Metabolic Pathway of Theobromine in the Rat and Identification of Two New Metabolites in Human Urine

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Theobromine, a metabolite of caffeine, is present in food and is also used in pharmacology as a diuretic. [7-*methyl*-^{14}C]Theobromine was synthesized and administered by stomach tube to six rats. Twenty-four hours later, the radioactivity collected in $^{14}CO_2$, feces, and urine amounted respectively to 6 ± 1, 11 ± 1, and 84 ± 8% of the total dose. Less than 3% of the radioactivity was found in the body of the animal. Compounds identified in the urine were theobromine (49% of the urine radioactivity), 7-methylxanthine (6%), and 7-methyluric acid (4%). Two new metabolites, 3,7-dimethyluric acid (2.7%) and 4-amino-5-(*N*-methylformylamino)-3-methyluracil (36%), were identified and also low amounts of dimethylallantoin and *N*-methylurea. 3-Methylxanthine, a pharmacologically active metabolite of theophylline, and 3-methyluric acid amounted to 5.8% and corresponded to a minor metabolic pathway in the case of theobromine. 3,7-Dimethyluric acid and the uracil derivative were identified in human urine.

Theobromine is 3,7-dimethylxanthine and shares in common with caffeine and theophylline several pharmacological actions of therapeutic interest. In contrast with caffeine and theophylline, the action of theobromine on the central nervous system is weak. Theobromine is used in therapy for its diuretic properties, explained by a direct action on the kidney and a mild cardiac stimulation (Richtie, 1970).

Theobromine can also be ingested daily through food and drinks containing cocoa. Defatted cocoa powder contains 1.7–3.3% theobromine and 0.1–1.4% caffeine. A concentration of 0.17% theobromine is found in tea (Czok, 1974). Moreover, theobromine enters the body through caffeine metabolism and appears in the urine of coffee drinkers (Arnaud, 1978, a).

Cornish and Christman (1956) did not detect theobromine in human urine after administration of caffeine but published the first metabolic study of humans after ingestion of 1 g of theobromine. The metabolic pathway proposed showed that 7-methylxanthine was the most important metabolite (28–30%). Unchanged theobromine (11–12%), 3-methylxanthine (14–21%), and 7-methyluric acid (3–4%) were also found. These results were incomplete and a maximum recovery of only 67% was reached after 48-h urine collection.

More recently, Van Gennip et al. (1973) identified 3-methylxanthine and 7-methylxanthine in the urine of children eating chocolate. These authors conclude that the methylxanthines found were of dietary origin and not produced endogenously from methylated purines of transfer RNA.

The metabolic pathway of theobromine is of importance because some metabolites may have pharmacological activity. This is the case for 3-methylxanthine, which has been compared to theophylline. However, the physiological effects of theophylline are always more potent than those of 3-methylxanthine (Williams et al., 1976; Persson and Andersson, 1977).

In the animal, the metabolic pathway of theobromine is unknown because no work has yet been carried out with a labeled molecule.

This work describes the synthesis of [7-*methyl*-^{14}C]-theobromine and its metabolism in the rat after oral administration. Theobromine from cocoa powder was also administered to a volunteer in order to identify in the urine the new metabolites discovered in the rat.

METHODS

[7-*methyl*-^{14}C]Theobromine was obtained by methylation of 8 mg of 3-methylxanthine (Cyclo Chemical) with 400 μCi of dimethyl sulfate in benzene solution (Schwarz/Mann, 8.32 mCi/mM). 3-Methylxanthine was solubilized with 1 mL of NaOH 0.1 N and 2 mL of dimethyl sulfoxide was added. This solution was put directly into the tube containing dimethyl sulfate and the methylation lasted for 2 h at 60 °C with continuous shaking of the sealed tube.

The entire reaction mixture was then put on a Whatman No. 3 paper for chromatography with the solvent 1-propanol/concentrated ammonium hydroxide/water (6:3:1, v/v). Theobromine was eluted and purified by preparative thin-layer chromatography (Merck, 2-mm thickness) with the solvent chloroform/methanol (8:2, v/v) and chloroform/acetone/butan-1-ol/concentrated ammonium hydroxide (3:3:4:1, v/v). Seventy-five microCuries of [7-*methyl*-^{14}C]theobromine (8.32 mCi/mM) was obtained with more than 98% radiochemical purity (Scanner Berthold).

Theobromine was administered in aqueous solution (1–2 mL, 5–6 μCi) by a stomach tube to six male Sprague-Dawley rats weighing 100–150 g. Unlabeled theobromine was added with the radioactive compound in order to administer doses in the range of 1 to 6 mg/kg body weight. Rats were immediately housed in individual glass metabolic cages where $^{14}CO_2$, urine, and stools were collected for 24 h after the administration.

