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EFFECTS OF DIFFERENT BILE SALTS UPON THE COMPOSITION AND MORPHOLOGY OF A LIVER PLASMA MEMBRANE PREPARATION

DEOXYCHOLATE IS MORE MEMBRANE DAMAGING THAN CHOLATE AND ITS CONJUGATES

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Summary

1. Rat liver plasma membrane preparations were incubated with various bile salts at 0 or 37°C. The bile salts caused the removal of various amounts of proteins, membrane enzymes and phospholipids; the extent and nature of these losses, and the morphological changes which accompanied them, varied with the detergent used.

2. Cholate, taurocholate and glycocholate removed appreciable amounts of protein from the saline-washed membranes, and considerable amounts of both phospholipids and the membrane enzymes, 5-nucleotidase, alkaline phosphatase, alkaline phosphodiesterase 1 and L-leucyl-β-naphtylamidase. These losses were greater at 37 than at 0°C. The material remaining contained membrane-like profiles, many of vesicular form, even when the preparation was almost completely devoid of phospholipids.

3. Deoxycholate, both at 0 and 37°C, removed more protein, membrane enzymes and phospholipids than did cholate and its conjugates. The material remaining was mainly granular and unorganised and the only remaining features were structures resembling the nexus, and occasional desmosomes.

4. Deoxycholate, a dihydroxy bile salt, therefore appears to cause greater perturbation of membrane structure than the trihydroxy bile salt, cholate, and its conjugates. The results may have implications for the effects of bile salts upon the membranes of liver cells during bile salt secretion and the production of bile.

Introduction

The bile salts are natural detergents; some, particularly deoxycholate and, to a lesser extent, cholate, have found considerable use in biochemistry as reagents

able to cause dissociation or dissection of membranes and thus have been used in the preparation of biologically active membrane components [1—3].

Each day the liver secretes large quantities of bile salts; the concentration of these may reach 0.5—1% (10—20 mM) in hepatic bile and, if concentrated in the gall bladder, may rise to about 7% [4,5]. During the production of bile in the liver, however, there is no gross disruption of liver cells, either morphologically or as judged by the relative absence of intracellular proteins from the bile [6]. There are however present, in bile, significant amounts of the enzymes 5′-nucleotidase, alkaline phosphatase, alkaline phosphodiesterase I and L-leucyl-β-napthylamidase, which are normally ascribed to the plasma membranes of liver cells [6].

Recent studies in this laboratory have been carried out in an effort to understand the process of bile salt secretion and its possible effects upon the membranes of liver cells, and to account for some of the specific components of bile. One of these studies [7] has shown that the various bile salts differ in their abilities to solubilize components of the membrane of the erythrocyte. Thus, whilst deoxycholate brought about total solubilization of the membrane at 37°C, cholate and its conjugates (glycocholate and taurocholate), though extracting some of the proteins and lipids, still left a residue composed of "membrane-like profiles". These could be clearly identified by phase contrast and electron microscopy. This led to the suggestion that one of the reasons why the liver appeared to be minimally damaged by bile salt secretion might be the presence, in bile, of quantities of "mild" i.e. less membrane-damaging, bile salts (e.g. conjugated derivatives of cholate) rather than the predominance of "stronger" types of bile salts such as free deoxycholate [7].

These studies have now been extended to the actions of various bile salts upon preparations of liver plasma membranes. We report a difference in the effects between deoxycholate, and cholate and its conjugates.

Materials and Methods

Materials. The sodium salts of cholate and deoxycholate, and most fine chemicals, were obtained from Sigma Chemical Co., London SW6, U.K. The sodium salts of glycocholate and taurocholate were "A" grade reagents supplied by Calbiochem Ltd., Hereford, U.K.; these were synthetic compounds made from pure cholate and contained at least 97% of the compound described. Male Wistar rats, 8—10 weeks old, were used throughout.

