Subcellular distribution of hepatic bile acid-conjugating enzymes

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1. The subcellular location of enzymes conjugating bile acids with glycine or taurine was investigated by centrifugation of rat liver homogenates. 2. [^{14}C]Cholic acid-conjugating activity was predominantly associated with the soluble-microsomal region of the gradient after centrifugation in a Ti-15 zonal rotor but the bulk of the conjugating activity sedimented with mitochondrial-lysosomal fractions in differential pelleting experiments. 3. Cholate:CoA ligase (EC 6.2.1.7) and cholytransferase (EC 2.3.1) were not enriched in purified Golgi or plasma-membrane fractions. Cholate:CoA ligase was distributed evenly between rough- and smooth-surfaced microsomal subfractions but cholytransferase showed a dual soluble-rough microsomal activity distribution. 4. Sedimentation of cholytransferase in mitochondria-enriched fractions prepared by differential centrifugation appears to be an artefact of sedimentation of rough microsomal membranes in mitochondrial fractions. 5. The subcellular distribution of bile acid-conjugating enzymes is discussed with reference to hepatic processing of bile acids.

Conjugation of bile acids in mammalian liver occurs via activation to the bile acyl-CoA derivatives followed by condensation of the bile acid carboxy group with the amino group of glycine or taurine. Activation is catalysed by the microsomal enzyme choloyl-CoA synthetase [EC 6.2.1.7; cholate:CoA ligase (AMP-forming)] in a reaction requiring CoA and ATP (Bremer, 1956; Elliot, 1956). The product, cholyl-CoA, undergoes reaction with glycine or taurine catalysed by bile acid:amino acid *N*-acyltransferase (EC 2.3.1; cholytransferase) (Vessey, 1979).

Bremer & Gloor (1955) found that liver microsomes reconstituted in soluble fraction conjugated [^{14}C]cholic acid at rates similar to those found in whole homogenates and Elliot (1956) located bile acid conjugation in the microsomal fraction of liver homogenates. Schersten (1967) reported stimulation of bile acid conjugation by a lysosomal-rich fraction added to human liver microsomes and concluded that cholytransferase might be a lysosomal enzyme. Vessey *et al.* (1977) recovered 90% of ox liver cholytransferase from liver postmicrosomal supernatant. Killenberg (1978) reported a dual microsomal-soluble localization of rat liver cholytransferase and concluded that the transferase was probably a soluble enzyme that non-specifically absorbed to microsomal membranes. All previous studies have relied on differential centrifugation in angle rotors to characterize the subcellular distribution of bile acid

conjugation with resulting heterogeneity of membrane fractions. Because there is circumstantial evidence for the role of lysosomal, Golgi and canalicular membranes and cytoplasmic vesicles in the biliary secretion of a variety of compounds (Renson *et al.*, 1980) we have studied the subcellular distribution of bile acid-conjugating enzymes in rat liver to see if conjugation was associated with a membrane fraction implicated in bile formation.

In the present paper we describe the use of centrifugation in a zonal rotor to clarify the results of differential-centrifugation experiments, report the distribution of bile acid-conjugating enzymes in liver plasma and Golgi membranes and microsomal subfractions and comment on the significance of these observations for hepatic processing of bile acids.

Experimental

Materials

Bile acids, CoA and substrates for enzyme assays were obtained from Sigma Chemical Company, St. Louis, MO, U.S.A., and AR grade sucrose was obtained from BDH Chemicals, Poole, Dorset, U.K. Cholyl-CoA was synthesized from CoA and the mixed anhydride of cholic acid with ethylchloroformate (Shah & Staple, 1968). [*Carboxy*- ^{14}C]cholic acid (sodium salt; sp. radioactivity 59.5 Ci/mol) and uridine diphospho-D-[U- ^{14}C]galactose (lithium salt; sp. radioactivity 347 Ci/mol) were purchased from

The Radiochemical Centre, Amersham, Bucks., U.K., and were greater than 97% pure when chromatographed in three separate t.l.c. systems.

