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URINARY EXCRETION OF CAFFEINE AND ITS METABOLITES IN THE MOUSE

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Abstract—The excretion of radioactive metabolites in urine, feces, and expired air after the oral administration of 5 or 25 mg/kg of [^{3}H,^{14}C]caffeine to male CD-1 mice was investigated. Of the total radioactivity, 64–90 per cent was recovered in the urine. The major identifiable urinary metabolites were 1,7-dimethylxanthine, 3-methylxanthine, 7-methylxanthine, 1,3-dimethyluric acid and 1-methyluric acid. Of the administered caffeine, 3–6 per cent was recovered from the urine. Excretion of identifiable metabolites occurred rapidly and was 80 per cent complete in 8 hr following the 25 mg/kg dose. No consistent difference in urinary excretion of radioactivity was seen when mice fed caffeine since weaning were compared to those not previously exposed to caffeine, although the fecal excretory route was enhanced in the former.

DURING the past few years, much work has been performed on the physiological effects of caffeine in experimental animals. The fact that caffeine is a pharmacologically active agent and is consumed by the general population in the form of coffee and other beverages has made such work of especial interest. However, little has been published concerning the metabolism of caffeine by such experimental animals so as to determine if the metabolites in these species bear resemblance to those reported in man nor to what extent these metabolites may be responsible for the pharmacological effects observed with caffeine. Neither have reports appeared to indicate the kinetics of metabolism in these species so as to allow a comparison of tissue exposure.

Work with both the dog[1] and rat[2] has indicated that caffeine is readily absorbed after oral administration. However, little caffeine can be identified in the urine of the dog,[1] rabbit,[3] pig,[4] rat,[5] and man,[6,7] indicating extensive metabolism. The nature and the quantity of metabolites have only been looked at briefly. Cornish and Christman[6] and Schmidt and Schoyerer[7] have each looked at the excretion patterns of xanthines and, in the former case, uric acids, after caffeine administration in two human subjects. A striking disparity exists between the results of these two pairs of investigators. The first found the major xanthine metabolite to be 1-methylxanthine,[6] while the other authors identified theobromine and 1,7-dimethylxanthine as the major metabolites.[7] The report that 1-methyluric acid is the major metabolite in man[6] is corroborated by the finding of 1-methyl and 1,3-dimethyluric acid in the urine of a single rabbit and man after caffeine ingestion.[3] Unfortunately, extensive metabolism of this compound makes it difficult to interpret results reported in terms of total radioactivity after the administration of labeled caffeine.[8,9]

In the light of this paucity of published data, we have investigated in detail the metabolism of radioactively labeled caffeine in the mouse. The mouse was chosen for this initial study, since this species has been used extensively in studies of caffeine in the dominant lethal system.[10,11]*

MATERIALS AND METHODS

Labeled compounds. [^{14}C]Caffeine-1-methyl was obtained from Tracerlab Corp. with a specific activity of 4 mc/m-mole. Paper chromatography followed by radioautography in our laboratory indicated this material to be greater than 99 per cent homogeneous. Caffeine generally labeled with tritium was obtained from Amersham/Searle with specific activity of 1·5 c/m-mole. Radioautography revealed only one radioactive spot upon paper chromatography. These labeled compounds were diluted with caffeine and used together in a ratio of 1 μc ^{14}C/50 μc of tritium in our experiments.

Scintillation fluids. TEN solution is prepared from 3% naphthalene, 0·5% 2,5-diphenyloxazole, 0·01% 1*p*–bis [5–phenyloxazolyl[benzene (Pilot Chemical) in 55:45 toluene–ethyl cellosolve.† It has the advantage over many scintillation solvents of superior water solubility. Modified Bray's solution is standard Bray's solution[12] made 2·5% in 2-ethylhexanoic acid (Eastman) and 1·0% in ethanolamine (Fisher).† The modification allows carbonate in strong base to exist as a soluble salt and thus be readily quantitated.

Other materials. Theobromine, theophylline and uric acid used as standards were obtained from Matheson, Coleman & Bell. Caffeine was obtained from Eastman Organic Chemicals. AG-2-X8 anion-exchange resin, 100–200 mesh, was obtained in the chloride form from Bio-Rad Laboratories. Other materials employed are described together with their application.

Dosage. Male CD-1 mice of approximately 30 g weight obtained from Charles River Breeding Laboratories were used throughout this study. Animals fed a diet of lab chow (Wayne) *ad lib.* were administered single oral doses of either 5 or 25 mg/kg of [^{3}H,^{14}C]caffeine by intubation of a 0·5- or 2·5- mg/ml solution containing 250 μc/ml of tritium and 5 μc/ml of ^{14}C. For the examination of the differential metabolism of a single dose of caffeine in animals chronically receiving caffeine, mice which had received 0·02 or 0·12 mg/ml of caffeine in their drinking water since weaning, at an average level of 4·1 or 25·2 mg/kg/day, and which were the third filial generation so treated, were given either 5 or 25 mg/kg of [^{3}H,^{14}C]caffeine respectively. Average chronic ingestion of caffeine was determined by measurement of water consumption during this 4-month period (time of weaning to time of dosing).

