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HEPATIC CLEARANCE OF GITOXIN

Metabolism and Biliary Excretion by Rabbit Isolated Liver

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ABSTRACT:

After *in vitro* perfusion of rabbit isolated liver with an emulsion of perfluorocarbon containing [^{3}H]gitoxin, the radioactivity in the liver and in the bile was the sum of that contained in a volatile fraction (tritiated water due to metabolism of gitoxin) and that contained in a nonvolatile fraction (gitoxin and its metabolites). This fraction was divided, by differential extraction, into two groups: liposoluble (dichloromethane soluble) compounds, including unchanged gitoxin and lipophilic metabolites (amounting to 50% in the liver and to 5% in the bile), and hydrosoluble (dichloromethane insoluble) metabolites (50% in the liver, 95% in the bile). Three types of metabolites were found in the hydrosoluble fraction. One type was sensitive to β-glucuronidase and arylsulfatase hydrolysis, a second type was insensitive to enzymatic hydrolysis but sensitive to acid hydrolysis, and a third type was insensitive to both enzymatic and acid hydrolysis. The liposoluble compounds and the conjugates sensitive to enzymatic hydrolysis were analyzed by reversed phase high performance liquid chromatography and found to comprise a wide variety of metabolites. The present study demonstrates that gitoxin uptake by the liver is followed by a fast and extensive metabolism to highly polar metabolites that are rapidly excreted into the bile.

In a previous study on rabbit isolated liver perfused with an emulsion of a perfluorocarbon containing gitoxin, a cardiac glycoside from *Digitalis purpurea*, we have shown that gitoxin rapidly disappeared from the perfusion medium. The distribution and elimination half-lives of gitoxin were estimated to be 0.22 and 0.70 hr, respectively, and the intrinsic metabolic clearance (Cl_{int}) to be 11 $ml \cdot min^{-1} \cdot g^{-1}$. Moreover, liposoluble and hydrosoluble metabolites appeared rapidly in the emulsion. These results suggested that hepatic uptake of gitoxin was followed by a rapid and extensive metabolism. In order to define the most important pathways of gitoxin metabolism in rabbit liver, we have analyzed the composition of both the metabolites present in the liver and those excreted into the bile, by high performance liquid chromatography.

Materials and Methods

Liver Perfusion. Rabbit isolated liver prepared as described by Pellegrin and Lesne[2] was perfused for 1- to 5-h periods, with an emulsion of perfluorobutyltetrahydrofuran (Mediflor FC80, 3M, Minneapolis) containing tritiated gitoxin (400 μg; 0.046 Ci/mmol). The bile was collected in 30-min fractions, weighed, and stored at −16°C. At the end of the perfusion period, the liver was rapidly immersed in ice-cold sucrose (0.25 M sucrose, 3 mM imidazole, HCl to pH 7.4), dissected free of gallbladder and other tissues, weighed, and then homogenized in buffered sucrose.

Liver Extraction Method. Gitoxin and its metabolites were extracted from liver tissue as follows. A 17-ml aliquot of ice-cold liver homogenate was rehomogenized three times (10 sec at 20,000 rpm) by means of an Ultra-Turrax homogenizer (Janke and Kunkel, Germany). Gitoxin and its metabolites were then extracted from the homogenate three times with 15 ml of acetone. The mean recovery of gitoxin in this extraction step amounted to 94%. The resulting pooled acetone-water phase was then evaporated to 0.5 ml and the residue was diluted with 5 ml of 0.1 M acetate buffer (pH 5.2 at 37°C). The liposoluble metabolites were then extracted three times with 10 ml of DCM.[3] The mean recovery of gitoxin at this step averaged 97%. The pooled DCM extracts were evaporated to dryness, the residue was taken up in 0.6 ml of 80% ethanol, and the metabolites were separated by HPLC. The hydrosoluble (DCM insoluble) material was incubated for 72 hr at 37°C in the presence of β-glucuronidase and arylsulfatase (Merck 4114). The hydrolyzed metabolites were extracted from the incubation mixture three times with 10 ml of DCM and the pooled DCM extracts were evaporated as indicated above and analyzed by HPLC.

The water phase was acidified (HCl) to pH 2 and incubated for 5 days at 30°C. Hydrolyzed metabolites were then extracted with DCM as described above.

Controls, in which tritiated gitoxin was added after homogenization, were extracted by the same procedure. Degradation of gitoxin was less than 3%.

Bile Extraction Method. Gitoxin and its liposoluble metabolites were extracted from a 0.5-ml aliquot of each bile sample three times with 1 ml of DCM (mean recovery for gitoxin was 97%) and the pooled DCM extracts were evaporated to dryness. The residue was taken up in 0.4 ml of 80% ethanol and analyzed by HPLC. The hydrosoluble metabolites were then extracted from the bile three times with 1 ml of ether-ethanol (3:1), after addition of ammonium sulfate (0.5 g), and the combined ether-ethanol extracts were evaporated to dryness. The residue was taken up in 0.6 ml of acetate buffer (pH 5.2) and incubated for 72 hr at 37°C with β-glucuronidase and arylsulfatase. Hydrolyzed metabolites were then extracted three times with 1 ml of DCM and the pooled extracts were treated and analyzed by HPLC as indicated above. The water phase was acidified (HCl) to pH 2 and incubated for 120 hr at 30°C. The hydrolyzed metabolites were subsequently extracted as described above.