Carbon dioxide expired by the animals was trapped with a 12% solution of ethanolamine in methanol. Time of collection was 30 min for each sample of 15 mL and radioactivity of an aliquot of 5 mL was counted by liquid scintillation. Urine samples were counted with Aquasol (New England Nuclear). At the end of the experiment, the organs, samples of feces, and samples of the homogenized carcass of the animals were burnt with an Oxymat (Intertechnique), and the radioactivity was counted on a liquid scintillation counter (Mark III, Searle).

Metabolites were separated and identified by two-dimensional, thin-layer chromatography on silica plates (Merck, 0.25-mm thickness) with the following solvent systems: (1) chloroform/methanol (8:2, v/v) and (2)

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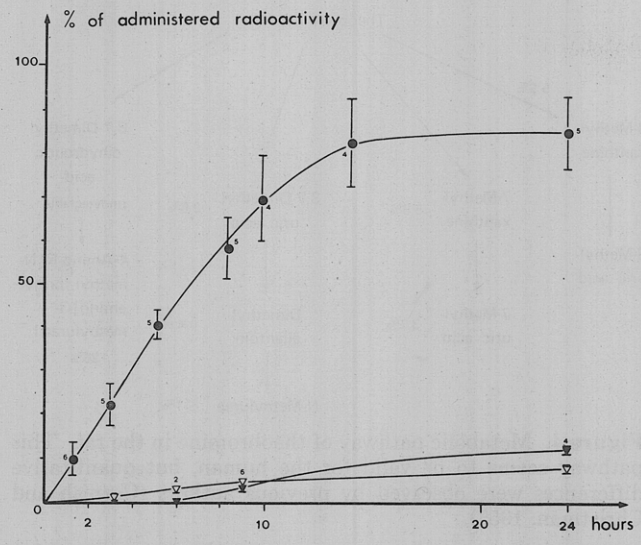

Figure 1. Cumulative curves of total radioactivity collected in the urine (●), carbon dioxide (▽), and the feces (▼) after rat administration of labeled theobromine. The values are expressed as mean ± SEM. The figures correspond to the number of rats.

chloroform/acetone/butan-1-ol/concentrated ammonium hydroxide (3:3:4:1, v/v). Whole urine was directly spotted on the thin-layer plate, while total radioactivity found in the feces was extracted with water and then chromatographed.

Standards used were theobromine (R_f 0.67 in solvent system 1 and R_f 0.47 in solvent system 2) (ICN Pharmaceuticals), 3,7-dimethyluric acid (R_f 0.37; R_f 0.04), 7-methylxanthine (R_f 0.40; R_f 0.04) 3-methylxanthine (R_f 0.47; R_f 0.07), 7-methyluric acid (R_f 0.01; R_f 0), and 3-methyluric acid (R_f 0; R_f 0) (Fluka A.G.).

RESULTS

The total amount of $^{14}CO_2$ expired within 24 h after stomach tube administration of [7-*methyl*-^{14}C]theobromine corresponded to 6 ± 1% (mean ± SEM) of the administered radioactivity. This result shows that demethylation of the 7-methyl group followed by CO_2 production is not quantitatively an important metabolic pathway of theobromine in the rat. Maximum $^{14}CO_2$ expiration occurred 3 h after the administration (Figure 1). Labeled theobromine incubated for 24 h in vitro with the gastrointestinal content produced no $^{14}CO_2$ and no radioactive derivative either with or without oxygen. Thus the $^{14}CO_2$ collected in vivo seemed to be produced in the rat organs and 3-methyl derivatives must have appeared in the blood and have been excreted in the urine: probably 3-methylxanthine and 3-methyluric acid.

A mean of 11 ± 1% of the radioactivity administered was excreted in the feces. The total radioactivity of the feces was extracted with water and was studied by thin-layer chromatography.

Theobromine corresponded only to 10% of the fecal radioactivity (1% of the administered radioactivity). The most important compound was the 3,7-dimethyluric acid. In some extracts, *N*-methylurea was found with a derivative which seemed to be a dimethylallantoin: 3,6- or 1,8-dimethylallantoin. The presence of these compounds in the feces could be explained by the degradation of the 3,7-dimethyluric acid by the flora of the intestine. The new metabolite discovered in the urine and described later was also found in the feces.

After 24 h, about 1–2% of administered radioactivity remained in the content of the intestine, cecum, and colon. The total radioactivity found in the kidney, testicle, spleen, gall bladder, pancreas, lungs, heart, thymus, brain, and liver amounted only to 0.3–0.6% of the administered radioactivity. The liver contained most of the radioactivity: 0.2–0.4%.

