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Cholyl-S-CoA as an Intermediate in the Conjugation of Cholic Acid with Taurine by Rat Liver Microsomes

JON BREMER*

Norsk Hydro's Institute for Cancer Research, The Norwegian Radium Hospital, Oslo, Norway

The conjugation of cholic acid with taurine has been investigated. The conjugation required the presence of coenzyme A (CoA) and adenosinetriphosphate (ATP) and was stimulated by magnesium, manganese, and fluoride and in some cases by cysteine and glutathione.

Cystine, pyrophosphate and adenosine-5-phosphate (AMP) inhibited the conjugation.

When hydroxylamine was added to the incubation medium, cholhydroxamic acid was identified as the reaction product.

A reaction mechanism, involving cholyl-S-CoA as an intermediate and AMP and pyrophosphate as reaction products, has been proposed.

In previous studies [1-3] we have shown that the enzyme system performing the conjugation of cholic acid with taurine or with glycine, was localized in the microsomic particles of the rat liver cell. When isolated microsomes were used, ATP and a heat stable factor in the particle free supernatant were found to be required in the conjugation. This factor was presumed to be CoA.

In the present paper further studies on the reaction mechanism are presented. A preliminary report on the results has been published [4].

MATERIALS AND METHODS

The biological conjugation of cholic acid with taurine was followed by means of ^{35}S-labelled taurine as earlier described [1]. The labelled taurine was synthesized according to Eldjarn [5], and the synthesis was kindly performed by L. Eldjarn and P. Fritzson in this institute.

A 75 % pure CoA, crystalline ATP (potassium salt), and ADP (sodium salt) were obtained from Nutritional Biochemicals Corporation, Cleveland, U. S. A.

A 2 % CoA preparation and tri-(hydroxymethyl)-amino-methane (TRIS) were obtained from Zigma Chemical Company, USA.

* Fellow of the Norwegian Cancer Society.

Preparation of rat liver microsomes

Livers from male rats of a mixed strain were used. The liver weights were usually 6—8 g. After preliminary slicing, the livers were homogenized in four parts 10 % sucrose for approximately 1 ½ minutes in a glass homogenizer with a plastic pestle doing 1 500 rev/min. Cell debris, nuclei and mitochondria were sedimented by centrifuging at 15 000 —18 000 × g for 15 minutes. The microsomic fraction was then sedimented at 25 000 × g for 110 minutes. The particle free supernatant was discarded, leaving behind the densely packed microsomic particles as a translucent cake of the typical red colour. All isolating operations were performed at 0° C. The sedimented microsomes were stored at —20° C until used.

Preparation of cholhydroxamic acid

The cholhydroxamic acid was synthesized from cholic acid ethyl ester prepared according to Bondi and Müller [6]. The cholic acid ethyl ester was converted to the hydroxamic acid by a modification of the method used by Kornberg and Pricer [7] in their preparation of fatty hydroxamic acids: 2.5 g of ethyl ester and 1 g of hydroxylamine hydrochloride were dissolved in 35 ml of absolute methanol in which 1.2 g metallic sodium had been dissolved beforehand. The mixture was refluxed for 30 minutes on a boiling water bath and neutralized with a mixture of one part concentrated hydrochloric acid in ten parts of absolute ethanol. Two volumes of ethyl ether were added and the precipitated salts filtered off. The filtrate was then evaporated to dryness under reduced pressure. The yellowish residue consisted of almost pure hydroxamic acid. The product was dissolved in 25 ml absolute ethanol, and 150 ml ethyl acetate was added. After storing overnight at 5° C, the precipitate which formed was filtered off and discarded. The clear filtrate was slowly evaporated to 1/5—1/10 of the original volume by passing a stream of air over the surface of the fluid. The hydroxamic acid then started to crystallize in rosettes. After storing overnight at —20° C, 2.15 g of the crystalline compound could be filtered off. When dried at 4 mm pressure and 80° C for 5 hours, the weight decreased by about 10 %. Recrystallization of the product with the same evaporation procedure gave no detectable change in purity as estimated from the colour reaction with ferric ions. The compound decomposed at 117° and was completely melted at 145° C. Elementary analysis showed (theoretical values in brackets): Carbon 68.22 % (68.39), hydrogen 9.54 % (9.39), nitrogen 3.31 % (3.64). When dissolved in concentrated sulphuric acid, the cholhydroxamic acid was shown to have light absorption spectrum and extinction coefficients between 220 and 600 mμ identical with those reported for cholic acid, taurocholic acid and glycocholic acid [8,9]. The compound gave the typical cherry-red colour of hydroxamic acids with ferric ions in acid solution [10]. The elaboration of two spectrophotometric methods for the estimation of biologically formed cholhydroxamic acid were based on these results.

Chromatographic and spectrophotometric estimation of cholhydroxamic acid

To estimate the cholhydroxamic acid formed in the incubation experiments, the acid was extracted from the incubation mixture with 1/3 volume n-butanol as described for the isolation of taurocholic acid [1].