High Performance Liquid Chromatography. HPLC was performed with a Hewlett-Packard 1084B apparatus equipped with a semi-automatic syringe injector (HP 79841A), a UV-visible variable wavelength detector (HP 79875A), and a radioactivity monitor (Berthold LB503). The following standards were used: gitoxin (Gt3), gitoxigenin bisdigitox-

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[3] Abbreviation used is: DCM, dichloromethane.

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oside (Gt2), gitoxigenin monodigitoxoside (Gtl), gitoxigenin (Gt0), diginatin (12β-OH gitoxine) (Dn3), diginatigenin bisdigitoxoside (Dn2), diginatigenin monodigitoxoside (Dnl), and diginatigenin (Dn0). The separation of these compounds was achieved on a C_8 reversed phase column (Hibar RT 250-4, RP8, 5 μm, Merck) with a gradient of acetonitrile in water (acetonitrile UV grade, Burdick and Jackson; Milli-Q, Millipore, deionized water), from 20 to 36% acetonitrile in 20 min at a flow rate of 1 ml/min. The absorbance was monitored at 220 nm. Unlabeled standards (Gt0, Gtl, Gt2, and Gt3) were added to each sample before injection. At the outlet of the UV-visible detector, the HPLC effluent was analyzed by continuous liquid scintillation counting in the radioactivity monitor. Two fractions were collected, from 0 to 8 min (unknown metabolites) and from 8 to 20 min (diginatin and its bisoside, monoside, and genin together). Thereafter, eight fractions corresponding to the radioactive peaks were collected separately.

Results

Liver. The proportion of liposoluble (DCM soluble) and of hydrosoluble (DCM insoluble) metabolites in the liver each amounted to about 50% of the nonvolatile radioactivity at each time (fig. 1, *top*). The ratio of hydrosoluble to liposoluble metabolites ranged from 0.7 to 1.4.

The hydrosoluble metabolites contained three fractions: metabolites sensitive to enzymatic hydrolysis ("conjugates A," conjugates with glucuronic or sulfuric acid), metabolites insensitive to enzymatic hydrolysis but sensitive to acid hydrolysis ("conjugates B," perhaps conjugates with other endogenous molecules), and hydrolysis-resistant metabolites ("polar fraction"). Conjugates A averaged 30 to 40% of the hydrosoluble fraction (fig. 1, *bottom*). Conjugates B averaged 23% after 1 hr of perfusion and 10 to 14% after longer perfusion times, and the polar fraction amounted to 46 to 58%.

The separation of gitoxin, diginatin, and their derivatives (bisoside, monoside, and genin) by HPLC is demonstrated in fig. 2. The gradient used produced a good separation of these eight standards and each compound could be collected separately. With this gradient, the liposoluble compounds and hydrolyzed conjugates A were separated into 10 fractions (fig. 3). Unchanged gitoxin (Gt3) represented 12 to 23% of the liposoluble radioactivity and 10 to 27% of the radioactivity in conjugates A. Gitoxigenin (Gt0), its monoside (Gtl) and bisoside (Gt2) together averaged 30 to 40% of the liposoluble fraction and 40 to 60% of conjugates A. Gtl and/or Gt3 were the most important conjugates. Diginatin derivatives (peak 2) averaged 13 to 20% of the liposoluble fraction and 4 to 30% of conjugates A. The unknown peaks 6, 8, and 10 were probably metabolites derived from Gtl, Gt2, and Gt3, respectively. They represented 20 to 27% of the liposoluble fraction and 2 to 20% of conjugates A.

Bile. Hydrosoluble metabolites (fig. 4, *top*) amounted to 92–97% of the nonvolatile radioactivity found in the bile at each time. The ratio of hydrosoluble to liposoluble metabolites ranged from 12 to 32. Metabolites sensitive to enzymatic hydrolysis (conjugates A) averaged 57 to 64% of the hydrosoluble radioac-