Plasma membrane preparations. A plasma membrane fraction including bile canaliculi was prepared by the method of Song et al. [8]. The membranes were then washed with 20 volumes of iso-osmotic saline (0.9% NaCl/10 mM Tris · HCl, pH 8) at 0°C, followed by centrifugation at 100 000 × g for 60 min to release adsorbed or occluded proteins from the pellets of membranes obtained after the first $NaHCO_3$ resuspension (see Song et al. [8]).

In the preparation the relative specific activities of the plasma membrane enzymes, alkaline phosphodiesterase I (EC 3.1.4.1.), 5-nucleotidase (EC 3.1.3.5.), L-leucyl-β-napthylamidase (EC 3.4.11.1) and alkaline phosphatase (EC 3.1.3.1) were increased 16—22, 20—24, 11 and 5 times, respectively, in relation to the homogenate. The intracellular enzymes glucose-6-phosphatase

(EC 3.1.3.9), alcohol dehydrogenase (EC 1.1.1.1) and 6-phosphogluconate dehydrogenase (EC 1.1.1.44) were undetectable in the preparation and the relative specific activities of lactate dehydrogenase (EC 1.1.1.27) and succinate dehydrogenase (EC 1.2.99.1) were 0.47 and 0.41 respectively. The lactate dehydrogenase was removed by the saline wash, suggesting that it had been adsorbed on the membranes. Thus the principle contaminants of the preparation appeared to be mitochondria. Electron micrographs of the freshly prepared membrane preparation showed it to resemble what Song et al. [8] described as plasma membrane fraction (M-2).

Incubation and separation of materials. The saline-washed membranes were homogenized by hand in a loose-fitting Potter-Elvehjem homogeniser in 0.9% NaCl/10 mM Tris · HCl, pH 7.5. 1-ml aliquots of this suspension (2 mg protein/ml) were added to, and incubated with, 9 ml of 0.9% NaCl/10 mM Tris · HCl, pH 7.5 containing appropriate amounts of bile salts for 10 min at 37, or 0°C, as appropriate. The bile salt solutions were prepared fresh on the day of use.

After incubation a known aliquot of the mixture was centrifuged at 100 000 × *g* for 60 min and the pellet obtained was resuspended in a known volume of 0.9% NaCl/10 mM Tris · HCl, pH 7.5.

Protein [9], phospholipid phosphorus [10] of lipid extracts [11], and enzymes were measured in both pellets and supernatants, unless otherwise stated.

Corrections were made for detergent activation or inhibition of enzyme activities by comparison with appropriate uncentrifuged detergent-containing and detergent-free controls.

Enzyme determinations. Most of the enzyme activities were determined in preparations stored at −20°C and no loss was observed in thawed membranes as compared with fresh membranes. The enzymes studied were: alkaline phosphodiesterase 1 [12], 5′-nucleotidase [13], L-leucyl-β-naphthylamidase [14] and alkaline phosphatase [15], all plasma membrane enzymes, and succinate dehydrogenase (mitochondria), [16]; lactate dehydrogenase (cytosol), [17], 6-phosphogluconate dehydrogenase, (cytosol), [18], alcohol dehydrogenase (cytosol) [19] and glucose-6-phosphatase (endoplasmic reticulum), (ref. 20, modified by Shephard and Hübscher [21]).

Electron microscopy. Specimens were prepared for electron microscopy by addition of buffered 6.25% glutaraldehyde to the pellet obtained by centrifuging the incubation mixture of untreated material at 14 000 × *g* for 2 min, or at 100 000 × *g* for 60 min at 0°C. These were then fixed with osmium tetroxide, embedded, sectioned and stained with 25% uranyl acetate in methanol or Reynold's lead citrate [22].

