# Ornithocholanic Acids—Abnormal Conjugates of Bile Acids.* (27506)

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Cholesterol and pigment precipitate occur in the gall bladders of guinea pigs after suppurative infections or following parenteral injection of toxic capsular polysaccharide from *Klebsiella pneumoniae*(1,2). In the study of the pathogenesis of experimental cholelithiasis, 2 significant observations have been made: a) of the 3 bile acids in adult guinea pigs, cholic acid appeared slowly but soon became the predominant bile acid(3); b) after injection of $C^{14}$-labeled polysaccharide, the label appeared in bile, but was not present as the original polysaccharide or as a component of the bile precipitate(2). In the present study some of the label in bile has been found to be in L-ornithine conjugated with all 3 bile acids of adult and with the 2 bile acids of immature guinea pigs. The unusual conjugation of L-ornithine with cholic acid was readily confirmed *in vitro* with homogenate of both rat and guinea pig liver. The excretion of abnormal bile acid conjugate in animals with disease or injury may afford a physicochemical explanation for formation of cholesterol and pigment precipitates.

*Materials and methods.* $C^{14}$-labeled bacterial polysaccharide was prepared as previously described(4). For the *in vivo* studies, 0.50 mg of the labeled polysaccharide, prepared as a 1% solution in 0.15 M NaCl, was administered by ear vein into young guinea pigs of either sex weighing 300 to 350 g each and into adult guinea pigs of either sex weighing 550 to 700 g each. Animals were maintained on Purina rabbit chow, leafy green vegetables and water *ad libitum*. The young guinea pigs were sacrificed 24 and 48 hours and adult guinea pigs 24 hours after administration of the polysaccharide. Gall bladder bile was collected as previously reported(2).

Conjugated bile acids were separated and identified by paper and column chromatography(5). A small aliquot of each bile sample was analyzed by paper chromatography while the larger portions of the bile samples were pooled according to age groups and time after injection of the polysaccharide and processed by column chromatography. Acidic fractions of column effluents were subjected to alkaline hydrolysis, and the resultant free bile acids were determined by paper and column chromatography(5). All columns were eluted with chloroform after 120 ml of effluent had been collected. $C^{14}$ content of the original bile samples, extracts, water phases after extractions, materials separated by column chromatography and ethanolic eluates of paper chromatographic zones (containing conjugated bile acids) was determined by applying small aliquots to aluminum planchets and counting in a windowless flow counter. The bile samples of the control, non-injected animals were processed in the same manner as the samples from polysaccharide injected animals.

A portion of a labeled material, bacterial product "B", isolated from polysaccharide by 42 transfers of counter current distribution between n-butyl alcohol and water, was further purified by partitioning between heptane and water and chloroform and water, and identified by paper chromatography in the systems: ethanol/$NH_4OH$/$H_2O$, n-butyl alcohol/$NH_3$ and n-butyl alcohol/acetic acid/$H_2O$(6). Paper strips were sprayed with 0.1% aqueous ninhydrin. $C^{14}$ was determined in the aqueous eluate of ninhydrin positive zones.

The incubation medium for *in vitro* study was supernatant of liver homogenates, obtained after centrifugation at 400 $\times$ *g* for 5 min., prepared as described by Bremer and Gloor(7). The livers of mature male guinea pigs weighing 500 to 600 g and mature male rats weighing 350 to 400 g were homogenized with approximately 4 volumes of homogenizing medium. Two moles of cholic acid were added to 3.0 ml aliquots of such fluid with addition of 2.0 $\mu$moles taurine, 2.0 $\mu$moles L-ornithine or 57 $\mu$g (2,000 d/m) bacterial product "B" in combinations shown on Tables

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TABLE I. *In vitro* Conjugation of Cholic Acid by Guinea Pig Liver.

