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The Effect of Bile Salts and some Bile-Salt Analogues on the Oxidation of Cholesterol by Liver Mitochondria

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Conjugated hydroxycholanic acids (bile salts) are the products of cholesterol catabolism in mammalian liver. Normally, most of the bile-salt output of the liver is returned to the liver after passage through the bile duct and absorption from the small intestine. If this enterohepatic circulation is interrupted by cannulation of the bile duct and removal of the bile, the daily bile-salt production in the rat is considerably increased (Thompson & Vars, 1953; Eriksson, 1957). Bergström & Danielsson (1958) showed that the output of bile salts through a cannula inserted in the upper half (i.e. proximal to the liver) of a bile duct ligated in the middle, was greatly reduced when a solution of bile salt was infused into the small intestine via the lower half of the duct. They therefore concluded that the concentration of bile salts supplied to the liver via the portal blood influences the rate of synthesis of bile salts in the liver. Further evidence for the operation of a 'negative-feedback' mechanism in this system was obtained by Beher & Baker (1958*a*, *b*) and Beher, Baker & Anthony (1959), who showed that feeding of bile acids to rats and mice reduces the rate of mobilization (and also the rate of synthesis) of liver cholesterol.

Conversion of cholesterol into bile acids has not yet been conclusively demonstrated *in vitro*. However, one step in the series of reactions must be the removal of the terminal isopropyl group of the cholesterol side chain. Oxidation of the terminal methyl groups to carbon dioxide by rat-liver slices *in vitro* was observed by Meier, Siperstein & Chaikoff (1952). Subsequently it was shown that this oxidation was carried out by liver mitochondria in the presence of a soluble cofactor ('supernatant factor') (Anfinsen & Horning, 1953; Whitehouse, Staple & Gurin, 1959) and was inhibited by the addition of taurocholate or glycocholate (Whitehouse & Staple, 1959).

Bile salts are surface-active agents and at high concentrations promote mitochondrial lysis. It is therefore questionable whether this inhibition is really a negative-feedback effect, since it could also be the consequence of impaired metabolism in subcellular particles due to the surface-active properties of bile salts. The results of further investigations into the effects on cholesterol oxidation *in vitro* of a range of natural bile salts and some bile-salt analogues are presented in this paper.

A preliminary account of this work has been published (Lee & Whitehouse, 1963).

EXPERIMENTAL

Materials. Sources of supply for special materials were as follows: [26-^{14}C]cholesterol, sodium [1-^{14}C]octanoate and sodium [2-^{14}C]propionate (The Radiochemical Centre, Amersham, Bucks.); Tween 20 (Atlas Powder Co., Wilmington, Delaware, U.S.A.); crystalline bovine serum albumin (C.S.I.R.O., Australia; given by Dr F. J. Hird, University of Melbourne); Asolectin (purified soya phosphatides, Associated Concentrates Inc., Long Island, N.Y., U.S.A.); polyethylene glycol (mol.wt. 1540; L. Light and Co. Ltd., Colnbrook, Bucks.); ATP (disodium salt), AMP, GSH and NAD (Sigma Chemical Co., St Louis, Mo., U.S.A.); Cab-o-Sil (thixotropic gelling agent) (Packard Instruments Ltd., Wembley, Middx.); various cholanic acids (British Drug Houses Ltd., Poole, Dorset; California Corp. for Biochemical Research, Los Angeles, U.S.A.; L. Light and Co. Ltd.; Mann Research Laboratories Inc., New York, U.S.A.; Zori Pharmaceutical and Chemical Industrial Co. Ltd., Tel Aviv, Israel).

Isolation of liver mitochondria. Suspensions of mitochondria in aq. 10% (w/v) sucrose were prepared from livers of 3- to 4-month-old white mice (Swiss Hygienic strain), 2- to 3-month-old Wistar rats or 2- to 3-month-old

albino guinea pigs, according to the procedure of Whitehouse *et al.* (1959), modified as follows to increase the yield of mitochondria. After the first centrifuging of the liver homogenate at 600*g* the supernatant was decanted, and the residue of unbroken cells, cell debris and nuclei resuspended in 10% sucrose and recentrifuged at 600*g* for 10 min. Mitochondria were isolated from each of the supernatants by centrifuging at 8500*g* for 12 min., then combined and washed, centrifuged again and resuspended as described by Whitehouse *et al.* (1959).

