Hyodeoxycholate-6-O-glucuronide cannot be quantitated with 3α-hydroxysteroid dehydrogenase

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Summary The reaction of the 6-hydroxylated bile acid, hyodeoxycholic acid, and its 6-O-glucuronide conjugate with 3α-hydroxysteroid dehydrogenase was examined. A standard end-point assay and determination of the initial rates of reaction showed only minimal activity of the enzyme toward hyodeoxycholate-6-glucuronide in spite of the presence of a free 3α-hydroxyl group. It was established that 6-hydroxylation itself did not significantly affect the enzyme reaction. It is concluded that the 6-glucuronide either blocks or hinders enzyme access to the 3-hydroxyl group.—**Little, J. M., P. Zimniak, A. Radominska, and R. Lester.** Hyodeoxycholate-6-O-glucuronide cannot be quantitated with 3α-hydroxysteroid dehydrogenase. *J. Lipid Res.* 1987. **28:** 1370–1372.

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For some time, several variations of the 3α-hydroxysteroid dehydrogenase assay have been used to quantitate bile acid concentrations in biological fluids and experimental samples (1–4). Recent interest in glucuronidation of bile acids has led to the use of the 3α-hydroxysteroid dehydrogenase reaction as a criterion for determining the position of bile acid hydroxyl glucuronidation (5, 6) using the rationale that glucuronidation of the 3α-hydroxyl group of a bile acid will block any substrate–enzyme interaction. This report demonstrates that the 6-O-β-D-glucuronide of hyodeoxycholic acid does not react with 3α-hydroxysteroid dehydrogenase to any significant extent even though it has a free 3α-hydroxyl group. Thus, caution must be exercised in the application of the 3α-hydroxysteroid dehydrogenase reaction to structure determination and negative results of a 3α-hydroxysteroid dehydrogenase assay are not necessarily evidence for conjugation of the 3α-hydroxy group.

MATERIALS AND METHODS

3α-hydroxysteroid dehydrogenase from *Pseudomonas testosteroni* (EC 1:1:1:50) was obtained from Cooper Biomedical (Malvern, PA); nicotinamide adenine dinucleotide (NAD), glycine, Tris, EDTA, and hydrazine hydrate were from Sigma Chemical (St. Louis, MO); and taurocholic (TC) and hyodeoxycholic (HDC) acids were from Calbiochem-Behring (La Jolla, CA). The 6-O-β-D-glucuronide of HDC (HDC-Glu) was prepared biosynthetically by incubation of HDC with human liver microsomes. The purification and structure determination are described in detail elsewhere (7). Briefly, the single glucuronidated product of incubation of HDC with human liver microsomes was isolated, purified, and the methyl ester-acetate derivative was analyzed by high field proton nuclear magnetic resonance spectroscopy (NMR) and electron impact mass spectrometry (MS). The compound was unequivocally identified as the 6α-O-β-D-glucuronide of HDC.

To conserve the limited amount of HDC-Glu available, a micro modification of a standard 3α-hydroxysteroid dehydrogenase assay was used. A reaction mixture consisting of 200 μl of 0.1 M glycine-NaOH buffer, pH 9.5, 200 μl of 1.0 M hydrazine hydrate, pH 9.5, 80 μl of 3α-hydroxysteroid dehydrogenase (0.5 mg/ml or 1 unit/ml in 3 mM Tris-HCl/1 mM EDTA, pH 7.5) and 40 μl of water were placed in a microcuvette (light path: 1 cm; total volume: 0.7 ml), 20 μl of a methanolic solution of bile acid standard (10–60 nmol of HDC) or sample (ca. 20 nmol of HDC or HDC-Glu) was added, the samples were mixed well and background absorbance was read at 340 nm. The enzyme reaction was started by addition of 60 μl of 6.0 mM NAD, pH 7.0. After 30 min at room temperature, absorbance was again read at 340 nm. Samples were read again at 45 min to ensure that all substrate had been oxidized. The amount of bile acid present in the samples was calculated from a standard curve generated using

known amounts of HDC. Standard curves obtained with HDC and TC were identical. For kinetic studies of HDC, the reaction was carried out in a volume of 3 ml containing 50 mM glycine-NaOH, 0.019 mg of enzyme, 0.025 to 5.0 μmol of bile acid, and 0.6 mM NAD. The reaction was started by the addition of NAD and was followed continuously with a recorder (Fisher Recordall, Fisher Scientific, Pittsburgh, PA). Initial velocities were calculated from the slope of the linear portions of the recorder tracings.

RESULTS AND DISCUSSION

Standard solutions (ca. 2.0 μmol/ml) of HDC and HDC-Glu were assayed first under conditions designed to measure the total 3α-hydroxysteroid in the sample (i.e., excess enzyme, long incubation time). From the amount of NADH generated in the course of the reaction, it was determined that the HDC solution contained 1.951 μmol/ml. An equivalent aliquot of the HDC-Glu solution, assayed simultaneously under identical conditions, was found to have an apparent concentration of only 0.097 μmol/ml, less than 5% of the known concentration.

The rate of the 3α-hydroxysteroid dehydrogenase reaction with each of the substrates is shown in **Fig. 1**. The very low rate of reaction with HDC-Glu could result from a small contamination (i.e., not detectable by TLC) of the HDC-Glu preparation with unconjugated bile acid, from very slow hydrolysis of the glucuronide moiety during incubation in an alkaline medium, or from a nonspecific increase in absorbance.

