BINDING OF CHOLIC ACID TO SOLUBLE PROTEINS FROM RAT LIVER

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1. Introduction

Bile acids are transported across the liver cell from blood to the bile canaliculus. The mechanisms of this transport have not yet been clearly defined. Uptake into the liver cell is a carrier-mediated process [1] involving a bile acid-binding receptor [2]. In addition, intracellular proteins capable of binding bile acids have been described. Strange et al. [3,4] have shown that bile acids bind to proteins present in high speed supernatant fractions from rat liver. Thus the transport of bile acids shows similarities to the transport of other organic anions. For example there is strong evidence that bilirubin transport is facilitated by the cytoplasmic binding protein, ligandin [5]. Certainly, the efficiency of bile acid transport is as great or greater than that of bilirubin [6].

Intracellular proteins capable of binding bile acids may also play a role in the regulation of synthesis of bile acids. It has been shown that the rate of synthesis appears to be regulated by the flux of bile acid through the liver cell [7]. A precedent for the involvement of an intracellular binding protein in the regulation of synthesis of a specific metabolite is the role of fatty acid-binding protein in hepatic triglyceride synthesis [8]. This study examines the binding of cholic acid to cytoplasmic proteins from rat liver.

2. Materials and methods

2.1. *Measurement of the capacity of subcellular fractions from rat liver to bind [^{3}H]cholic acid*

Rats were fasted overnight and killed by cervical dislocation. The livers were removed and placed in ice cold 250 mM sucrose containing 10 mM Tris (hydroxy methyl) amino methane hydrochloride and 5 mM $MgCl_2$. After mincing with scissors the tissue was homogenized using a Polytron shear sonicator (Kinematische GmBL). The homogenate was then incubated with 0.5 μCi [2,4-^{3}H]cholic acid (spec. act. 3.8 mCi/mmol, New England Nuclear) for 1 h at 4°C. It was then separated into nuclear (containing plasma membranes), mitochondrial, mixed (lysozymes, peroxisomes), ribosomal/microsomal and supernatant fractions according to Novikoff et al. [9].

The 100 000 × *g* supernatant fraction obtained by centrifugation was further fractionated by ultrafiltration through an Amicon CF50 centriflo filter (nominal molecular weight retention limit (NMWRL) 50 000, Amicon Corporation) to separate free cholic acid from bound cholic acid. [^{3}H]Cholic acid in each fraction was determined by counting an aliquot of each fraction in Bray's scintillant [10] using a liquid scintillation counter (Packard Tri-carb, model 3330).

2.2. *Gel filtration chromatography of [^{3}H]cholic acid incubated with supernatant*

Freshly prepared homogenate was spun at 100 000 × *g* for 70 min. The supernatant fraction was incubated for 1 h at 4°C with 0.5 μCi [^{3}H]cholic acid. A 2 ml aliquot was taken and applied to a Sephadex G-75 column (1.5 × 90 cm) and eluted with 5.0 mM sodium/potassium phosphate buffer, pH 7.4. Six ml fractions were collected and the protein profile determined by measuring absorbance at 280 nm. [^{3}H]Cholic acid in each fraction was determined as described above.

2.3. *Measurement of binding of [^{14}C]cholic acid to supernatant proteins*

An aliquot of freshly prepared supernatant fraction was applied to the Sephadex column and eluted as above. Binding studies on each column fraction were performed using the gel equilibration method of Hirose and Kano [11]. Sephadex G-15 gel was used with 0.05 μCi [2,4-^{14}C]cholic acid (spec. act. 46 mCi/mmol, New England Nuclear) as the tracer ligand. Results were calculated as described by Hirose and Kano [11]. To remove albumin from the supernatant fraction a DEAE cellulose column (Whatman DE50) was prepared at pH 7.4. Supernatant was added to the column (1.5 × 30 cm) and eluted with 50 mM phosphate buffer, pH 7.4, as preliminary experiments showed that these conditions effectively bind albumin to the DEAE cellulose column. The eluate was collected, concentrated by ultrafiltration and applied to a Sephadex G-75 column. Binding studies were done on the column fractions as described above. In order to check whether binding was to protein, fractions were also treated with pronase (Boehringer-Mannheim) before carrying out the binding assay. Molisch's test [12] for carbohydrates and Lowry [13] and tryptophan reactions [12] for protein were performed on each binding fraction.

3. Results

3.1. *Binding of bile acid to different subcellular fractions*

The distribution of [^{3}H]cholic acid bound to subcellular fractions of liver is shown in table 1. Most of the labelled bile acid was in the nuclei/membrane (31%) and supernatant (52%) fractions. In the supernatant fraction the percentage of [^{3}H]cholic acid bound to protein as determined by ultrafiltration varied between 30% and 45%.

