the rats except numbers 5 and 6 were given rather high doses of either nicotinamide or nicotinic acid, and no food was given during the 48-hour collection period. Rats 5 and 6 were given lower (approaching normal) levels of nicotinic acid and nicotinamide. These rats were kept in their metabolism cages on a synthetic nicotinic acid-free diet for four days. During this time rat 5 received 200 μg. of labeled nicotinic acid by injection per day and rat 6, the same amount of labeled nicotinamide. At the end of each collection period the animals were sacrificed, and the lungs, liver, heart, kidneys, spleen, blood and remainder of carcass were separately frozen for work on the intermediary metabolism of the radioactive vitamin, to be reported later. The expired CO_2 was caught in NaOH traps and precipitated and collected as $BaCO_3$. The feces were stored in a desiccator. The urine samples were adjusted to *p*H 6.8 and evaporated almost to dryness under a stream of air at approximately 40°.

The concentrated urine samples were chromatographed on paper strips or sheets using Whatman No. 1 filter paper.

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(2) Presented before the American Society of Biological Chemists at the Federation meeting held in Cincinnati, Ohio, April, 1951 (*The Federation Proc.*, **10**, 203 (1951)).

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TABLE II

RADIOACTIVE NICOTINIC ACID OR NICOTINAMIDE WITH C_{14}-LABELING AT CARBOXYL GROUP ADMINISTERED TO INDIVIDUAL ALBINO RATS UNDER DIFFERENT CONDITIONS

Rat no.	Wt. of animal, g.	Radioactive comp. injected intraperitoneally[a]	Dose per injection[b]	Collection period, days	Food intake[c]
1	325	Nicotinic acid	2.518 mg./48 hr.	2	None
2	240	Nicotinamide	2.4365 mg./48 hr.	2	None
3	260	Nicotinic acid	1.813 mg./48 hr.	2	None
4	230	Nicotinamide	1.493 mg./48 hr.	2	None
5	210	Nicotinic acid	230.6 gamma/day	4	Niacin-free
6	280	Nicotinamide	236.7 gamma/day	4	diet[d]
7	200	Nicotinamide	1.852 mg./48 hr.	2	None
8	195	Nicotinic acid	2.065 mg./48 hr.	2	None

[a] Radioactivity of nicotinic acid and nicotinamide was 37.03 μc./mg. and 38.70 μc./mg., respectively. [b] Compounds were dissolved into 0.9% saline solution and sterilized before use. [c] Water *ad libitum*, in every case throughout. [d] Niacin-free diet was prepared with sucrose 78; casein 15; salts mixture No. 446, 4; cystine 0.15; corn oil 5%; and synthetic vitamins (thiamine hydrochloride 5; riboflavin 5; pyridoxine hydrochloride 2.5; calcium pantothate 20; biotin 0.1; folic acid 1.0; 2-methyl-1,4-naphthoquinone 1.0; choline chloride 1000 gamma); vitamin A 20 U.S.P. units; vitamin D 2 U.S.P. units; and vitamin E (α-tocopherol acetate 0.1 mg.) for 1 g. of diet.

Two solvent systems were used for most of this work, the first consisting of the upper layer from an equilibrium mixture of 45 parts *n*-butanol, 5 parts acetone and 50 parts water (hereafter referred to as the acetone system), and the second of 80% of the above upper phase plus 20% ethyl cellosolve (cellosolve system). The latter was used to try to improve the separation of the slow moving compounds which overlapped one another when the acetone system was used.

After the paper strip chromatograms were completely dried, radioautographs were made using Eastman no-screen X-ray film either in strips $1^7/_8 \times 14''$ or sheets $11'' \times 14''$. The time of exposure varied markedly, but exposures of 20 to 30 days, or longer, were necessary to demonstrate all the metabolites. Using the pure radioactive vitamins it was found that a 24-hour exposure or less was sufficient for a chromatogram spot of 200 counts per minute (as read on a thin window, 1.4 mg. sq. cm., GM Monitor, Tracerlab Model SU3A), while 20 counts per minute required over 20 days. Radioautographs of paper strip chromatograms of the pure compounds and of the urines from all the treated rats using the "acetone" solvent systems are given in Fig. 1.

