The Biochemical Basis for the Conjugation of Bile Acids with either Glycine or Taurine

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All animals, except for the placental mammals, conjugate their bile acids exclusively with taurine. However, in certain of the placental mammals, glycine conjugates are also found. The basis for the appearance of glycine conjugation among the placental mammals was investigated. The reaction of choloyl-CoA with glycine and taurine, as catalysed by the soluble fraction from guinea-pig liver, had a high affinity for taurine and a poor affinity for glycine. The predominant synthesis of glycine conjugates in the guinea pig can be related to the fact that guinea-pig liver contains an unusually low concentration of taurine and a high concentration of glycine. Rabbits make exclusively glycine conjugates and their livers also contain low concentrations of taurine. However, the biochemical basis for their glycine conjugation is more straightforward than in the guinea pig in that the soluble fraction from rabbit liver has a high affinity for glycine and a poor affinity for taurine. Alternative-substrate-inhibition studies with glycine and taurine in soluble fractions from guinea-pig and rabbit liver revealed that glycine and taurine were mutually inhibitory. This suggests that there is only one enzyme for glycine and taurine conjugation in these tissues. The soluble fractions from bovine liver and human liver also made both glycine and taurine conjugates and evidence is presented that suggests that there is only one enzyme in these tissues too. Even the rat, which excretes mostly taurine conjugates, could make both glycine and taurine conjugates *in vitro*. However, in contrast with all of the placental mammals studied, the supernatant fraction from liver of the chicken, and other non-mammals, could not make glycine conjugates even in the presence of very high concentrations of glycine.

An important step in the formation of bile is the conjugation of the individual bile acids with an amino acid (O'Maille *et al.*, 1965; Hoffman & Small, 1967). This reaction is catalysed by an enzyme present in the soluble fraction of liver (Vessey *et al.*, 1977) and requires the prior activation of the bile acid to a CoA derivative. In most animals, bile acids are conjugated with taurine (Haslewood, 1962, 1967), and in fact conjugation of bile acids and arylacetic acids (Williams, 1974) is the only proven role for this highly abundant and ubiquitous amino acid. It is noteworthy that among the placental mammals are a number of species that can also make glycine conjugates of bile acids, and a few of these appear unable to make taurine conjugates (Haslewood, 1962, 1967).

We were interested in understanding the biochemical basis for the appearance of glycine conjugation among the placental mammals and for the disappearance of taurine conjugation in certain of these species. Toward this end, we have studied the kinetics of the choloyl-CoA–glycine *N*-acyltransferase and choloyl-CoA–taurine *N*-acyltransferase

activities in the soluble fraction of liver from a variety of different animals.

Materials and Methods

[1,2-¹⁴C]Taurine and [1-¹⁴C]glycine were obtained from New England Nuclear, Boston, MA, U.S.A. Choloyl-CoA and chenodeoxycholoyl-CoA were prepared by the method of Shah & Staple (1968).

The soluble cell fraction of liver was prepared as described by Vessey *et al.* (1977), and was dialysed at 0°C for 5 h against two changes of 25 mM-phosphate buffer, pH 8.0. Protein was determined by the biuret method (Gornall *et al.*, 1949). The α -oxoglutarate dehydrogenase complex was isolated from bovine heart mitochondria by the method of Kenney *et al.* (1972) and then further fractionated by ammonium acetate precipitation.

Choloyl-CoA–amino acid *N*-acyltransferase activities were determined either by a radiochemical assay or by spectrophotometric assay. The radioactive assays measure the synthesis of either [¹⁴C]taurocholate or [¹⁴C]glycocholate from [¹⁴C]-

taurine or [^{14}C]glycine and choloyl-CoA. The standard assay contained 50mM-potassium phosphate, pH 8.0, 0.8mg of soluble-fraction protein, 25 μM -choloyl-CoA and either [^{14}C]taurine or [^{14}C]glycine. Multiple time-point assays were conducted to allow for the determination of initial rates of reaction. The conjugated bile acid was separated from unchanged amino acid by extraction with butan-1-ol (Vessey *et al.*, 1977). The same assay techniques were used to measure chenodeoxycholoyl-CoA-amino acid *N*-acyltransferase activities.

