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Some Catalytic Properties of Human Liver Alcohol Dehydrogenase*

A. Huntley Blair† and Bert L. Vallee

ABSTRACT: Human liver alcohol dehydrogenase purified by means of DEAE- and CM-cellulose chromatography can be resolved into three chromatographically distinct, enzymatically active forms. One of these was studied in detail and found to exhibit broad substrate specificity characteristic of the unresolved material. In addition to ethanol, methanol, 1,2-ethanediol, 1,2-propanediol, 1,3-propanediol, 2-methoxyethanol, and 2-ethoxyethanol are oxidized. The oxidation of 1,2-propanediol is competitive with that of ethanol. The enzyme also oxidizes monohalo, but not di- or trihalo derivatives of ethanol, and reduces monochloro, dichloro, and trihalo derivatives of acetaldehyde. Both the polyhalo derivatives of ethanol and of acetaldehyde inhibit ethanol oxidation. These enzymatic findings are pertinent to the metabolism of these alcohols and aldehydes in man.

Alcohol dehydrogenase from human liver has been purified substantially (von Wartburg *et al.*, 1964). Zinc is a functional constituent of the molecule, and like other zinc metalloenzymes, the activity of alcohol dehydrogenase is inhibited in a characteristic manner by chelating agents such as 1,10-phenanthroline. Many of the catalytic properties of the human protein are similar to those of the enzyme from horse liver, but they differ in regard to the magnitudes of the Michaelis constants for substrates and coenzymes and in substrate specificity (von Wartburg *et al.*, 1964). In particular, human liver alcohol dehydrogenase oxidizes ethylene glycol and methanol at significant rates whereas the horse enzyme exhibits little or no activity toward these alcohols (Sund and Theorell, 1963; Winer, 1958; Theorell and Bonnichsen, 1951). These observations are of importance since the metabolic products of ethylene glycol and methanol are toxic to man and the enzyme may play a role in adverse reactions to these and other compounds (Wacker *et al.*, 1965). This paper reports further studies on the purification of human liver alcohol dehydrogenase and on its behavior toward other aliphatic diols and a number of halogenated derivatives of ethanol and acetaldehyde.

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Materials and Methods

Reagents. All chemicals used for isolation were reagent grade and employed without further purification. DEAE- and CM-cellulose were products of the Brown Co. and were prepared for use according to the method of Peterson and Sober (1962). NAD[1] and NADH were obtained from the Sigma Chemical Co. Reagent grade 95% ethanol was used without further purification while acetaldehyde and bromoethanol (Eastman Organic Chemicals) were freshly distilled before use. Ethylene glycol was obtained from the J. T. Baker Chemical Co. while the monomethyl and monoethyl ethers of ethylene glycol were products of the Fisher Scientific Co. 1,2-Propanediol and 1,3-propanediol were products of Eastman Organic Chemicals. Dichloro-, trifluoro-, and tribromoethanol were obtained from the Aldrich Chemical Co. Chloral hydrate and chloroethanol were products of Eastman Organic Chemicals and their purity was verified by gas chromatographic analysis. Fluoroethanol (K and K Laboratories) and trichloroethanol (Aldrich Chemical Co.) were distilled before use under nitrogen and under vacuum, respectively, and the purity of the products was also verified by gas chromatographic analysis. Mono- and dichloroacetaldehyde were prepared from the corresponding acetal derivatives by the methods of Natterer (1882) and Wohl and Roth (1907), respectively.

Protein concentration was determined by the method of Lowry *et al.* (1951), using bovine albumin, fraction V (Armour Laboratories), as a standard.

Conductivity was measured with a Radiometer conductivity meter, type CDM 2d, using a type CDC 114 electrode at 4°.

Assay of Alcohol Dehydrogenase Activity. Activity was measured spectrophotometrically at 20° using a Beckman DU spectrophotometer equipped with a Gilford Model 200 optical density converter, and a Brown 50-mv recorder. For ethanol oxidation, the reaction was started by addition of 0.1 ml of enzyme solution to 2.9 ml of the reaction mixture consisting of 1.7×10^{-2} M sodium pyrophosphate, pH 9.3, 1.7×10^{-3} M NAD, and 1.7×10^{-2} M ethanol. The rate of formation of NADH was constant for the 2 min of measurement at 340 mμ. For all steps in the enzyme preparation prior to chromatography on CM-cellulose, a blank without ethanol was run as a control. Enzymatic activity was calculated as the difference in the A_{340}/min of the two reactions, with and without ethanol.

The reaction mixture (2.9 ml) for measurement of acetaldehyde reduction consisted of 3.3×10^{-2} M sodium phosphate, pH 7.15, 1.6×10^{-4} M NADH, and 1.25×10^{-2} M acetaldehyde. The reaction was initiated by addition of 0.1 ml of enzyme and the constant rate of NADH disappearance was measured for 1 min at 340 mμ. Conditions deviating from those cited above are specified in the text.

