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2-FLUORO-β-ALANINE, A PREVIOUSLY UNRECOGNIZED SUBSTRATE FOR BILE ACID COENZYME A:AMINO ACID:*N*-ACYLTRANSFERASE FROM HUMAN LIVER

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Abstract—Our laboratory has demonstrated recently that conjugates of 2-fluoro-β-alanine (FBAL) and bile acids are the major biliary metabolites of 5-fluorouracil (FUra) in cancer patients. Bile acids are normally conjugated with glycine or taurine, and therefore the identification of the FBAL-bile acid conjugates suggested that FBAL may also be a substrate for the bile acid conjugating enzyme, bile acid CoA:amino acid:*N*-acyltransferase. Enzyme activity detected using glycine and taurine as substrates was purified 8-fold from human liver cytosol using a DEAE-cellulose column. This preparation when tested for its activity towards β-alanine and FBAL using cholyl CoA as the bile acid substrate only catalyzed the formation of FBAL-cholate. β-Alanine was not a substrate. Confirmation of FBAL-cholate as the enzymatic product was demonstrated by (1) coelution of the product of this reaction on HPLC with authentic FBAL-cholate, (2) specific hydrolysis of this product by cholylglycine hydrolase, and (3) molecular weight of the product (497) being identical to that of the authentic FBAL-cholate. Kinetic experiments demonstrated that the enzyme had an affinity for FBAL (K_m 1.45 mM) comparable to taurine (K_m 1.32 mM), but greater than glycine (K_m 6.45 mM). Formation of FBAL-cholate was inhibited competitively by taurine (K_i 1.27 mM) and glycine (K_i 4.47 mM), suggesting that a single enzyme is responsible for conjugation of glycine, taurine and FBAL with bile acids. These data indicate that the formation of the FBAL-bile acid conjugates in patients receiving FUra results from high affinity of the bile acid conjugating enzyme for FBAL.

Introduced over 30 years ago, 5-fluorouracil (FUra)† continues to be a widely used antineoplastic agent. The anabolism of FUra has been studied extensively and appears to be responsible for the observed cytotoxicity in host and tumor cells. However, catabolism is the major route of FUra metabolism with greater than 80% of the administered dose being rapidly degraded and eliminated [1]. The major catabolite of FUra in humans is the 2*R*-stereoisomer of the fluorinated amino acid 2-fluoro-β-alanine (FBAL) [2], which is excreted largely in the urine [3]. Studies from our laboratory have demonstrated, utilizing mass spectrometry, that greater than 90% of biliary FUra metabolites are conjugates of FBAL and bile acids [4, 5]. Other investigators have confirmed by NMR spectroscopy the presence of these conjugates in the bile of cancer patients receiving FUra [6].

In humans, bile acids are usually conjugated with glycine or taurine prior to their secretion into bile [7]. This amidation of bile acids is catalyzed by the hepatic enzyme bile acid CoA:amino acid:*N*-acyltransferase (EC 2.3.1) [8]. Since FBAL is structurally similar to taurine, the identification of the FBAL-bile acid conjugates suggests that FBAL is a substrate for this enzyme. However, this has not been demonstrated previously.

In the present study, a specific radioassay [9] was used to examine whether partially purified human hepatic bile acid CoA:amino acid:*N*-acyltransferase could catalyze the formation of FBAL-cholate, the major FBAL-bile acid conjugate in patients receiving FUra [4]. Kinetic experiments were also performed to determine the apparent K_m and V_{max} values of this enzyme for FBAL compared to the naturally occurring substrates glycine and taurine. In addition, experiments were performed to determine whether glycine or taurine is a competitive inhibitor of FBAL-bile acid conjugation since this would suggest that each amino acid is a substrate for the same enzyme.

MATERIALS AND METHODS

Chemicals. Unlabeled glycine, taurine, β-alanine and cholic acid were purchased from CalBiochem (San Diego, CA). (*R,S*)-FBAL was purchased from American Tokyo Kasei, Inc. (Portland, OR). Coenzyme A (CoA) and cholylglycine hydrolase were purchased from the Sigma Chemical Co. (St. Louis, MO). Cholyl CoA was synthesized by the method of Shah and Staple [10] and purified as previously described [9]. DEAE-cellulose was purchased from Whatman Ltd. (Clifton, NJ). All solvents were HPLC grade, and all other reagents were the highest grade available.

