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.

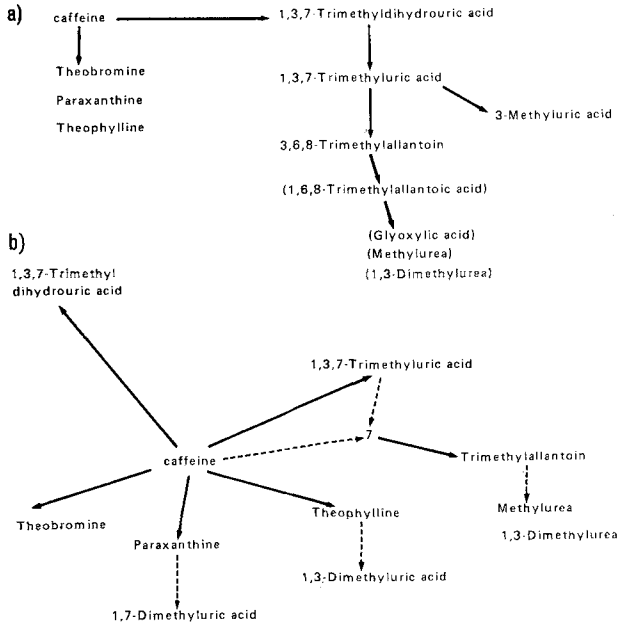


Fig 2. Metabolic pathway of caffeine proposed previously[2]. The products in brackets were not identified in the urine a). The new metabolic pathway taking into account the results of this work. The steps suggested by the identification of metabolites, but not directly proved, are shown with dotted lines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

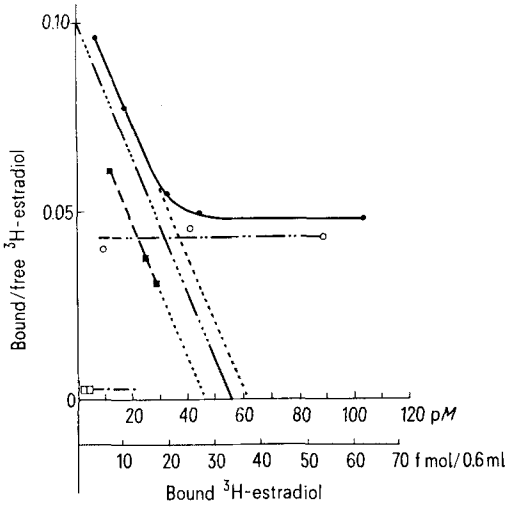
Binding of ^{3}H-estradiol to rat uterine and human blood leukocyte 24,000 *g* fractions. Effects of non-radioactive estradiol and hydrogen peroxide

Concentrations in the incubation tubes			Bound radioactivity (dpm/incubation tube) to		
^{3}H-estradiol (*M*)	Hydrogen peroxide (*M*)	Non-radioactive estradiol (*M*)	Eosinophil-rich blood-leukocyte 24,000 *g* fraction	Low-eosinophil-count blood leukocyte 24,000 *g* fraction	Rat uterine 24,000 *g* fraction
3×10^{-10}	–	–	810	90	1690
3×10^{-10}	1×10^{-6}	–	6165	900	2295
3×10^{-10}	1×10^{-6}	7.5×10^{-9}	1620	90	945
1×10^{-9}	–	–	1620	180	4320
1×10^{-9}	1×10^{-6}	–	9360	1260	4725
1×10^{-9}	1×10^{-6}	2.5×10^{-8}	2070	90	3750

Rat uterine 24,000 *g* fractions (corresponding to 0.15 uterine horn), eosinophil-rich blood-leukocyte 24,000 *g* fractions (corresponding to 2,250,000 eosinophils) or low eosinophil-count blood-leukocyte 24,000 *g* fractions (corresponding to 170,000 eosinophils) were used in each incubation tube. The protein content in each incubation tube was 88 µg.

sent experiments were pretreated for 3 days with 30 µg of non-radioactive estradiol per 100 g of body weight and per day. The rats were sacrified 24 h after the last injection. Each fraction was resuspended in a 0.05 *M Tris* – 0.4 m*M* EDTA buffer (pH 7.4) and was incubated at 37 °C for 1 h with various concentrations (between 1×10^{-10} *M* and 3×10^{-9} *M*) of ^{3}H-estradiol-17β (specific activity = 48 Ci per millimole), with or without a 25-fold concentration of non-radioactive estradiol-17β, and with or without 1×10^{-6} *M* hydrogen peroxide. At the end of the incubation, aliquots were taken for the separation of the bound and free ^{3}H-estradiol by filtration in G-25 coarse Sephadex-columns. Separate aliquots were used for the measurement of total radioactivity, proteins[12] and peroxidase[13]. The results obtained in the rat uterine and in the eosinophil-rich blood leukocyte 24,000 *g* fractions were expressed as a Scatchard plot[14].