Preparation of supernatant factor. The supernatant obtained after isolation of the mitochondria was centrifuged at 100 000*g* for 30 min. to remove microsomes. The supernatant from this operation was boiled and the precipitated protein removed by low-speed centrifuging and discarded.

Solubilization of cholesterol. (i) With Tween 20. Except where otherwise specified, [26-^{14}C]cholesterol emulsions were prepared with Tween 20 (polyoxyethylene sorbitan monolaurate). The cholesterol and Tween 20, in the ratio 1:10 by weight, were dissolved in the minimal volume of methanol, the methanol was evaporated at 40–50° and the residue dissolved in the appropriate volume of warm 0·25M-tris–hydrochloride buffer, pH 8·5. The resulting suspension contained 4 μg. of [26-^{14}C]cholesterol and 40 μg. of Tween 20/ml.

(ii) With serum albumin. [26-^{14}C]Cholesterol was dissolved in 95% ethanol to give a solution containing 80 μg. of cholesterol/ml.; 1 vol. of this was diluted with 19 vol. of 1% (w/v) bovine serum albumin in water.

(iii) With phospholipid (Fleischer & Brierley, 1961). Asolectin (purified soya phosphatides) was extracted with acetone for 18 hr. in a Soxhlet apparatus to remove sterols. A quantity (5 mg.) of the purified Asolectin was dissolved in 1 ml. of ether and added to a solution of 8 μg. of [26-^{14}C]-cholesterol in 1 ml. of ether. The mixture was transferred to a Potter homogenizer and the ether evaporated. The residue was suspended with homogenization in tris–hydrochloride buffer, pH 8·5, and the suspension was exposed to sonic irradiation (20 kcyc./sec.) for 5 min. The mixture was then centrifuged at 80 000*g* for 30 min. and the supernatant was drawn off for use.

(iv) With polyethylene glycol. [26-^{14}C]Cholesterol and polyethylene glycol, mol.wt. 1540, in the ratio 1:10 by wt. were dissolved in the minimal volume of methanol, the methanol was evaporated at 40–50° and the residue dissolved in the appropriate volume of warm tris–hydrochloride buffer, pH 8·5.

(iv) With ethanol. [26-^{14}C]Cholesterol was dissolved in 95% ethanol to give a solution containing 80 μg. of cholesterol/ml.; 1 vol. of this was diluted with 19 vol. of tris–hydrochloride buffer, pH 8·5. All solubilized [26-^{14}C]-cholesterol preparations had a radioactivity approx. 250 μmc/ml.

Other substrates. Sodium [1-^{14}C]octanoate and sodium [2-^{14}C]propionate were made up in tris–hydrochloride buffer, pH 8·5, to radioactivities 120 and 250 μmc/ml. respectively.

Cholanic acid conjugates. All conjugates used were synthesized in our Laboratory by the method of Norman (1955), and recrystallized until free of unconjugated bile acids, as shown by thin-layer chromatography with the solvent system propionic acid–isoamyl acetate–water–propan-1-ol (3:4:1:2, by vol.; Hofmann, 1962).

Details are given in Table 1 of seven new compounds synthesized by this method. The β-alanine and aminomethanesulphonic acid conjugates were synthesized by substituting these amino acids for glycine and taurine respectively in Norman's procedure. 4-Methylpentanoyltaurine was prepared from isocaproic acid and taurine.

Incubations. These were conducted in 25 ml. conical flasks fitted with 0·6 ml. centre wells. The centre wells contained 0·2 ml. of N-sodium hydroxide. The main compartments of the flasks contained: 0·5 ml. of 0·25M-tris–hydrochloride buffer, pH 8·5; 0·5 ml. of supernatant factor; 0·1 ml. of ^{14}C-labelled substrate solution; 0·6 ml. of a solution containing 0·2 mg. of citric acid, 8·0 mg. of disodium ATP, 1·5 mg. of NAD, 3·0 mg. of magnesium chloride hexahydrate, 2·0 mg. of AMP and 5·0 mg. of GSH; bile salts dissolved in 0·3 ml. of buffer, or 0·3 ml. of

