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Kinetic Studies on the Enzymes Conjugating Bile Acids with Taurine and Glycine in Bovine Liver

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Synthesis of glyco- or tauro-cholate from choloyl-CoA and the respective amino acid is shown to be catalysed by the soluble fraction of liver cells. Kinetic evidence supports the conclusion that there are separate enzymes for the synthesis of glycocholate and taurocholate. The kinetic parameters of these enzymes were determined.

Bile acids conjugated with glycine and taurine are constituents of mammalian bile. Unconjugated bile acids are not found in normal bile. Further, conjugated bile acids, as compared with unconjugated bile acids, are less toxic to the intestinal mucosa and are more efficient promoters of intestinal absorption (cf. Wheeler, 1975). Despite the physiological importance of conjugated bile acids, little is known about the enzymes responsible for their conjugation. The conjugation of bile acids involves two separate reactions (Elliott, 1955; Siperstein & Murray, 1955; Bremer, 1956*a*). The first reaction is the ATP-dependent formation of the CoA derivative of the bile acid. This reaction is catalysed by the microsomal fraction of liver and a preliminary characterization has been conducted by Vessey & Zakim (1977). The second step involves the reaction of the CoA derivative of the bile acid with either glycine or taurine. The kinetic properties of the second reaction have not been characterized. In addition, it is not known whether there are separate enzymes for conjugation with glycine and taurine, nor is there agreement on the intracellular localization of this enzyme(s). Thus Siperstein & Murray (1955) localized the enzyme(s) in the soluble fraction of liver cells. Bremer (1956*a*) and Elliott (1956) concluded that it was in the microsomal fraction, and Schersten (1967) in the lysosomal fraction. The purpose of the present paper is to resolve this discrepancy and to present preliminary kinetic data on these reactions in bovine liver. Bile from these animals contains both glycine and taurine conjugates (G. A. D. Haslewood, personal communication). Thus kinetic experiments were designed to provide evidence on whether there are separate enzymes for glycine and taurine conjugation in bovine liver.

Materials and Methods

[1,2-^{14}C]Taurine, [G-^{3}H]taurocholic acid, [1-^{14}C]-glycine and glyco[G-^{3}H]cholic acid were obtained from New England Nuclear, Boston, MA, U.S.A., and were used at a specific radioactivity of 5mCi/mmol. Choloyl-CoA was prepared by the method of Shah & Staple (1968). Bovine liver was obtained fresh from the slaughterhouse. Bovine or guinea-pig livers were homogenized in 4vol. of 0.25M-sucrose and the soluble fraction was obtained by centrifuging a low-speed supernatant at 100000*g* ($r_{av.}$ 5.9cm) at 2°C for 40min. The high-speed supernatants were dialysed at 0°C against two changes of 25mM-potassium phosphate buffer, pH 8.0.

Cholyl-CoA thiolase (EC 6.2.1.7) activity, assayed at pH 8, was determined by measuring the release of free CoA by the phosphotransacetylase assay (Michal & Bergmeyer, 1974). All enzyme preparations for this assay were obtained from Sigma, St. Louis, MO, U.S.A. The activity of choloyl-CoA–amino acid *N*-acyltransferase (EC 2.3.1.13) was assayed by following the synthesis of [^{14}C]taurocholic acid and [^{14}C]-glycocholic acid from choloyl-CoA and [^{14}C]taurine or [^{14}C]glycine. Assays, containing 50mM-potassium phosphate, pH 8.0, 0.8mg of soluble-fraction protein [measured by the biuret method of (Gornall *et al.*, 1949), with albumin as standard], and ^{14}C-labelled amino acid and choloyl-CoA as indicated, in a total volume of 0.1ml, were incubated at 30°C for 0, 20, 40 or 60s. The reaction was terminated by adding 0.5ml of butan-1-ol (water-saturated), and then 0.5ml of 0.1M-potassium phosphate, pH 4 (butan-1-ol-saturated). The two phases were separated after centrifugation. The butan-1-ol layer was washed with 0.5ml of the 0.1M-phosphate, pH 4, and then counted at 95% efficiency in a liquid-scintillation system [with Aquasol scintillator (New England Nuclear)]. Examination of blank reactions (i.e. without choloyl-CoA) revealed that the final butanol-1-ol phase contained less than 0.2% of the [^{14}C]taurine or [^{14}C]glycine originally present in the assay, but reproducibly retained 60% of added [^{3}H]taurocholic acid and 68% of added [^{3}H]glycocholic acid. Extraction blanks for [^{14}C]-taurine and [^{14}C]glycine

were run for each assay in the form of a zero-time reaction. The radioactivity in the final butan-1-ol phase was corrected for extraction losses and the extraction blanks. In all cases assays were conducted at 0, 20, 40 and 60s to obtain initial rates of reaction. The reaction products were identified as [^{14}C]taurocholic acid and [^{14}C]glycocholic acid on the basis of their R_F values on paper chromatography in aq. 70% (R_F 0.71 for both) and 85% (R_F 0.45 for taurocholate; R_F 0.38 for glycocholate) propan-2-ol.

