Inhibition of Hepatic Alcohol Dehydrogenase by Bilirubin*

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SUMMARY

Human bile is capable of inhibiting, in vitro, the action of human and equine liver alcohol dehydrogenase. The effect is attributable mainly to bilirubin conjugates. Sodium deoxycholate had negligible inhibitory ability.

Preliminary evidence indicates that the mechanism of this inhibition of alcohol dehydrogenase is related to the capability of bilirubin for complex formation with the zinc ion.

The clinical implication of these findings is discussed.

Inhibition of various types of enzyme activity by bile, bile constituents, or degraded bile is a recognized process in vitro and in vivo. During a study of the role of alcohol ingestion in diseases of the liver and pancreas, a possibility that some enzyme involved in the metabolism of alcohol might be similarly abnormally inhibited was considered. Accordingly, measurements of alcohol dehydrogenase activity were made on liver samples obtained at surgical operations performed on patients with various diseases. In one icteric patient, alcohol dehydrogenase activity was found to be strikingly diminished. Seven non-icteric patients showed a mean alcohol dehydrogenase activity of 100 units per mg of hepatic tissue. The icteric patient had 19.1 units of alcohol dehydrogenase activity per mg of liver. The liver sample was found to be congested with bile. This finding led to further investigations that suggest that bile or one of its constituents is inhibitory to this activity. The results of these investigations are reported here.

A survey of the literature disclosed many instances of enzyme inhibition by bile and bile components. In 1837, Itzioka reported the inhibition of casein hydrolysis by rabbit pancreatic juice by addition of large amounts of rabbit bile (1). The inhibition by bilirubin of trypsin, chymotrypsin, pancreatic amylase, and intestinal alkaline phosphatase has been described (2, 3).

The irreversible brain damage caused by high blood levels of bilirubin in the newborn (kernicterus) has stimulated several studies of the effect of bilirubin on brain metabolism. Inhibition of brain homogenate respiration by bilirubin has been noted, as well as uncoupling of oxidative phosphorylation in brain mitochondria (4–6). Similarly, the bilirubin inhibition of hepatic respiration in the resting human liver (7) has been reported, and confirmed in the resting (8) and regenerating (9) rat liver.

Bilirubin also uncouples oxidative phosphorylation in rat liver mitochondria in vitro (10). The administration of bilirubin parenterally to rats inhibited the incorporation of phosphate into mitochondria in vivo (11). In human liver homogenates, addition of bile reduced the incorporation of $^{32}P$-labeled phosphate into ribonucleic acid and phosphatides (12).

Prolongation of the prothrombin time by bile has been reported in vivo (13). The same effect has been reported for bilirubin glucuronide in vitro (14).

Inhibition of cholesterol esterase activity by bilirubin has been advanced as an explanation of the lowered cholesterol ester to free cholesterol ratio in hepatic disease with jaundice (15).

Bile salts have been recognized as enzyme inhibitors and have been reported to be capable of decreasing the adenosine triphosphatase activity of myosin (16), as well as bile phosphatase (17). The regeneration of rhodopsin in the frog retina can be markedly inhibited by the intraocular injection of 0.15 $\mu$g of bilirubin (18). Since alcohol dehydrogenase has been shown to play a vital role in this process (19), a plausible explanation may be found in the subject of this report.

EXPERIMENTAL PROCEDURE

Materials—Crystalline horse liver alcohol dehydrogenase was obtained from Sigma. Crude human alcohol dehydrogenase was prepared from liver samples obtained at laparotomy from patients requiring surgery for various reasons. There was no clinical or histological evidence of liver disease. After homogenization in 0.05 $\times$ Tris buffer, pH 7.5 (1 g of fresh liver per 10 ml of buffer), by a Virtis tissue homogenizer, the samples were centrifuged for 30 min at 22,000 $\times$ g at 0°C in an International refrigerated centrifuge, model PR-2. The supernatant fluid containing crude human alcohol dehydrogenase was assayed directly (average, 100 units of alcohol dehydrogenase activity per mg of protein). The assay for this enzyme is described below.

