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Biochemistry

Digitalis metabolism and human liver alcohol dehydrogenase

(substrate specificity/cardiac sterols/metalloenzyme inhibition)

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ABSTRACT **Human liver alcohol dehydrogenase (alcohol: NAD^+ oxidoreductase, EC 1.1.1.1) catalyzes the oxidation of the 3β-OH group of digitoxigenin, digoxigenin, and gitoxigenin to their 3-keto derivatives, which have been characterized by high-performance liquid chromatography and mass spectrometry. These studies have identified human liver alcohol dehydrogenase as the unknown NAD(H)-dependent liver enzyme specific for the free hydroxyl group at C3 of the cardiac genins; this hydroxyl is the critical site of the genins' enzymatic oxidation and concomitant pharmacological inactivation in humans. Several kinetic approaches have demonstrated that ethanol and the pharmacologically active components of the digitalis glycosides are oxidized with closely similar k_{cat}/K_m values at the same site on human liver alcohol dehydrogenase, for which they compete. Human liver alcohol dehydrogenase thereby becomes an important biochemical link in the metabolism, pharmacology, and toxicology of ethanol and these glycosides, structurally unrelated agents that are both used widely. Both the competition of ethanol with these cardiac sterols and the narrow margin of safety in the therapeutic use of digitalis derivatives would seem to place at increased risk those individuals who receive digitalis and simultaneously consume large amounts of ethanol or whose alcohol dehydrogenase function is impaired.**

Our earliest kinetic studies of partially purified human liver alcohol dehydrogenase (LADH; alcohol:NAD^+ oxidoreductase, EC 1.1.1.1) showed its broad substrate specificity (1), encompassing, *inter alia*, aldehydes, primary and secondary aliphatic and aromatic alcohols, polyenoates, and sterols.

We have found recently that highly purified human LADH inactivates digoxin, digitoxin, and gitoxin, glycosides employed widely in the treatment of cardiac failure. Specifically, human LADH catalyzes the oxidation of the 3β-OH group of digoxigenin, digitoxigenin, and gitoxigenin, the genins of digoxin, digitoxin, and gitoxin, to their 3-keto derivatives.

The 3 position of these sterols has proven to be an important site of enzymatic transformation (2), long thought to be under control of an unknown enzyme (3). After oxidation of the 3β-OH group of the genins, cardiac activity is decreased by more than 90%. Moreover, LADH is the only cytosolic enzyme in human liver, the known site of genin metabolism, to catalyze this oxidation, which is the first major step in the abolition of the pharmacological action of these cardiac glycosides. Subsequent enzymatic conversion of the 3-keto to the 3α-OH group abolishes activity. The participation of human LADH in the oxidation of ethanol and of the genins, the active principles of digitalis, has important biochemical, therapeutic, and toxicological implications because it establishes a direct link between the metabolisms of these important drugs. The finding is a result of our efforts to delineate the substrate specificities, to identify and characterize the human isoenzymes, and to further explore the biochemical basis for the metabolism and pathology of ethanol.

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METHODS

Human LADH, homogeneous by sodium dodecyl sulfate/polyacrylamide gel electrophoresis and ultracentrifugation but composed of multiple isoenzymes, was purified by double ternary complex affinity chromatography (4). Protein concentration was calculated from absorbance at 280 nm, using a value of $A_{280}^{0.1\%} = 0.58$ (5). Dehydrogenase activity was determined by following the rate of NADH formation at 340 nm with a Gilford 240 spectrophotometer (Gilford Instruments). Assays were performed at 25°C in 0.1 M NaP_i, pH 7.0, containing 2.4 mM NAD^+, 0.78 μM LADH, 0.1–25 mM ethanol, and alternatively 30–230 μM cardiac sterols or their oxidation products. The rate constant, k_{cat}, is expressed as μmol of NADH produced per min/μmol of enzyme. The cardiac sterols, 40 mM, were dissolved in dioxane and added to the assay mixture last. Digoxin, digitoxin, their genins, and 3-keto and 3α-OH derivatives employed were from Sigma and Boehringer Mannhein. All other chemicals were reagent grade. All solid compounds were dissolved in dioxane and lyopholized prior to use to remove any volatile contaminants, including alcohols. Glass-distilled deionized water was used throughout. Starch gel electrophoresis was performed at pH 7.7 (6). Activity stains were developed in the presence of either 140 mM ethanol or 0.27 mM cardiac sterols in 60 mM dioxane. Retention times of cardiac sterols and related derivatives were determined by using a Waters Associates high-performance liquid chromatographic (HPLC) system equipped with a model 440 absorbance detector. Mass spectra were determined by Paul Vorous, Northeastern University, Boston, MA. Spectra were recorded on a Nuclide 12-90-G magnetic mass spectrometer with an ionizing energy of 70 eV and an accelerating voltage of 4.5 kV.