Radiolabeled [6-^{3}H]FUra (26 Ci/mmol) was obtained from Moravek Biochemicals (Brea, CA). Radiochemical purity was determined by HPLC to be >99% [11]. The [2-^{3}H]taurine (20.9 Ci/mmol),

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† Abbreviations: FUra, fluorouracil; FBAL, 2-fluoro-β-alanine; and FAB-MS, fast atom bombardment mass spectrometry.

[2-^{3}H]glycine (20 Ci/mmol) and [3-^{3}H]β-alanine (87.2 Ci/mmol) were obtained from New England Nuclear (Boston, MA), and each was purified by binding to 1 mL of Dowex 50W-X8 [H^+] cation exchange resin (BioRad, Richmond, CA), previously equilibrated with distilled deionized water. The slurry was packed in a 2.5-mL Pasteur pipette and washed with 10 mL distilled deionized water. The amino acids were eluted from the resin with 7 N ammonium hydroxide.

Preparation of radiolabeled FBAL. The 2*R*-stereoisomer of FBAL, the isomer formed *in vivo* [2], is not available commercially and, therefore, was purified from the urine of patients receiving [6-^{3}H]FUra, coadministered with unlabeled FUra (obtained at the University of Alabama at Birmingham through an institutionally approved protocol) [3]. Urine was passed through a Sep-pak C_{18} cartridge (Waters Associates, Milford, MA), acidified with HCl to pH 2.0, and loaded onto a 10-mL/polypropylene econo-column packed with 2 mL of Dowex 50W-X8 [H^+] cation exchange resin. The resin was washed with 1.5 column volumes of distilled deionized water. The FBAL was eluted with 1 column volume of 7 N ammonium hydroxide, evaporated under N_2, and reconstituted in 1 mL of 0.01 M sodium citrate buffer containing 0.3% sodium dodecyl sulfate (SDS, w/v), pH 2.7. This solution was passed through a Sep-pak C_{18} cartridge and concentrated 10-fold by evaporation under N_2.

The concentrate was then injected on a model 1084B Hewlett–Packard high-performance liquid chromatograph fitted with a 25 × 1.2 cm reversed phase C_{18} column (Jones Chromatography, Littleton, CO). Elution was carried out isocratically at a flow rate of 1.5 mL/min with a mobile phase of 0.01 M sodium citrate buffer containing 0.3% SDS (w/v), pH 2.5. The retention time of FBAL was determined using authentic unlabeled FBAL. Fractions containing the radiolabeled FBAL were pooled, acidified to pH 2.0, and loaded onto a 10-ml Dowex 50W-X8 [H^+] cation exchange column. The column was washed with distilled deionized water followed by 7 N ammonium hydroxide as described above. The eluent from the ammonium hydroxide wash was passed through a Sep-pak C_{18} cartridge and lyophilized.

Chemical and radioactive purities of the [^{3}H]FBAL were confirmed following derivatization with phenylisothiocyanate [12] and analysis by reversed-phase HPLC using the PICO TAG System (Waters Associates, Milford, MA). Since there is no dilution *in vivo* by endogenous metabolites, the specific activity of the purified FBAL used in these experiments was 85.6 μCi/mmol. The [^{3}H]FBAL was reconstituted in 40% ethanol and stored at −20°. Under these conditions [^{3}H]FBAL was stable over a 6-month period as determined by reversed phase HPLC.

Partial purification of hepatic bile acid CoA:amino acid:N-acyltransferase. Human liver (obtained from the University of Alabama at Birmingham Organ Bank through an institutionally approved protocol), removed from transplant donors in less than 30 min after cessation of cardiac function, was cut into 100-g pieces, perfused with cold saline, and stored at −70°. Prior to use, the liver was set in a 4° cold room for 24 hr. The partially thawed liver was minced into small cubes and homogenized in an Osterizer blender at high speed in 4 vol. of 0.25 M sucrose. The homogenate was filtered through gauze and spun at 100,000 *g* for 60 min in a Beckman Type 45 Ti rotor. The resulting supernatant fraction was dialyzed at 4° against 10 L of 50 mM Tris–HCl buffer, pH 8.1, overnight.