Chromatography and scintillation counting

Bile acids were separated by t.l.c. on Kieselgel 60 GF₂₅₄ (Merck, Darmstadt, Germany) in butan-2-one/water/acetic acid (40:20:1, by vol.) and were detected visually by their blue reaction at 90°C with 2 mM-dodeca-molybdophosphoric acid in ethanol. R_F values were: cholate, 0.85; glycocholate, 0.50; taurocholate, 0.15. Radioactive bile acids separated chromatographically were scraped from t.l.c. plates into liquid-scintillation vials for radioactivity measurement. Radioactive extracts were counted in a Beckman LS-3100 liquid-scintillation counter (Beckman Instruments, Fullerton, CA, U.S.A.) in 10 ml of a cocktail containing 5 g of 2,5-diphenyl-oxazole and 0.3 g of 1,4-bis-(4-methyl-5-phenyl-oxazol-2-yl)benzene per litre of toluene/Triton X-100 (2:1, v/v).

Protein, RNA and enzyme assays

Protein was measured by Petersen's (1977) method and RNA as described by Ceriotti (1955).

[¹⁴C]Cholic acid conjugation was measured in 3 ml reaction mixtures containing up to 10 mg of liver protein, 0.5 mM-sodium [¹⁴C]cholate, 5.0 mM-glycine or -taurine, 17 mM-nicotinamide, 0.65 mM-MgCl₂, 0.75 mM-EDTA, 0.63 mM-ATP and 0.12 M-KH₂PO₄/0.12 M-K₂HPO₄, pH 7.4. Incubation mixtures were shaken in a rocking water bath at 37°C for 30 min and the reaction was terminated with 0.2 ml of conc. HCl. Radioactive bile acids were extracted into 2 ml of n-butanol and the extracts were concentrated in a stream of N₂. The extracts were chromatographed to separate cholic acid from its glycine and taurine conjugates and zones corresponding to the bile acids were removed for radioactivity determination.

Cholate:CoA ligase was measured by the reaction of FeCl₃ with the hydroxamate produced from choly-CoA and hydroxylamine (Bremer, 1956). A standard curve was prepared from the reaction of ethyl cholate with hydroxylamine and FeCl₃ reagents. Enzyme assays (1 ml) contained 25 mM-KH₂PO₄/25 mM-K₂HPO₄, pH 7.4, 10 mM-ATP, 5 mM-MgSO₄, 1.0 mM-sodium cholate, 0.2 mM-CoA, 500 mM-neutralized hydroxylamine and up to 2 mg of enzyme protein. After 30 min at 37°C the reaction was terminated with 3.7 ml of 0.3 M-HClO₄ at 4°C. Protein was removed by centrifugation and 0.5 ml of 0.4 M-FeCl₃ in conc. HCl was added to the supernatant. The absorbances of ferric hydroxamates were measured at 500 nm. Blanks showed no appreciable product formation when enzyme or cholate were omitted or in complete systems when HClO₄ was added before enzyme.

Cholyltransferase was measured with a continuous spectrophotometric assay. The amino-acid-dependent release of CoA from choly-CoA was detected by the reaction of the liberated CoA sulphhydryl group with 5,5'-dithiobis-(2-nitrobenzoic acid) at 37°C. The assay contained 0.04 mM-choly-CoA, 0.5 mM-5,5'-dithiobis-(2-nitrobenzoic acid), 50 mM-glycine or -taurine and up to 0.5 mg of enzyme protein in a total volume of 1 ml of 40 mM-Tris/HCl, pH 8.0. Enzyme and 5,5'-dithiobis-(2-nitrobenzoic acid) were added last to start the reaction. Blanks from which amino acids were omitted were always carried out to correct for amino-acid-independent hydrolysis of choly-CoA.

Marker enzyme assays (Fleischer & Kervina, 1974) used were: acid phosphatase, EC 3.1.3.2, using *p*-nitrophenyl phosphate, for lysosomes; succinate:cytochrome *c* oxidoreductase, EC 1.3.99.1, for mitochondria; urate oxidase, EC 1.7.3.3, for peroxisomes; glucose 6-phosphatase, EC 3.1.3.9, for microsomes; 5'-nucleotidase, EC 3.1.3.5, for plasma membranes; UDP-galactose:*N*-acetylglucosamine galactosyltransferase, EC 2.4.1.38, for Golgi membranes; and glutathione *S*-transferase, EC 2.5.1.18, using 1-chloro-2,4-dinitrobenzene, for soluble fraction (Clark *et al.*, 1967).

