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Species Differences in the Conjugation of Free Bile Acids with Taurine and Glycine

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Several investigations indicate that no free bile acids are normally found in the bile, but only acids conjugated with taurine and glycine (Haslewood & Sjövall, 1954; Norman, 1954). It is also known that the bile acids are reabsorbed mainly in the conjugated form from the intestine (Norman, 1955; Portman & Mann, 1955).

In different species the bile acids are found conjugated with taurine and glycine in various proportions (Haslewood & Sjövall, 1954; Haslewood, 1955). Thus rabbit and pig bile contain almost exclusively glycine-conjugated acids, whereas rat and bird bile contain only taurine-conjugated acids. In man and primates both conjugates are found in variable proportions.

Bergström & Gloor (1954) found that mainly taurine-conjugated acids were formed in human-liver homogenates when both taurine and glycine were available for conjugation with free bile acids. The same preference for taurine has been demonstrated in rat-liver homogenates (Bremer, 1955*a*). The enzyme system of rat liver performing the conjugation of free bile acids with taurine and glycine is confined to the microsomal particles of the liver cell (Bremer & Gloor, 1955; Bremer, 1956). The isolated microsomes perform this conjugation

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in the presence of adenosine triphosphate (ATP), coenzyme A and Mg^{2+} ions, probably with *S*-cholyl-coenzyme A (cholyl-*S*-Co A) as an intermediate (Bremer, 1956).

It is not known whether the species differences in the bile content of taurine- and glycine-conjugated acids are due to differences in the availability of taurine and glycine or to differences in the enzyme patterns of the livers.

In the present paper comparative studies on the conjugation of free bile acids with taurine and glycine are reported. The isolated microsomal fractions of rat, rabbit and chicken liver were used. The effect of conjugated bile acids on the conjugation has also been investigated. The results are discussed in relation to cholesterol catabolism.

EXPERIMENTAL

Materials. Taurine was labelled with ^{35}S according to Eldjarn (1951), and the synthesis was kindly performed by L. Eldjarn and P. Fritzson at this Institute. The [1-^{14}C]-glycine was obtained from the Joint Establishment for Nuclear-Energy Research, Norway. Crystalline ATP (potassium salt) and 75% pure coenzyme A were obtained from Nutritional Biochemicals Corporation, U.S.A. The pure bile acids were kindly supplied by Dr Arne Norman, University of Lund, Sweden.

Incubation experiments. The microsomal fractions from rat, rabbit and chicken liver were prepared as earlier described (Bremer, 1956). The isolated microsomes were suspended in 10% sucrose and pipetted as such. Microsomes from approximately 500 mg. of liver (0·3 ml. of suspension) were added to each vessel. The following concentrations were maintained during the incubation: potassium phosphate buffer (pH 7·4), 0·022M; ATP, 0·0067M; coenzyme A, 0·0001M (0·1 mg. of 75% pure coenzyme A/ml.); Mg^{2+}, 0·001M; potassium fluoride, 0·2M. Taurine, glycine and bile acids were added as stated under the individual experiments. Total volume per vessel was 1·5 ml., and incubation time was 90 min. at 37°. The reaction was stopped by immersing the vessels for 0·5 min. in boiling water, and the conjugated acids were extracted with ½ vol. of *n*-butanol as earlier described (Bremer, 1955*b*).

Estimation of amount of conjugated acids formed. The conjugation of the free bile acids was followed by means of the taurine labelled with ^{35}S and the [1-^{14}C]glycine as earlier described (Bremer, 1955*b*). No corrections for self-absorption were made. In the experiments in which the effect of adding conjugated bile acids was investigated (Figs. 2–5), these acids were extracted into the butanol. Control experiments showed that the resulting increase in self-absorption reduced the measured radioactivity of the butanol extracts by at most 10% when 10 μmoles of glycocholic acid were added to the incubation mixture before extraction. The inhibiting effect of the conjugated acids therefore could not be due to increased self-absorption in the radioactivity measurements.

To calculate the amounts formed of the respective conjugated acids, the partition coefficient of the different acids between butanol and water was determined as earlier described (Bremer, 1955*b*). The partition coefficients are shown in Table 1. From these coefficients and the radioactivity content of the butanol extracts, the formation of conjugated acids per vessel was calculated.