Results. The Table shows that a significant amount of bound ^{3}H-estradiol is found in the 24,000 *g* fraction after incubation with ^{3}H-estradiol when this fraction is obtained from rat uterine homogenates or from eosinophil-rich blood leukocyte preparations. Almost no binding of ^{3}H-estradiol is found in the 24,000 *g* fraction when it was obtained from low eosinophil count blood leukocyte preparations. A 25-fold concentration of non-radioactive estradiol-17β drastically decreases or almost abolishes the binding of ^{3}H-estradiol in the 24,000 *g* fractions. The presence of 1×10^{-6} *M* hydrogen peroxide greatly increases the amount of bound ^{3}H-estradiol in the 24,000 *g* fractions from rat uteri, from eosinophil-rich human blood leukocyte and from low eosinophil count blood leukocyte preparations. A 25-fold concentration of non-radioactive estradiol produces a significant decrease in the bound radioactivity in the 24,000 *g* fractions incubated in the presence of hydrogen peroxide.



Scatchard plots for binding of ^{3}H-estradiol to rat uterine and human blood leukocyte 24,000 *g* fractions.
Rat uterine 24,000 *g* fractions corresponding to 0.10 uterine horn (● and ○) and eosinophil-rich human blood leukocyte 24,000 *g* fractions corresponding to 2,250,000 eosinophils (■ and □), were used in each incubation with ^{3}H-estradiol-17β. ○ and □, the incubation media contained a 25-fold concentration of non-radioactive estradiol-17β; ● and ■, the incubation media was without non-radioactive estradiol. The total volume of the incubation medium was 0.6 ml, and the protein content was 88 µg in the incubation performed with the blood leukocyte fractions, and 58 µg in the incubation performed with the rat uterine fractions. The bound and free ^{3}H-estradiol were determined as described in the text.

[1] Acknowledgments. This work was supported in part by funds from the Unité de Recherches sur le Métabolisme Moléculaire et la Physio-Pathologie des Stéroïdes de l'INSERM, Hôpital de Bicetre, France, and by grant No. 2015 from the Oficina Técnica de Desarrollo Científico y Creación Artística of the University of Chile.

[2] A. Tchernitchin, Steroids *19*, 575 (1972).
[3] A. N. Tchernitchin, 7th World Congress of Obstetrics and Gynaecology, Moscow, Excerpta Medica, Amsterdam Int. Congr. Series (1973), No. 279, p. 12.
[4] A. Tchernitchin, J. Steroid Biochem. *4*, 277 (1973).
[5] A. Tchernitchin and A. Bongiovanni, Fedn. Proc. *32*, 242 (1973).
[6] A. Tchernitchin, J. Roorijck, X. Tchernitchin, J. Vandenhende and P. Galand, Nature Lond. *248*, 142 (1974).
[7] E. V. Jensen and E. R. DeSombre, A. Rev. Biochem. *41*, 203 (1972).
[8] A. Tchernitchin, Steroids *10*, 661 (1967).
[9] A. Tchernitchin and R. Chandross, J. Steroid Biochem. *4*, 41 (1973).
[10] A. Tchernitchin, Eur. J. Obst. Gyn. Reprod. Biol. Suppl. *4*, 99 (1974).
[12] O. H. Lowry, N. J. Rosebrough, A. L. Farr and R. J. Randall, J. biol. Chem. *193*, 265 (1951).
[13] S. R. Himmelhoch, W. H. Evans, M. G. Mage and E. A. Peterson, Biochemistry *8*, 914 (1969).
[14] G. Scatchard, Ann. N. Y. Acad. Sci. *51*, 660 (1949).

The amount of bound ^{3}H-estradiol in the nuclear, microsomal and cytosol fractions from rat uteri, was well as those from human blood leukocyte preparations, is much lower than that from the 24,000 *g* fractions. Non-radioactive estradiol does not decrease the amount of bound radioactivity in the nuclear, microsomal and cytosol fractions. Hydrogen peroxide does not increase the amount of bound radioactivity in those fractions, as was observed in the 24,000 *g* fractions. The guiacol reaction demonstrates only the presence of peroxidase in the uterine and blood leukocyte 24,000 *g* fraction, but not in the nuclear, microsomal and cytosol fractions.

The Figure shows the results expressed as SCATCHARD plots. The estrogen receptors from both the rat uterine 24,000 *g* fraction and the eosinophil-rich human blood leukocyte 24,000 *g* fraction have a constant of dissociation of 5.6×10^{-10} *M*. The total number of binding sites per eosinophil (from the blood leukocyte preparations) is 7,400 sites per cell. In both the rat uterine and the eosinophil-rich human blood leukocyte 24,000 *g* fractions, there is no detectable specific estrogen binding in the presence of non-radioactive estradiol-17β.

Discussion. The present report describes high affinity and low capacity estrogen receptors in the rat uterine and the human eosinophil-rich blood leukocyte 24,000 *g* fractions. No estrogen receptors were detected in the cytosol, microsomal and nuclear fractions from rat uteri or from human blood leukocyte preparations, probably because of the long incubation at 37 °C, which has been reported to destroy the uterine cytosol receptors[7].

