according to the method of BARBER and JAMIESON.[11] as modified by CAMERINI-DAVALOS et al.[12] Protein was determined by the biuret method[13] using serum albumin as standard.

Results and discussion. Table I shows the results of oral glucose tolerance tests in SA, KK and hybrid mice. No significant difference either in the oral glucose tolerance or in the glucose area was observed among the three groups of mice. However, the kidney glucosyltransferase activity was significantly higher in the KK when compared to that of both SA and hybrid mice (Table II). The enzyme activity, although elevated in the hybrids, was not statistically significant when compared to SA mice. It should, however, be noted that the glucosyltransferase activity in the hybrids was intermediary of both parental strains.

That the KK mouse is an ideal animal model for the studies of human genetic diabetes and microangiopathy has been suggested by us and other investigators[14–18].

These mice have normal tolerance to oral glucose until 100 days (prediabetes) and impaired tolerance later on (chemical diabetes)[12]. About 60% of the mice develop glomerular lesions by 2 months of age[19] at the same time when these mice still have normal tolerance to oral glucose. Determination of glucosyltransferase activity in the renal cortices showed that the KK mice have elevated levels between the 25th and 55th days of life when compared to age-matched SA mice[12, 20]. Later on, no difference in the enzyme activity between the two groups of mice was observed. It is, therefore, understandable that the significant elevation of glucosyltransferase activity in the prediabetic KK mice precedes detectable structural lesions in the kidney and abnormal tolerance to oral glucose.

The present results indicate that changes in the kidney glucosyltransferase activity can be observed in response to gene dosage without affecting glucose tolerance, suggesting that microangiopathy and carbohydrate tolerance are independent at the stage of early diabetes. The data are compatible with changes in muscle capillary basement membrane width in human prediabetics with normal oral glucose tolerance.[4, 5] In addition, the data suggest that enzymes involved in the basement-membrane synthesis are subject to changes depending upon the genetic make-up of the animal. Such an early enzyme change can be used as a possible genetic marker for the diagnosis and prognosis of microangiopathy in those individuals with a family history of diabetes and who had not yet developed intolerance to glucose.

[11] A. J. BARBER and G. A. JAMIESON, Biochim. biophys. Acta *252*, 533 (1971).
[12] R. A. CAMERINI-DAVALOS, C. A. VELASCO, A. S. REDDI and W. OPPERMANN, J. clin. Invest., submitted.
[13] A. G. GORNALL, C. J. BARADAWILL and M. M. DAVIS, J. biol. Chem. *177*, 751 (1949).
[14] W. OPPERMANN, G. TRESER, T. EHRENREICH, K. LANGE, R. LEVINE and R. A. CAMERINI-DAVALOS, Clin. Res. 16, 347 (1968).
[15] R. A. CAMERINI-DAVALOS, W. OPPERMANN, R. MITTL and T. EHRENREICH, Diabetologia *6*, 324 (1970).
[16] H. WEHNER, E. HOHN, U. FAIX-SCHADE, H. HUBER and P. WALZER, Lab. Invest. *27*, 331 (1972).
[17] P. KERN, M. LAURENT and F. REGNAULT, Revue Étud. clin. biol. *18*, 882 (1972).
[18] J. DUHAULT, F. LEBON, M. BOULANGER and R. DU BOISTESSELIN, Bibliophie anat. *11*, 453 (1973).
[19] W. OPPERMANN, T. EHRENREICH, D. PATEL, T. ESPINOZA and R. A. CAMERINI-DAVALOS, in *Vascular and Neurological Changes in Early Diabetes* (Eds. R. A. CAMERINI-DAVALOS and H. S. COLE; Academic Press, New York 1973), Suppl. 2, p. 281.
[20] C. A. VELASCO, W. OPPERMANN, N. MARINE and R. A. CAMERINI-DAVALOS, Horm. Metab. Res. *6*, 427 (1974).

Metabolism of 1,3,7-Trimethyldihydrouric Acid in the Rat: New Metabolic Pathway of Caffeine

M. J. ARNAUD

Nestlé Products Technical Assistance Co. Ltd., Research Department, P. O. Box 88, CH–1814 La Tour-de-Peilz (Switzerland), 5 May 1976.

Summary. [1-CH_3-^{14}C] 1,3,7-trimethyldihydrouric acid which, in quantity, is the most important caffeine metabolite, was isolated and purified from the urine of rats fed with [1-CH_3-^{14}C] caffeine. The oral administration of this metabolite to rats showed that 1,3,7-trimethyldihydrouric acid was excreted unchanged in urine and was therefore an end product of caffeine metabolism. This result implies a new metabolic pathway of caffeine.