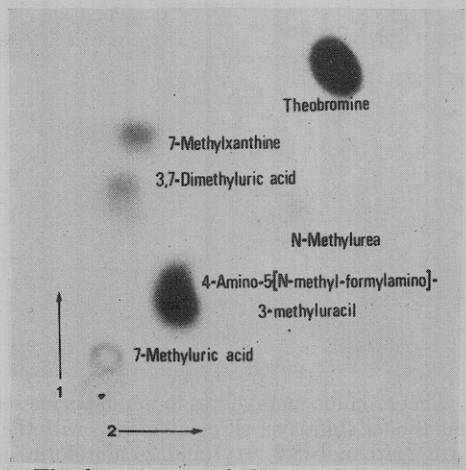

Figure 2. Theobromine metabolites in the urine separated by two-dimensional, thin-layer chromatography on silica plates and localized by autoradiography with X-ray film. The solvent system used is 1-chloroform/methanol (8:2, v/v) and 2-chloroform/acetone/butan-1-ol/concentrated ammonium hydroxide (3:3:4:1, v/v).

Table I. Quantitative Results of Theobromine Metabolites Excretion in Rats' Urine during 24-h Period after Stomach Tubing

compounds	% of urine radioactivity mean ± SEM
theobromine	49 ± 4
7-methylxanthine	6 ± 1
4-amino-5-(*N*-methylformylamino)-3-methyluracil	36 ± 4
3,7-dimethyluric acid	2.7 ± 0.2
7-methyluric acid	3.9 ± 0.5
dimethylallantoin	Tr
N-methylurea	<1

Figure 1 shows that the excretion in the urine was the main metabolic route of theobromine in the rat. Half of the radioactivity administered was collected in the urine after 9 h, and this value reached 84 ± 8% after 24 h.

The autoradiography of urinary metabolites separated by two-dimensional, thin-layer chromatography (Figure 2) shows that unchanged theobromine was quantitatively an important excreted compound. Theobromine appeared more rapidly than other metabolites and the relative proportion of theobromine in the urine samples decreased continuously with time during the experiments.

Table I shows that theobromine corresponded to 49 ± 4% of the total radioactivity excreted in the urine.

The 3,7-dimethyluric acid was not found previously in humans but traces were detected in the urine of the rat during metabolic study of [2-^{14}C]caffeine (Arnaud, 1976b). This compound was identified on our radiochromatograms and was not higher than 3%, while 7-methylxanthine, the most important metabolite in human according to Cornish and Christman (1957), represented only 6% of the urine radioactivity. Traces of *N*-methylurea were also present in the urine.

We have identified the 7-methyluric acid which corresponded to 3–5% of the urine radioactivity. This compound can be oxidized on the silica plates and partially transformed into 3-methylallantoin. About half of the 7-methyluric acid remained at the origin of the chromatogram with the solvent system used.

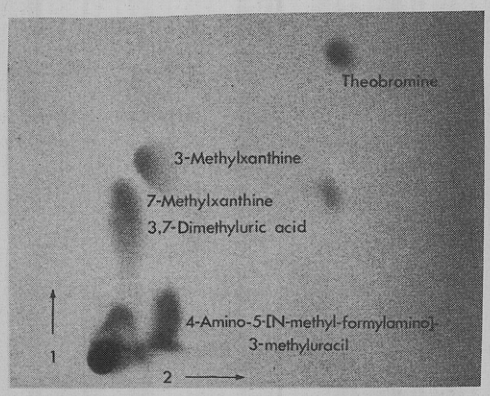

Figure 3. Theobromine metabolites in human urine separated by two-dimensional, thin-layer chromatography with the solvent 1-chloroform/methanol (8:2, v/v) and 2-chloroform/acetone/butan-1-ol/concentrated ammonium hydroxide (3:3:4:1, v/v) on silica plates and detected in the ultraviolet.

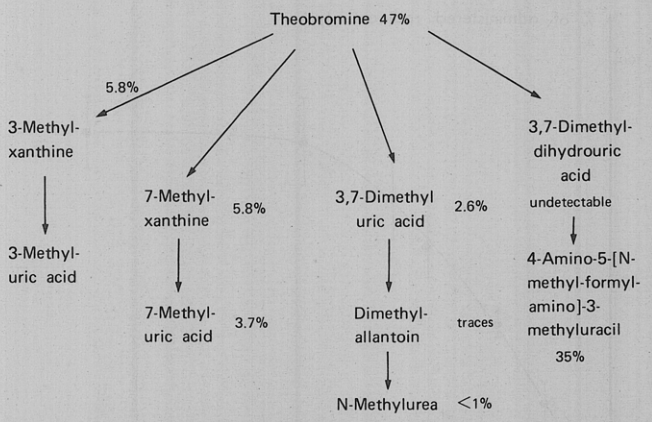

Figure 4. Metabolic pathway of theobromine in the rat. This pathway seems to be valid for the human, but quantitative differences were observed by previous authors (Cornish and Christman, 1957).