Method 1. When the cholhydroxamic acid was estimated from its light absorption in concentrated sulphuric acid, the acid was separated from the unchanged cholic acid using a modification of Sjövall's [11] quantitative paper chromatography for conjugated bile acids. Paper pretreatment and stationary phase were identical with those described by Sjövall [11, 12]. Aliquots of 0.05 ml of butanol extract were put on the paper. Descending chromatography was used. As moving phase a mixture of one part n-butanol and two parts petroleum spirit (b. p. above 120° C) saturated with 4 % acetic acid was used. In this system the cholhydroxamic acid moved with an R_F value of 0.20—0.25 and the cholic acid with an R_F value of 0.40—0.45. There was some tailing on the paper, but a complete separation of the compounds was easily obtained. Further details of the method have been described by Sjövall [11].

Table 1. The extraction of cholhydroxamic acid with n-*butanol.*

Each vessel contained 1.5 ml 0.1 M potassium phosphate buffer (pH 7.2) and 0.5 ml *n*-butanol. After equilibration in a shaking machine for 25 minutes, 0.25 ml butanol phase was pipetted off and the content of hydroxamic acid determined according to method 2 (see text).

μmoles cholhydroxamic acid added per vessel		Density read at 525 mμ	
0.19	0.19	0.087	0.090
0.47	0.47	0.184	0.191
1.18	1.18	0.447	0.449
2.36	2.36	0.870	0.873
1.18 + microsomes from 500 mg liver		0.433	0.426

Method 2. As cholic acid gave no colour reaction with ferric ions, no chromatographic separation of the cholic acid and the cholhydroxamic acid was necessary. Aliquots of 0.25 ml of the butanol extract were diluted to 2 ml with the colour reagent, consisting of 0.33 % ferric chloride and 0.2 % perchloric acid in absolute ethanol. The density of the solution was measured in a 1 cm cuvette at 525 mμ. A solution containing 1 μmole hydroxamic acid per ml gave an extinction of 1.26 when measured in a Hilger quartz spectrophotometer, *i. e.* the $\varepsilon^{525}_{\mu\text{mole}} = 1\,260$.

The two analytical methods gave results in good agreement as seen from Table 4.

The extraction method had to be calibrated. For this purpose a series of vessels containing 1.5 ml of 0.1 M potassium phosphate buffer (pH 7.2) and 0.5 ml *n*-butanol were prepared. Known amounts of hydroxamic acid were dissolved in the added butanol beforehand. After 25 minutes' equilibration in a shaking machine, the colour developed by 0.25 ml butanol phase was measured in the usual way. As seen from Table 1, the extinction coefficient was proportional to the amount of hydroxamic acid added, and it was not significantly affected by addition of rat liver microsomes in amounts used in the incubation experiments.

EXPERIMENTAL

Before use, the microsomes stored in the frozen condition were thawed at 37° C and resuspended in 10 % sucrose by means of the homogenizer and pipetted as such. Microsomes from 300—500 mg of fresh liver were added to each vessel in the incubation experiments. The following concentrations were maintained in the incubation mixture if not otherwise stated: Potassium phosphate buffer (pH 7.4) 0.0022 M, potassium fluoride 0.2 M, magnesium sulphate 0.001 M, coenzyme A approximately 0.001 M, cysteine 0.0067 M. Cholic acid and ^{35}S-taurine were added as stated under the individual experiments. The incubation was performed at 37° C. Total volume was 1.5 ml per vessel. The results are given as micromoles taurocholate formed per vessel.

The pH-effect was evaluated in the following way: A solution containing all the factors needed for the reaction except the microsomes was prepared, using a phosphate buffer of pH 6.0. This solution was made more and more alkaline by adding normal potassium hydroxide under the control of a pH-meter. Aliquots of 1.4 ml were pipetted off when suitable pH-values were reached. The microsome suspension was later added before incubation without any further adjustment of pH. The diluting effect of the added potassium hydroxide solution did not exceed 2—3 %.

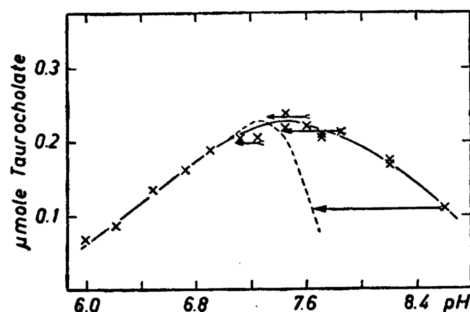


Fig. 1. The effect of pH. Microsomes from approx. 300 mg fresh liver per vessel. ^{35}S-taurine 0.8 µmoles/vessel, cholic acid 2 µmoles/vessel, ATP 4 µmoles/vessel, CoA (2 %) 3 mg/vessel. Potassium phosphate buffer 0.044 M, glutathione 0.0022 M, $MgSO_4$ 0.0025 M, NaF 0.15 M. Incubation time 90 minutes. The arrows indicate the fall of pH during incubation.