Human bile was obtained from T-tube drainage in patients convalescing from cholecystectomy and choledochostomy. This was stored under refrigeration and clarified by centrifugation for 15 min at 22,000 $\times$ g just prior to use. The bile samples after centrifugation had pH values in the neighborhood of 8.5 and had little buffering capacity. A crude preparation of watersoluble bile pigment conjugates was obtained from human bile by the method of Watson, Campbell, and Lowry (10).
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Inhibition of alcohol dehydrogenase by bile. The reaction components, in 1 ml of 0.1 M glycine buffer, pH 9.4, were 4.5 µmoles of DPN, 100 units of alcohol dehydrogenase activity, and 10 µmoles of ethanol. ○, bile inhibition of crude human liver alcohol dehydrogenase (specific activity, 100 units per mg of protein); ●, bile inhibition of crystalline horse liver alcohol dehydrogenase (specific activity, 7000 units per mg of protein). The abscissa is calibrated in both amount of bile added to the reaction mixture and total bilirubin level in the bile sample added. The ordinate is the ratio of observed initial velocity ($V_i$) to velocity without inhibitor ($V_0$).

Bilirubin was purchased from Fisher Scientific Company as the free unconjugated pigment; after being dissolved in 0.1 N NaOH, it was adjusted to pH 9.4 with concentrated hydrochloric acid. A stable, aqueous solution of bilirubin bound to albumin at pH 7.4 was prepared by the method of Shinowara (21). Ethanol was purchased as the 100% U.S.P. reagent from U. S. Industrial Chemicals Company, New York. DPN was purchased from Sigma at a stated purity of 98%.

Methods—The assay of alcohol dehydrogenase activity was conducted according to the method of Theorell and Bonnichsen (22). Assay of hepatic alcohol dehydrogenase levels was performed in a standard reaction volume of 3 ml, consisting of 3 mmoles of glycine buffer (pH 9.4), 0.1 ml of absolute ethanol, and 1 mg of DPN. In all cases, the reaction was started by the addition of enzyme to the reaction mixture, thereby avoiding prior incubation of enzyme and inhibitor. One unit of alcohol dehydrogenase activity was defined as the amount of enzyme causing an increase in absorbance of 0.001 per min at 340 mµ in a Beckman DU spectrophotometer. The first reading was made 30 sec after the addition of the enzyme-containing component. The details of other reaction mixtures are listed in the legends of the appropriate figures.

Bilirubin was assayed by the method of van den Bergh as adapted by Malloy and Evelyn (23). This measures the total reacting bilirubin by the amount of color generated in 50% methanol after 30 min. The bilirubin sample obtained from Fisher was used as the standard.

Protein was determined by the method of Lowry et al. (24) except where the biuret method (25) is specified.

RESULTS

Inhibition of Alcohol Dehydrogenase by Bile—The effect of bile on both the crystalline horse liver enzyme and a crude extract of human liver containing alcohol dehydrogenase is shown in Fig. 1. The extent of inhibition by bile is very nearly the same for each enzyme. The abscissa gives the volume of bile added per ml of reaction mixture, as well as the amount of total bilirubin present at each level of added bile. The 50% inhibition point occurs at 0.19 ml of bile per ml of reaction mixture, corresponding to 0.089 mg of total bilirubin (total reacting), or $1.55 \times 10^{-4}$ M free bilirubin.

Inhibition of Crystalline Horse Liver Alcohol Dehydrogenase by Bilirubin—Fig. 2 depicts the inhibition of the horse liver enzyme by an extract of bile containing the water-soluble pigments, presumably mainly the mono- and diglucuronides. It may be noted that the extent of inhibition relative to the amount...
of total reacting bilirubin is similar for both crude bile and impure bilirubin glucuronide (Fig. 1).

The inhibition of horse liver alcohol dehydrogenase by unconjugated bilirubin is shown in Fig. 2. While bilirubin is capable of inhibiting the reaction, it is only approximately one-half as effective as bile on the basis of concentration of total reacting bilirubin in the bile sample used, and bilirubin albuminate is still less effective. Thus, Fig. 2 reveals the 50% inhibition point for free bilirubin to be 0.184 mg per ml (3.2 × 10^{-4} M), and for bilirubin albuminate, 0.25 mg per ml (4.3 × 10^{-4} M).

Figs. 3 and 4 are Lineweaver-Burk plots for the inhibition of alcohol dehydrogenase by bilirubin, with varying amounts of DPN and ethanol, respectively. In the case of DPN, the kinetics is clearly competitive. Since DPN is believed to bind zinc in the enzyme, it would seem evident that bilirubin functions as an inhibitor in a similar manner. Pure competitive inhibition was not observed between ethanol and bilirubin in this system. These results are similar to those obtained for o-phenanthroline by Vallee, Williams, and Hoch (26).

Reversal of the bilirubin inhibition was attempted by addition of zinc ion (Fig. 5). It was possible to increase the initial velocity of the reaction relative to the control by addition of zinc, but a high concentration (0.02 M zinc sulfate) was required. Under comparable conditions, Vallee and Hoch (27) were able to reverse nearly completely the inhibition of alcohol dehydrogenase by o-phenanthroline with 0.004 M added zinc.

