Equilibrium-Dialysis Studies of the Interaction between Cholic Acid and 100000g-Supernatant Preparations from the Rat Liver

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(Received 12 December 1975)

1. The binding of cholic acid to 100000g supernatants from rat livers was investigated by equilibrium dialysis and gel-exclusion chromatography.
2. Supernatants were found to contain at least two classes of binding site for cholic acid.
3. These receptor molecules are probably proteins since incubation with proteolytic enzymes resulted in complete loss of cholic acid binding.
4. Supernatants were added to columns of Sephadex G-75, and two groups of fractions were shown to bind cholic acid. One of these contained low-affinity binding sites and the other contained both low- and high-affinity binding sites.
5. Feeding cholestyramine had no effect on cholic acid binding.
6. Increased cholic acid binding occurred after injection of phenobarbitone. There was an increase in the amount of the low-affinity component but no change in the high-affinity component.
7. The dissociation constants of the binding of cholic acid suggest that the binding proteins may be involved in bile acid transport.

The maintenance of the enterohepatic circulation of bile acids by the liver requires the removal of bile acids from the portal blood, their transport through the hepatocyte and finally biliary excretion. Bile acid synthesis appears to be linked to this circulation, since the formation of an external biliary fistula or the feeding of the anion-exchange resin, cholestyramine, leads to induction of cholesterol 7 α -hydroxylase (Boyd *et al.*, 1969; Shefer *et al.*, 1970; Boyd *et al.*, 1973) and an increase in the rate of synthesis of bile acids from cholesterol (Eriksson, 1957; Percy-Robb & Boyd, 1970).

Although the mechanism whereby the bile acids pass through the hepatocyte and apparently affect the activity of cholesterol 7 α -hydroxylase is not known, analogy with present knowledge of hepatic transport of many compounds and also of steroid-induced enzyme synthesis suggests that proteins present in liver 100000g supernatants might be involved. Thus it has been proposed that the transport of many compounds from the blood into the liver involves a group of cytoplasmic proteins, X, Y (ligandin) and Z (Levi *et al.*, 1969; Levine *et al.*, 1971; Litwack *et al.*, 1971). Further, the induction of hepatic enzymes by steroid hormones appears to require initial binding of the steroid to a cytoplasmic receptor protein (Rousseau *et al.*, 1972; Thompson & Lippmann, 1974). Such steroid-protein complexes can apparently interact with nuclear receptors and so affect the transcriptional process (O'Malley *et al.*, 1972; Lippmann & Thompson, 1973). The bile acids possess the perhydrocyclopentanophenanthrene ring structure and since over 50% of the bile acids in whole

homogenates of rat liver are found in the 100000g supernatant (Okishio & Nair, 1966), bile acids might also be bound by supernatant proteins. Binding might aid the passage of bile acids through the cytoplasm, to either the bile ducts for excretion or the nucleus.

We have therefore investigated the binding of cholic acid (3 α ,7 α ,12 α -trihydroxy-5 β -cholan-24-oic acid) to hepatic 100000g supernatants by equilibrium dialysis and gel-exclusion chromatography.

Materials and Methods

Chemicals

Cholic acid (Maybridge Chemical Co., Tintagel, Cornwall, U.K.) was recrystallized four times from methanol, and a 3.6mM solution was made up in ethanol/water (1:20, v/v). At monthly intervals the purity of this solution was checked by placing a portion (10 μ l) on a silica-gel 60 plate (Merck A.-G., Darmstadt, Germany) and developing in either trimethylpentane/di-isopropyl ether/acetic acid (2:1:1, by vol.) (Hamilton & Muldrey, 1961) or in chloroform/methanol/acetic acid/water (30:10:3:2, by vol.) (Kelly & Doisy, 1964). A single spot corresponding to cholic acid was seen after spraying the plate with anisaldehyde/acetic acid/conc. H₂SO₄ (1:100:2, by vol.) (Kritchevsky *et al.*, 1963).

[24-¹⁴C]Cholic acid (51 Ci/mol) was purchased from The Radiochemical Centre, Amersham, Bucks., U.K. A working solution (10mCi/l) was prepared in ethanol and its purity checked by t.l.c. as described above. After development, the plate was dried at 100°C for

20 min, left to cool and divided into 18 horizontal 1 cm strips, which were scraped into plastic scintillation vials for the determination of radioactivity. In all cases 99% of the radioactivity on the plate was found in a single spot corresponding in position to cholic acid.

