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Re-evaluation of the 3α-hydroxysteroid dehydrogenase assay for total bile acids in bile

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Summary A review of the 3α-hydroxysteroid dehydrogenase method for determining the concentration of total bile acids in bile is described. The optimum conditions for the assay were established with respect to pH, temperature, incubation time, amount of NAD^+, and units of enzyme activity required to obtain complete oxidation of the substrate under fixed conditions. Furthermore, the effect of hydrazine hydrate, methanol, and bile volume on the reaction was examined. It was also established that the bile acid concentration in bile samples with a high molar percentage of cholesterol would be overestimated if 3β-hydroxysteroid dehydrogenase were present with the 3α-enzyme.

Supplementary key words bile salt · enzymatic assay

Various methods are available for the quantitation of bile acids in biological fluids. One of these is an enzymatic assay involving the use of NAD^+-dependent 3α-hydroxysteroid dehydrogenase obtained from *Pseudomonas testosteroni.* As described by Talalay (1) this enzyme catalyzes the conversion of the 3α-hydroxyl group of steroids to a keto group and the NADH that is formed is measured either spectrophotometrically or spectrofluorometrically. Although this enzymatic method is now widely used as a rapid and simple means for determining the total bile acid concentration in bile (2–5) there has not been a comprehensive examination of the method for this specific purpose. In one study (6) which attempted to define the most suitable conditions for this assay, the ketone trapping agent, hydrazine, was excluded from the reaction mixture. A further limitation of that study was that the enzyme preparation used may have been contaminated with alcohol dehydrogenases which also reduce NAD^+, thereby interfering with estimation of bile acids (7). Admirand and Small (2) have briefly described their modification of the 3α-hydroxysteroid dehydrogenase assay; however, the details of their method have not been published.[1] Thus, the present study was undertaken to establish the optimum conditions for this enzymatic assay.

Materials and methods

Reagents. 3α-Hydroxysteroid dehydrogenase (EC 1:1:1:50) and a preparation containing both 3α- and

Abbreviations: 3α-HSD, 3α-hydroxysteroid dehydrogenase; C, cholic acid; TC, sodium taurocholate; DC, sodium deoxycholate; TCDC, sodium taurochenodeoxycholate.

[1] We are indebted to Dr. Donald M. Small for supplying us with the details of the method referred to in reference 2.

3β-HSD (EC 1:1:1:51) were obtained from Worthington Biochemicals Corp., Freehold, NJ. The enzymes were supplied as a purified powder prepared from cells of *Pseudomonas testosteroni*. In the preparation containing only the 3α enzyme the activity was 1.01 units per mg while in the preparation containing both hydroxysteroid dehydrogenases the activities of the 3α and 3β enzymes were 0.89 and 0.81 units per mg, respectively. Enzyme solution with an activity of 2 units per ml (1 unit is that amount of enzyme required to reduce 1 μmol of NAD^+ per minute) was prepared with cold 0.03 M Tris-HCl buffer, pH 7.2, containing 1 mM disodium ethylenediamine tetraacetate (EDTA) as described by Admirand and Small (2). The enzyme solution was stored at 4°C and used within one week of preparation. The enzyme powder was stored in a desiccator at −20°C. β-Nicotinamide adenine dinucleotide (NAD^+) was obtained from Sigma Chemical Company, St. Louis, MO, and stored in a desiccator at −20°C. A solution of NAD^+ (7 mM), adjusted to pH 7.0 with sodium bicarbonate, was stored at 4°C and prepared fresh every 2 weeks. Tris (tris [hydroxymethyl]-aminomethane), hydrazine hydrate (99%), and methanol (reagent grade) were supplied by Mallinckrodt Chemical Works, St. Louis, MO. EDTA was obtained from Sigma Chemical Company. A 1 M solution of hydrazine hydrate was prepared as described by Talalay (1). Sodium taurocholate (TC) and sodium deoxycholate (DC) were obtained from Calbiochem, San Diego, CA, sodium taurochenodeoxycholate (TCDC) from Sigma Chemical Company, and cholic acid (C) from Steraloids, Inc., Pauling, NY.