Homogenization and differential centrifugation

All centrifugation was carried out with Beckman rotors at 4°C in a Beckman L5-65 centrifuge (Beckman Instruments). Male white rats were of the Victoria strain fed a pellet diet *ad libitum*. Homogenates were prepared from the livers of 240 ± 20 g animals killed by cervical fracture. Livers were cut into small pieces and homogenized in 4 vol. of ice-cold buffered sucrose (0.25 M-sucrose/1 mM-di-sodium EDTA/5 mM-Tris/HCl, pH 7.4) in a size C Potter-Elvehjem homogenizer (Arthur H. Thomas Co., Philadelphia, PA, U.S.A.) driven at a speed of 900 rev./min. Three up-and-down strokes of the pestle were used during homogenization.

The homogenate was centrifuged at 4°C in a Beckman 60Ti fixed-angle rotor (r_{av} , 6.3 cm) at 750 *g* for 5 min. The sediment was resuspended in ice-cold buffered sucrose and recentrifuged for 5 min at 750 *g*. The combined pellets were designated nuclei and cell debris fraction (N). Further fractions were prepared by centrifuging the bulked 750 *g* supernatants in the 60Ti rotor. All pellets were washed by resuspension in ice-cold buffered sucrose followed by re-centrifugation. A heavy-mitochondrial pellet (M) was prepared by centrifuging the 750 *g* supernatant at 7000 *g* for 5 min. Centrifugation of the 7000 *g* × 5 min supernatant at 20 000 *g* for 15 min gave a light-mitochondrial pellet (L). Centrifugation of the 20 000 *g* × 15 min supernatant at 113 000 *g* × 60 min gave the microsomal

pellet (P) and soluble supernatant (S). Pellets were re-suspended and stored in ice-cold buffered sucrose.

Microsomal fractionation

A postmitochondrial supernatant produced by centrifuging liver homogenates at 20 000 g for 20 min in the 60Ti rotor was diluted with water to 0.17 M-sucrose and layered over 1.31 M-sucrose. Samples were centrifuged for 8 h at 113 000 g in the 60Ti rotor. Membranes enriched in smooth microsomes collected at the sucrose interface, whereas rough-surfaced vesicles collected at the bottom of the centrifuge tube (Rothschild, 1963). Membrane fractions were re-suspended in ice-cold buffered sucrose and re-sedimented at 113 000 g for 1 h in the 60Ti rotor.

Preparation of Golgi- and plasma-membrane fractions

Membrane fractions enriched in Golgi or plasma membranes were prepared by centrifugal flotation in a Beckman SW27 rotor (r_{av} , 11.83 cm).

Golgi membranes isolated by the method of Fleisher & Kervina (1974) showed mainly sacs and secretory vesicles with dense staining lipoprotein stores when examined by electron microscopy. The specific activity of the Golgi enzyme UDP-galactose : *N*-acetylglucosamine galactosyltransferase was about 120 times greater ($1.14 \mu\text{mol/min per g}$ of protein) in the purified Golgi fraction than in liver 750 g supernatant. The Golgi fraction contained 0.3% of the liver protein and had negligible 5'-nucleotidase and glucose 6-phosphatase activities.

Plasma-membrane-enriched fractions (Song *et al.*, 1969) contained about 2% of the liver protein and showed sheets of membranes often with tight junctions, although bile canaliculi were not seen. More than 30% of the 5'-nucleotidase was recovered in the plasma-membrane fraction with a specific activity 18 times greater than that of the whole homogenate. The specific activity of glucose 6-phosphatase in the plasma-membrane fraction was less than that of the homogenate, although succinate:cytochrome *c* oxidoreductase and UDP-galactose : *N*-acetylglucosamine galactosyltransferase activities were three to four times enhanced compared with the homogenate.

