REFERENCES

1. J. W. Lown, H. Chen and J. A. Plambeck, *Biochem. Pharmac.* **28**, 2563 (1979).
2. R. D. Olson, R. C. Boerth, J. G. Gerber and A. S. Nies, *Life Sci.* **29**, 1393 (1981).
3. H. Nohl and W. Jordan, *Biochem. biophys. Res. Commun.* **114**, 197 (1983).
4. S. S. Pan, L. Pedersen and N. R. Bachur, *Molec. Pharmac.* **19**, 184 (1981).
5. J. H. Doroshow and J. Reeves, *Biochem. Pharmac.* **30**, 259 (1981).
6. J. H. Doroshow, *Cancer Res.* **43**, 460 (1983).
7. A. L. Taylor and B. H. Bulkey, *Lab. Invest.* **47**, 459 (1982).
8. H. Muhammed, T. Ramasarma and C. K. Ramakrishna Kurup, *Biochim. biophys. Acta* **722**, 43 (1982).
9. D. A. Bates and C. C. Winterbourn, *FEBS Lett.* **145**, 137 (1982).
10. R. Goldman, T. Facchinetti, D. Bach, A. Raz and M. Shinitzky, *Biochim. biophys. Acta* **512**, 254 (1978).
11. C. L. Van Boxtel, R. D. Olson, R. C. Boerth and T. A. Oates, *J. Pharmac. exp. Ther.* **207**, 277 (1978).
12. P. Caroni, F. Villani and E. Carafoli, *FEBS Lett.* **30**, 184 (1981).
13. J. P. Reeves and J. L. Sutko, *Science* **208**, 1461 (1980).
14. J. M. J. Lamers and J. T. Stinis, *Cell Calcium* **4**, 281 (1983).
15. M. J. Edwards and M. H. Maguire, *Molec. Pharmac.* **6**, 641 (1970).
16. G. L. Sottocasa, B. Kuylenstierna, L. Ernester and A. Bergstrand, *J. Cell. Biol.* **32**, 415 (1967).
17. T. E. King, *Methods Enzymol.* **10**, 322 (1967).
18. M. M. Bradford, *Analyt. Biochem.* **72**, 246 (1976).
19. S. Gudbjarnason and B. Doell, in *Tocopherol, Oxygen and Biomembranes* (Eds. C. de Duve and O. Hayaishi), p. 297. Elsevier/North-Holland Biomedical Press, Amsterdam (1978).
20. A. Montfoort and J. M. J. Lamers, *J. molec. cell. Cardiol.* **15**, (suppl. 3) 24 (1983).
21. M. R. Bristow, N. E. Kantrowitz, W. D. Harrison, W. A. Minobe, W. S. Sageman and M. E. Billingham, *J. cardiovasc. Pharmac.* **5**, 913 (1983).
22. R. J. Jaenke, *Cancer Res.* **36**, 2958 (1976).
23. J. Zähringer, B. Höfling, W. Raum and R. Kandolph, *Biochim. biophys. Acta* **608**, 315 (1980).
24. M. R. Bristow, R. H. Scott, M. E. Billingham and J. R. Daniels, *Circulation* **58**, (suppl. II) 19 (1978).
25. A. Fleckenstein, in *Calcium and the Heart* (Eds. P. Harris and L. Opie), p. 135. Academic Press, London (1970).
26. P. K. Singal, K. S. Dhillon, R. E. Beamish, N. Kapur and N. S. Dhalla, *Br. J. exp. Pathol.* **63**, 167 (1982).

Biochemical Pharmacology, Vol. 33, No. 22, pp. 3709–3711, 1984.
Printed in Great Britain.

0006-2952/84 $3.00 + 0.00

A decrease in the capacity of hepatocytes isolated from aged male BN/BiRij rats to metabolize digitoxin

(*Received* 27 *March* 1984; *accepted* 12 *June* 1984)

Lipid soluble drugs are generally metabolized by the liver in two successive phases, the Phase I and Phase II reactions. Phase I reactions include oxidations, reductions, dealkylations and hydrolyses. Phase II metabolism involves those reactions in which the drug or its Phase I metabolites are transformed into less lipophilic metabolites by conjugation with small endogenous molecules such as glucuronic or sulphuric acid. Probably, the effect of age is different for the different metabolic pathways, which might explain the apparently contradictory literature data on the effect of age on the metabolism of lipid soluble drugs. To obtain insight into this problem, the effect of age on digitoxin (DT_3*) metabolism was investigated in the present study. DT_3 is metabolized via different metabolic pathways, viz. degraded by hydrolysis and hydroxylation reactions. The resulting active Phase I metabolites and DT_3 itself can be inactivated by conjugation with glucuronic or sulphuric acid. Therefore, by studying the effect of age on the metabolic pattern of DT_3, information on the influence of age on hydroxylation, hydrolysis and conjugation reactions can be obtained.

