Requirement for cysteine. Cysteine is necessary for full activity, presumably to maintain CoA in the reduced form. The requirement is not absolute, since the activity in the absence of cysteine is 45% of that in its presence.

Distribution of the enzyme in the liver fractions. Activity has been conclusively observed only in the microsome fraction. None was present in mitochondria. With the soluble fraction (Table 3) hydroxamic acid is formed from endogenous substrates; this formation is inhibited by cholic acid. The results obtained with this enzyme system do not indicate an activation of cholic acid.

DISCUSSION

The results suggest that the following reactions take place in liver microsomes:

(1) cholic acid + ATP + CoA → cholyl-CoA,

(2) cholyl-CoA + hydroxylamine → cholylhydroxamic acid + CoA.

Reaction 1 is probably the only enzyme-catalysed step in the synthesis of cholylhydroxamic acid. The final step in the reaction, that between cholyl-CoA and hydroxylamine, is most likely non-enzymic in nature and consequently high concentrations of the trapping agent are required to give a reasonably fast rate of reaction. The nature of the breakdown of ATP in reaction 1 has not yet been investigated.

Since this work was completed Siperstein (1955) has reported that CoA stimulates the synthesis of taurocholic acid in rat-liver microsomes plus the soluble fraction of liver.

The next paper (Elliott, 1956) will describe the participation of CoA in taurocholic acid synthesis in guinea-pig-liver microsomes.

SUMMARY

The synthesis of cholylhydroxamic acid by liver preparations has been demonstrated. This has been found to be due to two distinct mechanisms. The first is a simple condensation of cholic acid and hydroxylamine, and is probably lipase-catalysed. The second occurs in the microsome fraction of guinea-pig liver and requires the presence of CoA, ATP, cysteine and Mg^{2+} or Mn^{2+} for full activity. The properties of this cholic acid-activating system are described.

I wish to thank Mr K. M. Jones for useful criticisms and Dr D. C. Torpy for help in the preparation of CoA. I also wish to thank Miss Janet Payne for valuable technical assistance.

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The Enzymic Synthesis of Taurocholic Acid: A Qualitative Study

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(*Received* 28 *July* 1955)

Cholylhydroxamic acid has been found to be synthesized from cholic acid in the presence of adenosine triphosphate (ATP), hydroxylamine, coenzyme A (CoA) and Mg^{2+} by preparations of guinea-pig-liver microsomes (Elliott, 1955, 1956). It seemed likely that cholyl-CoA would prove to be an intermediate in the synthesis of taurocholic acid from cholic acid and taurine. This has been investigated, and the present publication describes evidence which supports this.

While this work was in progress several reports appeared on the synthesis of conjugated bile acids. Bergström & Gloor (1954) reported the synthesis of taurocholic acid in rat-liver slices and homogenates. More recently, Bremer (1955) has described the production of conjugated bile acids in the

presence of rat-liver microsomes plus the soluble fraction. No evidence about the mechanism of the synthesis has so far appeared. Siperstein (1955) has also reported the synthesis of taurocholic acid in rat-liver microsomes, the soluble fraction being found necessary. Mg^{2+}, glucose 6-phosphate, diphosphopyridine nucleotide, CoA and nicotinamide were all found to stimulate.

EXPERIMENTAL

Materials

The preparation of buffers, substrates and coenzymes has been described in the previous paper (Elliott, 1956).

Taurocholic acid. This was synthesized from cholylhydrazide by the method of Cortese (1937).

Methods

Preparation of washed microsomes. Unwashed microsomes were prepared as described earlier (Elliott, 1956). The pellet of microsomes was resuspended in about 10 vol. of 0·25 M sucrose and recentrifuged at 100 000 *g* for 30 min. at 0°. The washed microsomes were suspended in cold distilled water (1 ml./g. of original liver) and stored at −10°.