Zonal centrifugation

Zonal centrifugation was carried out in a Beckman Ti-15 rotor. All gradient solutions contained sucrose, 1 mM-EDTA, pH 7.4, 5 mM-Tris/HCl and 10⁻⁴% (v/v) Triton GR-5 (dioctyl sodium sulphosuccinate; Rohm and Haas, Philadelphia, PA, U.S.A.). Gradient solutions were loaded at approx. 40 ml/min with the rotor spinning at 2000 rev./min. The gradient was designed to give a good separation

of mitochondria from microsomes and was formed by the addition of sucrose solutions in the following order: 100 ml of 15% (w/w); 120 ml of 16% (w/w); 130 ml of 17% (w/w); 160 ml of 18% (w/w); 180 ml of 20% (w/w); 210 ml of 22% (w/w); 230 ml of 25% (w/w); 260 ml of 35% (w/w). The cushion solution was 400 ml of 40% (w/w) sucrose loaded through the edge line. Sample (100 ml of liver homogenate or subcellular fraction) and overlay [100 ml of 6.6% (w/w) sucrose] were added at a rate of 5 ml/min through the centre line. After centrifugation at 12 000 rev./min for 45 min the rotor was decelerated to 2000 rev./min and unloaded dynamically by pumping a 45% (w/w) sucrose solution through the edge line. Fractions (42, each of 40 ml) were collected at a rate of 1 fraction/min. Sucrose concentrations were measured at 4°C with a hand refractometer (Atago Optical Works Co., Tokyo, Japan).

Electron microscopy

Membrane pellets were fixed for 1 h at room temperature in a solution of 2.5% (v/v) glutaraldehyde in 0.25 M-sucrose buffered to pH 7.2 with sodium cacodylate (0.1 M after pH adjustment with HCl solution). Fixed pellets were washed in water, postfixed with aq. 1% (w/v) OsO₄, dehydrated with ethanol and embedded in Araldite. Thin sections were stained with uranyl nitrate and lead citrate and examined with a Zeiss EM9A electron microscope.

Results

Differential centrifugation

Rates of [¹⁴C]cholic acid conjugation in differential fractions were affected by sample preparation (Table 1). The bulk of the conjugating activity was

Table 1. *Differential centrifugation of [¹⁴C]cholic acid-conjugation activity*

Subcellular fractions were prepared by differential centrifugation as described in the Experimental section. Rates of [¹⁴C]cholic acid conjugation were measured on subcellular fractions equivalent to 400 mg of liver. Enzyme activities are means of four experiments. Membrane fractions were resuspended in homogenization medium with 1 mg of CoA or in soluble fraction from 100 mg of liver.

Subcellular fraction	Reconstituted in ...	Enzyme activity (nmol/min per g of liver)	
		Homogenization medium	Soluble fraction
750 g supernatant		37.8 ± 1.5	38.9 ± 1.3
M		3.4 ± 0.5	10.9 ± 0.7
L		3.3 ± 0.3	6.3 ± 0.5
P		1.3 ± 0.2	2.6 ± 0.2
S		2.3 ± 0.3	2.0 ± 0.2

recovered in the M and L pellets and not in the microsomes and soluble fraction as reported by previous workers. Only 30% of the total activity was recovered when fractions were re-constituted in buffered sucrose, but 60% of the activity was recovered when membrane pellets were re-constituted in soluble supernatant. The activity of M fraction re-constituted in soluble supernatant was enhanced 2.5-fold by addition of microsomes from an equivalent amount of liver. Electron microscopy showed that the M and L fractions were enriched in mitochondria but also contained rough-surfaced membranes apparently derived from granular endoplasmic reticulum. The distribution of cholytransferase and some marker enzymes in differential fractions is shown in Table 2. Sedimentation of some microsomal membranes in the M + L fractions was indicated by the distribution of glucose 6-phosphatase. Approx. 65% of the cholytransferase was found in the 113 000 g supernatant with smaller amounts in each of the particulate fractions. When washed M, L and P fractions were further washed by re-suspension in buffered sucrose and re-centrifuged approx. 50% of the M and L cholytransferase and approx. 75% of the microsomal enzyme were solubilized by two re-suspension-centrifugation cycles. In one experiment a microsomal pellet washed twice in buffered sucrose was re-suspended in soluble supernatant and centrifuged at 113 000 g for 1 h. Less than 4% of the soluble cholytransferase re-sedimented at 113 000 g with the microsomal pellet indicating little re-absorption of soluble enzyme to microsomes. Solubilization of the microsomal transferase was only partially enhanced by brief sonication or by freeze-thawing four times in liquid N₂. When membrane fractions were washed by resuspension and centrifugation in buffered sucrose/0.2 M-KCl approx. 45% of the M and L transferases and about 60% of the microsomal transferase were solubilized. Washing in 0.2 M-KCl without sucrose released 80% of the M, L and P

transferase. A single wash in 1.0 M-KCl removed more than 90% of the transferase from membrane fractions in the presence or absence of sucrose.