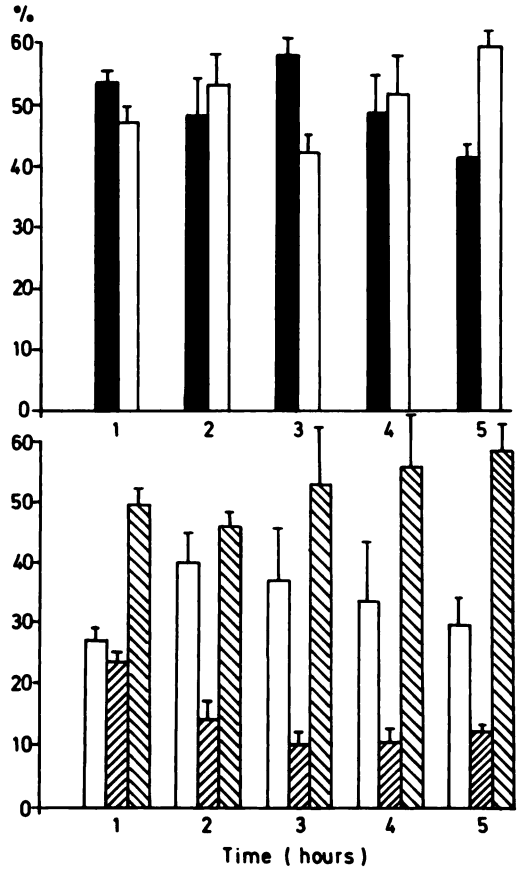

FIG. 1. *Nonvolatile radioactivity in the liver.*

Top, the nonvolatile radioactivity contained two fractions: a liposoluble fraction (■, in percentage of the nonvolatile radioactivity) and a hydrosoluble fraction (□). *Bottom*, the hydrosoluble fraction contained three types of metabolites: conjugates with glucuronic or sulfuric acid (conjugate A, □, in percentage of the hydrosoluble fraction), conjugates with other molecules (▨, conjugate B) and polar (hydrolysis-resistant) metabolites (▧). *Vertical bars* represent SEM ($N = 3$).

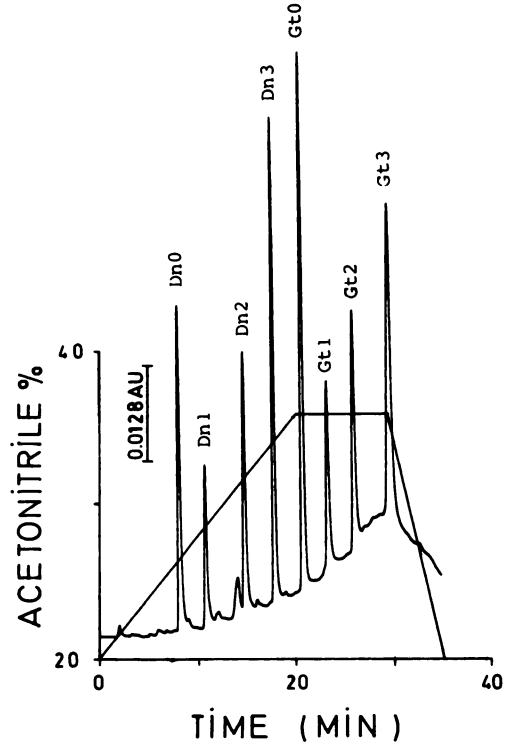

FIG. 2. *HPLC separation of gitoxin derivatives.*

Conditions, see text. *Dn0*, diginatigenin; *Dn1*, diginatigenin monodigitoxoside; *Dn2*, diginatigenin bisdigitoxoside; *Dn3*, diginatin; *Gt0*, gitoxigenin; *Gt1*, gitoxigenin monodigitoxoside; *Gt2*, gitoxigenin bisdigitoxoside; *Gt3*, gitoxin.