Proteolytic enzyme preparations were from *Streptomyces griseus* (Pronase; Koch-Light Laboratories, Colnbrook, Bucks., U.K.) and from *Bacillus subtilis* (Nagarse; Teikoku Chemical Industry Co., Osaka, Japan). Human albumin was obtained from the Protein Fractionation Centre, Scottish National Blood Transfusion Association, Edinburgh, Scotland, U.K., as a 200 g/l solution containing octanoic acid and mandelic acid. Polyethylene glycol (mol.wt. 25000) was obtained from Union Carbide, Southampton, Hants., U.K. The anion-exchange resin, cholestyramine (main constituent, polystyrene trimethylbenzyl ammonium chloride), was purchased as Cuemid from Merck, Sharp and Dohme, West Point, PA, U.S.A.

Animals

Male Wistar rats (300–330 g body wt.) fed *ad libitum* were used. In the cholestyramine-feeding experiments animals were maintained for 4 days on a soft diet (70% wholemeal flour, 25% skimmed-milk powder, 5% brewer's yeast, by wt.) to which was added 4% (w/w) cholestyramine. To confirm induction of cholesterol 7 α -hydroxylase, enzyme activity was assayed by the method of Boyd *et al.* (1973). Sodium phenobarbitone (25 mg) in sterile water (0.5 ml) was administered subcutaneously for 7 successive days and the animals were killed on day 8.

Preparation of supernatant fractions

Rats were anaesthetized with diethyl ether and the liver was perfused *in situ* via a portal-vein cannula with approx. 20 ml of ice-cold phosphate/NaCl buffer containing sucrose (250 mM). Phosphate/NaCl buffer was prepared by adjusting a solution of Na₂HPO₄ (20 mM) containing NaCl (100 mM) to pH 7.4 with KH₂PO₄ (20 mM) containing NaCl (100 mM). When seen to be free of blood the livers were removed, gently dried, weighed and homogenized with 2 vol. of the same buffer in a Teflon-glass homogenizer. The homogenate was centrifuged (2500 g; 4°C; 15 min), the cellular debris discarded and the supernatant re-centrifuged (100000 g; 4°C; 60 min). The resulting supernatant fraction was harvested after removal of the lipid layer and dialysed overnight at 4°C. This initial dialysis was performed to remove components of the supernatant with mol.wt. less than about 14000 and to decrease the content of endogenous cholic acid in the supernatant. T.l.c. (Hamilton & Muldrey, 1961) of portions (50 μ l)

of dialysed supernatants showed no detectable cholic acid. Since the sensitivity of the t.l.c. method is approx. 2.5 nmol of cholic acid, the concentration of cholic acid in the dialysed supernatant must have been less than 50 μ M. Before equilibrium dialysis against cholic acid the dialysed supernatant was further diluted fivefold. The contribution of endogenous cholic acid was therefore negligible.

Equilibrium dialysis

These studies were carried out in Teflon cells (Dianorm, Fisons Ltd., Crawley, Sussex, U.K.). Each cell consisted of two Teflon half-cells (volume 1.36 ml, membrane surface area 4.52 cm²) separated by a membrane (Spectrapor, Spectrum Medical Industries, Los Angeles, CA, U.S.A.) of mol.wt. cut-off 12000–14000. Five such cells were arranged in an assembly that rotated gently (10 turns/min) in a water bath (37°C).

In preliminary experiments cholic acid was dialysed against buffer to determine the time required to reach an equilibrium concentration. Phosphate/NaCl buffer (1 ml) containing [¹⁴C]cholic acid (100 nCi) and various amounts of unlabelled cholic acid (15–550 nmol) was injected into one half-cell and phosphate/NaCl buffer (1 ml) was injected into the other half-cell. During dialysis, samples (10 μ l) were taken through ports from both sides of the membrane and the radioactivity was determined. Equilibrium was reached after 120 min.

When [¹⁴C]cholic acid was dialysed against hepatic supernatants (1 ml, approx. 5 g of protein/l), comparison of the total amount of radioactivity used with the amounts found in the half-cells during dialysis showed that more than 98% of the radioactivity was recovered. The small loss of radioactivity was probably due to binding to the dialysis membrane. In every instance there was no significant difference between the amounts of ligand bound at 150 and 180 min suggesting that equilibrium had been attained by 150 min and that the binding capacity of supernatants was unchanged during the dialysis period.