The possibility that the mere presence of a 6-hydroxyl group on the bile acid turns it into a poor substrate for 3α-hydroxysteroid dehydrogenase was of some concern. If that were the case, it could be argued that glucuronidation of the 6-hydroxyl groups is significant only because the substrate was marginal in the first place. To explore this question, a kinetic analysis of the behavior of HDC and chenodeoxycholate was carried out. For HDC, the apparent kinetic constants (derived from an Eadie-Hofstee plot, **Fig. 2**) were: V_{max} = 2.1 μmol/mg • min, and K_m = 135 μM. The latter is similar to the K_m for chenodeoxycholate (109 μM, data not shown), proving that hydroxylation in position 6 does not, in itself, significantly affect the hydroxysteroid dehydrogenase reaction.

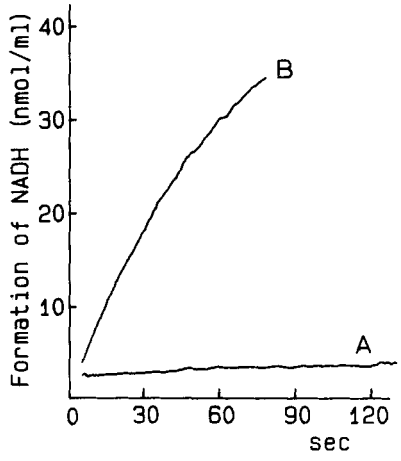

Fig. 1. Rate of reaction of hyodeoxycholate-6-O-glucuronide (A) and hyodeoxycholic acid (B) with 3α-hydroxystroid dehydrogenase. See text for incubation conditions.

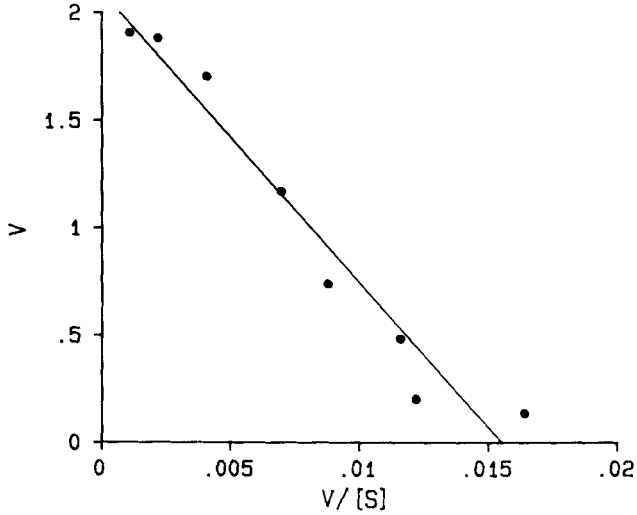

Fig. 2. Eadie-Hofstee plot of kinetic data obtained by incubation of hyodeoxycholic acid with 3α-hydroxysteroid dehydrogenase (see text for details of incubation). V is the initial reaction velocity in μmol/min • mg and [S] is the substrate concentration in μmol/l.

The difference in the initial rates of reaction between the two substrates (0.62 vs. 0.01 μmol/min • mg for HDC and HDC-Glu, respectively) and the small amount of 3α-hydroxy bile acid detected by the end-point assay indicated that the 3α-hydroxyl group of HDC-Glu was not available for oxidation by the enzyme under conditions typically used and sufficient for other substrates. A molecular model of the compound indicates why this might be so: a glucuronide moiety on the 6α-hydroxyl would most likely be positioned such that it would completely screen the 3α-hydroxyl and block enzyme access or at least hinder the binding of substrate to the enzyme.

The HDC-Glu used here, a product of human microsomal glucuronosyl transferase, has been rigorously identified by several independent means (7). This same glucuronide was identified several years ago in human urine (8). A more recent publication has assigned the structure of 3-O-β-D-glucuronide to a metabolite isolated from the bile or urine of patients fed hyodeoxycholate (6). This identification was based on the inactivity of 3α-hydroxysteroid dehydrogenase towards the metabolite. Over the years, several investigators have issued cautionary notes about the use of 3-hydroxysteroid dehydrogenase assay under several different sets of conditions

(9–11). The application of this enzymic reaction to structure determination is a fairly recent development, more or less coinciding with an increase of interest in bile acid conjugation with glucuronic and sulfuric acids. Recent reports from our laboratory have included data from 3α-hydroxysteroid dehydrogenase assays as one criterion for establishing the presence of the 3-O-glucuronides of etianic acid and cholic acid (5, 12). Indeed, in the case of etianic acid, lack of a reaction with 3α-hydroxysteroid dehydrogenase was the first clue to the identity of the conjugate form. However, in each case, confirmation of the structure of the conjugate was supplied by mass spectrometry and/or nuclear magnetic resonance spectroscopy.

The results of this report clearly demonstrate that while the 3α-hydroxysteroid dehydrogenase assay may provide an initial indication of the presence or absence of a conjugate group at the 3α-hydroxy position, corroboration of the proposed structure by another technique is essential, particularly when the bile acid being studied has more than one hydroxy substituent.

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