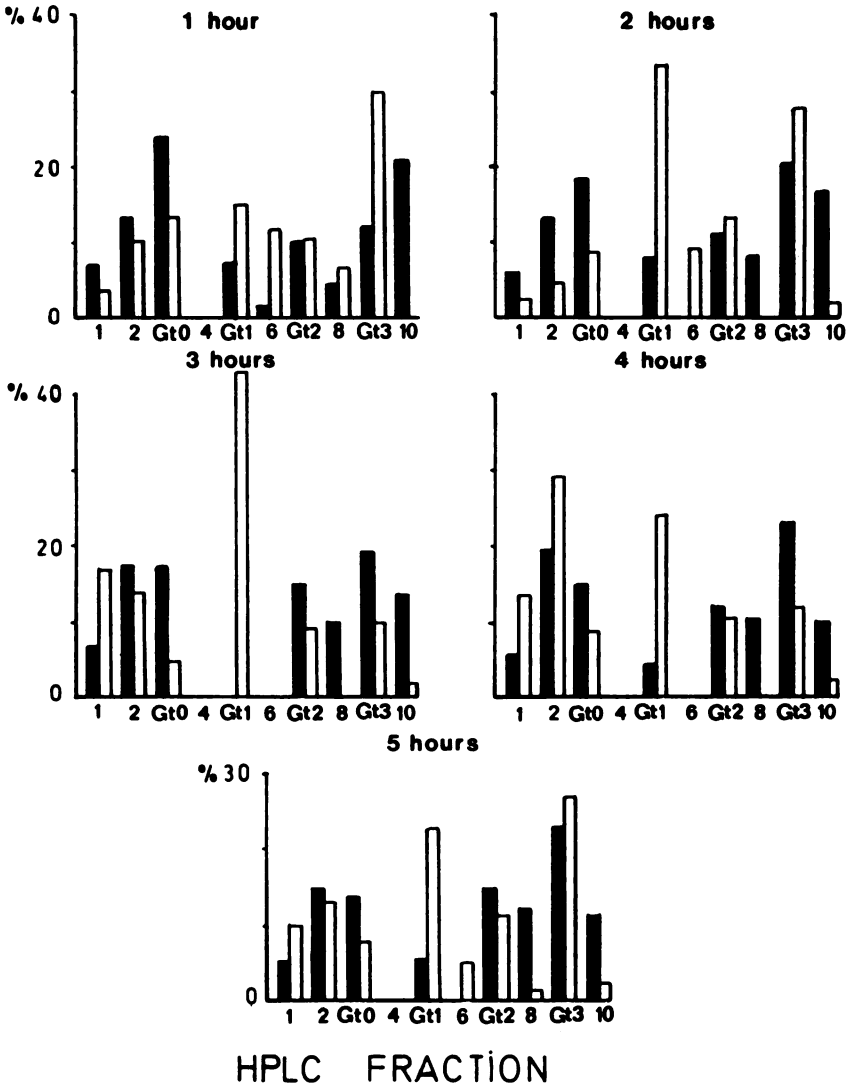

FIG. 3. *Metabolic pattern of gitoxin in the liver after 1 to 5 hr of perfusion.*

The liposoluble compounds (■) and the hydrosoluble metabolites sensitive to enzymatic hydrolysis (□) in the liver were separated by HPLC into 10 fractions. At each time, the sum of the 10 fractions represents 100% in each group ($N = 3$). *1, 4, 6, 8, 10,* unknown metabolites; *2,* diginatin derivatives; *Gt0, Gt1, Gt2,* and *Gt3,* see fig. 2.

tivity. Conjugates B averaged 15 to 20% and the polar metabolites averaged 20 to 24% (fig. 4, *bottom*).

The cumulative biliary excretion of gitoxin and its metabolites is illustrated in fig. 5. The total amount of radioactivity excreted into the bile averaged 39% of the dose (liposoluble compounds, 1.7%; hydrosoluble metabolites, 35.7%; tritiated water, 1.6%) and was calculated to be 46% of the dose at infinite time (B_∞). The biliary excretion rate (dB/dt) is shown in fig. 6. The maximum excretion rate (measured as total radioactivity, including volatile radioactivity) was reached between the 60th and the 90th min of perfusion and amounted to about 938 ng of gitoxin eq/min. The maximal excretion rate of the liposoluble compounds was about 30 ng/min and that of the hydrosoluble metabolites was about 886 ng/min. The elimination half-life calculated from the slope of the log-linear phase was 2.2 hr for the liposoluble compounds and 1.3 hr for the hydrosoluble metabolites.

The liposoluble compounds and hydrolyzed conjugates A were analyzed by HPLC. The biliary excretion rate and the cumulative biliary excretion of gitoxin and each metabolite are presented in fig. 7.

Unknown metabolites 6 and 8 were found only in the conjugated fraction. All other metabolites were excreted both as conjugates with glucuronic or sulfuric acid and in unconjugated form. Unknown conjugates 6, 8, and 10 together amounted to 22% of the total excretion during the perfusion period. The ratio of conjugate A to liposoluble metabolite, which can be considered as an index of conjugation intensity, amounted to 2 for unknown 1, 6 for Gt1, 10 for Gt2, Gt3, and unknown 2, 13.5 for Gt0, and 17 for unknown 10. Biliary excretion was higher for each conjugate than for any liposoluble metabolite.

Discussion

The metabolism of digitoxin, digoxin, and some related cardiac glycosides has been studied by several authors in man and in a wide variety of laboratory animals including the rabbit. Several different metabolic pathways have been reported (fig. 8).

Hydrolysis of the Sugar Chain. This leads to the bisoside, the monoside, and the genin (1–8). It is followed by epimerization from the β to the α orientation at the C-3 hydroxyl group by way of a 3-keto intermediate (8–11). The enzymatic basis for this