Procedure

The components of the incubation mixture were similar to those described by Admirand and Small (2) except that the pyrophosphate buffer was substituted with Tris-HCl buffer.[1] The standard incubation mixture, of final volume 3.0 ml, contained 1.5 ml of Tris-HCl buffer (0.133 M Tris, 0.666 mM EDTA, pH 9.5); 1.0 ml of hydrazine hydrate solution (pH 9.5); 0.3 ml of NAD^+ solution (7 mM, pH 7.0); 0.1 ml of methanol containing either pure bile acid or bile extract, and 0.1 ml of HSD solution in Tris-HCl buffer (0.03 M Tris, pH 7.2). The final pH of the incubation mixture was 9.5. Reagent blanks were prepared by adding 0.1 ml of methanol in place of the bile acid or bile extract while sample blanks were prepared by adding 0.1 ml of Tris-HCl buffer (pH 7.2) in place of the enzyme solution. Unless otherwise indicated, all incubations were carried out at 30°C for 60 min. The reaction mixtures, contained in disposable 12 × 75 mm glass culture tubes (American Hospital Supply Corp., McGaw Park, IL), were incubated in a Freas, Model 260 waterbath (GCA Precision Scientific, Chicago, IL). After incubation the optical densities (OD) of the samples and blanks were read at 340 nm using a Model 24 spectrophotometer (Beckman Instruments, Inc., Fullerton CA).

The standard assay system applied in all experiments except those on the effect of methanol and bile on the reaction. In those experiments the volume of Tris-HCl buffer was reduced to 1.0 ml (with an appropriate adjustment in the concentration of Tris and EDTA) so that increasing amounts of other components could be accommodated in the incubation mixture without altering the final volume of 3.0 ml. The final pH of the incubation mixture was always 9.5. For the experiment on the effect of varying the amount of bile in the incubation mixture, a sample of rat bile, low in bile acid concentration, was diluted 1 to 5 with methanol and the protein precipitate was removed by centrifugation. The methanol was then evaporated and the residue was taken up in a volume of methanol equivalent to the volume of the original bile sample. Three sets of assays were then carried out. In one set 0–0.10 ml of deproteinated bile was added to the incubation mixture, the volume of which was brought to 3.0 ml with water. The other two sets of assays were identical except that a fixed amount of TC (either 0.1 or 0.2 μmol) was added to the incubation mixture dissolved in the water.

One final experiment was carried out to determine the importance of enzyme specificity in the accuracy of the method. Four bile samples were obtained from a 3-month-old child undergoing T-tube drainage. Samples were taken on the 2nd, 5th, and 7th day of drainage. After saponification and extraction with petroleum ether, biliary cholesterol was precipitated as the digitonide and quantitated using the $FeCl_3$–H_2SO_4 method (8). The molar percentage of cholesterol in these samples averaged 19.3 ± 2.2 (mean ± SEM). To determine whether such high levels of cholesterol in the bile resulted in an overestimation of bile acid concentration when 3β-HSD was present with the 3α enzyme, assays of the bile acid concentration were carried out using both 3α-HSD and a preparation containing similar amounts of 3α- and 3β-HSD.

Results and discussion

The effect of varying incubation time, amount of enzyme activity, and amount of NAD^+ on the reaction is shown in **Fig. 1.** Complete oxidation of 0.2 μmol of TC was achieved within 60 min when 0.2 or more units of enzyme activity was present (Fig. 1*A*). When the amount of substrate was increased to 1.0 μmol, it was again found that beyond an incubation time of

