Laboratory of Neuroendocrine Regulation, Department of Nutrition and Food Science, Massachusetts Institute of Technology, Cambridge, MA 02139, *and* **Section of Neurology, Department of Medicine, Peter Bent Brigham Hospital, and Harvard Medical School, Boston, MA* 02115, *U.S.A.*

JOHN F. REINHARD, Jr.† YOSHISUKE OZAKI MICHAEL A. MOSKOWITZ*, ‡

REFERENCES

1. J. M. Saavedra, M. Brownstein and J. Axelrod, *J. Pharmac. exp. Ther.* **186**, 508 (1973.
2. B. B. Mrsulja, B. J. Mrsulja, T. Fujimoto, I. Klatzo and M. Spatz, *Brain Res.* **110**, 361 (1976).
3. O. H. Lowry, N. J. Rosebrough, A. L. Farr and R. J. Randall, *J. biol. Chem.* **193**, 265 (1951).
4. Y. Ozaki, R. J. Wurtman, R. Alonso and H. J. Lynch, *Proc. natn. Acad. Sci. U.S.A.* **75**, 531 (1978).
5. E. Stahl, in *Thin-Layer Chromatography* (Ed. E. Stahl), p. 296. Springer, Berlin (1965).
6. B. W. Wilson, H. J. Lynch and Y. Ozaki, *Life Sci.* **23**, 1019 (1978).
7. V. Chan-Palay, in *Neurogenic Control of Brain Circulation*, Wenner-Gren Center International Symposium Series (Eds. C. H. Owman and L. Edvinsson), Vol. 30, p. 39. Pergamon Press, New York (1977).
8. M. Brownstein, J. M. Saavedra and J. Axelrod, *Molec. Pharmac.* **9**, 605 (1973).
9. J. M. Saavedra, N. Palkovits, M. J. Brownstein and J. Axelrod, *Brain Res.* **77**, 157 (1974).

† Present Address: Yale University School of Medicine, Dept. of Pharmacology, New Haven, CT 06510, U.S.A.
‡ Correspondence and reprint requests should be addressed to: Dr. Michael A. Moskowitz, Rm. 37-311, M.I.T., Cambridge, MA 02139, U.S.A.

Biochemical Pharmacology, Vol. 29, pp. 3023–3025

0006-2952/80/1101-3023 $02.00/0

Metabolism of digitoxin by isolated rat hepatocytes

(*Received* 29 *August* 1979; *accepted* 27 *June* 1980)

The metabolism of drugs in the liver can be studied at different levels of biological organization including the intact organ, cellular and subcellular levels. Studies at each organizational level will give useful information on types of reactions or kinetic parameters, but these also have their own limitations. In *In vivo* studies, complicating extrahepatic factors such as neurological, endocrinological and circulatory phenomena may influence the results. In studies using homogenates or microsomal preparations, the composition of the incubation medium is quite different from that of the cytosol of intact hepatocytes [1]. In addition, liver homogenates or microsomes are prepared from different cell populations constituting the liver. In view of these limitations, an experimental system based on the use of isolated hepatocytes has the advantage that extrahepatic influences can be excluded and that the isolated hepatocytes are intact cells. Therefore, the data obtained with isolated hepatocytes can be expected to provide useful information on the role of the liver in the kinetics of drug metabolism *in vivo*. In this paper, a study on the metabolism of the cardiac glycoside digitoxin (DT_3)* using isolated hepatocytes will be described.

Male 3-month-old Wistar rats (304 ± 26 g body wt., mean ± S.D.) were used. The animals were purchased when they were 11 weeks old (Shizuoka Jikkendobutsu, Shizuoka, Japan) and maintained on commercial rat pellets in the animal laboratory of the institute (four animals to a cage). Room temperature was kept constant (22 ± 2°). [^{3}H]DT_3 (10.9 Ci·mmole^{-1}, generally labeled) was purchased from New England Nuclear, Boston, MA., U.S.A.; DT_3 and digoxin (DG_3) from Merck, Darmstadt, F.R.G.; digitoxigenin-bis-digitoxoside (DT_2), digitoxigenin-mono-digitoxoside (DT_1), digitoxigenin (DT_0), digoxigenin-bis-digitoxoside (DG_2), digoxigenin-mono-digitoxoside (DG_1) and digoxigenin (DG_0) from Roth, Karlsruhe, F.R.G.; Amberlite XAD-2 resin from Rohm and Haas Nederland BV, Dordrecht, The Netherlands; the enzymes collagenase (type 1) and hyaluronidase (type 1) from Sigma, St. Louis, MO, U.S.A. and Waymouth MB 752/1 medium from GIBCO, Grand Island, NY, U.S.A.