Hepatocytes isolated from rats represent a suitable system for studying the metabolism of DT_3 for the following reasons. It was previously observed that the pattern of active DT_3 metabolites formed by hepatocytes isolated from 3-month-old male Wistar rats was similar to that reported to be present in serum and urine of patients on DT_3 therapy, although the amounts of the inactive conjugated metabolites were smaller in the rat hepatocytes [1]. The amount of metabolized DT_3 was linear for at least 1 hr of incubation and was proportional to the number of hepatocytes present in the incubation medium [1]. In the present study, the effect of aging on the pattern of metabolites and on the kinetic characteristics (K_m, V_{max} and Cli ($=V_{max}/K_m$)) of DT_3 was determined with hepatocytes isolated from various age groups of male BN/BiRij rats.

A preliminary report incorporating some of these data has been previously published [2].

Materials and methods

Inbred 3-, 18-, 30- and 36-month-old male BN/BiRij rats were used. The 90%, 50% and 10% survival ages were 21, 32 and 39 months respectively. The rats were maintained under "clean conventional" conditions as described by Hollander [3].

Digitoxin (DT_3), 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) were obtained from Roth (Karlsruhe, G.F.R.), digoxin (DG_3) from Merck (Darmstadt, G.F.R.). (^{3}H) Digitoxin (13.8 C/mmole) was purchased from New England Nuclear (Boston, MA, U.S.A.). Collagenase (type 1) was purchased from Sigma (St. Louis, MO, U.S.A.), Waymouth MB 752/1 medium from GIBCO (New York, NY, U.S.A.) and Seppak Cartridges from Water Associates Inc. (Etten-Leur, The Netherlands).

Thin layer chromatography (t.l.c.) was performed with D.C. Fertigplatten, Kieselgel 60 of 20 × 20 cm (Merck, Darmstadt, G.F.R.). A Dunnschicht-Scanner LB 2723 Berthold was used for locating the radioactive spots on t.l.c. plates.

* 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; t.l.c., thin-layer chromatography.

The hepatocytes were isolated by perfusion and incubation of the liver with the enzyme collagenase as reported earlier [4]. The concentration of the cells was determined with a Coulter Counter or a hemocytometer. Hepatocytes isolated from the rats of different ages did not differ significantly in average yield (40×10^6 cells/g liver) and viability (at least 80% of the cells excluded trypan blue).

The hepatocytes suspended in Waymouth MB 752/1 medium were incubated with unlabelled DT_3 and with (^3H) DT_3. The method for determining the amount of DT_3 and its metabolites has been previously described in detail [1] and is summarized below. The pH of the medium was kept at 7.4. The incubation was carried out at 37° under an atmosphere of 95% O_2 and 5% CO_2 with constant shaking. After incubation, samples were withdrawn and added to ethanol. After centrifugation, the supernatant was evaporated, the residue dissolved in water and layered on a Seppak® column. The column was washed with water and eluted with ethanol. The ethanol fraction was evaporated and the pellet dissolved and applied to a t.l.c. "kieselgel" plate. The plate was developed once with cyclohexane:acetone:glacial acetic acid (49:49:2). Thereafter, development was repeated four times with isopropyl ether: methanol (6:1). Radioactive spots were traced with a Dünnschicht Scanner. After collecting each radioactive spot, the radioactivity was determined with a liquid scintillation counter.

Results

The relative pattern of DT_3 metabolites was determined by incubating hepatocytes isolated from 3-month-old male BN/BiRij rats in concentrations of about 10×10^6 cells/ml in the presence of 50 μM DT_3 for 3 hr. Under these incubation conditions, the types and relative amounts of metabolites can be determined by t.l.c. (Fig. 1). An important metabolic pathway of DT_3 metabolism is the stepwise cleavage of the sugar side chain which successively yields DT_2 and DT_1. In addition, 12β-hydroxylation of DT_3 to DG_3 was shown to take place. DG_2 and DG_1 which might be formed by hydrolysis of DG_3 and DG_2 respectively, or by hydroxylation of DT_2 and DT_1 respectively, were also present. The conjugate fraction occupied a small part of all metabolites. The distribution of DT_3 and its metabolites was also studied with hepatocytes isolated from 18-, 30- and 36-month-old male BN/BiRij rats. No significant changes in the pattern of digitoxin metabolites with age were observed (Table 1).

