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Plasma concentration, uptake by liver, and biliary excretion of tritiated cardiac glycosides in the isolated perfused guinea-pig liver

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Summary

1. Investigations were carried out on isolated perfused guinea-pig livers. Different doses of tritiated ouabain, digoxin, and digitoxin were added to the perfusion medium and the subsequent plasma elimination, hepatic uptake, and biliary excretion quantitatively measured. After the perfusion, extracts of liver, bile and plasma were subjected to thin layer chromatography in order to detect the radioactively labelled glycosides and their metabolites.

2. The ouabain concentration in the plasma approached the equilibrium stage within 45 minutes. At this time 40% of the administered dose had been taken up by the liver, and no further elimination occurred. The elimination curve for ouabain followed a simple exponential function. After 1 h the tissue medium (T/M) ratio was approximately 3. In bile hardly any radioactivity could be detected. Ouabain was therefore not excreted by the liver.

3. Up to 80% of the digitoxin was eliminated from the plasma within 4 hours. The elimination of radioactive material for the dose range studied could be described by a hyperbolic function. The T/M ratio in the liver varied with time. At the beginning it was as high as 10 and after 4 h reduced to approximately 3. After 45–60 min the concentration of radioactive material in the bile was 500 times as high as that in the plasma. Almost 70% of the administered radioactivity was excreted with the bile within 4 hours. At the end of the perfusion almost all the identifiable substances in plasma and bile were polar metabolites, as shown by thin layer radiochromatography.

4. Digoxin behaved similarly to digitoxin.

5. The findings led to the following hypothesis: uptake of cardiac glycosides into the liver cells occurs by a passive diffusion process and is related to their lipid solubility. On the other hand excretion in the bile occurs in general if polar metabolites are formed in the liver cells.

Introduction

Qualitatively, the effect of the clinically important cardiac glycosides ouabain, digoxin and digitoxin on heart muscle is approximately the same. Nevertheless considerable differences exist between the onset of action, the duration of the effect in the intact organism and the degree of accumulation in isolated organs. These differences are probably caused by the quite different fate of the three glycosides in

the intact organism; that is to say in their absorption, distribution, binding to plasma protein, metabolism, and excretion. These factors have been separately investigated over recent years in intact animals, in humans, and in isolated organ systems *in vitro*. The studies of Doherty (1968) on the distribution of digitalis after acute administration as well as the investigations by Repke (1963) on its metabolism should be particularly emphasized. However, important questions still remain unsolved: for example, the role of the liver in the excretion and the metabolic degradation of the cardiac glycosides still has to be elucidated quantitatively. The liver is the most significant organ for the metabolism of the cardiac glycosides (Okita, Talso, Curry, Smith & Geiling, 1955). Perfusing the isolated guinea-pig liver according to the method of Schimassek (1962, 1963, 1968) we investigated the excretion and metabolism of ouabain, digoxin, and digitoxin thus avoiding any interference by other factors, such as renal excretion, tissue distribution and so on. Guinea-pigs were chosen because the sensitivity of the heart muscle of this species to cardiac glycosides is similar to that of the human heart (Repke, Est & Portius, 1965). In the guinea-pig ouabain is chiefly excreted by the kidneys (Garbe & Novak, 1968) as it is in man (Marks, Dutta, Gauthier & Elliott, 1964, Lahrtz, Reinold & van Zwieten, 1969). Rats are less suitable for use since in this species ouabain is chiefly eliminated in the bile (Cox, Roxburgh & Wright, 1959, Kupferberg & Schanker, 1968).

This paper describes quantitative investigations of the fates of ouabain, digitoxin, and digoxin in the isolated guinea-pig liver perfused with oxygenated blood.

Methods

Surgical procedure and perfusion technique

Guinea-pigs of either sex weighing 500–600 g were used. The perfusion was started immediately after cannulation of the portal vein and the inferior vena cava *in situ*. The perfusion flow was maintained at approximately (0·7 ml/g liver)/minute. After cannulation of the bile duct the liver was dissected from the body and placed on a platform in a moisture chamber kept at 37° C. Further details of the perfusion technique and apparatus are described by Berg, Kolenda, Peters & Seiler (1970).

Perfusion medium

Blood from guinea-pigs was treated with heparin (5,000 I.U. of heparin/100 ml) and filtered through cheese cloth. It was diluted with Tyrode solution 2:1. Finally, 20 mg of Streptomycinesulphate/100 ml were added. The total volume of the perfusion system was maintained at 250 ml.