In experiments to determine the nature of the binding components, portions of 100000 g supernatants were incubated with proteolytic enzymes. A supernatant was divided into three portions (3 ml) and each portion was diluted with an equal volume of phosphate/NaCl buffer. One portion of diluted supernatant was used as a control solution, to the second portion was added 10 mg of the enzyme preparation from *Strep. griseus* (Pronase) and to the third portion was added 10 mg of the preparation from *B. Subtilis* (Nagarse). After the enzymes had been dissolved the solutions were placed in separate dialysis sacs and dialysed overnight against phosphate/NaCl buffer (4°C); the protein concentration

of each of the three solutions was then measured. In the control solution the protein concentration was 16 g/l, in the Pronase-treated solution 1 g/l, and in the Nagarse-treated solution 2 g/l. Each solution was then diluted with an equal volume of phosphate/NaCl buffer, and portions (1 ml) were dialysed against phosphate/NaCl buffer (1 ml) containing [^{14}C]cholic acid (100 nCi) and cholic acid (150 nmol).

Dialysis of known amounts of cholic acid against dilutions of a 100000g supernatant showed that the amount of cholic acid bound was inversely proportional to the supernatant dilution. To check the reliability of the system, the parameters were determined for the binding of cholic acid (15–130 nmol per half-cell) to human albumin (4.8–6.8 g/l). The values for the dissociation constant (71 μmol of cholic acid/l) and the binding capacity (25 μmol of cholic acid/g of protein) were similar to those obtained by Burke *et al.* (1971) for the primary cholic acid-binding sites of human albumin.

Sephadex column chromatography

Gel-filtration experiments were performed on columns (30 cm $\times$ 2 cm) of Sephadex G-75 equilibrated with phosphate/NaCl buffer. The columns were eluted at 4°C with the same buffer, the flow rate being 24 ml/h and the fraction volume 3.5 ml. The void volume, determined by using Dextran Blue, was 50 ml.

The binding of cholic acid to supernatant preparations was studied by mixing [^{14}C]cholic acid (1 μCi ; 20 nmol) with samples of supernatant (4 ml), and immediately adding the mixture to the Sephadex G-75 column. The radioactivity in portions (100 μl) of each fraction was measured to determine the amount of [^{14}C]cholic acid present in fractions containing protein.

The presence of the anion-binding proteins, X, Y and Z described by Levi *et al.* (1969), was demonstrated by adding bromosulphthalein (2.5 mg) and portions (2 ml) of supernatant to the Sephadex G-75 column and measuring the amount of bromosulphthalein in the protein-containing fractions. In some experiments the column fractions containing X protein (total volume 24.5 ml) and Y protein (total volume 28 ml) were combined separately and each was dialysed against cholic acid. The protein concentration in fractions containing X protein was sufficiently high (5–6.5 g/l) to allow immediate dialysis. In fractions containing Y protein, however, the protein concentration was low and the pooled fractions were first concentrated by dialysis at 1°C against polyethylene glycol (300 g made up to 1500 ml with water). When the volume of the protein-containing solution was decreased by 50% (approx. 8 h) it was removed, placed in a fresh dialysis sac and dialysed overnight at 4°C against phosphate/NaCl

buffer. The final solution (protein concentration 4.3–5.3 g/l) was dialysed to equilibrium against cholic acid.

Analytical methods

Total protein concentrations in liver supernatants were measured by the method of Lowry as described by Herbert *et al.* (1971), with bovine albumin (Armour Pharmaceutical Co., Chicago, IL, U.S.A.) as the standard. Protein concentrations in the fractions from the Sephadex G-75 column were calculated from the extinction values at 280 nm. Bromosulphthalein was measured by the extinction values at 580 nm after the addition of NaOH (100 μl ; 10 M) to each column fraction.

At the end of experiments (180 min), samples were regularly taken from both sides of the dialysis membrane and the integrity of both the [^{14}C]cholic acid and the unlabelled cholic acid was confirmed by t.l.c.

Radioactive counting was performed by using a toluene-based scintillant [2,5-diphenyloxazole, 7 g; 1,4-bis-(4-methyl-5-phenyloxazol-2-yl)benzene, 0.6 g; naphthalene, 150 g; 2-ethoxyethanol, 300 ml; toluene to 1 litre]. Samples (10 μl) of diffusate and non-diffusible material were counted with a standard deviation of less than 0.5%.

