

Metabolism of Caffeine-³H in the Rat^{1,2}

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Metabolism of Caffeine-³H in the Rat. KHANNA, K. L., RAO, G. S. and CORNISH, H. H. (1972). *Toxicol. Appl. Pharmacol.* 23, 720-730. The methodology for the unequivocal identification of caffeine and 13 possible metabolites (mono, di- and tri-*N*-methylated xanthine and uric acid derivatives) based on TLC, UV and mass spectrometry has been developed. Upon ip administration of caffeine-³H to the rat, 64-67% of the radioactivity was recovered in the urine over a period of 24 hr. The chloroform-methanol (9:1) extract of the urine accounted for about 37% of the administered radioactivity. Water soluble metabolites constitute approximately 30% of the injected caffeine-³H. With the aid of preparative TLC, 8.8% of unchanged caffeine and the following metabolites were isolated from chloroform-methanol extract of urine: theophylline (1.2%), theobromine (5.1%), paraxanthine (8.8%) and trace amounts of 1,3,7-trimethyluric acid and 3-methyluric acid. Two unidentified metabolites (metabolite A, 11.4% and metabolite B, 1.3%) have also been isolated.

Caffeine, a xanthine alkaloid, is being consumed daily by the general public in large quantities in beverages such as coffee, tea, cocoa and soft drinks as well as in a number of prescription and nonprescription drugs. While caffeine is the most powerful stimulant of the central nervous system among the methylxanthines, to a lesser degree it also acts on the kidney to produce diuresis, stimulates cardiac muscle and relaxes smooth muscle. Stimulation of the drug-metabolizing liver microsomal enzymes by acute doses of caffeine has been reported by Mitoma *et al.* (1968). Recently Lombrozo and Mitoma (1970) suggested that the effect of caffeine on cytochrome P-450 is similar to that of 3-methylcholanthrene. Caffeine causes mutations in human cells in culture (Osterstag *et al.*, 1965) and seems to pass into gonadal tissues as well as through the placenta into fetal tissues (Goldstein and Warren, 1962). While the inhibition of phosphodiesterase by caffeine and the consequent accumulation of cyclic AMP and

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lipolysis is well documented (Beavo *et al.*, 1971), its possible role as an inhibitor of DNA polymerase has also been pointed out (Wragg *et al.*, 1967). The factors affecting caffeine toxicity have been recently reviewed (Peters, 1967).

The absorption, distribution, metabolism and excretion of caffeine in human subjects as well as various experimental animals have been studied by several investigators. In general, it is rapidly absorbed from the intestinal tract and distributes in the body according to tissue water content (Schmidt, 1968; Czok *et al.*, 1968, 1969; Bertoli *et al.*, 1968). Warren (1969) has shown that it takes about 7 days to decaffeinate the blood of habitual coffee drinkers. This study suggests that there is a possibility of storage of caffeine in the tissues of habitual coffee drinkers and that the rate of caffeine metabolism differs between the habitual coffee drinkers and the noncoffee drinkers. The effect of diet and environment on the distribution of caffeine in rats has also been reported (Czok *et al.*, 1968).

Most of ingested caffeine is reported to be metabolized prior to its excretion. Cornish and Christman (1957) found paraxanthine, 1-methylxanthine and 1-methyluric acid as the major urinary metabolites of caffeine in man and the rabbit. Subsequently, theobromine was also reported to be a metabolite of caffeine in man (Schmidt and Schoyerer, 1966). In the dog, theophylline has been identified as an additional metabolite of caffeine (Parke, 1968).

