Enzymatic determination of bile acids from liver cells with 3α-hydroxysteroid dehydrogenase—a warning

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Summary 3α-Hydroxysteroid dehydrogenase was used to monitor bile acid production by rat hepatocyte suspensions. Several observations suggested that the method lacked specificity and detected some other metabolites besides bile acids. This was due to a malate dehydrogenase activity present in four different commercial enzyme preparations of 3α-hydroxysteroid dehydrogenase. It is concluded that considerable error can be caused by this contaminating malate dehydrogenase activity, especially in the case of low bile acid concentration in the sample.—**Barth, C. A. and K. Wirthensohn.** Enzymatic determination of bile acids from liver cells with 3α-hydroxysteroid dehydrogenase—a warning. *J. Lipid Res.* 1981. **22:** 1025–1027.

Supplementary key words substrate specificity · hepatocytes · bile acid synthesis · malate dehydrogenase

The discovery of a bacterial 3α-hydroxysteroid dehydrogenase (HSD) has resulted in a widely used and convenient optical assay of bile acids (1). It has been successfully applied to the analysis of bile, intestinal contents, and blood (2).

We, as others (3, 4), have recently tried to apply the analytical procedure to the study of bile acid secretion by isolated rat hepatocytes. Several observations made during these experiments left some doubts about the specificity of the method.

We report now on these observations, showing a basic drawback of the method that should be kept in mind, particularly when analyzing low concentrations of bile acids. While this work was in progress, data have been published by Whiting and Edwards (5) which are complementary to and perfectly compatible with our observations. Our data give a plausible explanation for the discrepancy these authors observed between results obtained by enzymatic assay and gas–liquid chromatography.

MATERIALS AND METHODS

Taurocholate was from Roth (Karlsruhe, Germany) and was used as standard for the HSD assay after two-fold recrystallization from ethanol–diethyl ether. Collagenase was from Worthington (Freehold, NJ type CLS II). HSD was obtained from the following suppliers: Worthington (Cat. Nr. STDHMP), Serva (Heidelberg; Cat Nr. 25885), Nygaard (Oslo; Sterognost) and Sigma (Cat. Nr. H-8879).

The assays shown in Figs. 1 and 2 were performed with the preparation from Sigma (Cat. Nr. H-8879). The results were comparable with the other enzyme preparations. All other enzymes and coenzymes including malate dehydrogenase (Cat. Nr. 127248) were from Boehringer (Mannheim, Germany).

Isolated rat hepatocytes were prepared according to Bischoff et al. (6). Criteria of viability and metabolic characteristics of the hepatocyte preparations used are given in detail elsewhere (7). Incubations of the hepatocytes were performed in 25-ml polycarbonate Erlenmeyer flasks under an atmosphere of 5% CO_2 in O_2. The incubation volume was 2 ml and the cell concentration 15 mg/ml (wet weight). Aliquots of the incubations were centrifuged at 1300 *g* and the resulting supernatants were deproteinized by adding an equal volume of methanol. After centrifugation of this methanol extract, bile acid analyses were performed according to Koss, Mayer, and Haindl (8). In the case of malate dehydrogenase assays, the same buffer and coenzyme concentrations were used as for the bile acid assay with HSD.

RESULTS

In an attempt to study bile acid synthesis and its stimulation by malate (4), isolated hepatocytes were incubated with 1.25–5 mM L-malate. There was an apparent rise of bile acid concentration in the incubation medium depending on L-malate concentration (**Fig. 1**). But, surprisingly, the amount of $NADH_2$ generated in the HSD assay at 60 min incubation time was higher than at 120 min (Fig. 1); when expressed in bile salt concentration, this would indicate a disappearance of bile acids during the incubation. Because of this finding, the specificity of the analytical procedure seemed questionable to us.

As there seemed to be roughly a stoichiometry between the $NADH_2$ signal and the L-malate added at the onset of incubation, we wondered whether L-malate caused the $NADH_2$ formation. HSD catalyzed a stoichiometric formation of $NADH_2$ from control incubations consisting of L-malate without cells (**Fig. 2**). An identical $NADH_2$ signal was obtained with malate dehydrogenase in these experiments. This suggested that the HSD preparation used contained a malate dehydrogenase activity. **Fig. 3** shows poly-

Abreviations: HSD, 3α-hydroxysteroid dehydrogenase (E.C.-1.1.1.50); MDH, malate dehydrogenase, L-malate: NAD^+ oxidoreductase (E.C.1.1.1.37).

