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Thiamine Metabolism. I. The Metabolism of Thiazole-2-C^{14}-thiamine in Rat[1,2]

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RECEIVED JUNE 17, 1957

This work was undertaken to study the metabolism of radiocarbon-labeled thiamine with particular reference to its metabolic excretion products. It was found that over 60% of the radioactivity of intraperitoneally injected thiamine was excreted by the rat in the urine and that only small amounts of radioactivity occurred in the feces and in the expired CO_2. While the majority (approximately 60%) of the radioactivity in the urine was present as unchanged thiamine, it was found that at least 15 other radioactive metabolites separable by paper chromatography were also present. Three of these have been tentatively identified as thiamine disulfide, thiochrome and the thiazole moiety of thiamine.

Introduction

The metabolic fate of thiamine has been studied on numerous occasions in various species of animals, but little emphasis has been placed on the separation and identification of urinary metabolic excretion products. Thiamine was first determined in urine by Wang and Harris[3] using the thiochrome method. Kraut and co-workers[4] demonstrated that some of the thiamine in urine is present in the form of cocarboxylase. The pyrimidine moiety of thiamine was reported present in urine by Pollack and collaborators.[5] Later Kraut and Wildemann[6] reported thiamine carboxylic acid to be the chief metabolite of thiamine in human urine, with small amounts of thiochrome also present. Borsook, *et al.*,[7] injected thiamine labeled with radiosulfur into human subjects and found neutral sulfur compounds and inorganic sulfate in urine, and later McCarthy and co-workers[8] obtained essentially the same results following the administration of S^{35}-thiamine to rats.

The present paper reports the metabolic fate of thiazole-2-C^{14}-thiamine in the rat and the separation of the urinary end-products by the paper chromatographic technique.

Experimental

Over-all Metabolism.—Thiamine labeled with C^{14} in the 2-position of the thiazole ring[9] and having a specific activity of 6 μc. per mg. (4.8×10^6 c.p.m. per mg.) was injected intraperitoneally into rats of the Sprague–Dawley strain which had been maintained on a complete diet of Purina Laboratory Chow. The individual rats were placed in an all-glass metabolism cage modeled after that of Roth, *et al.*,[10] for the separate collection of urine, feces and expired CO_2. During the collection period the rats were fasted but had access to water. CO_2-free air was drawn into the cage at the rate of approximately 500 ml. per minute. The urines were collected under toluene.

For each rat total radioactivity was estimated in urine, feces, expired CO_2 and carcass after a 24-hr. collection period. All measurements of radioactivity were carried out on a windowless gas-flow counter (Tracerlab, Inc., Boston, Mass., Model No. SC-16) and scaler (Nuclear Chem. and Instr. Lab., Chicago, Ill., Model No. 163), and all samples were corrected for self-absorption by the method of Yankwich, *et al.*[11]

Preparation of Chromatograms.—Whatman Nos. 1 and 3 MM paper were used for paper chromatograms. The upper phases of *n*-butanol/acetic acid/water (40:10:50) and *sec*-butyl alcohol/water, hereafter referred to as *n*-butanol and *sec*-butyl alcohol solvents, respectively, proved suitable for the separation of the known thiamine derivatives and were employed for the separation of the urinary metabolites of radiothiamine. Descending chromatography was used throughout.

Identification of Thiamine Compounds on Paper Chromatograms. A. Thiochrome Test.—Thiamine, cocarboxylase, thiamine carboxylic acid and the urinary radiometabolites of thiamine were detected by the thiochrome test as modified by Kraut, *et al.*[6] Alkaline ferricyanide solution was sprayed on the paper chromatogram and the chromatogram dried in a current of warm air. Under these conditions thiochrome gave a blue fluorescence under the ultraviolet lamp. The test is sensitive to <1.0 μg. of thiamine.

B. Bioautographs.—Bioautographic methods also were used to detect thiamine-active compounds. This method of identification is more sensitive than the thiochrome test and responds to more thiamine-active compounds, thus is less specific than the chemical test.

The microörganisms used for the bioautographic work were *L. fermenti* 36,[12] *L. mesenteroides* 9135[13] and *S. cerevisiae*.[14] Intact thiamine and its derivatives were detected with *L. fermenti*. Thiamine split products do not interfere with this test. *L. mesenteroides* 9135 was employed for the detection of thiazole derivatives of thiamine, since it responds to the thiazole moiety as well as to intact thiamine but not to the pyrimidine moiety. The pyrimidine moiety of thiamine was detected on paper chromatograms by the yeast, *S. cerevisiae*. Since thiamine was labeled in the thiazole ring, no further study was made of the pyrimidine part of the molecule. In the preparation of the bioautographs, the procedure employed was that of Johnson and Lin.[15] The R_f values of some known compounds related to thiamine are recorded on Table I.