[1] Abbreviations used: NADH and NAD, reduced and nicotinamide-adenine dinucleotide; TCE, trichloroethanol.

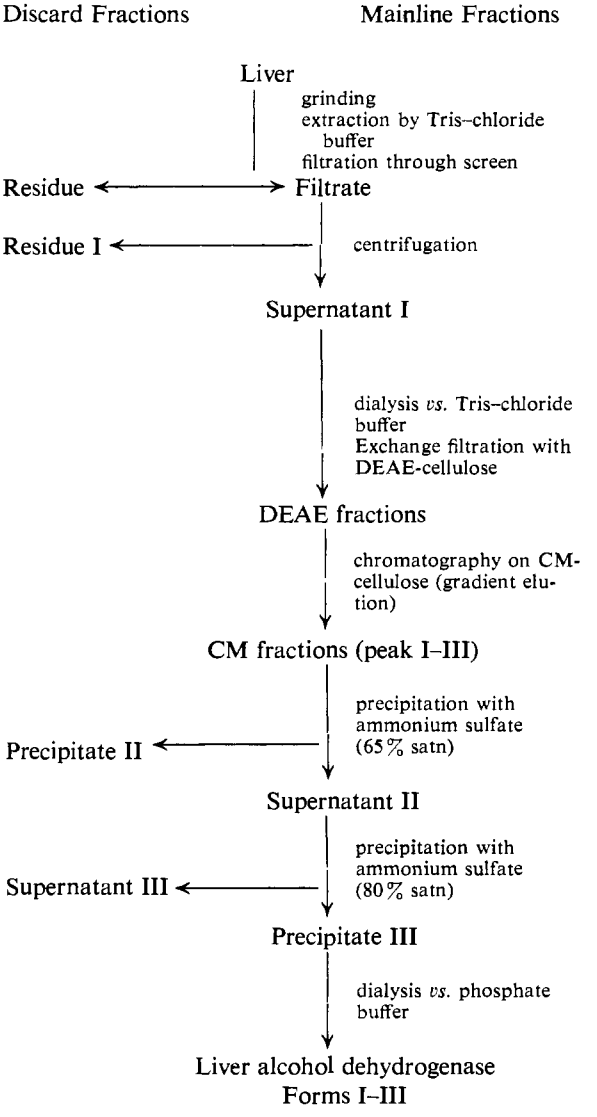

FIGURE 1: Purification of human liver alcohol dehydrogenase.

One unit of human alcohol dehydrogenase activity is defined as that amount of enzyme which results in an increase in A_{340} of 1.0/min under the conditions stated with ethanol as the substrate. Specific activity is expressed in units per milligram of protein.

Purification of Alcohol Dehydrogenase Activity. The procedure reported previously (von Wartburg *et al.*, 1964) was developed for small scale preparations from single livers. Using this method, attempts to process large amounts of material to obtain greater quantities of enzyme were not encouraging due to variable and low yields of activity. Modification of the scheme by employing DEAE-cellulose early in the procedure allowed processing of 10–20 kg of liver at one time.

A schematic diagram of the fractionation procedure

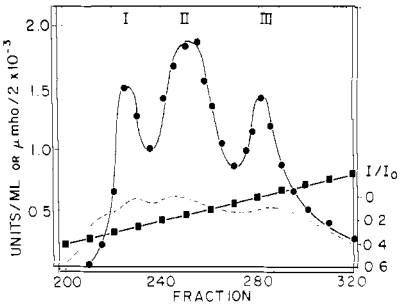

FIGURE 2: CM-cellulose column chromatography of human liver alcohol dehydrogenase. The detailed experimental procedure and assay method are described in the text. DEAE-cellulose-treated material (36 g) is applied to a column, 8 × 80 cm, and eluted with a linear gradient of 0.01–0.1 M Tris–chloride, pH 8, 4°. The fraction volume is 80 ml. Conductivity (■), enzymatic activity (●), and transmittance (I/I_0) at 280 mμ (- - - -) are indicated as is the resolution of the enzyme into peaks I–III.

is shown in Figure 1.[2] Livers were obtained at autopsy and stored frozen until used. In all instances, the case history, gross and histological criteria indicated the livers to be free of disease. Prior to processing, livers were screened for "normal" and "atypical" alcohol dehydrogenase pH rate profiles and those exhibiting an atypical pH optimum at 8.5 (von Wartburg *et al.*, 1965) were not utilized in this study.

