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Nicotinic Acid Metabolism. I. The Use of Paper Chromatography in the Study of Nicotinic Acid Metabolism[1,2]

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Nicotinic acid and its metabolites have been separated by paper strip chromatography using a water-saturated *n*-butanol–acetone solvent system. Chemical, physical, radio- and bioautographic methods were used to detect the various spots. These methods were applied to the study of the excretion products of nicotinic acid in the urine of various species.

The known metabolites of nicotinic acid and nicotinamide which have been found in the urine of one or more species include nicotinic acid,[3] nicotinamide,[4] nicotinuric acid,[4] N^1-methylnicotinamide,[5–8] or compound F_2,[5,9] and N^1-methyl-2-pyridone-5-carboxylamide.[10,11] All of these compounds can be determined by specific methods: N^1-methylnicotinamide[9,12] and its 2-pyridone[13] by fluorescent methods and the other three metabolites determined together as a total "nicotinic acid" value by using either the König reaction[14] or microbiological methods.[15,16] A differential microbial method for the separate determination of nicotinic acid, nicotinamide and nicotinuric acid in urine has also been used.[17]

Other workers[18–23] have shown that the metabolism of niacin differs in different species. Herviborous animals excrete little or no N^1-methylnicotinamide, although this is the chief metabolite of nicotinamide in dogs, rats and humans. The work reported in this paper is concerned with the identification of some of these different excretion products, using paper strip chromatographic techniques. In this work a solvent system consisting of the upper phase of a mixture of 45% *n*-butanol, 5% acetone, and 50% water (by volume) has proved satisfactory. The detection of nicotinic acid and its derivatives was made by chemical, bioautographical and radioautographical methods, and the *Rf* values of the various spots were determined.

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Experimental

Preparation of Chromatograms.—Approximately 0.01 ml. of solution of sample to be chromatographed was spotted 5 cm. from the top of each 2 × 34 cm. strip of Whatman No. 1 filter paper. After drying the spot, the strips were suspended from a solvent container in a closed equilibrated chamber (jar or cabinet) which was kept in a constant temperature (80°F.) room to minimize variations in rate of solvent flow. The chromatograms were normally allowed to develop for approximately 8 hours, after which the strips were removed and air-dried at room temperature under the hood for 30 minutes.

Detection Methods. A. Cyanogen Bromide Reaction ($CNBr–ArNH_2$).—The positions of nicotinic acid, nicotinamide and nicotinuric acid on the developed paper strip chromatogram were detected by a modification of the König reaction. The air-dried strip was sprayed with a 0.5% alcoholic solution of N-(1-naphthyl)-ethylenediamine dihydrochloride. It was then air-dried and immediately put into a closed chamber through which cyanogen bromide vapor was drawn for 5 minutes. The three nicotinic acid compounds appeared as yellow spots on the paper strip. When such strips were viewed under ultraviolet light, quinolinic acid showed up as a dark spot.

B. **Fluorescence** (FL–KOH).—The position of co-enzymes I and II (nicotinamide dinucleotides), nicotinamide ribosephosphate (nicotinamide mononucleotide), nicotinamide riboside (nicotinamide nucleoside), N^1-methylnicotinuric acid betaine and N^1-methylnicotinamide chloride were detected by a modification of the fluorometric technique of Huff and Perlzweig.[12] By streaking a 20% solution of KOH down the center of the strip and drying the strip, a violet fluorescent spot appeared along the edges of the alkali-treated area for each of the above compounds when the strips were observed under ultraviolet light (a Corning 587 filter was used in front of a mercury vapor lamp).

C. **Spectrophotometry.**—The position of N^1-methyl-2-pyridone-5-carboxylamide could be detected only following elution. The paper strip chromatograms were cut crosswise into 3-cm. long sections. Each section was then eluted with 10 ml. of water and the eluates read in the Beckman spectrophotometer at 260 mμ.

D. **Bioautographic Methods.**—Nicotinic acid, nicotinamide, nicotinuric acid, trigonelline and nicotinamide riboside and mononucleotide were detected on paper strip chromatograms by bioautographic methods using the yeast *Torula cremoris* as the test organism.[24] This bioautographic technique was more sensitive than the chemical methods and therefore is preferable for those compounds which are active for the microörganism used. Five μg. of nicotinic acid per ml. of the solution used to spot the paper can be detected as compared to 40 μg. per ml. which is the limit of sensitivity with the CNBr method. The medium used was that of Williams[25] with the addition of 1.5% agar. Two hundred fifty ml. of sterilized medium was inoculated with a 10-ml. 24-hour culture of *T. cremoris* and poured into a

(24) *American Type Culture Collector*, No. 2512.