C. Radioautographic Methods.—Radioautographs were prepared in the usual way using Eastman No-Screen X-ray film. The film was exposed to the chromatograms for periods varying from 3 days to 3 months depending on the radioactivity.

D. Separation of Radiometabolites.—During the early phases of this study, it became apparent that it would be necessary to remove salts and other interfering substances from the urine since streaking and tailing of radiometabolites occurred on one- and two-dimensional chromatograms with the various solvents employed. In order to obtain enough urine to separate and detect as many as possible of

(1) Supported in part by a grant-in-aid from the U. S. Atomic Energy Commission under Contract No. AT(11-1)-67, Animal Science Project 2.

(2) Some of the results reported here were presented before the Division of Biological Chemistry, American Chemical Society, New York, 1954: A. C. S. Abs., Sept., 1954, p. 38C; and before the American Society of Biological Chemists at the Federation meeting held in Chicago, 1953: *Federation Proc.*, **12**, 223 (1953).

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(13) B. C. Johnson, *ibid.*, **159**, 227 (1945).

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(15) B. C. Johnson and P.-H. Lin, THIS JOURNAL, **75**, 2971 (1953).

TABLE I

PAPER CHROMATOGRAPHIC BEHAVIOR OF THIAMINE AND SOME RELATED COMPOUNDS

Substance	R_f Values *n*-Butanol[a]	*sec*-Butyl alcohol[b]
Thiamine	0.30	0.20
Cocarboxylase	.06	.00
Thiamine carboxylic acid	.16	.18
4-Methyl-5-B-hydroxyethylthiazole	.81	.85
2-Methyl-5-ethoxymethyl-6-amino-pyrimidine	.51	...
Thiochrome	.56	.55
Thiochrome pyrophosphate	.20	.11
Thiamine disulfide	.26	.05

[a] *n*-Butanol/acetic acid/water (40:10:50). [b] *sec*-Butyl alcohol saturated with water.

the metabolites of radiothiamine, 4 rats were injected intraperitoneally with 6.0 μc. (1 mg.) of thiamine daily for 5 days. The rats were housed in cages designed for the separate collection of urine and feces and were fed a thiamine-free diet *ad libitum*. The cages were placed in a large hood behind glass doors to facilitate the evacuation of exhaled $C^{14}O_2$ without contaminating the room. Urine was collected under toluene in a large test-tube immersed in a Thermos containing a freezing mixture of Dry Ice and trichloroethylene. In this way, the urine was frozen immediately upon collection. At the end of the collection period the urine of the 4 rats was pooled and the inorganic salts removed by the phenol extraction procedure of Crammer.[16] Ninety-eight per cent. of the radioactivity was recovered by this method. The extraction was carried out in a separatory funnel by adding 60 ml. of water-saturated phenol to the urine which was previously diluted to 200 ml. with distilled water. The mixture was shaken vigorously and allowed to separate overnight. The phenol layer was drawn off and washed several times with water to remove the last traces of salt. Finally, 200 ml. of diethyl ether and 5 ml. of water were added to the phenol and the mixture was shaken. Within minutes, the water phase containing the radiometabolites settled to the bottom of the separatory funnel and was drawn off. The ether–phenol mixture was washed several times with small volumes of water, and the washings were added to the original extract. This extraction technique was convenient not only for removing inorganic salts from the biological mixture but also for concentrating the large volume of urine. In order to eliminate still more of the urinary solids and yet retain the radioactivity in solution, the urinary extract was concentrated *in vacuo* at 35° to about 4 ml. and about 35 ml. of absolute ethanol was added to it. The resulting precipitate was discarded. Less than 1% of the radioactivity of the extract was lost in this procedure.