The dialysate was mixed at 4° with 200 g (dry weight) DEAE-cellulose, previously equilibrated with 50 mM Tris–HCl, 40 mM NaCl, pH 8.1. The slurry was incubated under vacuum for 30 min at 4°, packed in a 2.6 × 40.0 cm column, and re-equilibrated with 5 column volumes of 50 mM Tris–HCl, 40 mM NaCl, pH 8.1. Enzyme activity was eluted during a linear gradient of NaCl (40–200 mM) in 50 mM Tris–HCl, pH 8.1. Fractions demonstrating bile acid conjugating activity with glycine and taurine were pooled and concentrated 10-fold in an Amicon Centriprep 10 concentrator. Protein concentration was determined by the method of Lowry *et al.* [13].

Assay of bile acid CoA:N-acyltransferase. Bile acid CoA : amino acid : *N*-acyltransferase activity was determined using a radioassay which directly measures the formation of ^{3}H-labeled bile acid conjugates [9]. To examine the fractions eluted from the DEAE column, the assay mixture contained 100 mM potassium phosphate buffer, 1.15 mM cholyl CoA and 0.25 mM unlabeled glycine or taurine, with 0.025 μCi of the corresponding ^{3}H-labeled amino acid, in a total volume of 100 μL (final pH 8.4). In assays using the concentrated, pooled fractions of bile acid CoA:*N*-acyltransferase activity from the DEAE column, the reaction conditions were identical, except that FBAL (0.025 μCi) and β-alanine (0.025 μCi) were the amino acid substrates. Reactions were terminated after 30 min and products were recovered by extraction into *n*-butanol [9]. Blank incubations were run in the absence of enzyme, in the absence of substrate, or in the presence of heat-inactivating enzyme. Under these conditions no enzymatic product formation could be detected.

Identification of FBAL-cholate. The product formed by bile acid CoA:amino acid:*N*-acyltransferase in the presence of FBAL and cholyl CoA was identified as follows: the *n*-butanol extract was dried under N_2, reconstituted in 50 μL of 50% methanol, and analyzed by HPLC using a 25 × 0.46 cm reversed phase C_{18} column (Jones Chromatography). Elution was carried out isocratically at 1 mL/min using a mobile phase consisting of 2 mM potassium phosphate buffer/64% methanol (pH 4.0). Timed 1-min fractions were collected over a 30-min period, and the radioactive profile was determined by liquid scintillation spectrometry using 50-μL aliquots of each fraction. The radioactive peak (retention time 13 min) was dried under N_2. The radioactive metabolite was incubated for 30 min at 37° in 2 mL of 0.1 M sodium acetate buffer (pH 5.6) in the presence and absence of cholylglycine hydrolase (an enzyme which specifically hydrolyzes *N*-acyl bile acid conjugates) [14]. Aliquots (100 μL) of the incubation mixtures were analyzed by HPLC using the same method described above.

The unhydrolyzed metabolite was also examined

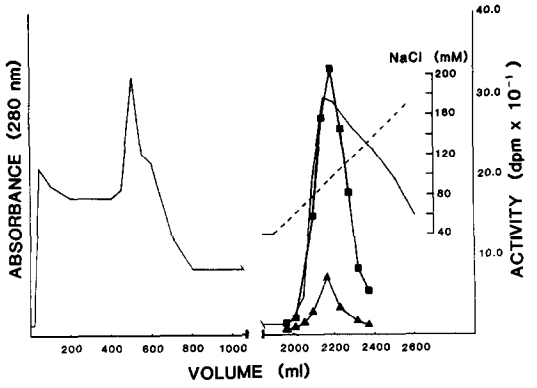

Fig. 1. Elution pattern of glycine and taurine conjugating activity from DEAE-cellulose. Dialyzed, particle-free, liver homogenate was incubated with 200 g DEAE-cellulose and packed in a 2.6 × 40 cm column. Enzyme activity was eluted during a 40 to 200 nM NaCl salt gradient (----), and was determined using glycine (▲) and taurine (■) as substrates.