Results

Effects of different bile salts upon the release of materials from the membrane preparation

Preliminary experiments were carried out to study the effects of various bile salts, at different concentration levels, at 37°C (Fig. 1). Deoxycholate caused the removal of components at lower concentrations than did glycocholate, taurocholate and cholate. In most cases the removal of alkaline phosphodiester-

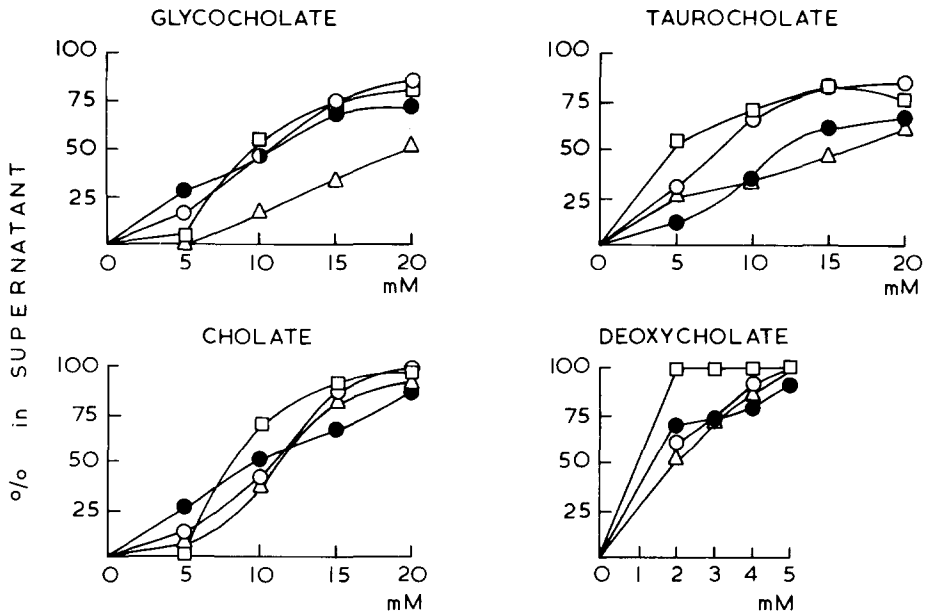

Fig. 1. The effect of the concentration range of different bile salts upon the release of components from liver plasma membrane preparations. The plasma membrane preparations were incubated at 37° C with the final bile salt levels shown. Four preparations were used, one for each detergent. Conditions of incubation, separation and analyses are described in Materials and Methods. Determinations of enzymes and phospholipids were made on both pellets and supernatants and the values recorded relate to total recoveries (which were 80—95%). Protein values were obtained from pellets only and relate to material in the incubated control pellet (100%). △, 5′-nucleotidase; □, alkaline phosphodiesterase; ●, protein; ○, phospholipid.

ase I slightly exceeded that of 5′-nucleotidase, phospholipids and protein. Protein removal had reached a plateau at 15—20 mM for glycocholate and taurocholate, and cholate had achieved most of its maximum effects at 20 mM. Thus, in subsequent studies, a concentration of 20 mM was chosen for glycocholate, taurocholate and cholate, and for deoxycholate, 5 mM.

At these levels the effects of the detergents upon the release of components from saline-washed membranes is shown in Table I. Some protein was released from the membranes upon incubation in the absence of detergent, more at 37 than at 0° C. The apparent protein solubilization brought about by the detergents must be judged in relation to this and therefore a second calculation has been made which relates the amount of protein remaining in the detergent-treated pellet to the control pellet after incubation; a truer estimate of the effect of the detergent can therefore be obtained. Deoxycholate removed more protein from the membrane preparation than did the cholate group, and this was especially marked at 0° C.

