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TISSUE DISTRIBUTION OF CAFFEINE AND ITS METABOLITES IN THE MOUSE

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Abstract—The disposition of a single oral dose of 5 or 25 mg/kg of [^{3}H, ^{14}C]caffeine in the brain, heart, kidneys, liver, lungs, spleen, testes, muscle, plasma and erythrocytes of the CD-1 mouse was investigated. Caffeine appeared in all tissues within 5 min. It distributed in proportion to tissue water only at 1 hr after dosing, entering and leaving various organs at different rates. The maximum organ half-life of caffeine was 3 hr. A shorter half-life was observed after the 5-mg/kg dose, but the disposition of both doses was otherwise similar. 1,7-Dimethylxanthine is the major tissue metabolite. Little methyluric acid was present in the tissues. No methylxanthine nucleotides were demonstrable in the liver. The exposure of organs to caffeine and 1,7-dimethylxanthine was calculated.

THE EFFECT of caffeine and theophylline on isolated tissue and cellular systems has been the subject of much investigation, expecially since the advent of interest in the mediating role of cyclic adenosinemonophosphate (c-AMP). These methylxanthines have been studied as to effects on perfused organs, tissue homogenates, tissue-cultured cells and a variety of nonmammalian test systems *in vitro*. The relevancy of any of this work to possible effects to be expected in the situation *in vivo* is dependent upon the extent to which any organ is perfused or contains caffeine and theophylline *in vivo* after oral administration. The actual exposure of individual tissues may differ widely.

Although work with the rat indicates that caffeine is poorly absorbed through the stomach,[1] Axelrod and Reichenthal[2] have shown that upon ingestion, it is completely absorbed from the gastrointestinal tract within 1 hr in man and distributes in the tissues of the dog in direct proportion to tissue water. A similar distribution between plasma and testes or ovaries within 2 hr after i.v. administration has been observed in man.[3] Subsequently, the plasma level falls to half its value in 3·5 and 5 hr in man and dog respectively.[2] A half-life of approximately 13 hr for caffeine in the plasma, liver, muscle and kidney (and 20 hr in the back fat) of the pig has been found.[4] A shorter serum half-life is reported for the rat with neither caffeine nor metabolites demonstrable 5 hr after dosing.[5] These findings represent the available reported data on the disposition of caffeine in mammalian tissues.

Techniques have been developed for the identification of metabolites of caffeine.[6] In the work reported here, these techniques have been applied to a complete study of the physiological disposition of caffeine in the mouse in order to determine the importance of the concentration and time of contact of caffeine metabolites within the tissues in relation to tissue levels of caffeine itself, and to the caffeine administered.

MATERIALS AND METHODS

Materials. Sodium heparin was a product of Riker Laboratories. Precoated cellulose thin-layer plates containing fluorescent indicator were purchased from Brinkmann. Analytical grade AG-1-X8 formate resin, 200–400 mesh, was obtained from Bio-Rad Laboratories; it was washed first with 6 N formic acid–1 N sodium formate and then with water before use. All other reagents were obtained from sources reported previously.[6]

Dosage. Male CD-1 mice of approximately 30 g weight obtained from Charles River Breeding Laboratories were used throughout this study. Male mice were used, since they have been the subject of previous experiments investigating the effects of caffeine.[7] Animals raised on a diet of lab chow (Wayne) were given single oral doses of either 5 or 25 mg/kg of [^{3}H,^{14}C]caffeine by intubation of a 100 g/ml solution in which the ratio of tritium to ^{14}C was 15·6:1. The large ratio decreased the opportunity for errors due to dual label counting and calculation.

Groups of three animals were used for each time period and each dose. Animals were sacrificed by cervical dislocation at 0·08, 0·5, 1, 3, 8 and 24 hr after dosing, and the brain, heart, kidneys, liver, lungs, spleen and testes were removed by immediate dissection. A muscle sample was excised from muscle adjacent to the femur. Whole blood was obtained by heart puncture with a heparinized hypodermic syringe.

For the radioautography experiment, 8-month-old male CD-1 mice which had received 0·02, 0·06 or 0·12 mg/ml of caffeine in their drinking water since weaning, estimated to correspond to 4·1, 12·2 or 25·2 mg/kg/day, were given a single 10-mg/kg dose of 1-methyl [^{14}C]caffeine. Twenty-four hr later the mice were sacrificed by immersion in a hexane–dry ice mixture. The frozen carcasses were stored at −26° for microtomy.

Tissue preparation. Whole blood was separated into plasma and red blood cells by centrifugation in an International desktop centrifuge. Erythrocytes and all other tissues were homogenized using a Potter–Elvehjem homogenizer to yield a 9% suspension in normal saline. Samples (0·05 ml) of homogenate were hydrolyzed overnight with 0·5 ml of Amersham/Searle NCS solubilizer and counted in TEN solvent[8] in the Nuclear Chicago Mark I scintillation spectrometer.

