Synthesis of conjugated bile acids by means of a peptide coupling reagent

LEON LACK, FLOYD O. DORRITY, JR., THOMAS WALKER, AND GAIL D. SINGLETARY

Department of Physiology and Pharmacology, Duke University Medical Center, Durham, North Carolina 22710

Summary The conditions for the preparation of conjugated bile acids by means of *N*-ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline are described. Conjugation is obtained in one step via the intermediary formation of mixed carbonic–carboxylic acid anhydrides.

Supplementary key words conjugated bile acid synthesis · taurine · glycine

BELLEAU AND MALEK (1) have reported that *N*-ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline (EEDQ) can induce the formation of peptide bonds. Carboxyl group activation was attained by the transient formation of mixed carbonic–carboxylic acid anhydrides.

The widely used method of Norman (2) for the synthesis of conjugated bile salts also involves the formation of this type of anhydride. This is an overall two-step procedure the first step of which involves the synthesis of the mixed anhydride by reacting the tributylamine salt of the unconjugated bile acid with ethyl chloroformate. This note describes conditions by which EEDQ, obtained from Aldrich Chemical Co., can be employed to conjugate bile acids with taurine, glycine, and other amino acids.

Taurine conjugates of bile acids. The general procedure involves the reaction of 1 mmole of unconjugated bile acid with 1.4 mmoles EEDQ and 1 mmole taurine (as the sodium salt) in aqueous alcohol. See below for specific conditions as well as the order of addition of reagents. The reaction mixture is shaken vigorously in a water bath maintained at 40°C. When the aqueous solution containing the taurine is added to the alcohol solution, a fine precipitate of taurine slowly forms. This is a useful indicator, since solubilization and the reaction proceed simultaneously, and completion of conjugation may be inferred when the reaction mixture becomes clear. The period of shaking is between 15 and 20 hr. Conjugation proceeds to the extent of 75–80%, but efficient purification may be obtained by partition of the reaction mixture between ethyl acetate and water (see below). The aqueous phase contains the sodium salt of the conjugated bile salt in chromatographically pure form while the unconjugated bile acids and quinoline products remain in the ethyl acetate phase. The product may be recovered from the water solution and recrystallized by one of the relevant methods described by Norman (2).

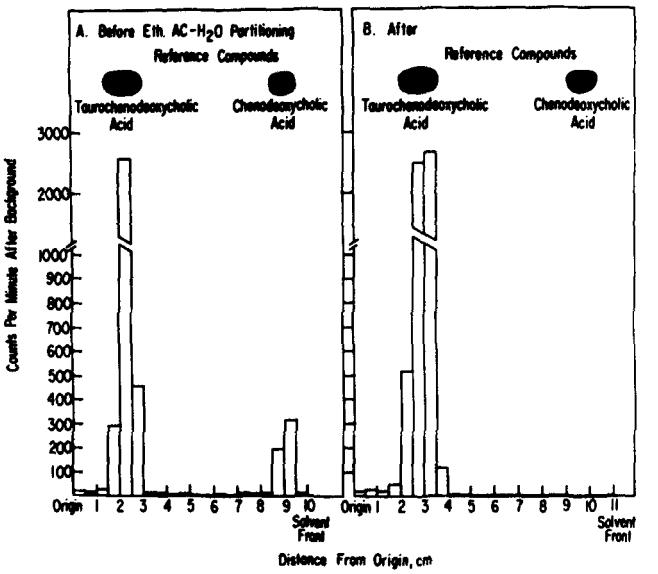

FIG. 1. Thin-layer chromatographic analysis of products after the reaction of [^{14}C]chenodeoxycholic acid and taurine in the presence of EEDQ. *A*, before purification; *B*, after one-step partition purification with ethyl acetate–water. Each bar represents the radioactivity observed in a 5-mm scraped zone. Reference compounds were run in parallel lanes. Solvent system 2 of Hofmann (4) was used. The scrapings were counted in a Beckman liquid scintillation counter, model LS-150.

As a specific example, a synthesis of sodium taurochenodeoxycholate is described in detail. In this case we employed [^{14}C]carboxyl-labeled chenodeoxycholic acid, obtained from Mallinckrodt, in order to demonstrate the degree of conjugation initially obtained as well as the effectiveness of the purification procedures.