After theobromine, the most important metabolite found quantitatively in the urine was unknown (36 ± 4%). This compound exhibited an ultraviolet absorption spectrum different from theobromine and 3,7-dimethyluric acid, with a maximum at 266–268 n, at pH 7. This labeled metabolite isolated from the urine of the rat and heated at 160 °C for 3 h was converted to theobromine with a 43% yield. The structure of 3,7-dimethyldihydrouric acid was assumed because the 1,3,7-trimethyldihydrouric acid has been shown to be unstable and to dehydrate at high temperature to give caffeine (Arnaud, 1976c). Rao et al. (1973) published that the 1,3,7-trimethyldihydrouric acid was in equilibrium with its open chain. Using a synthetic standard (Pfleiderer, 1971), the open-chain structure was demonstrated by cochromatography on TLC (R_f 0.20 in solvent system 1 and R_f 0.16 in solvent system 2) and using high-pressure liquid chromatography (RP-8, mobile phase: water/acetonitrile, 4%). By high-pressure liquid chromatography, the synthetic compound and the labeled metabolite showed two peaks corresponding to two isomers. The chemical structure of the 4-amino-5-(*N*-methylformylamino)-3-methyluracil obtained by synthesis was verified by nuclear magnetic resonance analysis (Philippossian and Arnaud, 1978).

Urine samples of a volunteer, previously deprived of food containing methylated xanthines (coffee, tea, chocolate), were collected before and after ingestion of 1 g of theobromine from 40 g of defatted cocoa powder solubilized in hot milk. Ultraviolet absorbing compounds appeared in the urine after administration and were separated by TLC as shown in Figure 3. Unchanged theobromine, 7-methylxanthine, 3-methylxanthine, 3,7-dimethyluric acid, and the 4-amino-5-(*N*-methylformylamino)-3-methyluracil were identified. Only 5 days after administration, these metabolites became undetectable on the urine chromatogram.

DISCUSSION

These results demonstrate that theobromine is almost completely absorbed as only 1% of the administered compound was collected in the feces of the rats. After 24 h, no organ accumulation of theobromine and metabolites could be seen by whole animal body autoradiography and the most labeled organ was the liver with 0.4% of the administered radioactivity. At that time, the content of the intestine contained 2% of the radioactivity localized in the cecum and the colon. The fecal excretion of metabolites like 3,7-dimethyluric acid and 4-amino-5-(*N*-methylformylamino)-3-methyluracil must have come from intestinal and bile secretions because theobromine was not metabolized in vitro by the intestinal flora. Although dimethylallantoin could be produced in the rat like trimethylallantoin from caffeine metabolism (Rao et al., 1973; Arnaud, 1976b), the detection of only traces in the urine could be explained by its formation, like *N*-methylurea, by degradation of the 3,7-dimethyluric acid in the intestines.

After intestinal absorption, theobromine and the metabolites produced are excreted in the urine. This excretion is almost complete in the rat after 1 day.

The metabolic pathway proposed for the rat (Figure 4) shows quantitative differences from that published for the human by Cornish and Christman (1957). The only agreement was obtained for the amount of 7-methyluric acid: 3–5%.

The formation of 3-methyl derivatives calculated from $^{14}CO_2$ collection are included in the rat metabolic pathway. These derivatives corresponded only to 5.8% in the rat instead of 14–21% in the human. Thus, the 3-methylxanthine which is now the only known metabolite with a pharmacological activity is a minor compound produced in the rat. Higher amounts of 7-methylxanthine were also reported for the human urine: 28–30% instead of 6% for the rat.

It appears from these results that theobromine, which represented 11–12% of excreted compound in human urine, was metabolized mainly to 3- and 7-methylxanthine, while in the case of the rat, demethylation of theobromine to methylxanthines is less important and about half of the compounds in the urine is unchanged theobromine. For the first time 3,7-dimethyluric acid is identified in the urine. The direct oxidation of theobromine into dimethyluric acid is a minor metabolic pathway, both in the human and the rat.