Sample collection. After the oral administration of [^{3}H,^{14}C]caffeine, groups of three animals each were placed in single Roth metabolism chambers.[13] Air was drawn continually into these chambers by vacuum pump, passing initially through Drierite calcium sulfate (W. A. Hammond Drierite Company) and then through Ascarite sodium hydrate asbestos absorbent (Arthur H. Thomas Company) before passing into the cage. The air drawn out of the chambers passed through a train of three towers each containing 100 ml of 8 N sodium hydroxide. The animals were provided with 375 mg/ml of "Get Up and Go" instant breakfast (Mead Johnson) *ad lib.* The system

* P. Thayer, in preparation.

† P. Barnowsky, personal communication.

was kept sealed except during the brief periods when samples were obtained. At these times, 0·1-ml samples of each of the tower contents, the urine diluted by the water necessary to wash the walls of the chamber, and the feces were removed. Collections of urine, feces and trap contents were made at 0·08, 0·5, 1, 2, 3, 5, 8, 24 and 32 hr after dosing with [^{3}H,^{14}C]caffeine. Samples were kept frozen until assayed.

Assay of radioactivity. Samples of the diluted urine and a 1 : 10 aqueous homogenate of feces were mixed with 10 ml TEN solution in scintillation vials. The 0·1-ml 8 N sodium hydroxide samples were diluted with 0·4 ml water and mixed with 10 ml of modified Bray's solution in vials.

Radioactivity was measured in a Nuclear Chicago Mark I scintillation spectrometer using external standard channel-ratio correction. Separate channels were optimized for detecting ^{3}H and ^{14}C emissions, and counting efficiencies were routinely determined. The quantitation of the two isotopes was effected by solution of the requisite simultaneous equation.

Location of ^{14}C radioactivity on developed paper chromatograms was accomplished by passing the paper strips through a Packard 4*Π* radiochromatogram scanner using ^{14}C-ink (Schwarz BioResearch) as a marker. Quantitation was effected by excising areas of the chromatogram, folding them accordion-fashion, placing them in vials with 10 ml TEN, and using the scintillation spectrometer.

Chromatographic purification. Urine as obtained and 1 : 10 aqueous fecal hemogenates were applied to 0·5 × 8 cm (Pasteur pipette) AG-2-chloride anion-exchange columns which had been prepared by first washing the resin with one bed-volume of 0·1 N HCl and rinsing to neutrality with water. The columns were developed by batch elution; first with distilled water until radioactivity eluting from the column approached background, and then with 0·01 N HCl until the radioactivity of the eluate again approached background. The water eluate contained all the xanthines as well as other basic and neutral species and was referred to as the "non-acid fraction". The acid eluate contained the uric acids and other acidic compounds[6] and was called the "acid fraction". All eluates were flash evaporated to dryness.

Preparation of standards. Only caffeine, theobromine theophylline and uric acid standards were obtained commercially. Other standards were prepared by biological synthesis. An aqueous solution of caffeine was administered orally over 5 hr to an 83-kg man, resulting in a total dose of 1 g caffeine. Urine was collected over the subsequent 24 hr. Aliquots (50 ml) of this urine were subjected to column chromatography as described above, except that a 2·5 × 18 cm column was used and batch elution volumes were determined by monitoring the ultra-violet optical density of the eluate. The eluates were concentrated by lyophilization and chromatographed in various paper chromatographic systems. Specific paper chromatographic systems described in the literature for the separation of methylxanthines and methyluric acids were utilized. The migration of xanthines and uric acids in these systems was visualized by staining developed chromatograms using the two-step fresh mercuric acetate–diphenylcarbazone spray system of Dikstein *et al.*[14] Methyluric acids and the methylxanthines other than caffeine itself can be visualized by this method.

Those compounds developing color when sprayed were ascertained to be known methylated xanthines and uric acids by chromatography of larger amounts of the concentrated column eluates on Whatman 3MM paper. The areas of these developed chromatograms corresponding to areas which developed color with spray were excised

TABLE 1. IDENTIFICATION OF COMPOUNDS IN HUMAN URINE*

Suspected identity	Observed R_f	Reported R_f†	Observed absorption max. (nm)	Reported absorption max.‡ (nm)
1-Methyluric acid	0·42	0·42	291 (NaOH)	292
7-Methylxanthine	0·44	0·41	268 (HCl)	267
1-Methylxanthine	0·58	0·55	246 (NaOH)	243
			279	277
1,3-Dimethyluric acid	0·60	0·54	294 (NaOH)	294
1,7-Dimethylxanthine	0·68	0·66	265 (HCl)	262

* Chromatograms were developed with *n*-butanol–water–acetic acid, 4:1:1, and R_f values determined after the visualization of compounds using the mercuric acetate–diphenylcarbazone spray.[16] Spectra were taken of compounds eluted from developed chromatograms with either 0·01 N HCl or 0·05 N NaOH. These compounds were then employed as standards where required.

† Reference 14.

‡ Reference 6.

and eluted with either 0·1 N HCl or 0·05 N NaOH. Ultra-violet spectra were taken and compared to values reported in the literature (Table 1). These eluates were then used as secondary standards where required.