The final extract was chromatographed on Whatman 3 MM paper by streaking approximately 1 ml. across the width of sheets of paper, 15 cm. from the top. The sheets were developed with the *n*-butanol solvent in a Chromatocab for about 16 hr., air-dried and radioautographed as usual. A typical radioautograph showing the presence of at least 12 radiometabolites is shown in Fig. 1. Although at least 12 bands could be detected on the radioautographs, the sheets were only cut into 8 sections for elution since some bands occurred too close together to be separated satisfactorily. The bands were eluted with water, the eluates concentrated *in vacuo* at 35° and chromatographed on Whatman 3 MM paper with the *sec*-butyl alcohol solvent system. The radioautographs of the sheets developed with the *sec*-butyl alcohol showed that each of the original eluates had separated into 2–6 bands. The procedure of elution and rechromatographing was repeated again, this time with the *n*-butanol solvent for each of the bands separated with the *sec*-butyl alcohol–solvent system. After the third separation of the radiometabolites, the number of fractions totaled 50. Figure 2 graphically illustrates the separation of one of the original radioactive bands through the repeated chromatographic procedure.

(16) J. L. Crammer, *Nature*, **161**, 349 (1948).

To determine which of the fractions were similar, an aliquot of each of the 50 fractions of approximately 300 c.p.m. was spotted on two separate sheets of Whatman No. 1 paper so that each spot would be developed with the *n*-butanol and with the *sec*-butyl alcohol solvent systems. The R_f values of all fractions were recorded from the radioautographs of the developed sheets and all fractions having the same behavior pattern in both systems were consolidated. For example, 9 fractions having an R_f value of 0.40 with the *n*-butanol solvent and 0.60 with the *sec*-butyl alcohol solvent were pooled and tested for chromatographic purity with the *sec*-butyl alcohol solvent. Figure 3 shows this consolidation to result in one chromatographically pure compound. Similar procedures were carried out for each compound listed in Table II and illustrated in Fig. 4. Identification tests for thiamine-related compounds were made on the consolidated fractions by chemical and microbiological methods. The chromatographic behavior and the test responses of these substances are given in Table II.

TABLE II

PAPER CHROMATOGRAPHIC BEHAVIOR OF URINARY METABOLITES OF THIAZOLE-2-C^{14}-THIAMINE

Spot No.	Solvent system: *n*-Butanol R_f Values	Solvent system: *sec*-Butyl alc.	Tests: *L. Fermenti* 36	Tests: Thiochrome	Tests: *L. Mesenteroides* 9135	Identification
1	10	12	+	+	+	
2	19	14	+	−	+	
3	25	12	+	+	+	
4	26	5		−		Thiamine disulfide
5	30	13				
6	30	20	+	+	+	Thiamine
7	30	25	+	−	+	
8	30	30	+	−	+	
9	40	60	−	−	−	
10	47	40	−	−	−	
11	47	50	+	−	+	
12[a]	55	61	−	+	−	Thiochrome
13	52	68	+	−	−	
14	72	19	−	−	−	
15[a]	81	35	−	−	−	
16	81	85	−	−	+	Thiazole moiety

[a] Fluorescent.

Results and Discussion

The results of the over-all metabolism studies are shown in Table III and reveal that urine is the main avenue for the excretion of radiothiamine with small amounts present in feces and expired CO_2. The oxidation of the thiazole moiety of thiamine to $C^{14}O_2$ is in agreement with the work of Borsook

TABLE III

OVER-ALL DISTRIBUTION OF RADIOACTIVITY 24 HOURS AFTER INJECTION OF C^{14}-THIAMINE

Rat no. and wt. (g.)	% recovery of injected dose 1 (122)	2 (185)	3 (215)
Amount injected, mg.	1[e]	10[e]	1[e]
Urine[a]	66.3	69.6	67.6
Feces[b]	0.8	1.6	1.2
Respiratory CO_2[c]	0.2	0.5	3.7
Carcass[d]	8.7	...	...

[a] Urine was taken to volume (25 ml.) and aliquots plated on aluminum discs and counted. [b] Feces were dried to constant weight, pulverized and a weighed amount was plated and counted. [c] Respiratory CO_2 was trapped in concentrated NaOH and converted to $BaCO_3$ for counting. [d] Carcass was ground, homogenized and taken to volume (2 l.). An aliquot was dried at 105° to constant weight and a weighed amount plated and counted. [e] 6.0 μc./mg.