Function tests

The mean bile secretion amounted to about (4 ml/100 g liver)/hour. All experiments with less than (2·5 ml/100 g liver)/hour were discarded. The ratio lactate/pyruvate in the perfusion medium can be regarded as a valid criterion of the functional status of the liver (Schimassek, 1962, 1963, 1968). This parameter should have a value of about 10 for isolated rat and guinea-pig livers (Berg *et al.*, 1970). Experiments in which this ratio exceeded a value of 15 throughout the perfusion were also discarded.

Measurement of radioactivity

Radioactivity was counted in a Packard-Tricarb liquid scintillation spectrometer, equipped with an automatic external radium standard.

Bile (0·1 ml) was added to 1 ml hyamine and left at room temperature for 4 hours. The mixture was diluted with 2 ml anhydrous ethanol and 10 ml of a liquid scintillation phosphor comprising: 4·0 g PPO $\pm$ 0·1 g POPOP dissolved in 1,000 ml toluene. Plasma samples were prepared in the same way as bile samples.

Extraction method and chromatography

At the end of perfusion the radioactive compounds were extracted initially from plasma and bile with absolute ethanol and subsequently with a chloroform/methanol mixture (1 : 1). Accordingly, it was possible to obtain in a single extract both the unchanged glycosides and their non-polar and polar metabolites. The extracts were separated on thin-layer silica gel plates prepared in this laboratory. Cyclohexane/acetone/acetic acid (49 : 49 : 2) was used as a solvent for the elution of digoxin and digitoxin (Stahl, 1967), and chloroform/methanol/H_2O (65 : 30 : 5) was used for ouabain (Kuschinsky, Lüllmann & van Zwieten, 1968). The radioactive spots were detected by means of a radiochromatogram scanner (Packard, Model 7201). The R_F values of the pure tritiated glycosides and some of the non-labelled metabolites were determined in both solvents and compared with the peaks obtained upon chromatography of the extracts. The non-radioactive metabolites used for reference were made visible by means of colouring with anisaldehyde sulphuric acid reagent (0·5 ml of anisaldehyde in 50 ml of acetic acid plus 1 ml of sulphuric acid: Stahl, 1967). For details see Kolenda, Lüllmann & Peters (1971).

Compounds and reagents

Tritiated ouabain, digoxin, and digitoxin were obtained from New England Nuclear Corp. The compounds were randomly labelled. The specific activities were: ouabain, 20 mCi/mg; digoxin, 11·5 mCi/mg; digitoxin, 7·5 mCi/mg.

For the chromatographic analysis and identification of the metabolites the following pure compounds supplied by Boehringer-Mannheim were used: digitoxin, digoxin, digitoxigenin, digitoxigenin-mono-digitoxoside, digioxigenin-bis-digioxoside, digoxigenin-mono-digitoxoside, digoxigenin-bis-digitoxoside. Silica Gel G according to Stahl (1967) was obtained from Merck-AG (Darmstadt). The ratio lactate/pyruvate was determined with the Biochemica-Test-Combination from Boehringer-Mannheim.

Results

Plasma concentration, biliary excretion and uptake by liver of the three tritiated cardiac glycosides

For each set of experiments the time course of the changes of the total radioactivity was determined disregarding the possible occurrence of metabolites.

Three different concentrations of ^{3}H-ouabain were used: 57 μg, 11·5 μg and 1·58 μg/250 ml of perfusion fluid (0·4 μmol, 0·8 μmol, 0·012 μmol respectively). The plasma concentration decreased to about 60% of the initial value independent of