Table 1
Binding of [^{3}H]cholic acid to subcellular fractions

Fraction	% [^{3}H]cholic acid/fraction[a]
Nuclei/membrane	30.5 ± 6.6
Mitochondrial	3.6 ± 1.5
Lysozyme/peroxisomes	4.4 ± 2.9
Ribosomal/microsomal	9.2 ± 0.7
Supernatant	52.4 ± 3.5

[a] Percent of total [^{3}H]cholic acid added to the initial homogenate present as a mean of 4 separate experiments ± SD

3.2. *Chromatography of rat liver supernatant fraction after incubation with [^{3}H]cholic acid*

In order to determine the protein species binding the [^{3}H]cholic acid an aliquot of incubated supernatant was applied to a Sephadex G-75 column. Greater than 98% of the [^{3}H]cholic acid applied to the column eluted as a single peak, at an elution volume identical to that of free [^{3}H]cholic acid (fig.1). Thus the [^{3}H]cholic acid did not remain bound to the supernatant protein during gel filtration. In contrast, the [^{3}H]cholic acid did not become separated from protein during ultrafiltration using a membrane with a NMWRL of 50 000.

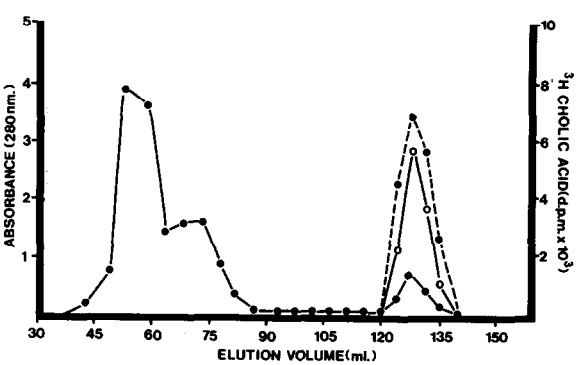

Fig.1. Elution profile of supernatant incubated with [^{3}H] cholic acid and then eluted from a Sephadex G-75 column as described in text. This figure shows the elution profile of protein (●——●), the elution position of the [^{3}H]cholic acid added to supernatant (○——○) and the elution position of [^{3}H]cholic acid in the absence of supernatant (●- - -●).

3.3. *Characterization of cholic acid-binding proteins*

Supernatant fractions from a Sephadex G-75 column showed two areas which bound [^{14}C]cholic acid. The first fraction corresponded to mol. wt approx. 65 000 as shown by calibration of the column with bovine serum albumin (mol. wt 65 000). The second fraction corresponded to mol. wt 30 000 – 40 000, estimated from the elution position of ligandin (mol. wt 44 000) [5]. Since the first fraction binding [^{14}C]cholic acid contains serum albumin, the experiments were repeated following the removal of serum albumin by DEAE cellulose chromatography. Conditions for the removal of albumin were worked out using standard bovine serum albumin solutions. At pH 7.4, 100% of the albumin was found to be bound to the DEAE column. Figure 2 shows the protein elution profile and binding of cholic acid in each fraction following Sephadex G-75 chromatography of supernatant fraction treated with DEAE cellulose. The two fractions which bound cholic acid were still present at the same molecular weight, showing that there are at least two proteins other than albumin which bind cholic acid. Proteolytic digestion with pronase completely destroyed all binding in these fractions and the biochemical tests were positive for protein and negative for carbohydrate. This suggests that the binding species are protein, not glycoprotein.

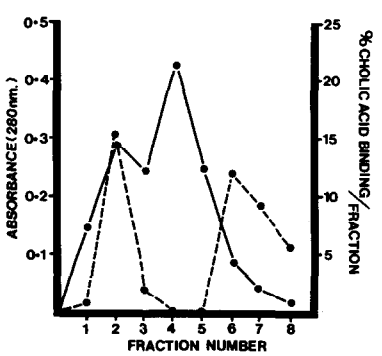

Fig.2. Binding peaks from DEAE cellulose treated supernatant eluted from a Sephadex G-75 column. This figure shows the protein profile (●——●) in the first eight column fractions and the binding (●- - -●) in each fraction determined by gel equilibration. Experimental conditions as described in text.

4. Discussion

We have shown the presence of 2 proteins from cytoplasm of rat liver which bind cholic acid. Besides the cytoplasmic binding, there was also significant binding to the nuclei/membrane fraction. This was probably due to binding to plasma membranes as Accatino and Simon [2] showed bile acid-binding sites on these structures. The molecular weights of the cytoplasmic proteins are approx. 65 000 for the first and 30 000–40 000 for the second. The 65 000 dalton species has different isoelectric properties from albumin since it is not removed by DEAE cellulose chromatography at pH 7.4. This species has not previously been identified, presumably because prior binding studies have been performed on fractions from which albumin has not been removed. For example, Strange et al. [3] divided rat liver supernatant into fractions corresponding to the X and Y fractions of Levi et al. [14] and found bile acid binding to both fractions using equilibrium dialysis.

More recently the same authors [4] found two macromolecular species capable of binding lithocholic acid, one of mol. wt 40 000 and the other of mol. wt 10 000. The 40 000 mol. wt protein may well be the same as the one reported in this paper. The 10 000 mol. wt species would not be identified in the present study as this is very close to the limit of effective fractionation for Sephadex G-75 gel and our separations were not taken to the fractionation limit. The tightness of binding of cholic acid to the two protein fractions has not yet been determined. However, the binding was not tight enough for the ligand and protein to remain together on gel filtration chromatography, but tight enough to remain together on ultrafiltration using a centriflo membrane.

Acknowledgements

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