Purification. For the effective recovery of metabolites, extraction was required. Although less than 20 per cent of a 60 mM caffeine, theophylline or theobromine solution is bound by 2% bovine serum albumin, 83 per cent of 1,3,7-trimethyluric acid is so bound.[9] The samples of homogenate were therefore mixed with an equal volume of 0·4 M perchloric acid so as to extract all such bound compounds. The precipitate resulting was collected, centrifuged and washed twice with a half-volume of 0·2 M perchloric acid before being discarded. The washes and the original supernatant were combined and neutralized with 5 M potassium hydroxide. Potassium perchlorate was removed by centrifugation. The acid extract was then applied to a 0·5 × 8 cm AG-2-chloride column and the column was developed as reported in the previous paper.[6]

Quantitation. The acidic and non-acidic radioactive metabolites present in the column eluates were identified by paper chromatography in multiple chromatographic systems.[6] The location of any ^{14}C-metabolite in a developed chromatogram was determined by scanning with a Packard radiochromatogram scanner. Quantitation was then effected using a Keuffel and Esser compensating polar planimeter to determine

the areas of peaks appearing on the scanner recording ratemeter chart output. The ratio of any peak area to the total area obtained by scanning the entire chromatogram was assumed to be proportional to the ratio of the radioactivity of the specific compound responsible for the peak to the total radioactivity of the sample applied. This assumption was shown to be correct for the quantitation of metabolites present in urine.

Nucleotide analysis, Livers obtained from mice at 1, 3 and 8 hr after p.o. administration of [^{3}H,^{14}C]caffeine at 25 mg/kg and at 3 hr after a 5-mg/kg dose were analyzed. The chromatographic method used was a modification of the gradient elution technique of Chandhuri *et al.*[10] The perchloric acid-soluble fraction of the livers was loaded on to AG-1-formate anion-exchange columns (18 × 0·5 cm) and were eluted with: (1) 20 ml of H_2O; (2) 80 ml of 0·05 M formic acid; (3) approximately 220 ml of an asymptotic formic acid gradient, with 4 M formic acid in the reservoir and 100 ml of H_2O in the mixing chamber; (4) 50 ml of 2·0 M ammonium formate. Fractions of 3 ml were collected and the radioactivity in each fraction was determined.

To determine if any of the peaks eluted by the formic acid gradient were nucleotides, fractions comprising each potential nucleotide peak were pooled, evaporated to dryness and were taken up into 3·0 ml of 0·1 M glycine buffer, pH 10·5. To one-half of this solution, 10 μl of alkaline phosphatase was added, and to the other half, 10 μl water was added. The mixtures were incubated at 34° for 2 hr. Preliminary experiments had shown that under these conditions 9 μmoles of inosinic acid was converted to inosine. The reaction mixtures were evaporated to dryness, taken up into water, and desalted by passing through Dowex-50 cation-exchange resin. The eluate was dried, redissolved in water, and applied to precoated cellulose plates. Thin-layer chromatography was finally used to determine if alkaline phosphatase altered the chromatographic mobility of the suspect radioactive materials.

Acid-insoluble radioactivity. Samples of the acid-insoluble fraction of livers were extracted twice with 95% ethanol and once with 95% ethanol–ether, 3:1. The residues were suspended in 0·3 M potassium hydroxide and centrifuged to remove the insoluble potassium perchlorate. The radioactivity in the ribonucleic acid (RNA) extract was determined in TEN scintillator solution. The acid-insoluble precipitate was extracted twice with 0·5 N perchloric acid at 90° for 15 min. The extracts were combined and neutralized with KOH and centrifuged to remove the insoluble potassium perchlorate. The radioactivity in the resulting deoxyribonucleic acid (DNA) extract was determined in TEN scintillator solution.

RESULTS

The concentrations of caffeine, its major metabolites and of unidentified radioactive species within the organs and tissues of the mouse after oral administration of [^{3}H,^{14}C]caffeine are presented in Tables 1, 2 and 3. The values for organs are expressed as the per cent of the administered ^{14}C present in the whole organ. The values for tissues have been calculated for the total muscle mass based on the per cent of muscle present in man,[11] and for the total body complement of blood plasma and erythrocytes based on the volumes reported in the literature for the mouse.[12] The results presented are the combined data obtained with three animals at each time period.

It is apparent that caffeine radioactivity rapidly entered these organs and tissues within 5 min after administration of a 25- or 5-mg/kg (Tables 1 and 3) oral dose.