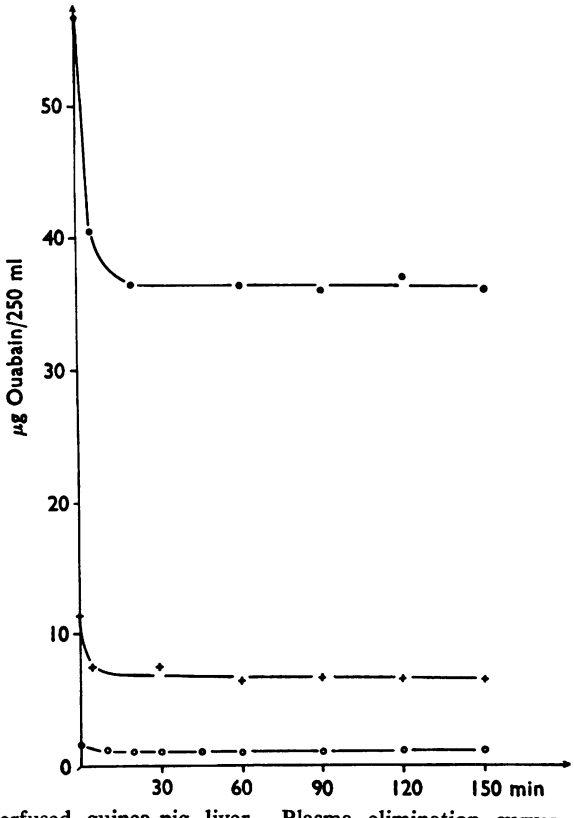

FIG. 1. Isolated perfused guinea-pig liver. Plasma elimination curves for three different concentrations of ^{3}H-ouabain. They are from above downwards: 57 μg/250 ml; 11·4 μg/250 ml; 1·58 μg/250 ml. Ordinate, μg ouabain/250 ml of perfusion fluid. Abscissa, time of perfusion in minutes. The points represent the means of three different experiments.

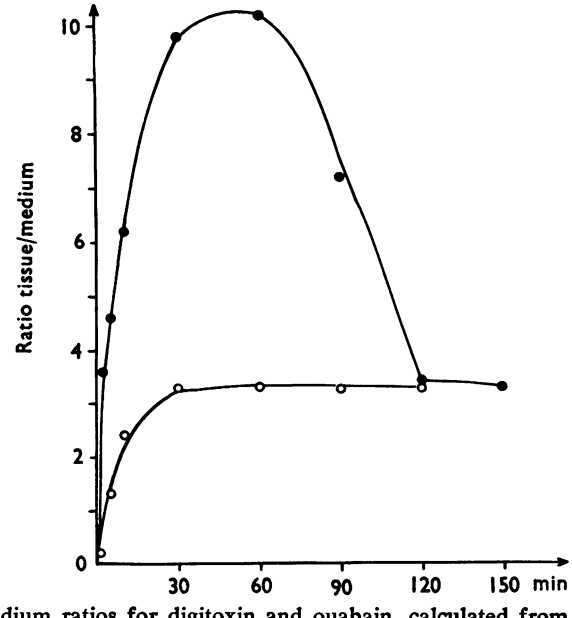

FIG. 2. Tissue/medium ratios for digitoxin and ouabain, calculated from plasma elimination and biliary invasion curves. Upper curve, digitoxin; lower curve, ouabain. Ordinate: T/M. Abscissa, time in minutes. The points represent the means of six different experiments.

the plasma concentrations within about 30 min (Fig. 1). As little as 0·5% of the administered amount could be recovered from the bile.

At each plasma concentration the tissue/medium (T/M) ratio was about 3 (w/v), that is the concentration of the drug in the liver was three times that in the perfusate (Fig. 2). The value remained constant throughout the experiment. The fact that the tissue/medium ratio did not decrease with increasing ouabain concentrations