Zonal centrifugation

When 750 g supernatant was centrifuged in the Ti-15 rotor at 12 000 rev./min for 45 min the bulk of the [¹⁴C]cholic acid-conjugating activity migrated slowly and was associated near the sample region with soluble supernatant and the microsomal enzyme glucose 6-phosphatase (Fig. 1). These results

Fig. 1. Distribution of [¹⁴C]cholic acid-conjugating activity and marker enzymes after centrifugation at 12 000 rev./min for 45 min in the Ti-15 zonal rotor. Units of enzyme activity are $\mu\text{mol}/\text{min}$ per fraction.

Table 2 Subcellular distribution of enzymes after differential centrifugation

Subcellular fractions were prepared and enzyme activities were measured as described in the Experimental section. Both glycine-dependent and taurine-dependent cholytransferase activity was assayed. Results are means of four experiments.

Enzyme	Specific activity of 750 g supernatant ($\mu\text{mol}/\text{min}$ per g of protein)	Subcellular fraction ...	Activity (% of activity in 750 g supernatant)			
			M	L	P	S
Choly transferase						
Glycine-dependent	4.3		6	14	15	63
Taurine-dependent	3.3		14	11	11	68
Succinate:cytochrome c oxidoreductase	40.9		77	7	1	0.2
Glucose 6-phosphatase	8.9		14	22	63	8
5'-Nucleotidase	4.2		22	15	48	23
Protein (mg/g of liver)	172		11	7	22	63

suggest that the previous mitochondrial-lysosomal distribution of cholic acid conjugation in differential-centrifugation experiments resulted in part from sedimentation of microsomal conjugating enzymes in the M and L pellets.

The distribution of cholate:CoA ligase, cholytransferase and some marker enzymes after centrifugation in the Ti-15 rotor is shown in Fig. 2. Cholate:CoA ligase migrated with the microsomal enzyme glucose 6-phosphatase and cholytransferase was collected from the fractions near the rotor core with glutathione *S*-transferase, a mainly cytoplasmic enzyme used in our study as an enzyme marker for soluble supernatant.

When fractions collected from the zonal rotor were adjusted to 0.25 M-sucrose by the addition of water or solid sucrose and centrifuged at 113 000 *g* for 1 h in the 60Ti rotor to separate membrane associated from soluble enzymes approx. 85% of the cholytransferase was found in the soluble supernatant with cytoplasmic glutathione *S*-transferase. Membrane-bound cholytransferase was recovered mainly from zonal fractions 2–8 with microsomal glucose 6-phosphatase.

Fig. 2. Distribution of enzyme activities after centrifugation of liver 750 *g* supernatant in the Ti-15 rotor at 12 000 rev./min for 45 min

Units of enzyme activity are $\mu\text{mol}/\text{min}$ per fraction. Shaded areas represent membrane-associated enzymes assayed after zonal fractions were adjusted to 0.25 M-sucrose and centrifuged at 113 000 *g* for 60 min in the 60 Ti rotor.

The subcellular origin of membrane-bound cholytransferase that was distributed in M and L pellets on differential centrifugation was studied by zonal centrifugation of a combined M and L fraction. The 750 *g* supernatant from eight rat livers was centrifuged in the 60Ti rotor at 25 000 *g* for 25 min and the pellet was resuspended in 60 ml of buffered sucrose by hand homogenization in a Potter/Elvehjem homogenizer. The re-suspended M + L fraction was centrifuged in the Ti-15 zonal rotor at 12 000 rev./min for 45 min. Cholate:CoA ligase migrated with microsomal glucose 6-phosphatase but the distribution of cholytransferase was slightly modified with approx. 70% of the M + L activity distributed in fractions 3–7 with glucose 6-phosphatase and approx. 20% of the M + L cholytransferase (about 3% of the total liver activity) distributed in a peak around fraction 25. Peaks of activity for peroxisomal, lysosomal and plasma-membrane enzymes were in fractions 12, 15 and 28 respectively. Mitochondrial succinate:cytochrome *c* oxidoreductase activity was found as a broad peak in fractions 22–32 with maximum activity in fraction

