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Studies on the Conjugation of Cholic Acid with Taurine in Cell Subfractions. Bile Acids and Steroids 23

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The conjugation of cholic acid with taurine has been studied in subfractions of rat liver homogenate.

The full conjugating activity of the total homogenate was retained in the crude supernatant (III) after centrifugation at 12 000 × g for 10 minutes. The microsomic fraction (IV) and the particle-free supernatant (V) were each inactive, but recombination restored the full activity when ATP was added. Partial reactivation was achieved when fresh microsomes were incubated with boiled supernatant and ATP, or with an acetone powder of the particle-free supernatant (V), DPN and ATP.

The conjugation in crude supernatant (III) was stimulated by nicotinamide, DPN, ATP, Mg^{++}, the complex forming agent versene and possibly by TPN.

At low taurine and cholic acid concentrations, the conjugation was almost quantitative.

When ATP was omitted cyanide strongly inhibited the conjugation. When ATP was added, cyanide had no effect.

Recently we have published a simple method to follow the formation of taurocholic acid from cholic acid and ^{35}S-labelled taurine together with preliminary results on the conjugation in liver homogenates [1]. The present paper deals with some further studies on this process in cell subfractions.

EXPERIMENTAL

The experimental procedure previously reported [1] has been slightly modified for these studies.

Due to the higher taurine content in the female rat liver [1], we have used male albino rats weighing 200—250 g throughout the study. The homogenization of the liver was performed as previously described [1,2]. Four parts of homogenizing medium were used to one part of liver (crude homogenate = I). The homogenizing medium consisted of 5 parts of 0.11 *M* potassium phosphate buffer of pH 7.7 and 7 parts of 10 % sucrose.

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In the fractionation experiments, nuclei and cell debris were taken down by centrifugation at 400 × g for 5 minutes (homogenate = II), mitochondria at 12 000 × g for 10 minutes (crude supernatant = III), and the microsomic fraction at 100 000 × g for 30 minutes (IV), leaving in solution the particle-free supernatant (V). The mitochondria were washed once by resuspension in homogenizing medium and recentrifugation. When recombination of the different fractions was undertaken, the microsomic and the mitochondrial fractions were resuspended in homogenizing medium to 1/10 of the original homogenate volume and pipetted as such.

In all the experiments 1 ml of homogenate was used per vessel. Additions to the following final concentrations were made: nicotinamide 0.017 *M*, MgCl 0.00065 *M*, versene (ethylenediamine tetraacetate) 0.00075 *M*. The mixture was finally brought to a volume of 1.75 ml with a 1.15 % solution of potassium chloride. Incubation, protein precipitation and butanol extraction were performed as previously described [1].

In order to evaluate the relative amount of taurocholic acid formed, ^{35}S-labelled taurine * was added to the incubation mixture, and the radioactivity of taurocholic acid formed was determined. The absolute amount of taurocholic acid formed was evaluated from the amount of preformed taurine present in the homogenate batch used [1]. The results are given in micromoles taurocholic acid formed per vessel, *i. e.* per 200 mg of liver. Duplicates were always run.

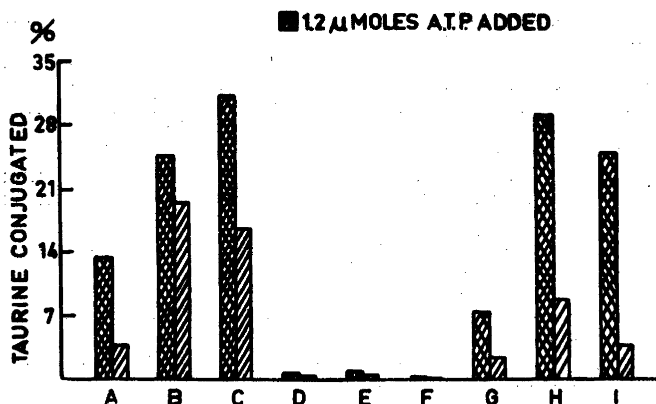

Fig. 1. Conjugation of cholic acid with taurine in liver cell subfractions.

Substrate additions: 2.5 μmole cholic acid, 2 μmole taurine. Further 1.2 μmole ATP where noted.