To a solution containing 393 mg (1 mmole) of chenodeoxycholic acid in 20 ml 95% ethanol was added 346 mg EEDQ (1.4 mmoles) dissolved in 14 ml 95% ethanol, followed by 125 mg (1 mmole) of taurine dissolved in 2 ml 0.5 N NaOH. The mixture was shaken in a 40°C water bath until all the formed solid was dissolved (in this case 19 hr). Care must be taken that the shaking procedure does not cause any solid to splatter beyond the reach of the agitated fluid. Thin-layer chromatography (see Fig. 1*A*) of an aliquot demonstrated that approximately 80% of all radioactivity could be accounted for as conjugated bile acid.

This clear solution was transferred to a separatory funnel to which was then added 360 ml of ethyl acetate and 54 ml water. After shaking, an aqueous phase separated (41 ml), which was recovered and washed once with 41 ml ethyl acetate and once with 10 ml ethyl ace-

Abbreviations: EEDQ, *N*-ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline.

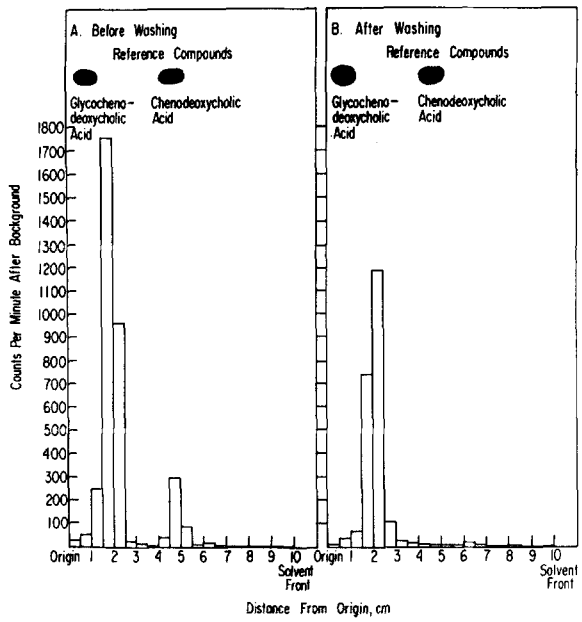

FIG. 2. Thin-layer chromatographic analysis of the reaction products observed after the reaction of [^{14}C]chenodeoxycholic acid and glycine ethyl ester in the presence of EEDQ. *A*, before purification; *B*, after sequential extractions with aqueous HCl, bicarbonate, and sodium hydroxide. Solvent system S11 of Eneroth (5) was used. Experimental conditions for counting were the same as for Fig. 1.

tate. This aqueous phase, now 30 ml, contained only conjugated sodium taurochenodeoxycholate (see Fig. 1*B*). The solution containing the product was evaporated to dryness in a rotary vacuum evaporator, care being taken that the bath temperature did not exceed 35°C. The product was dissolved in 10 ml warm ethanol containing 0.1 ml water and then filtered. The flask and filter paper were then washed with two 5-ml portions of ethanol. Excess ethyl acetate was added to the combined filtrate, and the crystalline suspension was then allowed to stand overnight in an ice bath. The product was recovered by centrifugation in the cold, washed once with ether, and dried in vacuo. The yield was 203 mg. Additional material (106 mg) was obtained from the mother liquor by recrystallization from smaller volumes (mp 193–196°C; reported, 165–175°C [2] and 182–184°C [3]).

Instead of shaking the reaction mixture in a 40°C water bath for the time necessary to obtain a clear solution (see below), one may achieve the same results by boiling the mixture under reflux with stirring for 90 min. Upon cooling, the solution is clear and may be worked up in the manner already described.

Glycine conjugates of bile acids. The synthesis of this type of conjugate requires the use of the amino acid ethyl ester present as the free base. A solution containing 1.0 mmole unconjugated bile acid, 1.1 mmoles EEDQ and 1.5 mmoles ethyl glycinate in ethyl acetate is refluxed overnight. The ethyl acetate solution contains the products of conjugation as the ester, unreacted starting materials, and quinoline materials derived from EEDQ. Sequential extraction with acid, bicarbonate, and sodium hydroxide removes the impurities from the organic solvent leaving behind the desired product, which is recovered and saponified by the method of Hofmann (3). Acidification of the hydrolyzed ester precipitates the product as the free acid, which, when feasible, may be recrystallized by the specific methods described by Norman (2). In the case of glycochenodeoxycholic acid, the free acid must be converted to the sodium salt prior to crystallization (see below).