Analysis of results

The binding of a ligand to a protein where all the binding sites are identical and independent of each other is described by a rectangular hyperbola. The characteristic parameters of binding are K (dissociation constant) and N (mol of ligand bound/mol of protein). These parameters can be estimated by determining the amount of ligand bound per mol of protein (r) at different concentrations of free ligand (f). Values for K and N can be calculated from the linear regression of $1/r$ on $1/f$. If, however, two proteins bind the ligand, one with a relatively high affinity (K_1 , N_1) and the other with a relatively low affinity (K_2 , N_2) the binding process is described by the equation:

$$r = \frac{N_1 \cdot f}{K_1 + f} + \frac{N_2 \cdot f}{K_2} \quad (1)$$

provided that $f \ll K_2$. This equation was fitted to the experimental data by the method of least squares to give values of the constants K_1 , N_1 and N_2/K_2 . The non-linear equation was solved by the method of Davidenko as described by Broyden (1969).

Results

Untreated animals

Fig. 1 shows how the amount of cholic acid bound by the whole supernatant increased with the concentration of the ligand. The broken line, which

Fig. 1. Concentration-dependence of cholic acid binding by hepatic supernatants from untreated rats

The binding of cholic acid to hepatic 100000g supernatants was studied by equilibrium dialysis as described in the Material and Methods Section. ----, Best-fit rectangular hyperbola; —, sum of a straight line and rectangular hyperbola described by the equation $r = N_1 f / (K_1 + f) + N_2 f / K_2$ (see the text). ●, Experimental points.

Table 1. Values for the parameters of binding of cholic acid to rat liver supernatant preparations

Cellular 10000g supernatants were prepared from liver homogenates taken from untreated animals and from animals given a diet containing 4% (w/w) cholestyramine and also from rats given subcutaneous injections of phenobarbitone. Supernatants from untreated animals were eluted from Sephadex G-75 columns, and the X and Y fractions were collected separately. The parameters of binding of cholic acid to supernatants and fractionated supernatants were determined by equilibrium dialysis.

	K_1 (μM)	N_1 ($\mu\text{mol/g}$)	N_2/K_2 (ml/g)
Untreated animals (nine animals)	14	0.44	14
Cholestyramine-fed animals (seven animals)	19	0.58	11
Phenobarbitone-treated animals (six animals)	34	0.53	22
X fractions (four animals)	499	0.78	11
Y fractions (ten animals)	6	0.76	19

represents the best-fit rectangular hyperbola calculated from a double-reciprocal plot, does not fit the experimental results. Consequently the results cannot be explained in terms of a single binding protein with identical non-interacting sites. Inspection of Fig. 1 suggests that the experimental points could be described by the sum of a straight line and a rectangular hyperbola as would occur if two binding proteins were present, one with K approx. $30\mu\text{M}$ and the other the $K > 300\mu\text{M}$. The continuous line (Fig. 1) shows the way in which such an equation corresponds to the experimental data; there is good agreement between this line and the experimental points. The

best-fit values of the parameters were $K_1 = 14\mu\text{M}$, $N_1 = 0.44\mu\text{mol/g}$, $N_2/K_2 = 14\text{ml/g}$ (Table 1).

When 100000g supernatants were treated with the proteolytic enzyme preparations (Pronase or Nagarse) the control solution bound the expected amount of cholic acid (Fig. 1), whereas the solutions incubated with the proteolytic enzymes bound no cholic acid. This suggests that protein is an integral part of the cholic acid-binding component.

To determine whether more than one component in the 100000g supernatant was capable of binding cholic acid, two series of experiments were performed. First, a mixture of supernatant and [^{14}C]cholic acid was eluted from a column of Sephadex G-75; the elution pattern (Fig. 2) shows that little radioactivity was associated with the protein-containing fractions, suggesting that any [^{14}C]cholic acid-protein complex dissociated during passage through the column. More than 95% of the radioactivity was recovered in the eluate. Secondly, portions of 100000g supernatant were eluted from columns of Sephadex G-75, and the fractions described by Levi *et al.* (1969) as X and Y were identified, collected and combined separately (Fig. 2). The binding characteristics of these fractions are shown in Figs. 3(a) and 3(b) and are summarized in Table 1. The combined X fraction only contains binding proteins with low affinities, whereas the pooled Y fraction, besides containing a protein with low affinity, also contains one with a high affinity. The whole supernatant therefore contains at least two distinct components that can bind cholic acid.

Cholestyramine-treated animals

After feeding rats for 4 days on a diet containing 4% (w/w) cholestyramine, microsomal cholesterol 7α -hydroxylase activity was increased 8–10-fold. The unfractionated supernatants gave a binding plot that was virtually indistinguishable from that in Fig. 1. The parameters were similar to those found for the untreated animals (Table 1).