Table 1. *Properties and analyses of new conjugates synthesized*

Compound	Recrystallized from	m.p.	R_F (Hofmann system: cholic acid R_F 0·67)		Analysis (%) C	H	N	S
N-Cholyl-β-alanine	Ethanol–light petroleum (b.p. 60–80°)	206–207°	0·37	Found	67·0	9·4	3·0	—
				Calc. for $C_{27}H_{45}NO_6$	67·6	9·4	2·9	—
N-Deoxycholyl-β-alanine	Acetone	192	0·62	Found	69·9	9·6	3·0	—
				Calc. for $C_{27}H_{45}NO_5$	69·9	9·7	3·0	—
Taurohyodeoxycholic acid	Ethanol–ethyl acetate	145–155	0·10	Found	—	—	2·7	—
				Calc. for $C_{26}H_{45}NO_6S$	—	—	2·8	—
Tauro-3β-hydroxy-allocholanic acid	Ethanol	199–202	0·16	Found	—	—	2·8	—
				Calc. for $C_{26}H_{45}NO_5S$	—	—	2·9	—
N-Cholylaminomethane-sulphonic acid	Ethanol–ethyl acetate	165–170	0·05	Found	—	—	2·8	—
				Calc. for $C_{25}H_{43}NO_7S$	—	—	2·8	—
N-Deoxycholylamino-methanesulphonic acid	Ethanol–ethyl acetate	175–180	0·16	Found	60·5	9·1	3·0	6·6
				Calc. for $C_{25}H_{43}NO_6S$	61·8	8·9	2·9	6·6
4-Methylpentanoyl-taurine	Ethanol–ethyl acetate	226–232	—	Found	36·6	6·6	5·3	12·5
				Calc. for Na salt monohydrate $C_8H_{18}NO_5SNa$	36·5	6·8	5·3	12·2

buffer alone in the control incubations. Mitochondria, isolated from two-thirds of a mouse liver, one-fifth of a rat liver or one-seventh of a guinea-pig liver and suspended in 0·5 ml. of 10% sucrose, were added finally to each flask. The flasks were then stoppered and shaken in a water bath at 37° for 6 hr.

At the end of the incubation period 0·2 ml. of 12N-sulphuric acid was added to each flask. Flasks were then shaken for 1 hr. at room temperature to displace dissolved carbon dioxide from the medium.

Assay. The radioactive carbon dioxide absorbed in the centre wells was assayed by direct scintillation-counting, with the Diotol phosphor system of Herberg (1960), which tolerates the addition of 6% of water without losing homogeneity, with the addition of 2% (w/v) of Cab-o-Sil as a suspending agent.

A portion (0·1 ml.) of the centre-well contents was added to 5 ml. of the Diotol–Cab-o-Sil mixture and the radioactivity was counted in an Ekco liquid scintillation counter. The volume of liquid remaining in the centre well was noted to determine what fraction had evaporated.

Measurements of turbidity. A portion (0·5 ml.) of the incubation mixture was withdrawn at the appropriate time and diluted to 2·5 ml. by the addition of 10% sucrose. The extinction was measured at 520 mμ in 1 cm. glass cells with a Hilger and Watts Uvispek spectrophotometer.

Partition of bile salt–methylene blue complexes between water and chloroform. A portion (1 ml.) of Roy's (1956) methylene blue reagent, pH 1·5, was added to 2 ml. of 0·1 mM-bile salt solution in a stoppered test tube. Chloroform (5 ml.) was added and the tube was shaken vigorously by hand for 30 sec. After centrifuging, the upper (aqueous) layer was removed and discarded. A portion (1 ml.) of the chloroform layer was added to 5 ml. of 95% ethanol, and E at 655 mμ was measured in 1 cm. glass cells against the chloroform layer from a water blank containing Roy's reagent but no bile salt.

RESULTS

Where no bile salts were added, the percentage conversion of the ^{14}C of the radioactive substrates into $^{14}CO_2$ by liver mitochondria was of the following order: [26-^{14}C]cholesterol, 20–30%; sodium [1-^{14}C]octanoate, 40–50%; sodium [2-^{14}C]-propionate, 75–80%.

The amounts of $^{14}CO_2$ evolved in duplicate incubations normally differed by less than 5%. The effects of adding bile salts to incubations are shown in the Tables in terms of $^{14}CO_2$ evolved expressed as a percentage of the $^{14}CO_2$ evolved in control incubations. The experimental variation in cholesterol oxidation in the presence of a given bile salt at a given concentration (relative to controls) was generally less than 10% in serial experiments.