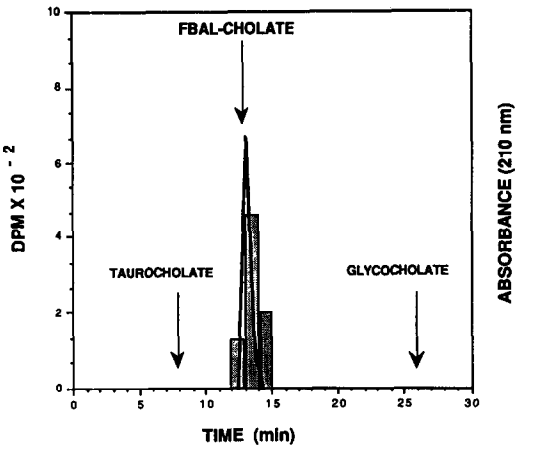

Fig. 2. HPLC elution pattern of FBAL-cholate formed by bile acid CoA:amino acid:*N*-acyltransferase. An aliquot (50 μL) of the concentrated fractions from the DEAE column having glycine and taurine conjugating activity was incubated with cholyl CoA and FBAL. The reaction was terminated, the reaction mixture was extracted with *n*-butanol, and HPLC analysis was performed as described in Materials and Methods. Elution times for authentic taurocholate and glycocholate are shown.

by FAB-MS. Samples were dissolved in glycerol and run at 8 kV with a 1 mA current on a Varian (San Jose, CA) MAT 311A mass spectrometer equipped with an Ion Tech FAB gun.

*Kinetic and inhibition studies of bile acid CoA:amino acid:*N*-acyltransferase.* Initial reaction rates were determined at various concentrations of each amino acid (0.5, 0.75, 1.0, 2.0, and 10.0 mM) in the presence of a saturating concentration (1.15 mM) of cholyl CoA by incubation at 37°. The incubation time (30 min) and the amount of protein (10 μg) were selected so linear initial reaction rates were obtained. Estimation of the apparent K_m and V_{max} for each amino acid was performed by fitting these data for several concentrations of glycine, taurine or FBAL to the Michaelis–Menten equation by non-linear regression analysis. Comparisons of parameter estimates were made using pooled asymptotic standard errors in a Z-test [15], assuming an asymptotic normal distribution [16]. Enzyme activity is expressed as nanomoles of product formed per minute per milligram of protein.

Inhibition studies were performed by varying the concentration of FBAL (0.7, 1.0, 1.5, 3.0, and 6.0 mM) in the presence of fixed concentrations of taurine (0, 1.0 and 3.0 mM) or glycine (0, 2.0, 4.0 and 8.0 mM). These data were interpreted using the programs COMPET and NONCOMPET devised by Cleland [17], as described elsewhere [18].

RESULTS

*Partial purification of bile acid CoA:amino acid:*N*-acyltransferase.* The bile acid CoA:amino acid:*N*-acyltransferase in the 100,000 *g* supernatant fraction of human liver homogenate was purified 8-fold by binding the enzyme to a DEAE-cellulose column, washing with buffer, and eluting activity with a linear NaCl gradient. A single peak of bile acid:amino acid:*N*-acyltransferase activity using both glycine and taurine as substrates eluted at an average NaCl concentration of 100 mM (Fig. 1). There was no systematic variation in the ratio of activity with glycine versus taurine through the peak of enzyme activity. These data suggest co-elution of glycine and taurine conjugating enzyme activity.

Isolation and identification of FBAL-cholate as an enzymatic product. The partially-purified bile acid CoA:amino acid:*N*-acyltransferase from human liver was incubated with [^{3}H]FBAL and [^{3}H]β-alanine in the presence of cholyl CoA. After termination of the reaction, the assay mixture was extracted with *n*-butanol. In the case of β-alanine, no radioactivity was extracted into the *n*-butanol. HPLC analysis of the radioactivity recovered in *n*-butanol from the FBAL assay mixture demonstrated a single peak of radioactivity whose elution time (13 min) corresponded to authentic FBAL-cholate (Fig. 2).