A new metabolite, identified as the 4-amino-5-(*N*-methylformylamino)-3-methyluracil and corresponding to 35% of the excreted derivatives in the urine of the rat is detected also in human urine. We assume that this compound is obtained directly by the hydration of the 8,9 double bond of theobromine, giving the 3,7-dimethyldihydrouric acid which is unstable and thus undetectable in the urine (Figure 5). The formation of this intermediate can explain the rapid conversion of the metabolite into theobromine on heating.

We have previously shown (Arnaud, 1976b) that the 1,3,7-trimethyldihydrouric acid is quantitatively the most important caffeine metabolite in the rat. Now with the synthetic standard (Pfleiderer, 1971) we have verified that the structure of the compound isolated from urine by TLC

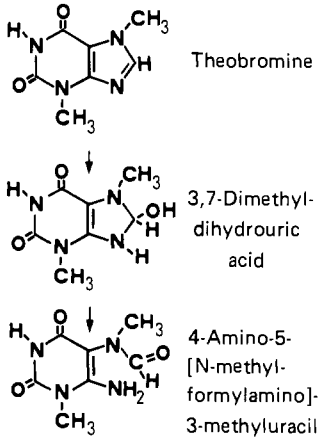

Figure 5. Pathway of 4-amino-5-(*N*-methylformylamino)-3-methyluracil formation from theobromine through the hypothetic 3,7-dimethyldihydrouric acid.

was in fact the 4-amino-5-(*N*-methylformylamino)-1,3-dimethyluracil.

Blood analysis using the less drastic technique of high-pressure liquid chromatography and the study of the chemical structure of these compounds may be of value in the understanding of the biological mechanism of the theobromine and caffeine transformation into these polar compounds rapidly excreted in the urine.

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We thank Professor W. Pfleiderer for providing us with a sample of 4-amino-5-(*N*-methylformylamino)-3-methyluracil and I. Bracco for the technical assistance in performing the whole animal body autoradiography.

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A Convenient Preparation of Pure Stearoyl-2-lactylic Acid

Carl A. Elliger

A preparation of pure stearoyl-2-lactylic acid is described in which benzyl lactylate is allowed to react with stearoyl chloride. The benzyl ester, a key intermediate, is prepared from lactide and benzyl alcohol.

Stearoyl-2-lactylic acid, the stearate ester of the linear lactic acid dimer, and its sodium or calcium salts find use as surfactants in dough conditioner mixtures (Griffin and Lynch, 1972). The effect of these compounds in modifying rheological properties of doughs, altering crumb structure in breads, and improving loaf volume has been attributed to complexation with the starch components, especially amylose (Krog, 1971). The involvement of amylopectin was pointed out by De Stefanis et al. (1977) and the role of binding of these surfactants to cereal proteins has also been established (Chung and Tsen, 1975; Greene, 1975). Fullington (1974) isolated a protein from wheat flour that binds calcium and which also forms a complex with the stearoyl-2-lactylate ion.

Investigations in this laboratory on protein interactions with various surfactants necessitated pure samples of stearoyllactylic acid and its salts. Since commercially obtainable materials consist of a mixture of mainly mono-, di-, and trimeric lactic acid derivatives which resist purification it was necessary for us to develop a satisfactory synthesis for the desired dimeric compounds.

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EXPERIMENTAL SECTION

General. Melting points were taken on a Thomas-Hoover capillary melting point apparatus and are corrected. Infrared (IR) spectra were recorded on a Perkin-Elmer Model 257 instrument and proton magnetic resonance (^{1}H NMR) spectra were determined with a Varian A-60 spectrometer and are reported as parts per million (δ) relative to tetramethylsilane (internal standard).

Lactide. Lactic acid (87%) was heated under aspirator vacuum until water was no longer evolved (pot temperature to 200 °C). The pressure was then reduced to ca. 0.1 mm and crude product was collected as a very viscous distillate, bp 80–90 °C. Crystallization from diethyl ether followed by 95% ethanol gave a 20% yield of cyclic dimer, mp 124–125 °C (lit. mp 125 °C for the *d,l* form; Dietzel and Krug, 1925). IR ($CHCl_3$): 1770 cm^{-1} (cyclic ester carbonyl); NMR ($CDCl_3$) δ 1.64 (d, J = 7 Hz, 6 H, $CHCH_3$), 5.13 (q, J = 7 Hz, 2 H, $CHCH_3$).

Benzyl Lactylate. In a 500-mL flask equipped with stirrer and condenser were placed 28.8 g (200 mmol) of lactide, 43.2 g (400 mmol) of benzyl alcohol, 0.5 g of *p*-toluenesulfonic acid and 150 mL of dry dioxane. The mixture was refluxed 19 h and most of the dioxane was