As a specific example of this type of synthesis, the preparation of sodium glycochenodeoxycholate is described. [^{14}C]Carboxyl-labeled chenodeoxycholic acid was used in this instance for the illustrative purposes mentioned earlier.

209 mg (1.5 mmoles) glycine ethyl ester hydrochloride was placed in a 50-ml centrifuge tube together with 20 ml ethyl acetate and 0.5 ml water, which dissolved the ester hydrochloride. The tube was then cooled in an ice bath, and small amounts of anhydrous K_2CO_3 were added, with shaking, to saturate the aqueous phase. Additional potassium carbonate was then added to remove the water and to dry the ethyl acetate. The ultimate appearance of the potassium carbonate salt as a free-flowing powdery suspension indicated that the ethyl acetate phase was dry. The ethyl acetate was removed after centrifugation, and the remaining salt was washed successively with 10–15-ml portions of dry ethyl acetate which were added to the original ethyl acetate until a total of 50 ml was obtained. This solution (50 ml), containing the glycine ester as the free base, was filtered and added to a dry 100-ml round-bottomed flask containing 273 mg EEDQ (1.1 mmoles) and 392 mg chenodeoxycholic acid (1.0 mmole). When all reagents were solubilized (slight warming may be necessary), the contents of the flask were boiled overnight under reflux. After refluxing, thin-layer analysis of the solution revealed that 12% of all the radioactivity migrated as free chenodeoxycholic acid; 83% of the radioactivity migrated as a spot less polar than glycochenodeoxycholic acid and was presumed to be the ethyl ester of the conjugate. The remainder of the radioactivity was found in regions above that of the unconjugated acid (see Fig. 2*A*).

The ethyl acetate solution was transferred to a separatory funnel and extracted sequentially: once with 30 ml 0.1 N HCl, once with 30 ml water, twice with 30 ml 1% $NaHCO_3$, once with 30 ml 0.1 N NaOH and once with 30 ml water. When an aliquot of the material remaining in the ethyl acetate was chromatographed,

only radioactivity corresponding to the ethyl ester of glycochenodeoxycholic acid was observed (see Fig. 2*B*).

The ethyl acetate solution was taken to dryness with the rotary evaporator and then dissolved in 30 ml ethanol. After the addition of 30 ml 0.2 N NaOH, the solution was permitted to stand at room temperature overnight. 60 ml of water was added to this solution and evaporated to approximately 30 ml in a rotary evaporator with the bath temperature at 25°C. This solution was extracted once with 15 ml ethyl acetate and once with 15 ml ether. The alkaline solution was placed in a 50-ml centrifuge tube and acidified with HCl; an oily precipitate formed. The tube was placed in an ice bath for several hours, centrifuged in the cold, and then washed with 30 ml water. After drying in vacuo at room temperature, the amorphous solid was dissolved in 40 ml of 50% ethanol in water and neutralized to pH 8 with 0.1 N sodium hydroxide. The sodium salt of the product was recovered by evaporation on the rotary evaporator, bath temperature 25°C. The product was dissolved in 10 ml warm alcohol and precipitated with an excess (40 ml) of ether. The material, after standing overnight in the ice bath, was recovered by centrifugation, washed with ether, dried in vacuo and dissolved in 5 ml ethanol. Addition of an excess (40 ml) of ethyl acetate caused the precipitation of the product as crystals. The product was allowed to stand in the ice bath several hours, centrifuged, and finally washed with ether prior to drying in the vacuum desiccator. The yield was 284 mg (mp 164–173°C; reported, 160–170°C [2]). Thin-layer chromatography demonstrated the absence of unconjugated bile acid as well as materials more polar than glycochenodeoxycholic acid (see Fig. 3).

Discussion. The general and specific descriptions of the syntheses of conjugated bile salts and acids have all been based on starting with 1 mmole of unconjugated bile acid. It should be noted that these preparations can be conveniently scaled in either direction.