After removal from storage, 12 kg of livers is thawed overnight at 4°, rinsed to remove blood, and passed through a meat grinder. All subsequent steps are carried out at 0–4°. The ground liver tissue is extracted by stirring for 1 hr with a volume of 0.004 M Tris–chloride buffer, pH 8, equal to twice the weight of the liver, followed by filtration through a plastic screen to remove coarse debris. The screened suspension (31 l.) is centrifuged for 20 min at 10,000*g* using International HR1 centrifuges. The supernatant solution (27 l.) is adjusted to pH 8.5 with Tris base and dialyzed *vs.* 125 l. of 0.002 M Tris–chloride buffer, pH 8.5, which is changed every 6–8 hr. When the conductivity of the turbid, red enzyme solution is <200 μmho, the dialyzed extract (pH 8.2–8.5) is divided into two equal portions and passed through DEAE-cellulose columns with a total bed volume of 20 l. each preequilibrated with the eluting buffer, 0.004 M Tris–chloride, pH 8.2. Effluent fractions (2 l.) are collected and those containing 5% or more of the activity are combined.

CM-Cellulose Column Chromatography of Human Liver Alcohol Dehydrogenase. The clear, colorless, DEAE-cellulose-treated extract (41 l.) is brought to pH 7.6–7.8 with dilute HCl and the conductivity is adjusted to *ca.* 200 μmho by addition of distilled water. The extract is then adsorbed on a CM-cellulose column (8 × 80 cm; 4 l. of bed volume) equilibrated with 0.004 M Tris–chloride buffer, pH 8. The column is washed with 10 l. of 0.01 M Tris–chloride buffer, pH 8, and the material containing the activity eluted by a linear salt gradient obtained with 10 l. of 0.1 M Tris–chloride buffer, pH 8, in the reservoir and 10 l. of 0.01 M Tris–chloride buffer, pH 8, in the mixing chamber. Activity may be concentrated at this stage by elution from the CM-cellulose column by 0.1 M Tris–chloride buffer, pH 8. Activity transmittance at 280 mμ and conductivity are measured on 80-ml fractions. Fractions (i) 220–230, (ii) 242–268, and (iii) 280–320 (Figure 2) are combined and the enzyme in each pooled extract is concentrated by precipitation with ammonium sulfate. The fraction precipitating between 65 and 80% of saturation with ammonium sulfate is dissolved in a minimum amount of 0.03 M sodium phosphate buffer, pH 7, and the resulting enzyme solution dialyzed against the same buffer to remove ammonium sulfate. The dialyzed enzyme stock solutions are kept at 0°. Variable losses in activity are observed upon thawing if the enzyme solution is frozen.

Results

Fractionation of Human Liver Alcohol Dehydrogenase. Human liver alcohol dehydrogenase has been purified by a procedure designed to process 10–20-kg quantities of liver. The results are summarized in Table I. After

TABLE I: Large-Scale Purification of Human Liver Alcohol Dehydrogenase.[a]

Fraction	Vol (l.)	Act. (ΔA_{340}/min)	Protein (g)	Sp Act. (ΔA_{340}/min/mg)
Crude extract	27	24,000	760	0.03
Dialyzed extract	32	23,300	770	0.03
DEAE-cellulose-treated extract	41	19,200	36	0.53
Concentrated CM-cellulose eluates				
Peak I	0.16	1,000	0.78	1.1
Peak II	0.19	2,700	2.6	1.0
Peak III	0.21	1,920	2.4	0.8

[a] The total activity recovered from CM-cellulose column chromatography, which represents 60–70% of that present in the DEAE-cellulose-treated extract, exceeds, of course, that exhibited by the selected fractions from peaks I–III which were combined and concentrated.

[2] We are indebted to the New England Enzyme Center, Tufts Medical School, Boston, Mass., where this procedure was carried out. The Center's large-scale equipment and multiple high-speed centrifuges are capable of processing rapidly the large volumes encountered in this procedure prior to the stage of elution from CM-cellulose.

exchange filtration with DEAE-cellulose the product, obtained in 80% yield, had a specific activity of 0.53 unit/mg. The DEAE-cellulose-treated enzyme was fractionated further by linear gradient elution chromatography on CM-cellulose. Activity was resolved into three distinct peaks having similar specific activity toward ethanol (Table I and Figure 2). After concentration with ammonium sulfate, peaks I–III had specific activities of 1.1, 1.0, and 0.8 units/mg, respectively, comparable to those previously achieved in small scale preparations (von Wartburg *et al.*, 1964). Further purification and physical–chemical characterization of each of these species is in progress (D. E. Drum and B. L. Vallee, in preparation). It is of interest in this regard that preliminary measurements on such more highly purified material yield a zinc content in excess of 2.5 g-atoms/provisional 87,000 mol wt, the highest concentration seen previously (von Wartburg *et al.*, 1964), suggesting that the ultimate zinc content will be higher. This might be expected if the impurities which were present did not contain zinc (D. E. Drum and B. L. Vallee, in preparation).