Based on the differential migration of standards, three chromatographic systems were selected for use in the identification of caffeine metabolites. These were: system I, *n*-butanol–water–acetic acid, 4:1:1, v/v;[6] system II, pyridine–25% aqueous ammonia, 94:6, v/v;[15] and system III, 95% ethanol–pyridine–water, 70:20:10, v/v.[15] All paper chromatograms were developed in the descending direction on Whatman No. 1 in. wide paper strips. Both standards and compounds obtained from human urine migrated in these systems in our hands with R_f values similar to those reported in the literature. Major differences were noted for system II in which most standards migrated with an R_f less than that reported and for uric acids in system III, which were also retarded.[15]

Identification of metabolites. The metabolites and other compounds obtained from the mouse which were eluted from the chromatographic columns were applied to Whatman No. 1 paper strips and developed in duplicate in system I. After chromatographic development and drying of the strips, the paper was cut into 1 × 2·5 cm pieces and placed in scintillation vials in the presence of 10 ml TEN scintillation solvent. The quantity of ^3H and ^{14}C at any R_f values was calculated using a computer program to evaluate the external standard counting efficiency and to solve the simultaneous equation. In later work it became apparent that those metabolites labeled with tritium alone were of minor significance. The developed paper chromatograms were thereafter scanned with the Packard radiochromatogram scanner which was adjusted such that tritium did not contribute to instrument response.

After the location of peaks had been determined by counting the paper pieces in scintillation vials, those areas corresponding to peak radioactivities were cut from the duplicate strip, eluted with water, spotted on a second Whatman No. 1 paper strip and developed in system II to aid further in identification. Only when results obtained with these two systems were questionable as to identification of metabolite were additional strips developed, peak materials eluted, and system III employed.

Three criteria were used in the identification of metabolites. Co-chromatography of radioactive materials with unlabeled standards (detected by ultra-violet light absorption) was the strongest evidence for the identification of an unknown. Second, the value of $^{3}H/^{14}C$ was helpful in corroborating identity, since it was known that the tritium was distributed between the ring nucleus of caffeine and the methyl groups whereas ^{14}C was only present as the 1-methyl group. Finally, the migration of a compound in accordance with published R_f values in multiple systems was used. Other radioactive compounds obtained which did not meet these criteria are described in this paper in terms of R_f values alone with only suggestions as to their possible identity.

RESULTS

In Fig. 1 are presented results on the eventual disposition of radioactivity after the administration of [^{3}H,^{14}C]caffeine, p.o., to male CD-1 mice. No large differences were observed between the disposition of a 5- and 25-mg/kg dose. The recovery of tritium parallels recovery of ^{14}C. A greater amount of tritium is found in the urine, presumably because some tritium eventually equilibrates with body water and is

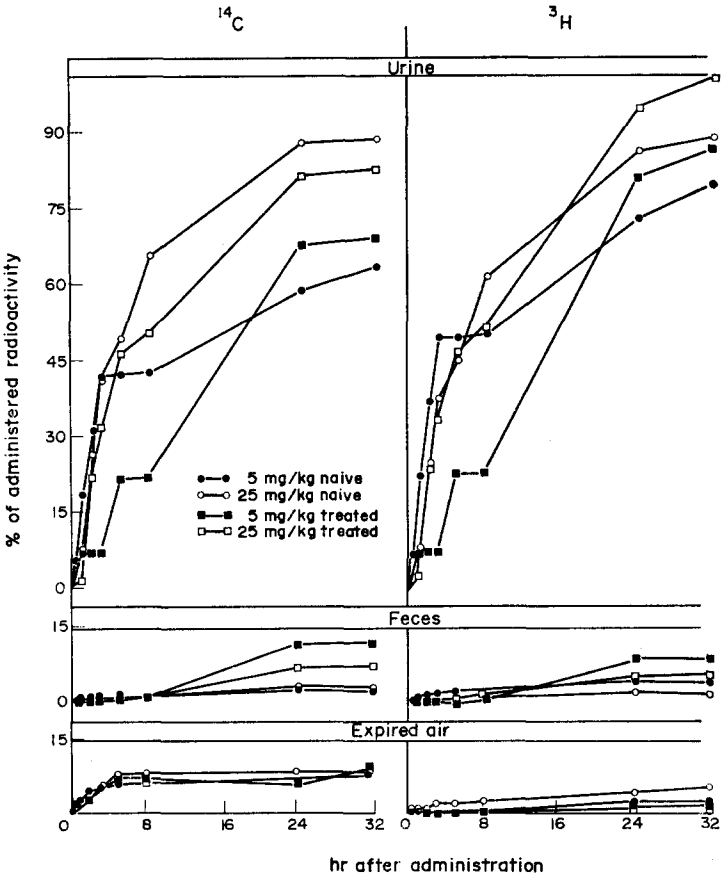

FIG. 1. Excretion of radioactivity after the administration of a single dose of [^{3}H,^{14}C]caffeine, p.o. Naive animals had not previously been exposed to caffeine. Treated animals had received an average of either 4·1 or 25·2 mg/kg/day caffeine since weaning.

excreted as ^3H_2O regardless of intermediary metabolism. At the later time periods, the tritium is indeed present as water, as shown later in this paper. More ^{14}C is recovered in the expired air than tritium. This, too, is to be expected, since the expired air route selectively disposes of carbon dioxide which can easily result from the metabolism of the 1-methyl group of caffeine after demethylation.