Age-related changes in the kinetic characteristics of DT_3 biotransformation were studied with hepatocytes isolated from the same rats used for determining the metabolite pattern. The hepatocytes were incubated for 1 hr at cell concentrations of about 1.0×10^6 cells/ml, since under these incubation conditions the amount of metabolized DT_3 was proportional to the cell concentration in the incubation medium [1]. Hepatocyte suspensions isolated from 3-, 18-, 30- and 36-month-old rats were incubated with six DT_3 concentrations of 20, 25, 30, 40, 60 and 100 μM and Lineweaver–Burke plots were made. From these plots, the apparent kinetic constants K_m and V_{max} could be calculated. The apparent K_m values did not change with age (Table 2). The V_{max} was constant at between 3 and 18 months of age, but was decreased sharply at between 18 and 36 months of age. The value for the hepatic intrinsic clearance ($Cli = V_{max}/K_m$) observed at 36 months of age was significantly decreased in comparison with the 3- and 18-month values (Table 2).

Discussion

The objective of this study was to determine the effect of age on hydroxylation, hydrolysis and conjugation reac-

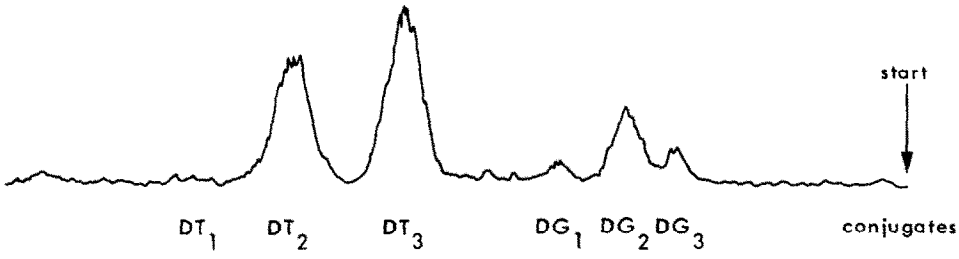

Fig. 1. Thin layer chromatogram of digitoxin and its metabolites. Hepatocytes were isolated from a 3-month-old male BN/BiRij rat. The incubation conditions were: cell concentration, 9.2×10^6 cells/ml; digitoxin concentration, 50 μM; incubation time 3 hr.

Table 1. The effect of age on the relative distribution pattern of digitoxin metabolites in hepatocytes isolated from male BN/BiRij rats of various ages

	3 months (N = 4)	18 months (N = 5)	30 months (N = 5)	36 months (N = 6)
Conjugates	3.5 ± 2.6	9.9 ± 3.9	3.6 ± 2.1	6.8 ± 3.0
DG_3	21 ± 14	11.2 ± 1.4	15.7 ± 4.0	18.5 ± 5.2
DG_2	23.5 ± 9.8	20.6 ± 5.3	28.8 ± 4.3	19.0 ± 3.7
DG_1	9.1 ± 2.7	3.8 ± 2.1	7.2 ± 8.0	4.5 ± 3.6
DT_2	40.5 ± 6.0	49.2 ± 9.4	42.3 ± 9.5	46.2 ± 3.6
DT_1	1.7 ± 2.0	5.3 ± 0.6	2.4 ± 2.3	4.9 ± 0.8

Cell concentration: $\pm 10 \times 10^6$ cells/ml; digitoxin concentration: 50.10^{-6} M; incubation time: 3 hr. Values representing % of the total metabolite fraction are expressed as mean ± S.D.

Table 2. The effect of age on the kinetic characteristics of digitoxin biotransformation by hepatocytes isolated from male BN/BiRij rats of various ages

	3 months (N = 4)	18 months (N = 5)	30 months (N = 4)	36 months (N = 6)
V_{max} (pmol/min · 10^6 cells)	82 ± 14	88 ± 34	57 ± 15*	37 ± 11*†‡
Apparent K_m (μM)	41 ± 18	47 ± 20	43 ± 26	34 ± 18
Cli (V_{max}/K_m)	2.25 ± 0.88	1.95 ± 0.33	1.61 ± 0.75	1.30 ± 0.50*†

Cell concentration: ±1.0 × 10^6 cells/ml; Digitoxin concentrations 20, 25, 30, 40, 60 and 100 μM; incubation time 1 hr. Values are expressed as mean ± S.D.

* Value differs significantly ($P < 0.05$) from the 3-month value.

† Value differs significantly ($P < 0.05$) from the 18-month value.