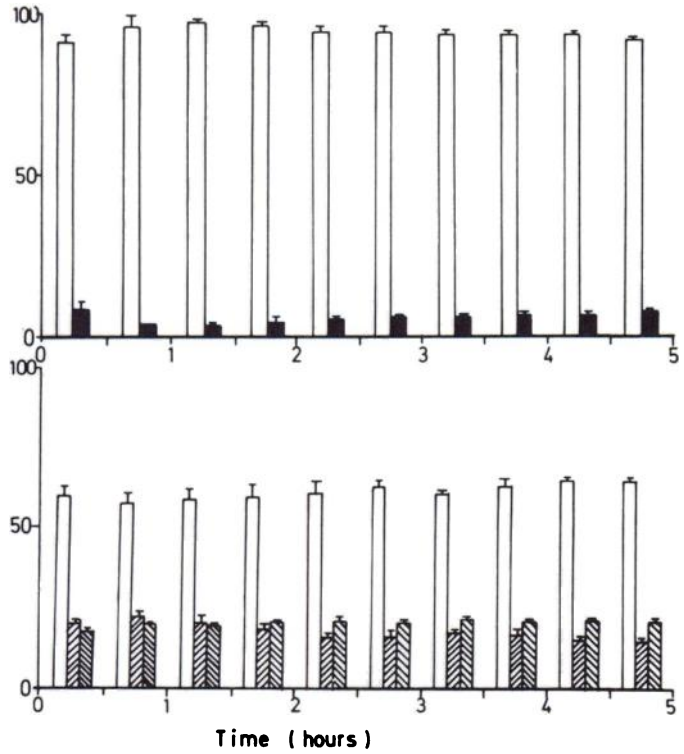

FIG. 4. *Nonvolatile radioactivity in the bile.*

Top, the nonvolatile radioactivity in bile was divided into two fractions: a liposoluble fraction (■, in percentage of the nonvolatile radioactivity) and a hydrosoluble fraction (□). *Bottom*, the hydrosoluble fraction contained three types of metabolites: conjugates with glucuronic or sulfuric acid (conjugate A, □, in percentage of hydrosoluble fraction), conjugates with other molecules (■, conjugate B) and polar (hydrolysis-resistant) metabolites (■). *Vertical bars* represent SEM ($N = 6$).

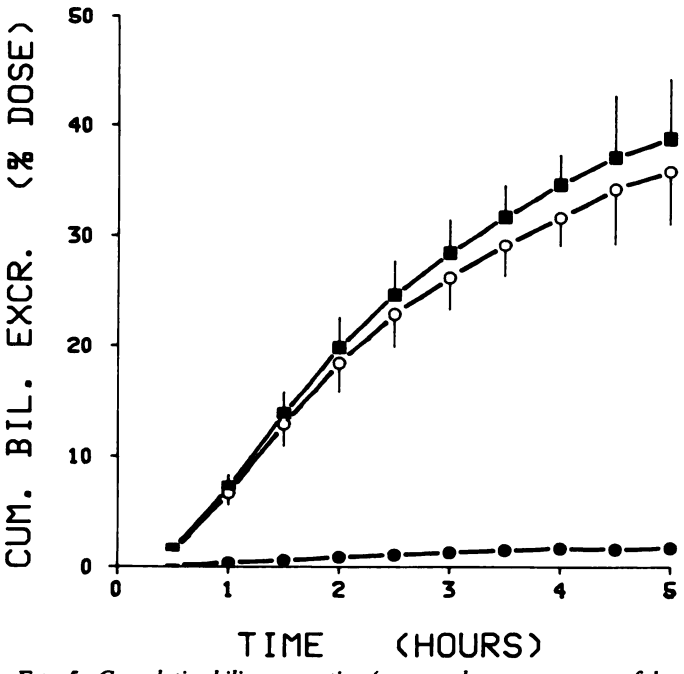

FIG. 5. *Cumulative biliary excretion (expressed as a percentage of the dose).*

Liposoluble fraction (●), hydrosoluble fraction (○), and the total radioactivity (■, including volatile radioactivity). *Vertical bars* represent SEM ($N = 6$).

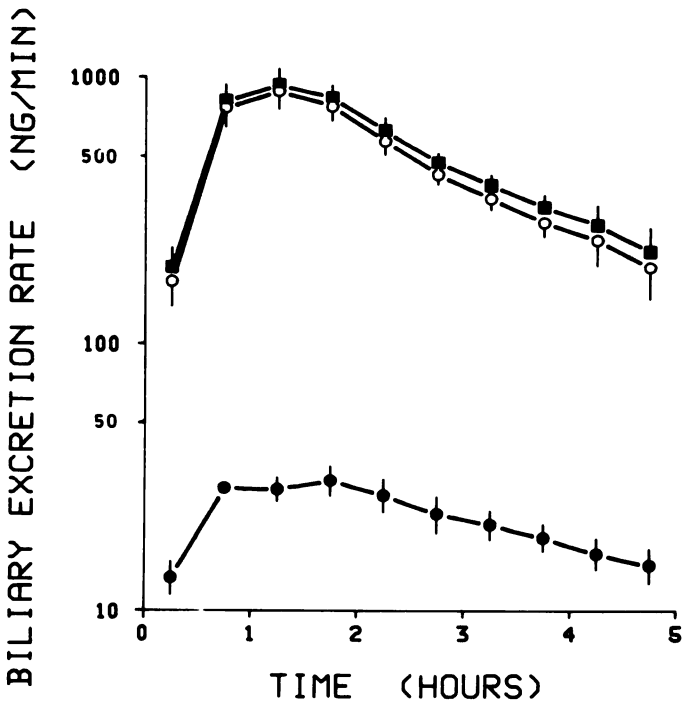

FIG. 6. *Biliary excretion rate (expressed as ng of gitoxin eq/min).*

Liposoluble fraction (●), hydrosoluble fraction (○), and the total radioactivity (■). *Vertical bars* represent SEM ($N = 6$).

epimerization has been demonstrated by Repke and Samuels (12). The bisoside, the monoside, and the genin have cardioactive properties (13–18) but epimerization leads to inactivation of the genin (9, 10).