DISCUSSION

While both free bilirubin and its water-soluble conjugates inhibit alcohol dehydrogenase, the latter are nearly twice as effective. This observed discrepancy may be a solubility phenomenon. Evidence exists that free bilirubin does not form a true solution at a pH of 9.4 but is present instead as a very fine colloidal dispersion. Another explanation is the possibility that the sample of bilirubin conjugates are contaminated with biliverdin. Biliverdin also forms zinc complexes (28, 29), and causes inhibition of alcohol dehydrogenase as observed in this laboratory.

Besides bilirubin, other inhibitors may be found in bile. Bile salts are often responsible for enzyme inhibition by virtue of their ability to denature proteins. However, sodium deoxy-

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**Fig. 3.** Lineweaver-Burk reciprocal plots for the relation between activity and DPN concentration. The ordinate is reciprocal change in absorbance units per min, and the abscissa is reciprocal millimolar DPN concentration. The reaction components, in 1 ml of 0.1 M glycine buffer, pH 9.4, which were not varied, are 100 units of alcohol dehydrogenase activity and 10 μmoles of ethanol. $K_n (DPN) = 1.46 \times 10^{-4}$ M; $K_T$ (free bilirubin) = $3.38 \times 10^{-4}$ M.

**Fig. 4.** Lineweaver-Burk reciprocal plot for the relation between activity and ethanol concentration. The ordinate is reciprocal change in absorbance units per min, and the abscissa is reciprocal millimolar ethanol concentration. The reaction components, in 1 ml of 0.1 M glycine buffer, pH 9.4, which were not varied, are 55 units of alcohol dehydrogenase activity and 0.15 μmole of DPN. $K_n$ (ethanol) = $4.14 \times 10^{-4}$ M.

**Fig. 5.** Reversal of bilirubin inhibition of alcohol dehydrogenase with zinc sulfate. The reaction components, in 0.2 M glycine buffer, pH 9.4, were 0.15 μmole of DPN, 10 μmoles of ethanol, 0.13 μmole of unconjugated bilirubin, and 65 units of crystalline horse liver alcohol dehydrogenase. The ordinate is the ratio of velocity with zinc sulfate and bilirubin to velocity with zinc sulfate but without bilirubin. The abscissa is concentration of zinc sulfate present in the mixture at the start of the reaction.
cholate at a concentration of 20 mg per ml had a negligible effect on the rate of the alcohol dehydrogenase-catalyzed reaction. This amount is approximately 5 times the total bile salt level present in the reaction mixture with crude bile added to the 50% inhibition point.

Alcohol dehydrogenase is a zinc-containing enzyme, and its activity depends on the presence of zinc in the molecule (27). The inhibition of the enzyme by organic agents capable of complexation with zinc, such as o-phenanthroline, has been studied in detail (26, 30). Since it was known that substances related to bilirubin can form complexes with zinc ions (for example, biliverdin, as noted above, and urobilinogen (31)), an analogous mechanism for bilirubin seemed likely. It has been reported that bilirubin itself can form a complex with zinc (32).

The studies of the effect of o-phenanthroline on alcohol dehydrogenase revealed competitive inhibition with respect to the coenzyme in either direction of the reaction (26, 30). These same studies revealed noncompetitive inhibition with aldehyde as the substrate and mixed competitive inhibition with alcohol as the substrate.

Bilirubin may also exert its inhibitory effect by means other than the chelation of zinc. It is known to bind to proteins (21), and bilirubin inhibition of enzyme systems that do not contain zinc has been noted above (2, 3). The observation that high concentrations of zinc are required for reversal of the reaction might support this conjecture.

The bilirubin inhibition of rhodopsin regeneration in frog retina has been mentioned above (18). Alcohol dehydrogenase plays a central role in the regeneration of rhodopsin, and while the amount of bilirubin necessary to inhibit rhodopsin regeneration in vitro is far less than that required to inhibit liver alcohol dehydrogenase in vivo, the two observations may nevertheless be related.

Patients in the advanced stages of Laennec's cirrhosis often have low serum zinc levels and increased urinary excretion of zinc (33, 34). These patients have been reported to have decreased levels of hepatic alcohol dehydrogenase (35, 36). These patients have been reported to have decreased levels of hepatic alcohol dehydrogenase (35, 36). These patients have been reported to have decreased levels of hepatic alcohol dehydrogenase (35, 36). These patients have been reported to have decreased levels of hepatic alcohol dehydrogenase (35, 36). These patients have been reported to have decreased levels of hepatic alcohol dehydrogenase (35, 36).

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