The spectrophotometric assay is based on the CoA-dependent oxidation of α -oxoglutarate catalysed by the α -oxoglutarate dehydrogenase complex. The reactions are followed continuously by measuring the reduction of NAD^+ at 340nm. The assay mixture contained 100mM-phosphate, pH 8.1, 0.3mM- α -oxoglutarate, 0.4mM- NAD^+ , choloyl-CoA, 1 unit of α -oxoglutarate dehydrogenase complex, taurine or glycine and approx. 1 mg of soluble fraction protein. Vessey *et al.* (1977) reported that the soluble fractions from most livers contained choloyl-CoA thiolase activity. Therefore the rate of production of CoA from choloyl-CoA, in the absence of added glycine or taurine, was measured at each concentration choloyl-CoA used and this rate was subtracted from those obtained in the presence of amino acid. Rates determined with the spectrophotometric assay were comparable with rates determined by the radiochemical assay.

Results

Bile acid conjugation in the guinea pig

The bile acids in guinea-pig bile are conjugated predominantly with glycine (Schoenfield & Sjoval, 1966; Spaeth & Schneider, 1973). The bile collected from the guinea pigs used in our studies contained approx. 70% glycochenodeoxycholic acid, 15–20% taurochenodeoxycholic acid and 10–15% unidentified bile acids. However, when the soluble fraction from guinea-pig liver was assayed for choloyl-CoA-glycine *N*-acyltransferase or choloyl-CoA-taurine *N*-acyltransferase activities by the radioassays, glycine-conjugating activity was barely detectable. Taurine conjugation, on the other hand, proceeded at a rate comparable with that reported for bovine liver (Vessey *et al.*, 1977). The assay conditions used for measuring glycine and taurine conjugation were those that gave optimal activity in the work with the bovine liver enzymes (Vessey *et al.*, 1977); the concentration of glycine was 0.4mM and of taurine, 0.75mM. Further, glycine conjugating activity was not present in an undialysed whole-cell homogenate of guinea-pig liver nor in any of the associated particulate cell fractions.

The subcellular fractions of guinea-pig liver were

also tested for glycine conjugating activity with chenodeoxycholoyl-CoA, since chenodeoxycholic acid is the predominant bile acid in the guinea pig. Once again, activity was barely detectable and was restricted to the soluble cell fraction. Taurine conjugating activity with chenodeoxycholoyl-CoA was found in the soluble cell fraction and the rates were the same as with choloyl-CoA. Attempts to enhance the glycine conjugating activity towards either choloyl-CoA or chenodeoxycholoyl-CoA by varying the pH or changing the buffer only resulted in a diminution of activity. However, increasing the concentration of glycine did enhance the rate of glycine conjugation. The rate of glycine conjugation became comparable with the rate of taurine conjugation for both choloyl-CoA and chenodeoxycholoyl-CoA. For such high concentrations of glycine, it is not possible to use the [^{14}C]glycine radiochemical assay. The extraction procedure is not able to completely separate the unchanged [^{14}C]glycine from the [^{14}C]glycocholate, and at high concentrations of [^{14}C]glycine the radioactivity in unextracted [^{14}C]glycine is higher than the radioactivity in the product. Alternatively, we have developed a spectrophotometric assay that measures the CoA released in the conjugation reaction by means of the α -oxoglutarate dehydrogenase complex (see the Materials and Methods section).

From the spectrophotometric assay, a kinetic analysis of both glycine and taurine conjugation was conducted on the soluble fraction from guinea-pig liver. Since the activities with chenodeoxycholoyl-CoA and choloyl-CoA parallel one another,

Fig. 1. Rate of the *N*-acyltransferase reaction catalysed by the soluble fraction from guinea-pig liver as a function of the concentration of either glycine or taurine

Rates of reaction were determined with either glycine (■) or taurine (●), in the spectrophotometric assay at a choloyl-CoA concentration of 80 μM .

Fig. 2. Rate of the *N*-acyltransferase reaction catalysed by the soluble fraction from rabbit liver as a function of the concentration of either glycine or taurine

Rates of reaction were determined with either glycine (■) or taurine (●), in the spectrophotometric assay at a choloyl-CoA concentration of 25 μ M.

we used choloyl-CoA in the studies that follow to facilitate comparison with other species in which choloyl-CoA is the standard. Double reciprocal plots of $1/v$ versus $1/\text{amino acid concentration}$ were non-linear. The kinetic data are therefore presented in the form of straight v versus $[S]$ plots, which are shown in Fig. 1 for both glycine and taurine in the presence of saturating amounts of choloyl-CoA (80 μ M). Glycine and taurine conjugation have about the same activity at $V_{\max}$, but the affinity for taurine is 50–100 times higher than that for glycine.