Incubation of the preparation in the absence of detergent did not cause the release of any of the enzymes studied. The detergents released greater amounts of enzymes at 37 than at 0° C. In all cases deoxycholate appeared to be more effective than cholate and its conjugates. Alkaline phosphodiesterase release was greater than the removal of 5′-nucleotidase which, at both temperatures,

TABLE I

THE EFFECT OF DIFFERENT BILE SALTS UPON THE COMPOSITION OF SALINE-WASHED PLASMA MEMBRANE PREPARATIONS

The plasma membrane preparations used had been washed with 0.9% NaCl/10 mM Tris · HCl, pH 8.0 as described in Materials and Methods, and incubated in 0.9% NaCl/10 mM Tris · HCl, pH 7.5 for 10 min at 0 or 37° C as indicated, with glycocholate, taurocholate or cholate (20 mM) or deoxycholate (5 mM). All determinations were made on pellets and supernatants. The results given represent means of two separate experiments (with the range indicated), with exception of L-leucyl-β-naphthylamidase, which at 0° C was determined once only. Recoveries of all parameters were in the range of 81—105%.

Temperature	Detergent	% of control recovered in pellet						Protein % of control pellet remaining
		5′-Nucleotidase	Alkaline phosphodiesterase	Alkaline phosphatase	L-Leucyl-β-naphthyl-amidase	Phospholipid	Protein	
0° C	none	100	100	100	100	100	80 ± 2	100
	glycocholate	67 ± 15	17 ± 7	67 ± 3	85	22 ± 7	56 ± 4	70 ± 4
	taurocholate	68 ± 5	18 ± 3	83 ± 4	88	20 ± 2	50 ± 2	62 ± 4
	cholate	69 ± 6	24 ± 1	83 ± 3	89	23 ± 2	51 ± 2	63 ± 3
	deoxycholate	51 ± 2	8 ± 2	60 ± 5	46	11 ± 2	32 ± 2	40 ± 5
37° C	none	100	100	100	100	100	65 ± 3	100
	glycocholate	23 ± 4	4 ± 4	37 ± 10	53 ± 7	8 ± 1	37 ± 2	56 ± 4
	taurocholate	15 ± 3	3 ± 3	23 ± 3	55 ± 3	1 ± 1	37 ± 2	56 ± 4
	cholate	14 ± 5	0 ± 0	20 ± 4	47 ± 3	3 ± 3	30 ± 4	47 ± 5
	deoxycholate	12 ± 5	0 ± 0	9 ± 0	26 ± 1	0 ± 0	25 ± 3	38 ± 6

was removed faster than alkaline phosphatase. The release of L-leucyl-β-naphthylamidase was similar to alkaline phosphatase at 0 and less effective at 37° C.

No phospholipid was lost in the absence of detergent. All the bile salts removed more phospholipid at 37 than at 0° C. Deoxycholate was the most effective bile salt at both temperatures, but at 37° C phospholipid removal was essentially complete for deoxycholate, cholate and taurocholate, within the accuracy of the methods used at these low phospholipid levels.

Effect of bile salts on morphology

From the results above it is clear that not all the protein has been solubilized even though there was extensive, at 0° C, and essentially complete, at 37° C, removal of phospholipid in some cases. The morphology of these lipid-depleted materials has been studied.

Pellets obtained at 14 000 × *g* for 2 min gave, by electron microscopy, a better representation of the detergent-treated material than pellets obtained at 100 000 × *g* for 60 min; the tight packing of the detergent-treated specimens in the latter case made visualization and identification difficult. The lower-speed (14 000 × *g*, for 2 min) pellets contained strips of "membranes" and vesicles. When the supernatants of such pellets were then centrifuged at 100 000 × *g* for 60 min, they yielded only small pellets (≈5% of the protein of the original), which contained mainly granular material; it is therefore safe to assume that the low speed material gives a fair representation of the residue remaining after bile salt treatment. Moreover, the electron micrographic sections were cut to be representative of the material in the pellet; low power scans were then conducted to establish representative areas of the material which were then photographed.

The original membrane preparation contained membranes in the form of long strips, some joined together by desmosomes, bile canaliculi and vesicles of various sizes. Incubation at 0° C, or saline-washing at 0° C, had only minimal effects upon the morphology of the preparation but incubation at 37° C caused the preparation to undergo fragmentation and vesiculation (Fig. 2a). This therefore is the control against which detergent-treated at 37° C must be judged.