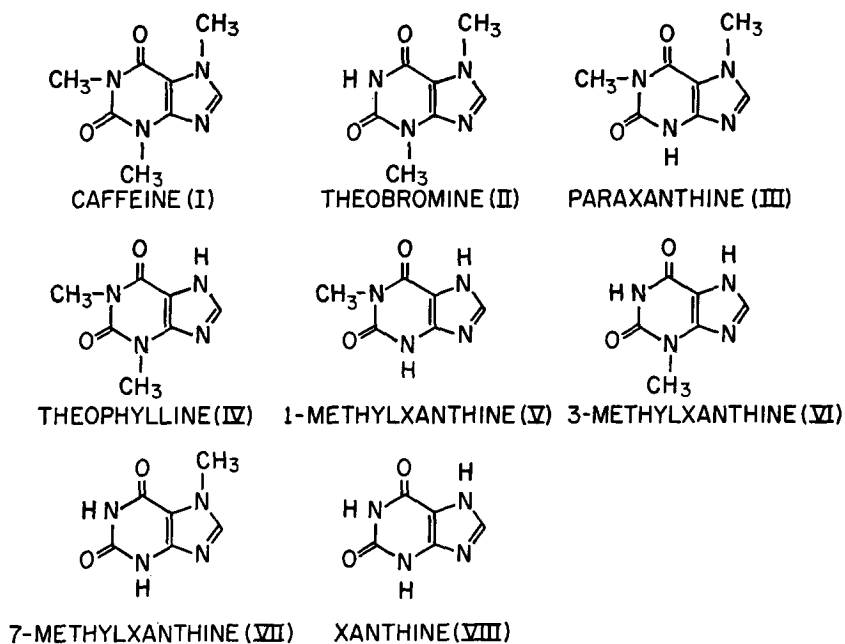
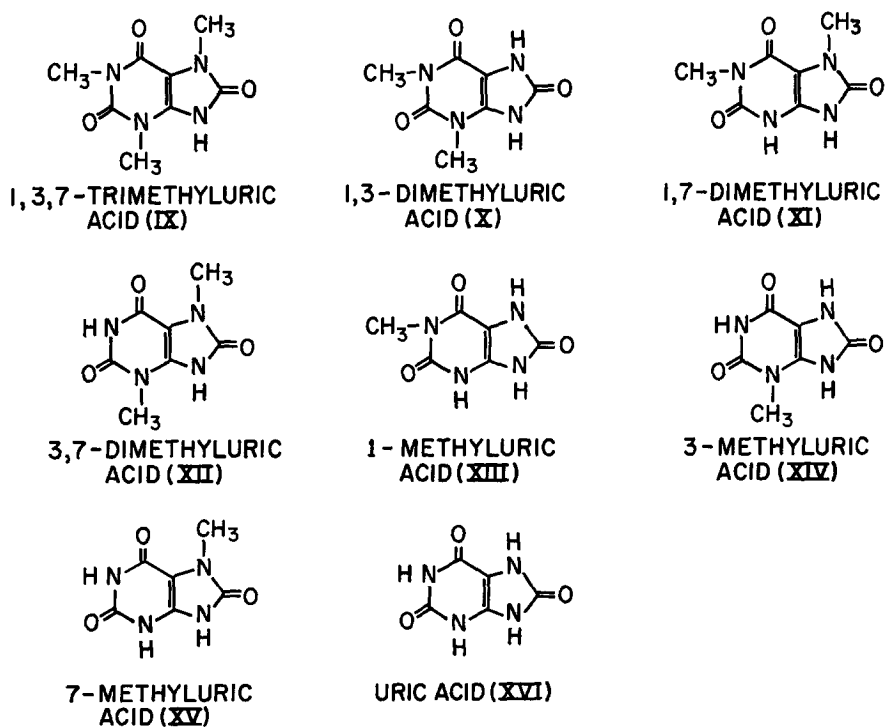
Otomo (1959) studied the metabolism of caffeine in the rat by injecting it continually into the abdominal cavity for a period of 10 days. Otomo found 1-methylxanthine, theophylline, 1,3-dimethyluric acid, 3-methyluric acid and 1-methyluric acid in the serum, and 1-methylxanthine, 1,3,7-trimethyluric acid, 3-methyluric acid and three unknown compounds in the urine as the metabolites of caffeine.