Hydroxylation of the Steroid Moiety. The intensity of this hydroxylation and the position at which it occurs, are dependent on the cardiac glycoside and the species (1, 3, 5, 7, 8, 10, 11, 19–24).

Saturation of the Lactone Ring. This leads to cardioinactive 20,22-dihydro derivatives (7, 18, 25, 26).

Opening of the Lactone Ring. This leads to both some liposoluble and some polar metabolites (27, 28).

Conjugation with Glucuronic or Sulfuric Acid. According to Refs. 2, 7, 8, 24, and 29–33, this leads to cardioinactive metabolites (15, 18).

Only a few studies have been devoted to the metabolism of gitoxin (7, 8, 19, 24, 28). Since the rabbit has been shown to metabolize other digitalis glycosides extensively (5, 23, 34–37), it seemed to be of interest to study gitoxin metabolism in that species.

Total Excretion. Biliary excretion of gitoxin and its metabolites (20% after 2 hr) was less than that observed after iv injection of randomly tritiated digitoxin and digoxin in the whole rabbit (31 and 39%, respectively; 34), but much higher than that observed for ouabain (1.5% after 2 hr, 4.4% after 12 hr). It was also less important than that of gitoxin in the guinea pig (82.6% of the dose during the first 6 hr; 24). These differences could be due to a lower bile flow rate *in vitro* (2.3 ml/hr/kg of body weight) than *in vivo* (3.6 ml/hr/kg of body weight in the rabbit (34) and 14 ml/hr/kg of body weight in the guinea pig (7)).

The ratio of hydrosoluble to liposoluble metabolites in the liver (0.7 to 1.4) indicates that uptake of gitoxin by the liver was quickly followed by metabolism to highly polar DCM-insoluble derivatives. A similar observation was made in an *in vitro* study