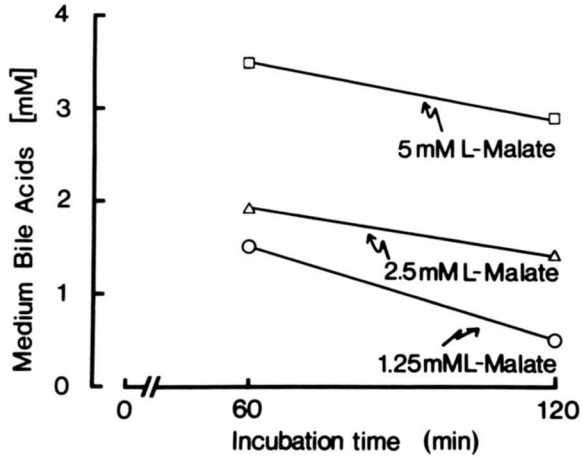

Fig. 1. Secretion of bile acids by isolated hepatocytes in the presence of different concentrations of L-malate. Aliquots of the same hepatocyte preparation were incubated at a concentration of 15 mg wet weight per ml in a total volume of 2.0 ml. The incubation buffer was 20 mM Tris-HCl, pH 7.4, supplemented with 100 mM KCl, 2 mg/ml bovine serum albumin, 6.7 mM nicotinamide, 1.7 mM glucose-6-phosphate, and 40 μg/ml ampicillin (4). Shaking frequency was 100 per min and the temperature was 37°C. Aliquots were taken at times indicated and analyzed with 0.2 U of HSD (for further details see Methods).

acrylamide slab gels of a commercial HSD preparation; when stained with Coomassie Blue, a marked heterogeneity appeared (Fig. 3A). If the same gels

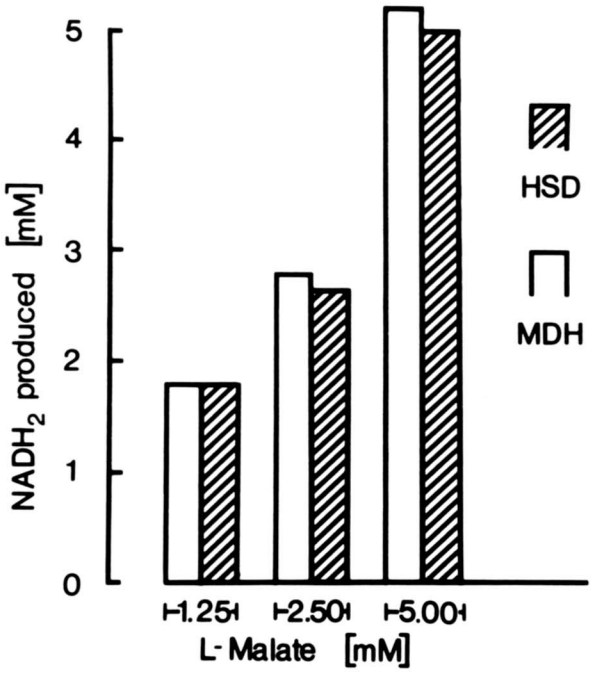

Fig. 2. Identity of $NADH_2$ formation from L-malate by HSD or malate dehydrogenase. Three vials with different concentrations of L-malate (abscissa) without cells were incubated, as described in Fig. 1, for 1 hr. Then appropriate samples were taken and analyzed, as described in Methods, with either 0.2 U HSD (hatched columns) or 30 U of malate dehydrogenase (white columns). The ordinate gives the concentration of medium metabolites as calculated from the respective $NADH_2$ signal.