In all the investigations cited above, caffeine metabolites were identified on the basis of chromatographic and UV methods. There appears to be no report in the literature on the application of tracer methods to the study of caffeine metabolism encompassing the isolation and characterization of the metabolites. The present paper describes the metabolic fate of caffeine-³H in the rat. As an adjunct to this study, we have developed methods for the isolation of metabolites as well as their unequivocal identification based on TLC, UV and mass spectrometry. The 15 possible xanthine and uric acid metabolites of caffeine include six mono- and di-*N*-demethylated products (II-VII); xanthine (VIII); seven mono-, di and tri-*N*-methyluric acids (IX-XV) and uric acid (XVI) (Figs. 1 and 2).

METHODS

Caffeine-³H, specific activity 1.5 Ci/mole, uniformly labeled, was purchased from Amersham/Searle, Arlington Heights, Illinois. The purity of caffeine-³H was determined by TLC in two solvent systems. It was diluted with nonradioactive caffeine to contain 0.4 μ Ci/mg (2 μ Ci/ml).

Male rats (Sprague-Dawley strain), weighing 250-300 g were used for this study. The rats were injected ip with 4 μ Ci (10 mg) caffeine in 2 ml of distilled water and were housed in individual metabolism cages. They were not fed during the course of the experiment, but water was given ad libitum. Twenty-four hour urine samples were collected and monitored for radioactivity using a liquid scintillation counter.

FIG. 1. Caffeine and its *N*-demethylated derivatives.FIG. 2. Uric acid and its *N*-methylated derivatives.

Radioactivity assay. A Packard TriCarb scintillation spectrometer, Model 3375, and a Beckman LS-150 liquid scintillation counter were used for radioactive assays. These instruments were standardized; the former had an efficiency of 28% whereas the latter was 43% efficient. A mixture containing Packard scintillation grade naphthalene (80 g), POP (14.0 g, 2,5-diphenyloxazole), POPOP (50 mg, 1,4-bis-2-(4-methyl-5-phenyloxazolyl)benzene), and 1,4-dioxane reagent grade (to make 1 liter) was used as scintillation fluid. A Packard Radiochromatogram Scanner, Model No. 7201 was used for screening TLC plates for radioactivity.

Chromatography. Precoated TLC sheets, silica gel F-254 on aluminum (Brinkman Instruments, Inc.) were used for chromatographic studies. Solvent system A contained chloroform-ethanol (9:1) and solvent system B was a mixture of chloroform-acetone-*n*-butanol-concentrated ammonium hydroxide (30:30:40:10). Multiple-development technique was used to separate the compounds having close R_f values. Authentic samples of known compounds were run on the same TLC plate along with the unknown metabolites. Ultraviolet light was used to mark the compounds on the TLC plates, and the plates were also scanned for radioactive areas.

Ultraviolet analysis. The radioactive areas were scraped off the thin-layer plates, eluted with ethanol-concentrated NH_4OH (8:2) and purified by rechromatography on TLC plates using solvent system A. The compounds were eluted with ethanol and screened for UV absorption in the range 220–340 nm with the aid of a Beckman DK-2A Spectrophotometer. The reference cell contained an ethanol eluent of blank silica gel F-254. A drop of 0.01 N sodium hydroxide solution was added to the ethanol solution of the samples for the measurement of absorptions in the alkaline media.

Mass spectrometry. The mass spectra of the unknown metabolites were recorded on an LKB 9000 gc-mass spectrometer at 70 eV electron beam voltage as described earlier (Rao *et al.*, 1972).