‡ Value differs significantly ($P < 0.05$) from the 30-month value.

tions by studying the DT_3 metabolism with hepatocytes isolated from rats of different ages. The capacity of isolated hepatocytes to metabolize DT_3 decreased with age as judged from the decrease in hepatic intrinsic clearance in the hepatocytes isolated from old rats. The decreased intrinsic clearance resulted from a decrease in V_{max}, suggesting a decline in the capacity of the metabolizing system. However, the efficiency of the metabolizing system and its affinity for the substrate, did not change with age as indicated by the unchanged K_m.

From these data and from the observation that the relative distribution pattern of DT_3 metabolites did not change with age, it can be concluded that all mechanisms involved in DT_3 metabolism, namely, hydroxylation, hydrolysis and conjugation, equally decrease with age. A study by Kitani and co-workers [5, 6], however, revealed that *in vivo* the conjugation reaction itself is not decreased with aging, but, that as a consequence of a decrease in hydrolysis, less DT_1, the preferred substrate for glucuronidation [7], is available for conjugation. A similar situation may arise in isolated hepatocytes.

In a previous study, the influence of age on DT_3 metabolism was investigated *in vivo* by determining the amount of DT_3 and its metabolites in the bile of 24-month-old male and female Wistar [6] and 30-month-old female BN/BiRij rats [5]. The excretion of DT_3 itself did not decrease with age. However, a decrease in the amounts of DT_3 metabolites excreted via the bile was observed, which in the female BN/BiRij and the female Wistar rat was more pronounced for the hydroxylation products (DG_3, DG_2, DG_1 and DG_0) in comparison with the metabolites resulting from hydrolysis alone (DT_2, DT_1 and DT_0) [5, 6]. The reverse was observed for male Wistar rats [6]. In addition, Kitani *et al.* [5, 6] also determined the plasma disappearance curves of the total of DT_3 and its metabolites. A slower disappearance was observed in the 24- and 30-month-old rats. All these findings suggest an age-related decline in the capacity of the liver to metabolize DT_3. These observed *in vivo* changes might be due to age-related changes in physiological factors such as the governing nervous and endocrine systems and the blood supply of the liver. The present study reveals that, in addition to possible changes in these physiological factors, age-related changes in the intrinsic hepatic clearance ($Cli = V_{max}/K_m$) of the individual hepatocytes play an important role.

Summarizing, the DT_3 biotransformation capacity of hepatocytes isolated from male BN/BiRij rats of 3, 18, 30 and 36 months of age was determined. With respect to the relative distribution pattern of the digitoxin metabolites no change with age was observed. Kinetic characterization of the DT_3 biotransformation revealed that the K_m did not change with age, but that after 18 months of age the V_{max} sharply decreased. The intrinsic hepatic clearance ($Cli = V_{max}/K_m$) was significantly decreased at 36 months of age. The *in vitro* data obtained can explain the *in vivo* observations that with age the plasma disappearance for DT_3 and its metabolites is slower and the biliary excretion of digitoxin metabolites decreases [5, 6].

Acknowledgements—The authors wish to thank Prof. Dr. C. F. Hollander for his critical reading of the manuscript, Dr. A. C. Ford for editing the English text and Mrs. Lutien Vermeer for carefully typing the manuscript.

Institute for Experimental Gerontology TNO
151, Lange Kleiweg, P.O. Box 5815
2280 HV Rijswijk, The Netherlands

C. F. A. VAN BEZOOIJEN
I. H. J. BOONSTRA-NIEVELD
A. N. SAKKEE
D. L. KNOOK

REFERENCES

1. C. F. A. van Bezooijen, Y. Soekawa, M. Ohta, M. Nokubo and K. Kitani, *Biochem. Pharmac.* **29**, 3023 (1980).
2. C. F. A. van Bezooijen, A. N. Sakkee, I. H. J. Boonstra-Nieveld, J.M. Begué, A. Guillouzo and D. L. Knook, In *Liver and Aging, Liver and Drugs* (Ed. K. Kitani), p. 167, Elsevier/North-Holland Biomedical Press, Amsterdam (1982).
3. C. F. Hollander, *Lab. Animal Sci.* **26**, 320 (1976).
4. C. F. A. van Bezooijen, T. Grell and D. L. Knook, *Mech. Age Dev.* **6**, 293 (1977).
5. K. Kitani, Y. Sato and C. F. A. van Bezooijen, *Arch. Gerontol. Geriatr.* **1**, 43 (1982).
6. K. Kitani, Y. Sato, S. Kanai and M. Nokubo, in *Liver and Aging, Liver and Drugs* (Ed. K. Kitani), p. 179, Elsevier/North-Holland Biomedical Press, Amsterdam (1982).
7. A. Schmoldt and J. Promies, *Biochem. Pharmac.* **31**, 2285 (1982).