Unlabelled and [^{3}H]DT_3 were added to a dimethyl sulfoxide (DMSO) solution. The final concentration of DMSO in the incubation medium was 0.5%. No damage to the isolated hepatocytes was to be expected at this concentration [2]. The degree of dissolution of DT_3 at this concentration was determined by means of ultracentrifugation and the millipore filter method. Thin-layer chromatography (t.l.c.) was performed with DC Fertigplatten Kieselgel 60 of 20 × 20 cm dimension (Merck, Darmstadt, F.R.G.). The hepatocytes were isolated by perfusion and incubation of the liver with the enzymes collagenase and hyaluronidase as reported earlier [3, 4]. The concentration of the cells was determined with a hemocytometer.

The method for determining DT_3 biotransformation was as follows. A known number of hepatocytes in 4 ml Waymouth MB 752/1 medium were incubated with various amounts of unlabelled DT_3 mixed with [^{3}H]DT_3. The pH of the medium was kept at 7.4, which was found to be optimal for digitoxin metabolism [5]. The incubation was carried out at 37° under an atmosphere of 95% O_2 and 5% CO_2 with constant shaking (100 oscillations·min^{-1}). Immediately after the addition of DT_3 and after various incubation periods, 0.5-ml samples of the cell suspension were withdrawn and added to 1.5 ml ethanol [6]. The mixture was centrifuged at 3000 *g* for 10 min [6]. The supernatant fraction was evaporated and then layered on a column packed with Amberlite XAD-2 resin using 6 ml water. The column was washed with 20 ml distilled water to remove the constituents of the incubation medium such as salts and sugars. DT_3 and its metabolites were eluted from the column with 20 ml ethanol. The ethanol fraction was evaporated *in vacuo* at 37°; the residue was dissolved in about 100 μl of chloroform:methanol, 1:1. The solution

* Abbreviations used: DT_3, digitoxin; DT_2, digitoxigenin-bis-digitoxoside; DT_1, digitoxigenin-mono-digitoxoside; DT_0, digitoxigenin; DG_3, digoxin; DG_2, digoxigenin-bis-digitoxoside; DG_1, digoxigenin-mono-digitoxoside; DG_0, digoxigenin; DMSO, dimethyl sulfoxide; t.l.c., thin-layer chromatography.

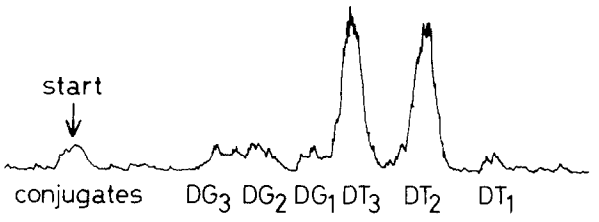

Fig. 1. Thin layer chromatogram of digitoxin and its metabolites. Hepatocytes were isolated from 3-month-old male Wistar rats. The incubation conditions were: cell concentrations, 7.4×10^6 cells $\cdot ml^{-1}$; digitoxin concentration, 2.5×10^{-6}M; incubation time, 3 hr.

was applied to a t.l.c. 'kieselgel' plate. The plate was developed once with cyclohexane:acetone:glacial acetic acid (49:49:2) [6]. After this, development was repeated four times with isopropyl ether:methanol (6:1). The migration distance of the solvents was about 16 cm. The radioactivities on t.l.c. plates were traced using a Dünnschicht Scanner (LB 2723, Berthold). Each radioactive metabolite of DT_3 was identified by referring to the R_f values of the unlabeled standards (DT_3, DT_2, DT_1, DT_0, DG_3, DG_2, DG_1 and DG_0). After collecting each radioactive spot on t.l.c. the radioactivities of digitoxin and its metabolites were determined with a liquid scintillation counter. The recovery of radioactivity from the developed t.l.c. plate exceeded 80 per cent of the applied activity of the incubation sample. The major loss of activity during the procedures was in the process of the transfer of the evaporated ethanol fraction to a t.l.c. plate with a small amount of solvent. Since the final quantitation of DT_3 metabolism was done using the relative decrease of DT_3 radioactivity in the sample, the loss of radioactivity during these procedures does not enter into the error of the results.

The radiochemical purity of [^{3}H]DT_3 determined by the same t.l.c. procedure as described above was 95.9 ± 1.8 per cent (mean ± S.D.) and the major contaminant was DT_2 (1.6 ± 1.1 per cent). The stability of DT_3 during the procedures from incubation to t.l.c. development was checked with hepatocytes previously heated at 95° for 5 min. The t.l.c. procedure revealed only a single peak of radioactivity corresponding to DT_3, indicating that DT_3 remains unchanged during the incubation, extraction and separation procedures, unless the cells were enzymatically active.