When single livers were processed, the number of peaks obtained on CM-cellulose has varied from one to three. When the enzyme is prepared on a large scale from a mixed batch of livers, three major peaks have always been observed. However, the areas under the three peaks vary somewhat from batch to batch, as might be expected on the basis of the chromatography performed on single livers. Similarly, the total, unresolved activity per gram of tissue extracted from individual livers has varied substantially in different preparations. In a sequential series of 117 liver specimens, all normal by histological criteria, the activity ranged from 0.2 to 34 units/g; 113 of these had activities ranging from 0.2 to 10 units/g with a mean of 2.5 units/g. Four livers had unusually high activities of 18, 20, 21, and 34 units/g. While artifacts due to storage, activation, or inhibition cannot be excluded, these data suggest the existence of an exceptionally active enzyme in the livers of some individuals. Similarly high activities in human liver extracts have been reported (von Wartburg *et al.*, 1965).

The studies of substrate specificity of alcohol dehydrogenase reported here were carried out with material from peak II (Figure 2), the predominant chromatographic form. This material, when rechromatographed under the same conditions on CM-cellulose, was eluted as a single peak at the same gradient position, as determined by conductivity, as was peak II in the original chromatogram. Enzymatic activity peaks, measured with chloroethanol, acetaldehyde, and chloral hydrate as substrates, corresponded to the peak measured with ethanol.

Each of the three forms of human liver alcohol dehydrogenase separated by chromatography exhibit both the broad substrate specificity characteristic of unresolved fractions and similar substrate kinetics. Michaelis constants for ethanol and chloral hydrate determined for peaks I and III (Figure 2) were closely similar to the corresponding values obtained for peak II (Tables II and III). Inhibition by 1,10-phenanthroline was also similar for all three peaks. Inhibition (50%) of activity with ethanol at pH 9.3 was obtained at inhibitor concentrations of 1.4×10^{-4}, 1.4×10^{-4}, and 1.0×10^{-4} M, for peaks I–III, respectively. In another series of experiments, *two* forms isolated from a *single* liver had k_m values of 8×10^{-4} and 9×10^{-4} M for ethanol, 6×10^{-4} and 7×10^{-4} M for acetaldehyde, and 2×10^{-5} and 1×10^{-5} M for NADH.

TABLE II: Alcohols as Substrates for Human Liver Alcohol Dehydrogenase.[a]

	k_m (M)	V_{max} (ΔA_{340}/min/mg)
Ethanol	1×10^{-3}	1.0
Methanol	3×10^{-2}	0.11
1,2-Ethanediol	3×10^{-2}	0.40
1,2-Propanediol	1×10^{-2}	0.55
1,3-Propanediol	1×10^{-2}	0.71
2-Methoxyethanol	1×10^{-3}	0.40
2-Ethoxyethanol	6×10^{-4}	0.44

[a] Activities were determined at pH 9.3 as described in the text for ethanol. For all other alcohols shown the concentration of substrate in the reaction mixture was varied over a 10-fold range to permit determination of the Michaelis constant (k_m) and the maximal reaction velocity (V_{max}) from Lineweaver–Burk plots.

TABLE III: α-Chloroacetaldehydes as Substrates for Human Liver Alcohol Dehydrogenase.[a]

	k_m (M)	V_{max} (ΔA_{340}/min/mg)
Acetaldehyde	6×10^{-4}	24
Monochloroacetaldehyde	2×10^{-4}	20
Dichloroacetaldehyde	9×10^{-4}	8.4
Trichloroacetaldehyde	6×10^{-3}	6.5

[a] Activities were determined at pH 7.15 as described in the text for acetaldehyde. For all other aldehydes shown the concentration of substrate in the reaction mixture was varied over a 10-fold range to permit determination of the Michaelis constant (k_m) and the maximal reaction velocity (V_{max}) from Lineweaver–Burk plots.

Catalytic Functions. Human liver alcohol dehydrogenase oxidizes methanol as well as ethanol, and also readily oxidizes ethylene glycol and its analogs. Michaelis constants and maximal velocities for a series of related alcohols are shown in Table II. Maximal reaction velocities for the alcohols studied range from 11 to 71%

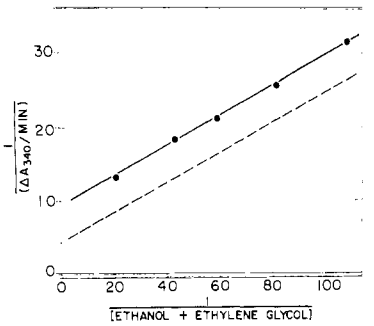

FIGURE 3: Competition of ethanol with ethylene glycol for human liver alcohol dehydrogenase. Reaction mixtures contained ethylene glycol plus ethanol at the indicated total concentration. The molar ratio of ethylene glycol/ethanol was 37. Reciprocal velocity, $1/V_t$ (ΔA_{340}^{-1} min) *vs.* reciprocal total substrate concentrations, $1/S_t$ (M^{-1}). The solid line is calculated for competition for a single site, the dashed line for two separate, independent sites, according to Foster and Niemann (1951). The individual points, (●), identify experimentally determined values.