RESULTS

Effect of pH

The effect of pH on the conjugation of cholic acid with taurine is shown in Fig. 1. The pH optimum was found to be in the range 7.3—7.6 which is in good agreement with our earlier results when a system of microsomes and particle free supernatant was used [2]. As the buffering capacity of phosphate rapidly decreases at pH values above 7.5, the fall of pH during incubation was measured in some vessels. As indicated by the arrows in Fig. 1, there was a rather large fall in the pH values in the alkaline end of the curve. The decrease in conjugating capacity was therefore probably more abrupt with increasing pH than shown in the curve. A similar pH optimum has been found in the hippuric acid synthesis [13] and in the acetate activation [14]. In all later experiments, the incubation mixtures were adjusted to a pH of 7.40—7.45.

Effect of coenzyme A, glutathione, cysteine and cystine

Fig. 2 shows that there was an absolute requirement for CoA in the conjugation of cholic acid with taurine, and the results obtained with the two different CoA preparations used, showed that probably no other components of the CoA concentrates had any stimulating effect on the process.

Figs. 3 and 4 show that glutathione and cysteine had no significant stimulating effect when the 75 % pure CoA was used. The sulphydryl content of this preparation was titrated according to the method of MacDonnell *et al.*[15]. A sulphydryl content of 1.1 µmoles/mg was found, in good agreement with a content of 75 % CoA in the reduced form. The effect of cysteine and glutathione was therefore probably due to their reducing properties which kept the sulphydryl group of CoA in the reduced form. This sulphydryl group is known to be the functioning group of CoA [16], and other sulphydryl containing com-

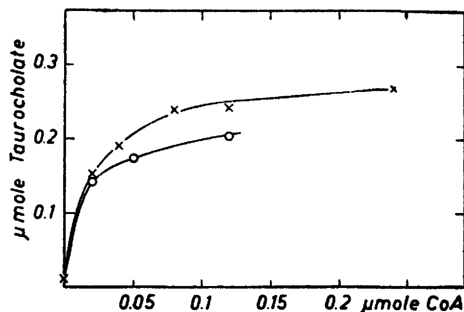


Fig. 2. Effect of CoA. Microsomes from approx. 500 mg liver. ^{35}S *taurine 2 μmoles/vessel, cholic acid 2 μmoles/vessel, ATP 4 μmoles/vessel. Potassium phosphate buffer 0.026 M, glutathione 0.0022 M,* $MgSO_4$ *0.0025 M, KF 0.15 M. Incubation time 120 minutes.*
+—+—+—+— *2 % CoA,* O—O—O—O— *75 % CoA.*

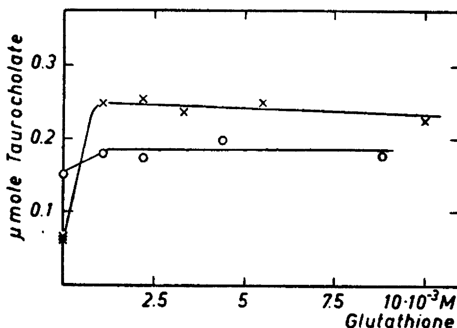


Fig. 3. Effect of glutathione. Microsomes from approx. 500 mg liver. ^{35}S*-taurine 2 μmoles/vessel, cholic acid 2 μmoles/vessel, ATP 4 μmoles/vessel, CoA approx. 0.1 μmoles/vessel. Potassium phosphate buffer 0.022 M,* $MgSO_4$ *0.0025 M, KF 0.15 M. Incubation time 120 minutes.* +—+—+—+—+ *2 % CoA,* O—O—O—O—O *75 % CoA.*

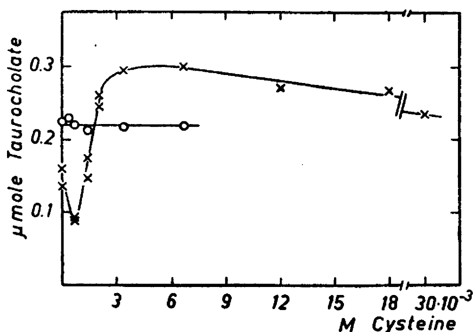


Fig. 4. Effect of cysteine. Microsomes from approx. 500 mg liver. ^{35}S*-taurine 2 μmoles/vessel, cholic acid 2 μmoles/vessel, ATP 4 μmoles/vessel, CoA approx. 0.15 μmoles/vessel. Potassium phosphate buffer 0.024 M,* $MgSO_4$ *0.0025 M, KF 0.15 M. Incubation time 120 minutes.* +—+—+—+—+ *2 % CoA,* O—O—O—O *75 % CoA.*

Table 2. Effect of cysteine and cystine.