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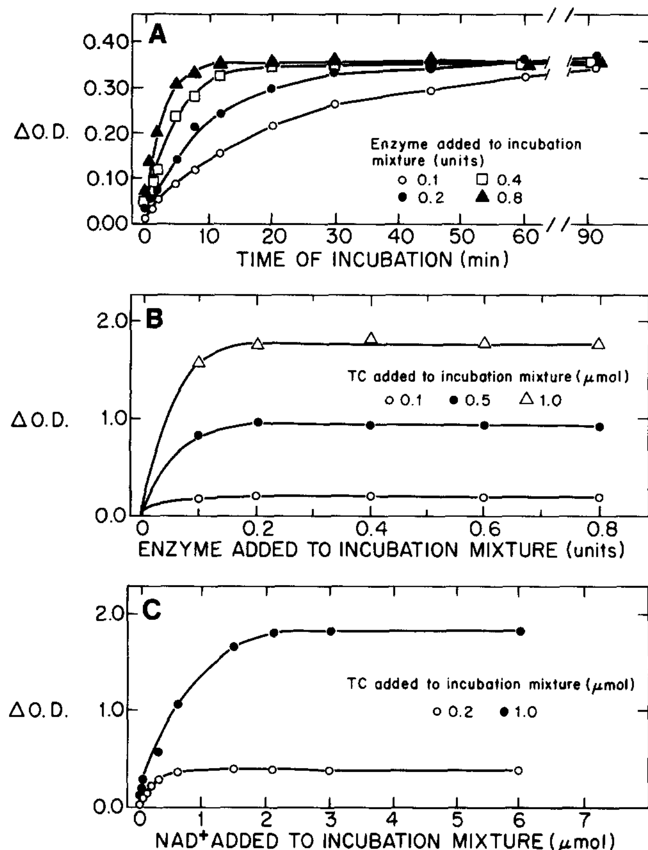

Fig. 1. Effect of time of incubation, amount of enzyme activity, and amount of NAD^+ on the rate of oxidation of TC by 3α-HSD. In panel *A*, 0.2 μmol of TC was incubated in the presence of 2.1 μmol of NAD^+ and different amounts of 3α-HSD activity. The time of incubation was 0–90 min. In panel *B*, 0.1, 0.5, or 1.0 μmol of TC was incubated in the presence of 2.1 μmol of NAD^+ and different amounts of 3α-HSD activity for 60 min. In panel *C*, 0.2 or 1.0 μmol of TC was incubated in the presence of 0.2 units of 3α-HSD activity and different amounts of NAD^+ for 60 min. In the various assays described in each panel the TC was added to the incubation mixture in 0.1 ml of methanol and the final volume of the incubation mixture was 3.0 ml. All incubation mixtures contained 1 mmol of hydrazine hydrate and the final pH was 9.5. The incubation temperature was 30°C. In panels *A* and *B* the ΔOD represents the difference between the OD for the complete incubation mixture and the OD for a blank prepared by substituting 0.1 ml of methanol for the TC. In panel *C* the ΔOD was calculated in the same way except that the blank contained 0.1 ml of Tris-HCl buffer (pH 7.2) in place of the enzyme.

60 min, 0.2 units of activity was sufficient to completely oxidize the substrate (Fig. 1*B*). The effect on the reaction rate of varying the amount of NAD^+ is shown in Fig. 1*C*. In this experiment 0.2 or 1.0 μmol of TC was incubated in the presence of 0.2 units of enzyme activity and varying amounts of NAD^+ (0–6.0 μmol). This had no effect on the final pH of the incubation mixture. It was found that 0.2 μmol of TC was completely oxidized in 1 hr in the presence of 1.5 μmol of NAD^+. However, complete oxidation of 1.0 μmol of TC within 60 min required the presence of 2.1 μmol of NAD^+.