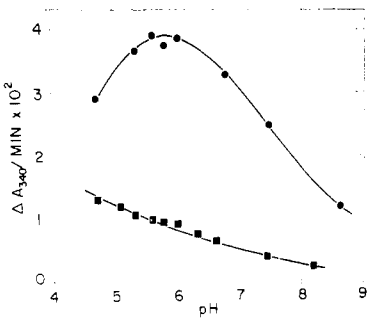

FIGURE 4: pH dependence of acetaldehyde and chloral hydrate reduction by human liver alcohol dehydrogenase. Activity (ΔA_{340}/min), measured instantaneously, *vs.* pH of the reaction mixture. Reaction mixture contained sodium phosphate, 3.3×10^{-2} M; acetaldehyde, 1.25×10^{-2} M (●), or chloral hydrate, 5×10^{-2} M (■); protein, 7.0 μg.

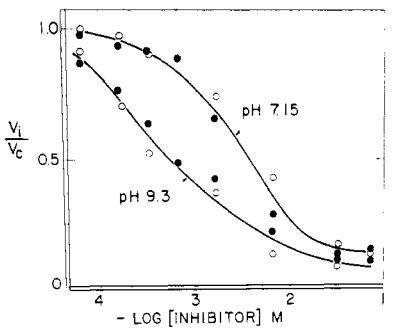

FIGURE 5: Instantaneous inhibition of human liver alcohol dehydrogenase by chloral hydrate (●) and trichloroethanol (○). Fractional residual activity, (V_i/V_c), of the enzyme *vs.* the negative logarithm of the inhibitor concentration, where V_i is the inhibited activity and V_c is the uninhibited control activity. Reaction mixtures contained: sodium pyrophosphate, 1.7×10^{-2} M, pH 9.3, or sodium phosphate, 3.3×10^{-2} M, pH 7.15; protein, 110 μg (pH 9.3) or 210 μg (pH 7.15).

of that observed with ethanol, 1,3-propanediol being oxidized most readily and methanol least readily among the compounds studied. Michaelis constants for the diols are 10–30 times greater than that of ethanol. Ethylene glycol monomethyl or monoethyl ethers have maximal rates close to that of ethylene glycol itself but k_m values are close to that of ethanol.

It has been reported that ethanol inhibits oxidation of ethylene glycol by human liver alcohol dehydrogenase (von Wartburg and Vallee, 1963). Detailed evidence on the competitive nature of this inhibition has now been obtained. Study of competition between ethylene glycol and ethanol for the substrate site of the enzyme can be shown only indirectly by means of the present assay, however, because activity is monitored by measuring the increase in absorbance at 340 mμ. Since both alcohols are substrates, only the *total activity*, due to their concurrent oxidation, can be determined. Nevertheless, competition can be demonstrated by the procedure of Foster and Niemann (1951), assuming (a) competitive behavior for a single enzyme and (b) noncompetitive behavior for two separate, independent sites either on the same or different enzymes. The results of this calculation, using the k_m and V_{max} values obtained with each substrate alone, are consistent with competitive behavior of both substrates for a single site (Figure 3).

Human liver alcohol dehydrogenase catalyzes the oxidoreduction of a number of α-substituted halogen derivatives of acetaldehyde and ethanol. The reaction rates obey Michaelis–Menton kinetics as indicated by linear reciprocal plots. Maximal velocities and k_m values for reduction of the α-chloro derivatives of acetaldehyde at pH 7.15, in 3.3×10^{-2} M phosphate buffer, are given in Table III. Tribromoacetaldehyde is also reduced, although at a lower rate than the corresponding trichloro compound. Successive substitution of chlorine atoms in the α position of acetaldehyde decreases the maximal reaction velocity. Like acetaldehyde itself, the chloro derivatives cause substrate inhibition at very high concentrations. The Michaelis constant for monochloroacetaldehyde is lower than that for acetaldehyde whereas the k_m values of di- and trichloroacetaldehyde are higher, that for trichloroacetaldehyde (chloral hydrate) being 10-fold greater than the k_m for acetaldehyde.

The pH–rate profile with chloral hydrate as a substrate differs from that for acetaldehyde (Figure 4). Since zinc is likely to dissociate from alcohol dehydrogenase on prolonged exposure to pH values <6, thereby

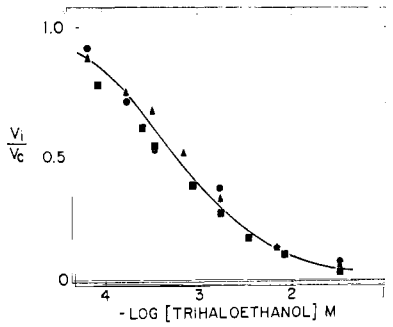

FIGURE 6: Instantaneous inhibition of human liver alcohol dehydrogenase by trihaloethanols at pH 9.3. Fractional residual activity, (V_i/V_c), of the enzyme *vs.* the negative logarithm of the inhibitor concentration. Reaction mixtures contained: protein 110 μg; trifluoro- (■), trichloro- (■), or tribromoethanol (▲) at the concentrations indicated.

