Disparate Na^+-requirement of taurocholate and indocyanine green uptake by isolated hepatocytes[1]

E. Minder and G. Paumgartner[2]

Department of Clinical Pharmacology, University of Berne, Murtenstrasse 35, CH-3010 Berne (Switzerland), 1 March 1979

Summary. Uptake of both taurocholate and indocyanine green (ICG) by isolated rat hepatocytes was saturable and obeyed Michaelis-Menten kinetics. Only uptake of taurocholate exhibited Na^+-dependence, a phenomenon compatible with active membrane transport. These findings suggest that the mechanisms responsible for hepatocellular uptake of bile acids and anionic dyes differ fundamentally.

Hepatocellular uptake of bile acids such as taurocholate, cholate and chenodeoxycholate[3,4], and anionic dyes such as indocyanine green (ICG)[5,6], bromosulphophthalein[7] and bilirubin[8,9] obeys Michaelis-Menten kinetics and is thought to be carrier mediated. Absence of competition for uptake between the 2 groups of organic anions suggests the presence of at least 2 different pathways[6,9]. Demonstration of energy dependence for bile acid uptake[10], but not for dye uptake[11], supports the assumption that not only the carrier sites but also the mechanisms responsible for uptake differ fundamentally. Further to test this hypothesis, it was investigated whether hepatocellular uptake of ICG exhibits Na^+-dependence, an important feature of many active transport processes. This characteristic has previously been demonstrated for bile acid uptake[4,10].

Material and methods. Male SPF rats of the Sprague-Dawley strain, weighing 265±13 g, were maintained on a standard rat diet (Altromin 300 R, Altromin GmbH, Lage, BRD) and tap water ad libitum. For the isolation of the liver cells the following enzymes were used: Collagenase (E.C.3.4.4.19) type III (Worthington, Biochemical Corporation, Freehold, N.J., USA), hyaluronidase (E.C.3.2.1.35) type I (Sigma, St. Louis, Mo., USA) and soybean trypsin inhibitor (Worthington, Biochemical Corporation). Dried chromatographically pure bovine serum albumin was obtained from Behringwerke AG (Marburg/Lahn, BRD), HEPES (N-2 hydroxyethylpiperazine-N′-2-ethanesulfonic acid) from Fluka AG (Buchs/St. Gallen, Switzerland), taurocholate sodium (grade A), from Calbiochem (Lucerne, Switzerland), 26-^{14}C taurocholate sodium (sp. act. 5 mCi/mmole) from International Chemical and Nuclear Corporation (Irvine, Cal. USA) and indocyanine green (ICG) from Hynson, Westcott and Dunning Inc. (Baltimore, Md. USA). Whatman glass fibre filters were type GF-D (W. and R. Balston Ltd., England).

Hepatocytes were isolated from rat livers according to a modification of the method of Seglen[12]. The portal vein and the inferior vena cava were cannulated in pentobarbital anaesthesia (5 mg/100 g b.wt) and the liver was perfused in situ as described previously[4]. Unless otherwise stated, all procedures were carried out at 37 °C. During the first 10 min of perfusion, non-recirculating calcium-free Krebs Ringer bicarbonate buffer gassed with 95% O_2 and 5% CO_2 was used (flow rate: 34±1.2 ml/min; pressure: 13.3±0.3 cm water). Thereafter, a recirculating perfusion with 250 ml Krebs Ringer bicarbonate buffer containing 62,500 IU/l collagenase, 78,000 NF units/l hyaluronidase, 93.8 mg/l trypsin inhibitor and 4 mmoles/l calcium was started (flow rate: 34.5±0.8 ml/min; perfusion pressure: 14.2±0.2 cm water). After 14-22 min of recirculating perfusion, the liver desintegrated. To stop further digestion, the liver was flushed with ice cold HEPES buffer (30 mmoles/l). It was then rapidly removed from the animal and transferred into a plastic petri dish on ice. The capsule was pealed off using fine forceps, and the cells were suspended, shaking the liver gently in 150 ml of ice cold HEPES buffer containing 2 g% bovine serum albumin. The resulting suspension was filtered through a nylon filter (mesh size 250 µm), and incubated at 37 °C for 30 min under gassing with 95% O_2 and 5% CO_2. Thereafter, the cells were once more filtered through 2 nylon filters (mesh size 250 and 60 µm), centrifuged at 20×g for 2.5 min, resuspended in HEPES buffer, centrifuged a 2nd time at the same conditions and resuspended in the appropriate buffer as mentioned below. Trypan blue exclusion was greater than 90%. Uptake studies were either performed in Krebs Ringer bicarbonate buffer with 2 g% albumin and 5.5 mmoles/l glucose (Na^+ concentration: 148 mmoles/l) or in the same medium in which Na^+ had been replaced by equimolar amounts of lithium or potassium. Although the albumin used in these experiments had been dialyzed against isotonic lithium chloride, the final medium still contained 1-6 mmoles/l sodium. All media were checked for equal osmolarity (290-300 mosm/kg H_2O). The cell suspensions were made up to a concentration of about 1 mg protein/ml and kept on ice. Prior to the uptake studies,