Chromatography of taurocholic acid. After incubation, the tubes were placed in boiling water for about 3 min., then cooled and acidified with 0·5 ml. of 2 N-HCl. The protein and the precipitated cholic acid were centrifuged off. The supernatants were poured into small evaporating dishes and neutralized with N-KOH and the solutions dried in a vacuum desiccator over P_2O_5 and NaOH. The dry solids were then extracted by grinding in small glass mortars with 1·5 ml. of ethanol. The precipitates were removed by centrifuging, and 0·5 ml. samples of the supernatants applied to paper for chromatography. The papers used for chromatograms were Whatman no. 3 which had been washed twice, first in acetic acid as described earlier (Elliott, 1956) and secondly by running the solvent used for chromatography down the paper. After this chromatographic wash the papers were dried, soaked in water and again dried. The second wash was found to reduce the background colour after development of the spots. The solvent used was made as follows: 45 ml. of the top layer of a mixture of *n*-butanol, acetic acid and water (80:20:100, by vol.) was mixed with 0·5 ml. of water and 54·5 ml. of acetone. The spots were developed by spraying with 50% $SbCl_3$ in glacial acetic acid, followed by heating of the papers. Taurocholic acid gives the rose-pink colour which is given by cholic acid derivatives.

RESULTS

Synthesis of taurocholic acid by microsome preparations

Fig. 1 shows the results of experiments with unwashed microsomes. Incubations were carried out at 37° in centrifuge tubes. The complete system contained the following: 0·2 ml. of 0·25 M phosphate buffer, pH 7·4; 0·1 ml. of 0·1 M-$MgSO_4$; 100 units of CoA; 0·25 ml. of 0·08 M potassium ATP; 0·1 ml. of 0·04 M potassium cholate; 0·1 ml. of 0·1 M taurine; 0·05 ml. of 0·2 M cysteine; 0·1 ml. of 0·3 M-KF; 0·5 ml. of microsome suspension. Volumes were made up to 2·0 ml. with water where necessary. The 13% pure preparation of CoA (Elliott, 1956) was used.

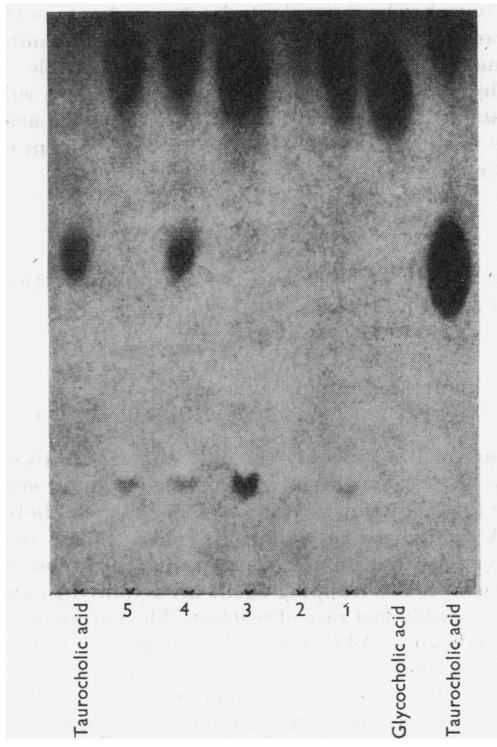

Fig. 1. Taurocholic acid synthesis by unwashed guinea-pig-liver microsomes. Reaction mixtures were incubated for 150 min. under conditions described in the text. (1), No taurine added; (2), no cholic acid added; (3), no ATP added; (4), complete system; (5), glycine added instead of taurine.

It can be seen from Fig. 1 that taurocholic acid is present on the chromatogram when the complete system (tube 4) had been incubated. None was detected when the incubation mixture lacked taurine (tube 1) or cholic acid (tube 2). Tube 3 shows the absolute requirement for ATP. Tube 5, in which taurine was replaced by glycine, again confirms the necessity for the presence of taurine in the system. Glycocholic acid runs very close to cholic acid (Fig. 1), and any synthesis of glycocholic acid might therefore be masked.

Effect of the soluble fraction of a guinea-pig-liver homogenate on taurocholic acid synthesis

Although the amount of soluble fraction in unwashed microsomes must be relatively small, experiments were carried out on washed micro-

somes. Synthesis of taurocholic acid was found to occur in such preparations, as can be seen from the chromatograms shown in Fig. 2. Experiments were also performed in which the microsome fraction was supplemented by the soluble fraction of liver. Within the limits of visual observation of chromatograms there was no stimulation by the soluble fraction.