The only significant difference in disposition of a single dose of [^3H,^{14}C]caffeine between animals naive to caffeine as compared to those which had experienced daily caffeine ingestion from weaning is in the disposition by the fecal route. The percent of both tritium and ^{14}C excreted in the feces by animals ingesting caffeine daily was greater than twice and as much as 4·4 times the amount observed with naive mice. However, as to whether this is due to a change in the uptake of caffeine, an increased activity of caffeine-metabolizing organisms in the intestinal tract, or an increase in biliary secretion of caffeine metabolites has not been determined. As is shown later, little of the radioactive material which appears in the feces is caffeine.

The plateaus which are seen in the urinary excretion profile (Fig. 1) are best explained as resulting from the diuretic effect of caffeine. After the oral administration of either a 5- or 25-mg/kg dose, diuresis occurs. There follows an apparent compensation such that little urine is often produced at time periods from 1 to 8 hr. Since the animals are not catheterized and only that urine is collected which proceeds out the urethra, the plateaus seen in some of the curves reflect the decreased urine production during those time periods.

Metabolites in expired air. It was presumed that the ^{14}C trapped in the 8 N sodium hydroxide towers was present as $^{14}CO_2$ and that the tritium was present as water. This identification was further tested. Aliquots were obtained from each of the three towers connected for 32 hr to a Roth metabolism chamber containing three animals which had received a dose of 5 mg/kg [^3H,^{14}C]caffeine. The 10-ml aliquots were distilled in glass distilling apparatus. Samples (0·5 ml) of the distilled water recovered were dissolved in 10 ml TEN and counted. Less than 0·4 per cent of the administered ^{14}C dose could be accounted for in the distillate. Thus a maximum of 5 per cent of the ^{14}C present in the expired air is a volatile non-acidic compound.

The reverse experiment was also performed. Aliquots of the first tower were made acidic with concentrated HCl and then refluxed for 2 hr. At the end of that time, no ^{14}C-radioactivity could be found in the presence of the original high level of tritium, indicating that the ^{14}C present in the expired air after 32 hr following a 5-mg/kg dose is all acid volatile while the tritiated molecular species does not have a higher boiling point than that of water. This is consistent with $^{14}CO_2$ and ^3H_2O accounting for 95 per cent of the expired radioactivity.

Feces. The 1:10 fecal homogenates obtained from animals receiving 5 mg/kg [^3H,^{14}C]caffeine were centrifuged to determine if the radioactive material present exists in an insoluble form. A 0·05-ml sample of each homogenate was placed in a vial and dissolved in 0·5 ml of NCS solubilizer (Amersham-Searle) overnight before the addition of TEN and counting. The radioactivities of the centrifugal supernatant fraction and the whole homogenate at each time period were identical (within 10 per cent) such that it was possible to then subject this fraction alone to the usual column and paper chromatographic fractionation.

As presented in Table 2, "non-acidic" compounds predominated at early times with an increase in acid metabolites at later time periods. It can be seen from Table 2 that

TABLE 2. RECOVERY OF RADIOACTIVE COMPOUNDS FROM THE FECES

Fraction	Per cent administered dose of $^{14}C(^{3}H)$*						
	(0·5 hr)	(1 hr)	(2 hr)	(3 hr)	(5 hr)	(24 hr)	(32 hr)
Total radioactivity	0·35 (0·41)	0·80 (0·91)	0·51 (0·57)	0·11 (0·15)	0·23 (0·26)	2·20 (2·35)	0·05 (0·04)
Insoluble radioactivity	0·04 (0·02)	0·01 (0·06)	0·00 (0·00)	0·01 (0·01)	0·02 (0·02)	0·12 (0·00)	
Acidic compounds		0·19 (0·16)	0·13 (0·10)	0·02 (0·02)	0·10 (0·09)	0·88 (0·66)	
R_f 0·70 unknown		0·11	0·05	0·01	0·05	0·27	
1,3-Dimethyluric acid		0·06	0·04	0·01	0·04	0·31	
Non-acidic compounds		0·40 (0·56)	0·54 (0·35)	0·04 (0·06)	0·08 (0·11)	0·72 (1·12)	
Caffeine		0·17	0·16	0·01			
Feces/animal (g)	0·24	0·12	0·13	0·07	0·03	0·24	0·02

* Five mg/kg [^{3}H,^{14}C]caffeine, p.o., in three mice.

most of the material appearing in the feces was not identifiable as specific methylxanthines and methyluric acids. Whether this was due to the effect of bacterial action on known metabolites or to the presence of conjugates originating in the bile is not known. However, it is apparent that the fecal route was not a major excretory pathway for caffeine metabolites in the mouse and that apparently caffeine itself was almost entirely absorbed from the gastrointestinal tract and did not pass directly into the feces.

Urine. Many radioactive metabolites were found in the urine. Volatile radioactive constituents as well as methylxanthines and methyluric acids were demonstrable. The concentration of volatile materials was determined by placing urine samples in triplicate in scintillation vials. One scintillation vial of each grouping was made 0·01 N in HCl. The other two were left untreated. The acidified sample and one of the two remaining samples were allowed to air dry while the third was not. The comparison of the values obtained after adding scintillation solvent and counting in the scintillation spectrometer indicated the amount of volatiles and of acid-volatiles. For our purposes, the ^{14}C-acid-volatiles were considered to be CO_2, although exhaustive identification of the volatile constituents was not made. That tritium which was volatile with and without the addition of acid was considered to be water.