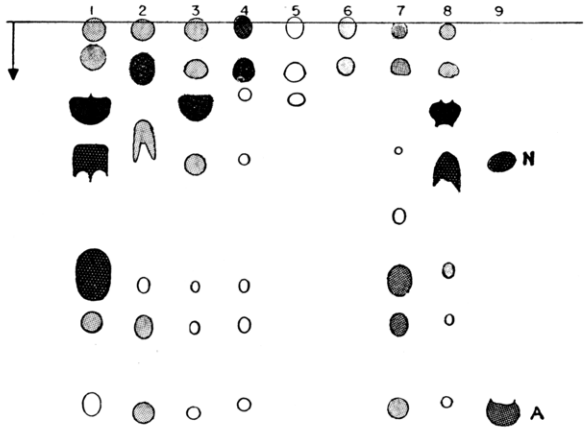

Fig. 1.—Schematic drawings of chromatographic radioautograms of pure mixture of radioactive nicotinic acid (N) and its amide (A) (No. 9); and urine samples from rats receiving high (No. 1, 3, and 8) or "normal" (No. 5) doses of radionicotinic acid, and from rats receiving high (No. 2, 4, and 7) or "normal" (No. 6) doses of radionicotinamide. Descending chromatograms; solvent system: *n*-butanol 45, acetone 5, water 50.

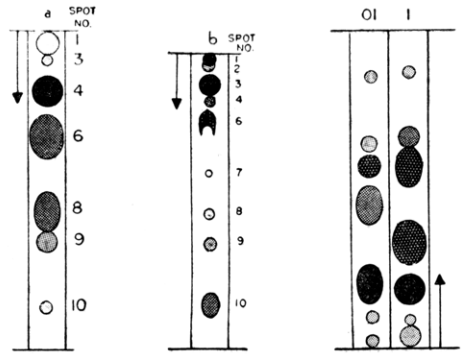

Fig. 2.—Schematic drawings of chromatographic radioautograms of urine from rat 1 (a) and rat 2 (b). Relative radioactivities of each spot are listed in Tables III and IV. Descending chromatograms; solvent system: *n*-butanol 45, acetone 5, water 50.

Fig. 3.—Schematic drawings of chromatographic radioautographs of a urine sample hydrolyzed (strip 01) and unhydrolyzed (strip 1) from rat No. 1 given C^{14}-nicotinic acid. Descending chromatograms; solvent system: *n*-butanol 45, acetone 5, water 50.

The relative radioactivities of the various compounds separated on the paper strip chromatograms of the urines from rats 1 and 2 (rat 1 received nicotinic acid and rat 2 nicotinamide) were determined on a Q gas counter and scaler (Nuclear Chemical and Instrument Lab., Chicago, Model No. 163). In order to do this, a radioautograph was first made of each paper strip chromatogram; then the paper strip was cut according to the indicated spots and the sections of the strip were counted. The relative activities of each spot are given along with the radioautographs and identification of the spots in Figs. 2a and 2b and Tables III and IV.

TABLE III

RELATIVE RADIOACTIVITIES OF CHROMATOGRAMS OF URINE FROM RAT 1 GIVEN C_{14}-NICOTINIC ACID, DETERMINED BY Q-GAS COUNTER

Spot no.	Counts/min.	Relative activities of C^{14} (%)	Corresponding to the position of
1	291.2	3.50	CoI (tentative)
3	227.3	2.73	N′-Methylnicotinamide (trigonelline)
4	5064.5	60.93	Nicotinuric acid
6	2024.5	24.36	Nicotinic acid
8	643.2	7.74	Unknown
9	40.0	0.48	N′-Methyl-2-pyridone-5-carboxylamide
10	20.0	0.26	Nicotinamide