Examination of the pellets obtained after extensive treatment with cholate, taurocholate or glycocholate showed that the predominant material in the residues had a "membrane-like" appearance. This "membranous" material was largely present as vesicles of various sizes and small strips (Fig. 2b,c). Material treated at 0° C showed some longer strips and larger vesicles, though few structures resembling the overall shape or dimensions of bile canaliculi could be seen. Occasionally desmosomes and structures resembling the nexus could be seen.

The material resulting from deoxycholate treatment, either at 0 or at 37° C (Fig. 2d), was mostly composed of granular material which was largely unorganised, though in some areas of the electron-micrograph the granular material appears to form faint lines, suggesting the previous existence of a membrane. Occasionally structures resembling desmosomes and the nexus could be seen, these were more prominant in the material treated at 0 than at 37° C.

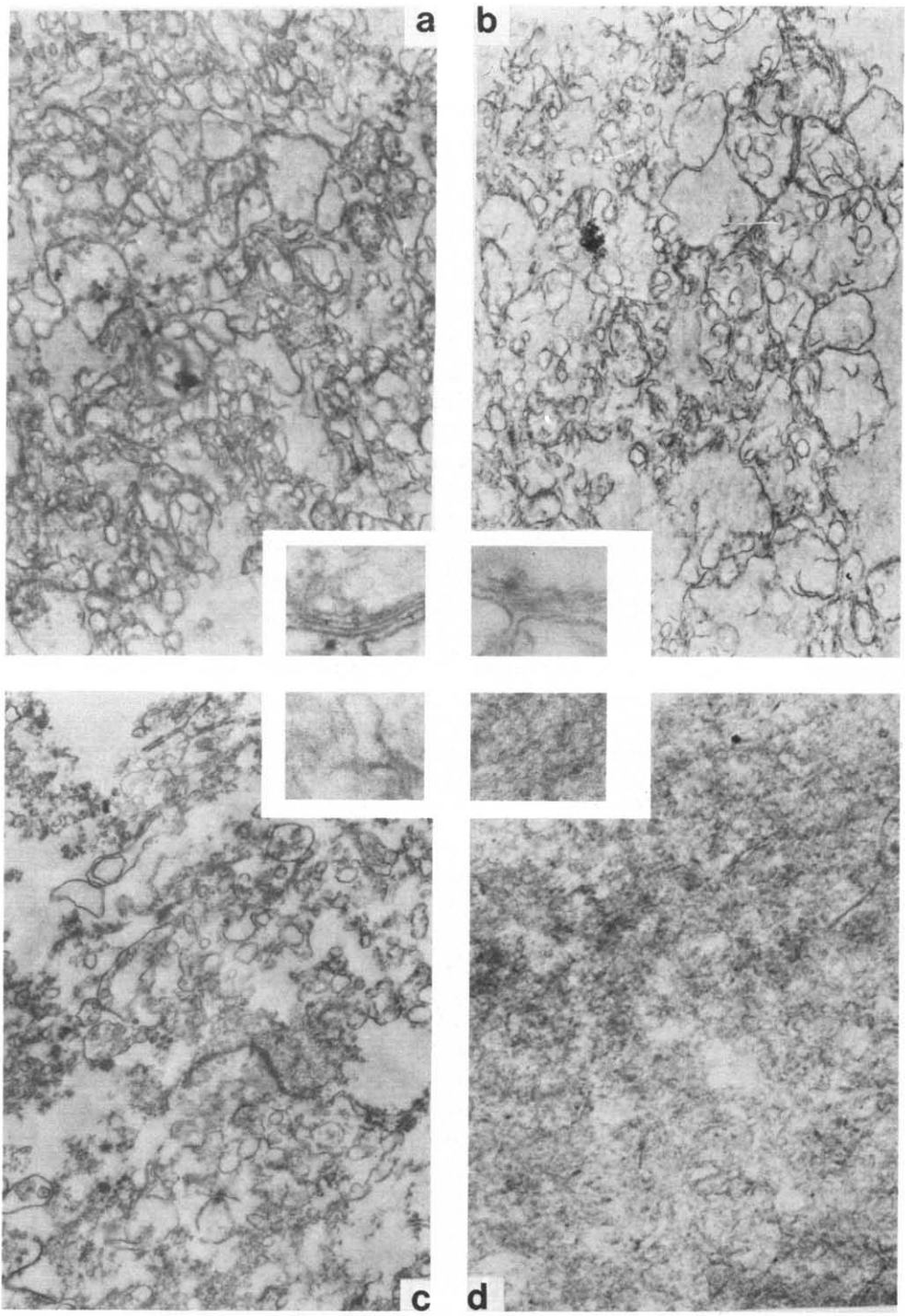

Fig. 2. Effect of detergents at 37° C. (a) Control, 37° C, 10 min; (b) taurocholate, 20 mM, 37° C, 10 min; (c) cholate, 20 mM, 37° C, 10 min; (d) deoxycholate, 5 mM, 37° C, 10 min. All preparations were saline washed before detergent treatment. The magnification of all specimens is ×17 000; the insets show the detail of the residual "membranes" and are magnified ×76 000.

Discussion

The proteins released during detergent-free incubation from the saline-washed preparation probably represent intravesicular or loosely associated membrane proteins, since their loss was not accompanied by the loss of membrane phospholipids. The further loss of protein which occurred as a result of the action of the bile salts was accompanied by the removal of membrane phospholipids indicating greater penturbation of the hydrophobic region of the membrane.

Cholate and its conjugates left behind material which still retained a membrane-like morphology. In the case of material treated at 0° there was still an appreciable amount of phospholipid in the preparation and the "membranes" therefore could involve a contribution of phospholipid. Membrane-like profiles were predominant features of the preparation even after the more extensive phospholipid removal at 37° C; the phospholipid levels had been reduced to as low as 1% of the original. Membrane-like trilamellar profiles have also been observed in other extensively lipid-depleted systems such as acetone-treated mitochondria, [23], solvent-extracted sciatic nerve myelin [24] and cholate-extracted erythrocyte membranes [7].