| Additions to incubation medium | Cholic acid recovered as conjugate of: Bacterial product "B" μmoles | % of total | Taurine μmoles | % of total |
|---|---|---|---|---|
| Bacterial product "B" | .54 | 91.5 | .05 | 8.5 |
| | .53 | 93.0 | .04 | 7.0 |
| Taurine | — | — | .52 | 100.0 |
| | | | .51 | 100.0 |
| Bacterial product "B" and taurine | .53 | 88.3 | .07 | 11.7 |
| | .56 | 90.3 | .06 | 9.7 |

I and II. The mixtures were incubated for 150 min. at 37°C and pH 7.4. The incubation was followed by acidification with HCl to pH 1.0 and extraction with n-butyl alcohol. An aliquot of the extract was analyzed by paper chromatography in the same systems as used for the *in vivo* study. The rest of the extract was hydrolyzed by boiling for 5 hours in 50.0 ml 1.0 N NaOH. The hydrolysate was acidified to pH 1.0 with HCl, extracted with ethyl ether and cholic acid isolated by paper chromatography. The zones of paper corresponding to cholic acid on parallel strips were eluted with ethanol and cholic acid recovered was quantitated by spectrophotometric procedure described by Eriksson and Sjövall(8). When labeled bacterial product "B" was used, $C^{14}$ content was determined in the various samples by the same procedure as reported for *in vivo* study.

*Results.* Only taurine conjugation was observed in bile of normal guinea pigs, but, in animals given labeled bacterial polysaccharide, a small portion of bile acids was conjugated with material other than taurine (Fig. 1 and 2, top). The unusual conjugate contained approximately 84% of total biliary $C^{14}$, the remaining activity being found in water phase (6.7%) after butanol extraction and in chloroform eluate of the columns (3.8%). No isotope was detected in taurine conjugates or in any other fractions of the column effluents. Paper chromatography confirmed the findings of column chromatography. Two phosphomolybdic acid positive spots were detected on paper chromatograms of bile of polysaccharide-treated animals. The weaker spot indicated material less polar than taurocholate and more polar than glycocholate. All $C^{14}$ applied to paper was found in ethanolic eluates of such areas.

Free bile acids were isolated from conjugated materials in both chromatographic zones (Fig. 1, 2). None of the $C^{14}$ was present in free bile acids after their recovery by extraction with ethyl ether. The combined values of free 3*a*, 7*a*-dihydroxycholanic (chenodeoxycholic) and 3*a*-hydroxy, 7-ketocholanic (7-ketolithocholic) acid in immature (Fig 1), and free 3*a*, 7*a*, 12*a*-trihydroxycho-

TABLE II. *In vitro* Conjugation of Cholic Acid by Rat Liver.

| Additions to incubation medium | Cholic acid recovered as conjugate of: Bacterial product "B" μmoles | % of total | L-ornithine μmoles | % of total | Taurine μmoles | % of total |
|---|---|---|---|---|---|---|
| Bacterial product "B" | .54 | 87.1 | — | — | .08 | 12.9 |
| | .56 | 86.1 | | | .09 | 13.9 |
| | .57 | 89.0 | | | .07 | 11.0 |
| Taurine | — | — | — | — | .51 | 100.0 |
| | | | | | .50 | 100.0 |
| | | | | | .53 | 100.0 |
| Bacterial product "B" and taurine | .53 | 73.6 | — | — | .19 | 26.4 |
| | .55 | 78.5 | | | .15 | 21.5 |
| | .54 | 74.0 | | | .19 | 26.0 |
| L-ornithine | — | — | .56 | 86.1 | .09 | 13.9 |
| | | | .57 | 85.1 | .10 | 14.9 |
| | | | .58 | 85.3 | .10 | 14.7 |
| L-ornithine and taurine | — | — | .53 | 79.1 | .14 | 20.9 |
| | | | .57 | 81.4 | .13 | 18.6 |
| | | | .56 | 75.7 | .18 | 24.3 |