RESULTS

Digitoxigenin, digoxigenin, and gitoxigenin—the pharmacologically active constituents of digitoxin, digoxin, and gitoxin—are oxidized by human LADH to the corresponding keto derivatives, thereby reducing pharmacological activity (2) by more than 90%. Oxidation occurs solely at the 3β-OH group; the tertiary OH group at position 14 remains unchanged.

Comparison of the kinetic constants of human LADH for the parent digitoxigenin, digoxigenin, and gitoxigenin with those for ethanol indicate that the enzyme binds the sterols more tightly than it binds ethanol. In terms of k_{cat}/K_m, the catalytic efficiency of the digitoxigenin oxidation reaction is virtually the same as that of ethanol (Table 1). In contrast, horse LADH (Boehringer) has a k_{cat} for digitoxigenin that is just 1% that of the human enzyme. Yeast alcohol dehydrogenase (Boehringer) is completely inactive.

More detailed studies were confined to those genins that are

Abbreviations: LADH, liver alcohol dehydrogenase; HPLC, high-performance liquid chromatography.

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Table 1. Kinetic parameters for cardiac genins, their oxidation products, and ethanol*

Substrate	k_{cat}, min^{-1}	K_m, mM	k_{cat}/K_m
Digitoxigenin	2.1	0.07	30
Digoxigenin	1.2	0.21	5.7
Gitoxigenin	0.6	0.07	8.6
3-Ketodigitoxigenin	0.39	0.12	3.3
3-Ketodigoxigenin	0.41	0.30	1.4
Ethanol	29	0.86	34

* Digitoxin, digoxin, gitoxin, 3-epidigitoxigenin, and 3-epidigoxigenin are not oxidized.

employed therapeutically. The products of the human LADH-catalyzed oxidation of digoxigenin and digitoxigenin were identified as the corresponding 3-keto derivatives by both HPLC and mass spectrometry. The HPLC retention times of the 3β-OH, 3α-OH, and 3-keto derivatives of digoxigenin and digitoxigenin were 2.7, 3.2, 3.7 min and 6.7, 7.2, and 8.7 min, respectively. The values for the oxidation products were identical with those of the authentic 3-keto derivatives (7). The mass spectra of the oxidation products and those of the authentic samples were identical and verified the structure as that of the 3-keto sterols (Fig. 1).

A number of approaches have confirmed that both ethanol and these cardiac sterols interact at the same site on human LADH. First, the chelating agents 1,10-phenanthroline, α,α′-bipyridine, and EDTA inhibited the oxidation of ethanol and of these sterols in exactly the same fashion (Table 2). Thus, 1,10-phenanthroline inhibited the oxidation of both ethanol and digitoxigenin instantaneously and reversibly, indicating formation of a ternary enzyme·metal·chelator complex (8). The $pK_{i_{app}}$ values obtained were 4.8 and 4.4, respectively. Dilution with buffer or addition of Zn^{2+} to the assay mixture restored activity to the values expected (Fig. 2). The number of moles of inhibitor bound per active site, $\bar{n}$, is consistent with this mode of inhibition (9). On the other hand, α,α′-bipyridine and

Table 2. Inhibition of digitoxigenin oxidation by metal-chelating agents

Inhibitor	Inhibitor concentration, mM	Inhibition, %
1,10-Phenanthroline	0.1	52
α,α′-Bipyridine	30	42
EDTA	30	38

EDTA, which also inhibited the oxidation of both substrates by human LADH, did so in a time-dependent irreversible manner, in line with past observations (5, 8). This mode of inhibition is thought to be due to metal removal by the chelating agent (10).