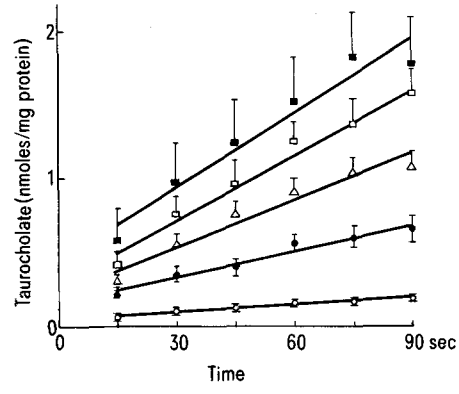

Fig. 1. Taurocholate uptake by isolated hepatocytes. Means±SEM of 8 experiments. Extracellular taurocholate concentration: ○ 5 µmoles/l; ● 20 µmoles/l; △ 50 µmoles/l; □ 200 µmoles/l; ■ 500 µmoles/l.

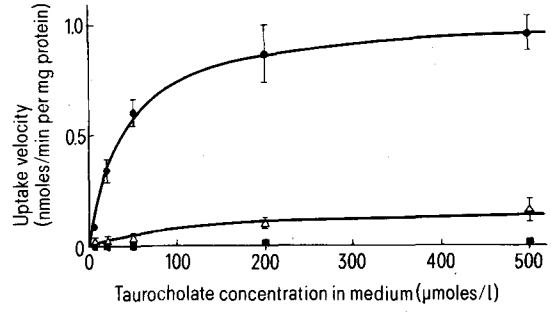

Fig. 2. Relationship between the velocity of taurocholate uptake by isolated hepatocytes and the extracellular concentration of taurocholate (means±SEM). ● Physiological (148 mmoles/l) concentration of extracellular sodium (n=8); △ extracellular sodium replaced by lithium (n=6); ■ extracellular sodium replaced by potassium (n=2).

they were incubated at 37 °C in a shaking water bath and gassed with 95% O_2 and 5% CO_2 for 30 min.

Taurocholate uptake was measured by mixing 4 ml of the cell suspension with 1 ml of Krebs Ringer bicarbonate buffer containing an appropriate concentration of taurocholate labelled with 25–250 nCi ^{14}C-taurocholate. After 15, 30, 45, 60, 75 and 90 sec, aliquots of 0.2 ml were filtered through a glass fibre filter. This was immediately followed by a washing with 4 ml of ice cold isotonic magnesium chloride solution to terminate the uptake process. ^{99m}Tc-albumin was used as a reference substance for extracellular taurocholate. After decay of the ^{99m}Tc for at least 10 half-life-times, the filters were transferred into counting vials, the cells were lyzed with 0.5 ml hyamin and 10 ml of Scintisol was added. ^{14}C radioactivity was counted in a Packard Tri Carb 3380 liquid scintillation counter.