The addition of albumin (2.5 per cent) to the medium reduced the rate of DT_3 biotransformation to about 50 per cent of that of incubation sample without albumin. Therefore, for all other experiments in the present study, albumin-free medium was used.

An example of the separation of DT_3 and its metabolites by t.l.c. is illustrated in Fig. 1. The hydrophilic metabolites are located at the start. According to previous studies [2, 7], these hydrophilic compounds are considered to be inactive metabolites conjugated with glucuronic acid or sulfuric acid. The relative amounts of DT_3 and its metabolites in the incubated samples were calculated from the percentage of radioactivity of each fraction using the sum of radioactivities of all fractions as 100 per cent, with the assumption that all metabolites had the same molar specific radioactivity as the parent drug, DT_3. An example of a time course study of DT_3 biotransformation by isolated hepatocytes using the above-mentioned quantitation is shown in Fig. 2. This shows a linear relationship between the amount of DT_3 which is metabolized and incubation time for a period of about 1.5 hr. Table 1 shows the relative amounts of DT_3 and its metabolites found after 30 and 180 min of incubation. The predominant metabolite of DT_3 produced by isolated hepatocytes was DT_2, representing about 70 per cent of total metabolites at 30 min after the start of incubation. DT_1, DG_3 and DG_2 were also detected, though in much smaller amounts as compared with DT_2. The conjugate fraction was also present but occupied only 3 per cent of all metabolites at 30 min.

The metabolites of DT_3 found in the present study employing isolated rat hepatocytes have also been identified in patients on digitoxin therapy. The results obtained indicate that the main metabolic pathway of DT_3 in isolated hepatocytes is the stepwise cleavage of the sugar side chains which successively yield DT_2 and DT_1. In addition, 12β-hydroxylation of DT_3 to DG_3 was shown to take place. However, this hydroxylation appeared to occur to only a limited extent in the isolated hepatocytes; this has also been reported in man by determining the metabolites in urine and feces [8, 9]. Our data obtained with isolated rat hepatocytes are also in agreement with those obtained with rat liver microsomes [5]. In these studies, it was found that the main metabolites were DT_2 and DT_1, while direct hydroxylation of DT_3 to DG_3 also occurred. Castle and Lage [10], who determined the DT_3 metabolites in the bile of rats, previously injected with DT_3, also found that the main metabolites of DT_3 were DT_2 and DT_1. However, they also reported that the conjugated metabolites constituted about 40 per cent of all metabolites found in the rat bile [10]. In our own experience with rats of the same strain, sex and age as used in the present study, conjugated metabolites also accounted for 30–40 per cent of all radioactivity found in the bile of rats previously injected i.v. with [^{3}H]DT_3 (unpublished observation). In contrast, conjugated metabolites represented less than 5 per cent of all metabolites in isolated hepatocyte preparations. Castle [11] also reported very recently that with isolated rat hepatocytes as well as with liver homogenates and microsomal

Table 1. Digitoxin biotransformation by isolated rat hepatocytes*

Incubation time	Conjugates	DG_3	DG_2	DT_3	DT_2	DT_1	DT_0
30 min†	0.93 ± 0.30	2.46 ± 0.52	3.76 ± 0.89	217.6 ± 4.6	20.6 ± 5.0	1.78 ± 0.59	0.96 ± 0.37
180 min‡	2.7	23	21	134	64	4.5	3.5

* The initial DT_3 concentration was 2.5×10^{-6}M in all experiments, the concentrations of DT_3 and its metabolites are expressed as 10^{-8}M. Values are means ± S.D. of duplicate determinations of six different incubation experiments.
† Cell concentration of 1×10^6 cells $\cdot ml^{-1}$.
‡ Cell concentration of 4.3×10^6 cells $\cdot ml^{-1}$.

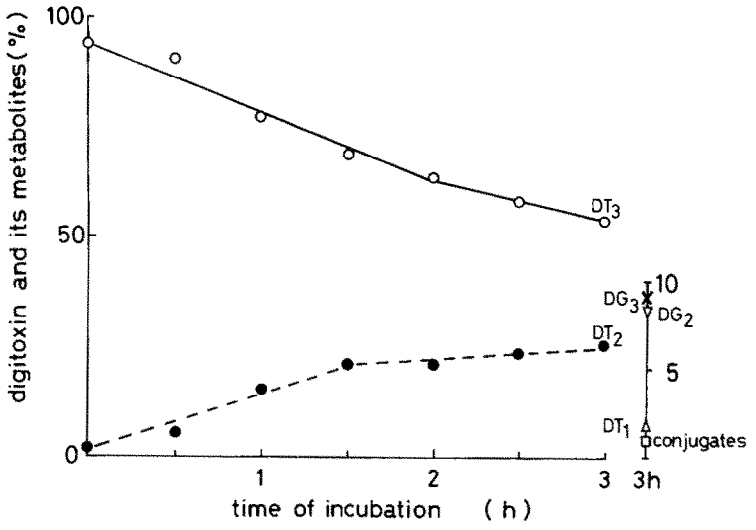

Fig. 2. An example of time course study of DT_3 biotransformation by isolated rat hepatocytes. Cell concentration, 4.0×10^6 cells · ml^{-1}; DT_3 concentration 2.5×10^{-6} M. For the quantitation of metabolites, see text.