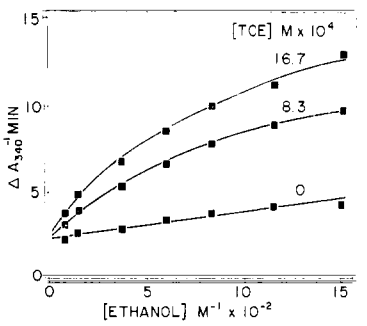

FIGURE 7: Kinetics of inhibition of human liver alcohol dehydrogenase by trichloroethanol when the ethanol concentration is varied in the reaction mixture. Reciprocal velocity (ΔA_{340}^{-1} min) *vs.* reciprocal ethanol concentration ($M^{-1} \times 10^{-2}$). Reaction mixtures contained: sodium phosphate, pH 7.15, 4×10^{-2} M; NAD, 3.3×10^{-3} M; ethanol and trichloroethanol (TCE) as indicated; protein, 1.4 mg, in a total volume of 15 ml; optical path length 5 cm.

nactivating the system, instantaneous[3] measurements were made to obviate this effect on activity. In phosphate buffer, the rate of chloral hydrate reduction continues to increase with decreasing pH at least to pH 4.7. Under more acid conditions, the enzyme and NADH become so unstable that reliable measurements could no longer be carried out. No maximum in the rate of chloral hydrate reduction was observed either at a substrate concentration of 5×10^{-2} M (Figure 4) or when the substrate concentration was reduced to 1.25×10^{-2} M. Thus, these data differ somewhat from those for acetaldehyde reduction which shows a pH maximum at pH 5.8.[4]

As would be expected, chloral hydrate also effectively inhibits ethanol oxidation. The degree of instantaneous inhibition is plotted as a function of the logarithm of the chloral hydrate concentration at pH 7.15 and at 9.3 in Figure 5.

In contrast to the results obtained with the polyhaloaldehydes, human liver alcohol dehydrogenase oxidizes only monohaloethanols; it exhibits little or no activity toward polyhalo alcohols.[5] Maximal velocities for monohaloethanols, however, are lower than that of ethanol. In pyrophosphate buffer, pH 9.3, maximal rates, relative to ethanol, for fluoro-, chloro-, and bromoethanol were found to be 0.10, 0.20, and 0.15, respectively. Under the same condition, horse liver alcohol dehydrogenase did not oxidize fluoroethanol and was only slightly active toward chloroethanol.

Although the polyhalo alcohols are not effective substrates, they are potent inhibitors of ethanol oxidation. This is exemplified by di- and trichloroethanol, both of which inhibit human liver alcohol dehydrogenase. Like chloral hydrate, trichloroethanol is a better inhibitor at pH 9.3 than at 7.15 (Figure 5).

The effects of increasing concentrations of trifluoro-, trichloro-, and tribromoethanol at pH 9.3 are compared in Figure 6. All of these compounds are similarly effective inhibitors, 50% inhibition being attained at about 5×10^{-4} M.

Trichloroethanol inhibition at pH 7.15 was also studied by varying the substrate concentration at fixed inhibitor concentrations. Reciprocal reaction velocity as a function of reciprocal ethanol concentration for different constant concentrations of trichloroethanol yielded nonlinear plots (Figure 7).

Inhibition by chloral hydrate and by trichloroethanol, the pharmacologically active reduction product presumed to be responsible for its sedative effects (Butler, 1948; Marshall and Owens, 1954), may provide the biochemical basis for the observation that chloral hydrate potentiates the toxic effects of ethanol in man (Sharpless, 1965).

Discussion

The preparative procedure for isolating larger quantities of enzyme reported here takes advantage of the observation that the isoelectric point of human liver alcohol dehydrogenase, in Tris or phosphate buffer, is above pH 9; the value is substantially higher than that re-

[3] "Instantaneous" is defined as the shortest time in which the reaction rate could be measured after all the components of the reaction, including inhibitors when used, have been brought into contact, commencing within *ca.* 5 sec. This is to be differentiated from time-dependent inhibition, not here employed, where enzyme and inhibitor are preincubated.

[4] This pH optimum for acetaldehyde reduction by peak II enzyme alone is slightly lower than that reported for unresolved enzyme using an acetaldehyde concentration sevenfold higher than that used here (von Wartburg *et al.*, 1964, 1965), a reflection of either substrate concentration or perhaps the characteristics of the different enzyme species.