Identification of reaction products. The butanol extracts were chromatographed on paper according to Sjövall (1954). The spots on the paper were located by means of a strip counter, and the localization of the radioactivity was compared with the localization of spots obtained in parallel runs with pure synthetic conjugated bile acids. Fig. 1 shows that almost all the extractable radioactivity in the experiments with deoxycholic and cholic acid was located at the correct R_F values in the chromatograms. The identity of the reaction products in the experiments with cholanic and dehydrocholic acid was not verified with certainty.

RESULTS

Efficiency of taurine and of glycine as substrates

Table 2 shows that with rat-liver microsomes the conjugation of cholic acid with taurine was more than twice as efficient as the conjugation with

Table 1. *Extraction of different conjugated bile acids with* n-*butanol*

Portions of the butanol solutions (0·5 ml.) of biologically labelled conjugated bile acids were equilibrated with 1·5 ml. of 0·1M phosphate buffers. The total radioactivity of the butanol portions was assumed to be in the water phase before equilibration. The counts are given as duplicate determinations in this and other tables.

Acid	pH	Counts/min. in 0·1 ml. of H_2O before extraction	Counts/min. in 0·1 ml. of butanol after extraction	Partition coeff. butanol–H_2O	Concn. in butanol phase/initial concn. in H_2O
Glycocholic	1	169 176	510 454	~18	2·8
Glycocholic	6·5	169 176	440 417	~10	2·5
Glycocholic	7·2	169 176	426 415	~ 9	2·4
Glycocholic	8·0	169 176	457 399	~10	2·5
Glycodeoxycholic	7·2	1200 1190	3660 3775	~48	3·1
Glycodehydrocholic	7·2	296 281	780 707	~12	2·6
Glycocholanic	1	230 238	692 705	~23	3·0
Taurocholic	7·2	77 77	175 154	~ 6	2·1*
Taurodeoxycholic	7·2	232 231	694	~32	3·0
Taurodehydrocholic	7·2	49	122	~10	2·5

* Formerly found: 2·0 (Bremer, 1955*b*).

glycine. The conjugation with glycine was almost totally depressed when enough taurine was available, although addition of glycine did not significantly decrease the conjugation with taurine. These results confirm earlier work on the taurine–glycine relationship in rat-liver homogenates (Bremer, 1955*a*). Rat liver has been shown to contain considerable amounts of free taurine (Awapara, 1955; Bremer, 1955*b*). The results therefore seem to explain why rat bile contains almost exclusively taurine-conjugated acids (Norman, 1954).

A different picture was found with rabbit-liver microsomes. The conjugation with glycine was of the same order as in the rat, but only traces of taurine were conjugated. The glycine conjugation was not significantly depressed by added taurine. Awapara (1955) has reported that rabbit liver does not contain any detectable amounts of taurine. This