Second, studies with nonchelating inhibitors that compete with ethanol showed that they also compete with these genins. Thus, 4-methylpyrazole is specific and selective for this and other alcohol dehydrogenases (4); it competitively inhibited the oxidation of digitoxigenin with a K_i of ≈0.9 μM (Fig. 3), which can be compared with the K_i of ≈1 μM for ethanol oxidation (5). Again, this indicates that ethanol and these genins bind to the same active site.

Third, these substrates can be shown to compete directly with each other by comparison of their rates of oxidation when either is present alone or when they are present together. A mixture of equal concentrations of digitoxigenin and ethanol suitable for studies of competition (50 μM) results in an activity of 2.7×10^{-2} μmol/min per ml, intermediate between the rates observed for either substrate alone—i.e., 1.2×10^{-2} and 4.7×10^{-2} μmol/min per ml, respectively (7).

Finally, when the purified enzyme is subjected to starch gel electrophoresis and then visualized by an activity stain sensitive to the production of NADH, the isoenzyme patterns are identical with either ethanol or digitoxigenin as the substrate (7). Importantly, both substrates give identical staining patterns not only with the purified enzyme but also with the crude liver homogenate. Jointly, such data indicate that human LADH is

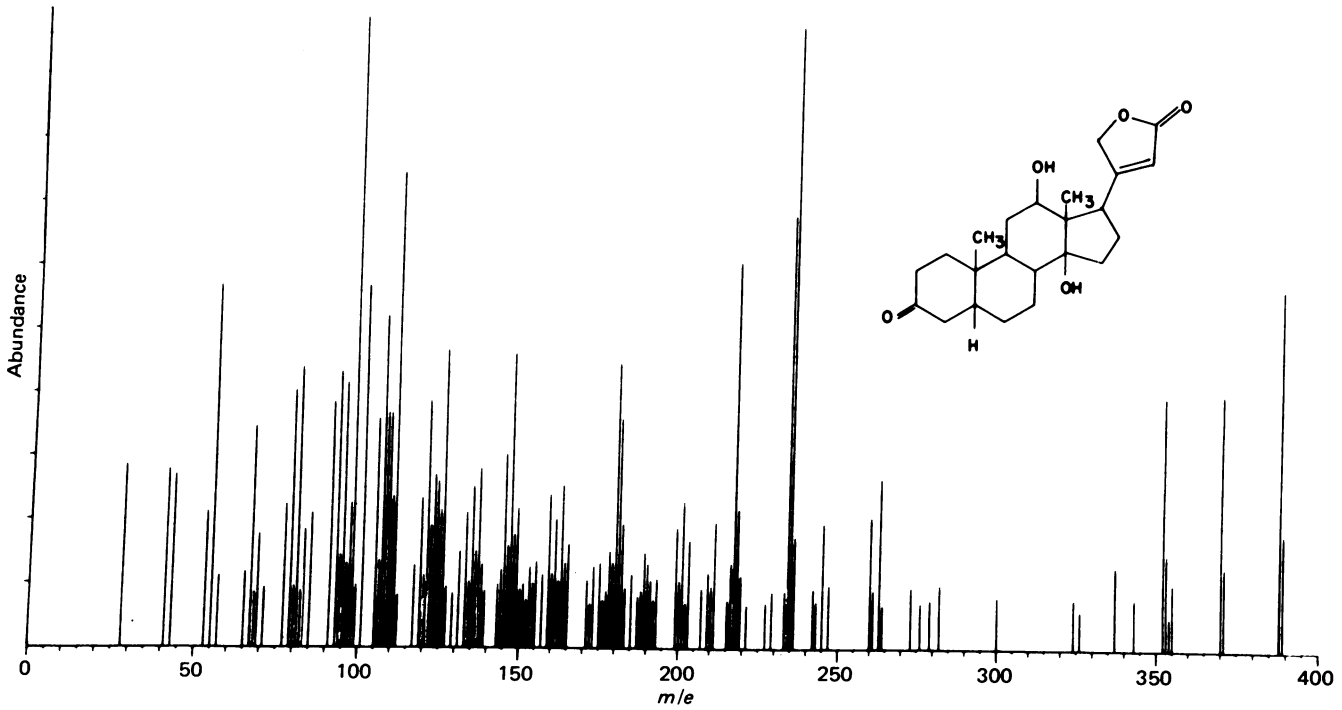

FIG. 1. Mass spectrum of digoxigenin oxidized by human LADH. This spectrum is identical to that obtained for the authentic 3-keto-digoxigenin (Boehringer).

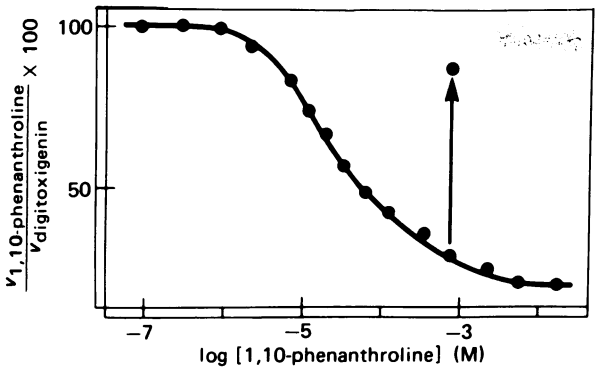

FIG. 2. Instantaneous inhibition of human LADH by 1,10-phenanthroline added to the assay mixture containing 0.1 mM digitoxigenin and 2.4 mM NAD^+ in 0.1 M NaP_i, pH 7.0, at 25°C. Addition of 70 mM zinc chloride (arrow) reversed the inhibition.