Specific effect of bile salts on cholesterol oxidation. When bile salts were incubated together with mouse-liver mitochondria and various oxidizable radioactive substrates, it was found that cholesterol oxidation was inhibited, whereas propionate oxidation was hardly affected, and octanoate oxidation was actually stimulated (Table 2). Deoxycholic conjugates inhibited cholesterol oxidation to a greater degree than cholic conjugates.

Effect of different species. Table 3 compares the effects of bile salts on cholesterol oxidation by liver mitochondria of four species: mouse, rat, guinea pig and rabbit. Cholesterol oxidation by mitochondria from larger species was less susceptible to a given concentration of added bile salt than oxidation by mouse-liver mitochondria.

Effect of different methods of solubilizing cholesterol. The addition of bile salts might have inhibited cholesterol oxidation in these experiments *in vitro* simply by altering the accessibility of the emulsified radioactive cholesterol to the mitochondrial cholesterol oxidase, such as by competing with Tween and forming less accessible cholesterol-containing micelles. Several alternative methods of solubilizing cholesterol were therefore tried, with serum albumin, phospholipid, polyethylene glycol (mol. wt. 1540) or ethanol.

Table 4 shows the effect of some bile salts on oxidation of these different cholesterol preparations by mouse-liver mitochondria. The results demonstrate that bile salts inhibit cholesterol

Table 2. *Effect of bile salts on the oxidation of* [26-^{14}C]*cholesterol,* [1-^{14}C]*octanoate and* [2-^{14}C]-*propionate by fortified mouse-liver mitochondria*

Bile salt concentration: 0·5 mM. $^{14}CO_2$ evolved is expressed as a percentage of $^{14}CO_2$ evolved in control incubations (no bile salt added).

	$^{14}CO_2$ evolved (%)		
Additions	From cholesterol	From octanoate	From propionate
None	100	100	100
Glycocholate	68	113	98
Taurocholate	65	113	98
Glycodeoxycholate	0	114	94
Taurodeoxycholate	6	125	96
Taurochenodeoxycholate	12	—	99

Table 3. *Effect of bile salts on the oxidation of* [26-^{14}C]*cholesterol by liver mitochondria of mouse, rat, guinea pig and rabbit*

Bile salt concentration: 0·5 mM. $^{14}CO_2$ evolved is expressed as a percentage of $^{14}CO_2$ evolved in control incubations (no bile salt added).

	$^{14}CO_2$ evolved (%)			
Source of liver mitochondria ...	Mouse	Rat	Guinea pig	Rabbit
Additions				
None	100	100	100	100
Glycocholate	68	71	69	92
Taurocholate	65	84	62	88
Glycodeoxycholate	0	35	41	50
Taurodeoxycholate	6	36	42	53

Table 4. *Effect of bile salts on the oxidation of* [26-^{14}C]*cholesterol solubilized by five different methods*

Bile salt concentration: 1 mM. Mouse-liver mitochondria were used for these experiments. For details of solubilizing procedures see the Experimental section. $^{14}CO_2$ evolved is expressed as a percentage of $^{14}CO_2$ evolved in control incubations (no bile salt added).

	$^{14}CO_2$ evolved (%)				
Solubilizing agent	Tween 20	Serum albumin	Phospho-lipid	Polyethylene glycol	Ethanol
Bile salt					
None	100	100	100	100	100
Glycocholate	41	28	21	16	19
Taurocholate	38	34	28	23	24
Glycodeoxycholate	0	0	1	5	4
Taurodeoxycholate	1	1	1	5	3

Table 5. *Comparison of bile salts with some bile-salt analogues in their effect on the oxidation of* [26-^{14}C]*cholesterol by mouse-liver mitochondria*

Concentration of bile salts and analogues: 0·2 mM. $^{14}CO_2$ evolved is expressed as a percentage of $^{14}CO_2$ evolved in control incubations.

Bile salt	$^{14}CO_2$ evolved (%)	Bile salt analogue	$^{14}CO_2$ evolved (%)
None	100	None	100
Glycocholate	72	Cholyl-β-alanine	79
Taurocholate	80	Cholylaminomethanesulphonate	79
Glycodeoxycholate	30	Deoxycholyl-β-alanine	24
Taurodeoxycholate	24	Deoxycholylaminomethanesulphonate	23
Taurolithocholate	1	Tauro-3β-hydroxyallocholanate	1

Table 6. *Effect of taurine conjugates on the oxidation of* [26-^{14}C]*cholesterol by mouse-liver mitochondria*

$^{14}CO_2$ evolved is expressed as a percentage of $^{14}CO_2$ evolved in control incubation (no bile salt added).