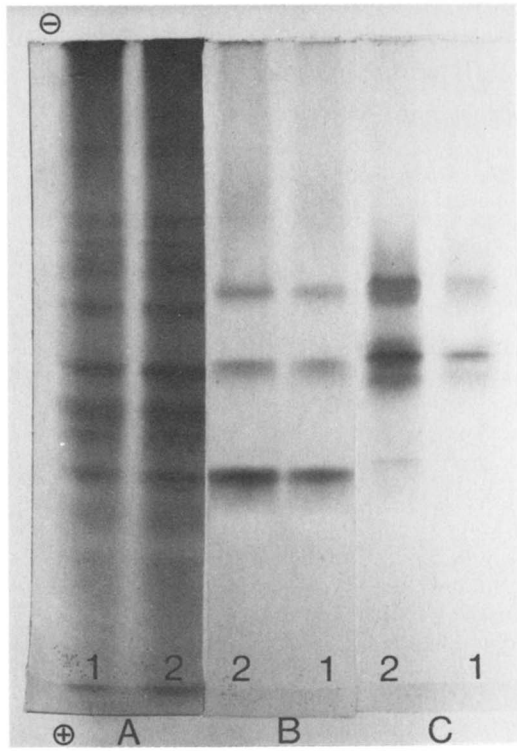

Fig. 3. Polyacrylamide slab gel electrophoresis of the crude enzyme preparation. 100 μg (lane 1) and 200 μg (lane 2) of the lyophilized powder (Worthington Cat. Nr. STDHMP) was run for 12 hr on 7.5% gel in 38 mM Tris-glycine, pH 8.3, at 10 V/cm. A, gel was stained with Coomassie Blue; B and C, gels were stained for enzymatic activity according to Doman and Koide (15), except that 8-dimethylamino-2,3-benzophenoxazin (1.25 mg/100 ml) (Boehringer Cat. Nr. 258504) and *p*-iodonitrotetrazolium violet (Sigma Cat. Nr. 18377) were used. Gel B was incubated 30 min at 37°C with L-malate (1 mM) and gel C with taurocholate (1 mM) as substrate. The control gel incubated without substrate showed no staining (not shown here).

were stained for dehydrogenase activity with taurocholate as substrate, more than three bands were observed (Fig. 3C). In the case of L-malate as substrate, three major bands were detected (Fig. 3B) with different electrophoretic mobility of the major bands compared with slab gel C.

DISCUSSION

We reported herein an apparent malate dehydrogenase activity in 3α-hydroxysteroid dehydrogenase. Because this contamination was detected in all four commercial HSD preparations, there seems to be a general lack of specificity of the enzyme preparations now available. Two kinds of evidence may be cited to support the view that this is due to the presence of a contaminating malate dehydrogenase enzyme in the cell extract. First, there were not only multiple protein bands, as evidenced by Coomassie Blue staining,

but the major bands of enzyme activities with the substrates taurocholate or L-malate, respectively, showed different electrophoretic mobility. Second, enzyme activity assayed in the presence of both substrates was nearly additive if compared to measurements in the presence of one of the substrates separately (data not shown). This can be taken as evidence that a HSD and a malate dehydrogenase enzyme are present in the extract (9). Furthermore, the fact that another contaminating activity, e.g., alcohol dehydrogenase, has been observed in a commercial HSD preparation (10) lends support to this conclusion.

This malate dehydrogenase activity impairs substantially the validity of the widely used procedure to determine bile acids with HSD. Particularly in cases of low bile acid concentrations to be analyzed, it becomes liable to errors unless a purification step is performed prior to the assay, as suggested by several procedures (1, 2, 11). For example, in human venous blood plasma, L-malate concentrations of 3.2 μM have been observed (12). Considering serum bile acid concentrations of 2.7 μM in human venous blood serum (13), values may be found that are too high by 130%, due to L-malate in the sample.

Recently, Whiting and Edwards (5) have observed a considerable discrepancy of bile acid secretion by isolated hepatocytes, depending on whether the medium was analyzed with HSD or by gas–liquid chromatography. From their data, these authors concluded that very probably the enzymatic procedure lacked specificity for bile acids and that some other substances besides bile acids contributed to their results. The results reported now are perfectly compatible with this conclusion and give an explanation of the above findings.

Extracellular concentrations of more than 10 μM L-malate have been reported for freshly incubated hepatocytes (50 sec incubation time) (14) because of leakage of metabolites to the medium. Therefore, even if no malate or malate precursors, such as fumarate of succinate (4), are added, too high values can be expected if the HSD method is applied for detection of bile acids.

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