Microsomes from 500 mg liver. ^{35}S-taurine 2 μmoles/vessel (= 16 500 c/min/vessel), cholic acid 2 μmoles/vessel, ATP 4 μmoles/vessel, CoA approx. 0.15 μmoles/vessel. Potassium phosphate buffer 0.024 M, $MgSO_4$ 0.0025 M, KF 0.15 M. Incubation time 120 minutes.

Addition	75 % CoA		2 % CoA	
	Net c/min/0.1 ml butanol extract	μmoles taurocholate/vessel	Net c/min/0.1 ml butanol extract	μmoles taurocholate/vessel
None	247 252	0.23	177 151	0.15
Cysteine 6.7×10^{-4} M	242	0.22	104 100	0.09
Cysteine 3.3×10^{-3} M	280 240	0.24	322	0.29
Cystine in substance	65	0.06	78 104	0.08

pounds have been shown to protect it [17, 18]. Fig. 4 shows a peculiar inhibiting effect of cysteine in low concentrations. This inhibition was reversed by cysteine in higher concentrations. A correct explanation of this phenomenon cannot be given with any certainty, but from Table 2 it is seen that cystine had an inhibiting effect of the same magnitude. We have therefore presumed that cysteine in low concentrations catalyzes the oxidation of the CoA sulphydryl groups by some oxidizing contaminant in the 2% CoA preparation.

In higher concentrations both cysteine and glutathione had a weakly inhibiting effect, as is evident also from Table 4.

Effect of fluoride

Fluoride has been used in ATP-dependent reactions to depress ATP-ase and pyrophosphatase activity [19, 20]. Fig. 5 shows that fluoride had a profound stimulating effect in our experiments, and the optimal concentration was found to be astonishingly high (0.15—0.20 M). The ATP-ase activity of microsomes has been reported to be moderate [21]. We have therefore made some experiments to elucidate any possible additional effect of fluoride. Fig. 6 shows the time curves with and without fluoride. The initial reaction rate seemed to be nearly the same in both cases. To exclude the possibility that fluoride prevented an inactivation of the enzyme system, the experiment shown in Table 3 was undertaken. Inactivation could not explain the rapidly de-

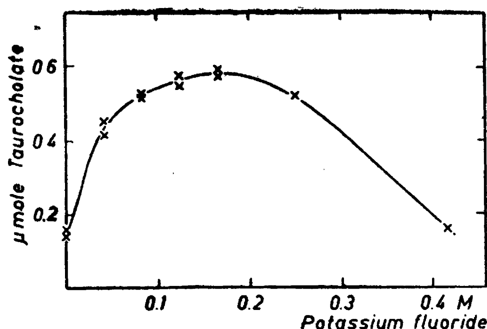


Fig. 5. Effect of fluoride. Microsomes from approx. 500 mg liver. ^{35}*S-taurine 2 μmoles/vessel, cholic acid 2 μmoles/vessel, ATP 4 μmoles/vessel, CoA (2 %) approx. 0.1 μmole/vessel. Potassium phosphate buffer 0.020 M,* $MgSO_4$ *0.002 M, cysteine 0.0055 M. Total volume 1.8 ml. Incubation time 120 minutes.*

creasing reaction rate when fluoride was omitted, as addition of another batch of ATP after 25 minutes resulted in an increased amount of taurocholic acid, whereas addition of another batch of microsomes had no effect. The effect of fluoride therefore seemed to be a saving of ATP, probably through the inhibition of ATP-ase present in the microsomes [21]. In an unpublished experiment an equivalent amount of potassium chloride was added instead of potassium fluoride. No effect on the conjugation was observed, neither stimulating nor inhibiting, showing that the stimulation was due to the fluoride ion, and not to an osmotic effect or to the potassium.

Effect of microsomes

When microsomes from less than approximately 500 mg of fresh liver were added to the vessels, the amount of taurocholate formed was proportional to

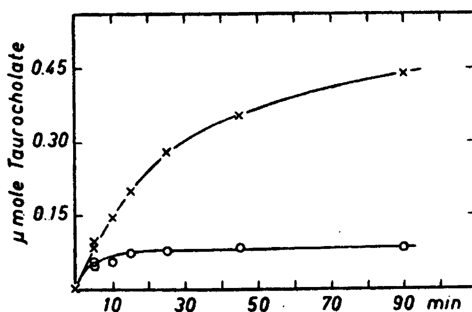


Fig. 6. Time curves with and without fluoride. Microsomes from approx. 500 mg liver. ^{35}*S-taurine 0.8 μmoles/vessel, cholic acid 2 μmoles/vessel, ATP 10 μmoles/vessel, CoA (75 %) 0.1 μmole/vessel. Potassium phosphate buffer 0.022 M,* $MgSO_4$ *0.001 M.*
+—+—+—+—+ 0.2 M KF, O—O—O—O—O No KF.

Table 3. Effect of ATP-ase present in the microsomes.