(25) W. L. Williams, *J. Biol. Chem.*, **166**, 397 (1946).

sterile 8 × 14" Pyrex dish, covered with a glass plate, and allowed to solidify. The paper strips were laid on the agar surface for 10 minutes and then removed. The covered plates were incubated at 37° for 24 hours.

The zones of growth appeared after approximately 14 hours and were usually pronounced by 24 hours. Shadowgraphs of representative bioautographs are shown in Fig. 1.

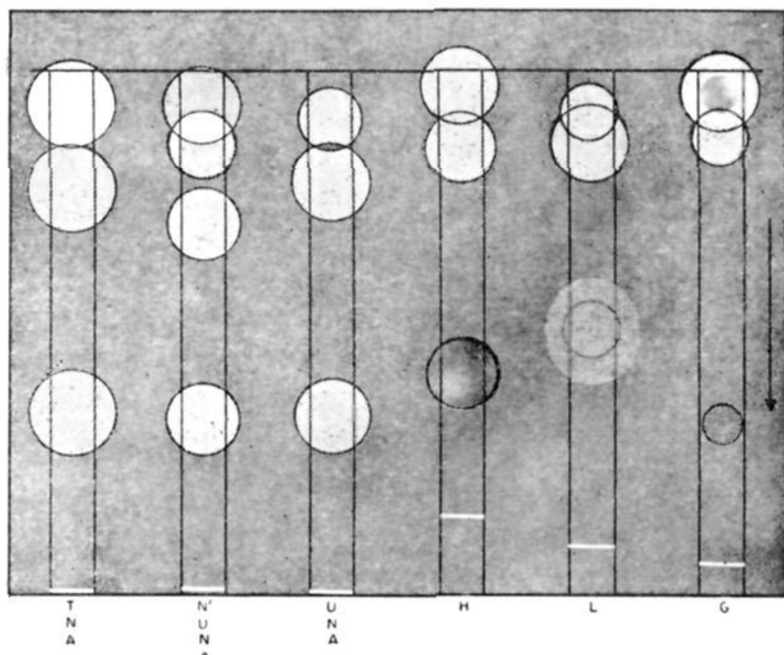

Fig. 1.—Schematic drawings of shadow photographs of *T. cremoris* bioautographs. Bioautographic chromatograms of human (H), lamb (L), and guinea pig (G) urine are shown along with mixtures of known compounds: trigonelline (T), N^1-methylnicotinamide (N^1), nicotinic acid (N), nicotinuric acid (U), and nicotinamide (A), upon which the descending order of the letters corresponds to the descending order of zones of growth. Solvent system: *n*-butanol 45, acetone 5, water 50; descending chromatograms.

E. Radioautographic Method.—Radioactive nicotinic acid and nicotinamide labeled in the carboxyl group[26] were paper strip chromatographed as before and the compounds detected by radioautography or by scanning with a G–M counter. To prepare the radioautographs the air-dried paper strips were clamped between two sheets (1⅞" × 16") of Eastman Kodak no-screen X-ray film between two pieces of heavy glass. In this way two identical films could be obtained at each exposure. The exposure time depended on the amount of radioactivity; an activity of 200 counts per minute (thin-window G–M tube)[27] produced sufficient radiation so that the strips could be removed after 36 hours' exposure. Radioautographs of radionicotinic acid and radionicotinamide are shown in Fig. 2.

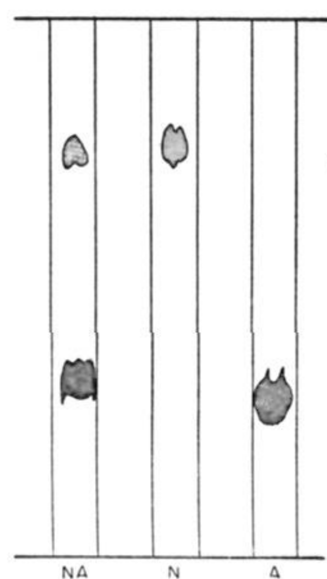

Fig. 2.—Radioautographs of pure nicotinic acid (N) and nicotinamide (A). Solvent system: *n*-butanol 45, acetone 5, water 50; descending chromatograms.