The estrogen receptors from the blood leukocyte 24,000 *g* fraction were only clearly demonstrated when the blood was obtained from patients with a high count of blood eosinophils, suggesting that these estrogen receptors are only present in the eosinophil leukocytes.

The estrogen receptors from the 24,000 *g* fraction of rat uterus and those from the 24,000 *g* fraction of eosinophil-rich blood leukocyte preparations have three characteristics in common: They have a similar constant of dissociation, they increase the binding of ^{3}H-estradiol in the presence of a low concentration of hydrogen peroxide and they are found in the 24,000 *g* fraction. These characteristics suggest that the 24,000 *g* fraction eosinophil-rich blood leukocyte receptors are identical with the uterine estrogen receptors found in the 24,000 *g* fraction.

An increase in estrogen binding by the blood leukocyte and the uterine 24,000 *g* fractions occurs in the presence of a low concentration of hydrogen peroxide. Similarly, an increase in estrogen binding and retention by the uterine eosinophils in the presence of a low concentration of hydrogen peroxide has been reported in radioautographic studies[15,16], suggesting that the estrogen receptors found in the 24,000 *g* fractions correspond to the estrogen receptors previously described in uterine eosinophils[2-6,8-11,15-17]. Furthermore, electron microscope radioautography demonstrated the localization of the estrogen eosinophil receptors in the 'specific lysosomes' of the eosinophil leukocytes[17], known as 'peroxidasosomes'[18], which sediment in the 24,000 *g* fraction[19].

Experimental data has led us to conclude that the estrogen receptors described in the blood leukocyte and the uterine 24,000 *g* fractions correspond to the uterine eosinophil estrogen receptors previously found in uterine radioautograms, confirming their existence in the uterus, in addition to the cytosol-nuclear receptor system.

[15] A. TCHERNITCHIN, Acta endocr. Copenh. Suppl. *177*, 219 (1973).
[16] A. TCHERNITCHIN, X. TCHERNITCHIN and A. M. BONGIOVANNI, Acta endocr. Copenh. Suppl. *177*, 246 (1973).
[17] J. BROKELMANN, J. Histochem. Cytochem. *17*, 394 (1969).
[18] V. V. ROGOVIN, V. M. FROLOVA, N. G. GERANINA, R. A. MURAVIEFF and L. A. PIRUZYAN, Izv. Akad. Nauk SSSR, Ser. Biol. *1*, 144 (1973).
[19] G. T. ARCHER and J. G. HIRSCH, J. exp. Med. *118*, 277 (1973).

The Desulphation of Hexosamine Sulphates by Arylsulphatase B[1]

A. A. FAROOQUI

Centre de Neurochimie, 11, rue Humann, F-67085 Strasbourg, Cedex (France), 13 April 1976.

Summary. The sulphation of Carbobenzoxyglucosamine by chlorosulphonic acid resulted in formation of *N*-carbobenzoxyglucosamine-4,6-disulphate. UDP-galactosamine 4-sulphate and glucosamine 4,6-disulphate were the competitive inhibitors of arylsulphatase B. Arylsulphatase B can hydrolyze UDP-galactosamine 4-sulphate and glucosamine 4,6-disulphate but not galactosamine 6-sulphate.

Lysosomal arylsulphatase B has been extensively purified from various mammalian tissues[2-4]. It can be clearly distinguished from the corresponding arylsulphatase A both by its lower molecular weight and higher isoelectric point[2]. Although the physiological role of arylsulphatase A has been well established[5-7], the role of arylsulphatase B is still obscure. However, it has been suggested[8] that the latter may have a role in mucopolysaccharide metabolism. Further, it has recently been reported that arylsulphatase B activity is markedly diminished in the tissues of patients with Maroteaux-Lamy syndrome, which is characterized by the abnormal excretion of dermatan sulphate[9,10]. In a preliminary report STEVENS et al.[11] have reported that a partially purified preparation of human placenta arylsulphatase B could desulphate UDP-N-acetylgalactosamine 4-sulphate.

In the present work, attempts were made to prepare hexosamine sulphates for using them as substrates for arylsulphatase B. The hexosamine sulphates were also coupled with CH-sepharose in an attempt to make an affinity chromatography column for arylsulphatase B.

Methods. The homogeneous preparation of arylsulphatase B was prepared by the method of FAROOQUI and ROY[4], and assayed by the method of ALLEN and ROY[2] using nitrocatechol sulphate as a substrate. The purified enzyme preparation had a specific activity of 130 µmole/min/mg.

Preparation of hexosamine sulphates. Carbobenzoxyhexosamines were prepared by the method of CHARGAFF and BOVARNICK[12]. They were crystallized 3 times with 30% methanol before sulphation. The sulphation of carbobenzoxyhexosamines was done by the method of ADAM[13]