TABLE 1. MAJOR ^{14}C-COMPOUNDS IN THE ORGANS OF THE MOUSE WITHIN 1 hr AFTER A 25-mg/kg DOSE OF [^{3}H,^{14}C]CAFFEINE

Compounds	Per cent of administered ^{14}C-radioactivity in									
	Brain (0·40 g)*	Heart (0·15 g)	Kidneys (0·47 g)	Liver (1·70 g)	Lungs (0·23 g)	Spleen (0·17 g)	Testes (0·22 g)	Muscle (12·9 g)	Blood plasma (1·46 ml)	Erythrocytes (0·87 ml)
At 5 min										
Total radioactivity	0·63	0·31	1·18	7·2	0·56	0·48	0·14	13·1	4·1	0·52
Extractable radioactivity	0·65	0·31	1·17	6·7	0·58	0·50	0·14	13·1	4·1	
Total acids	0·01	0·00	0·02	0·06	0·00	0·01	0·00	0·66	0·06	
Total non-acids	0·58	0·28	1·20	5·5	0·48	0·45	0·13	12·5	2·82	
Caffeine	0·58	0·28	1·20	5·5	0·48	0·45			2·13	
At 30 min										
Total radioactivity	0·94	0·42	1·54	5·1	0·61	0·46	0·67	29·9	4·2	0·69
Extractable radioactivity	0·97	0·47	1·49	5·8	0·65	0·49	0·67	29·4	3·5	
Total acids	0·01	0·01	0·04	0·25	0·01	0·01	0·01	0·49	0·18	
1-Methyluric				0·12						
Total non-acids	0·88	0·37	1·37	5·5	0·64	0·36	0·54	30·9	2·94	
Caffeine	0·65	0·27	1·01	4·8	0·52	0·28	0·54	23·5	2·07	
1,7-CH_3-xanthine	0·23	0·10	0·35	0·65	0·12	0·08			0·69	
At 1 hr										
Total radioactivity	0·87	0·40	2·02	5·9	0·56	0·43	0·95	35·8	4·8	0·62
Extractable radioactivity	0·85	0·38	1·89	5·8	0·58	0·52	1·21	3·52		
Total acids	0·01	0·01	0·11	0·41	0·01	0·02	0·14	0·00		
1-Methyluric			0·04	0·24	0·00	0·00				
Total non-acids	0·70	0·33	1·69	4·7	0·51	0·39	0·60	34·0		
Caffeine	0·61	0·26	0·98	2·7	0·38	0·25	0·38	22·7		
1,7-CH_3-xanthine	0·09	0·07	0·49	1·6	0·04	0·14	0·22			

* The average weight of each organ is given in parentheses.

TABLE 2. MAJOR ^{14}C-COMPOUNDS IN THE ORGANS OF THE MOUSE 3 hr AFTER A 25-mg/kg DOSE OF [^{3}H,^{14}C]CAFFEINE

Compounds	Per cent administered ^{14}C-radioactivity in									
	Brain (0·37 g)*	Heart (0·15 g)	Kidneys (0·47 g)	Liver (1·50 g)	Lungs (0·23 g)	Spleen (0·16 g)	Testes (0·25 g)	Muscle (12·9 g)	Blood plasma (1·46 ml)	Erythrocytes (0·87 ml)
At 3 hr										
Total radioactivity	0·32	0·19	1·82	3·9	0·30	0·26	0·27	13·4	2·07	0·42
Extractable radioactivity	0·35	0·19	1·65	3·6	0·28	0·21	0·26	16·9	2·07	
Total acids	0·01	0·01	0·24	0·53	0·02	0·02	0·02	1·17	0·33	
1-Methyluric			0·11	0·36	0·00	0·01			0·15	
Total non-acids	0·31	0·15	1·42	2·4	0·19	0·17	0·21	13·4	1·44	
Caffeine	0·22	0·08	0·47	1·14	0·12	0·10	0·12		0·69	
1,7-CH_3-xanthine	0·09	0·06	0·53	0·90	0·08	0·08	0·09		0·42	
At 8 hr										
Total radioactivity	0·04	0·03	0·33	1·15	0·10	0·08	0·14	6·6	0·60	
Extractable radioactivity	0·06	0·03	0·26	0·86	0·08	0·05	0·16	6·6	0·57	
Total acids		0·00	0·03	0·19	0·01	0·02	0·03			
1-Methyluric			0·02	0·17						
Total non-acids		0·02	0·19	0·44	0·03	0·03	0·09			
At 24 hr										
Total radioactivity	0·03	0·02	0·22	0·60	0·07	0·05	0·03	1·47	0·24	0·07
Extractable radioactivity	0·06	0·01	0·12	0·28	0·07	0·03	0·05	1·47	0·24	
Total acids		0·00			0·00	0·01	0·00			
Total non-acids		0·01			0·01	0·02	0·01			

* The average weight of each organ is given in parentheses.