1,3,7-Trimethyldihydrouric acid was isolated[1] and then identified[2] in extracts from rats' urine. Although the exact amount of this caffeine metabolite was unknown, it was reported that about 12% of injected caffeine was excreted in this form. A quantitative study of all of the caffeine derivatives excreted in the urine has shown that this compound corresponds to 20% of caffeine administered orally[3].

The metabolism of 1,3,7-trimethyldihydrouric acid has never been studied, because this product appears to be unstable and readily dehydrates to give caffeine[1].

In this study, thin-layer chromatography was used with success to isolate and purify [1-CH_3-^{14}C] 1,3,7-trimethyldihydrouric acid from the urine of rats which had received [1-CH_3-^{14}C] caffeine orally. The metabolism of this caffeine derivative in the rat was studied, and, according to the results, a new metabolic pathway of caffeine is proposed.

Material and methods. Male Sprague-Dawley rats weighing 200 g were directly fed by stomach tube 100 μCi of [1-CH_3-^{14}C] caffeine (specific activity: 24.0 μCi/mg, New England Nuclear). Urine was collected during the 48 h after administration and immediately frozen. The urine samples were then chromatographed on preparative silica plates (Merck, 2 mm thickness) and the 1,3,7-trimethyldihydrouric acid isolated and purified by chromatography with the following solvents: 1. Chloroform-methanol (4:1, v/v), 2. Chloroform-acetone-butan-1-ol-concentrated ammonium hydroxide (3:3:4:1, v/v), 3. Chloroform-methanol (9:1 v/v). The radiochemical purity of the [1-CH_3-

[1] K. L. KHANNA, G. S. RAO and H. H. CORNISH, Toxic. appl. Pharmac. *23*, 720 (1972).
[2] G. S. RAO, K. L. KHANNA, H. H. CORNISH, Experientia *29*, 953 (1973).
[3] M. J. ARNAUD, Biochem. Med., in press (1976).

^{14}C] 1, 3, 7-trimethyldihydrouric acid used for metabolic studies was higher than 99.5% (counted with a 2 Π scanner, Berthold).

The compound was administered by stomach tube (9 µCi, 3–4 mg/kg body wt.) to 3 rats weighing 100 to 150 g. Each animals was placed in an individual metabolic glass cage, and $^{14}CO_2$, urine and faeces were collected.

Results. Isolation of [1-CH_3-^{14}C] 1, 3, 7-trimethyldihydrouric acid. The investigation of isolation techniques confirmed the instability of the 1, 3, 7-trimethyldihydrouric acid at high temperature[1] but thin-layer chromatography under our conditions proved to be suitable. The radioactive impurities present in the compound (Figure 1a) were caffeine, resulting from the dehydration of 1, 3, 7-trimethyldihydrouric acid, trimethylallantoin, methylurea and an unknown derivative called compound 3. These last 3 derivatives were not completely eliminated during purification because they have Rf values close to those of 1, 3, 7-trimethyldihydrouric acid.

The 1, 3, 7-trimethyldihydrouric acid was easily detected in urine by its UV-absorption, which showed absorption maxima at 264 nm for pH 2 (0.01 *M* HCl), 7 (0.02 *M* sodium phosphate) and 11 (0.002 *M* NaOH) and at 268 nm for pH 1 (0.10 *M* HCl). These maxima are very different from those of methylxanthines and methyluric acids[4] and are characteristic of this compound.

[4] H. H. Cornish, University Microfilms, Ann. Arbor, Michigan *21, 165*, p. 145.