Deoxycholate appeared to have a far more drastic effect upon the membrane preparation than the cholate group. The loss of membrane morphology may have been due to either extraction of further proteins, or to marginally better removal of phospholipids, or to a greater interaction with some of the intrinsic membrane proteins. Such effects may be due to the lower hydrophilic character of the dihydroxy bile salt compared to the trihydroxy bile salts. This difference in relative hydrophilic character is probably indicated by the concentrations of bile salt at which phospholipid began to be removed.

Previous work on the effects of detergents upon liver plasma membranes has been restricted to deoxycholate alone [25—31], synthetic detergents [27,29, 30, 32,33] or both together, and usually at temperatures lower than 37° C. In these studies it was shown that at temperatures ranging from 0 to 23° C, 1% deoxycholate removed 70—80% of protein together with 70—100% of the phospholipids and 70—80% of the 5′-nucleotidase activity. Human [25] and mouse [30] liver plasma membranes behaved in essentially similar fashion to those of the rat. The residues gave morphological results in general accord to those observed here for deoxycholate-treated material [25,26,28].

It appears from the present study and from that of a similar one with erythrocyte membranes [7], therefore, that cholate and its conjugates are much milder in their effects upon membranes than is deoxycholate. The ability of the cholate group to solubilize several membrane-bound enzymes without causing the loss of a membrane-like profile may be related to the relative depth of insertion of these glycoprotein ectoenzymes into the hydrophobic region of the lipid layer [34,35]. It may be that inserted proteins can be removed along with the phospholipids but that spanning proteins are more resistant to solubilization by cholate [36—38]. These same enzymes occur in the bile of many mammalian species during bile salt secretion, in the absence of evidence of more extensive membrane disruption [6]. This is discussed more fully elsewhere [37, 39].

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