After a 25-mg/kg dose of [^{3}H,^{14}C]caffeine, 20 per cent of the tritium administered appears in the urine in a volatile form. Most of this tritium is probably present as water, but 6·6 per cent is volatile only when the urine is acidified. The tritiated water and other volatile ^{3}H materials are excreted more slowly than the xanthine and uric acid metabolites and presumably are the end products of metabolic action or result from exchange reactions which allow the incorporation of tritium into normal urinary compounds. Over 12 per cent of the ^{14}C administered as [^{3}H,^{14}C]caffeine appearing in the urine was also volatile. Of that a maximum of 1·5 per cent of the initial dose can be accounted for as CO_2. Most volatile ^{14}C radioactivity present in the urine first appeared in the 8-hr sample. The extent to which the 6·6 per cent dose of ^{3}H and the 10·7 per cent dose of ^{14}C correspond to one or more defined volatile species is not known.

The identity and amounts of nonvolatile urinary metabolites were determined by multiple chromatography as described in Methods. The actual results obtained with two acid fractions (Fig. 2) and two non-acid fractions (Fig. 3) are illustrated.

The large peak migrating 21 cm from the origin in the paper chromatogram of the acid fraction of the 1-hr urine sample (Fig. 2) was an unknown denoted as R_f 0·70 unknown as a result of its behavior in system I. The material migrating approximately 16 cm from the origin with the high ratio of ^{14}C to tritium was identifiable as 1,3-dimethyluric acid. The high ratio was the result of the elimination of the 8-hydrogen by oxidation to the uric acid and the fact that the 3-methyl group of the generally tritiated caffeine apparently contains little label. The small peak migrating approximately 12 cm from the origin was identifiable as 1-methyluric acid. The compound responsible for the peak preceding this, however, is not identifiable at the 1-hr time period.

The pattern observable in the acid fraction of the urine 24 hr after the administration of [^{3}H,^{14}C]caffeine was different from that at 1 hr. It is presented at the bottom of Fig. 2, slightly displaced from the 1-hr pattern above it. Thus the large peak migrating 18 cm from the origin was again 1,3-dimethyluric acid, this being the major con-

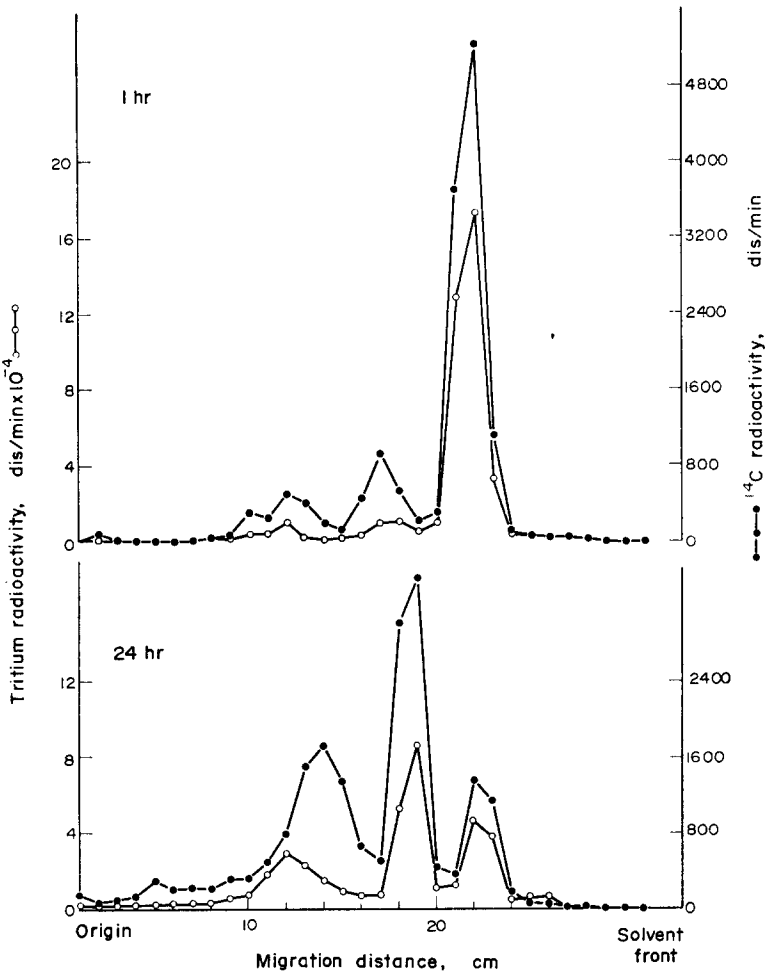

FIG. 2. Chromatographic distribution of radioactivity in the acid fraction of mouse urine at 1 and 24 hr after dosing. Urines were obtained from three male CD-1 mice at time periods after the administration of 25 mg/kg [^{3}H,^{14}C]caffeine, p.o., and the acid fractions were prepared as described in the text. Samples of these fractions were chromatographed on paper in system I. The developed chromatograms were cut into 1-cm segments and radioactivity was determined by scintillation counting.

stituent of the acid fraction at later times. Migrating further was the R_f 0·70 unknown, and just beyond this material was what may be a trace of 3,7-dimethyluric acid. The identity of this small amount of material, however, could not be confirmed. The material with the very high ratio of ^{14}C to tritium migrating 14 cm from the origin in this chromatogram was 1-methyluric acid and the tritium peak at 12 cm from the origin was 7-methyluric acid. The small ^{14}C peak at 5 cm from the origin is described as R_f 0·28 unknown, this being its normal R_f in system I.