Vessels A. Total homogenate.
- *B. Homogenate centrifuged at 400 g for 5 min.*
- *C. Homogenate centrifuged at 12 000 g for 10 min.*
- *D. Homogenate centrifuged at 100 000 g for 30 min. (particle-free).*
- *E. Sediment after centrifugation at 12 000 g (mitochondria).*
- *F. Sediment after centrifugation at 100 000 g (microsomes).*
- *G. Particle-free supernatant + mitochondria.*
- *H. Particle-free supernatant + microsomes.*
- *I. Particle-free supernatant + mitochondria + microsomes.*

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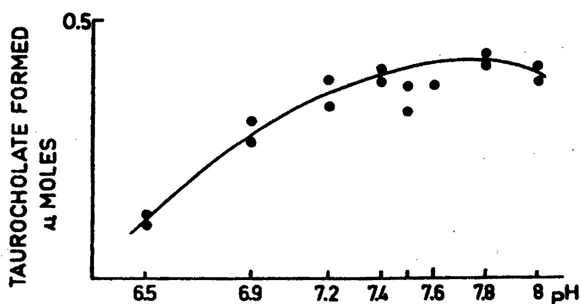

Fig. 2. Effect of pH. Crude supernatant + the following additions: 0.4 µmole ^{35}S-taurine, 1.0 µmole cholic acid, 1.2 µmole ATP.

RESULTS

The results from experiments with cell subfractions obtained by differential centrifugation are shown in Fig. 1.

The conjugating enzyme system was found to be localized in the crude supernatant fraction (III) of the liver cell, as the homogenate freed from nuclei and mitochondria by centrifugation at 12 000 × g for 10 minutes showed full activity (Fig. 1 C).

Neither the microsomic fraction alone (F) nor the particle-free supernatant (D) showed any activity. Full activity was obtained after recombination of these fractions in the presence of ATP (H).

We have performed several experiments with isolated microsomes without obtaining any conjugation of the added cholic acid and taurine. Addition of ATP, magnesium and coenzyme A was without any influence. Unfortunately the only preparation of coenzyme A available was a very crude extract. The possible role of coenzyme A will be subject to further studies.

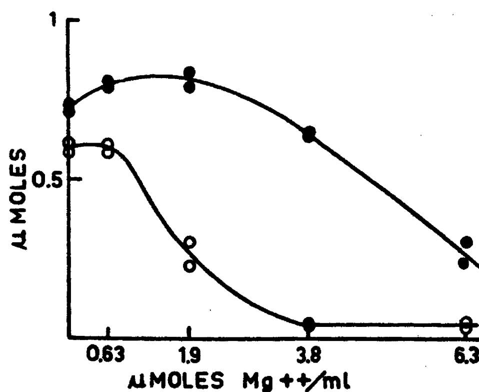

Fig. 3. Effect of Mg^{++}. Crude supernatant + the following additions: 0.4 µmole ^{35}S-taurine, 1.0 µmole cholic acid. Further with 1.2 µmole ATP (●), and without ATP (○). Magnesium concentrations 0—0.0065 M.

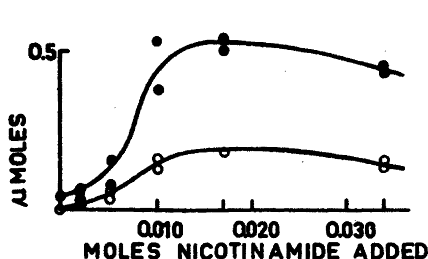

Fig. 4. Effect of nicotinamide. Crude supernatant + the following additions: 0.4 µmole ^{35}S-taurine, 1.0 µmole cholic acid, 1.2 µmole ATP were indicated (●). No ATP (○), nicotinamide concentrations 0—0.034 M.

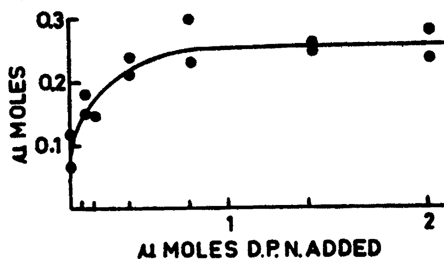

Fig. 5. Effect of DPN. Crude supernatant + the following additions: 0.4 µmole ^{35}S-taurine, 1.0 µmole cholic acid. 0—2.25 µmole DPN per vessel.