Isolation and identification of the metabolites. Urine samples were evaporated to dryness *in vacuo*. The residue was suspended in 100 ml of chloroform-methanol (9:1), and the suspension was filtered. The filtrate was concentrated by evaporation and streaked on two silica gel F-254 plates (20 × 20 cm). The plates were developed in solvent system A. Multiple pass techniques were used for better resolution of the metabolites. A strip (5 × 20 cm) was cut from each of the plates and scanned for radioactive areas (Fig. 2). The radioactive bands were designated with capital letters starting with A at the bottom of the plate. These bands were then scraped off the plate and eluted with ethanol-concentrated NH_4OH (8:2). The eluents were concentrated by evaporation and purified by rechromatography using solvent system B. The isolated metabolites were identified with the aid of TLC, UV and mass spectrometric methods described above.

Quantitation of the metabolites. The pooled urine samples were evaporated to dryness in a flash evaporator *in vacuo*. The dried residue was suspended in 100 ml chloroform-methanol (9:1), and a 10 ml aliquot was concentrated under a stream of nitrogen. The concentrated extract was then chromatographed on a silica gel plate (5 × 20 cm) using solvent system A. The plate was developed three times (multiple pass) for effective resolution of the metabolites, and the radioactive areas were eluted with ethanol-concentrated NH_4OH (8:2). The eluents were evaporated to dryness and counted for radioactivity.

RESULTS

Metabolite Pattern

The thin-layer radiochromatogram (Fig. 3) prepared from the chloroform-methanol (9:1) extract of urine from the caffeine injected rats revealed the presence of at least 5 major radioactive areas corresponding to the 6 bands A-F visible under UV light. These radioactive areas upon elution with ethanol-concentrated NH_4OH (8:2) and rechromatography in solvent system B were found to be homogeneous except for the radioactive areas corresponding to the bands C and D which indicated the presence of two radioactive compounds.

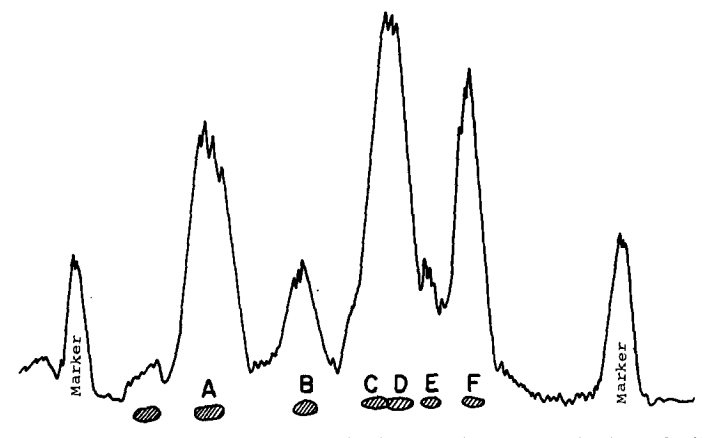


FIG. 3. Radiochromatogram scan of caffeine metabolites separated from rat urine by extraction with chloroform-methanol (9:1). Developed in solvent system A (CHCl_3 -EtOH, 9:1) on precoated TLC sheets of silica gel F-254 on aluminum.

Thin-Layer Chromatography

Table 1 lists the R_f values of caffeine and 13 possible metabolites in the solvent systems A and B. Comparison of the R_f values of the isolated metabolites of caffeine and the known compounds (Tables 1 and 2) indicated the presence of caffeine, theophylline, theobromine and paraxanthine in the chloroform-methanol extract of rat urine. Metabolite A (R_f 0.06 in solvent system A and R_f 0.40 in solvent system B) and metabolite B (R_f 0.08 in solvent system A and 0.50 in solvent system B) do not seem to have R_f values comparable with any of the known methylated xanthine and uric acid derivatives (II-XIV) examined.