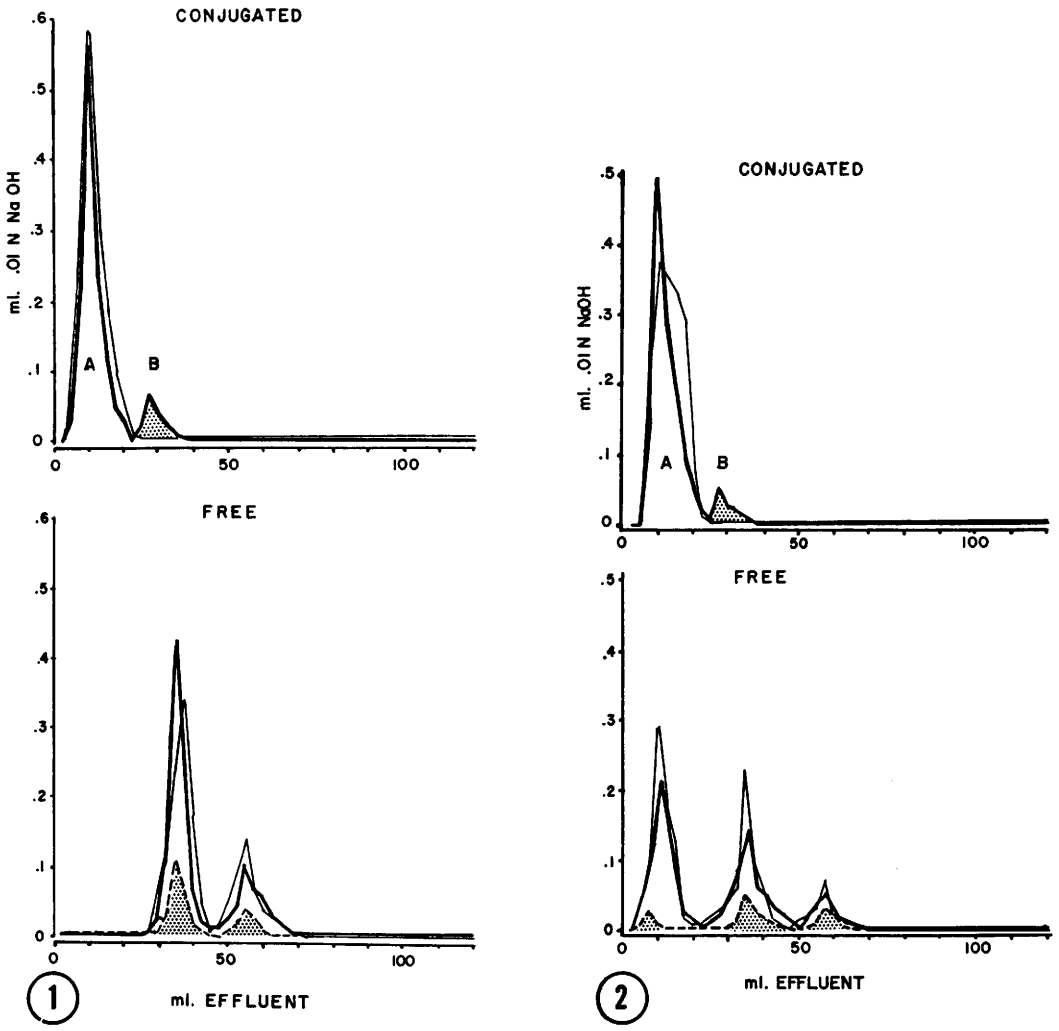


FIG. 1. Separation of bile acids by column chromatography in immature guinea pigs. Polysaccharide injected animals (——); controls (——). Shaded area: unusual conjugate (above) and free bile acids (below) obtained therefrom.