Since the conjugation reactions in guinea-pig soluble-fraction are characterized by a high affinity for taurine and a low affinity for glycine, we wondered if glycine conjugation and taurine conjugation were taking place on the same enzyme. If glycine and taurine conjugation are occurring at the same active site, then they will show competitive-binding kinetics. To test for this, we used the radiochemical assay to determine the rate of production of [^{14}C]taurocholate from choloyl-CoA and [^{14}C]taurine in the presence and absence of unlabelled glycine. When [^{14}C]taurine was present at its apparent K_m concentration (0.2 mM), the rate of reaction was 1.13 nmol of [^{14}C]taurocholate formed/min per mg of protein. The addition of glycine to the assay at its apparent K_m concentration (20 mM) decreased this rate to 0.83 nmol/min per mg. Thus glycine can bind at the [^{14}C]taurine active site.

Similarly, one would expect that taurine should be able to inhibit conjugation with [^{14}C]glycine. Unfortunately, this cannot be validated directly because the radiochemical assay cannot be used at

the high concentrations of glycine required to obtain adequate rates of conjugation. As an alternative, one can use the spectrophotometric assay to measure the rate of release of CoA from choloyl-CoA when only glycine is present and when only taurine is present. The sum of these two rates can then be compared with the rate of CoA production when both glycine and taurine are present in the same assay. The rate of CoA release in the presence of 50 mM-glycine, a concentration at which approx. 74% of the glycine active sites would be occupied, was 3.48 nmol/min per mg of protein. The rate of CoA release in the presence of 0.4 mM-taurine ($2 \times K_m$, or 67% of the sites occupied) was 2.27 nmol/min per mg of protein. If the taurine and glycine were non-competitive in their binding, the rate in the presence of both should be additive or 5.75 nmol/min per mg of protein. However, it is already known that glycine binds at the taurine site (see above). This inhibition would lower the taurine rate to 1.6 and the additive rate to 5.1 nmol/min per mg of protein. The actual rate in the presence of 0.4 mM-taurine and 50 mM-glycine is 3.20 nmol/min per mg of protein. This rate indicates that 0.4 mM-taurine is able to inhibit the synthesis of glycocholate at 50 mM-glycine. These data suggest that taurine and glycine conjugation takes place at the same active site in guinea-pig liver.

Bile acid conjugation in the rabbit

The predominant bile acids found in the rabbit are cholic acid and deoxycholic acid (Gregg & Poley, 1966). In bile, they are present almost exclusively as glycine conjugates (Spaeth & Schneider, 1973; Gregg & Poley, 1966). The soluble cell fraction was obtained from rabbit liver and assayed for taurine and glycine conjugating activity toward choloyl-CoA. The spectrophotometric assay was used. A high rate of glycine conjugation was observed at a glycine concentration of 1 mM. The rate of conjugation at 1 mM-taurine was extremely low.

A kinetic analysis of glycine and taurine conjugation was conducted on the soluble fraction of rabbit liver by using the spectrophotometric assay. Double-reciprocal plots of $1/v$ versus $1/[\text{substrate}]$ were non-linear, but appeared to extrapolate to similar values for $V_{\max}$ for taurine and glycine. The results of the kinetic analyses plotted in v versus $[S]$ form are shown in Fig. 2. The data reveal that, although the rate of taurine conjugation at $V_{\max}$ is nearly the same as the rate of glycine conjugation at $V_{\max}$, the affinity for glycine is 20–30 times higher than the affinity for taurine.

Alternate substrate inhibition was used to study whether the glycine and taurine conjugations occurred at the same active site. Radiochemical assays were necessary for this type of study in order to measure the rate of synthesis of a specific conjugated

Table 1. *Alternative-substrate-inhibition studies with glycine and taurine on the soluble fraction of rabbit liver*

By using the standard radiochemical assays and the soluble fraction of rabbit liver, the initial rate of reaction of choloyl-CoA with 0.9 mM-[14 C]glycine was determined in the presence and in the absence of 15 mM-*taurine* and the initial rate of reaction of choloyl-CoA with 4.7 mM-[14 C]*taurine* was determined in the presence and in the absence of 0.3 mM-glycine. Activity is expressed as nmol of 14 C-labelled bile acid conjugate formed/min per mg of soluble-fraction protein. The data are representative of results from a number of animals.