Phenobarbitone-treated animals

All animals given phenobarbitone had a greater liver weight (10.2–11.6g) than similar untreated animals (7.8–9.6g), but body weight was unchanged.

The concentration-dependence of binding was similar in form to that shown for untreated animals (Fig. 1) but differed from it in that, at any concentration, the supernatants from the phenobarbitone-treated animals bound more cholic acid per g of protein than did those from the untreated ones. The results (Table 1) indicate that treatment with phenobarbitone increased the amount of the low-affinity component but not that of the high-affinity one.

Mixtures consisting of the supernatant from phenobarbitone-treated animals plus either [^{14}C]-

Fig. 2. Elution pattern of liver supernatant, bromosulphthalein and [^{14}C]cholic acid

Liver 100000g supernatants (4.0 ml) were mixed with either bromosulphthalein (2.5 mg) or [^{14}C]cholic acid (1 μCi), placed on a Sephadex G-75 column and eluted at 4°C with sodium potassium phosphate buffer (20 mM; pH 7.4) containing NaCl (100 mM). Fractions (3.5 ml) were collected, and the radioactivity, protein concentration and bromosulphthalein absorbance were measured. $\square$, Radioactivity; $\circ$, protein concentration; $\blacktriangle$, bromosulphthalein absorbance.

Fig. 3. Concentration-dependence of cholic acid binding by X and Y fractions

Supernatants (100000g) were prepared from livers taken from untreated animals, and the X and Y fractions were isolated by Sephadex G-75 column chromatography. The binding of cholic acid to the X fraction (a) and to the Y fraction (b) is shown by the experimental points ($\circ$). —, Sum of rectangular hyperbolas and straight lines.

cholic acid or bromosulphthalein were eluted from columns of Sephadex G-75. No difference was found between the binding of [^{14}C]cholic acid or bromosulphthalein by supernatants from phenobarbitone-treated or untreated animals.

Discussion

Our experiments show that 100000g supernatants prepared from rat livers contain at least two components able to bind cholic acid. Although one pro-

tein was apparently not saturated by the amounts of cholic acid used, the other appeared to be tending towards saturation. Separation of supernatants into fractions containing the anion-binding proteins X and Y showed that although both fractions contained a component(s) with a low binding affinity, the Y fraction also contained a component with a higher binding affinity.

Equilibrium dialysis allows estimation of free and bound ligand concentrations without the use of

adsorbents. Although buffer solutions of relatively high ionic strength and relatively low macromolecule concentration are used to overcome the Donnan effect (Hughes & Klotz, 1956), this technique has two advantages over methods using agents such as talc or charcoal to separate free from bound ligand. First, since separation cannot be achieved instantaneously, the estimated amount of bound ligand must be less than that obtaining at equilibrium. Secondly, if the amount of free ligand is calculated from the difference between the amount of ligand bound and the total amount of ligand used, the results will be susceptible to the sort of systematic error and misinterpretation described by Swillens & Dumont (1975).

The proteins present in liver 100000g supernatants able to bind cholic acid were further studied by eluting supernatants from columns of Sephadex G-75 and separately collecting the X and Y fractions. Although X protein has been reported to bind anions non-specifically (Levi *et al.*, 1969), Y protein or ligandin (Litwack *et al.*, 1971), a protein with a molecular weight of approx. 40000, may be involved in the hepatic removal of anions such as bromosulphthalein from plasma (Levine *et al.*, 1971). Studying binding of cholic acid by these fractions can be criticized since the mechanism of bromosulphthalein excretion from hepatocyte into the bile duct appears to be different from that of bile acids (Heaton, 1972). Further, Levi *et al.*, (1969) have shown that taurocholic acid does not displace bound bromosulphthalein from Y protein. Work by Ketterer *et al.* (1975), however, indicated that purified ligandin can bind cholic acid with a dissociation constant of 1×10^{-4} M. We obtained a similar value for the dissociation constant of the binding between supernatants and cholic acid using a double-reciprocal plot, although, as shown in Fig. 1, this means of measuring K and N appears to be inappropriate for our results. Our Y fractions appeared to contain a protein with a high binding affinity as well as one with a low affinity. The X fractions only contained components of relatively low affinity. Results also suggest that the dissociation constants of the components in the whole supernatant have been altered by fractionation. This may be an apparent rather than a real effect, for two reasons. First, the constants K_1 and N_1 are difficult to determine precisely from our results and valid standard errors cannot be calculated for them; consequently a value for K_1 of $6 \mu\text{M}$ may not be significantly different from one of $14 \mu\text{M}$ (experience shows that values for N_2/K_2 can be estimated with greater confidence). Secondly, if a fraction contains several binding proteins with dissociation constants of within an order of magnitude they will not be distinguished from one another by our mathematical analysis. Consequently the values of the parameters in Table 1 are descriptive rather than definitive. A