TABLE 3. MAJOR ^{14}C-COMPOUNDS IN THE ORGANS OF THE MOUSE AFTER A 5-mg/kg DOSE OF [^{3}H,^{14}C]CAFFEINE

Compounds	Per cent administered ^{14}C-radioactivity in									
	Brain (0·37 g)*	Heart (0·15 g)	Kidneys (0·47 g)	Liver (1·77 g)	Lungs (0·26 g)	Spleen (0·17 g)	Testes (0·24 g)	Muscle (12·9 g)	Blood plasma (1·46 ml)	Erythrocytes (0·87 ml)
At 5 min										
Total radioactivity	0·57	0·31	1·06	6·6	0·65	0·60	0·17	21·2	3·54	0·60
Extractable radioactivity	0·56	0·28	1·06	6·7	0·44	0·64	0·17	20·4	3·46	
Total acids	0·01	0·01	0·02	0·10	0·01	0·00	0·00	0·29	0·15	
Total non-acids	0·48	0·23	1·11	5·7	0·41	0·53	0·14	17·4	2·54	
Caffeine	0·41	0·23	1·01	5·7	0·41	0·53		17·4	2·54	
1,7-CH_3-xanthine			0·10							
At 1 hr										
Total radioactivity	0·58	0·29	2·40	6·9	0·73	0·29	0·32	23·8	4·52	0·58
Extractable radioactivity	0·58	0·36	2·38	6·4	0·47	0·32	0·36	23·4	4·43	
Total acids	0·01	0·02	0·19	0·62	0·20	0·01	0·02	0·73	0·63	
1-Methyluric					0·02					
Total non-acids	0·54	0·28	2·24	5·4	0·36	0·27	0·31	20·6	3·09	
Caffeine	0·25	0·14	0·85	1·63	0·19	0·16	0·18	12·7	0·86	
1,7-CH_3-xanthine	0·10	0·14	1·27	1·50	0·17	0·11	0·13	8·0	1·06	
At 8 hr										
Total radioactivity	0·04	0·04	0·35	2·0	0·20	0·10	0·08	3·6	0·60	0·10
Extractable radioactivity	0·09	0·03	0·24	1·2	0·11	0·05	0·09	5·1	0·40	
Total acids	0·01	0·01	0·03	0·18	0·01	0·00	0·01	0·55		
1-Methyluric										
Total non-acids	0·04	0·02	0·18	0·76	0·03	0·03	0·06	2·3		
1,7-CH_3-xanthine								2·3		

* The average weight of each organ is given in parentheses.

It is also apparent from the seven organs for which the data are given that caffeine was responsible for almost all of the radioactivity present at this early time. All of the radioactivity was extractable. At this early time period, very little radioactivity was present in the acid fraction of the extracted tissue and only in plasma were acid metabolites observable. These compounds were present in low concentration and have not been identified.

Thirty min after administering the 25-mg/kg dose, the total radioactivity of the organs and tissues had increased over the 5-min level, but not by a very large margin. The amount of caffeine, expressed as a per cent of the administered dose, remained relatively constant within the organs. Practically all of the radioactive material present at the 30-min time period was extractable. 1,7-Dimethylxanthine became apparent here as the major metabolite. A non-acid fraction material with the chromatographic property of a dimethylxanthine was also present in low concentration in muscle and plasma. The acid fraction contained low concentrations of three hydrophobic compounds which were not identified. 1-Methyluric acid was the only identifiable acid metabolite present in measurable amount. A trace of 1,3-dimethyluric acid may have existed in certain organs. The difference between extractable radioactivity and the sum of total acids plus total non-acids indicates the presence of volatile constituents. This was especially noticeable in the blood plasma.

By 1 hr after the administration of [^{3}H,^{14}C]caffeine, the concentration of caffeine in most organs accounted for only half of the total radioactivity. 1,7-Dimethylxanthine remained as the major metabolite. The ratio of 1,7-dimethylxanthine to caffeine varied widely between organs, but at no time did the caffeine metabolite exceed caffeine in concentration after the 25-mg/kg dose and only rarely after the 5-mg/kg dose. At this time, the highest recovery of radioactivity from these organs was affected; 52 per cent of the administered dose. Since only about 10 per cent of the administered dose had been excreted by this time,[6] the remaining 38 per cent of the radioactivity was presumably in the 38 per cent of the body weight unsampled in this study, i.e. the bladder, fat bodies, intestines.

Table 2 and the 8-hr data of Table 3 reflect the presence of radioactive materials within the organs of the mouse at later time periods. Besides caffeine, 1,7-dimethylxanthine and 1-methyluric acid, after 3 hr, a material which may have been 1,7-dimethyluric acid was present in liver, lungs, kidneys, testes and plasma. However, as can be determined by comparing the total acid fraction at this time to the dose administered, only a very small amount of radioactivity can exist as this metabolite. By 3 hr after dosing, considerable material in the metabolically active organs, such as the liver, spleen and kidneys, existed as nonextractable radioactivity. This amount of nonextractable material increased through 8 and 24 hr as a percentage of the total radioactivity at each time. Nonextractable ^{14}C-radioactivity presumably results from the reincorporation of the radioactive methyl group into insoluble body constituents such as protein.