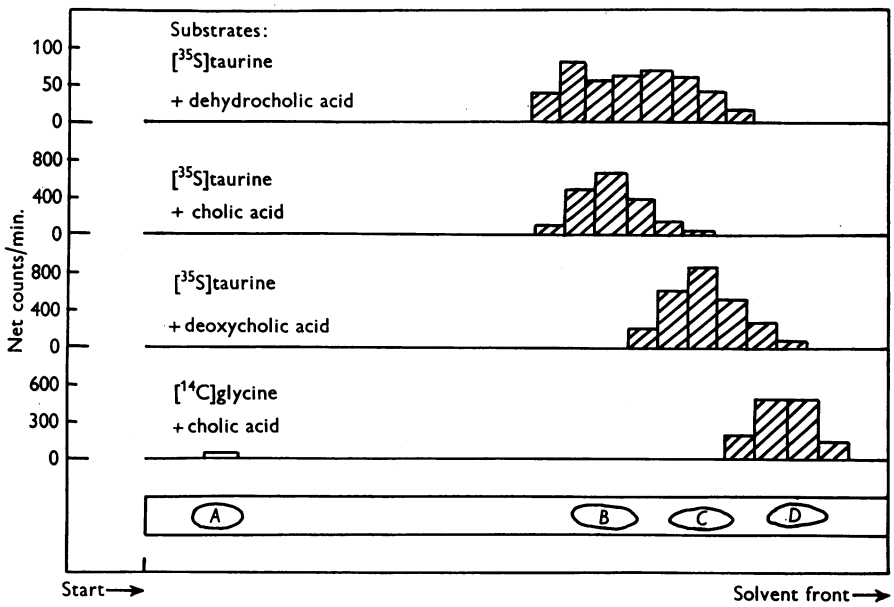

Fig. 1. Paper chromatographic identification of the radioactivity of the butanol extract. Volumes of 0·1 ml. were put on the paper. *A*, *B*, *C* and *D* show the positions of taurine (and glycine), taurocholic, taurodeoxycholic and of glycocholic acid respectively in the reference chromatograms.

Table 2. *Conjugation of cholic acid with taurine and glycine in the presence of rat-, rabbit- and chicken-liver microsomes*

Microsomes from approx. 500 mg. of liver were incubated in 0·022 M potassium phosphate buffer (pH 7·4) with potassium fluoride (0·2 M), magnesium sulphate (0·001 M), coenzyme A (0·15 μmole/vessel), ATP (10 μmoles/vessel), cholic acid (2 μmoles/vessel) and taurine and glycine (as tabulated). Total volume, 1·5 ml.; incubation time, 90 min. at 37°.

Animal	Taurine and glycine additions	Net counts/min./0·1 ml. of butanol extract		Cholic acid conjugated with [35S]taurine or with [14C]glycine (μmole)	
Rat	0·8 μmole of [35S]taurine = 17 400 counts/min./vessel	1445	1370	0·49	0·46
	0·8 μmole of [35S]taurine + 2·5 μmoles of inactive glycine	1170	1170	0·40	0·40
	1 μmole of [14C]glycine = 130 000 counts/min./vessel	4040	4280	0·18	0·19
	1 μmole of [14C]glycine + 2·5 μmoles of inactive taurine	330	290	0·02	0·01
Rabbit	0·8 μmole of [35S]taurine = 17 400 counts/min./vessel	53	60	0·02	0·02
	0·8 μmole of [35S]taurine + 2·5 μmoles of inactive glycine	27	34	0·01	0·01
	1 μmole of [14C]glycine = 130 000 counts/min./vessel	2840	2990	0·13	0·14
	1 μmole of [14C]glycine + 2·5 μmoles of inactive taurine	2575	2735	0·12	0·13
Chicken	0·8 μmole of [35S]taurine = 28 000 counts/min./vessel	125	139	0·03	0·03
	0·8 μmole of [35S]taurine + 2·5 μmoles of inactive glycine	118	139	0·03	0·03
	1 μmole of [14C]glycine = 230 000 counts/min./vessel	0	0	0	0
	1 μmole of [14C]glycine + 2·5 μmoles of inactive taurine	0	0	0	0

might explain why only traces of taurine-conjugated acids are found in rabbit bile (Ekdahl & Sjövall, 1955), but the present results indicate that the rabbit also lacks the specific enzyme for taurine conjugation.

With microsomes from chicken liver there was a small but significant formation of taurocholic acid. Haslewood & Sjövall (1954) have reported that no glycine-conjugated acids could be detected in bird's bile. The enzyme for glycine conjugation appears to be lacking in the chicken liver.

It is impossible to state whether the small activity found in chicken liver expresses a lower conjugating capacity in this species. The incubation conditions used have been found to be optimum for rat-liver microsomes, but they have not been systematically tested for chicken and rabbit liver. A different localization of the conjugating-enzyme system within the chicken-liver cell could not explain the low activity in the chicken experiment, as addition of particle-free supernatant or of mitochondria had no significantly stimulating effect.