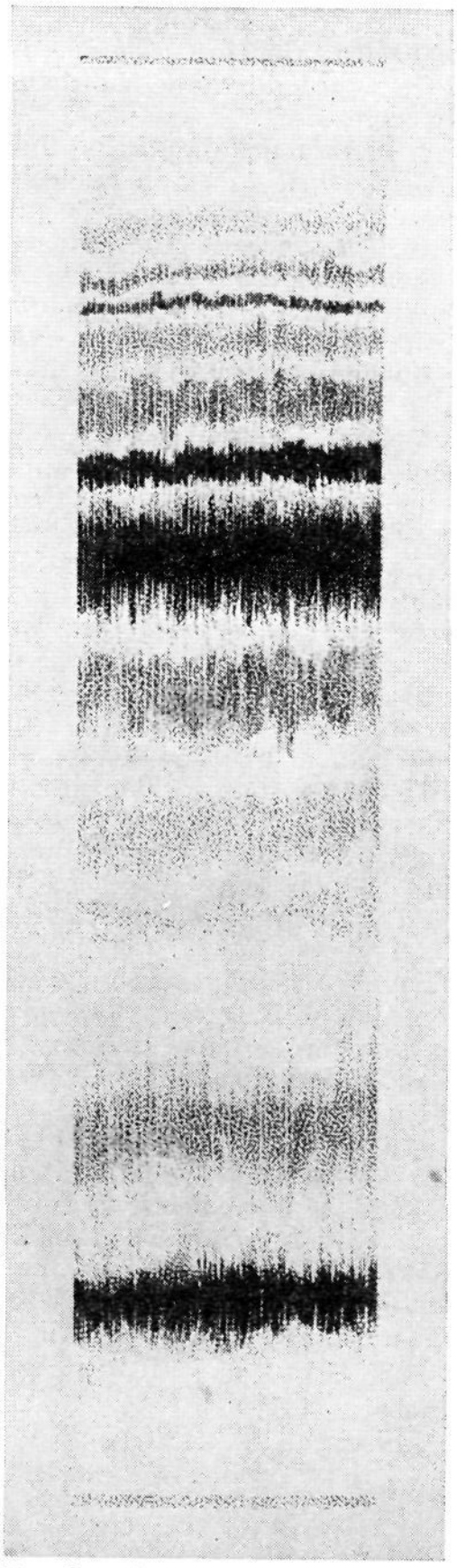

Fig. 1.—Drawing of radioautograph of paper chromatogram of urine of rat which received radio-thiamine. A typical radioautograph of rat urine following intraperitoneal injection of thiazole-2-C^{14}-thiamine. Solvent system: upper phase of butanol, 40:acetic acid, 10:water, 50. Whatman 3 MM paper. Exposure time 3 months. Upper line of drawing indicates origin, bottom line of drawing indicates solvent front.

and co-workers[7] and McCarthy, *et al.*,[8] who both reported the urinary excretion of inorganic sulfate following the administration of S^{35}-thiamine.

In order to obtain a complete metabolic picture (since some metabolites are excreted in very small amounts), the pooled urine from 5-day collections from 4 rats which had received radiothiamine throughout the 5-day period was used. By this means at least 16 different urinary metabolites

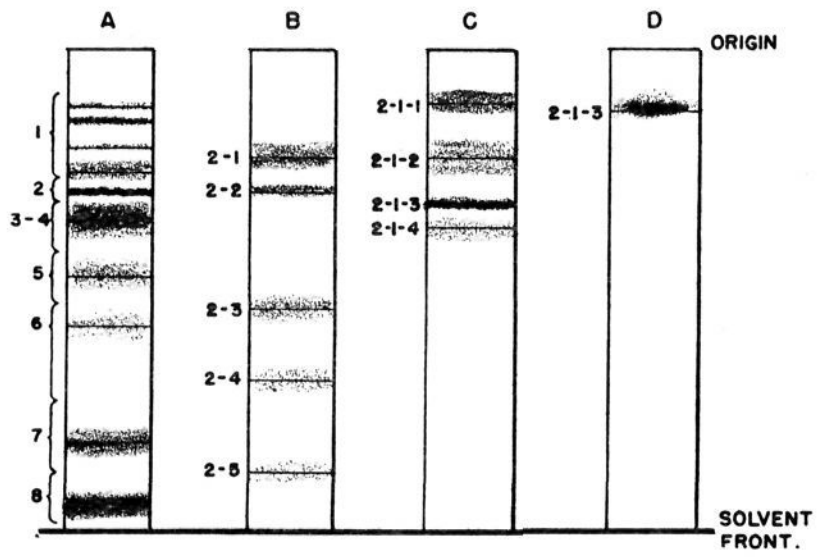

Fig. 2.—Schematic radioautographs of urinary metabolites of thiazole-2-C^{14}-thiamine: A, urinary extract developed with *n*-butanol solvent; B, piece 2 of chromatogram A cut-out, eluted with water and rechromatographed with *sec*-butyl alcohol solvent; C, piece 2-1 of chromatogram B cut-out, eluted with water and rechromatographed with *n*-butanol solvent; D, piece 2-1-3 of chromatogram C cut-out, eluted with water and rechromatographed with *sec*-butyl alcohol solvent.