By the 8-hr and 24-hr time periods, the level of radioactivity had become so low as to make it difficult to identify metabolites. 1-Methyluric acid was present in certain organs. Its apparent absence in other organs, however, may be due to the sensitivity limitations of our method of analysis. The absence of specific xanthine and other non-acid metabolites at these last two sampling times is due to the low level of radioactivity available in the organs at this time for chromatographic identification. By 24 hr, it is

presumed that the 0·01–0·03 per cent of the administered dose of ^{14}C-radioactivity which remained as acid or non-acid metabolites within the organs was present not as xanthines or uric acids, but as normal cellular compounds.

The disposition in the organs of the tritium administered paralleled that for ^{14}C. The per cent of tritium as total radioactivity, extractable radioactivity, total acids and total non-acids throughout the early time periods was as shown in Tables 1 and 3. However, at the 8-hr and 24-hr time periods, the ratio of tritium to ^{14}C present as total or extractable radioactivity increased greatly. The total tritium recovered after 8 hr represented 20 per cent of the administered dose. It could be presumed that much of this tritium was present as body water resulting from tritium exchange with the generally labeled caffeine molecule and from labeled metabolites. Such water would not have been selectively excreted as were the ^{14}C metabolites.

In Table 3 are presented the results obtained after the administration of a 5-mg/kg dose of [^{3}H,^{14}C]caffeine at three selected time periods. Since the data are presented as a percentage of radioactive dose administered and not in terms of concentration, the data are directly comparable to those presented in Tables 1 and 2. It may be observed that the 5 mg/kg dose was distributed within the organs of the mouse and as defined metabolites in a manner similar to that for the 25-mg/kg dose. In percentage terms, however, the 5-mg/kg dose was metabolized somewhat more rapidly. This can be adjudged by comparing the 1-hr values for caffeine presented in Table 3 to those in Table 1. In absolute terms, of course, a greater number of micrograms was metabolized within the same time period after the 25-mg/kg dose.

The disposition of trace metabolites after the 5-mg/kg dose of [^{3}H,^{14}C]caffeine was also nearly identical to that for the 25-mg/kg dose. However, the greater specific activity of the 5-mg/kg dose allowed a few additional trace peaks to be observed. None of these corresponded in R_f to 1-methylxanthine. The additional uric acid chromatographic peaks observed were mainly unidentifiable hydrophobic compounds, and the additional non-acid chromatographic peaks were non-xanthine hydrophilic compounds, presumably resulting from further catabolic reaction of the caffeine molecule. In all of the organs examined (Tables 1, 2 and 3), it was apparent that only a small percentage of the administered radioactivity could be found as "acids," in contrast to the results obtained with urine, where these "acids" often make up 50 per cent of the nonvolatile radioactivity.[6]

Rather than repeat the entire quantitative analysis of the concentration of caffeine and its metabolites within specific organs for animals which had received caffeine in their drinking water since weaning, the distribution of caffeine-derived radioactivity in these animals 24 hr after the administration of 10 mg/kg of 1-methyl[^{14}C]caffeine was determined by whole body radioautography.[13] Frozen carcasses were sectioned to yield whole body sections and these were placed in contact with X-ray film for 3·5 months. The developed radioautograms were compared to similar radioautograms prepared from animals which had received 10 mg/kg of [^{14}C]caffeine 24 hr prior to sacrifice but which had not previously been exposed to caffeine.*

The results obtained by radioautography with animals receiving a chronic oral dose of 4, 15 or 30 mg/kg/day of caffeine as compared to those receiving caffeine for the first time indicated no significant difference in the distribution of caffeine-derived ^{14}C-radioactivity. Some evidence was seen for a generally longer retention of radioactivity

* R. Liss, unpublished observations.

in the chronically treated animals. However, the relative distribution and tissue retention between organs as determined in the 24-hr sections were identical in both experiments. Specifically, no difference in localization as determined by the tissue/blood image density ratio was observed. The chronic administration of caffeine apparently did not result in an altered concentration of caffeine-derived radioactivity in any organ or tissue of the mouse.

Although the major metabolites of caffeine had been identified and the disposition of the administered caffeine had been reasonably described, it was important to us to determine whether an amount of caffeine even less than 0·01 per cent of the administered radioactivity was metabolized to a methylxanthine nucleotide such that incorporation into nucleic acid could occur. A separate specific investigation of liver homogenate was thus conducted to determine the presence of radioactivity in nucleic acid and nucleotides after the administration of [^{3}H,^{14}C]caffeine, using gradient elution column chromatographic techniques as described in Methods.