preparations, the formation of conjugated metabolites was limited; the present observations are in accordance with those findings. The reason why isolated hepatocytes form fewer conjugates than the liver *in vivo* is currently under investigation in our laboratory. With regard to DT_3 metabolites, different data have been reported for guinea pig liver. With the isolated perfused guinea pig liver [6] and with isolated guinea pig hepatocytes [2], no DT_2, DT_1 or DG_3 could be identified and major metabolites were DG_2, DG_1 and conjugates. To explain the absence of DT_2 and DT_1, Kolenda *et al.* [6] and Klompe [2] suggested that these metabolites are quickly converted to DG_2 and DG_1 in guinea pig liver.

A further attempt was made to obtain kinetic parameters for DT_3 biotransformation with isolated hepatocytes. For this purpose, cell concentrations lower than 1.3×10^6 cells/ml were used, since under this condition the velocity of DT_3 biotransformation expressed per unit cell concentration was independent of cell concentration in the medium.

For the Lineweaver–Burke plots from which the apparent kinetic constants K_m and V_{max} could be calculated, the initial velocity for DT_3 biotransformation was calculated from the linear part of the DT_3 disappearance curve. An apparent K_m value of 25 ± 8 μM (mean ± S.D.) and a V_{max} of 58 ± 13 pmoles/min/10^6 cells (mean ± S.D.) were calculated from five different hepatocyte preparations. Calculation of such kinetic parameters for DT_3 metabolism by isolated hepatocytes is only valid if the uptake process of DT_3 by hepatocytes is not rate limiting. The data of Klompe [2] revealed that the apparent K_m values for the metabolism of DT_3 were the same in isolated hepatocytes and in microsomes, indicating that the uptake of DT_3 by isolated hepatocytes is probably not rate limiting. The uptake of DT_3 by isolated rat hepatocytes takes place by means of diffusion which is practically accomplished in the first few minutes after the start of incubation (Schwenk, personal communication). This supports our assumption that uptake process is not rate-limiting. Thus, kinetic characteristics of the type obtained in the present study can provide a basis for a comparison of rats of different physiological state, i.e. age, sex and strain, in future studies.

Acknowledgement—We thank Dr. D. L. Knook for his helpful suggestion in the preparation of this manuscript.

Tokyo Metropolitan Institute of Gerontology, Tokyo, Japan.

C. F. A. VAN BEZOOIJEN*
Y. SOEKAWA
M. OHTA
M. NOKUBO
K. KITANI

REFERENCES

1. D. J. Stewart and T. Inabe, *Biochem. Pharmac.* **28**, 461 (1979).
2. M. Klompe, The role of cytochrome P-450 in the biotransformation of digitoxin and its aglycone. Thesis, Amsterdam (1977).
3. C. F. A. van Bezooijen, M. J. van Noord and D. L. Knook, *Mech. Age. Dev.* **3**, 107 (1974).
4. C. F. A. van Bezooijen, T. Grell and D. L. Knook, *Mech. Age. Dev.* **6**, 293 (1977).
5. A. Schmoldt and C. Rohloff, *Naunyn-Schmiedeberg's Archs Pharmac.* **305**, 167 (1978).
6. K. D. Kolenda, H. Lüllmann and T. Peters, *Br. J. Pharmac.* **41**, 661 (1971).
7. M. C. Castle and G. L. Lage, *Toxic. appl. Pharmac.* **27**, 641 (1974).
8. H. F. Vöhringer and N. Rietbrock, *Clin. Pharmac. Ther.* **16**, 796 (1974).
9. L. Storstein, *Clin. Pharmac. Ther.* **21**, 395 (1977).
10. M. C. Castle and G. L. Lage, *Res. Commun. Chem. Path. Pharmac.* **6**, 601 (1973).
11. M. C. Castle, *J. Pharmac. exp. Ther.* **211**, 120 (1979).

* Present address: Institute for Experimental Gerontology TNO, P.O. Box 5815, 2280 HV Rijswijk, The Netherlands.