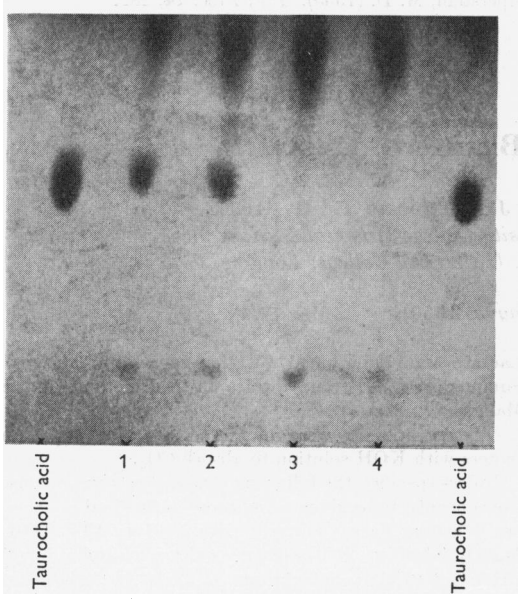

Fig. 2. Requirement for CoA in taurocholic acid synthesis by washed guinea-pig-liver microsomes. Reaction mixtures were incubated as described in the text for 80 min. (1) and (2) are duplicate experiments containing the complete system; (3) and (4) are duplicates from which CoA was omitted.

Requirement for CoA in taurocholic acid synthesis

Fig. 2 also shows the results of duplicate experiments designed to test whether taurocholic acid synthesis in washed guinea-pig-liver microsomes is dependent on added CoA. It can be seen from (1) and (2) that taurocholic acid is synthesized in the presence of added CoA; in (3) and (4), which lacked the coenzyme, none was formed. This suggests that CoA is completely removed from the system by washing the microsomes. This is in contrast to the somewhat analogous synthesis of hippuric acid in liver mitochondria, where the CoA is very tightly bound (Chantrenne, 1951). Further experiments in which a 70% pure preparation of CoA was used instead of the 13% pure preparation confirmed that, as with cholic acid activation, the active principle in the coenzyme preparation is CoA.

DISCUSSION

The results show that taurocholic acid is synthesized in guinea-pig-liver microsomes from cholic acid and taurine by a mechanism requiring ATP and CoA. The pathway is therefore probably as follows:

(1) cholic acid + CoA + ATP → cholyl-CoA,

(2) cholyl-CoA + taurine → taurocholic acid + CoA.

Evidence in support of reaction 1 was reported in the previous paper (Elliott, 1956), when hydroxylamine was used as a trapping agent for cholyl-CoA. The two reactions are presumably catalysed by different enzymes, though evidence for this must await purification of the system.

The proposed mechanism is analogous to the synthesis of the peptide-like bonds of acetylsulphanilamide (Lipmann, Kaplan, Novelli, Tuttle & Guirard, 1947) and of hippuric acid (Chantrenne, 1951).

The observation that the synthesis of taurocholic acid is unaffected by the addition of the soluble fraction of liver, so far as can be detected by the visual examination of chromatographic spots, is at variance with the results of Siperstein (1955) and of Bremer (1955), who find that the soluble fraction is essential for taurocholic acid synthesis by rat-liver microsome preparations. It is not possible at present to explain this difference. It may be that there is a species difference, since different animals were used. However, it is also possible that the effect of the supernatant on taurocholic acid synthesis may have been due not to the participation of a soluble enzyme but to the supply of essential metabolites. In one case the soluble fraction may have been a source of CoA, since the addition of this was not reported (Bremer, 1955). In Siperstein's experiments, which have not yet been described in detail, DPN, nicotinamide and glucose 6-phosphate were found to stimulate. This suggests that the glycolytic activity of the soluble fraction may have been necessary to rephosphorylate adenosine phosphates. In the present work ATP breakdown was minimized by the addition of fluoride, which would suppress ATP-ase activity.