the primary cytosolic enzyme in liver responsible for the oxidation of cardiac sterols and further that all the isoenzymes detected by those starch gels are active towards both substrates.

DISCUSSION

It has become apparent that pure human LADH, available since 1976 (4), has broader substrate specificity and greater polymorphism than found for the corresponding enzyme from other species, including that of horse liver. The substrate specificity of the three major horse isoenzymes has been studied extensively and has led to their classification by Theorell as being ethanol-active (EE), steroid-active (SS), or active towards both (ES) (11). In particular, the activity of the horse SS isoenzyme is apparently directed towards sterols. All molecular forms of the human enzyme inspected thus far seemingly share this property (12).

We have recently examined additional sterol substrates to further define possible specificity requirements of the human enzyme. This systematic survey of biologically active sterols focused on those that are characterized by *cis* A/B ring fusion and have a 3β-OH group. This, in turn, directed attention to the 3β-OH sterol constituents of the cardiac glycosides. A closer and more detailed examination of these substrates seemed important, in terms of both the mechanism of action of human LADH and their use in cardiac therapeutics. Indeed, these particular 3β-OH sterols have proven to be excellent substrates of human LADH (Table 1).

Numerous reports have described the tissue distribution, sites

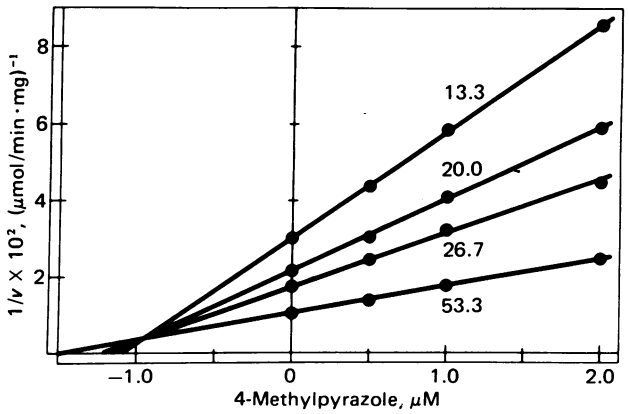

FIG. 3. Inhibition of human LADH digitoxigenin oxidation by 4-methylpyrazole. The inhibitor concentration was varied in assay mixtures containing 2.4 mM NAD^+ and 13.3–53.3 μM digitoxigenin (as indicated on the lines) in 0.1 M NaP_i, pH 7.0, at 25°C.