Additions	No. of nuclear oxygen substituents	$^{14}CO_2$ evolved (%) 0·5 mM-Taurine conjugates	0·2 mM-Taurine conjugates
None	—	100	100
Taurocholate	3	65	80
Taurodehydrocholate	3	61	—
Taurodeoxycholate	2	6	22
Taurochenodeoxycholate	2	12	—
Taurohyodeoxycholate	2	14	—
Taurolithocholate	1	0	1
Taurocholanate	0	1	2
4-Methylpentanoyltaurine	—	96	—

Table 7. *Partition of bile salts between water and chloroform*

Results are measured as E at 655mμ of co-partitioned methylene blue (see Experimental section) in the chloroform phase.

Solute	E_{655}
Taurocholate	0·01
Taurodehydrocholate	0·31
Taurodeoxycholate	0·30
Taurochenodeoxycholate	0·29
Taurolithocholate	0·61
Lauryl sulphate	0·58

oxidation regardless of the method used to solubilize the cholesterol.

Relation of structure to inhibitory activity of bile salts and their analogues. The following 'unnatural' bile salts were synthesized to test whether inhibition by bile salts was dependent on the nature of the conjugated amino acid: *N*-cholyl-β-alanine, *N*-cholylaminomethanesulphonic acid, *N*-deoxycholyl-β-alanine and *N*-deoxycholylaminomethanesulphonic acid. In addition, the taurine conjugate of 3β-hydroxyallocholanic acid was prepared to determine whether inhibitory activity was dependent on the configuration of the A/B ring junction. The results (Table 5) show no significant differences between the activities of the corresponding 'natural' and 'unnatural' compounds. The ability to inhibit cholesterol oxidation increases as the number of oxygen substituents on the nucleus is decreased (Table 6).

Some experiments were carried out to determine whether the keto groups of taurodehydrocholate

Table 8. *Comparison of effects of bile salts and sodium lauryl sulphate on the oxidation of* [26-^{14}C]*cholesterol and on mitochondrial swelling*

Mouse-liver mitochondria were used in this experiment. *E*, Percentage of extinction of controls (no bile salt added). $^{14}CO_2$ evolved is expressed as a percentage of $^{14}CO_2$ evolved from controls.

	Taurodeoxycholate		Taurolithocholate		Lauryl sulphate	
Concn. (mM)	*E* (%)	$^{14}CO_2$ evolved (%)	*E* (%)	$^{14}CO_2$ evolved (%)	*E* (%)	$^{14}CO_2$ evolved (%)
0	100	100	100	100	100	100
0·1	90	55	39	6	45	35
0·2	84	22	35	1	33	9
0·3	41	6	34	0	30	5
0·5	35	1	31	0	30	2

were reduced by mitochondria under the conditions required for cholesterol oxidation. No reduction products could be detected. 4-Methylpentanoyltaurine, which contains the side chain of a taurine-conjugated bile acid, did not inhibit cholesterol oxidation.

Partition studies. A possible explanation of the greater inhibitory effect of the bile salts with fewer nuclear substituents is that these compounds, being less hydrophilic, would enter the mitochondria more easily. A measure of the differences in hydrophilic character of a number of bile salts was obtained by adapting Roy's (1956) method for determining steroid sulphates. The partition of bile-salt anions between choroform and water at pH 1·5 was measured by adding methylene blue and determining colorimetrically the quantity of methylene blue extracted into the organic phase. The results (Table 7) show a definite decrease in hydrophilic character with loss of hydroxyl groups.

Effect of bile salts on mitochondria. Table 8 presents the results of experiments in which the flask contents were sampled for measurements of *E* at the end of the incubation period, to compare the effect of bile salts (and a typical anionic detergent, sodium lauryl sulphate) on cholesterol oxidation with their observable physical effect (swelling or possibly lysis) on the mitochondria (Lehninger, 1959). Extinction and cholesterol-oxidation values are expressed as percentages of the values for controls.