Figure 1 illustrates the elution profile of ^{14}C-radioactivity from a column to which had been applied a sample of liver homogenate acid extract obtained from a mouse 3 hr after the administration of 25 mg/kg of [^{3}H,^{14}C]caffeine. Samples obtained 1 and 8 hr after dosing with caffeine were also prepared. Peaks with identical elution characteristics were observed with each of these samples. In the case of the 1-hr sample, the

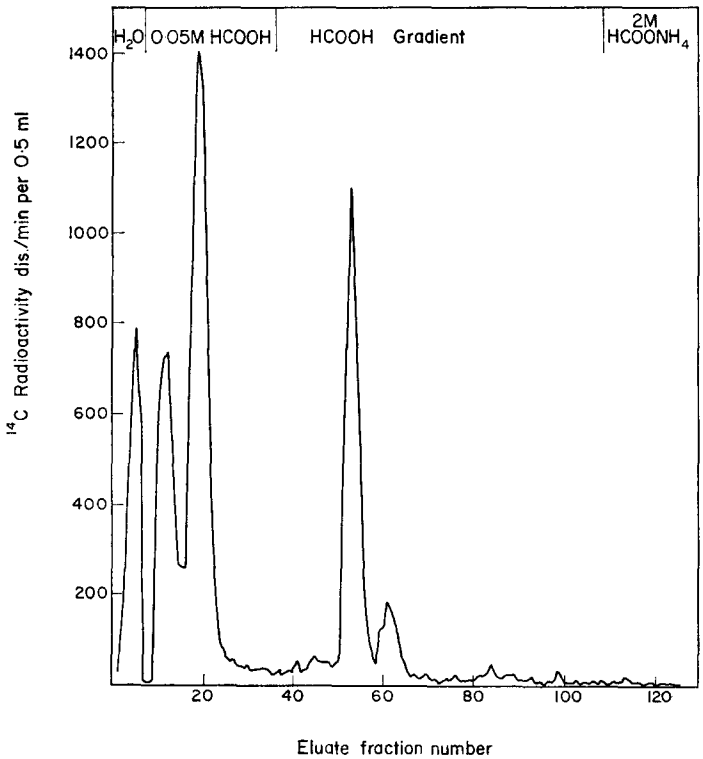

FIG. 1. Elution pattern from an AG-1-formate column to which an acid extract of liver had been applied. The liver was obtained from three mice which had been given 25 mg/kg of [^{3}H,^{14}C]caffeine, p.o., 3 hr previously. The composition of eluant is indicated; 3·0-ml fractions were collected.

second and third peaks were twice the size of those seen in Fig. 1, however, and with the 8-hr sample all peaks were smaller, as would be expected, based on the low level of radioactivity present in the liver 8 hr after dosage. Tritium radioactivity eluted from the column directly paralleled ^{14}C-radioactivity except that the peak observed eluting at tube 61 contained no tritium, and with the 8-hr sample, two small peaks containing tritium but no ^{14}C were eluted in the vicinity of tube 80. Only the 3-hr 25 mg/kg sample resulted in radioactivity being eluted by the 2·0 M ammonium formate final eluant.

Nucleotide di- and triphosphates would be expected to be eluted from the columns between tubes 110 and 126. As can be seen from Fig. 1, only an insignificant amount of radioactivity was found in this area. The peak at tube 114 was only twice background and was not present at all in the 1-hr or 8-hr samples. Nucleotides might appear anywhere in the formic acid gradient. A considerable portion of the radioactive material present in the liver homogenates (25 per cent) does elute here. It is also possible, however, that the radioactivity in this area might be due to other acids such as methyluric acids. Uric acid itself, however, would be expected to elute earlier from the column.

To determine if the large peaks eluted by the formic acid gradient represented nucleotides or uric acids, a sample of the acid fraction of urine obtained 3 hr after the administration of a 5-mg/kg dose of [^{3}H,^{14}C]caffeine was placed on an AG-1-formate column and chromatographed in the same fashion as the liver homogenate. Most of the radioactivity eluted in the water and formic acid washes, as it did with the liver preparation. However, two additional peaks in the formic acid gradient, representing 26 and 1·4 per cent of the ^{14}C-radioactivity were observed. The peaks corresponded closely to the two "nucleotide" peaks observed with the liver acid extract. The appearance of compounds resulting in such peaks in the urine indicated that the two peaks from liver are not associated with nucleotide material, since it is highly unlikely for nucleotides to exist in any abundance in the urine. It is most probable that these peaks are, in fact, methylated uric acids. When the total acid fraction obtained from the various liver homogenates was compared to the amount of material in the AG-1-formate eluate in tubes 44–69, a constant ratio of 1·4–1·7 was observed, further strengthening the conclusion that these two peaks result from uric acids.

To investigate further the possibility that methylxanthine nucleotides by coincidence are eluted concomitantly with methyluric acids, the material in tubes 44–69 was chromatographed on a cellulose thin-layer system with 95% ethanol–1 M ammonium acetate, pH 7·5, 35:70, both before and after treatment with alkaline phosphatase. In this system, inosine migrated with an R_f value of 0·65, inosinic acid with an R_f of 0·47, and uric acid with an R_f of 0·30. The thin-layer plate was scanned for radioactivity with the Packard strip scanner. The plate was then divided into small areas which were scraped into scintillation vials, eluted with water, and counted in the scintillation spectrometer in TEN scintillator solution. No significant differences were seen between the developed chromatograms with and without alkaline phosphatase treatment.