In Fig. 3 are presented the analogous results for the non-acid fraction. Here the peak which migrates just behind the solvent front in the 1-hr urine sample was caffeine. The probable reason that the tritium and ^{14}C plots for caffeine did not coincide is

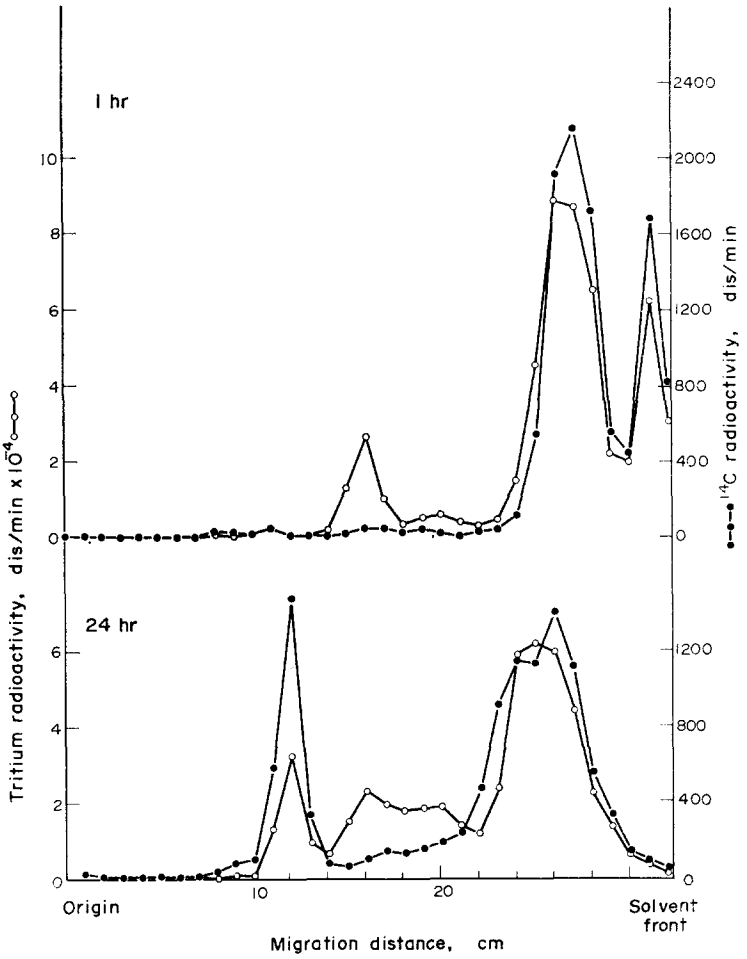

FIG. 3. Chromatographic distribution of radioactivity in the non-acid fraction of mouse urine at 1 and 24 hr after dosing. Urines were obtained from three male CD-1 mice at time periods after the administration of 25 mg/kg [^{3}H,^{14}C]caffeine, p.o., and the non-acid fractions were prepared as described in the text. Samples of these fractions were chromatographed on paper in system I. The developed chromatograms were cut into 1-cm segments and the radioactivity in each segment was determined by scintillation counting.

that some tritium has been lost from the caffeine by exchange in the body. The ratio of ^{14}C to tritium in caffeine is similar to that observed for the R_f 0·70 unknown in Fig. 2. The large peak centered at a migration distance of approximately 28 cm from the origin was a complex peak which was only resolved by subsequent chromatography. The leading edge contained a trace of what might have been theophylline, but was most especially an unknown compound, termed R_f 0·93 unknown, from its behaviour in system II. The trailing edge of this peak, which had a lower ratio of ^{14}C/^{3}H, was 1,7-dimethylxanthine. The very slight peak composed of tritium alone which has migrated 20 cm from the origin was 3-methylxanthine and the larger peak with a migration distance of 17 cm was 7-methylxanthine.

A different chromatographic pattern was found for the non-acid fraction of the 24-hr urine sample obtained from mice after a 25-mg/kg [^{3}H,^{14}C]caffeine dose (Fig. 3). In the urine collected 8 and 24 hr after the administration of [^{3}H,^{14}C]caffeine, no caffeine was detectable. Some 1,7-dimethylxanthine was still present, having migrated 26 cm from the origin. Just behind (22–25 cm) the 1,7-dimethylxanthine there was evidence for two compounds, one with a $^{14}C/^3H$ ratio of <1 and one existing as a shoulder on that peak with a ratio of >1. The identity of this material was not resolved by chromatography in other systems. 3-Methylxanthine and 7-methylxanthine again were identifiable, but this time contaminated by some ^{14}C-containing material. The large sharp peak which had migrated 12 cm from the origin was not identifiable as a methylated xanthine and has been denoted as R_f 0·29 unknown, based on its relative migration characteristics in system I. It is apparent from Fig. 3 that no 1-methylxanthine was present at either of the two time periods shown, as this material would have existed as a ^{14}C-containing peak almost halfway between the peaks identified as 3-methylxanthine (20 cm) and 1,7-dimethylxanthine (26 cm).