ICG uptake was measured by mixing 0.5 ml of the cell suspension with 0.5 ml Krebs Ringer bicarbonate buffer containing an appropriate concentration of ICG. After 10, 20, 30 and 40 sec the cells were rapidly separated from the supernatant by centrifugation. The pellet was suspended in 1 ml of distilled water and sonicated. ICG was extracted by addition of 2 ml acetone to 0.5 ml of the homogenate and read spectrophotometrically at 790 nm. ^{14}C-dextran was used as reference substance to correct for extracellular ICG. Apparent half-saturation constant, K_m, and maximal uptake velocity, V_{max} of hepatocellular uptake were computed non-linearly according to Wilkinson[14]. All results were expressed as means±SEM.

Results. 15–90 sec after addition of taurocholate, a linear increase of hepatocellular taurocholate content was observed with all taurocholate concentrations employed (figure 1). The relationship between extracellular taurocholate concentration and initial uptake velocity was compatible with saturation kinetics, which could be described by the Michaelis-Menten equation (figure 2). Maximal uptake velocity (V_{max}) and apparent half-saturation constant (K_m) were 1.04±0.08 nmoles/min per mg protein and 39±11 µmoles/l, respectively. Uptake velocities of taurocholate were greatly reduced, when Na^+ in the medium was replaced by lithium. At all substrate concentrations, uptake in the lithium medium did not exceed 15% of the values obtained in the sodium medium (figure 2). To exclude a toxic effect of lithium, potassium was used for sodium replacement in 2 experiments. In this medium, uptake velocities were even more reduced than in the lithium medium.

10–40 sec after addition of ICG, the hepatocellular concentration of the dye increased linearly (figure 3). Initial uptake velocity increased with increasing concentration of extracellular ICG in a non-linear fashion, obeying the Michaelis-Menten kinetics (figure 4). V_{max} and K_m were 10.4±1.9 nmoles/min per mg protein and 547±249 µmoles/l, respectively. In contrast to taurocholate uptake, ICG uptake rates were increased in the lithium medium (V_{max}: 22.8±4.6 nmoles/min per mg protein; K_m: 963±382 µmoles/l).

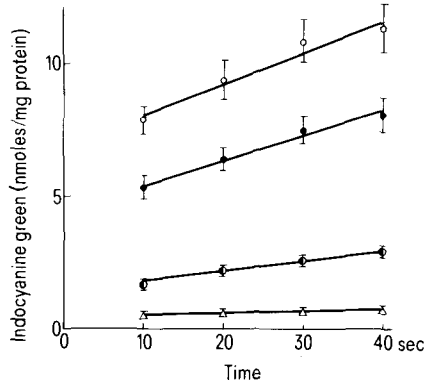

Fig. 3. Indocyanine green uptake by isolated hepatocytes. Means±SEM of 7 experiments. Extracellular indocyanine green concentration: △ 24.5 µmoles/l; ◐ 125.5 µmoles/l; ● 639 µmoles/l; ○ 1121 µmoles/l.

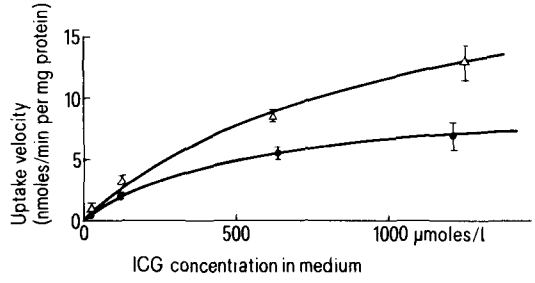

Fig. 4. Relationship between the velocity of indocyanine green uptake by isolated hepatocytes and the extracellular concentration of indocyanine green (means±SEM). ● Physiological extracellular sodium concentration (n=7); △ extracellular sodium replaced by lithium (n=3).