Finally, the low level of radioactive material existing between tubes 73 and 93 was combined and chromatographed in a 5 M ammonium acetate (pH 9·0)–saturated sodium tetraborate–95% ethanol–0·5 M versene (40:160:360:1) thin-layer system. Once again, no difference was observed in the distribution of radioactivity between the control and the alkaline phosphatase-treated eluate. The absence of radioactivity at the origin of the control sample where the nucleotide would remain also led to the

conclusion that the material eluting in tubes 73–93 was probably not a methylxanthine nucleotide.

Significant radioactivity was incorporated into the DNA and RNA fractions of liver. Three hr after administration of 25 mg/kg of [^{3}H,^{14}C]caffeine, p.o., 0·02 per cent of the ^{14}C and 0·01 per cent of the ^{3}H was associated with DNA in this organ, and the liver RNA contained 0·04 and 0·03 per cent of the respective administered radioactivity. After 8 hr, the liver nucleic acid fractions still contained 0·01 per cent of the administered radioactivity in DNA, and 0·02 per cent ^{14}C and 0·04 per cent ^{3}H in RNA. This radioactivity represented less than one-fifth of the radioactivity associated with the acid-insoluble fraction at these times and is probably the result of random incorporation of labeled compounds resulting from the extensive metabolic decomposition of caffeine. This view was supported by the finding that, whereas the distribution of radioactivity (both ^{14}C and ^{3}H) between RNA and DNA remained constant from 3 to 8 hr after dosing, the ratio of ^{3}H/^{14}C increased over 2-fold in this same time period in both nucleic acid fractions. No attempt was made to determine which monomeric elements of the nucleic acid were radioactive.

DISCUSSION

The data of Tables 1, 2 and 3 were recalculated to compare the concentration of caffeine in each organ to the water content of that organ (Fig. 2). In the interests of clarity, the results for all organs investigated are not presented graphically. Heart and muscle, for instance, gave results almost identical to those for lung. At the 1-hr period following dosage, the milligrams of caffeine per kilogram of tissue water in the lung, heart, muscle, testes and liver was 17·1, 17·4, 17·5, 17·6 and 17·8 respectively. Such results tend to support the finding in the dog and man that caffeine distributes with body water.[2,3] The more detailed analysis presented in Fig. 2, however, indicates that at the earlier and later time periods the value of caffeine/tissue water did differ from one organ to another. The uptake and excretion of caffeine from these organs did vary and a direct correspondence between caffeine concentration and tissue water resulted only during a short plateau period.

Specifically, it would seem that a barrier to the accumulation of caffeine in the brain and testes existed, while the liver, spleen and perhaps kidney actively removed caffeine from the plasma. This is in agreement with published data on the distribution of total radioactivity in the liver, brain and testes of mice after the injection of tritiated caffeine.[16] The kidney may maintain a higher concentration of caffeine because of the presence of compound in the tubules. However, the low value for caffeine concentration in the plasma, apparent after both the 5- and 25-mg/kg doses, indicates that in the body of the mouse caffeine did not have an opportunity to establish a true equilibrium concentration.

Caffeine had a half-life of less than 3 hr in all organs investigated and apparently the rapid metabolism and excretion of caffeine metabolites were responsible for this.

After a 5-mg/kg oral dose of caffeine, a shorter tissue half-life for caffeine was observed than after a 25-mg/kg oral dose. This decreased half-life of caffeine was independent of the half-life for total radioactivity administered, which was the same after each dose. Specifically, a plasma half-life of 2·5 hr for total ^{14}C radioactivity was observed after both oral doses. The half-life of caffeine, however, was calculated

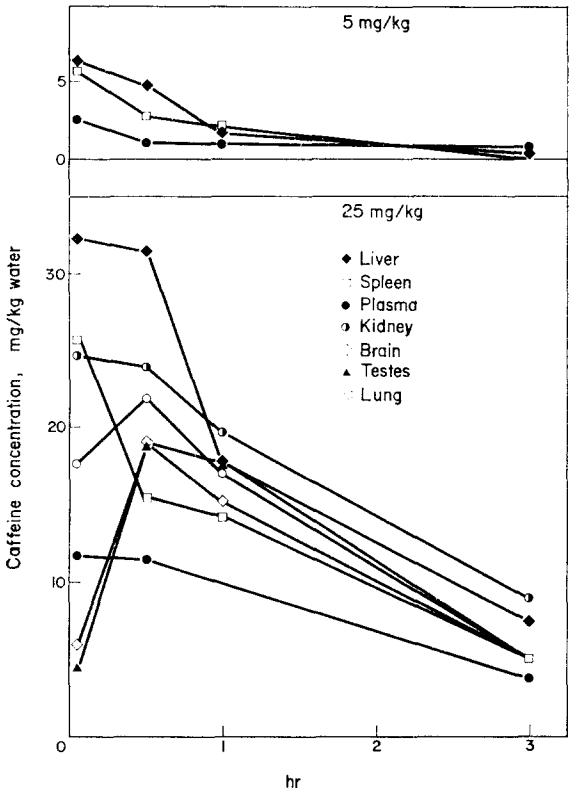

FIG. 2. Caffeine in organs of the mouse expressed as the concentration in tissue water. Caffeine was determined by multiple chromatography of tissue homogenates obtained from groups of three mice sacrificed at various times after the administration of 5 or 25 mg/kg of [^{3}H,^{14}C]caffeine, p.o. Tissue water was calculated based on organ wet weights and values obtained from the literature.[14,15]