Microsomes from 500 mg liver. ^{35}S-taurine 0.8 μmoles/vessel (= 17 000 c/min/vessel), cholic acid 2 μmoles/vessel, ATP 10 μmoles/vessel, CoA (75 %) 0.15 μmoles/vessel. Potassium phosphate buffer 0.022 M, total volume: first 25 minutes 1.2 ml, next 50 minutes 1.5 ml.

Additions and incubation conditions	Net c/min/0.1 ml butanol extract		μmoles taurocholate formed per vessel	
Reaction stopped after 25 min.	438	441	0.15	0.15
0.3 ml 1.15 % KCl added after 25 min. Reaction stopped after 75 min.	473	495	0.17	0.17
10 μmoles ATP added after 25 min. Stopped after 75 min.	784	843	0.28	0.30
Microsomes from 500 mg liver added after 25 min. Stopped after 75 min.	343	404	0.12	0.14
300 μmoles KF added after 25 min. Stopped after 75 min.	477	524	0.17	0.18

the amount of microsomes added (Fig. 7). Microsomes from 300—500 mg of fresh liver were therefore used in the experiments.

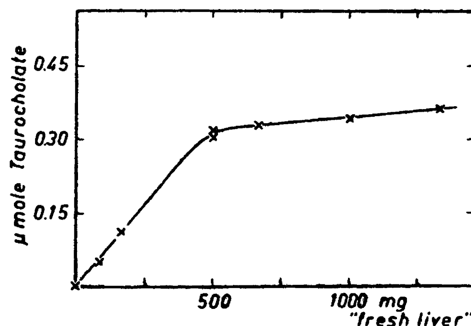


Fig. 7. Effect of microsomes. ^{35}*S-taurine 2 μmoles/vessel, cholic acid 2 μmoles/vessel, ATP 4 μmoles/vessel, CoA (75 %) 0.15 μmoles/vessel. Potassium phosphate buffer 0.022 M, KF 0.2 M. Incubation time 120 minutes.*

Cholhydroxamic acid formation

The requirement for CoA in the conjugation of cholic acid with taurine made it probable that an "activated cholic acid" in the form of cholyl-S-CoA represented an intermediate in the reaction. As hydroxylamine is known to react spontaneously with acyl-S-CoA at *ca.* pH 7 to form hydroxamic acids [7,19,22] the hydroxylamine represents a trapping agent for such activated carboxyl groups. Table 4 shows that cholhydroxamic acid was formed during the incubation when hydroxylamine was added to the incubation mixture instead of taurine. There was the same dependance on CoA, ATP and fluoride in this reaction as in the formation of taurocholic acid.

As expected cysteine was found to have no effect on the hydroxamic acid formation in this experiment, as the 75 % pure reduced CoA was used.

The same formation of cholhydroxamic acid has been reported in a short communication by Elliott [23].

Effect of preincubation without taurine

Further support for the existence of cholyl-S-CoA as an intermediate was obtained from the experiment cited in Table 5. An intermediate must have

Table 4. The formation of cholhydroxamic acid.

Complete system: Microsomes from approx. 500 mg liver, ATP 0.0067 M, CoA (75 %) 0.0001 M, cysteine 0.0067 M, potassium phosphate buffer 0.022 M, $MgSO_4$ 0.002 M, KF 0.2 M. Expt. I: ^{35}S-taurine 0.8 µmoles/vessel (= 25 000 c/min/vessel), cholic acid 2 µmoles/vessel. Total volume 1.5 ml. Expt. II: Hydroxylamine 50 µmoles/vessel, cholic acid 1 µmole/vessel. Total volume 1.75 ml. Incubation time 90 minutes.

Factor *excluded* from complete system	Experiment 1 µmoles taurocholate formed per vessel		Experiment 2 µmoles hydroxamic acid formed per vessel			
			$FeCl_3$-method		H_2SO_4-method	
None	0.27	0.31	0.73	0.77	0.63	0.65
Cholic acid	0	0	0.07	0.07	0	0
Potassium fluoride	0.12	0.12	0.43	0.43	0.38	0.37
ATP	0	0	0.05	0.05	0	0
CoA	0	0	0.10	0.10	0.06	0.13
Cysteine	0.34	0.35	0.77	0.78	0.77	0.76
Mg^{++}	0.36	0.41	0.68	0.67	0.67	0.66
Hydroxylamine	—	—	0.02	0.02	0	0.05

Table 5. Effect of preincubation without taurine.

Microsomes from approx. 500 mg liver. ^{35}S-taurine 0.8 μmoles/vessel (= 16 900 c/min/vessel), cholic acid 2 μmoles/vessel CoA (75 %) 0.3 μmoles/vessel, ATP 20 μmoles/vessel. Potassium phosphate buffer 0.022 M, KF 0.2 M. Total volume during preincubation 1.4 ml, after preincubation 1.5 ml.