Efficiency of different bile acids as substrates

The main bile acid of the rat is cholic acid, and deoxycholic acid is the main bile acid in the rabbit. It was of interest, therefore, to investigate whether the nature of the free bile acid might influence the speed of the conjugation. Table 3 shows that in the rat and in the rabbit there were no great differences in the amounts of conjugated acids obtained when cholic and deoxycholic acids were used as substrates; with trioxocholanic acid smaller amounts of conjugated acids were obtained. Table 3 also shows that cholanic acid with no substituents in the steroid nucleus may serve as substrate in the conjugation. The amounts of conjugated cholanic acid obtained cannot be compared with the amounts obtained with the other free bile acids, since cholanic acid is almost completely insoluble in water at pH 7·4. It was not possible to identify the reaction products, but in analogy with the behaviour of deoxycholic acid (Fig. 1) it may be presumed that no hydroxylation occurred.

Table 3. *Conjugation of different free bile acids with taurine and glycine in the presence of rat- and rabbit-liver microsomes*

Microsomes from approx. 500 mg. of liver were incubated in 0·022 M potassium phosphate buffer (pH 7·4) with potassium fluoride (0·2 M), magnesium sulphate (0·001 M), coenzyme A (0·15 μmole/vessel), ATP (10 μmoles/vessel), bile acid (2 μmoles/vessel) and [^{35}S]taurine (0·8 μmole/vessel = 17 400 c/min./vessel) or [^{14}C]glycine (1 μmole/vessel = 130 000 c/min./vessel). Total volume, 1·5 ml.; incubation time, 90 min. at 37°.

Animal	Substrates added	Net counts/min./0·1 ml. of butanol extract	Conjugated acids formed (μmole)
Rat	Cholic acid Taurine	862 865	0·30 0·30
	Deoxycholic acid Taurine	1050 1175	0·24 0·27
	Dehydrocholic acid Taurine	283 280	0·08 0·08
	Cholanic acid Taurine	79 80	0·02 0·02*
	Cholic acid Glycine	2125 2150	0·10 0·10
	Deoxycholic acid Glycine	3575 3680	0·13 0·14
	Dehydrocholic acid Glycine	800 885	0·04 0·04
	Cholanic acid Glycine	260 235	0·01 0·01
Rabbit	Cholic acid Glycine	1865 1795	0·09 0·08
	Deoxycholic acid Glycine	1205 2740	0·05 0·10
	Dehydrocholic acid Glycine	925 945	0·05 0·05
	Cholanic acid Glycine	575 575	0·02 0·02

* The relation $\frac{\text{concn. in butanol phase}}{\text{initial concn. in } H_2O}$ was assumed to be 3·0.

In the paper chromatogram (Fig. 1) an elongated spot of the reaction product of trioxocholanic acid and taurine was obtained. This spot was eluted from the chromatogram and the ultraviolet absorption spectrum in concentrated sulphuric acid was determined (Sjövall, 1955). The eluted compound (or compounds) gave a distinct absorption maximum at 305–310 mμ.; the trioxocholanic acid used did not give this absorption maximum. Keto bile acids do not give any characteristic absorption spectra in concentrated sulphuric acid, but hydroxy bile acids give absorption maxima at about 310 or 390 mμ. or both (Eriksson & Sjövall, 1955). These results indicate that a reduction of one or more keto groups in the trioxocholanic acid had taken place during the incubation. Such reductions of keto bile acids are known to take place in liver slices (Kataoka, 1941).

Influence of conjugated bile acids on conjugation

It has previously been found that taurocholate inhibits the conjugation of cholic acid with taurine in rat-liver homogenates (Gloor, 1954; Bremer & Gloor, 1955). Fig. 2 shows that glycocholate also inhibited the formation of taurocholic acid.

The conjugation presumably involves at least two steps with cholyl-S-Co A as an intermediate (Bremer, 1956; Elliott, 1955). Hydroxylamine is known to be a trapping agent of coenzyme A-activated carboxyl groups, and the formation of cholohydroxamic acid in the presence of hydroxylamine will therefore be a measure of the cholyl-*S*-Co A formation. Fig. 3 shows that the formation of cholohydroxamic acid was depressed by the added tauro- and glyco-cholic acids, indicating that the conjugated acids interfered with the first step of the reaction. The inhibition of hydroxamic acid formation also indicates that the transfer of the cholyl group from coenzyme A to taurine or glycine is irreversible, since in the presence of conjugated acids and coenzyme A a reversal of this transfer should lead to an increased formation of cholohydroxamic acid. Fig. 2 also shows that addition of cholic acid did not diminish the inhibiting effect of taurocholate.