In Table I is given a complete listing of the *Rf* values which we have obtained in our experimental work with the various compounds tested by the methods already given in the descriptions recorded above.

Application of Methods to Urines from Various Species.—The occurrence of different nicotinic acid derivatives in the urine of the rat, the guinea pig, the calf, the lamb, and of man was studied. The urine samples were adjusted to *p*H 6.8 and were usually concentrated tenfold on a water-bath. 0.01 to 0.02 ml. of the concentrated urine was then spotted on the paper strips as before. After chromatographing, the paper strips were examined using the detection methods described above. Bioautographs of representative paper strip chromatograms from several species are shown in Fig. 1.

The *Rf* values of the spots detected by bioautography with *Torula cremoris* and/or by fluorescence are given in Table II. The spots were identified by comparison with the *Rf* values and activity of known compounds given in Table I.

It was observed that a spot with an *Rf* of 0.06 obtained with urine from several individual species and corresponding to pure N^1-methylnicotinamide not only gave the fluorescent reaction of the latter compound but also gave a bioautograph with *Torula cremoris*. Pure N^1-methylnicotinamide[28] is inactive for the growth of *Torula cremoris*, but can be converted to an active compound by autoclaving with 1 *N* H_2SO_4 or 1 *N* NaOH (Williams[25]). Several methods of forming a *T. cremoris*-active compound from pure N^1-methylnicotinamide were tried, and the results are summarized in Table III. The most satisfactory methods were those employing molar disodium phosphate.

TABLE I

Rf VALUES OF NICOTINIC ACID AND RELATED COMPOUNDS (Solvent system: *n*-butanol 45, acetone 5, water 50)

Compound	Detection methods used	*Rf* value
Nicotinic acid	Chemical	0.301
	Bioautographic	(0.220–0.469)
	Radioautographic	
Nicotinamide	Chemical	0.665
	Bioautographic	(0.534–0.897)
	Radioautographic	
Nicotinuric acid	Chemical	0.134
	Bioautographic	(0.096–0.186)
N^1-Methylnicotinamide chloride	Fluorometric	0.060 (0.047–0.089)
N^1-Methyl-2-pyridone-5-carboxylamide	Spectrophotometric	0.5 (0.41–0.55)
Trigonelline	Bioautographic	0.064 (0.060–0.068)
Co-enzymes I and II[a]	Fluorometric, Bioautographic	0.00
Nicotinuric acid–N^1 methylbetaine[b]	Fluorometric	0.036 (0.031–0.041)
Nicotinamide-ribose phosphate[c]	Fluorometric, Bioautographic	0.02
Nicotinamide-riboside[c]	Fluorometric, Bioautographic	0.059
Quinolinic acid	Chemical	0.00

[a] From Sigma Chemical Company. [b] Kindly supplied by Dr. E. Kodicek, Cambridge University, England. [c] Kindly supplied by Dr. Arthur Kornberg, National Institutes of Health, Bethesda, Maryland.

Discussion

When applied to urine the bioautographic technique using the yeast *Torula cremoris* was by far the most useful detection method because of the variety of compounds detectable and the great sensitivity. The pattern of growth zones found on *T. cremoris* bioautographs of paper strip chromatograms of urine was characteristic for each species. The growth zones could be identified with the exception of one with an *Rf* of 0.50 which occurred in lamb urine. This *Rf* is similar to that of the 2-pyridone of N^1-methylnicotinamide, but this compound is inactive for *T. cremoris*.

(26) Obtained from the Oak Ridge National Laboratory, U. S. Atomic Energy Commission, Oak Ridge, Tennessee.

(27) Tracerlab, Model SU-3A Laboratory Monitor with TGC-2 G–M Tube.

(28) Three commercial samples of N^1-methylnicotinamide chloride were chromatographed and gave a fluorescent spot at *Rf* 0.4–0.6µ due to N^1-methylnicotinamide. In two samples the spot was also active for *Torula cremoris*, indicating contamination. One sample also gave a *T. cremoris*-active spot corresponding to nicotinamide. This was confirmed by an *L. arabinosus* assay.