[5] At high enzyme concentrations, trichloroethanol exhibited a low rate (1% that of ethanol) of reduction of NAD, proportional to the amount of human enzyme added and maximal at pH 9. Under these conditions, the horse enzyme was inactive.

ported for the horse liver enzyme (Dalziel, 1958) and unusual for a protein with a molecular weight of *ca.* 87,000. This property has permitted appreciable purification of the enzyme by a single chromatographic step. Thus, the bulk of the anionic and neutral proteins present in the liver extract are removed effectively by adsorption onto DEAE-cellulose, leaving only the cationic components for subsequent fractionation on CM-cellulose.

This use of DEAE-cellulose early in the procedure has several advantages. Even in large-scale preparations, 10–20-fold purification is achieved and the yield at this stage is *ca.* 80%. In fact, close to quantitative recoveries of activity have been attained.[6]

Under suitable conditions of pH and ionic strength, alcohol dehydrogenase activity has been resolved into multiple zones on CM-cellulose (Figure 2). The occurrence of three chromatographically distinct enzyme fractions is commonly observed in preparations starting with several livers, but heterogeneity may also be encountered in preparations obtained from individual organs where one, two, or more commonly, three chromatographically distinct forms have been observed in a number of experiments. The number of observations are not as yet sufficient to determine the frequency of occurrence and distribution of these forms in different individuals. The variability in the occurrence of the enzymatic species and the appreciable individual variation in the total activity present in extracts of single livers may well account for the range of chromatographic patterns obtained in the large-scale preparations using a number of livers.

The occurrence of multiple, chromatographic forms of the enzyme is reminiscent of the microheterogeneity of lactic dehydrogenase. In that protein all possible combinations of two different types of tetramer subunits give rise to five electrophoretically distinct species (Kaplan, 1963). Horse liver alcohol dehydrogenase also has enzymatically active variants (Dalziel, 1958), binds 2 moles of NADH/mole of protein, and is thought to consist of at least two subunits, based upon the number of peptides obtained by tryptic digestion (Li and Vallee, 1964). Preliminary data suggest that human liver alcohol dehydrogenase similarly binds 2 moles of NADH/mole of protein (von Wartburg *et al.*, 1964) and, hence, may also contain two subunits. If two different subunits should be demonstrated, then all possible combinations would give rise to three separate species, the number found in this study. Multiple forms of active enzyme could also arise due to partial proteolytic degradation into species of different charge, or alternatively, due to the binding of other molecules by the enzyme to yield complexes with different chromatographic behavior. Further studies are in progress to delineate the molecular basis for the properties of these species.

The wide variation in the activity per gram of liver from different individuals is also of interest. The present data do not indicate as yet whether this increased activity is due to greater specific activity of one of the three forms here identified, to other forms yet unrecognized, or to the removal of inhibitors. Although the presence of artifacts cannot be ruled out, these observations may be significant in relation to the biochemical and/or genetic basis of ethanol metabolism. Out of 117 liver extracts examined only four exhibited markedly greater intrinsic ability to metabolize ethanol, implying that these individuals might have differed in their tolerance to ethanol on this basis. Unfortunately, pertinent historical information regarding this point could not be obtained. These data, however, call for continued studies in this direction.

All of the chromatographically distinct peaks exhibited similar kinetic constants for the ethanol–acetaldehyde system and were inhibited in the same manner by 1,10-phenanthroline, indicating that all are metalloenzymes. The only kinetic difference noted thus far relates to the reduction of chloral hydrate. While the Michaelis constants for chloral hydrate were similar for all three forms the maximal activity of peak III toward this substrate was greater than that of peaks I and II. Since final purification of the system has not yet been achieved, the significance of such comparative values must await further evaluation. The preparative procedure reported here will permit the isolation of larger quantities of the different chromatographic forms of the enzyme for further purification and study.

The present investigation extends awareness of the range of substrate specificity of human liver alcohol dehydrogenase which now encompasses several compounds such as ethylene glycol and chloral hydrate, which become pharmacologically and toxicologically significant when metabolized. Methyl substitution of ethylene glycol (1,2-ethanediol), to yield 1,2-propanediol results in a lower Michaelis constant (Table II). This is analogous to the decrease of k_m on lengthening of the hydrocarbon chain of simple straight-chain monohydroxy alcohols (von Wartburg *et al.*, 1965). Blocking of one of the two hydroxyl groups of ethylene glycol, either with a methyl or an ethyl group (Table II), also decreases k_m, in these instances to values similar to that of ethanol. However, these substitutions do not materially affect the maximal rate which remains approximately equal to that of ethylene glycol. The hydrophobic group of these ether derivatives apparently induce better substrate binding without affecting the catalytic rate.

Ethanol and ethylene glycol compete for the active site of human liver alcohol dehydrogenase (Figure 3). Thus, when the two substrates are present, each at a concentration of 0.01 M, the rate of ethylene glycol oxidation is 15% of that in the absence of ethanol, calculated by the procedure indicated in Figure 3. When ethylene glycol is ingested either accidentally or intentionally, it is ultimately oxidized to oxalate, which is

[6] The high fat content of most human livers, in contrast to the negligible amounts found in commercially available horse livers, presents a significant obstacle to the isolation of the enzyme. The effective retention of fat on the DEAE-cellulose bed constitutes yet an additional advantage of this method of preparation.

precipitated as the calcium salt in the kidney with fatal consequences. Based on the competition between the two substrates ethylene glycol poisoning has recently been treated successfully with ethanol (Wacker *et al.*, 1965).