FIG. 2. Separation of bile acids by column chromatography in adult guinea pigs. Explanation of graph as in Fig. 1.

lanic (cholic) acid and the other 2 acids in adult guinea pigs (Fig. 2), approximated the absolute values and ratios observed in untreated control guinea pigs(5). Cholic acid, the major cholanic acid in adult guinea pigs, formed a relatively small portion of the abnormal conjugates.

After counter current distribution of $C^{14}$-labeled bacterial polysaccharide, $C^{14}$ was distributed within 3 zones of tubes. Material in zone "A", (distributed in initial 4 tubes) contained 96.2%, zone "B" (6th to 11th tube) 3%, and zone "C" (37th to 43rd tube) 0.8% of the isotope. The paper chromatography of material "B" revealed a ninhydrin positive spot of chromatographic mobility identical to that of L-ornithine. The aqueous eluate of this area contained approximately 85% of $C^{14}$ applied to the paper.

The results of *in vitro* studies are presented on Tables I and II. The conjugation of cholic acid with taurine and bacterial product "B" was accomplished with comparable effectiveness by both guinea pig and rat livers. Bacterial product "B" conjugated with cholic acid more readily than did taurine. The same

relation existed between L-ornithine and taurine when incubated with rat livers. Cholic acid, conjugated *in vitro* with bacterial product "B", had the same chromatographic mobility as the unusual conjugate excreted in the bile of polysaccharide-treated animals. Both conjugates had $R_f$ values identical to that of cholic acid conjugated with L-ornithine. Ninety to 94% of $C^{14}$ was detected in ethanolic eluates of zones of paper chromatograms containing cholic acid conjugated with bacterial product "B". The label was in the conjugated amino acid and not in the free bile acids, all $C^{14}$ remaining in water phase after alkaline hydrolysis and extraction of hydrolysate with ethyl ether.

*Comments.* The demonstration of L-ornithine-cholanic acid, in guinea pig, *in vivo* and *in vitro,* as well as in rat *in vitro,* refutes the exclusive role of glycine and taurine in bile acid conjugation.

Although absent in immature guinea pig (5), taurocholic acid has been shown to be the predominant conjugated bile acid in adult animal. When present, taurocholic acid apparently underwent continued enterohepatic cycling. The other 2 taurine-conjugated bile acids not only appeared in but disappeared from bile more rapidly than did taurocholic acid(3). In the present *in vivo* study, despite the larger amount of cholic acid in adult bile, less labeled L-ornithine (bacterial product "B") was found in cholic acid than in the other 2 bile acids. This may reflect the slower production of cholic acid which after conjugation with taurine is not hydrolyzed in the liver(9) and remains unavailable for ornithine conjugation. Further studies are needed to determine the possible enterohepatic cycling of ornithocholanic acid. Prior studies on biliary excretion of labeled constituents after parenteral injection of $C^{14}$-labeled Klebsiella polysaccharide, suggested that no recycling is to be anticipated(2). The excretion of label in the bile after introduodenal injection of $C^{14}$-Klebsiella polysaccharide probably represents the L-ornithine admixed with the bacterial polysaccharide. About 3% of the $C^{14}$ in the polysaccharide preparation was in L-ornithine and, since the latter was much more abundant in the ethanol-soluble fraction, would represent an inadvertant admixture rather than a constituent of the glucuronic acid-glucose-rhamnose polymer (unpublished studies—Jones, Radcliffe, Linker). Since .3% of the injected label of polysaccharide preparation was excreted in bile, it would appear that about 10% of the injected L-ornithine was conjugated with cholanic acids and excreted.

Since the polysaccharide was injected together with L-ornithine, the exact role of the toxic polysaccharide in excretion of ornithocholanic acids is uncertain. However, we have observed the ornithocholanic acid in guinea pigs with suppurative infections, and after surgical procedures, suggesting that the abnormal conjugate is a non-specific effect of hepatic injury. The preferential conjugation of L-ornithine with cholic acid *in vitro* is not incompatible with a non-specific metabolic alteration in homogenates. As obvious from Tables I and II, the same bile acyl transferase (10) would appear to be involved in the conjugation of both L-ornithine and taurine.