The effect of temperature, pH, hydrazine hydrate, and methanol on the reaction is shown in **Fig. 2.** In these studies the optimum incubation time and amount of enzyme and NAD^+ were utilized as given in the legend. The effect on the reaction rate of varying the incubation temperature was studied with four different substrates. As shown in Fig. 2*A*, there was a marked difference between bile acids with respect to the effect of temperature. The oxidation of both TCDC and DC showed no temperature dependence over the range 10–40°C. In contrast, the oxidation of TC and C was much slower at 10°C and, to a lesser extent, at 20°C than at either 30°C or 40°C. These differences were observed whether the incubations were carried out using either 0.2 or 1.0 μmol of bile acid. This suggests that the slower rate of oxidation of TC and C at 10–20°C was not the result of limited solubility at these temperatures. The effect of pH on the reaction, using TC as the substrate, is shown in Fig. 2*B*. Although there was not a marked dependence on pH over the range 8–11, the highest rate of oxidation occurred at pH 9.0–9.5. As shown in Fig. 2*C* the presence of the ketone trapping agent, hydrazine hydrate, in amounts less than 0.2 mmol had little effect on the reaction when only 0.2 μmol of TC was present. However, incomplete oxidation occurred when 1.0 μmol of bile acid was assayed. At least 0.5 mmol of hydrazine hydrate was required to attain complete oxidation of this amount of bile acid. Fig. 2*D* describes the effect of the bile acid solubilizer, methanol, on the reaction. Although there was no effect when 0.1 ml of methanol was present, a decrease in the rate of oxidation of TC was found when the volume of methanol was increased to either 0.2 or 0.5 ml. The mechanism of this apparent inhibition was not explored further.

In all of the preceding studies, except that on the effect of temperature, optimal conditions were established using TC. An additional study was therefore necessary to determine whether these various conditions were suitable for several other bile acids. Different amounts of TC, TCDC, DC, and C (up to 1.5 μmol) were added to the incubation mixture and the assays were carried out using the optimal conditions described above. As shown in **Fig. 3*A*,** the relationship between rate of oxidation and amount of bile acid added was identical for all four bile acids tested.

It was found that to precipitate the protein in bile samples a dilution of at least 1 to 5 with methanol was necessary. Since the standard volume of this methanolic solution in the reaction mixture was usually 0.1 ml, the volume of deproteinated bile actually present in the sample was 0.02 ml. For bile samples with extremely low bile acid concentrations it would be

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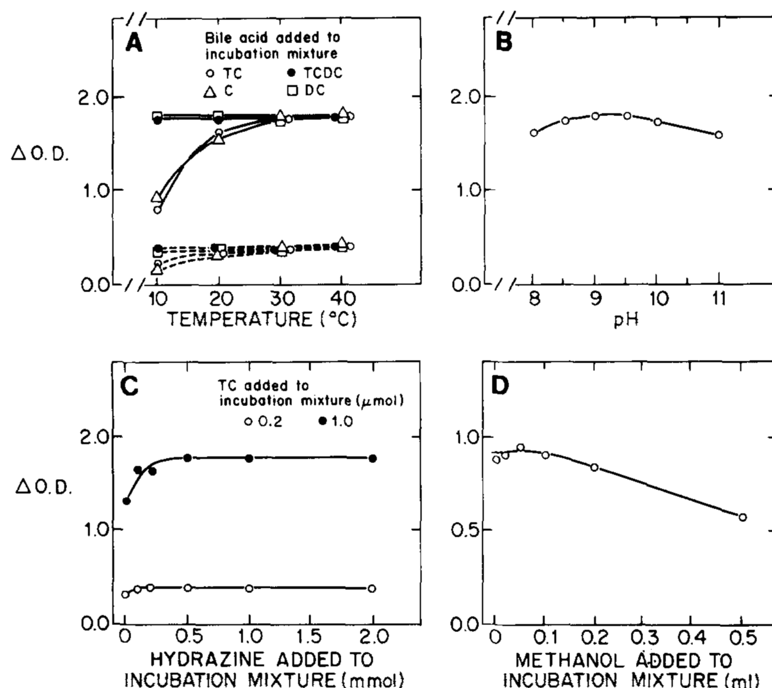