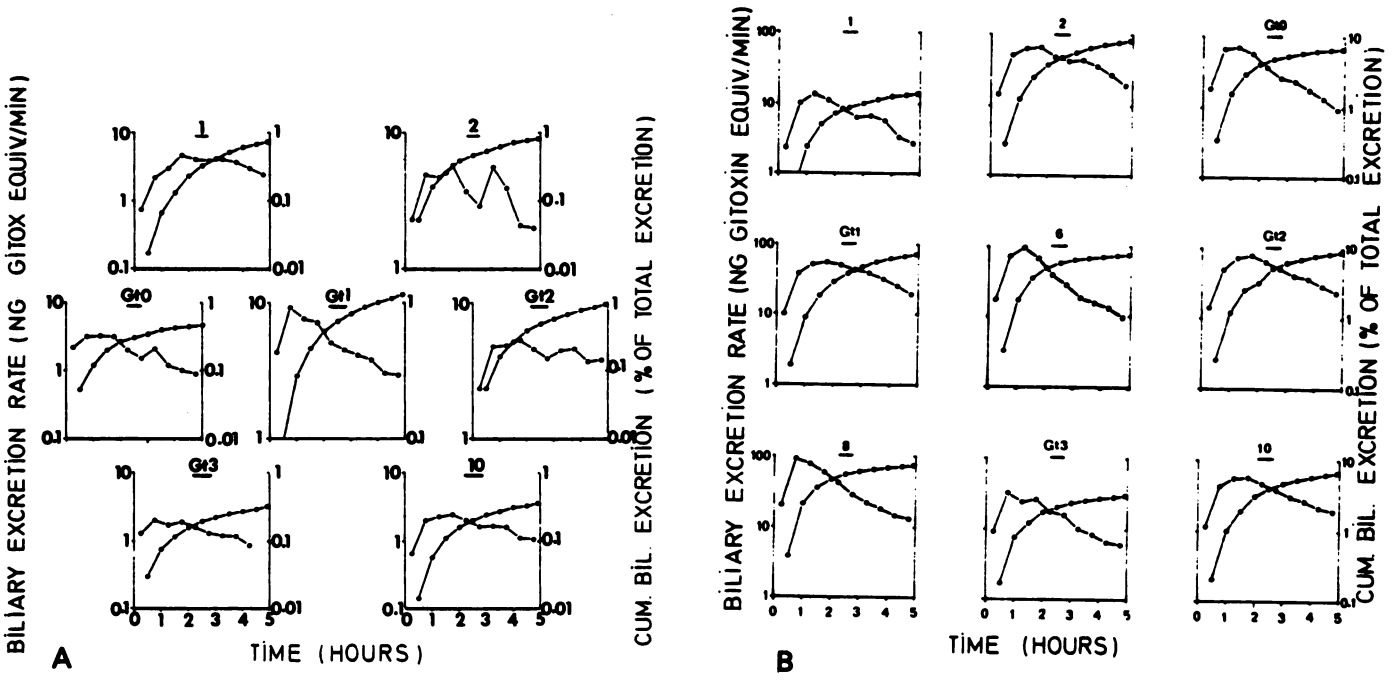

FIG. 7. *Biliary excretion.*

A, liposoluble compounds. Biliary excretion rate (●) and cumulated biliary excretion (○, in percentage of total biliary excretion) for each liposoluble compound separated by HPLC. (For identification of the fractions, see fig. 3). Each *point* is the mean of three experiments. *B*, conjugates A. Biliary excretion rate (●) and cumulative biliary excretion (○) for each conjugate A. See *A* above for other details.

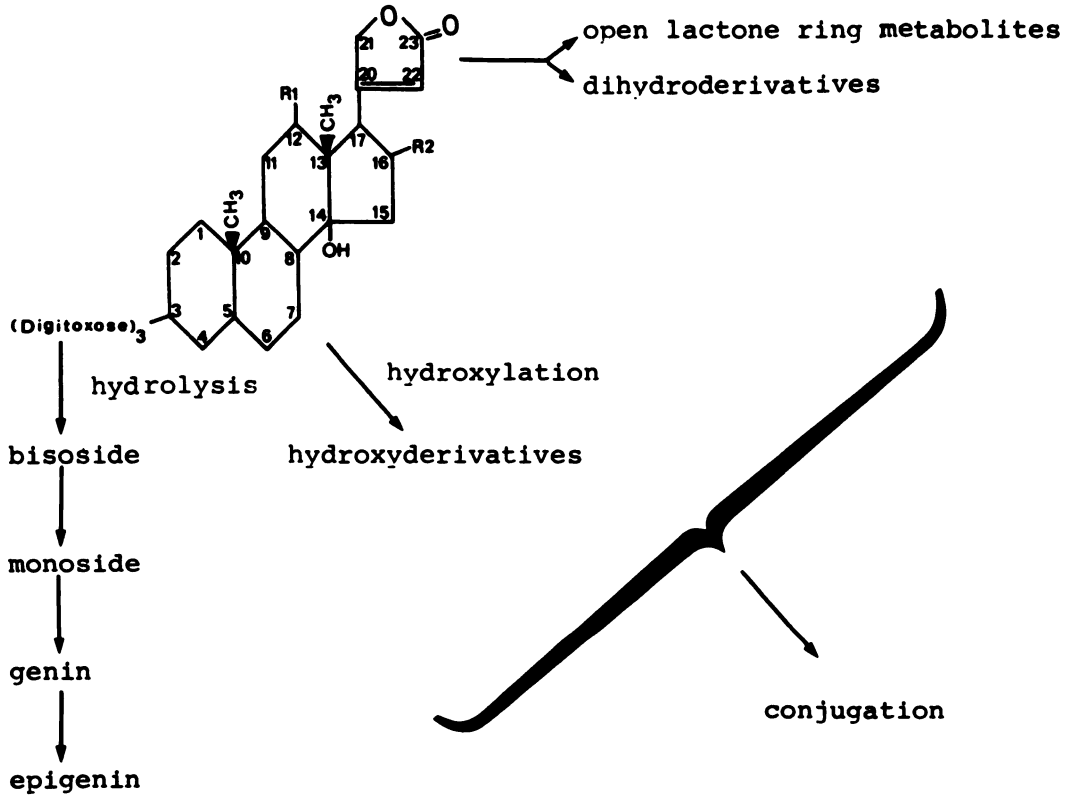

FIG. 8. *Metabolic pathways of cardiac glycosides derived from Digitalis.*

of digitoxin and digoxin metabolism in the rabbit (5). These highly polar metabolites are preferentially excreted into the bile, as indicated by the higher ratio of hydrosoluble to liposoluble metabolites in the bile (12 to 32) than in the liver (0.7 to 1.4). Russell and Klaassen (34) found that approximately 63% of the metabolites in the bile were hydrosoluble 0.5 hr after administration of digoxin to the rabbit, 70% after 1 hr, 73% after 2 hr, and more at later times. For digitoxin, they found that most of the

metabolites excreted into the bile at different time intervals were hydrosoluble. In the guinea pig, gitoxin is also extensively excreted into the bile, mainly as hydrosoluble metabolites (7).

In the isolated perfused liver model, it is assumed that biliary excretion at infinite time (B_∞) is equal to the dose (D). However, in our experiments B_∞ was estimated to be 46% of the dose. This difference could be due to the production of tritiated water which has a very high elimination half-life and to a progressive decrease in the ability of the liver to metabolize gitoxin and/or to excrete the metabolites from the cells.