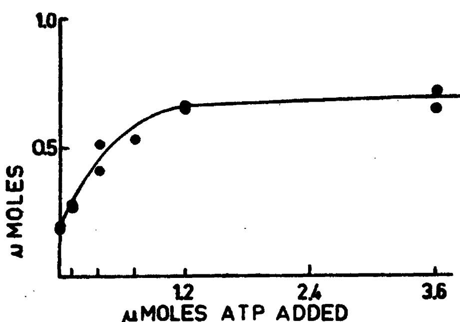

Fig. 6. Effect of ATP. Crude supernatant + the following additions: 0.4 µmole ^{35}S-taurine, 1.0 µmole cholic acid. Nicotinamide conc. 0.017 M, *0—3.6 µmole ATP per vessel.*

Effect of pH. No distinct pH-optimum was found (Fig. 2). Homogenates were almost equally effective when homogenizing media of pH 7 to pH 8 were used. At lower and higher pH values the activity decreased. Therefore a potassium phosphate buffer of pH 7.7 was used throughout. In these experiments the pH changed from 7.7 to 7.3—7.2 during the incubation period.

Effect of Mg-ions. Magnesium had a weak but distinct stimulating effect on the conjugation in the presence of ATP. Without addition of ATP the effect was purely inhibiting (Fig. 3). We also tried the effect of manganese chloride, since Ernster [3] and Lindberg have recently shown manganese to have a stimulating effect on the regeneration of energy rich phosphate bonds. In our experiment the addition of 1 µmole $MnCl_2$ gave a 90 % inhibition.

Effect of nicotinamide, DPN, ATP, and TPN. Figs. 4, 5, 6 and Table 1 show the effects of nicotinamide, DPN and ATP on the conjugation when the three other factors were excluded. As ATP had no, or only a very small effect when nicotinamide was omitted, the effect of ATP was investigated in the presence of this compound.

All these experiments are made with a crude supernatant(III). Consequently no mitochondria were present. Since nicotinamide and DPN show such a marked effect, however, there must still be enzymes present in the microsomes

or the particle free supernatant capable of producing the necessary energy for the conjugation.

We have also tried the effect of TPN and found it to have a stimulating effect of approximately the same order as DPN. Our TPN preparation was very impure, containing only 6 % of the compound. Presumably DPN was one of the main contaminants.

Table 1 shows the effect of the different combinations of nicotinamide, DPN and ATP. The data indicate that ATP requires the presence of nicotinamide or DPN in order to show any significant effect. In the presence of nicotinamide and ATP, the addition of DPN gave no further stimulation. We did not find any significant effect when cytochrom c or fumarate were added alone or in combinations with the other factors tried.

Table 1. Effect of stimulating and inhibiting factors. Crude supernatant — 2 μmole taurine, 2 μmole cholic acid.

1.2 μmole ATP	1.5 μmole DPN	Nicotinamide 0.017 *M*	Cyanide (8.5×10^{-4} *M*)	Dinitrophenol (2.8×10^{-4} *M*)	1 μmole Taurocholate	μmole Taurocholate formed	
—	—	—	—	—	—	0.06	0.06
+	—	—	—	—	—	0.05	0.06
—	+	—	—	—	—	0.39	0.39
—	—	+	—	—	—	0.40	0.40
—	—	+	+	—	—	0.12	—
—	—	+	—	+	—	0.38	0.27
+	+	—	—	—	—	0.45	0.49
—	+	+	—	—	—	0.72	0.70
+	—	+	—	—	—	0.85	0.85
+	+	+	—	—	—	0.84	0.84
+	—	+	+	—	—	—	0.77
+	—	+	—	+	—	0.74	0.77
+	—	+	—	—	+	0.37	0.31

When compared with the results of Cohen and McGilvery [4] in their work on the *p*-aminohippuric acid synthesis in rat liver homogenates, several dissimilarities appear. These authors found a purely inhibiting effect with nicotinamide. DPN and ATP added together inhibited their system if fumarate was omitted, but both DPN and ATP stimulated when added alone.