Fig. 2. Effect of temperature, pH, hydrazine hydrate, and methanol on the rate of oxidation of bile acids by 3α-HSD. In panel *A*, 0.2 μmol (dashed lines) and 1.0 μmol (solid lines) of TC, TCDC, and C was incubated at different temperatures. All substrates were added to the incubation mixture in 0.1 ml of methanol. The incubation mixture contained 1 mmol of hydrazine hydrate and the pH was 9.5. In panel *B*, 1.0 μmol of TC was incubated at different pH values. The substrate was added to the incubation mixture in 0.1 ml of methanol and the incubations were carried out in the presence of 1 mmol of hydrazine hydrate at 30°C. In panel *C*, 0.2 or 1.0 μmol of TC was incubated in the presence of different amounts of hydrazine hydrate. The substrate was added to the incubation mixture in 0.1 ml of methanol and the incubations were carried out at pH 9.5 at 30°C. In panel *D*, 0.5 μmol of TC was incubated in the presence of different amounts of methanol. The incubation mixture contained 1 mmol of hydrazine hydrate and the pH was 9.5. The incubations were carried out at 30°C. For this study the proportion of some components of the incubation mixture was modified as described in Materials and Methods. In the various assays described in each panel the final volume of the incubation mixture was 3.0 ml and the incubations were carried out in the presence of 2.1 μmol of NAD^+ and 0.2 units of 3α-HSD activity for 60 min. In all panels the ΔOD represents the difference between the OD for the complete incubation mixture and the OD for a blank prepared by substituting 0.1 ml of methanol for the bile acid.

necessary to concentrate the sample so as to be within the range of sensitivity of this assay. This would result in higher amounts of chromagens and other methanol-soluble compounds in bile being added to the incubation mixture. The results of a study designed to test possible interference by these biliary constituents on the reaction are shown in Fig. 3*B*. The reaction rate remained linear as a function of the volume of deproteinated bile added between the limits of 0 and 0.075 ml. At 0.1 ml there was some inhibition of the reaction. The same result was obtained when a fixed amount of exogenous bile acid (0.1 or 0.2 μmol of TC) was added together with the deproteinated bile to each incubation. This clearly established that bile samples could be concentrated up to approximately 4-fold without any effect on the reaction.

Based upon these various experimental results, optimal conditions were established for this assay and are summarized in **Table 1.**

In the experiment designed to determine the importance of enzyme specificity in this assay, it was found that the bile acid concentration in bile samples saturated with cholesterol was overestimated when the enzyme preparation used contained both 3α- and 3β-HSD. However the overestimation was only about half that expected. The average cholesterol concentration in the four bile samples was 0.8 ± 0.13 μmol/ml and the average bile acid concentration, determined using 3α-HSD, was 2.4 ± 0.05 μmol/ml. If all the cholesterol present had been oxidized by 3β-HSD, then the average bile acid concentration determined using the preparation containing both enzymes should have been 3.2 μmol/ml. However, the observed value was 2.8 ± 0.13 μmol/ml. Since the molar percentage of cholesterol in these samples averaged 19.3 ± 2.2, a substantial amount of cholesterol was probably not solubilized and therefore was unavailable for reaction with 3β-HSD. These findings thus emphasize

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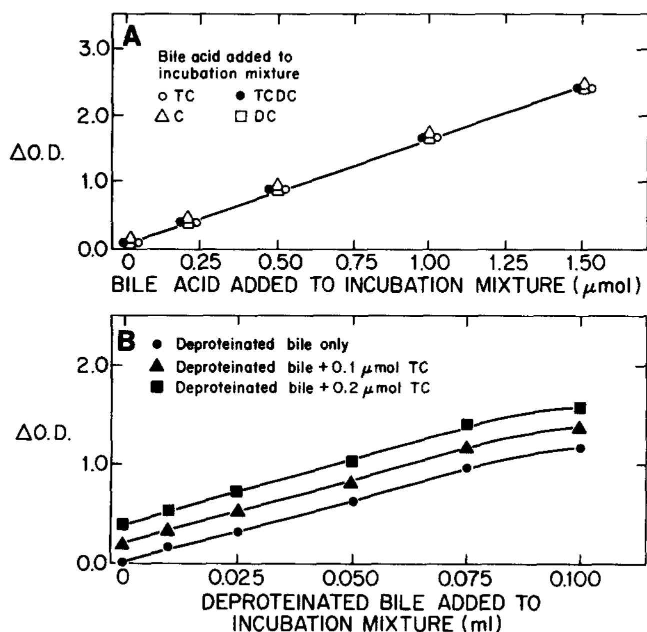