Ultraviolet Absorption

The ultraviolet absorptions of caffeine and 13 possible metabolites in ethanol and ethanolic sodium hydroxide are given in Table 1. The ultraviolet absorptions of the compounds C to F showed absorption maxima and minima consistent with that of paraxanthine, theobromine, theophylline and caffeine, respectively, thereby indicating these compounds as the metabolites of caffeine in the rat. Metabolite A showed an absorption maximum at 262.5 nm which did not change in the alkaline medium. The

TABLE 1
TLC AND UV CHARACTERISTICS OF POSSIBLE METABOLITES OF CAFFEINE

Compounds (number)	$R_f \times 100$ in solvents		UV absorption spectra (nm)			
			EtOH		EtOH + 0.01 N NaOH	
	A ^a	B ^b	max	min	max	min
7-Methyluric acid (XV)	0	0	296	266	296	266
3-Methyluric acid (XIV)	0	0	296	266	297	267
1-Methyluric acid (XIII)	3	3	295	266	295	267
3,7-Dimethyluric acid (XII)	9	4	297	265	297	265
7-Methylxanthine (VII)	11	4	268	241	271	243
3-Methylxanthine (VI)	12	5	272	242	274	245
1,3-Dimethyluric acid (X)	15	4	297	267	297	267, 240 (S)
1,7-Dimethyluric acid (XI)	15	4	295	266.5	295	266.5
1-Methylxanthine (V)	16	7	268	240	275, 245	260, 231
Paraxanthine (III)	21	14	269.5	242	293	260
Theobromine (II)	23	46	270	245	270	255
Theophylline (IV)	28	19	272	242	274	244
1,3,7-Trimethyluric acid (IX)	37	6	296	262	297	263, 242 (S)
Caffeine (I)	47	73	273	244	273	244

^a Solvent system A: chloroform-ethanol (9:1).

^b Solvent system B: chloroform-acetone-*n*-butanol-concentrated NH₄OH (30:30:40:10).

TABLE 2
TLC AND UV CHARACTERISTICS OF THE CAFFEINE METABOLITES EXTRACTED FROM RAT URINE BY CHLOROFORM-METHANOL (9:1)

Metabolites	$R_f \times 100$ in solvent		UV absorption spectra (nm)			
			EtOH		EtOH + 0.01 N NaOH	
	A ^a	B ^b	Max	Min	Max	Min
A	6	40	262.5	237	262.5	240
B	8	50	270	254	279	260
C (Paraxanthine)	21	14	269.5	242	293	260
D (Theobromine)	23	46	270	245	270	255
E (Theophylline)	28	19	272	242	274	244
F (Caffeine)	47	73	273	244	273	244

^a Solvent system A: chloroform-ethanol (9:1).

^b Solvent system B: chloroform-acetone-*n*-butanol-concentrated NH₄OH (30:30:40:10).

absorption maximum at 270 nm exhibited by metabolite B was found to shift to 279 nm upon basification, suggesting the presence of an ionizable hydrogen.

Mass Spectrometry

The mass spectra of the isolated metabolites C to F are listed in Table 3, and these are identified as paraxanthine, theobromine, theophylline and caffeine, respectively, by

TABLE 3
MASS SPECTROMETRIC IDENTIFICATION OF THE CAFFEINE METABOLITES

Metabolite	Mol. wt.	Base peak	Major peaks <i>m/e</i> (relative intensity)										
			1	2	3	4	5	6	7	8	9	10	
C (paraxanthine)	180	180 (100)	68 (82)	123 (47)	53 (24)	42 (13)	95 (12)	150 (10)	151 (9)	67 (9)	41 (8)		
D (theobromine)	180	180 (100)	55 (64)	43 (37)	109 (34)	82 (33)	67 (30)	41 (28)	69 (22)	97 (15)	137 (13)	136 (8)	
E (theophylline)	180	180 (100)	95 (60)	68 (44)	41 (34)	43 (22)	53 (20)	57 (19)	55 (18)	123 (15)	83 (10)	151 (9)	
F (caffeine)	194	194 (100)	109 (56)	55 (38)	67 (29)	82 (23)	43 (13)	137 (7)	165 (6)	136 (5)			
3-Methyluric acid	182	182 (100)	68 (60)	139 (50)	83 (40)	53 (36)	54 (35)	125 (20)	111 (15)	97 (15)	41 (15)		
1,3,7-Trimethyluric acid	210	210 (100)	82 (76)	67 (40)	153 (28)	125 (24)	42 (20)	55 (10)					

comparison with the spectra of the known reference compounds (Rao *et al.*, 1972). Trace quantities of 3-methyluric acid and 1,3,7-trimethyluric acid were also identified by mass spectrometry (Table 3) in the fraction designated metabolite B.