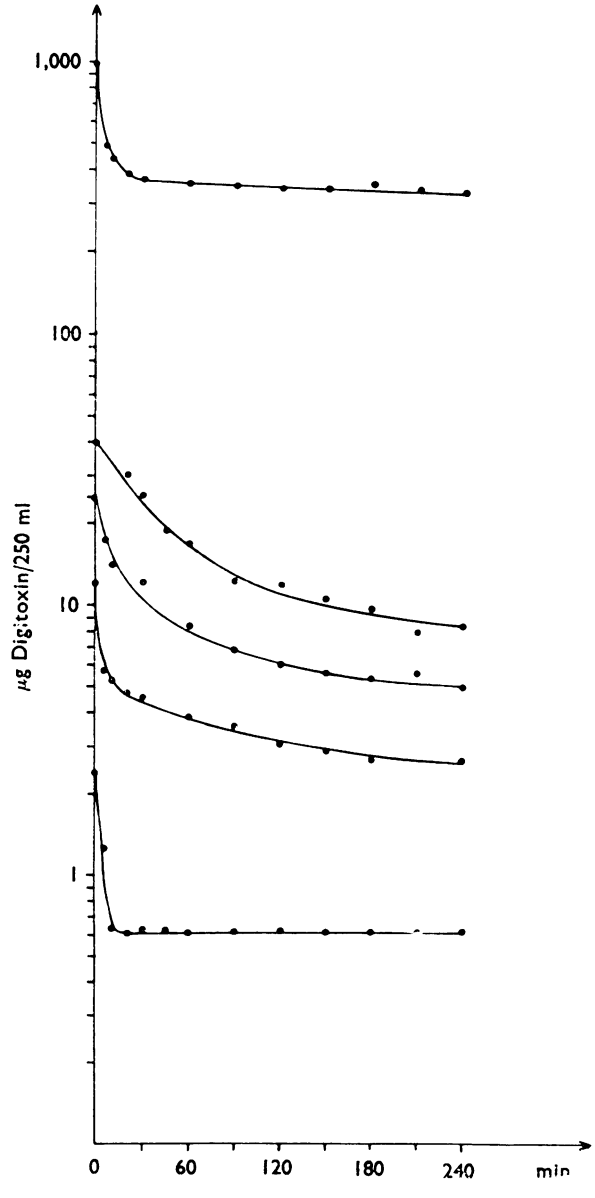

FIG. 3. Isolated perfused guinea-pig liver. Plasma elimination curves for different concentrations of ^{3}H-digitoxin. Curves from above downwards represent the following concentrations: 1,000 µg, 40 µg, 25 µg, 11 µg and 2·3 µg/250 ml. Ordinate, µg digitoxin/250 ml of perfusion fluid (log). Abscissa, time of perfusion in minutes. Each point of the curves represents the means of at least two different experiments.

indicates that the saturation of an uptake mechanism had not been reached in the concentration range involved.

Concentrations of ^{3}H-digitoxin, ranging betwen 2·3 μg and 1,000 μg/250 ml of perfusion fluid were used thus yielding a concentration range of 500-fold. The plasma elimination curves for all concentrations of digitoxin are shown in Figs. 3 and 4. Figure 3 shows the absolute concentrations of the drug measured at 30 min intervals over 4 h, while in Fig. 4 the same measurements are depicted as a percentage of the administered drug doses. In the lower dose range (10–50 μg/250 ml of perfusion fluid) the plasma concentration decreased to 20% of the initial value, whereas with the highest dose used (1,000 μg/250 ml) the residual concentration in plasma amounted to 50% of the initial value (Fig. 4).

In some experiments a second, identical dose of digitoxin was added to the perfusion fluid at a time when the initial plasma concentration had fallen to approximately 20%. As shown in Fig. 5, the plasma concentration measured 5 h after the first and 2 h after the second administration was 40% of the second dose given. In other words the final plasma concentration was the same whether the total amount was applied by a single administration at the beginning of the experiment, or was divided into two consecutive doses.

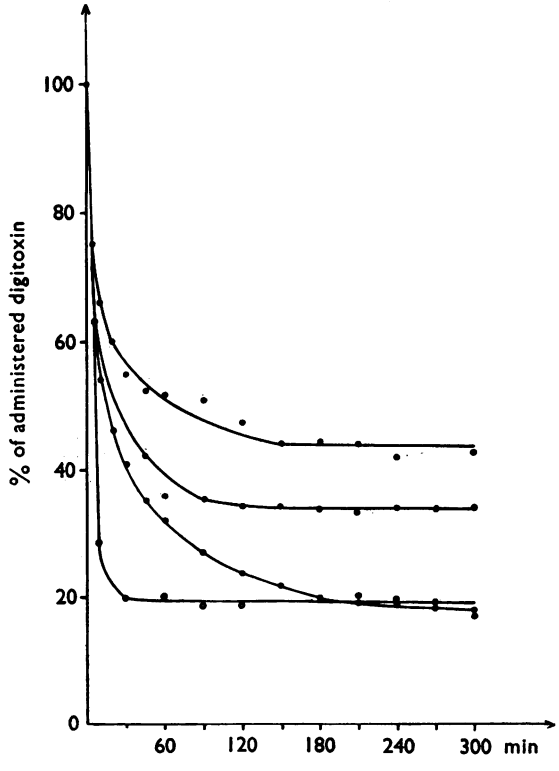

FIG. 4. Plasma concentration as percentage of the administered digitoxin dose. This figure shows the different elimination of low, medium, and high concentrations. Ordinate, plasma concentration as a percentage of the administered drug concentration. The curves from above downwards represent the following administered concentrations: 1,000 μg/250 ml, 5000 μg/250 ml, 11–100 μg/250 ml and 2·3 μg/250 ml. Abscissa, time in minutes. Each point of the curves represents the means of at least two different experiments.