TABLE II

FLUOROMETRIC AND BIOAUTOGRAPHIC DETECTION OF NICOTINIC ACID METABOLITES IN THE URINE OF VARIOUS SPECIES

(Solvent system: *n*-butanol 45, acetone 5, water 50)

Species	Detection method	*Rf* value	Corresponding to
Human	Fluorometric + Bioautographic	0.045	N^1-Methylnicotinamide + *T. cremoris* active cpd.
	Bioautographic	.172	Nicotinuric acid
	Bioautographic	.678	Nicotinamide
Rat	Fluorometric + Bioautographic	.0454	N^1-Methylnicotinamide + *T. cremoris* active cpd.
	Bioautographic	.177	Nicotinuric acid
	Bioautographic	.650	Nicotinamide
Pig	Fluorometric + Bioautographic	.045	N^1-Methylnicotinamide + *T. cremoris* active cpd.
	Bioautographic	.153	Nicotinuric acid
	Bioautographic	.623	Nicotinamide
Guinea pig	Fluorometric + Bioautographic	.0522	N^1-Methylnicotinamide + *T. cremoris* active cpd.
	Bioautographic	.14	Nicotinuric acid
	Bioautographic	.699	Nicotinamide
Lamb	Bioautographic and CNBr reaction	.133	Nicotinuric acid
		.50	(?)
		.596	Nicotinamide
Calf	Bioautographic and CNBr reaction	.166	Nicotinuric acid
		.696	Nicotinamide

TABLE III

BIOAUTOGRAPHIC RESPONSE OF N^1-METHYLNICOTINAMIDE CHLORIDE[a] TOWARD *T. cremoris* AFTER VARIOUS TREATMENTS

1000γ N^1-methylnicotinamide chloride/100 ml.	Treatment	Biological activity	FL-KOH[e]	*Rf* value	Remarks[f]
H_2O	None	−	+	0.058	Bright violet concentric spot
3 *N* H_2SO_4	Heat[b]	+	+	.053	Faint
Dilute acetic acid	[d]	−	+	.054	Bright
1 *N* NaOH	[d]	+	+	.056	Faint
2 *N* NaOH	[d]	−	+	.056	Very faint
3 *N* NaOH	[d]	−	+	.056	Very faint
1 *M* Na_2HPO_4.	Heat[b]	+	+	.068	Widely spread
$12H_2O$	Heat[c]	+	+	.060	Widely spread
	[d]	+	+	.068	Widely spread
1 *M* NaH_2PO_4.	Heat[b]	−	+	.060	Faint
H_2O	Heat[c]	+	+	.050	Widely spread
	[d]	−	+	.048	Widely spread

[a] From Bios Laboratories, New York, N. Y. [b] Adjusted to *p*H 6.8 after 1 hour of autoclaving under 15 lb. pressure. [c] Adjusted to *p*H 6.8 before 1 hour of autoclaving under 15 lb. pressure. [d] 10 minutes heating in 120° oven. [e] FL-KOH = the appearance of fluorescence after treatment with 20% KOH. [f] Description of fluorescence observed under FL-KOH.

In the case of the calf and the lamb, no fluorescent or biologically active spot corresponding to N^1-methylnicotinamide was found. This indicates that the small amounts of fluorescence obtained when one assays the urines from these species for N^1-methylnicotinamide is due to some other fluorescence probably unrelated to nicotinic acid metabolism. Fluorometric and bioautographic chromatograms both indicated that the guinea pig does excrete N^1-methylnicotinamide type compounds. From Table I it is apparent that both N^1-methylnicotinamide and trigonelline move at the same rate on chromatograms developed with our solvent system. N^1-Methylnicotinamide is fluorescent when treated with alkali, but inactive for *T. cremoris*, while trigonelline does not give the fluorescent reaction, but is active for *T. cremoris*. At the time of the discovery of N^1-methylnicotinamide by Huff and Perlzweig,[12] they reported that all of the metabolite previously thought to be trigonelline could be accounted for as N^1-methylnicotinamide. Our work indicates that a compound similar to N^1-methylnicotinamide but active for *T. cremoris* is excreted by several species. Since the possibility existed as shown in Table III that N^1-methylnicotinamide might be converted to trigonelline or some other biologically active compound during the tenfold concentration of the urine, chromatograms were run on freshly-voided urine. These gave the same spot which was both fluorescent and active for *T. cremoris*.

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