Hydrosoluble Fraction. Among the hydrosoluble metabolites, conjugates with glucuronic or sulfuric acid were proportionally lower in the liver (30 to 40%) than in the bile (60%); this difference also suggests that they are preferentially excreted into the bile. In the guinea pig, these metabolites amounted to only 12% of the hydrosoluble metabolites excreted into the bile (24).

Conjugates insensitive to enzymatic hydrolysis but sensitive to acid hydrolysis were present in similar proportions in the liver and the bile (20%). Such conjugates have also been found in the guinea pig by Kadima and Lesne (7) who suggested that conjugation might occur at carbon 16 in these compounds. However such a hypothesis doesn't account for the insensitivity of these conjugates to β-glucuronidase and arylsulfatase. Therefore, it might be suggested that they are conjugated to another endogenous molecule such as glycine, glutathione, or tauric acid, for example. Such conjugation has not yet been demonstrated for other cardiac glycosides.

The polar (hydrolysis-resistant) fraction was present in higher proportions in the liver than in the bile (50 and 20% of the hydrosoluble metabolites, respectively). Further investigation will be necessary to explain such a difference. The chemical structure of these highly polar metabolites has been investigated by Benthe (27) and Kadima *et al.* (28) and it is thought that they could arise from the opening of the lactone ring at carbon 17, which would lead to the production of some liposoluble products as well as to some highly polar DCM-insoluble molecules. Some of these metabolites could then be conjugated with glucuronic or sulfuric acid or with another endogenous molecules.

Metabolic Pattern of Gitoxin in the Liver. The pattern of liposoluble derivatives and of conjugates with glucuronic or sulfuric acid is very complex since eight or nine fractions were distinguished in each group. The contribution of Gt3 to the liposoluble group of compounds amounted to 12 to 23%, which represented only 6 to 10% of the total amount present in the liver. Hydrolysis of the sugar chain seems to be the fastest degradation pathway since Gt0, Gt1, and Gt2 represented the most important pool of both liposoluble metabolites and of conjugates. Cleavage of the sugar residues of Gt3 has also been demonstrated in the guinea pig (7, 8, 24).

Peak 2 has been considered in this study as diginatin (12β-OH-gitoxin) and its derivatives, since the retention time of that fraction corresponded to that of diginatin, its bisoside, monoside, and its genin. 12β-Hydroxylation of gitoxin has also been demonstrated in the rat (19) and guinea pig (7, 8, 24) and Megges and Repke (38) found diginatin in the urine of rats after ip injection of pentaacetylgitoxin. However, the rabbit has been shown to hydroxylate cardiac glycosides at other positions as well (22, 23).

The chemical structure of derivatives 6, 8, and 10 is unknown. Since they were eluted after Gt1, Gt2, and Gt3, respectively, we suggest that they might be metabolites derived from these compounds. Lage and Spratt (5) found that incubation of rabbit liver slices in the presence of digoxin produced an unknown metabolite of Dg3 which, on chromatography was located between Dg2 and Dg3. They did not find such a metabolite after incubation of Dg3 with dog, cat, rat, mouse, or guinea pig liver slices. It is likely that this metabolite is similar to those of gitoxin described here. Three hypothesis can be made about the structure of these unknown metabolites.

1) They could be intermediates in the cleavage of the sugar chain. Schmoldt *et al.* (39) have shown that the cleavage of the glycosyl residue requires an intermediate oxidation step of the terminal glycosyl group. These intermediates are more lipophilic than their parent molecules and should have higher retention times in reversed phase HPLC. However, it would be puzzling to find such intermediate metabolites in the bile. 2) They could be 20,22-dihydro derivatives of Gt1, Gt2, and Gt3, respectively. 3) They could arise from the opening of the lactone ring, like the Mt3 metabolite described in the guinea pig by Kadima *et al.* (28), without C-12 hydroxylation. Such an isomerization leads to slightly more lipophilic metabolites.

Metabolic Pattern of Gitoxin in the Bile. The pattern of metabolization in bile was as complex as that seen in the liver. Gt2 was the most extensively excreted metabolite, followed by unknown metabolites 2, 6, and 8. However, unknown metabolites 6, 8, and 10 were the most extensively conjugated since 6 and 8 were not found in the liposoluble fraction and 10 was characterized by the highest ratio of conjugated to liposoluble metabolite. This extensive conjugation might be due to the high reactivity of their corresponding liposoluble compounds, including perhaps some molecules with an open lactone ring as indicated above. Peak 1 was probably a bis- or polyhydroxylated metabolite with relatively high polarity, which would explain its relatively lower degree of conjugation.

The present study on rabbit isolated liver demonstrates that gitoxin uptake by the liver is followed by a fast and extensive metabolism to a wide variety of derivatives which can be conjugated to a large extent with glucuronic and sulfuric acid or other molecules. However, some highly polar metabolites were excreted into the bile in an unconjugated form. These observations are in agreement with those of Kadima and Lesne (7, 24) in the guinea pig and they provide an explanation for the very high elimination constant of gitoxin previously described (40).[2]

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