further point is that the relatively high concentrations of cholic acid used in equilibrium-dialysis experiments might prevent the detection of a high-affinity binding protein present in low concentration in supernatants. No [^{14}C]cholic acid, however, was associated with protein in fractions from the Sephadex column, suggesting that liver supernatants do not contain a protein with high affinity for cholic acid.

To investigate a physiological role for the hepatic binding of cholic acid, rats were treated with cholestyramine or phenobarbitone. The first of these agents sequesters bile salts in the intestine, thus increasing the faecal excretion of acidic steroids (Grundy *et al.*, 1971). There is also induction of cholesterol 7α -hydroxylase, the rate-limiting enzyme in bile acid synthesis (Boyd *et al.*, 1973). If, as has been suggested, bile salts have a feedback inhibition effect on their own synthesis, analogy with other steroids (Rousseau *et al.*, 1973) suggests that normally interaction between nuclear receptors and steroid molecules bound to cytoplasmic proteins represses synthesis of cholesterol 7α -hydroxylase. Induction of the enzyme by the feeding of cholestyramine to animals might alter the amount of cytoplasmic protein available for binding, thus producing a change in the binding characteristics of supernatants for cholic acid. The cholic acid-binding capacity of these supernatants, however, was similar to that of supernatants prepared from untreated animals. The second agent used, phenobarbitone, has a number of effects on bile acid metabolism and has been used to treat patients with intrahepatic cholestasis (Stiehl *et al.*, 1972). Phenobarbitone can affect the activity of cholesterol 7α -hydroxylase, although this effect appears to be species-dependent (Shefer *et al.*, 1972). It seemed important to study the effects of phenobarbitone on cholic acid binding to the partially saturated protein in Y fractions since Reyes *et al.* (1971) have shown that administration of phenobarbitone to rats results in an increase in the concentration of Y protein and a simultaneous increase in the clearance of bromosulphthalein. In our experiments rats were given amounts of phenobarbitone similar to those administered by Reyes *et al.* (1971). The liver weights of our animals were significantly higher than those of untreated animals, but we were unable to show an increase in the bromosulphthalein-binding capacity of these supernatants. There was, however, an increase in the cholic acid-binding capacity of these supernatants, occurring only in the low-affinity component.

The physiological role of the cholic acid-binding proteins is unknown. They may be involved in the transport of cholic acid to the bile duct, in the regulation, at the nuclear level, of bile acid synthesis or in the storage of the bile acids within the liver. In this context the magnitude of the dissociation constants may be important. Presumably a component that

binds a ligand with a high affinity constant is an inefficient vehicle for transport, because the ligand (once bound) is unlikely to dissociate, whereas tight binding would be an expected property if the complex were to be active in the nucleus. If so, the magnitude of the dissociation constants for the binding of cholic acid to supernatant components are more in keeping with the first of these possibilities for they are approximately of the same order of magnitude as the concentrations of cholic acid in portal blood (Okishio & Nair, 1966; Cronholm & Sjövall, 1967). They are several orders of magnitude greater than the dissociation constants (2×10^{-10} M) reported by Higgins *et al.* (1973) for the association of the receptor protein-dexamethasone complex from hepatoma cells to isolated nuclei or for the dissociation constants of several steroids thought to affect the transcription process, and hepatoma-cell cytosol proteins (Rousseau *et al.*, 1973). The concept that the liver has a storage capacity for anions such as bromosulphthalein was introduced by Wheeler *et al.* (1960). Storage of bile acids is suggested by the demonstration that the maximum capacity for taurocholate uptake from plasma is considerably in excess of the maximum rate of secretion into bile (Glasinovic *et al.*, 1975). It is possible that this storage function involves bile acid-binding proteins similar to those described for cholic acid, particularly since treatment with phenobarbitone increases the size of the bile acid pool (Mok *et al.*, 1974) and the binding capacity of 100000g supernatants.

We thank Professor G. S. Boyd and Dr. A. Ryle for advice, and Dr. W. A. Telfer Brunton and Miss P. MacDonald for considerable help in these experiments.

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