were found. A factor encountered in the chromatographic separation of the urinary radiometabolites was the impurity of the apparently distinct bands following unidimensional separation with the solvent systems used. This was a disturbing factor since the solvents employed for the known thiamine compounds were selected on the basis of

Fig. 3.—Drawing of radioautograph of metabolite 9 (Table II). An example of chromatographic purity of a radioactive metabolite after repeated chromatography. Solvent system: water saturated *sec*-butyl alcohol. R_f value 0.60. Upper line indicates origin, bottom line indicates solvent front.

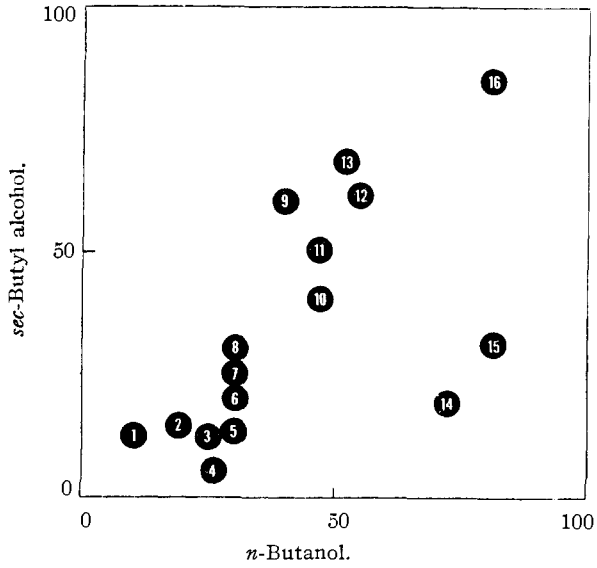

Fig. 4.—Schematic chromatogram of rat urine. A schematic two-dimensional chromatogram of rat urine showing the separation of 16 different radioactive metabolites following the administration of thiazole-2-C^{14}-thiamine. The spot numbers correspond to those given in Table II. Thus, spot 6, for example, is thiamine, and spot 12 is thiochrome.

clear-cut separations. It became apparent, in the process of rechromatographing, that distinct bands turned out on rechromatographing to be mixtures of 2 to 6 radiometabolites. It became necessary, therefore, to rechromatograph each band until no further separation occurred. Since two solvent systems were employed and hence R_f values are recorded for each metabolite in the two systems, the final effect was that of a two-dimensional paper chromatogram (Fig. 4) on enough urine so that those compounds excreted in small amounts could be detected at the same time as purification was being effected. Unfortunately, in rechromatographing the area at and just below the origin of each sheet, only small amounts of radioactivity could be separated from the solids and radioactivity localized in that area; hence, the number of metabolites reported may be less than the number actually excreted.

A total of 16 radiometabolites of thiamine are recorded. Of these, 8, shown on Table II, respond to *L. fermenti* 36 on a thiamine-deficient medium indicating the presence of the intact thiamine molecule. Four metabolites of the 8 responding to *L. fermenti* 36 give positive thiochrome tests. One metabolite (spot no. 1, Table II) which gives a positive thiochrome test behaves like a phosphorylated thiamine derivative, since the corresponding thiochrome compound is not soluble in isobutyl alcohol. On the basis of chromatographic behavior, spot no. 6 corresponds to thiamine and responds positively to thiamine tests. Spot no. 4 takes the same position on the paper chromatogram as thiamine disulfide, and spot no. 12 corresponds to thiochrome and emits the typical bright blue fluorescence of thiochrome under the ultraviolet lamp. Radioactive spot no. 15 also emits a brilliant blue fluorescence similar to that of thiochrome but does not behave like any known compound. Spot no. 16 behaves in the same manner as the thiazole moiety of thiamine and gives a positive *L. mesenteroides* 9135 bioautographic test for thiazole. Figure 4 represents a schematic two-dimensional radioautograph of the metabolites of thiamine.

The radioactive thiamine used gave, on chromatography and radioautography, spots corresponding to those for non-labeled thiamine, as given in Table I, and no other spots, thus indicating that the compound injected was radiochemically pure thiamine.

Chromatograms of the type illustrated in Fig. 1 were eluted and each band counted. Since subsequent work showed the bands obtained to be impure, the data are not presented. However, they did indicate that approximately 60% of the excreted radioactivity occurred in the thiamine band.

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