Substrate	Inhibitor	Activity
[14 C]Glycine	—	2.57
[14 C]Glycine	Taurine	1.76
[14 C]Taurine	—	0.639
[14 C]Taurine	Glycine	0.394

bile acid. Unlike the situation in the guinea pig, we were able to use the radiochemical assay to measure both glycine and taurine conjugation (although the highest [14 C]*taurine* concentration that could be used was still probably far below K_m). The initial rate of reaction with [14 C]glycine was determined with [14 C]glycine present at its apparent K_m concentration (0.9 mM). When unlabelled *taurine* was added, it was present at 15 mM. The results (Table 1) reveal that under these conditions, the addition of *taurine* causes a 32% inhibition. When the initial rate of [14 C]*taurine* conjugation was determined at 4.7 mM-[14 C]*taurine* (approx. $0.3 \times K_m$), in the presence and absence of 0.3 mM-unlabelled glycine ($0.3 \times K_m$), glycine led to a 38% inhibition of *taurine* conjugation (Table 2). Thus glycine is able to bind at the *taurine* active site and *taurine* can bind at the glycine active site.

Bile acid conjugation in the chicken and the croaker fish

The predominant bile acids in the chicken are chenodeoxycholic acid, isolithocholic acid and cholic acid, and in the croaker fish (Family, Sciaenidae) they are cholic acid and chenodeoxycholic acid. These are conjugated exclusively with *taurine* in both species (Haslewood, 1962). Examination of the soluble cell fraction of chicken liver *in vitro* revealed the expected *taurine* conjugating activity with choloyl-CoA and chenodeoxycholoyl-CoA. Conjugating activity with glycine was not detectable with either choloyl-CoA or chenodeoxycholoyl-CoA, even at concentrations of glycine as high as 75 mM. Also glycine at a concentration of 50 mM did not affect the rate of conjugation of 0.5 mM-*taurine*. The soluble cell fraction from croaker fish liver also contained *taurine* conjugating activity towards choloyl-CoA, but glycine con-

Table 2. *Taurine and glycine concentrations in the liver of several animals*

The concentrations are expressed as μ mol of amino acid/g of fresh liver. References: ^a, Vehvelainen *et al.* (1966); ^b, Spaeth & Schneider (1976); ^c, Awapara (1956); ^d, Krueger (1950); ^e, Christensen *et al.* (1948); ^f, Dubreuil & Timiras (1953); ^g, Fujihira *et al.* (1971).

Species	Concentration (μ mol/g of liver)	
	Taurine	Glycine
Guinea pig	0–1.2 ^{a,b,c}	13.8–22.9 ^{d,e}
Rabbit	0–3.0 ^{b,c,f}	6.28 ^d
Rat	4.0–7.7 ^{b,c,g}	2.7–6.0 ^d

jugating activity was not detectable even at 75 mM-glycine.

Bile acid conjugation in rat, man and beef cattle

The primary bile acid in the rat is cholic acid (Haslewood, 1967) and over 80% of the conjugates found in rat bile are *taurine* conjugates (Spaeth & Schneider, 1976). Examination of the soluble fraction from rat liver revealed the expected situation *in vitro*, that is, there was a high affinity for *taurine* and a low affinity for glycine. It is interesting that the relative affinities for glycine and *taurine* in the rat are quite similar to that for the guinea pig (Fig. 1), but the V_{max} value is twice as high in the rat.

Bile from both beef cattle and man contains primarily cholic acid, deoxycholic acid and chenodeoxycholic acid, all conjugated with both glycine and *taurine* (Haslewood, 1962; Hafkenschied & Hectors, 1975). In a study on the supernatant fraction from bovine liver (Vessey *et al.*, 1977), the radiochemical assay was used to examine the kinetics of the *N*-acyltransferase reactions. However, the radiochemical assay is limited to substrate concentrations that are less than 1–2 mM. We have subsequently examined the kinetics over a much broader range of substrate concentrations using the spectrophotometric assay and found that they are biphasic. The double-reciprocal plots of $1/v$ versus $1/\text{amino acid concentration}$ extrapolate to a low K_m value for low substrate concentrations and a high K_m value for high substrate concentrations. The alternative-substrate-inhibition studies with glycine and *taurine*, which were reported in the previous publication, were thus done at concentrations of amino acid that were not half-saturating the enzyme active site, as thought, but were in fact far less than that. Thus those studies cannot be taken as evidence for separate glycine and *taurine* conjugating enzymes. In fact, glycine and *taurine* conjugating activities co-purify from bovine liver, suggesting that there is only one enzyme (D. A. Vessey, unpublished work).

Human liver obtained at autopsy was also investigated. Since the *N*-acyltransferase activity in liver of all other species studied is quite stable, the delay involved in obtaining liver at autopsy probably does not greatly alter the *N*-acyltransferase of the soluble fraction from the human liver *in vitro*. Kinetic studies with this tissue revealed a slightly greater affinity for taurine relative to glycine. Moreover, glycine and taurine were mutually inhibitory, which suggests a common enzyme for both conjugations.