Fig. 3. Distribution of protein, RNA and enzymes after centrifugation at 27 000 rev./min for 2.5 h in the Ti-15 zonal rotor

The loaded sample was a postmitochondrial supernatant prepared by homogenizing four livers in buffered sucrose and centrifuging at 7000 *g* for 20 min in the 60Ti rotor. Units of enzyme activity are $\mu\text{mol}/\text{min}$ per fraction.

27. Approx. 2–5% of the M+L glutathione *S*-transferase peaked in fraction 25 with cholytransferase. Electron microscopy of membranes from fractions 25–27 showed mainly mitochondria with small amounts of endoplasmic reticulum. It is possible that this region contained aggregated microsomes or endoplasmic reticulum-derived membranes in association with mitochondria (Pickett *et al.*, 1980).

The microsomal/soluble location of bile acid-conjugating enzymes was further investigated by centrifuging a postmitochondrial supernatant in the Ti-15 rotor at 27000 rev./min for 2.5 h (Fig. 3). Under these conditions remaining lysosomes, mitochondria and peroxisomes migrated to the rotor edge and a good separation of soluble supernatant and microsomes was achieved. RNA was concentrated in two peaks, one near the sample region with soluble glutathione *S*-transferase, the other with rough-surfaced microsomes near the rotor edge. Acid nitrophenyl phosphatase showed a dual soluble/lysosomal activity distribution. Glucose 6-phosphatase and cholate:CoA ligase activities coincided, each as two peaks. One peak around fraction 20 (sucrose density 1.12 g/ml) was shown by electron microscopy to be enriched in smooth-surfaced microsomes and contained free ribosomes, whereas the other around fraction 36 (sucrose density 1.22 g/ml) was enriched in rough-surfaced microsomal vesicles. More than 80% of the cholytransferase was found in fractions 3–7 with the cytoplasmic marker. A minor peak of cholytransferase around fraction 37 coincided with the distribution of rough-surfaced microsomes.

Distribution of bile acid-conjugating enzymes in Golgi and plasma membranes and in microsomal subfractions

Table 3 shows enzyme, RNA and protein distributions between rough- and smooth-surface microsome subfractions prepared by centrifugation of

postmitochondrial supernatants. Accumulation of ribosome-studded vesicles in the rough microsomal fraction was indicated by electron microscopy and by the distribution of RNA (Table 3). Cholytransferase was concentrated with RNA in the rough microsomal fraction, but cholate:CoA ligase, like glucose 6-phosphatase, was more equally distributed between rough and smooth-surfaced vesicles. The results in Table 3 have not been adjusted for the contribution of ribosomal protein to the rough microsomes. Assuming that one-third of total rough-microsomal protein is derived from ribosomes (Fleischer & Kervina, 1974) the specific activities of cholate:CoA ligase in smooth and rough microsomes are similar.

Neither cholate:CoA ligase nor cholytransferase were concentrated in Golgi or plasma-membrane fractions (Table 4). Activity distributions in these experiments were consistent with a microsomal location for cholate:CoA ligase and a soluble/microsomal distribution for cholytransferase. Less than 1% and 3% respectively of liver choly-

Table 4. *Cholytransferase and cholate:CoA ligase distribution in purified membranes*

Purified membrane fractions were prepared as described in the Experimental section. Enzyme activities in microsomal subfractions are those shown in Table 3. Cholate:CoA ligase was not determined (n.d.) in 750 g supernatant.

Subcellular fraction	Specific activity ($\mu\text{mol/min per g of protein}$)	
	Cholytransferase	Cholate:CoA ligase
750 g supernatant	17.6	n.d.
Golgi membranes	1.0	0
Plasma membranes	1.4	0.24
Smooth microsomes	2.6	4.7
Rough microsomes	12.7	3.1

Table 3. *Enzyme activities in smooth and rough microsomes*

A postmitochondrial supernatant was separated into smooth- and rough-microsomal subfractions in the 60Ti rotor as described in the Experimental section. Glycine-dependent cholytransferase and marker enzyme specific activities ($\mu\text{mol/min per g of protein}$) were measured on the postmitochondrial supernatant and microsome subfractions.