Results and Discussion

As noted above, cholic acid is conjugated with glycine and taurine in liver by a two-step process. Assay of the second reaction requires choloyl-CoA. However, the rate of reaction of choloyl-CoA with glycine or taurine has never been measured directly, presumably because choloyl-CoA is not available from commercial sources. The activities of the *N*-acyltransferases have been determined instead in a system containing cholate, Mg^{2+}, ATP, CoA, the respective amino acid and portions of the soluble and microsomal fractions of liver cells. The microsomal fractions were added to generate choloyl-CoA from cholate, CoA and ATP. This assay system would give valid information on the activities and properties of the *N*-acyltransferase, if the rate of synthesis of choloyl-CoA were at least an order of magnitude greater than the activity of the *N*-acyltransferase, and if the choloyl-CoA synthesized were not metabolized in alternative pathways. Neither of these conditions apply. Thus the reported activities of the enzymes catalysing each of the reactions are nearly the same. In addition, we have studied bovine and guinea-pig liver homogenates and found that the soluble fraction of each hydrolyses added choloyl-CoA to cholic acid and CoA. By using an assay that measured released CoA, the specific activity of the dialysed bovine soluble fraction, when assayed at 50μM-choloyl-CoA, was 0.6nmol of CoA formed/min per mg of protein. The soluble fraction from guinea-pig liver has a specific activity of approx. 0.3nmol/min per mg of protein.

The presence of thiolase activity in the soluble fraction may explain why Bremer (1956*a,b*), Elliott (1956) and Schersten (1967, 1970) failed to find the *N*-acyltransferase in the soluble fraction, whereas Siperstein & Murray (1955) did. The latter investigators added a low concentration of [^{14}C]choloyl-CoA directly to the assay rather than trying to generate it *in situ*, although they did not measure rates of conjugation. The discrepancies in the results obtained by the indirect and direct assay systems can be accounted for by a competition between *N*-acyltransferase and thiolase for the relatively small amounts of choloyl-CoA generated in the assay by the microsomal fraction. Clearly, this problem can be avoided if the enzyme is assayed and characterized in a direct manner.

We have synthesized choloyl-CoA chemically, and used it to determine initial rates of glycine/taurine *N*-acyltransferase activity in different cell fractions of guinea-pig and bovine liver. In support of the findings of Siperstein & Murray (1955), we find that over 90% of the *N*-acyltransferase activity is in the soluble fraction of the cell. The results of these studies mean that the vast number of clinical investigations that were conducted by the indirect assay system (cf. Schersten, 1970) must all be viewed cautiously.

We have conducted a preliminary characterization of the glycine/taurine *N*-acyltransferase reaction in a dialysed supernatant fraction from bovine liver. Activity was determined by a radioassay with either [^{14}C]taurine or [^{14}C]glycine. Enzymic activity towards both glycine and taurine was very stable, withstanding incubation at 50°C for several minutes. The reaction rates were extremely sensitive to the nature of the buffer. Rates of taurine conjugation in phosphate buffer were twice as high as in Tris, nearly three times higher than in Hepes* or collidine, and 10 times higher than in borate buffer. Rates of glycine conjugation were somewhat less sensitive to the nature of the buffer, but phosphate still gave the highest rates. The reactions were unaffected by the presence or absence of bivalent cations. Each reaction had a pH optimum between pH 8 and 8.1, measured in either phosphate or Tris.

Because of interference from the thiolase activity in the soluble fraction, it was not possible to determine accurately initial rates of reaction at concentrations of choloyl-CoA less than 10μM. However, there was no difference in the initial rates of glycine or taurine conjugation at 10μM- and 25μM-choloyl-CoA. It appears therefore that these concentrations of choloyl-CoA are sufficient to saturate the enzyme(s). As a routine, we used 25μM-choloyl-CoA in the assays. At this concentration, the reaction with [^{14}C]taurine had a K_m for taurine of 0.75mM and an extrapolated maximum velocity of 5.9nmol of conjugate synthesized/min per mg of soluble-fraction protein. The reaction with [^{14}C]glycine had a K_m for glycine of 0.4mM and an extrapolated maximum velocity of 2.0nmol/min per mg of protein. Thus in bovine liver the maximum potential for taurine conjugation is greater than that for glycine, although glycine has a greater affinity for the enzyme.

The question of whether there are separate enzymes for glycine and taurine conjugation was investigated by the technique of alternative-substrate inhibition. The initial rate of reaction of choloyl-CoA

* Abbreviation: Hepes, 4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid.

Table 1. *Alternative-substrate-inhibition studies of cholic acid conjugation with glycine and taurine*

Initial rates of reaction were determined by the standard assay described in the Materials and Methods section. Conjugation with taurine was measured at a [^{14}C]taurine concentration of 0.75mM and glycine conjugation at a [^{14}C]glycine concentration of 0.4mM. Unlabelled glycine and taurine were tested as inhibitors at concentrations approx. 3 times the K_m for their own conjugation.

Substrate	Addition	Activity (nmol of conjugated cholic acid formed/min per mg of protein)
[^{14}C]Taurine	—	2.56
[^{14}C]Taurine	1.2mM-Glycine	2.56
[^{14}C]Glycine	—	0.82
[^{14}C]Glycine	2.0mM-Taurine	0.82

with [^{14}C]taurine was determined with [^{14}C]taurine present at its K_m concentration (0.75mM). Unlabelled glycine was added at a concentration of 1.2mM, which is 3 times its K_m concentration in the glycine-conjugation reaction. The data in Table 1 reveal that this concentration of glycine caused no inhibition of the conjugation of [^{14}C]taurine. The data in Table 1 reveal also that the initial rate of conjugation of [^{14}C]glycine (present at its K_m concentration) was unaffected by the addition to the assay of a concentration of unlabelled taurine, that is 3 times its K_m in the taurine reaction. These data demonstrate that there are separate active sites for glycine and taurine in the conjugation reactions. This result is consistent with the conclusion that there are separate enzymes for the conjugation of cholic acid with glycine and taurine.

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