The metabolites A (molecular ion at m/e 212) and B (molecular ion at m/e 200) do not appear to be identical with any of the known methylated xanthine and uric acid derivatives (II–XIV) examined (Rao *et al.*, 1972). The molecular ion in the mass spectrum of the metabolite A readily loses a molecule of water as indicated by the presence of the fragment ion at m/e 194. Further fragmentations of the ion of m/e 194 is reminiscent of caffeine (molecular ion at m/e 194), in particular the presence of the ion at m/e 109 (Table 3). The mass spectrum of the metabolite B appears to be quite different from the spectra of the known methylxanthines and methyluric acids, which suggests the absence of intact purine ring system in this metabolite.

Quantitation of the Metabolites

The percent urinary excretions of caffeine and its metabolites, after a single ip dose (40 mg/kg) of caffeine- ^3H in the rat, are given in Table 4. Metabolite A (11.4%) is the major metabolite, followed in concentration by paraxanthine (8.8%), theobromine (5.1%), and theophylline (1.2%). Trace quantities of 1,3,7-trimethyluric acid and 3-methyluric acid were detected as impurities in metabolite B. The excretion of caffeine (8.8%) may be artificially elevated due to the conversion of metabolite A to caffeine under isolation conditions.

TABLE 4
PERCENT URINARY EXCRETION OF CAFFEINE- ^3H
AND ITS METABOLITES AFTER A SINGLE
INTRAPERITONEAL DOSE (40 mg/kg) OF
CAFFEINE- ^3H IN THE RAT

Metabolites	Activity recovered (% of dose) ^a
A	11.4
B	1.3
C (paraxanthine)	8.8
D (theobromine)	5.1
E (theophylline)	1.2
F (caffeine)	8.8
3-Methyluric acid	Trace ^b
1,3,7-Trimethyluric acid	Trace ^b

^a The values are averages of 4 rats.

^b Detected by mass spectrometry as trace constituents in the fraction designated metabolite B.

Total radioactivity of the compounds reported here constitute about 37% of injected caffeine- ^3H . Approximately 30% of the radioactivity excreted in rat urine was not extracted by chloroform–methanol and therefore is not included in this study.

DISCUSSION

The chloroform-methanol (9:1) extract of rat urine contained about 37% of the radioactivity from the ingested caffeine-³H. Approximately 13% of the extracted radioactivity was accounted for by the three mono-*N*-demethylated products, paraxanthine (8.8%), theobromine (5.1%) and theophylline (1.2%). In this regard, caffeine metabolism in the rat parallels that observed in man (Cornish and Christman, 1957; Schmidt and Schoyerer, 1966; Warren, 1969), and in both cases the 3-*N*-demethylated product, paraxanthine, is the major metabolite among the dimethylxanthine products. The dimethylxanthines, theophylline, theobromine and paraxanthine, have not been reported earlier as metabolites of caffeine in rat urine. However, Otomo (1969) has reported the presence of theophylline in the serum of the rats receiving caffeine ip for a period of 10 days. In man and the rabbit, caffeine is known to be metabolized to paraxanthine and theobromine (Cornish and Christman, 1957; Schmidt and Schoyerer, 1966), while theophylline has been previously reported as a metabolite of caffeine in dog urine (Parke, 1968).