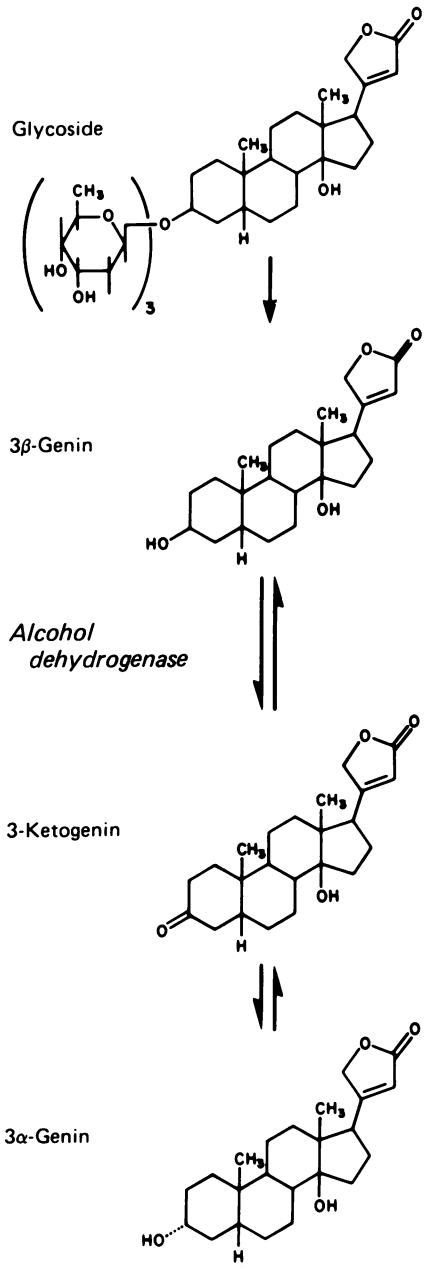

FIG. 4. Catabolism of cardiac glycosides.

of degradation, products, and excretory pathways of various digitalis preparations and, importantly, these reports have shown that the liver is the primary site of their catabolism (2). However, the specific enzymes involved in the stepwise degradation of cardiac glycosides and in digitalis pharmacokinetics have not been identified. Cardiac glycosides generally include a cardiac steroid—i.e., a genin linked through the 3β-OH group to a trisaccharide. The pharmacologic activity and, hence, both therapeutic and toxic effects reside exclusively in the genin but are prolonged by glycosylation, which also affects water solubility, membrane permeability, and potency (13). As would be expected on the basis of their chemistry, we have found that digoxin and digitoxin do *not* serve as LADH substrates (Table 1), because the 3β-OH group is blocked by a trisaccharide composed of three molecules of digitoxose (Fig. 4). The major metabolic pathway for the cardiac glycosides consists of stepwise cleavage of the sugar moieties, oxidation of the 3β-OH group, reduction of the resultant 3-keto function to a 3α-OH group and, finally, conjugation to sulfates or glucuronates and excretion. The pharmacologic activity is nearly identical for

the tris-, bis-, and monoglycosides as well as the 3β-genins, but the 3-keto derivatives are less than 10% as active and the 3α-OH products are totally inactive (2). Thus, human LADH is the hitherto unknown enzyme (3) that performs the important first and major step abolishing the activity of digitalis—i.e., the oxidation of digoxigenin and digitoxigenin to the corresponding 3-keto derivatives (Fig. 4). As a consequence and apart from any of its other functions, this enzyme has a vital role in the metabolism of digitalis.