Our results seem to be in good accordance with the findings of Mann and Quastel [5] who showed that nicotinamide protects DPN against a DPN-splitting enzyme in liver and brain, and with the findings of Rowen and Kornberg who showed that the following reactions take place in the liver

1. Nicotinamide + ribose-1-phosphate → nicotinamideriboside
2. Nicotinamideriboside + ATP → nicotinamidemononucleotide [6]
3. Nicotinamidemononucleotide + ATP → DPN + pyrophosphate [7]

The form of the curve illustrating the nicotinamide effect (Fig. 4) also indicates that in this case a more complex mecanism was involved than when DPN was added (Fig. 5).

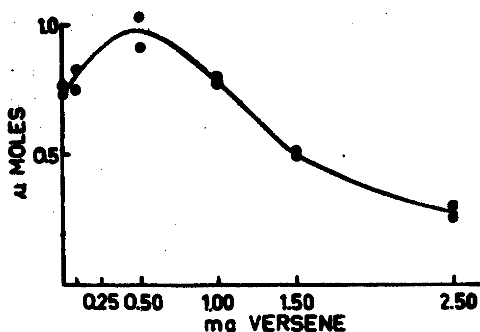

Fig. 7. Effect of versene. Crude supernatant + the following additions: 0.4 μmole ^{35}S-taurine, 1.0 μmole cholic acid, 1.2 μmole ATP, versene 0—2.5 mg per vessel.

Table 1 also shows that cyanide had a strongly inhibiting effect when ATP was omitted, whereas 2,4-dinitrophenol was almost without any effect in the concentration used (2.8×10^{-4} *M*). The addition of sodium taurocholate (1 μmole) gave a 50 % inhibition. The icteruspromoting compound icterogenin [8] was tried, but was found to have no effect.

Effect of versene. Fig. 7 shows that the addition of the complexforming agent versene had a positive effect on the formation of taurocholic acid, presumably by binding heavy metal impurities in the medium. Addition of more than 0.5 mg (7.5×10^{-4} *M*), gave an inhibiting effect. A similar effect of versene on the 7 α-hydroxylation of taurodesoxycholic acid has earlier been observed [9].

Effect of taurine and cholic acid. In experiments previously reported [1], taurine was found to be without effect on the conjugation efficiency when added to the homogenate system used. This was found to be due to the relatively high taurine content of the rat liver. However, when added to the

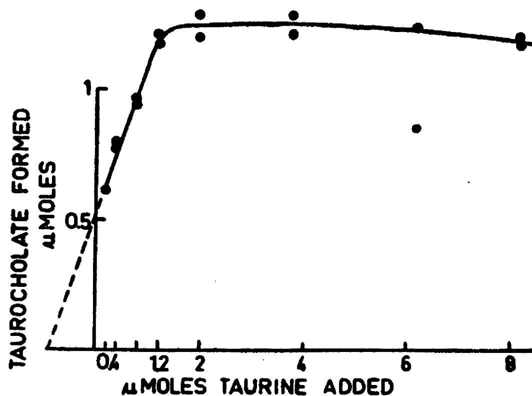

Fig. 8. Effect of taurine. Crude supernatant + the following additions: 2.5 μmole cholic acid, 0.2—0.4 μmole ^{35}S-taurine, 0—8 μmole unlabelled taurine. The conjugation was calculated from the amount of taurine added, the counting data and the calculated taurine content of the liver.

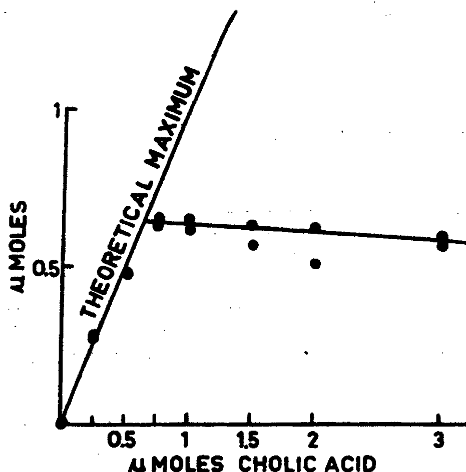

Fig. 9. Effect of cholic acid. Crude supernatant + the following additions: 0.4 µmole ^{35}S-taurine, 1.2 µmole ATP, 0—3 µmole cholic acid.

fortified system described in the present report, the formation of taurocholic acid was more than doubled (Fig. 8). The rise is almost linear up to the optimal taurine concentration (> 1.2 µmole added). Beyond this point, further addition had no effect.