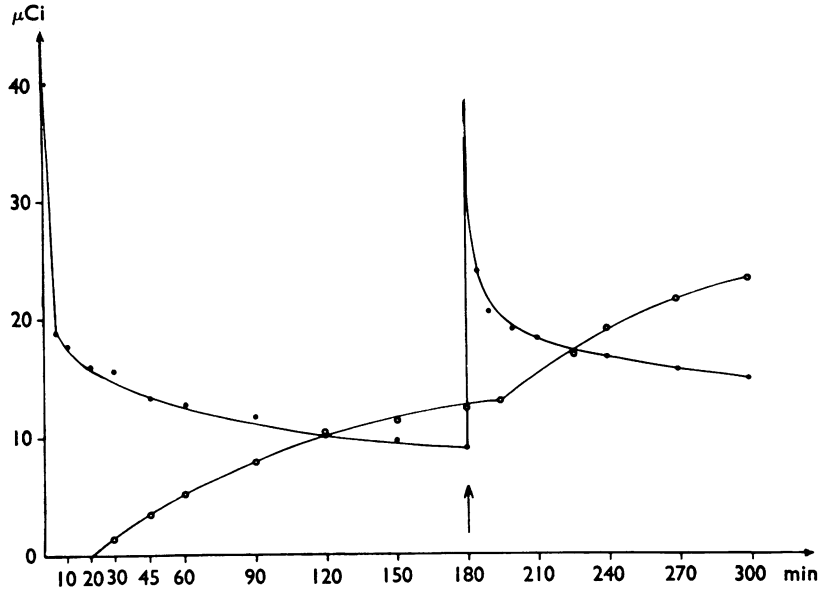

FIG. 5. Plasma elimination curves and biliary invasion curves after repeated addition of ^{3}H-digitoxin to the perfusion fluid. Ordinate, radioactivity in the plasma and bile (μCi). Abscissa, time in minutes. Upper curve, plasma content of radioactivity. Lower curve, radioactivity, excreted via the bile. ↑, Repeated addition of ^{3}H-digitoxin. The points of the curves represent the mean values from two different experiments.

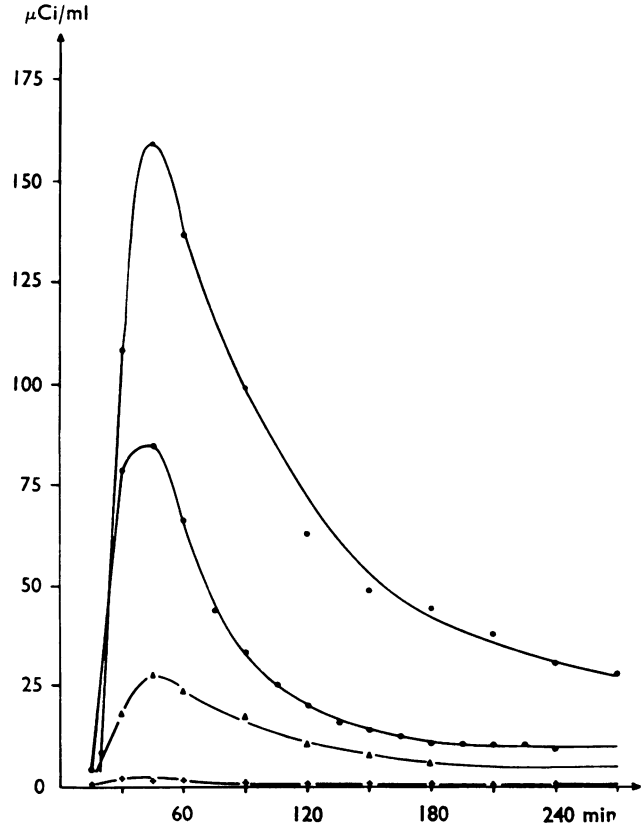

FIG. 6. Biliary excretion of digitoxin by the isolated liver. Biliary concentration of radioactivity after four different concentrations of digitoxin included in the perfusion fluid. Ordinate, μCi/ml of bile. Abscissa, time in minutes. Means of at least two experiments. The curves from above downwards represent the following administered concentrations of digitoxin: 150 μg, 70 μg, 25 μg and 2·3 μg/250 ml.