TABLE IV

RELATIVE RADIOACTIVITIES OF CHROMATOGRAMS OF URINE FROM RAT 2 GIVEN C_{14}-NICOTINAMIDE, DETERMINED BY Q-GAS COUNTER

Spot no.	Counts/min.	Relative activities of C_{14} (%)	Corresponding to the position of
1	628.4	6.08	CoI (tentative)
2	1031.4	10.78	Unknown
3	6406.4	66.94	N′-Methylnicotinamide (trigonelline)
4	90.0	0.90	Nicotinuric acid
6	631.2	0.59	Nicotinic acid
7	80.0	0.84	Unknown
8	75.0	0.99	Unknown
9	232.3	2.43	N^1-Methyl-2-pyridone 5-carboxylamide
10	392.7	4.10	Nicotinamide

Identification of the Spots on the Paper Strip Chromatograms.—On the basis of bioautographs with *Torula cremoris*, König reaction tests, fluorometric examination, spectrophotometric examination of eluates, and radioautographs of known compounds, the positions on the paper strips of known samples of coenzyme I, N-methylnicotinamide, nicotinuric acid, nicotinic acid, N^1-methyl-2-pyridone-5-carboxylamide, nicotinamide and its mononucleotide and nucleoside had been established.[15] The same methods were used to identify positively most of the spots occurring on the radioautographs. In the case of the 2-pyridone, since there are two spots close together, pure pyridone was added to the sample of the urine, which was then chromatographed and radioautographed. Each spot as detected on this radioautograph was then eluted and read in the Beckman spectrophotometer at 260 mμ, to give positive identification of the spot nearest to that of nicotinamide as being due to the pyridone. This was done using both solvent systems.

In order to distinguish between coenzyme I (and/or II) and carbonate and quinolinic acid, all of which have *Rf* values of approximately 0 under our conditions, a urine sample was evaporated to dryness and hydrolyzed with glacial acetic acid. This procedure would eliminate carbonate and convert quinolinic acid to nicotinic acid. As can be seen from Fig. 3, the acid treatment had no effect on the spot at the origin (although the lower *p*H of the solution applied to the paper did cause the nicotinic acid spot to migrate at a faster rate). Thus the spot is not due to quinolinic acid or carbonate.

In order to check the identification of these spots further and to study the identity of the unknown ones, a degradation and rechromatographing of some of the spots was carried out based on the König[18] reaction. Each paper strip chromatogram used was radioautographed to determine the exact position of every spot. It was then treated with N^1-naphthylethylenediamine dihydrochloride and cyanogen bromide vapor as described previously[17] (hereafter referred to as CNBr–$ArNH_2$). The spots (as determined by the radioautographs) were eluted separately with 8 ml. of 95% ethanol, and each ethanol extract was evaporated and rechromatographed on a paper strip. Some of these chromatograms are compared with those obtained following the same treatment of pure radionicotinic acid or nicotinamide, in Fig. 4. The glutaconic aldehyde derivatives[19] derived from the degradation of nicotinic acid or nicotinamide or from similar compounds with a free alpha-position and unsubstituted ring nitrogen establish new positions on the strip, while those compounds which do not react with cyanogen bromide, such as N^1-methylnicotinamide, its 2-pyridone, compound 8, etc., remain unaltered following this treatment. In some cases, incomplete degradation was obtained (*e.g.*, strips D, F and G, Fig. 4), some of the original compound remaining.