To confirm that the product of the enzyme reaction was FBAL-cholate, the peak of radioactivity was divided, and incubated with or without cholylglycine hydrolase. Analysis by HPLC demonstrated that hydrolysis of the putative FBAL–cholate conjugate occurred in the presence of cholylglycine hydrolase (Fig. 3A), but not in its absence (Fig. 3B).

The putative FBAL-cholate was also analyzed by negative ion FAB-MS. A single molecular species was identified (M-H) at $m/z = 496$ (Fig. 4). The molecular weight (497) corresponded to that of chemically synthesized FBAL-cholate, and to the FBAL-cholate isolated from the bile of patients given FUra [4].

*Kinetic experiments on partially purified bile acid CoA:amino acid:*N*-acyltransferase.* When various amounts of glycine, taurine or FBAL were incubated with the partially purified enzyme in the presence of saturating amounts of cholyl CoA, reaction rates conformed to Michaelis–Menten kinetics, as

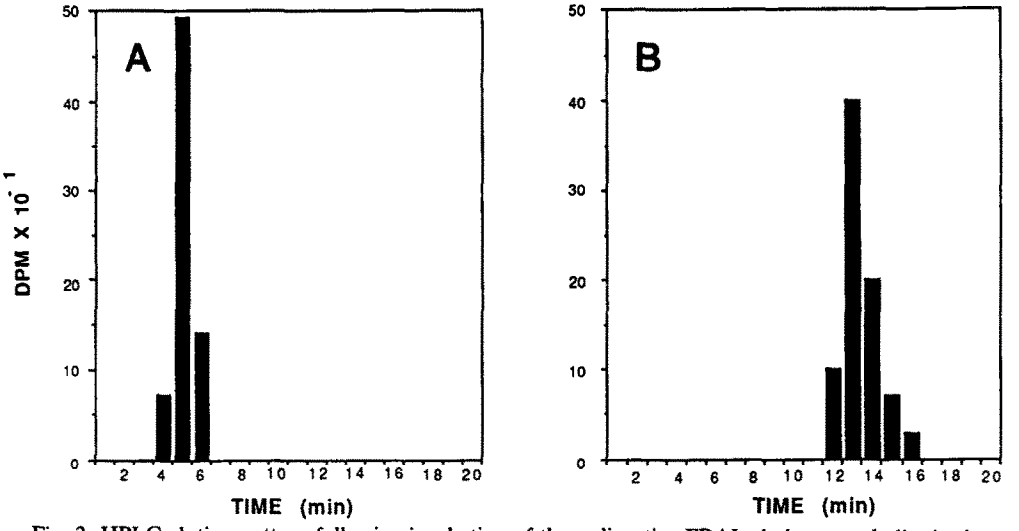

Fig. 3. HPLC elution pattern following incubation of the radioactive FBAL-cholate metabolite in the presence (A) and absence (B) of cholylglycine hydrolase.

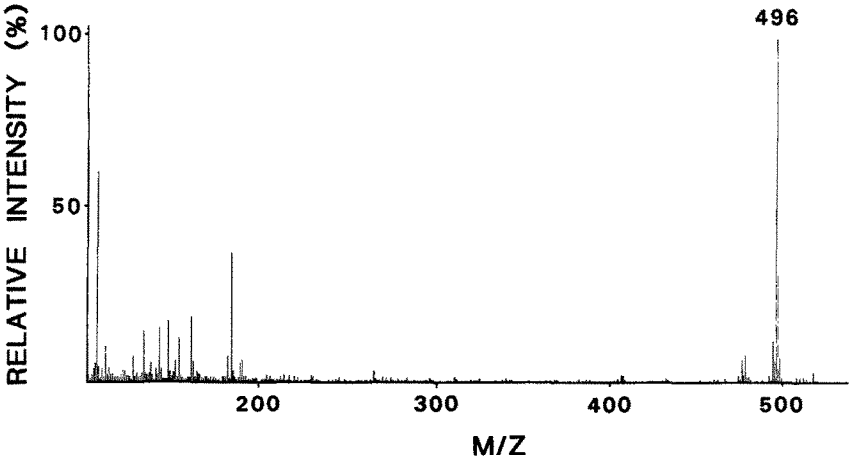

Fig. 4. Negative ion FAB-mass spectrum of the HPLC purified FBAL-cholate conjugate.