Figure 6 shows the time course of the excretion of radioactivity in the bile after various doses of digitoxin. The glycosides appeared in the bile fluid after a time lag of 15–20 min and reached a maximum value 40–60 min after administration of the drug. The biliary concentration of radioactivity was closely correlated with the concentration of the drug in the perfusate (Fig. 6). The maximal value obtained was 500 times higher than that measured simultaneously in the plasma. The total excretion of radioactivity in the bile was calculated by multiplying the biliary concentrations by the corresponding bile volumes. These figures were expressed as a percentage of the administered doses and plotted against time as shown in Fig. 7. The amount of drug excreted depended upon the plasma concentration of ^{3}H-digitoxin. The cumulative curves flattened after 3 h as biliary excretion diminished with decreasing plasma concentrations. This holds true for low and medium concentrations of digitoxin. (Fig. 4).

The total amount of radioactive material in the bile and in the plasma measured at any time did not add up to the dose originally administered, indicating the existence of a third compartment of distribution, namely the liver itself. It was therefore necessary to calculate the uptake by the liver by subtracting of the total plasma and bile content at a given time from the originally administered dose. The calculated figures for the hepatic compartment could be checked at the end of each experiment by determining the radioactivity of the liver. The calculated and measured values, completely agreed. As shown in Fig. 2 the T/M ratios for the liver were dependent on the perfusion time. Within the first 2 h of perfusion the ratios increased to about 10 and then decreased to as low as 3.

No marked differences were found between digoxin and digitoxin as far as plasma elimination, biliary excretion, and uptake by liver are concerned (cf. Figs. 2 and 8).

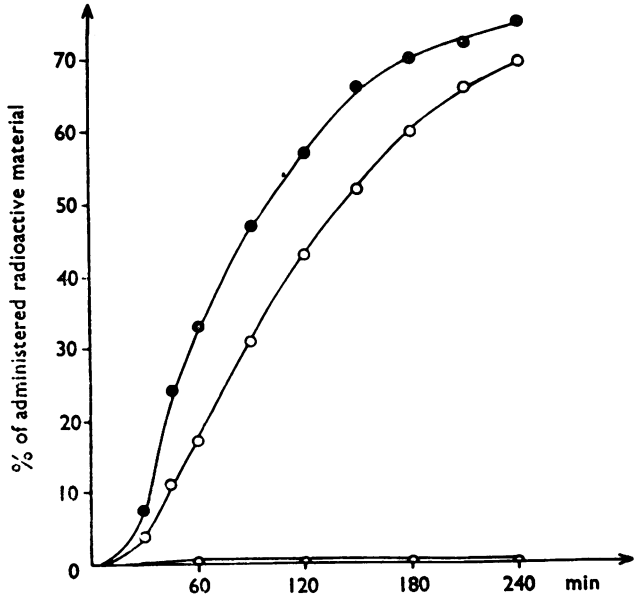

FIG. 7. Totally excreted radioactivity in the bile after addition of the glycosides to the perfusion medium. Ordinate, % of the administered drug concentration. The curves from above downwards represent the following perfusion concentrations: 2·3 μg digitoxin/250 ml, 1·8 μg digoxin/250 ml, and 1·58 μg ouabain/250 ml. The points represent the means of at least three different experiments.

Kinetics

Graphical analysis was carried out in order to define more exactly the kinetics of elimination from the plasma and entry into the bile (Dost, 1968).

The plasma elimination of ouabain could be described by a simple exponential function $y=a.e^{-k_2t}$ (a=initial concentration in the plasma ; y=plasma concentration at time t ; k_2=elimination constant). The values thus obtained for k_2 and $t_{0.5}$ were 0·01 min^{-1} and 7 min, respectively.

However, the elimination curves of digitoxin were characterized by a hyperbolic function of the general type $y=a-\frac{x\ t}{b+t}$ where x=fraction maximally eliminated, b=time at which a fraction $x/2$ is eliminated.

The transition of digitoxin and digoxin into the bile also follows a simple exponential function described by the equation $y=a\ (1-e^{-k_1t})$ (k_1=invasion constant). Regarding the time lag of biliary excretion, the k_1 and $t_{0.5}$ values of the biliary invasion of digitoxin yielded 0·017 min^{-1} and 40 min, respectively.