These results show that a non-steroid detergent, sodium lauryl sulphate, selectively inhibits cholesterol oxidation at the same concentrations as do bile salts (octanoate oxidation was not inhibited by lauryl sulphate at 0·5 mM). Inhibition of cholesterol oxidation was accompanied by decrease in *E* of the mitochondrial suspension, indicative of mitochondrial swelling. At a given concentration, taurodeoxycholate has less physical effect on mitochondria than taurolithocholate or lauryl sulphate.

DISCUSSION

These studies were restricted to bile acid conjugates, since any free bile acids present in the enterohepatic circulation are normally rapidly conjugated with glycine or taurine on passage through the liver *in vivo*.

According to Olivecrona & Sjövall (1959), the concentration of bile acids in rat portal blood is about 2 mg./100 ml., or of the order 0·05 mM. The concentration within the liver cells responsible for absorption of bile salts from the blood, and secretion of bile salts into the bile, would very probably be rather higher than this, so that the concentrations at which bile salts have been used in our experiments (0·1–0·5 mM) would appear to be physiologically realistic for experiments with small animals.

The bile salts tested in these experiments, when added at 0·1–0·5 mM, were found to inhibit oxidation of the cholesterol side chains by liver mitochondria *in vitro*. Those tested at the same concentration in incubations with octanoate or propionate as substrate did not inhibit the oxidation of these two compounds. Propionate has been implicated as an intermediate in the oxidation to carbon dioxide of the terminal three carbon atoms of at least one sterol (Suld, Staple & Gurin, 1962). Hence these results indicate that the bile salts probably inhibit some transformation of the cholesterol molecule preceding either the cleavage from the sterol side chain or the oxidation of a C_3 fragment.

Most of the experimental work was carried out with liver mitochondria from mice, but where other species (i.e. rats, guinea pigs, rabbits) were used, inhibition of cholesterol oxidation by bile salts was also observed. In all four species the deoxycholates were more powerful inhibitors than the cholates. However, the ratio for cholesterol oxidation (oxidation in the presence of 0·5 mM-taurocholate/oxidation in the presence of 0·5 mM-taurodeoxy-

cholate) ranges from 11 in the mouse system to 1·5 in the guinea-pig system (see Table 3).

Inhibition by bile salts was observed with cholesterol emulsions prepared by five different procedures, including the use of two 'natural' solubilizers of cholesterol (serum albumin and phospholipid). This suggests that *in vitro* the bile salts exert their inhibitory effect upon the mitochondria rather than rendering the added radioactive cholesterol less accessible to the mitochondrial cholesterol-oxidase system.

Relation between structure and inhibitory activity of bile salts

This can be considered in two parts, concerning the side chains and the nucleus (plus substituents) respectively.

Differences in inhibitory power of glycine and taurine conjugates of a given bile acid were generally small. Further alteration of the side chain, by preparing conjugates with β-alanine ('homo'-glycine) and aminomethanesulphonic acid ('nor'-taurine) had little effect on the inhibitory activity of a given cholanic acid (Table 5). It therefore seems unlikely that the length of the side chain is critical for the binding of a bile salt to a specific enzyme site, for in that case lengthening or shortening the chain might be expected to alter the inhibitory activity.

That the side chain on its own is not responsible for inhibition of cholesterol oxidation is demonstrated by the inactivity of 4-methylpentanoyltaurine, which can be regarded as the side chain plus C-17 of a taurine-conjugated bile acid (Table 6).

Alteration of the configuration at position 5 of the steroid nucleus (and the configuration of the 3-hydroxyl group) produced no change in inhibitory activity: the taurine conjugate of 3β-hydroxyallocholanic acid possessed the same activity (at 0·2 mM) as the taurine conjugate of 3α-hydroxycholanic acid (lithocholic acid) (Table 5). However, comparison of the inhibitory activities of seven taurine-conjugated bile acids of the common 5β-configuration (at 0·5 mM) with different numbers and arrangements of oxygen substituents on the nucleus (Table 6) shows that activity increases with decreasing number of such substituents. This increase is particularly striking on passing from the two trisubstituted compounds, which inhibit 35–39 % of oxidation, to the group of three disubstituted compounds, inhibiting 86–94 %. Taurolithocholate, with only one hydroxyl group, is in turn more potent than the dihydroxy compounds. This difference is more obvious when the activities of taurodeoxycholate and taurolithocholate are compared at 0·1 mM (see Table 8).