Based on the chromatographic evidence presented in Figs. 2 and 3, and through the use of the migration characteristics of compounds in systems II and III, it is possible to identify and quantitate the urinary radioactive metabolites occurring in the urine of the CD-1 mouse after the administration of a 5- or 25-mg/kg dose of [^{3}H,^{14}C]caffeine. The results of such an analysis for the three mice given a 25-mg/kg dose of radioactive caffeine are presented in Fig. 4. This figure details the presence of metabolites in urine expressed as a per cent of administered radioactivity versus time.

It can be readily observed that in this case only 6 per cent of the caffeine administered passes into the urine. A greater amount of 1,7-dimethylxanthine, 1,3-dimethyluric acid, and 1-methyluric acid was found (11, 10·5 and 8·5 per cent respectively). It is apparent that the methyluric acids continue to appear in the urine after 24 hr following caffeine administration, whereas the xanthines have mostly been excreted at shorter time periods. The quantities of 3-methylxanthine and 7-methylxanthine excreted are somewhat tenuous, since the extent to which the tritium within these compounds has been lost is not known. The fact that both tritium and ^{14}C are expressed as per cent of administered radioactivity allows a cumulative recovery of material greater than that expressed as total radioactivity of either tritium or ^{14}C alone.

As can be determined from Fig. 4, the second most abundant radioactive compound appearing in the urine was the material denoted as the R_f 0·70 unknown. This material was eluted from the column as an acid and was found in samples of both mouse urine and mouse feces as well as in monkey urine (work in progress). It had the highest R_f of all acid fraction radioactive species. As seen in Fig. 4, the contribution of radioactivity associated with this compound declined relative to the rest of the sample with increasing time. Thus, although after 1 hr it made up approximately 50 per cent of the acid fraction, in the 24-hr sample it accounted for only 5 per cent. Initially it was thought that this material might be 1,3,7-trimethyluric acid based on its hydrophobic nature. However, when it was eluted from system I and run in system II, two peaks resulted. The major one of these had a low R_f (0·18), indicating that the compound became more hydrophilic in base. The second peak (R_f 0·90) represented 20 per cent of the material. Subsequent rechromatography revealed that the R_f 0·90 peak was a breakdown product of the R_f 0·18 material. Thus the best indications are that the

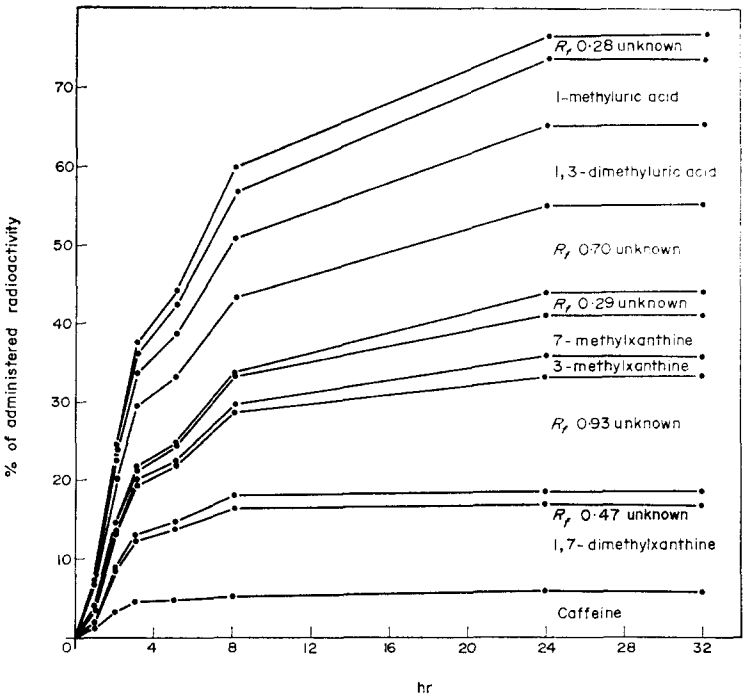

FIG. 4. Major radioactive metabolites in the urine of the mouse after the administration of 25 mg/kg [^{3}H,^{14}C]caffeine, p.o. Urine of three male CD-1 mice was pooled at each time period. The identity and quantity of metabolites were determined by multiple chromatography as described in the text. Data are presented as summated cumulative excreted per cents of administered radioactivity.

unknown identified as R_f 0·70 unknown was an acidic compound which was especially hydrophobic in acid and which was somewhat unstable. It was not a methyluric acid.

Those compounds identified in Fig. 4 as the R_f 0·93 and R_f 0·47 unknowns are denoted by the R_f values which these compounds demonstrate in system II. In system I, they co-chromatograph with 1,3-dimethylxanthine and 1,7-dimethylxanthine. Based on their behaviour in system I, it was presumed that they shared solubility characteristics with the dimethylxanthines. However, the hydrophobicity of the R_f 0·93 unknown in system II dispels this idea. Neither the R_f 0·93 unknown nor the R_f 0·47 unknown could be identified as any known methylxanthine. It was further demonstrated that the two major unidentified metabolites, the R_f 0·70 and R_f 0·93 unknowns, did not co-chromatograph with authentic carbonate, urea, methionine, thymidine or any of the 15 amino acids of an algal protein hydrolysate.

The R_f 0·29 unknown eluted with the acid fraction and the R_f 0·28 unknown eluted with the non-acid fraction were both minor metabolites. Based on their hydrophilicity and later appearance in the urine they were presumed to be further degradation products of caffeine still maintaining the labeled 1-methyl carbon. As such, these compounds could be any common excretory product in which this particular carbon had been reincorporated by the metabolic pathways of the mouse.