The k_{cat}/K_m values for the oxidation of digitoxigenin and ethanol are nearly the same in spite of their considerable structural dissimilarity (Table 1). Indeed, the K_m values of all three genins indicate that the enzyme binds them in preference to ethanol (Table 1). The fact that these interactions are also competitive is potentially of major physiological and pharmacological significance. Importantly, the rate of oxidation of digitoxigenin and digoxigenin by human LADH *in vitro* is consistent with their rates of inactivation as observed *in vivo*, supporting the conclusion that the enzyme activity of human liver is adequate to perform this critical step in their catabolism.

The beneficial and virtually unique pharmacological properties of cardiac glycosides are mitigated by their characteristically narrow toxicological margins.† While the ratio of a therapeutic to a toxic dose is identical for all cardiac glycosides, the lethal dose is thought to be only twice as high as that which causes minimal toxicity. Thus, toxicity, manifesting variously as metabolic, gastrointestinal, neurological, and cardiac abnormalities, is at least as disconcerting and often becomes a truly lethal threat. The recognition of variables that would significantly shift the balance between the pharmacological and toxicological effectiveness of cardiac glycosides and ethanol could have an important impact on the management of patients undergoing digitalis treatment, particularly if such patients were also to be consumers of ethanol. The present data indicate competition of the two substrates for the enzyme. The extent to which this pertains *in vivo* will ultimately be determined by their K_m values, relative concentrations, distribution, equilibria, and other physiological parameters. This dual specificity of human LADH towards ethanol and the genins of digitalis and the competition of these substances could account for some of the frequently observed and often unexplained individual variations in response to digitalis and its derivatives. Ignoring any other variables, competition by ethanol for LADH could significantly retard the oxidation of the cardiac glycosides. These could then reach concentrations sufficient to cause toxicity. Considering the narrow margin of safety in digitalis therapy, this biochemical relationship suggests clinical investigations regarding the induction of digitalis toxicity by ethanol consumption.‡ Moreover, the competition between ethanol and genins here uncovered would seem to place at increased risk those individuals on digitalis therapy whose alcohol intake is high or whose alcohol dehydrogenase activity in particular and liver function in general are impaired. Such predictions clearly require experimental examination.

In this regard, previous studies have revealed marked abnormalities of zinc metabolism in individuals afflicted with postalcoholic cirrhosis and have suggested subsequent adverse effects on the zinc-dependent human LADH (14). An impairment of this enzyme in cirrhosis could also bear importantly on the degradative pathway of digitalis in that disease.

An assessment of the potential implications of these findings for problems arising from the pharmacologic and toxicologic action of digitalis derivatives must consider the isoenzymes of human LADH. The distribution of such molecular forms in different racial groups, individuals, and organs, and their characteristic and markedly different substrate specificities and kinetic and inhibition constants (15) may be important to the oxidation of the genins. Thus, it can be appreciated that the response to cardiac glycosides is highly individualized. Rather than being given standard amounts of these drugs, patients are "digitalized," i.e., titrated to their own optimal dose response (16). Isoenzyme patterns might provide an at least partial biochemical basis for this observed variability. Whatever the origin of these isoenzymes, inherited or induced, their multiplicity and functional variety are likely to have a distinctive role in the normal metabolism and pathological consequences of human LADH substrates.

It is already apparent that many human isoenzymes differ markedly in their kinetic properties (17), and this difference might bear importantly on the pharmacology and toxicology of the cardiac glycosides. The present investigation requires extension to include human LADH isoenzymes so that a firm biochemical basis for the pharmacology and toxicology of digitalis action can be secured. Such studies might then lead to the recognition or prediction of deleterious interactions of the drug and perhaps even better diagnostic and preventive measures.

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† In the United States, some five million individuals are thought to be receiving digitalis therapy at any given time. For hospitalized patients receiving digitalis, the incidence of toxicity has been reported to vary from 10% to 20%, with a concomitant mortality of two-thirds among those with digitalis-induced cardiac arrhythmias.

‡ Adverse effects of "liver disease" on digitalis therapy, tolerance, and toxicity have been commented upon in very general terms; definitive clinical investigation is needed, and its results would fill an important void.