SUMMARY

Chromatographic evidence for the synthesis of taurocholic acid by washed microsome preparations of guinea-pig liver is presented. The synthesis

is completely dependent on the presence of ATP, taurine, cholic acid and CoA. The mechanism of the synthesis is discussed.

I wish to thank Mr K. M. Jones for helpful criticism and Miss Janet Payne for valuable technical assistance. The cholylhydrazide used for the synthesis of taurocholic acid was kindly supplied by Dr P. W. Kent. I also wish to thank Professor G. D. Haslewood for gifts of taurocholic and glycocholic acids.

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Glucose Oxidation by Brain Mitochondria

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(*Received* 10 *August* 1955)

Christie, Judah & Rees (1953), working with rat-brain mitochondrial preparations, measured the rates of oxidation of substrates of the tricarboxylic acid cycle and the process of oxidative phosphorylation. These measurements were employed as indicators of the survival of mitochondria as effective units. The survival time is short, but may be prolonged by the addition of a number of soluble factors including adenosine triphosphate, coenzyme I, glutathione, cobalt, and what was believed to be a new cofactor present in liver and yeast autolysates. In the present paper, experiments are described which show that the activity observed in tissue autolysates was due to glucose or glycolytic intermediates. It was found that rat-brain mitochondrial preparations, in contrast to liver and kidney mitochondrial preparations, are capable of oxidizing glucose completely to carbon dioxide and water.

METHODS

Enzyme preparations. Mitochondria were prepared in 0·25 M sucrose (Schneider, 1948).

Reagents. Adenosine triphosphate (ATP) was prepared by the method of Dounce, Rothstein, Beyer, Meier & Freer (1948). Adenylic acid was a commercial preparation, recrystallized from hot water. All adenine nucleotides were at least 95% pure as determined by a spectrophotometric method (Kalckar, 1947). Cytochrome *c* was prepared by a method of Keilin & Hartree (1937) and dialysed against distilled water. Coenzyme I (CoI) was prepared by an unpublished method of Ochoa and coenzyme II by the method of LePage & Mueller (1949). Glutathione and DL-*iso*citric acid were commercial preparations.

Methods of analysis. α-Oxoglutarate and pyruvate were estimated by the method of Friedemann & Haugen (1943). Lactate was determined by the method of Barker & Summerson (1941), and citrate by the method of Weil-Malherbe & Bone (1949).

Oxygen uptakes were measured by Warburg manometers with KOH solution to absorb CO_2.

Unless specified the following *standard medium* was used, the concentrations given being those in the final mixture in the Warburg flasks: adenylic acid 0·001 M; ATP 0·001 M; $MgSO_4$ 0·0067 M; KCl 0·025 M; sodium phosphate buffer pH 7·4, 0·0133 M; cytochrome *c* 2×10^{-5} M; glutathione $6{\cdot}6 \times 10^{-4}$ M; enzyme was added in 0·25 M sucrose, followed by water to 3 ml. total volume. Gas phase, air; temperature, 38°.

In experiments where citrate analyses were carried out, the enzyme preparation was added in 0·5 ml. of 0·15 M-KCl, and KCl was omitted from the standard medium.

All the experiments reported were carried out in duplicate and the results given are mean values.

Preparation of yeast extract. Yeast was autolysed for 6 hr. at 38° in the presence of toluene. The proteins were precipitated by the addition of equal volumes of 0·3 M-$Ba(OH)_2$ and 5% (w/v) $ZnSO_4$ solution. The protein precipitate was removed by filtration. The filtrate was placed in the cathode chamber of a three-compartment electrodialysis apparatus and the other compartments were filled with water. After electrodialysis the required activity was located in the centre compartment, and every 10 g. of fresh yeast yielded on freeze-drying 5·5 mg. of solid material. In the experiments described in the text 150 μg. of this material in 0·5 ml. of water was added to each Warburg flask.

RESULTS

In an earlier publication (Christie *et al.* 1953) it had been observed that the oxidation of L-glutamate by brain mitochondrial preparations fell off after a period of 1·5 hr. This decline in respiration was prevented by preparations from autolysed liver or brain or yeast.