In addition to those compounds shown in Fig. 4, traces of other constituents were found. Less than 1 per cent of a compound which might be theophylline is present in

urine through 3 hr after the administration of caffeine. Less than 0·1 per cent of material possibly identifiable as theobromine has been observed. This latter material was first detected 8 hr after administration of caffeine, in contrast to the earlier appearance of the other methylxanthines. The identification of both dimethylxanthines is questionable as a result of the small amount of compound present in the urine. Less than 0·5 per cent of the administered ^{14}C radioactivity in both the acid and non-acid fractions remains at the origin during chromatography in system I.

The pattern of metabolites in the urine after administration of a 5-mg/kg dose was similar to that reported (Fig. 4) after a 25-mg/kg dose. The percentage of caffeine, however, was only half that seen with the 25-mg/kg dose and it appeared more quickly in the urine, with less than 0·1 per cent appearing in the urine after 3 hr. No large differences in the amount of the other non-acid fraction metabolites found in the urine after the 5-mg/kg caffeine dose were observable. More than two-thirds of these materials were excreted within 8 hr after dosing as was all of the caffeine. For comparison, the total amount of acid fraction metabolites appearing in the urine over 32 hr after a 5-mg/kg dose, expressed as a percentage of the administered ^{14}C radioactivity, was 3·0, 8·2, 4·4 and 9·0 for the R_f 0·28 unknown, 1-methyluric acid, 1,3-dimethyluric acid and the R_f 0·70 unknown, respectively. Only the dimethyluric acid value was at variance with the values obtained after a 25-mg/kg dose (Fig. 4). No attempt was made to identify the metabolites produced by those mice which were given [^{3}H,^{14}C]-caffeine after having received caffeine in their drinking water since weaning.

DISCUSSION

The chronic administration of caffeine to mice did not noticeably alter the respiratory excretion of either a 5- or 25-mg/kg oral dose of [^{3}H,^{14}C]caffeine. The same percentage and rate of $^{14}CO_2$ production was observed with both doses and in both chronic and naive animals, indicating that a 1-methyldemethylase is not induced by chronic administration of caffeine or inhibited by high concentrations of caffeine, as has been suggested.[16] An increased amount of fecal radioactivity was observed in chronically treated animals, but the feces still remained a minor excretory route. No consistent difference in total urinary excretion of radioactive metabolites was demonstrable.

A difference in the percentage of radioactivity excreted in urine between those animals receiving the 5- and 25-mg/kg dose was obtained. A greater percentage of administered radioactivity (both ^{3}H and ^{14}C) was excreted after a 25-mg/kg dose. Presumably catabolic reactions allow some caffeine-derived radioactivity to be incorporated into normal body constituents which remain in the carcass. This amount of incorporation probably remains relatively constant whether a 5- or 25-mg/kg dose is given. This can explain the higher recovery of total radioactivity on a percentage basis after the 25-mg/kg dose.

In these experiments, a slightly greater percentage of caffeine also appeared in the urine after 25 mg/kg of oral caffeine. The male CD-1 mouse excretes 6 per cent of a 25-mg/kg oral caffeine dose as caffeine but only about 3 per cent as caffeine after a 5-mg/kg dose. This compares with the reported 0·5–1·5 per cent recovery of caffeine in the urine of man,[1,6,7] 2 per cent in rat,[5] 6 per cent in pig[4] and 13·3 per cent in rabbit urine.[3]

The mouse did not excrete 1-methylxanthine, as does man,[6,7] nor the theobromine

reported by Schmidt and Schoyerer.[7] However, 1,7-dimethylxanthine and 7-methylxanthine are produced in greater quantity by the mouse than by man, 11 and 5 per cent compared to 0·2–0·7 per cent and 0·4–0·9 per cent in man.[6] No measurable theophylline was observed with either species and the 3-methylxanthine found in mouse urine has not been reported for man.[6,7] Increased uric acid excretion after caffeine ingestion would not have been observed by our method, since none of the labeled atoms incorporated into our [^{3}H,^{14}C]caffeine remain in uric acid. The ^{14}C-methyl label would be lost by demethylation and the general tritium label would be lost by demethylation and oxidation. None of the four hydrogen atoms in uric acid derive from caffeine.

1-methyluric acid has been reported to be the major metabolite resulting from caffeine ingestion in man, accounting for 25–30 per cent of a 1 g oral dose.[6] In the mouse, 1-methyluric acid accounts for only 8 per cent of the administered caffeine radioactivity after either a 5- or 25-mg/kg dose. The absence of 1-methylxanthine and decreased production of 1-methyluric acid probably indicate the presence of a more active 1-methyldemethylase in the mouse. Differences in activity of such an enzyme would help explain the decreased recovery of identifiable xanthines and uric acids in the mouse; less than 44 per cent compared to 62–75 per cent in man.[6]

In rabbit urine, 1-methyluric acid plus 1,3-dimethyluric acid accounted for 25 per cent of the administered caffeine.[3] Thus, the CD-1 mouse, with a combined total of 13–21 per cent for these two metabolites, is more like the rabbit in this regard than like man (35–40 per cent). However, with the exception of 1 methylxanthine, the differences in urinary metabolites produced by the CD-1 mouse and man relate to quantity and not identity.

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