Chromatographic analysis

The results obtained by thin layer radiochromatography of the plasma, liver, and bile extracts are communicated as far as they are pertinent to the data described in this paper.

Ouabain was not metabolized by the isolated perfused guinea-pig liver, since no metabolites could be detected in the plasma, the liver tissue or the bile fluid. Only polar metabolites of digoxin were excreted in the bile, some residual pure digoxin being present as well. A similar pattern was found in the plasma. Digitoxin, however, was rapidly and completely metabolized by the liver. Polar metabolites were recovered from the bile, no unchanged digitoxin being left. Also in the plasma most of the radioactive material originated from polar metabolites.

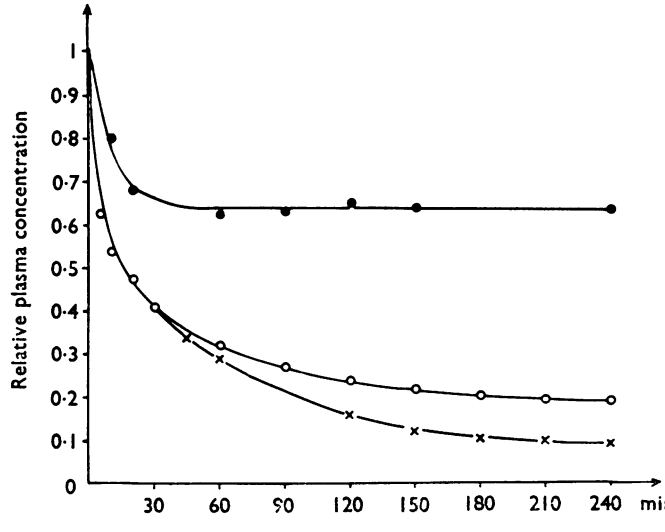

FIG. 8. Comparison of the plasma elimination curves for the three cardiac glycosides. Curves from above downwards are for ouabain, digitoxin and digoxin. Ordinate, plasma concentration relative to the administered drug dose (=1). Abscissa, time in minutes.

Discussion

Marked differences in the plasma elimination, uptake by liver and biliary excretion of ouabain, digoxin and digitoxin can be demonstrated in the isolated perfused liver system. The different lipid solubilities of these cardiac glycosides strongly influence their absorption, protein binding, tissue distribution, and renal excretion. Moreover, the lipid solubility determines the different rates of accumulation by isolated organs and in the intact organism (Brodie, Kurz & Schanker, 1960; Brodie and Maickel, 1963; Haass & Peters, 1970; Harmann & Repke, 1964; Kuschinsky *et al.*, 1968).

Our results emphasize that the uptake of the cardiac glycosides into the liver and their elimination by bile are determined by their physical and chemical properties. It may be assumed that the liver contains two different compartments (Fig. 9): (1) an aqueous compartment which is, with respect to the degradation of cardiac

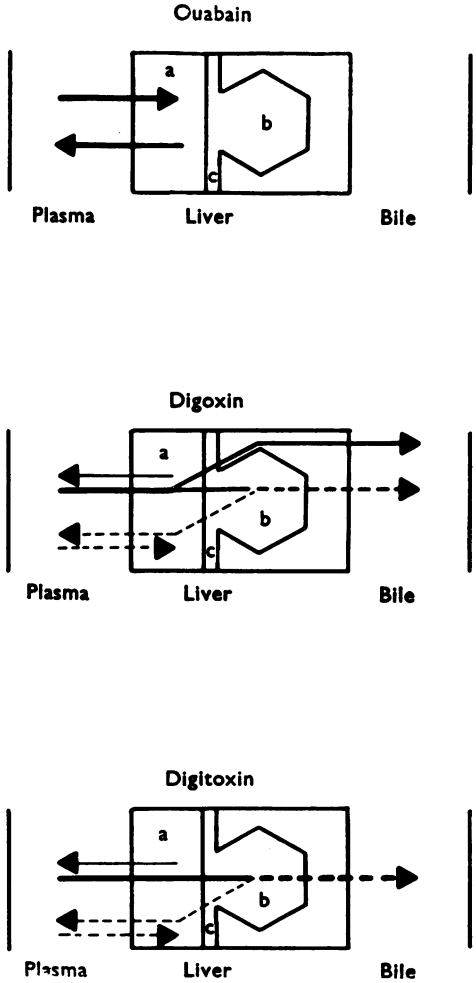

FIG. 9. Schematic presentation of the distribution of ouabain, digoxin and digitoxin between plasma, liver, and bile in the isolated, perfused guinea-pig liver system. Solid line, pure glycosides; broken line, metabolites; a, metabolically inactive compartment; b, metabolically active compartment; c, lipid barrier.