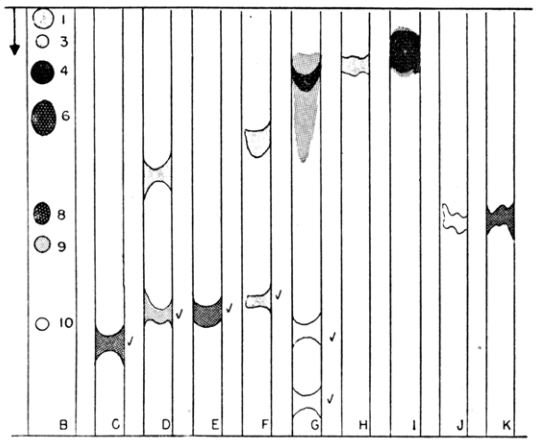

Fig. 4.—Examples of schematic drawings of chromatographic radioautograms illustrating the eluted spots being treated with CNBr–$ArNH_2$.* The degraded products are marked with "√." Descending chromatograms; solvent system; *n*-butanol 45, acetone 5, water 50. Strips: B, chromatographic radioautograms of urine from rat No. 1 receiving C^{14}-nicotinic acid; C, rechromatogram of pure C^{14}-nicotinamide following treatment with CNBr–$ArNH_2$; D, rechromatogram of pure C^{14}-nicotinic acid following treatment with CNBr–$ArNH_2$; E, rechromatogram of spot 10 (of rat 2 urine) following treatment with CNBr–$ArNH_2$; F, rechromatogram of spot 6 (of rat 2 urine) following treatment with CNBr–$ArNH_2$; G, rechromatogram of spot 4 (of rate 1 urine) following treatment with CNBr–$ArNH_2$; H, rechromatogram of spot 3 (of rat 1 urine) following treatment with CNBr–$ArNH_2$; I, rechromatogram of spot 3 (of rat 2 urine) following treatment with CNBr–$ArNH_2$; J, rechromatogram of spot 8 (of rat 2 urine) following treatment with CNBr–$ArNH_2$; K, rechromatogram of spot 8 (of rat 1 urine) following treatment with CNBr–$ArNH_2$. Strips D, F and G show the incomplete degradation. Strips H, I, J and K illustrate the spots not affected by CNBr–$ArNH_2$ treatment.

* CNBr–$ArNH_2$ = the treatment of N^1naphthylethylene-diamine-dihydrochloride and cyanogen bromide vapor (17).

Results and Discussion

The patterns of the radioautographs of paper strip chromatograms made from the urine of rats injected with various levels of radionicotinic acid and nicotinamide are shown for the acetone–butanol solvent system in Fig. 1. Considering all the samples, there appear to be at least ten different excretion products which can be separated by the paper chromatography method. These have been identified as listed in Tables III and IV.

Some other compounds are present in very small amounts and hence show up only when long exposure times up to 39 days are used and are not apparent in the figures. Figure 1 has the advantage of having all spots run on the same sheet at the same time and thus makes easier the identification of the compounds which occur in each urine, and those which do not (*e.g.*, spot 5 from rat 2).

From Tables III and IV it is apparent that at high dosage levels the chief excretion product of nicotinic acid is nicotinuric acid, with large amounts of nicotinic acid also being excreted. The chief excretion product following nicotinamide administration, on the other hand, is N^1-methylnicotinamide, with the nucleotides accounting for a considerable proportion of the C^{14} excreted.

The effect of level of vitamin dosage is shown in Fig. 1. It is apparent that at the more nearly "normal" intake level of about 200 μg. per day (rats 5 and 6), a chief excretory product of both nicotinamide and nicotinic acid is now N^1-methylnicotinamide. Nicotinic acid, however, still leads to some nicotinuric acid excretion, and the nicotinamide still gives a higher excretion of N^1-methylnicotinamide (than does nicotinic acid) and also gives some N^1-methyl-2-pyridone-5-carboxylamide (Fig. 1); many of the spots which were readily visible under high dosage are only faintly visible, or not visible at all, along the strips of the urines from rats 5 and 6. These two urine samples also appear to contain the nucleotides (see particularly rat 6, Fig. 1).

This work confirms the earlier work of Johnson, *et al.*,[4] and of Huff, *et al.*,[6] that nicotinuric acid is

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not a normal metabolic product but appears as a detoxification product following a high intake of nicotinic acid (not amide).

It appears that at normal levels of intake both nicotinic acid and nicotinamide are excreted by the rat chiefly as N^1-methylnicotinamide and/or a similar compound active toward *T. cremoris* (see ref. 17) as originally stated by Huff, *et al.*,[20] and by Rosen, *et al.*,[21] while when large doses of nicotinic acid or nicotinamide are given, much higher amounts of the other metabolites appear, some of which should be regarded as "detoxification products."