The comparatively low potency of the trisubstituted compounds could be one reason for the primary bile acids in very many species being trihydroxy acids. In contrast, the highly potent lithocholic acid is found only in traces and in a few species, and then probably as the result of the action of intestinal micro-organisms (Haslewood, 1962).

Mouse and rat livers contain a 7α-hydroxylase system that rapidly converts taurodeoxycholate into taurocholate (Bergström & Gloor, 1955; Danielsson & Kazuno, 1959). This could be a protective mechanism, in view of our findings that deoxycholates are much more potent inhibitors of mitochondrial oxidation of cholesterol than are cholates.

The increasing potency with loss of hydroxyl groups could be due to greater ability of the bile salts to enter mitochondria. The experiment with chloroform extraction of aqueous solutions of bile salt–methylene blue complexes (Table 7) indicates a progressive loss of hydrophilic character with loss of hydroxyl groups. Attempts to use a natural lipid (e.g. olive oil) or oleyl alcohol as an extractant instead of chloroform, to gain a better idea of the partition of bile salts between aqueous and lipid phases, were unsuccessful.

Possible changes in mitochondrial structure induced by bile salts

The contents of flasks in which mitochondria had been incubated with taurodeoxycholate often appeared less turbid than those of control flasks, or in flasks in which taurocholate was present. Since this probably indicated swelling or even lysis of mitochondria due to the surface activity of taurodeoxycholate, experiments were conducted to correlate this effect with inhibition of cholesterol oxidation, and to discover whether a non-steroid detergent would inhibit cholesterol oxidation. It was found (see Table 8) that increasing concentration of bile salts not only increased inhibition, but that this increase was closely associated with the magnitude of the decrease in E of the mitochondria. The anionic detergents, sodium lauryl sulphate and Manoxol OT (sodium dioctyl sulphosuccinate), produced rather similar results. Thus one possible explanation of the inhibition of cholesterol oxidation by bile salts *in vitro* could be that these compounds cause structural changes in the mitochondria such that the complex of enzymes necessary for oxidation of cholesterol is disrupted, whereas other enzymes required for octanoate oxidation, for example, remain intact.

On the other hand, at a given value of E, e.g. 45 % of control, the inhibition of cholesterol oxidation by taurodeoxycholate or taurolithocholate is greater than the inhibition by lauryl sulphate. This could be evidence for a fairly specific inhibition of the cholesterol-oxidase system by the bile salts as opposed to the lauryl sulphate.

Should this inhibition of cholesterol oxidation by bile salts *in vitro* be due entirely to physical disorganization of mitochondrial structure, it is conceivable that this might occur *in vivo* when there are high concentrations of circulating bile salts. If so, the apparent inhibition of new synthesis of bile salts from cholesterol by bile salts in the enterohepatic circulation would not then be a specific negative feedback as generally understood (Umbarger, 1961).

SUMMARY

1. The following new compounds were synthesized: taurohyodeoxycholic acid, tauro-3β-hydroxyallocholanic acid, *N*-cholyl-β-alanine, *N*-deoxycholyl-β-alanine, *N*-cholylaminomethanesulphonic acid, *N*-deoxycholylaminomethanesulphonic acid, 4-methylpentanoyltaurine.

2. The effect of these compounds and of taurine and glycine conjugates of natural bile (cholanic) acids on the oxidation of cholesterol, octanoate and propionate *in vitro* by isolated liver mitochondria has been studied.

3. Cholesterol oxidation by rat- and mouse-liver mitochondria was more sensitive to added bile salts than was oxidation by rabbit- and guinea-pig-liver mitochondria.

4. Cholanic acid conjugates selectively inhibited cholesterol oxidation. The degree of inhibition increased as the number of oxygen functions on the steroid nucleus was decreased, but was independent of the configuration at C-5 (A/B ring junction) or the conjugated amino acid.

5. Anionic detergents mimicked bile salts in selectively inhibiting cholesterol oxidation *in vitro*.

6. The relative lipophilic character of these anionic detergents and of various taurocholanates was estimated by partition between water and a lipid solvent (chloroform) in the presence of methylene blue.

7. The degree of mitochondrial swelling induced by inhibitors of cholesterol oxidation was measured optically.

8. The findings are discussed in relation to proposals that cholesterol oxidation to bile salts *in vivo* is subject to negative-feedback control.

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