Activity measured	Specific activity			Activity in microsomes (% of activity in postmitochondrial supernatant)
	Postmitochondrial supernatant	Smooth microsomes	Rough microsomes	
Choly transferase	15.0	2.6	12.7	21
Cholate:CoA ligase	1.6	4.7	3.1	79
Glucose 6-phosphatase	23.7	85.1	99.7	86
Glutathione <i>S</i> -transferase	276.9	38.1	51.7	7
5'-Nucleotidase	30.4	124.8	35.7	63
RNA (mg/g of protein)	54.4	34.7	120.2	59
Protein (mg)	742.9	78.7	74.5	21

transferase was recovered in the Golgi and plasma-membrane fractions.

Discussion

Membrane-associated cholytransferase was found mainly in rough-surfaced microsomes and to a lesser extent in smooth-surfaced vesicles. Small amounts of cholytransferase recovered from Golgi and plasma membranes were probably due to microsomal contamination. Similarly cholate:CoA ligase was not found in Golgi fractions and the small amount located in plasma membranes may represent contaminating microsomal activity. Cholate:CoA ligase is thought to be distributed at the cytoplasmic surface of hepatic microsomes (Polokoff *et al.*, 1979) and we have shown that cholate:CoA ligase is found in both smooth- and rough-microsomal fractions.

The apparent mitochondrial/lysosomal distribution of cholytransferase after differential centrifugation probably resulted from contamination of the M+L pellets with microsomal and soluble enzymes. When 750g supernatant was centrifuged in the zonal rotor only 5–17% of the total cholytransferase migrated away from the soluble/microsomal region near the rotor core and when an M+L fraction prepared by differential pelleting was centrifuged in the zonal rotor more than 80% of the cholytransferase distributed with the soluble and microsomal enzyme markers. From the results of zonal centrifugation it can be calculated that 3% of the total cholytransferase migrated near the mitochondrial succinate:cytochrome *c* oxidoreductase and that about 18% of the cholytransferase migrated with microsomal glucose 6-phosphatase near the rotor core. Microsomal contamination of mitochondrial/lysosomal fractions prepared by differential centrifugation may be a result of several processes. Dallner & Ernster (1968) calculated that about 10% of microsomal vesicles may coalesce to form larger particles as a result of side-wall impaction during centrifugation in angle rotors. Rough-surfaced microsomal vesicles predominate in mitochondrial fractions prepared by differential centrifugation. Adelman *et al.* (1974) recovered 20% of liver RNA in mitochondrial fractions and demonstrated RNA solubilization by puromycin indicating release from granular endoplasmic reticulum. Microsomal contamination of heavy- and light-mitochondrial fractions prepared by differential centrifugation in our study was evident on electron-microscope examination and was estimated to be 12–17% from the distribution of glucose 6-phosphatase.

Microsomal cholytransferase was readily released by washing subcellular fractions in 0.2–1.0M-KCl at pH 7.4. Transferase solubilisation was

greatest in the absence of sucrose, which promotes microsomal absorption of some proteins (de Pierre & Dallner, 1976). The enrichment of cholytransferase in rough-surfaced microsomes suggests that the enzyme may be bound to ribosomes but as the enzyme is isoelectric at pH 7.1 (Jordan *et al.*, 1980) and ribosomes have a net negative charge electrostatic interaction between the transferase and membrane-bound ribosomes may require cation bridges. The observation that microsomal cholytransferase was progressively solubilized by washing in buffered sucrose suggests that the 113000g supernatant enzyme may arise by solubilization of microsomal transferase during homogenization and centrifugation. As the 113000g supernatant transferase was not significantly re-absorbed by washed microsomes we tentatively conclude that at least a fraction of the hepatic cholytransferase may be a loosely associated peripheral microsomal protein. The possibility that the microsomal transferase represents newly synthesized enzyme deserves further attention.