Incubation conditions	Net c/min/0.1 ml butanol extract		μmoles taurocholate formed per vessel	
Complete system incubated for 9 min.	209	219	0.077	0.074
Preincubated for 10 min. without taurine, further for 9 min. with taurine	253	260	0.089	0.092

accumulated during the preincubation, as the reaction rate after addition of taurine was greater in the preincubated vessels. No isolation of the intermediate was attempted, as the amounts accumulated were evidently very small.

Effect of magnesium, manganese and calcium

Table 4 shows that magnesium had no effect when the isolated microsomes were used without any further treatment. This was probably due to magnesium (or manganese) following the microsomes. When the microsomes were dialyzed for 36 hours against 0.0025 M potassium phosphate buffer (pH 7.5), addition of magnesium had a stimulating effect both on the hydroxamic acid — and the taurocholic acid formation (Table 6). Any absolute requirement for

Table 6. Effect of Mg^{++}.

Microsomes from approx. 500 mg liver, dialyzed for 36 hours against 0.005 M potassium phosphate buffer (pH 7.4) at 5° C. ^{35}S-taurine 0.8 μmoles/vessel (= 17 000 c/min/vessel) or hydroxylamine 50 μmoles/vessel, cholic acid 2 μmoles/vessel, ATP 10 μmoles/vessel, CoA 0.15 μmoles/vessel. Potassium phosphate buffer 0.022 M, KF 0.2 M, glutathione 0.003 M. Incubation time 60 minutes.

Additions	μmoles taurocholate formed per vessel		μmoles cholhydroxamic acid formed per vessel	
None	0.16	0.16	0.29	0.31
Mg^{++} 0.4×10^{-3} M	0.23	0.23	0.45	0.44
Mg^{++} 1×10^{-3} M	0.27	0.26	—	—
Mn^{++} 0.4×10^{-3} M	0.17	0.18	0.38	—

Table 7. Effect of Mg^{++}, Mn^{++} and Ca^{++}.

0.8 ml solubilized microsomes dialyzed for 24 hours against 0.005 M potassium phosphate buffer (pH 7.5). ^{35}S-taurine 0.8 µmoles/vessel (= 20 000 c/min/vessel), cholic acid 2 µmoles/vessel, ATP 10 µmoles/vessel, CoA (75 %) 0.15 µmoles/vessel. Potassium phosphate buffer 0.015 M, KF 0.2 M. Incubation time 45 minutes.

Additions	Net c/min/0.1 ml butanol extract	µmoles taurocholate formed per vessel
None	152 153	0.046 0.046
Mg^{++} 0.2 × 10^{-3} M	245	0.074
Mg^{++} 1 + 10^{-3} M	228	0.068
Mn^{++} 0.2 × 10^{-3} M	265	0.080
Mn^{++} 1 × 10^{-3} M	223	0.067
Ca^{++} 1 × 10^{-3} M	115	0.034

magnesium could not be demonstrated, as further prolongation of the dialysis led to increasing inactivation of the enzyme system, and this inactivation could not be reversed by magnesium. It can be seen from Table 7 that manganese had a stimulating effect on the conjugation in contrast to our previously reported results with the microsomes-particle free supernatant system [2]. Solubilized microsomes (see later) dialyzed for approximately 24 hours against 0.0025 M potassium phosphate buffer were used in this experiment. Also in this case increasing inactivation of the enzyme system during dialysis was observed. Calcium had some inhibiting effect.

Effect of ATP, ADP, AMP, pyrophosphate and phosphate

As ATP was required in the reaction, it was found of interest to investigate the effects of the possible reaction products of ATP. Fig. 8 shows that ADP could substitute for ATP in the reaction, but ATP proved to be more than twice as effective in lower concentrations. AMP had, as expected, no effect. From the participation of ATP in the formation of cholyl-S-CoA it may be concluded that either ADP and phosphate or AMP and pyrophosphate must be reaction products. Fig. 9 shows that pyrophosphate had a stronger inhibiting effect on the taurocholate formation than had phosphate. The effect of pyrophosphate was also tested when fluoride was omitted from the incubation

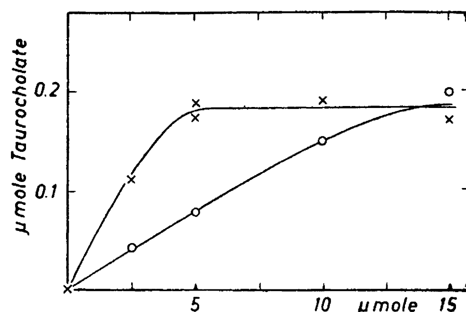


Fig. 8. Effect of ATP, ADP and AMP as energy donors. Microsomes from 300 mg liver. ^{35}S-taurine 0.8 μmoles/vessel, cholic acid 1 μmole/vessel, ATP 5 μmoles/vessel, CoA (75 %) 0.15 μmoles/vessel. TRIS-buffer 0.1 M, KF 0.2 M. Incubation time 60 minutes. + — + — + — + — + *ATP,* O—O—O—O *ADP,* ▲—▲—▲— *AMP.*