glycosides, metabolically inactive. It might for instance consist of the extracellular space, of the cell surface, and some particular part of the cell plasma. It does not have direct contact with the bile-capillaries and is separated from the main part of the cell by a lipid barrier. (2) A cellular compartment containing a lipid phase which includes the metabolically active sites with respect to degradation of the cardiac glycosides. The transition of compounds from the first to the second compartment depends upon their lipid solubility.

This hypothetical model would explain why the hydrophilic glycoside, ouabain, was eliminated from the plasma by simple distribution into the first compartment, yielding an equilibrium of 60:40 between the plasma and the liver. Penetration into the bile is impossible owing to the lipophobic nature of ouabain. This is supported by the following facts: (1) equilibrium is reached after 45 min; (2) the plasma concentration decreases according to a simple exponential function; (3) the T/M ratio is relatively low in comparison with those for digitoxin and digoxin; and (4) the T/M ratio remains constant. The present T/M ratio is still surprisingly high as compared with the ratio found for the distribution of ouabain between blood and isolated atrial tissue and Tyrode solution and isolated atrial tissue (Dutta, 1968; Kuschinsky *et al.*, 1968; Lüllmann, Peters & van Zwieten, 1969; Lüllmann & van Zwieten, 1969). Basic differences in the cellular structures of the two tissues may be the determining factors. On the other hand a T/M ratio of 33 to 117 has been found in rat liver, which is able to excrete ouabain into the bile (Kupferberg & Schanker, 1968).

Digoxin and digitoxin differ from ouabain in their lipid solubility. Eighty per cent of digoxin and digitoxin were elminated from the perfusion fluid within 4 h obeying a hyperbolic function. The drugs were chiefly excreted in the form of polar metabolites in the bile. Their lipophilic nature allows penetration into the metabolically active lipid compartment where conversion can occur. Digitoxin was completely metabolized (Fig. 9) whereas part of the digoxin was excreted in the unchanged form into the bile (Fig. 9). This difference may be caused by the different lipid solubility of the two glycosides as well as by their different liability to metabolic degradation. Accumulation of the hydrophilic metabolites in the aqueous bile fluid may simply be caused by a partition based upon their hydrophilic character or by more complicated processes. The assumption presented in Fig. 9 is further supported by the finding that the T/M ratio of the digitalis glycosides changes throughout the perfusion: in the first 2 h it reaches a value of about 10 and subsequently decreases to approximately 3, thus indicating a continuous increase of the ratio hydrophilic metabolites/lipophilic glycosides in the plasma.

The polar metabolites found in the plasma must have been redistributed from the liver into the blood since it has been shown that a conversion of these glycosides does not occur in the blood itself (Kolenda *et al.*, 1971; Lüllmann *et al.*, 1969). The newly appearing metabolites are distributed between plasma and liver in the same way as ouabain (Fig. 9) and are responsible for the rest of the plasma radioactivity. The metabolites reach a T/M ratio of about 3 and cannot be eliminated via the liver into the bile, thus resembling ouabain once more. These polar metabolites are probably conjugates of the aglycones with glucuronic and sulphuric acids (Hermann & Repke, 1964). The hyperbolic plasma elimination curves of digoxin and digitoxin are probably composed by the interaction of the following factors: at the beginning of the perfusion a more rapid elimination occurs as compared with ouabain as a

result of the lipophilic nature of these digitalis glycosides, and also because of their rapid biliary excretion. After longer perfusion times the increasing concentration of the polar metabolites will cause a flattening of the elimination curve.

Our results lead to the following conclusions: the uptake into the 'metabolically active' compartment of the liver cells depends upon the lipophilic character of the cardiac glycosides and is governed by a passive diffusion process. In this particular compartment the conversion into polar metabolites takes place. The newly formed hydrophilic metabolites are distributed into the bile and the perfusion medium. The hydrophilic agent ouabain can neither enter the 'metabolically active' cell compartment nor penetrate into the bile.

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