Discussion. The kinetics of both taurocholate and ICG uptake by isolated hepatocytes were compatible with carrier-mediated transport. These findings substantiate previous investigations in vivo[3,5,8], in the perfused rat liver[4,6] and in isolated rat hepatocytes[10]. It must, however, be noted that considerable differences exist between isolated hepatocytes and intact liver in respect to absolute values of organic anion uptake. Thus, V_{max} and K_m of taurocholate uptake in the present study agreed well with the data of other authors[10,15] who also used isolated hepatocytes, but were considerably smaller than the respective values obtained in the isolated perfused rat liver[4]. By contrast, V_{max} of ICG uptake was higher in isolated hepatocytes than in the perfused rat liver[6]. This resulted in higher transport rates for ICG than for taurocholate in isolated hepatocytes, a finding contrary to that in the intact organ[16]. Obviously, the loss of cell polarity and/or other alterations caused by the isolation of the cells affect the uptake of taurocholate and ICG in an opposite way. It must also be considered that the apparent uptake rates in isolated cells may represent the net result of uptake and secretion. Although this possibility cannot be excluded, it appears unlikely that secretion played a significant role. This view is supported by the finding that the increase of hepatocellular content of taurocholate and ICG during the period of uptake measurements was linear. Another difference between isolated cells and intact organ may be the thickness of the unstirred water layer surrounding the cell. Thus, a decrease of the unstirred water layer has been observed when ileal cells were isolated. It has been suggested that this change was responsible for the observed decrease of the K_m of taurocholate uptake[17].

The differential effects of cell isolation on taurocholate and ICG uptake point towards basic differences in the mechanisms responsible for uptake of these 2 organic anions. These differences become even more evident when the sodium dependence of the uptake mechanisms is studied. While uptake of taurocholate was nearly abolished when sodium was removed from the medium, uptake of ICG was increased. Na^+-dependence is an important feature of many active transport processes[18]. The dependence of bile acid uptake on extracellular Na^+ may be interpreted as evidence for co-transport of bile acid with Na^+. This concept

implies that the uptake process is driven by the concentration gradient of Na^+ across the plasma membrane of the hepatocyte. It appears reasonable to assume that such a secondary active process reacts sensitively to cell damage caused by the isolation procedure and that, as a consequence, bile acid uptake is diminished in isolated hepatocytes. The demonstration that ICG uptake does not depend on extracellular Na^+ concentration suggests a different mechanism and is compatible with facilitated transfer across the membrane[8] followed by binding to ligandin[19]. Such a mechanism may be relatively insensitive to cell damage. An increase in cell surface and possibly an increased exposure of cell proteins which bind ICG could then lead to enhanced ICG uptake. This view is supported by the observation that unspecific damage increases the uptake of the anionic dye sulfobromophthalein by isolated hepatocytes[20] and by liver surface membranes[21]. Thus, the basic differences in the postulated mechanisms responsible for hepatocellular uptake of bile acids and anionic dyes could explain why the uptake capacity for taurocholate is larger in intact liver than in isolated hepatocytes, and why the reverse is true for ICG.