When *T. cremoris* bioautographs were run on the strips from these rat urines, zones of growth occurred at the nicotinic acid and nicotinamide positions and at the N^1-methylnicotinamide position. This again indicates that the spot identified as N^1-methylnicotinamide is a mixture of at least two compounds, one of which is active for *T. cremoris* (N^1-methylnicotinamide is not active for *T. cremoris;* see reference 17).

A study of the identity of the unknown spot 8 is at present under way. From Fig. 4 it is seen that this spot is due to a compound which does not react with cyanogen bromide.

There is considerable variation in the amount of radioactivity occurring in the feces; however, the values are comparatively small, indicating that only a small portion of the intraperitoneally injected vitamin was secreted into the intestine and excreted in the feces (see Table I).

The presence of C^{14} in the expired CO_2 demonstrates that decarboxylation of both nicotinic acid and its amide does occur in the body. It also appears that the excretion of $C^{14}O_2$ is higher in rats receiving C^{14}-nicotinamide than in those receiving C^{14}-nicotinic acid, indicating that a greater degree of decarboxylation has taken place with nicotinamide than with nicotinic acid. This observation is in accord with the data of Roth, *et al.*, using mice.[14]

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[CONTRIBUTION FROM THE DEPARTMENT OF CHEMISTRY, KANSAS STATE COLLEGE]

An Anomaly Among Interactions Involving Certain Azo Dye Anions and Bovine Serum Albumin[1,2]

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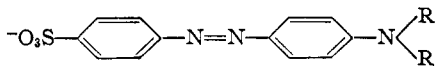
The 4′-aminoazobenzene-4-sulfonate anion is bound very slightly to bovine serum albumin at 25° and *p*H 7.3. If alkyl groups are placed on the amino nitrogen atom of this parent anion, or if the amino group of this parent anion is removed completely then the extent of interaction with bovine serum albumin increases. The interaction involving the 4′-dibutylaminoazobenzene-4-sulfonate anion (butyl orange) and bovine serum albumin exhibits an anomalous temperature dependence. This phenomenon is believed to be due to a temperature dependent association and dissociation of the butyl orange anions.

Recent investigations involving the interactions of various N,N-dialkylaminoazobenzene sulfonate anions with bovine and human serum albumins



have revealed that these two proteins evidently form similar complexes with certain azo dye anions when in solutions of *p*H 6.8, but form radically different complexes when in solutions of *p*H 9.2.[3,4] In the case of the complexes involving bovine serum albumin evidently the negatively charged sulfonate group of the azo dye is attracted to a positively charged ammonium group of the protein at both *p*H values. However, with human serum albumin this type of binding occurs only at the lower *p*H value, and when the *p*H is raised to 9.2 a second type of binding site becomes available in which the basic amino nitrogen atom of the azo dye can also be involved in the interaction by virtue of its ability to participate in hydrogen bonding to a phenolic tyrosine residue of the protein molecule.

One can thus easily account for the marked drop in the extent of interaction of various N,N-dialkylaminoazobenzene sulfonate anions with human serum albumin at *p*H 9.2 for as the size of the alkyl group is increased the reaction site involving the basic amino nitrogen atom of the dye is blocked. In the case of the interactions involving bovine serum albumin, however, this effect may not occur if only the charged sulfonate group of the dye is involved in complex formation.

It would appear then that additional support for the contention that the complexes involving bovine serum albumin and azo dyes related to methyl orange utilize only one reaction site of the dye, namely, the charged sulfonate group, could be gained by studying the interactions involving homologs of methyl orange and related compounds with bovine serum albumin in more detail. Accordingly, a study was undertaken to determine the influence which N-alkyl groups in N-alkylated aminoazobenzenesulfonate anions might have on complex formation with bovine serum albumin.

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(2) Presented in part at the Meeting in Miniature sponsored by the Kansas City Section of the American Chemical Society, Kansas City, Missouri, November 18, 1952.

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