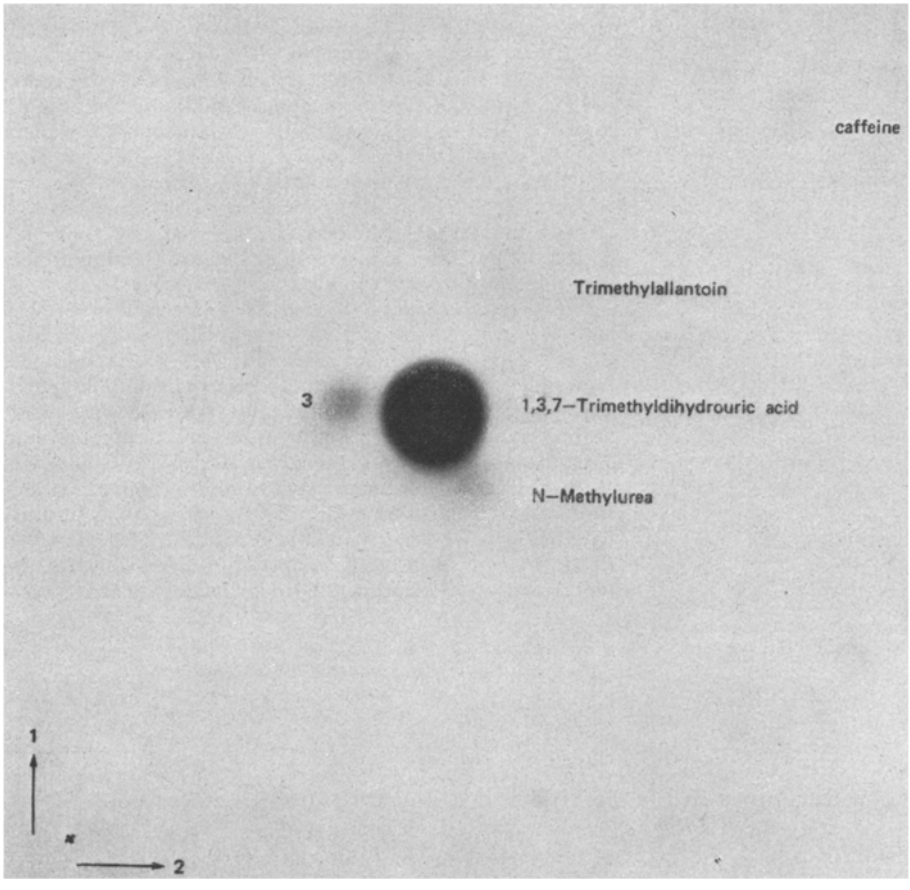
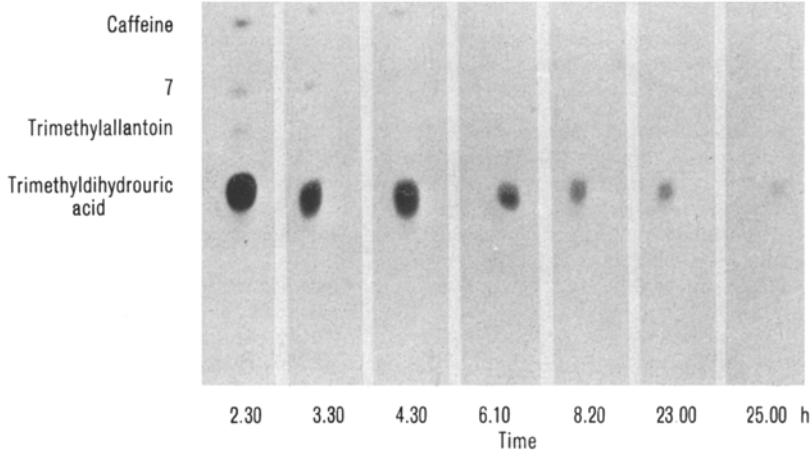


Fig. 1. a) Autoradiogram of [1-CH_3-^{14}C] 1,3,7-trimethyldihydrouric acid obtained after two-dimensional chromatography on silica plate with respectively the solvent (1) chloroform-methanol (4:1, v/v) and (2) chloroform-acetone-butan-1-ol-concentrated ammonium hydroxide (3:3:4:1, v/v).



b) Autoradiogram of radioactive compounds excreted in the urine after administration of [1-CH_3-^{14}C] 1,3,7-trimethyldihydrouric acid. Each urine sample was collected during 25 h and studied by mono-dimensional TLC on silica plates with the solvent chloroform-methanol (4:1, v/v).

Metabolism of [1-CH_3-^{14}C] 1, 3, 7-trimethyldihydrouric acid. The distribution of radioactivity in the organs, $^{14}CO_2$, urinary and faecal excretions, was studied. After 25 h, less than 1% of administered radioactivity was expired as $^{14}CO_2$ and the animal's body contained only 0.7%. Radioactivity collected in faeces represented 2% after 8 h and 9% after 25 h. For all of the experiments, between 75 and 90% of the radioactivity was recovered in the urine. Excretion of radioactivity in the urine was approximately complete after the first 5 h following administration. These results showed that, when caffeine was orally ingested and gave rise to 1,3,7-trimethyldihydrouric acid, this compound and its own metabolites were rapidly excreted in the urine. This fact established, it remained to demonstrate the metabolism undergone by the 1,3,7-trimethyldihydrouric acid.