1 This work was supported by the Swiss National Science Foundation.
2 The authors thank Mr Th. Weber for technical assistance.
3 V.C. Glasinovic, M. Duval and S. Erlinger, J. clin. Invest. *55*, 419 (1975).
4 J. Reichen and G. Paumgartner, Am. J. Physiol. *231*, 734 (1976).
5 G. Paumgartner, Schweiz. med. Wschr. *105*, suppl. (1975).
6 G. Paumgartner and J. Reichen, Experientia *31*, 306 (1975).
7 C.A. Goresky, Am. J. Physiol. *207*, 13 (1964).
8 B.F. Scharschmidt, J.G. Waggoner and P.D. Berk, J. clin. Invest. *56*, 1280 (1975).
9 G. Paumgartner and J. Reichen, Clin. Sci. mol. Med. *51*, 169 (1976).
10 L.R. Schwarz, R. Burr, M. Schwenk, E. Pfaff and H. Greim, Eur. J. Biochem. *55*, 617 (1975).
11 M. Schwenk, R. Burr, L. Schwarz and E. Pfaff, Eur. J. Biochem. *64*, 189 (1976).
12 P.O. Seglen, in: Methods in Cell Biology, vol. 13, p. 29. Ed. D.M. Prescott. Academic Press, New York 1976.
13 R. Herz, B. Cueni, J. Bircher and G. Paumgartner, Naunyn-Schmiedebergs Arch. Pharmac. *277*, 297 (1973).
14 G.N. Wilkinson, Biochem. J. *80*, 824 (1961).
15 M.S. Anwer, R. Kroker and D. Hegner, Biochem. biophys. Res. Commun. *73*, 63 (1976).
16 G. Paumgartner and J. Reichen, in: The hepatobiliary system, p. 287. Ed. W. Taylor. Plenum Press, New York 1976.
17 F.A. Wilson and L.L. Treanor, Biochim. biophys. Acta *406*, 280 (1975).
18 S.G. Schultz and P.F. Curran, Physiol. Rev. *50*, 637 (1970).
19 I.M. Arias and P. Jansen, in: Jaundice, p. 175. Ed. C.A. Goresky and M.M. Fisher. Plenum Press, New York 1975.
20 M. Schwenk, R. Burr and E. Pfaff, Naunyn-Schmiedebergs Arch. Pharmac. *295*, 99 (1976).
21 J. Reichen, B.L. Blitzer and P.D. Berk, Clin. Res. *25*, 468 A (1977).

Effects of low doses of ochratoxin A after intratesticular injection in the rat

J. Moré and R. Camguilhem

Laboratoire de Pharmacologie et Toxicologie, INRA, 180, chemin de Tournefeuille, F-31300 Toulouse (France), 6 November 1978

Summary. The toxic effect of various doses of ochratoxin A on the rat testis was investigated after a single intratesticular injection. At time of sacrifice (day 10) degenerating changes occur in the testicular tissues: seminiferous tubules dilatation, cytolysis of the seminiferous epithelium, hyperplasia of the interstitial tissue, vascular thrombosis. The relations between the blood supply disturbances and the observed lesions are discussed.

The testis of many mammals reacts to various natural or synthetic chemical compounds[1]. This action varies, for a definite substance, according with the route of injection (general and/or topical in the testis). For ochratoxin A, the different toxicological trials made p.o., i.p. or i.v. on swine[2], dog[3], rat and mice[4] do not mention the least damage on the male gonad. We have established[5] that administration of this toxin by gastric tubing (16 mg/kg i.e. MTD p.o.) does not modify libido, fecundity or the morphology of testicular tissues in the rat. Intratesticular injection gives similar results to i.v. injection and in the rat, the LD 50 of ochratoxin A injected i.v., is 12.75 mg/kg[4].

The aim of this work is to define the testicular effects of low doses (about $\frac{1}{3}$ of the LD 50 i.v.) of ochratoxin A on the rat testis receiving a single injection of this mycotoxin.

Material and methods. Male rats (Sprague-Dawley) weighing 282.18±5.33 g were housed 3 to a cage and received food (pellets UAR - A 304) and tap water ad libitum. Experimental animals were randomly divided into 4 groups. Each group received ochratoxin at different dose level (4.0, 4.2, 4.6 and 5.0 mg/kg) for each rat. Injections (0.1 ml/testis) were made into both testes under light ethyl ether anaesthesia. The control animals (20 rats) each received the same volume of the solvent of ochratoxin A (CO_3HNa, 14 g/l). All the animals were euthanized the 10th day following injection (day 0). The animals are weighed at day 0 and at time of sacrifice. The testis were weighed. For each animal, the percentage of testes weight (mg/per 100 g b.wt) was calculated. Testes and epididymis were fixed in Orth fluid and paraffin imbedded. Tissue sections were then stained with the PAS-hematoxylin of Leblond and Clermont[6] for histological study.

Weight results. Mean values of testes weight per 100 g b.wt are shown in the table. Means for treated animals do not

Testes weight results

Doses (mg/kg)	Mean testis weight (mg/100 g b.wt)
4	987.5 ± 64
4.2	1002.5 ± 42.4
4.6	985.0 ± 20
5	1032.5 ± 88.8
Control	1112.5 ± 70