The procedures for the preparation of taurine conjugates of bile acids have been, in the authors' experience, more rapid and convenient than those heretofore available. The methodology for the preparation of conjugates with amino acids containing a carboxylic acid group is somewhat more involved. However, there appears to be an advantage in that the product is attained as the ethyl ester while any unreacted starting bile salt is present as the free acid. Purification by alkaline extraction of the starting material from the ethyl acetate phase is relatively simple. This provides an additional advantage in the preparation of bile acid derivatives where the solubility properties of the product are sufficiently similar to those of the unconjugated bile acids to thwart purification by partition column chromatography. An example of this is provided in a recent publication (6); this work required that the bile acids conjugated with norleucine be prepared. The only practical approach was the utilization of the above procedure employing the ethyl ester of norleucine.

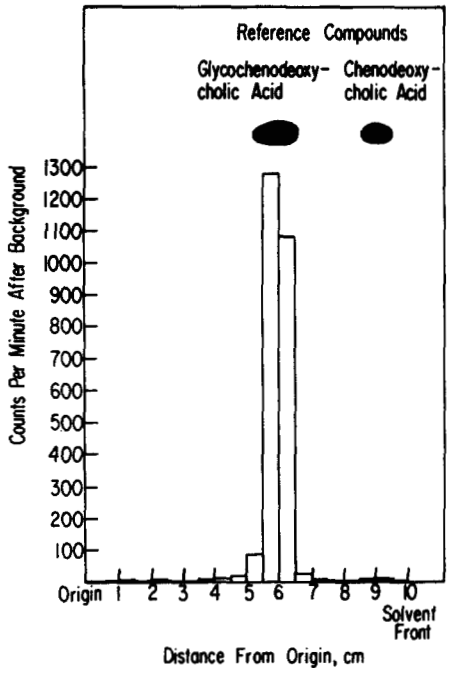

FIG. 3. Thin-layer chromatographic analysis of the sodium salt of glycochenodeoxycholic acid. Solvent system 2 of Hofmann (4) was used. Experimental conditions for counting were the same as for Fig. 1.

The following additional materials have been prepared by this procedure: sodium taurocholate, sodium taurodeoxycholate, sodium glycocholate, and sodium glycodeoxycholate. The melting points, chromatographic properties, and infrared spectra are comparable with those of authentic bile salt samples. The average yield was 60%. As indicated, all these products appear pure by thin-layer chromatography employing the solvent systems of Hofmann (4) and Eneroth (5). Although there was no indication of the presence of quinoline materials derived from EEDQ, we routinely purify all of our conjugated bile acids by the reversed-phase chromatographic methods of Norman (7) prior to their employment in biological experiments.

This work was supported by research grant AM-09582 from the National Institutes of Health, and in part by a grant (RR-30) from the General Clinical Research Centers Program of the Division of Research Resources, National Institutes of Health.

Manuscript received 14 June 1972; accepted 1 December 1972.

REFERENCES

1. Belleau, B., and G. Malek. 1968. A new convenient reagent for peptide syntheses. *J. Amer. Chem. Soc.* **90:** 1651–1652.

2. Norman, A. 1955. Preparation of conjugated bile acids using mixed carboxylic acid anhydrides. Bile acids and steroids. *Ark. Kemi.* **8:** 331–342.
3. Hofmann, A. F. 1963. The preparation of chenodeoxycholic acid and its glycine and taurine conjugates. *Acta Chem. Scand.* **17:** 173–186.
4. Hofmann, A. F. 1962. Thin-layer adsorption chromatography of free and conjugated bile acids on silicic acid. *J. Lipid Res.* **3:** 127–128.
5. Eneroth, P. 1963. Thin-layer chromatography of bile acids. *J. Lipid Res.* **4:** 11–16.
6. Singletary, W. V., Jr., J. T. Walker, and L. Lack. 1792. Ileal transport of bile acids conjugated with norleucine and lysine. *Biochim. Biophys. Acta.* **266:** 238–245.
7. Norman, A. 1953. Separation of conjugated bile acids by partition chromatography. Bile acids and steroids. *Acta Chem. Scand.* **7:** 1413–1419.