Figs. 4 and 5 show the effect of coenzyme A on the taurocholate inhibition of the glycocholate synthesis. Curve *A* (Fig. 4) shows that taurocholate had the same inhibiting effect on the glycocholate synthesis as on the taurocholate synthesis (Fig. 2). Curve *B* shows that an increase of the coenzyme A concentration from 0·05 to 0·4 μmole/vessel did not increase the glycocholate synthesis, while curve *C* shows that the same increase of the coenzyme A concentration almost doubled the glycocholate formation in the presence of 1·5 μmoles of taurocholate. In Fig. 5 a similar experiment is plotted according to Hunter & Downs's (1945) method, which distinguishes between competitive and non-competitive inhibition. The product $I.\alpha/(1-\alpha)$ increased with increasing concentration of coenzyme A. These results indicate that taurocholate at least partly inhibited the formation of glycocholate by competing with coenzyme A.

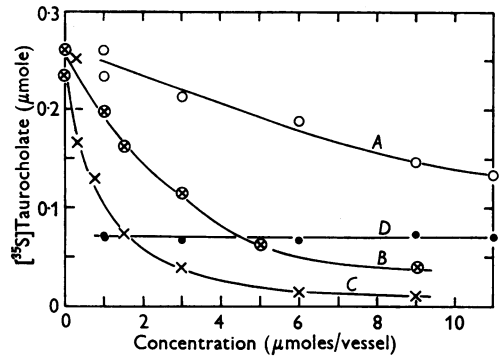

Fig. 2. Effect of increasing concentrations of taurocholate, glycocholate and of cholate on the formation of [^{35}S]-taurocholate. Microsomes from approx. 500 mg. of liver were incubated in 0·022M potassium phosphate buffer (pH 7·4) with potassium fluoride (0·2M), magnesium sulphate (0·001M), coenzyme A (0·1 μmole/vessel), ATP (10 μmoles/vessel), cholic acid (1 μmole/vessel) and [^{35}S]-taurine (0·8 μmole/vessel = 50 000 c/min./vessel). Curve *A*: 1–11 μmoles of cholic acid/vessel; curve *B*: 0–9 μmoles of glycocholic acid/vessel; curve *C*: 0–9 μmoles of taurocholic acid/vessel; curve *D*: 1·5 μmoles of taurocholic acid + 1–10 μmoles of cholic acid/vessel. Total volume, 1·5 ml.; incubation time, 90 min. at 37°.

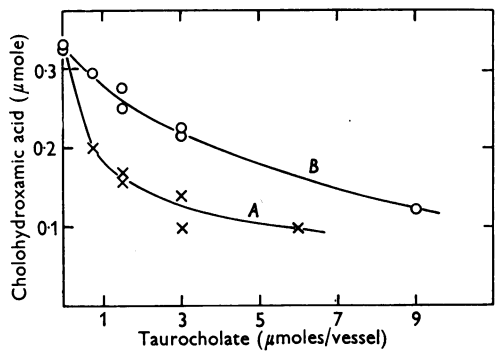

Fig. 3. Effect of increasing concentrations of taurocholate and of glycocholate on the formation of cholohydroxamic acid. Microsomes from approx. 500 mg. of liver were incubated in 0·022M potassium phosphate buffer (pH 7·4) with potassium fluoride (0·2M), magnesium sulphate (0·001M), coenzyme A (0·1 μmole/vessel), ATP (10 μmoles/vessel), cholic acid (1 μmole/vessel) and hydroxylamine hydrochloride (50 μmoles/vessel). Curve *A*: 0–6 μmoles of taurocholic acid/vessel; curve *B*: 0–9 μmoles of glycocholic acid/vessel. Total volume, 1·5 ml.; incubation time, 90 min. at 37°.