Extrapolation of the curve gave a taurine content of approximately 0.8 µmole per 200 mg liver, *i. e.* 0.5 mg/g fresh liver. This is in good agreement with our earlier results on the normal taurine concentration in male rat liver [9].

In Fig. 9 is shown the effect of cholic acid on the formation of taurocholic acid in the presence of 0.4 µmole ^{35}S-labelled taurine. The conjugation of the cholic acid was quantitative until 90 % of the taurine was conjugated. Further addition had no effect. (Probably a still greater fraction of the taurine was conjugated, since the labelled taurine synthesized according to Eldjarn's procedure [10] has been shown to contain 3—4 % sulphate [11]). The form of the curve shows that the conjugation occurs almost quantitatively when equimolar amounts of taurine and cholic acid are added in small concentrations.

Stability of the enzyme system. The homogenate showed a rapid decrease of the activity when stored at 0° C. After 24 hours it was completely inactive. When stored for the same length of time in the frozen condition at —20° C, it had about 70 % of the activity left. A homogenate frozen and thawed again twice in rapid succession had still an activity of 85 %.

The isolated microsomes were completely unaffected by freezing, and they also showed full activity after 60 minutes' treatment with distilled water. No enzyme went into solution neither by this treatment, nor when the microsomes were frozen twice in distilled water.

Precipitation of the crude supernatant (III) or the isolated microsomes (IV) with acetone produced completely inactive powders, even when recombined

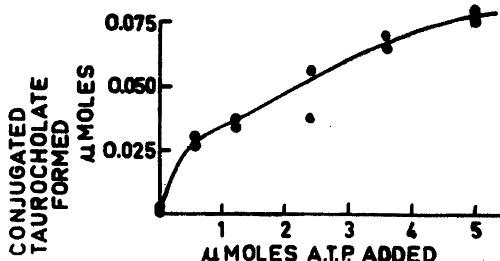

Fig. 10. Effect of ATP in the presence of boiled supernatant. Total volume 1.75 ml. 0.1 ml microsome suspension + 0.9 ml boiled supernatant from a 20 % homogenate kept in boiling water for five minutes and centrifuged free from precipitated proteins.

with fresh particle-free supernatant (V). The results were the same whether the homogenate or the microsomes were precipitated at 0° C or in the frozen condition.

On the other hand, when fresh microsomes from 1 ml homogenate were combined with acetone powder from 1—2 ml particle-free supernatant in the presence of ATP and DPN approximately 65 % of activity was restored. The possible influence of nicotinamide was not investigated in this preparation.

In Fig. 10 is shown that the boiled supernatant also was able partly to restore the activity of the microsomes when ATP was added. Thus in the presence of 5 µmole ATP approximately 20 % of the original activity was achieved. The possibility that fresh supernatant contaminated the isolated microsomes was excluded by an experiment where ATP was added also to isolated microsomes suspended in homogenizing medium. Only traces of activity could be detected. The ATP therefore had a greater effect when boiled supernatant was used than when fresh particle-free supernatant (V) was used. In the latter case ATP required addition of nicotinamide to give any effect (*cf.* Table 1). In the microsome+boiled supernatant system nicotinamide and DPN showed no effect.

DISCUSSION

Peptide bond synthesis has been studied by several workers in different systems.

Cohen and McGilvery [12] have published a series of works on the *p*-aminohippuric acid synthesis from *p*-aminobenzoic acid and glycine in rat liver slices and rat liver homogenate. They found the conjugation to be performed on the expence of phosphate bond energy by an enzyme system confined to the insoluble particles of the liver and kidney cell. Anaerobically the conjugation was strongly depressed if ATP was omitted from the reaction mixture. The conjugation was also depressed aerobically by substances known to inhibit oxidative processes, such as cyanide, azide and malonate.