mixture. In this case no inhibiting effect was observed at the pyrophosphate concentration used (0.04 M). As fluoride has been shown to inhibit pyrophosphatase [20, 24], this result indicated that the pyrophosphate ion itself was the inhibiting agent and not the phosphate resulting from its splitting by pyrophosphatase. From Table 8 it is seen that pyrophosphate and AMP together had an inhibiting effect stronger than could be explained by the inhibitions effected by the two compounds when added alone, indicating that the effect of one of them was dependent on the presence of the other. No such effect was observed with ADP and phosphate. These results strongly indicate AMP and pyrophosphate as reaction products. The action of ADP as energy donor (Fig. 8) must then be explained as a result of the myokinase reported to be present in liver [25, 26]. This assumption is contradicted by the fact that liver myokinase is claimed to be inhibited by fluoride [26, 27]. Further purification of the enzyme system is desirable for the clarification of this problem.

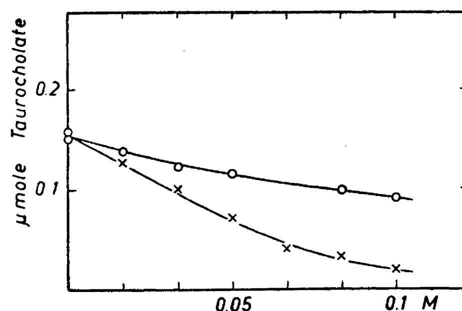


Fig. 9. Effect of orthophosphate and pyrophosphate. Microsomes from approx. 300 mg liver. ^{35}S-taurine 0.8 μmoles/vessel, cholic acid 1 μmole/vessel, ATP 5 μmoles/vessel, CoA (75 %) approx. 0.15 μmoles/vessel. TRIS-buffer 0.1 M, KF 0.2 M. Incubation time 60 minutes. O—O—O—O—O—O *orthophosphate,* + — + — + — + — + *pyrophosphate.*

Table 8. Effect of AMP, ADP, orthophosphate and pyrophosphate.

Microsomes from approx. 300 mg liver. ^{35}S-taurine 0.8 μmoles/vessel (= 65 000 c/min/vessel), cholic acid 1 μmole/vessel, ATP 5 μmoles/vessel, CoA (75 %) 0.15 μmoles/vessel. TRIS-buffer 0.1 M, KF 0.2 M. Incubation time 60 minutes.

Additions/vessel	Net c/min/0.1 ml butanol extract		μmoles taurocholate formed per vessel	
None	1 485 1 610	1 495 1 690	0.14 0.15	0.14 0.16
20 μmoles ADP	1 595	1 540	0.14	0.14
50 μmoles PO_4^{---}	1 165	1 265	0.11	0.12
20 μmoles ADP 50 μmoles PO_4^{---}	1 240	1 310	0.11	0.12
20 μmoles AMP	1 110	1 225	0.10	0.11
50 μmoles $P_2O_7^{----}$	945	1 080	0.09	0.10
20 μmoles AMP 50 μmoles $P_2O_7^{----}$	375	365	0.03	0.03

Table 9. Solubilization of microsomes.

Microsomes from 5 g liver suspended in 9 ml 0.1 M potassium phosphate buffer (pH 7.4) containing 0.5 % cholic acid. Aliquots of 1.5 ml treated as tabeled and centrifuged at 25 000 × g for 90 minutes. 1.0 *ml* of the slightly turbid supernatant added to the vessels. Other additions: ^{35}S-taurine 2 μmoles/vessel (= 7 600 c/min/vessel), cholic acid (from the microsome suspension) approx. 10 μmoles/vessel, ATP 10 μmoles/vessel, CoA (75 %) 0.15 μmoles/vessel, KF 0.2 M, glutathione 0.002 M. Incubation time 90 minutes.

Treatment of microsomes	Net c/min/0.1 ml butanol extract	μmoles taurocholate formed per vessel
None	178	0.35
Incubated for 40 min. at 37° C	89	0.18
Frozen and thawed once	184	0.36
Frozen and thawed twice	179	0.35
Frozen and thawed three times	227	0.44
Frozen and thawed six times	194	0.38

Stability and solubilization of the enzyme system

For a more careful investigation of the reaction mechanism, a further purification of the enzyme system was considered to be desirable. In preliminary experiments we have apparently succeeded in liberating the enzyme system from the microsomes. We used a method similar to that employed by Wainio *et al.*[28] for the liberation of cytochrome oxidase from cell particles. The microsome fraction was suspended in 0.1 M potassium phosphate buffer (pH 7.4) containing ½—1 % cholic acid. The suspension was frozen at —80° C and re-thawed at 37° C. After freezing and thawing once, the suspension got translucent. When centrifuged at 25 000 × g for 90 minutes, a small colourless sediment and a slightly turbid supernatant were formed, the latter containing at least 70—80 % of the enzyme activity. In Table 9 is shown an experiment where the total enzyme activity was recovered in the supernatant. It must be noted that both phosphate and cholic acid must be added to liberate the enzyme system from the particles. If cholate or phosphate were used alone, a practically inactive supernatant was obtained.