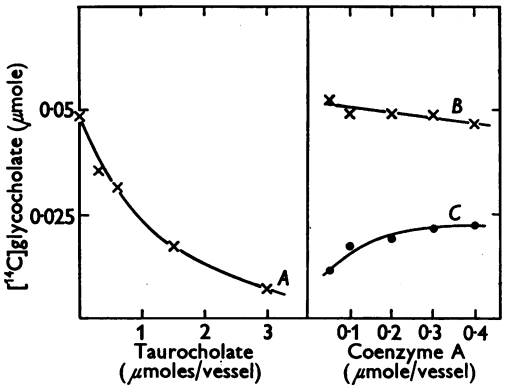

Fig. 4. Effect of taurocholate and of coenzyme A on the formation of [^{14}C]glycocholate. Microsomes from approx. 300 mg. of liver were incubated in 0·022 M potassium phosphate buffer (pH 7·4) with potassium fluoride (0·2 M), magnesium sulphate (0·001 M), ATP (6 μmoles/vessel), cholic acid (1 μmole/vessel), [^{14}C]glycine (1 μmole/vessel = 230 000 counts/min./vessel). Curve *A*: 0·1 μmole of coenzyme A + 0–3 μmoles of taurocholic acid/vessel; curve *B*: 0·05–0·4 μmole of coenzyme A/vessel; curve *C*: 1·5 μmoles of taurocholic acid + 0·05–0·4 μmole of coenzyme A/vessel. Total volume, 1·5 ml.; incubation time, 30 min. at 37°.

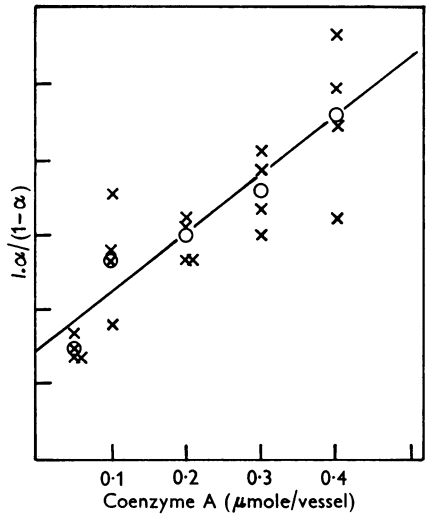

Fig. 5. Effect of coenzyme A on the inhibition by taurocholate of the formation of [^{14}C]glycocholic acid. Microsomes from approx. 300 mg. of liver were incubated in 0·022 M potassium phosphate buffer (pH 7·4) with potassium fluoride (0·2 M), magnesium sulphate (0·001 M), ATP (6 μmoles/vessel), cholic acid (1 μmole/vessel), [^{14}C]glycine (1 μmole/vessel = 230 000 counts/min./vessel), coenzyme A (0·05–0·4 μmole/vessel). Total volume, 1·5 ml.; incubation time, 30 min. at 37°. The inhibiting effect of taurocholate in different concentrations (0·3, 0·6, 1·5 and 3·0 μmoles/vessel) was determined in the presence of increasing concentrations of coenzyme A. The amounts of glycocholate formed during the incubation (0·055–0·006 μmole/vessel) were so small that they did not interfere significantly with the formation of cholyl-*S*-Co A. The fractional activity α (reaction velocity in the presence of inhibitor/reaction velocity without inhibitor present) was determined as the relation:

$$\frac{\text{glycocholate formed in 30 min. in presence of taurocholate}}{\text{glycocholate formed in 30 min. without taurocholate present}}$$

I = inhibitor concentration = taurocholate concentration. The circles denote the average of the four individual values (×) obtained with the different inhibitor (taurocholate) concentrations used at each concentration of coenzyme A.

The similarity in the configuration of the side chain in taurocholate and the end group in coenzyme A may possibly explain this competition.

Coenzyme A:

R-CO-NH-CH_2-CH_2-CO-NH-CH_2-CH_2-SH.

Taurocholate:

R′-CH(CH_3)-CH_2-CH_2-CO-NH-CH_2-CH_2-SO_3H.

It was not investigated whether glycocholate also inhibited by competing with coenzyme A.

DISCUSSION

These results indicate that two different acyl-transferring enzymes catalyse the transfer of the bile acyl group from coenzyme A to taurine and to glycine. If the transfer were catalysed by one enzyme only, the specificity of this enzyme would be totally different in rat, rabbit and chicken. A more reasonable explanation, therefore, would be the existence of two different transferring enzymes, both of which are found in rat liver, and one in rabbit and the other in chicken liver.