Conjugation of [14 C]cholic acid with glycine or taurine requires sequential action of microsomal cholate:CoA ligase and soluble/microsomal cholytransferase. Measurements *in vitro* indicate that cholate:CoA ligase action is the slower step so that conjugation occurs when rate-limiting amounts of cholate:CoA ligase are supplemented by sufficient transferase to conjugate choly-CoA with glycine or taurine. Greatest rates of [14 C]cholic acid conjugation were found in the microsomal/soluble region of the gradient after zonal centrifugation, where both ligase and transferase were concentrated. The low recovery of [14 C]cholic acid-conjugating activity from subcellular fractions prepared by differential centrifugation may arise from separation of the microsomal ligase from soluble or solubilized cholytransferase. The preferential distribution of [14 C]cholic acid-conjugating activity in differential mitochondrial pellets appears to be an artefact of reconstitution of microsomal ligase with rough microsomal transferase or soluble transferase trapped in interparticulate water. The enhancement of mitochondrial conjugation by soluble fraction and further enhancement on addition of microsomes may reflect reconstitution of transferase and ligase as a conjugating complex.

The location of bile acid-conjugating enzymes at the surface of microsomal vesicles derived from the endoplasmic reticulum (Polokoff *et al.*, 1979; the present study) indicates that newly formed bile acids and bile acids re-absorbed from portal blood are conjugated on the endoplasmic reticulum. Alternatively bile acids may be activated to the acyl-CoA derivatives at the endoplasmic-reticulum surface followed by cytoplasmic cholytransferase-mediated conjugation with amino acids. We have found no

evidence for the participation of Golgi-membrane- or plasma-membrane-bound bile acid-conjugating enzymes and conclude that the conjugating systems are not packaged on Golgi or plasma membranes as part of the processes responsible for secretion of bile acids across the canalicular membrane into the bile ductules.

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References

- Adelman, M. R., Blobel, G. & Sabatini, D. D. (1974) *Methods Enzymol.* **31**, 201–215
- Bretner, J. (1956) *Acta Chem. Scand.* **10**, 56–71
- Bremer, J. & Gloor, U. (1955) *Acta Chem. Scand.* **9**, 689–698
- Cerioti, G. (1955) *J. Biol. Chem.* **214**, 59–70
- Clark, A. G., Darby, F. J. & Smith, J. N. (1967) *Biochem. J.* **103**, 49–54
- Dallner, G. & Ernster, L. (1968) *J. Histochem. Cytochem.* **16**, 611–632
- de Pierre, J. & Dallner, G. (1976) in *Biochemical Analysis of Membranes* (Maddy, A. H. ed.), pp. 79–131, Chapman and Hall, London
- Elliot, W. H. (1956) *Biochem. J.* **62**, 427–433
- Fleischer, S. & Kervina, M. (1974) *Methods Enzymol.* **31**, 6–41
- Jordan, T. W., Lee, R. & Lim, W. C. (1980) *Biochem. Int.* **1**, 325–330
- Killenberg, P. G. (1978) *J. Lipid Res.* **19**, 24–31
- Petersen, G. L. (1977) *Anal. Biochem.* **83**, 346–356
- Picket, C. B., Montisano, D., Eisner, D. & Cascarano, J. (1980) *Exp. Cell Res.* **128**, 343–352
- Polokoff, M. A., Coleman, R. A. & Bell, R. M. (1979) *J. Lipid Res.* **20**, 17–21
- Renson, R. H., Mälöney, D. G., Jones, A. L., Hradek, G. T., Wong, K. Y. & Goldfine, I. D. (1980) *Gastroenterology* **78**, 1373–1388
- Rothschild, J. (1963) *Biochem. Soc. Symp.* **22**, 4–28
- Schärsten, T. (1967) *Acta Chirurg. Scand. Suppl.* **373**, 1–38
- Shah, P. P. & Staple, E. (1968) *Steroids* **12**, 571–576
- Song, C. S., Rubin, W., Rifkind, A. B. & Kappas, A. (1969) *J. Cell Biol.* **41**, 124–132
- Vessey, D. A. (1979) *J. Biol. Chem.* **254**, 2059–2063
- Vessey, D. A., Crissey, M. H. & Zakim, D. (1977) *Biochem. J.* **163**, 181–183