reflected by linear double-reciprocal plots for each substrate (Fig. 5). The apparent K_m and V_{max} values were calculated using non-linear regression analysis. As shown in Table 1, the enzyme has similar K_m values for taurine and FBAL, which were 4–5 times lower than that for glycine. However, the V_{max} for glycine conjugation was 2.0-fold and 1.3-fold higher than that for either FBAL or taurine respectively. A Z-test performed on these data yielded P values less than 0.001. This demonstrates a significant difference between the K_m values obtained for FBAL vs glycine and taurine vs glycine and between the V_{max} values for each amino acid.

Inhibition of FBAL-cholate formation by glycine and taurine. Formation of FBAL-cholate by bile acid CoA : amino acid : *N*-acyltransferase was inhibited by both taurine (Fig. 6A) and glycine (Fig. 6B). The double-reciprocal plots obtained at different concentrations of glycine or taurine intersect on the abscissa, indicating that these amino acids are competitive inhibitors of FBAL. Non-linear regression analysis using the program COMPET yielded inhibition constants of 1.27 ± 0.18 mM and 4.47 ± 0.50 mM for taurine and glycine respectively. Attempts to fit the data to a noncompetitive model were unsuccessful.

DISCUSSION

In the present study we have shown that FBAL-cholate, a major biliary metabolite of FUra in cancer patients, is formed by the hepatic enzyme bile acid CoA : amino acid : *N*-acyltransferase. The product formed after incubating FBAL and cholyl CoA with partially purified human bile acid CoA : amino acid : *N*-acyltransferase was identified as FBAL-cholate on the basis of (1) coelution from HPLC with

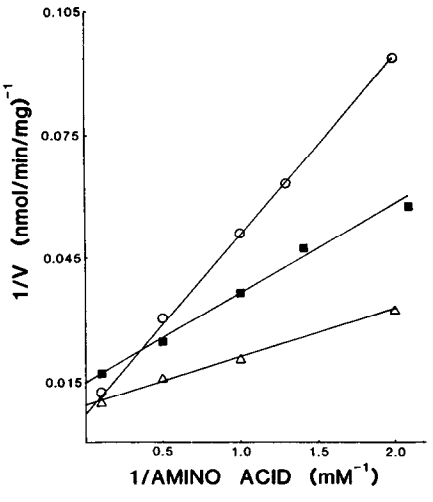

Fig. 5. Double-reciprocal plot of the rate of reaction as a function of the concentration of glycine, taurine and FBAL. The rates of bile acid conjugate formation were determined in the presence of 1.15 mM cholyl CoA using the following fixed concentrations (0.5, 0.75, 1.0, 2.0, and 10.0 mM) of glycine (○), taurine (△) and FBAL (■).

Table 1. Kinetic parameters for partially purified bile acid CoA:amino acid:*N*-acyltransferase

	K_m (mM)	V_{max}(nmol/min/mg)
Glycine	6.45 ± 0.35	142.0 ± 1.16
Taurine	1.32 ± 0.14	111.0 ± 1.35
FBAL	1.45 ± 0.10	69.0 ± 0.56

The kinetic parameters were calculated using non-linear regression analysis. Results are the means ± SE of five replicates.

authentic standard, (2) specific hydrolysis by cholylglycine hydrolase, and (3) identical molecular weight (497) to authentic FBAL-cholate as determined by FAB–MS.

The substrate specificity of bile acid CoA:amino acid:*N*-acyltransferase is such that glycine and taurine are usually the only amino acids that are conjugated to bile acid *in vivo*, although ornithine conjugates have been reported [19]. Other α-amino acids, including α-alanine, are not substrates. β-Alanine, the carboxy analog of taurine, has been reported as a poor substrate in some species [20], but was not a substrate for human hepatic bile acid CoA:amino acid:*N*-acyltransferase in this study. It was surprising, therefore, that the 2*R*-stereoisomer of FBAL (2-fluoro-β-alanine) was a good substrate, having a K_m (1.45 mM) similar to taurine (1.32 mM). It is possible that the greater acidity of the carboxyl group (FBAL pK_a = 1.58 vs β-alanine pK_a = 3.60) [6] is an important factor controlling substrate specificity, although other factors such as steric effects may contribute.