Discussion

There is a clear-cut separation between placental mammals and all other animals in the nature of the excreted bile acids (Haslewood, 1962). In the latter group, bile acids are excreted solely as taurine conjugates. However, among the placental mammals are many species that excrete glycine-conjugated bile acids, either entirely or in addition to taurine conjugates. The biochemical studies reported in the present paper reveal that there is a fundamental difference in the conjugating capacity of the five placental mammals studied as opposed to the two non-mammals. The livers of the chicken and the croaker fish contain an enzyme that makes only taurine conjugates. The supernatant fractions from these livers were unable to make glycine conjugates even in the presence of 75 mM-glycine. On the other hand, the supernatant fraction from the liver of all placental mammals tested could make glycine conjugates to at least some degree; even that from liver of the rat, which is primarily a taurine conjugator. This suggests that either a separate glycine-conjugating enzyme arose among the mammals or that the taurine enzyme underwent a change of specificity. There is no evidence at present to indicate the existence of a separate glycine conjugating enzyme. The fact that glycine and taurine were mutually inhibitory in the conjugation reactions for guinea pig, rabbit and human liver suggests that there is only one enzyme for bile acid conjugation. This is supported by the copurification of glycine and taurine conjugating activity from bovine liver (D. A. Vessey, unpublished work). Also, it has been found that the previous data suggesting a separate enzyme for glycine (Vessey *et al.*, 1977) can be interpreted in a different way (see the Results section).

It was also the purpose of the present study to try to account for the glycine:taurine ratio in the bile of the individual species studied. The guinea pig makes predominantly glycine conjugates so we expected that guinea-pig liver would contain an efficient enzyme for glycine conjugation. Instead, the data suggest the presence of an enzyme that was efficient at taurine conjugation and relatively devoid of glycine-conjugating activity. Why then does

the guinea pig make primarily glycine conjugates? The answer to this question, we believe, is that guinea-pig liver contains little or no taurine and an unusually high concentration of glycine (Table 2). The relatively inefficient glycine conjugation proceeds because there is little taurine available to compete with glycine for binding to the active site of the enzyme.

The rabbit makes exclusively glycine conjugates and, like the guinea pig, has a very low concentration of taurine in the liver (Table 2). In appearance, the situation looks to be the same in both species. However, the studies on rabbit revealed that the enzymology is quite different. Glycine conjugation by the soluble fraction from rabbit liver is characterized by a relatively high affinity for glycine, and taurine conjugation showed a relatively poor affinity for taurine. The rabbit appears to possess only one enzyme and it is most efficient at glycine conjugation.

This study has suggested that the appearance of glycine-conjugated bile acids in the bile of placental mammals is the result of two contributory factors. One is by the appearance of a new or altered enzyme, an enzyme that is able to utilize glycine as well as taurine. The other is that, in certain species, there is a deficiency of hepatic taurine, so that they were unable to synthesize taurine-conjugated bile acids and thus made an unusually high amount of glycine conjugates. Perhaps glycine conjugation arose in the eutherians as an adaptive response to taurine deficiency in the liver.

The above discussion is concerned with the relative rates of glycine versus taurine conjugation. However, bile acid conjugation is a two-step reaction and the overall rate is determined by the slowest step. Choloyl-CoA-glycine *N*-acyltransferase activity in the soluble fraction from guinea-pig liver is 3 nmol/min per mg of soluble fraction protein (Fig. 1). The choloyl-CoA synthetase activity for guinea-pig liver is approx. 2 nmol/min per mg of microsomal fraction protein (Vessey & Zakim, 1977). Since the yield of soluble-fraction protein in a subcellular fractionation of guinea-pig liver is 3 times the yield of microsomal protein, the *N*-acyltransferase activity per liver is 4–5 times higher than the choloyl-CoA synthetase activity. For rat liver, the *N*-acyltransferase activity per liver is over 15 times higher than the choloyl-CoA synthetase activity (D. A. Vessey, unpublished work). Thus the overall rate of bile acid conjugation in these species is controlled by the choloyl-CoA synthetase.

Note Added in Proof (Received 17 April 1978)

A recent paper by Paul Killenberg (1978) on 'The measurement and sub-cellular distribution of choloyl-CoA synthetase and bile acid-CoA:amino acid *N*-acyltransferase activities in rat liver' substantiates our data for the rat.

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