Whether the treatment resulted in a real solubilization or only in a decreased particle size could not be stated, as a faster centrifuge was not available. In preliminary experiments we did not obtain any further purification of the enzyme system, as both ammonium sulphate and acetone precipitation gave almost completely inactive precipitates. Whether this inactivation was due to denaturation or to loss of an unknown co-factor is not known. Thioctic acid has been reported to take part in the acetate activation [29], but this compound did not reactivate our ammonium sulphate — or acetone precipitates.

The enzyme system was very stable in the cold. Storing of the microsomes at —20° C for a fortnight did not lead to any appreciable loss in activity. The microsomes were also lyophilized, and the resulting dry powder showed full activity. Heating to 100° C gave immediately full inactivation.

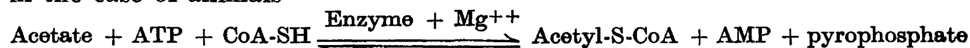
DISCUSSION AND CONCLUSION

Several biological mechanisms are known by which peptide bonds are formed [30]. CoA is required in the formation of the peptide bond in acetylsulfanilamide [19] and in hippuric acid [31]. Acetyl-S-CoA and benzoyl-S-CoA have been proved to be intermediates in these two reactions [19, 31], and in the acetylation of sulfanilamide two enzymes, one acetate activating and one acetyl transferring enzyme, are involved [19].

In all CoA-dependent reactions, the sulphydryl group of CoA forms thioester bonds with acyl groups [7, 31, 35], and these acyl-S-CoA compounds are known to react spontaneously with hydroxylamine to form hydroxamic acid [7, 18, 22]. The absolute requirement of CoA both in the conjugation of cholic acid with taurine and in the formation of cholhydroxamic acid from cholic acid and hydroxylamine therefore strongly indicates that cholyl-S-CoA is an intermediate in these reactions. The accumulation of an intermediate during the pre-incubation without taurine also supports this conclusion.

Admittedly, hydroxamic acids are also formed in other biological reactions, but in these cases CoA is not required [30, 36, 28].

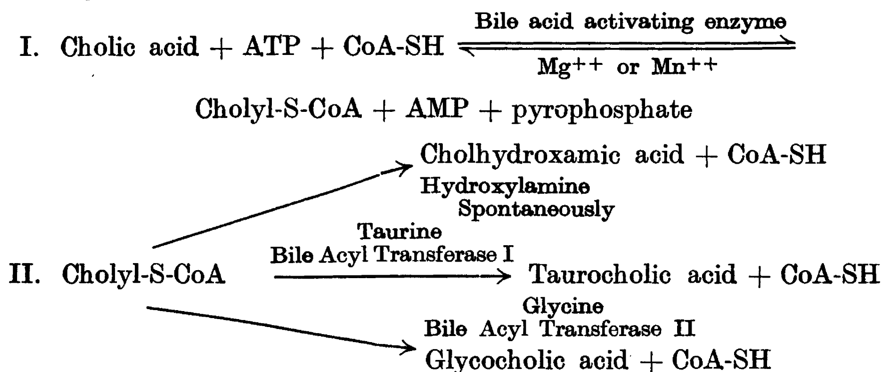
In the acetate activation the following reaction mechanism has been found in the case of animals [32]



A different reaction mechanism has been found in the activation of succinate [33]

Both these reactions have been proved to be reversible [32, 33]. The acetate activation is inhibited by AMP and pyrophosphate [14], whereas the succinate activation is inhibited by ADP and orthophosphate [38]. Our results, which showed a much stronger inhibition of the taurocholate formation by AMP and pyrophosphate than by ADP and orthophosphate, therefore indicate a reaction mechanism in this process identical with that found in the acetate activation. This similarity to the acetate activation is also confirmed by the stimulating effect of magnesium ions on the taurocholate- and cholhydroxamic acid formation.

Recently we have carried out experiments indicating that the transfer of the cholyl group from CoA to taurine and glycine is irreversible [39], as the conjugated acids were not converted to cholhydroxamic acid in the presence of microsomes and CoA. We have further shown that rabbit liver microsomes conjugate free bile acids almost exclusively with glycine, whereas chicken liver microsomes conjugate exclusively with taurine. Rat liver microsomes conjugate both with taurine and glycine. These results probably indicate that two different enzymes transfer the cholyl group from CoA to taurine and glycine. The following reaction scheme therefore seems to be a reasonable conclusion on the experimental results:



A further purification of the enzyme system will probably be needed for a closer elucidation of the reaction mechanism. Thioctic acid has been reported to take part in the acetate activation [29], and results have been published which indicate that the acetate activation proceeds with enzyme-AMP and enzyme-S-CoA as intermediates.

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