Although monomethylxanthines such as 1-methylxanthine are reported in the literature as the urinary metabolites of caffeine in the rat (Otomo, 1959), the rabbit and man (Cornish and Christman, 1957; Schmidt and Schoyerer, 1966), we have not detected monomethylxanthines in the chloroform-methanol extracts of rat urine. Trace quantities of 3-methyluric acid and 1,3,7-trimethyluric acid, however, were found to be present in the fraction containing metabolite B, and this is in agreement with earlier studies (Otomo, 1959).

In addition to the known metabolites described above, we have isolated two unknown metabolites, A (11.4%) and B (1.3%) from the chloroform-methanol (9:1) extract of rat urine. Metabolite A appears to be a polar compound, and it readily dehydrates to caffeine under TLC and mass spectrometric conditions. Based upon its mass spectral fragmentation pattern, we have tentatively identified metabolite A as 1,3,7-trimethyl-dihydrouric acid. Metabolite B exhibits mass spectral characteristics quite different from those of methylxanthines and methyluric acids. This may be taken as an indication of the absence of the intact purine ring system in metabolite B. Further work to establish the structures of these unknown metabolites is in progress and will be reported separately.

The proposed pathways for the metabolism of caffeine in the rat are given in Fig. 4. Caffeine is metabolized to paraxanthine, theobromine and theophylline by the appropriate mono-*N*-demethylation reaction. The oxygenation of the C-8 carbon in the caffeine molecule may proceed via the 1,3,7-trimethyl-dihydrouric acid intermediate (metabolite A). Dehydrogenation of this intermediate would lead to 1,3,7-trimethyluric acid, which then undergoes demethylation to form di- and monomethyluric acid.

In all earlier investigations reported in the literature, caffeine metabolites have been generally identified on the basis of chromatographic and UV methods. The application of TLC in conjunction with mass spectrometry appears to be a convenient and rapid method for the identification of caffeine and its metabolites. Further, microgram quantities of these metabolites isolated from TLC plates could be directly identified by the mass spectrometric method without further purification or derivatization.

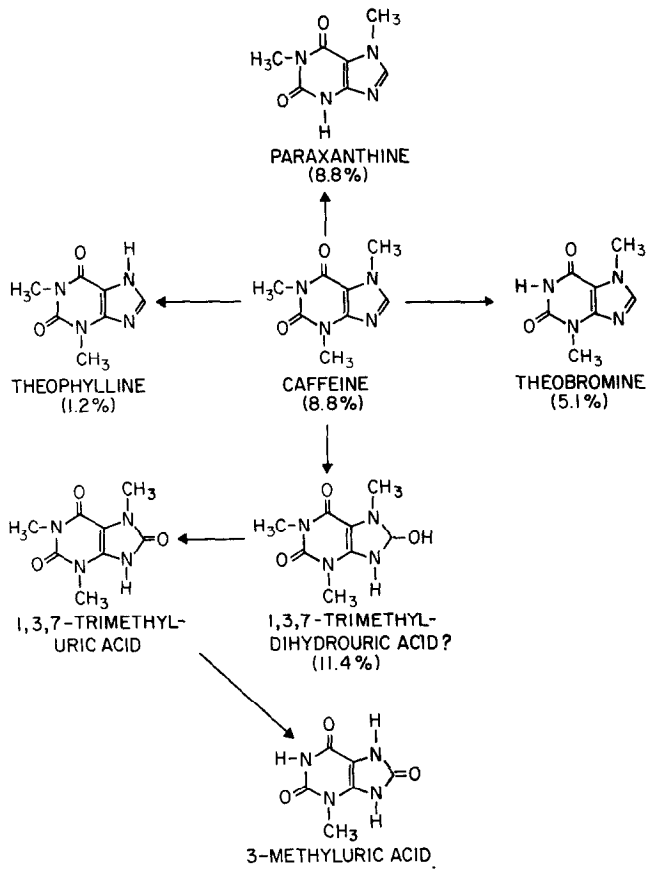


FIG. 4. Proposed pathways for the metabolism of caffeine-³H in the rat.

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