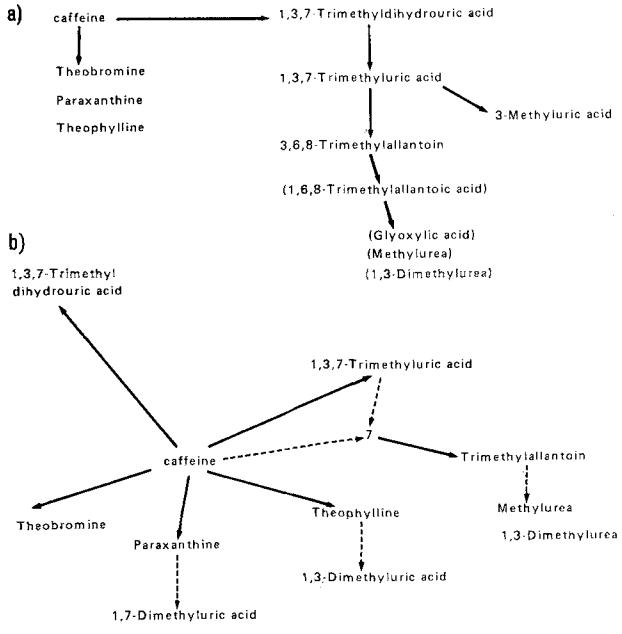


Fig 2. Metabolic pathway of caffeine proposed previously[2]. The products in brackets were not identified in the urine a). The new metabolic pathway taking into account the results of this work. The steps suggested by the identification of metabolites, but not directly proved, are shown with dotted lines (b).

The study of urine by thin-layer chromatography showed that [1-CH_3-^{14}C] 1,3,7-trimethyldihydrouric acid was not transformed but found unchanged in urine. The autoradiogram of each urine sample collected during the experiment lasting 25 h is presented in Figure 1b. The impurities were mainly excreted in the first 2 urine samples and after 4 h, the 1,3,7-trimethyldihydrouric acid was the single radioactive product in urine. These analyses of urine demonstrated that 1,3,7-trimethyldihydrouric acid was a final product of caffeine metabolism and not an intermediate in the formation of trimethylallantoin[2].

Discussion. New Metabolic Pathway of Caffeine. Although a large proportion of the population consumes caffeine every day in drinks and in medicines, the metabolic pathway of this molecule is little known in animals and even less in humans. The latest and most complete metabolic pathway previously proposed is presented in the Figure 2a. As a large number of caffeine derivatives found in urine are unknown, they were not shown in this pathway. It must, however, be pointed out that this metabolic pathway is interesting, because the derivatives reported were quantitatively the most important.

This study, showing that 1,3,7-trimethyldihydrouric acid was an end-product of caffeine metabolism, introduces some important modifications to the known metabolic pathway of caffeine. The new metabolic pathway proposed in Figure 2b takes into account this result and also the existence of an unstable product, compound 7, which is an isomer or a precursor of trimethylallantoin[3]. With this new metabolic pathway, it becomes evident that research must be undertaken in order to determine whether 1,3,7-trimethyluric acid is an intermediate in the formation of trimethylallantoin, and to identify the metabolites formed from the dimethylxanthines[5].

[5] The skilful technical assistance of Miss DORIS ROLLI and Miss MARLYSE HOLDEN is gratefully acknowledged.

Characterization of the Estrogen Receptors in the Uterine and Blood Eosinophil Leukocytes[1]

A. TCHERNITCHIN and X. TCHERNITCHIN

Laboratory of Experimental Endocrinology, Department of Experimental Morphology University of Chile Medical School, Casilla 21104, Correo 21, Santiago (Chile), 9 February 1976.

Summary. Estrogen receptors are found in the rat uterine and in the eosinophil-rich human blood leukocyte 24,000 *g* fractions, but not in the low-eosinophil count human blood leukocyte 24,000 *g* fraction. The total number of binding sites per blood eosinophil leukocyte is 7,400 sites per cell, and the $K_D = 5.6 \times 10^{-10}$ *M*.

Previous radioautographic studies have shown that two separate receptor systems for estrogens, thought to be involved in independent mechanisms of estrogen action, exist in the uterus: the cytosol-nuclear and the eosinophil receptor systems[2-6]. The cytosol-nuclear receptor system is responsible for the genomic response to estrogens, i.e., the increases in uterine RNA and protein synthesis[7]. The eosinophil receptor system is considered to be involved in some of the early non-genomic estrogenic responses in the uterus, such as water imbibition and the increase in vascular permeability[2-6].

The eosinophil receptor system for estrogens have been demonstrated in vitro and in vivo in radioautographic studies[3, 8-10], but there have not been attempts to study it biochemically. The present report describes the preliminary characterization of the estrogen receptors in the uterine eosinophils and also demonstrates the presence of a similar receptor in the circulating blood eosinophil leukocytes.

Material and methods. Estrogen receptors were investigated in the nuclear, the 24,000 *g*, the microsomal and the cytosol fractions from rat uterine tissue and from blood-leukocyte preparations taken from both patients with a high count of blood eosinophils and patients with a low number of blood eosinophils. To increase the number of uterine eosinophils[6, 11], the mature rats used in the pre-