Fig. 3. Effect of the amount of bile acid and of the volume of bile in the incubation mixture on the rate of oxidation of the bile acid by 3α-HSD. In panel *A*, different amounts of TC, TCDC, C, and DC were incubated in the presence of 2.1 μmol of NAD^+ and 0.2 units of 3α-HSD activity for 60 min at 30°C. The substrate was added in 0.1 ml of methanol and the final volume of the incubation mixture was 3.0 ml. The incubation mixture contained 1 mmol of hydrazine hydrate and the pH was 9.5. In panel *B*, 0.1 or 0.2 μmol of TC was incubated in the presence of increasing amounts of deproteinated rat bile. For this study the proportion of Tris-HCl buffer in the incubation mixture was modified as described in Materials and Methods, otherwise the conditions of assay were the same as those described for the study in panel *A*.

that the accuracy of this method, particularly when applied to bile with a high molar percentage of cholesterol or to other biological solutions with relatively high cholesterol concentrations, does depend upon the use of an enzyme preparation containing only 3α-HSD.

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TABLE 1. Summary of reagents for the 3α-HSD assay for total bile acids in bile and the respective proportions of each in the incubation mixture[a]

Reagent	Volume in Incubation Mixture	Mass in Incubation Mixture
	ml	*μmol*
Tris-HCl buffer (0.133 M Tris; 0.666 mM EDTA) pH 9.5	1.50	200 (Tris) 1 (EDTA)
Hydrazine hydrate (1 M) pH 9.5	1.00	1000
NAD^+ (7 mM) pH 7.0	0.30	2.1
Methanolic extract of bile[b]	0.10	Up to 1.5 μmol of bile acid
3α-HSD[c] (2 units of activity per ml)	0.10	0.2 units of activity

[a] After addition of the enzyme, reaction mixtures were incubated at 30°C in air for 1 hr. The absorbance at 340 nm was then measured. For each bile sample an appropriate blank was prepared by adding 0.1 ml of Tris-HCl buffer (pH 7.2) in place of the enzyme. For each series of assays a reagent blank, prepared by adding 0.1 ml of methanol in place of methanolic extract of bile, and a set of bile acid standards were also included.

[b] A dilution of approximately 1 to 5 with methanol was necessary to precipitate all protein.

[c] Enzyme solution was prepared using Tris-HCl buffer (0.03 M Tris) containing 1 mM EDTA, pH 7.2.

REFERENCES

1. Talalay, P. 1960. Enzymic analysis of steroid hormones. *Methods Biochem. Anal.* **8:** 119–143.
2. Admirand, W. H., and D. M. Small. 1968. The physicochemical basis of cholesterol gallstone formation in man. *J. Clin. Invest.* **47:** 1043–1052.
3. Hardison, W. G. M., and J. T. Apter. 1972. Micellar theory of biliary cholesterol excretion. *Am. J. Physiol.* **222:** 61–67.
4. Strasberg, S. M., C. N. Petrunka, and R. G. Ilson. 1976. Effect of bile acid synthesis rate on cholesterol secretion rate in the steady state. *Gastroenterology* **71:** 1067–1076.
5. Wheeler, H. O., and K. K. King. 1972. Biliary excretion of lecithin and cholesterol in the dog. *J. Clin. Invest.* **51:** 1337–1350.
6. Turnberg, L. A., and A. Anthony-Mote. 1969. The quantitative determination of bile salts in bile using thin-layer chromatography and 3α-hydroxysteroid dehydrogenase. *Clin. Chim. Acta* **24:** 253–259.
7. Engert, R., and M. D. Turner. 1973. Problems in the measurement of bile acids with 3α-hydroxysteroid dehydrogenase. *Anal. Biochem.* **51:** 399–407.
8. Tonks, D. B. 1967. The estimation of cholesterol in serum: A classification and critical review of methods. *Clin. Biochem.* **1:** 12–29.

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