It is of interest that the conditions associated with excretion of ornithocholanic acid also have been associated with precipitate formation in guinea pig bile. The less effective binding of cholesterol by ornithocholanic acid than by the usual taurocholic acid, would provide an attractive hypothesis for formation of cholesterol and bile pigment precipitate.

*Summary.* L-ornithine conjugate of the 2 cholanic acids of the immature and the 3 cholanic acids of the adult guinea pig have been identified in bile following injection of a toxic, $C^{14}$-labeled capsular polysaccharide of *Klebsiella pneumoniae.* Preferential conjugation of L-ornithine with cholic acid by rat and guinea pig liver has been demonstrated *in vitro.*

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## Conversion of Cholest-4-en-3-one* to 5α-Cholestan-3β-ol† by Centrifugal Fractions of Rat Liver.‡ (27507)

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The double bond between carbon atoms 4 and 5 conjugated to the 3 carbonyl group of ring A is a molecular configuration essential for physiological activity of many 19-carbon and 21-carbon steroid hormones(1). These hormones are inactivated in part by 2 enzymatic reactions: A) a 5α- or 5β-hydrogenase reduces the double bond between carbon atoms 4 and 5, thus producing a saturated 3-ketosteroid; and B) a 3α- or 3β-dehydrogenase reduces the latter to a secondary steroid alcohol. The localization of these reducing enzymes in cell fractions prepared by differential centrifugation of liver homogenates has been investigated extensively(2-6).

Although the reductions of the 4-5 double bond and the 3-keto group of the 27-carbon steroid, cholest-4-en-3-one, have been studied *in vivo*(7,8), no attempt has been made to localize the enzymes responsible for these reactions in cellular compartments. Since this unsaturated 3-ketosteroid may be an intermediate in conversion of cholesterol to 5α-cholestan-3β-ol, we studied the localization of the 5α-hydrogenase and the 3β-dehydrogenase that act on cholest-4-en-3-one in rat liver cells.

*Experimental.* Livers of female rats weighing 200-230 g were homogenized with 2.5 volumes of 0.25 M sucrose in a Potter-Elvehjem homogenizer provided with a loose fitting Teflon pestle. The homogenate was separated into cellular debris, mitochondria, microsomes and supernatant fractions by the centrifugation procedures of Hogeboom(9). The mitochondria and the microsomes were suspended separately in 0.25 M sucrose, by gentle hand homogenization, before being incubated with the substrate, labeled cholest-4-en-3-one.

The cholest-4-en-3-one-4-$C^{14}$ was purchased from the Nuclear-Chicago Corporation and was purified by chromatography on silicic acid(10). About 0.4 to 0.8 μc was added to each flask along with 0.55 mg of unlabeled cholest-4-en-3-one. The steroid was solubilized with Tween 20 as described in(11). To each incubation flask were added 10 ml of a centrifugal fraction containing about 90 mg of protein, which was assayed by the method of Gornall(12). The final incubation mixture, prepared with 0.025 M phosphate buffer of pH 7.4, contained $2 \times 10^{-4}$ M oxidized triphosphopyridine nucleotide, $0.7 \times 10^{-3}$ M potassium isocitrate, $0.6 \times 10^{-3}$ M magnesium chloride, and 50 units of isocitric dehydrogenase (Sigma) in a total volume of 20 ml. Incubations were carried out with shaking for 2 hours at 37° in a nitrogen atmosphere.

The enzymatic reaction was stopped by addition of 2 volumes of ethyl alcohol containing enough KOH to provide a 15% concentration of KOH in the mixture. After 300 mg of unlabeled 5α-cholestan-3β-ol were added to each flask, the contents were gently refluxed on a steam bath for 3 hours. The

* This sterol is also known as cholestenone.

† This sterol is also known as dihydrocholesterol and 3β-cholestanol.

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