Kielly and Schneider [13] have shown that the enzyme responsible for the conjugation of *p*-aminobenzoic acid is located in the mitochondria of the mouse liver cell, and Chantrenne [14] has succeeded in making an acetone extract from

mitochondria which catalyzes hippuric acid synthesis from benzoic acid and glycine when ATP, Mg^{++} and coenzyme A are added.

Schachter and Taggar [15] prepared benzoyl-coenzyme A and showed that this compound is active as a precursor to hippuric acid without any addition of ATP, Mg^{++} or coenzyme A. Their results strongly indicate that benzoyl-CoA is the "activated benzoic acid".

The biological synthesis of glutathion has been studied by Bloch [16,17]. Speck [18] has investigated the formation of glutamine from glutamate and ammonia. The participation of ATP and magnesium were also reported by these workers.

Coenzyme A has only been found to be necessary in the hippuric acid synthesis.

The results described in the present report show that the conjugation of taurine with cholic acid is very similar to the conjugation of glycine with benzoic acid or *p*-aminobenzoic acid, though the localization of the enzyme systems within the cell is different. The processes are all stimulated by factors concerned with oxygenation processes which procure energy for phosphate bond formation, and they are inhibited by substances interfering with these processes (cyanide).

Where probably most of the ATP-synthesizing enzymes have been omitted by combining fresh microsomes with boiled supernatant, no conjugation is achieved if ATP is omitted. In this case DPN and nicotinamide had no effect, whereas the effect of ATP was greater than when it was added alone to intact crude supernatant. At least one unknown factor from the supernatant must be necessary, as the isolated microsomes were inactive with all additions tried. This factor seems to withstand immersion in boiling water for five minutes and to follow the proteins when precipitated with acetone. In several isolation methods for coenzyme A, both boiling and precipitation with acetone are parts of the procedure [19]. Although we did not find any stimulating effect on the conjugation by the addition of a 6 % coenzyme A preparation, this compound may well be the unknown supernatant factor. Further work on this question is in progress.

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REFERENCES

1. Bremer, J. *Acta Chem. Scand.* **9** (1955) 683.
2. Bergström, S. and Gloor, U. *Acta Chem. Scand.* **8** (1954) 1373.
3. Ernster, L. and Lindberg, O. *Acta Chem. Scand.* **8** (1954) 1096.
4. Cohen, P. P. and McGilvery, R. W. *J. Biol. Chem.* **169** (1949) 119.
5. Mann, P. J. G. and Quastel, J. H. *Biochem. J. (London)* **35** (1941) 502.
6. Rowen, J. W. and Kornberg, A. *J. Biol. Chem.* **193** (1951) 497.
7. Kornberg, A. *J. Biol. Chem.* **182** (1950) 779.
8. Rimington, C., Quin, J. I. and Roets, G. C. S. *Onderstepoort J. Vet. Sci. Animal Ind.* **9** (1937) 225.
9. Bergström, S. and Gloor, U. *Acta Chem. Scand.* **9** (1955) 34.
10. Eldjarn, L. *Acta Chem. Scand.* **5** (1951) 677.
11. Shapiro, B. *Norsk Hydro's Institute for Cancer Research, Oslo.* Unpublished data.

12. Cohen, P. P. and McGilvery, R. W. *J. Biol. Chem.* **171** (1947) 121.
13. Kielley, R. K. and Schneider, W. C. *J. Biol. Chem.* **185** (1950) 869.
14. Chantrenne, H. *J. Biol. Chem.* **189** (1951) 189.
15. Schachter, D. and Taggart, J. V. *J. Biol. Chem.* **203** (1953) 203.
16. Bloch, K. *J. Biol. Chem.* **179** (1949) 1245.
17. Johnston, R. B. and Bloch, K. *J. Biol. Chem.* **188** (1951) 221.
18. Speck, J. F. *J. Biol. Chem.* **179** (1949) 1405.
19. Kaplan, N. O. and Lipmann, F. *J. Biol. Chem.* **174** (1948) 37.

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