to be 1·7 and 0·6 hr after 25 and 5 mg/kg of caffeine respectively. Such findings illustrate the difficulty in drawing conclusions concerning the dynamics of tissue distribution solely from total radioactivity data.[17] In this case the smaller dose was more extensively metabolized, but these products of metabolism were not, in general, excreted more rapidly.

One class of compounds was excreted rapidly. Very little methyluric acid could be demonstrated in the tissues of the mouse, although 1-methyluric acid and 1,3-dimethyluric acid have been shown to be major urinary metabolites in this species[6] as well as in man.[18] The uric acid metabolites must readily pass into the blood and be removed by the kidney. Thus the high concentration of uric acids in the urine gave no true indication of the exposure of tissue to these particular caffeine metabolites.

In contrast, the 1,7-dimethylxanthine which is found in the urine does not reveal that 1,7-dimethylxanthine is the major caffeine metabolite to which the mouse organs were exposed. Other dimethylxanthines absent from the urine were also absent in the tissues, although low levels of theobromine (as well as 3-methylxanthine and 7-methylxanthine) could have been missed due to the difficulties of measuring trace quantities of tritium-labeled metabolites in tissue by our methods. No 1-methylxanthine, a major

urinary metabolite in man[18] but not in the mouse,[6] was detectable. Neither could the presence of methylated nucleotides resulting from caffeine be shown. The presence of such compounds would have had implications for the relationship of caffeine and nucleic acid function.

The only metabolite of caffeine which had been previously reported in a mammalian tissue is 1,7-dimethylxanthine. The administration of 500 mg caffeine to human subjects by Warren[19] resulted in the appearance of 1,7-dimethylxanthine in the plasma and erythrocytes within 3 hr. Theophylline and theobromine were not observed. 1,7-Dimethylxanthine is the major metabolite of caffeine present in the tissues of the male CD-1 mouse and may prove to have a like role in other mammalian species.

TABLE 4. CONCENTRATION OF CAFFEINE AND 1,7-DIMETHYLXANTHINE IN THE ORGANS OF THE MOUSE OVER 24 hr

	C × t (mg hr/kg)			
	5 mg/kg dose		25 mg/kg dose	
Organ	Caffeine	1,7-CH_3-xanthine	Caffeine	1,7-CH_3-xanthine
Brain	3·5	<2	38	10
Heart	2·4	4·5	50	<10
Kidneys	5·9	9·9	55	30
Liver	5·4	2·8	45	24
Lungs	3·6	3·3	39	10
Spleen	2·6	<2	35	<10
Testes	5·0	<2	46	19

Table 4 presents a comparison of the exposure of organs of the mouse to caffeine and its major metabolite. The data in this table were obtained by actually measuring the areas under the concentration vs. time curves obtained for caffeine and 1,7-dimethylxanthine. These caffeine values (Table 4) should be compared to the theoretical C × t values of 120 and 600 mg hr/kg for a 5- and 25-mg/kg administered dose in the absence of excretion or metabolism.

Even considering the C × t exposure of organs over the 8 hr during which caffeine concentration was measurable or could be directly extrapolated, the exposure is less than one-fourth the theoretical value, assuming uniform equal distribution in body water. With two exceptions, the organs experienced an even lower exposure to 1,7-dimethylxanthine. The between organ variation in 1,7-dimethylxanthine exposure was much greater than that for caffeine exposure, indicating a different rate of formation or absorption between organs for this compound. No evidence for accumulation of 1,7-dimethylxanthine in any organ was apparent. The lack of organ-specific accumulation of any other ^{14}C metabolite was demonstrated by the radioautographic experiment. This experiment also revealed that prior chronic administration of caffeine did not significantly alter the level of residual ^{14}C-radioactivity in the mouse 24 hr after administration of 10 mg/kg of [^{14}C]caffeine.

The ability of caffeine to stimulate the activity of hepatic drug-metabolizing enzymes in the rat has been reported.[20] In the mouse, active microsomes from liver and the cytosol-microsome fraction from kidney failed to demethylate caffeine or theobromine

(unpublished observation). Mazel and Henderson[21] have observed less than a 0·4 per cent demethylation of caffeine, theobromine or theophylline, a level which would have been readily detected in our experiments. These low conversions seem inconsistent with the rapid metabolism of caffeine in the mouse. Likewise, we and others[22] have demonstrated the inability of bovine milk xanthine oxidase to oxidize caffeine or theophylline to the corresponding uric acids (1-methylxanthine is oxidized), although methyluric acids are a major metabolite.

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