The observation that when glycine or taurine was used as a substrate, one coincident peak of bile acid CoA:amino acid:*N*-acyltransferase activity was eluted from the DEAE-cellulose column, is consistent with previous reports that a single enzyme is responsible for both glycine and taurine bile acid conjugation in other species [21]. This can be extended to the conjugation of FBAL since FBAL-cholate formation was competitively inhibited by glycine and taurine. Because all these amino acids are substrates for the same enzyme, the apparent K_m for FBAL *in vivo* would be expected to be higher than that measured in these *in vitro* experiments because of competition from glycine and taurine. However, regardless of the concentrations of these other amino acids, the high affinity of bile acid CoA:amino acid:*N*-acyltransferase for FBAL assures that FBAL bile acid conjugation will occur *in vivo* as is observed in patients receiving FUra [4].

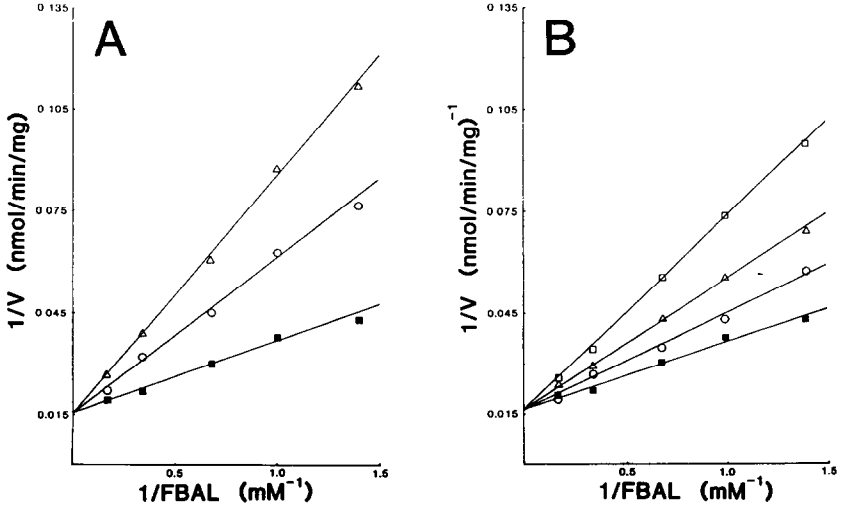

Fig. 6. Inhibition of FBAL-cholate formation by taurine (A) and glycine (B) as a function of the concentration of FBAL. Partially purified bile acid CoA:amino acid:*N*-acyltransferase was incubated with cholyl CoA (1.15 mM), various concentrations of FBAL, and fixed concentrations of taurine [(■) 0, (○) 1.0, and (△) 3.0 mM] and glycine [(■) 0, (○) 2.0, (△) 4.0, and (□) 8.0 mM].

Since glycine and taurine conjugated bile acids are secreted efficiently into bile [7], rapid biliary excretion could account for the predominance of FBAL bile acid conjugates in bile and their virtual absence in serum and urine [3]. Whether FBAL-cholate or other FBAL-bile acid conjugates undergo the efficient enterohepatic recycling, as is observed with glycine or taurine conjugated bile acids, remains to be investigated. The enterohepatic recycling of FBAL bile acid conjugates would be interrupted if they were hydrolyzed in the intestine by pancreatic peptidases, as occurs for other synthetic amino acid bile acid conjugates, but not for glycine and taurine conjugates [22]. Therefore, it is not possible to determine whether the biliary concentration of FBAL-bile acid conjugates during FUra therapy is a reflection of their accumulation due to enterohepatic recycling, or is simply a function of the rate of formation of FBAL-bile acid conjugates (i.e. with no conservation). The clinical significance of the FBAL-bile acid conjugates in the biliary toxicity found in patients after receiving FUra by intrahepatic infusion remains to be determined.

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