In the closely related hippuric acid synthesis, glycine cannot be replaced by taurine or β-alanine (Sarkar, Fuld & Green, 1951). [^{14}C]-β-Alanine has also been tested as substrate in the conjugation of cholic acid (Fritzson & Bremer, unpublished results) and it was found that rabbit-liver microsomes did not conjugate this amino acid with cholic acid, whereas rat-liver microsomes, which conjugate the structurally related taurine, also conjugated cholic acid with β-alanine, to a small degree. These results support our two-enzyme theory in the conjugation of free bile acids.

The preference for taurine in the conjugation of cholic acid seems to be of physiological importance. White (1935–6) found that feeding cholic acid to rats led to an increased requirement for the taurine precursors cystine and methionine. Mann, Andrus, McNally & Stare (1953) fed cebus monkeys a diet high in cholesterol and low in organic sulphur, and showed that both cystine and methionine lowered the hypercholesteraemia resulting from this diet.

Portman & Mann (1955) cite unpublished experiments performed by Mann, showing that the hypercholesteraemia in these animals could be lowered also by taurine feeding. These results also raise the question whether the absence of taurine conjugation in the rabbit may be of importance in the cholesterol catabolism in this animal. The bile acids are known to be the main degradation product in cholesterol catabolism (Bloch, Berg & Rittenberg, 1943; Siperstein & Chaikoff, 1952; Bergström & Norman, 1953).

The inhibiting effect of the conjugated bile acids on the coenzyme A-dependent conjugation of the free bile acids may explain observations made by several workers. Hummel (1929) produced cholesterol fatty liver in mice by feeding a high cholesterol diet. When this diet was replaced with a cholesterol-free diet, the liver cholesterol fell to normal values within a few days, but if bile acids were added to this cholesterol-free diet, the liver cholesterol remained unchanged for weeks. Cholic and glycocholic acid but not trioxocholanic acid had this effect. Friedman & Byers (1952) found that biliary obstruction led to hypercholesteraemia and accumulation of bile acids in the blood in rats. These bile acids must be assumed to be in the conjugated form, as only conjugated bile acids are found in the urine from rats with ligated bile ducts (Norman, 1955).

Pihl (1954, 1955) fed free bile acids to normal rats on a diet with high cholesterol and low fat content and found increased cholesterol deposition in the liver under conditions where the intestinal absorption was not increased. The results could not be explained by an increase in the endogenous synthesis of cholesterol, and the conclusion was drawn that bile acid feeding interfered with the cholesterol degradation. Trioxocholanic acid had a much smaller effect than had cholic and deoxycholic acids.

It is reasonable to assume that the conjugated acids mediate their effect through their inhibition of the coenzyme A-dependent conjugation of the free bile acids, and possibly also other coenzyme A-dependent steps in the cholesterol degradation. The free bile acids, after being conjugated, will enter the enterohepatic circulation and presumably give the same effect. They may also inhibit the cholesterol degradation by depleting the taurine 'pool'.

The lower efficiency of trioxocholanic acid as substrate in the conjugation then might explain the small or absent effect of this acid on the cholesterol degradation.

SUMMARY

1. The conjugation of free bile acids with taurine and glycine performed by rat, rabbit and chicken liver microsomes has been studied.

2. Rat-liver microsomes are shown to conjugate free bile acids with both glycine and taurine, but taurine is preferred in the conjugation.

3. Rabbit-liver microsomes conjugate the free bile acids almost exclusively with glycine.

4. Chicken-liver microsomes conjugate cholic acid exclusively with taurine.

5. Cholic, deoxycholic, 3:7:12-trioxocholanic and cholanic acids have been shown to serve as substrates in the conjugation, both in the rat and in the rabbit. Trioxocholanic acid was shown to be less efficient than the cholic and deoxycholic acids.

6. Taurocholic and glycocholic acids were shown to inhibit the conjugation by preventing the formation of cholyl-*S*-Co A, presumably by acting as antimetabolite to coenzyme A.

7. The results are discussed in relation to cholesterol metabolism.

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