Ethanol apparently protects against methanol toxicity in a similar fashion. Methanol is a poorer substrate for human liver alcohol dehydrogenase than is ethylene glycol, its maximal rate being lower than that of the other alcohols tested. The maximal velocity for methanol, reported to be approximately equal to that of ethanol (von Wartburg *et al.*, 1964), has been found to be *ca.* 12% (von Wartburg *et al.*, 1965) and 11% (Table II) of that of ethanol in other preparations. The possibility that these findings denote individual variability in the capacity to detoxify methanol requires further examination.

Monohalo derivatives of ethanol, *e.g.*, fluoroethanol, which yield toxic metabolic products, *e.g.*, fluoroacetate, are also oxidized by human liver alcohol dehydrogenase, although at rates much reduced when compared with ethanol. The human enzyme exhibits little or no activity toward the dihalo and trihalo derivatives and is similar to the horse and rabbit liver enzymes in this regard (Friedman and Cooper, 1960; Owens and Marshall, 1955).

The di- and trihaloethanols, however, inhibit ethanol oxidation. Trifluoro-, tribromo-, and trichloroethanol are about equally effective. Thus, changes both in the size and the inductive effect of the halogen atoms in the α position apparently do not affect the binding of these polyhalo compounds to the enzyme markedly. Chloral hydrate (trichloroacetaldehyde hydrate) while it is also a substrate for the enzyme exhibits the same inhibition kinetics as trichloroethanol, indicating similar binding to the enzyme. The results with trichloroethanol or chloral hydrate obtained at pH 7.15 reflect the interaction of 1 mole of inhibitor/mole of binding site when plotted according to the method of Kistiakowsky and Shaw (1953). At pH 9.3, however, the results are more complex and do not conform to this stoichiometry. Similarly, the curved double reciprocal inhibition plots obtained for trichloroethanol in the presence of varying amounts of ethanol (Figure 7) do not permit a simple interpretation of the mechanism of interaction of this agent with the enzyme. These results are similar to those for chloroethanol (D. E. Drum, personal communication) and methanol (Wratten and Cleland, 1965) inhibition of the horse enzyme when the ethanol concentration is varied. Wratten and Cleland (1965) interpreted such data for methanol to indicate the formation of an active enzyme–coenzyme–inhibitor complex.

In contrast to the halo derivatives of ethanol, human liver alcohol dehydrogenase reduces not only monobut also di- and trihaloacetaldehydes. Successive addition of electronegative chlorine atoms in the α position progressively decreases the affinity of these substrates for the enzyme: monochloroacetaldehyde has the highest maximal velocity and the lowest k_m while trichloroacetaldehyde exhibits the lowest maximal velocity and the highest k_m (Table III).

Horse (Owens and Marshall, 1955) and rabbit liver (Friedman and Cooper, 1960) alcohol dehydrogenases also catalyze reduction of chloral hydrate while the yeast enzyme does not (Friedman and Cooper, 1960). The k_m for the horse enzyme (Owens and Marshall, 1955) is somewhat lower, 1.4×10^{-3} M, while the maximal rate is substantially lower than the respective values for the human enzyme. The reduction of trichloroacetaldehyde or chloral hydrate by human liver alcohol dehydrogenase is of particular interest since this derivative exists in aqueous solution predominantly in the form of the hydrate with no free aldehyde group. Its distinctive pH–rate profile suggests that its reduction may proceed by different or additional mechanisms.

The present and previous studies (von Wartburg *et al.*, 1964) indicate differences in the catalyic properties of alcohol dehydrogenases from different species. The substrate specificity of the human liver enzyme seems broader than those of other organisms studied so far. Further, the high alcohol dehydrogenase activities found in a few human livers have not previously been noted in studies of other species. Both these findings may well bear importantly on the ultimate understanding of the human metabolism of alcohols.

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Structural Studies of Ribonuclease. XXII. Location of the Third Buried Tyrosyl Residue in Ribonuclease*

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ABSTRACT: Ribonuclease was iodinated with 6 moles of I_2/mole of ribonuclease at pH 6.7 and 4°. The major component, peak C, was isolated by chromatography on carboxymethylcellulose. Spectrophotometric titrations and amino acid analysis of the iodinated material showed the presence of about three uniodinated and still abnormal tyrosines. The ultraviolet difference spectrum indicated that three tyrosines had been diiodinated.

The oxidized iodinated protein was subjected to enzymatic digestion by trypsin, chymotrypsin, and pepsin, respectively, and the resulting peptides were chromatographed on Dowex 50-X2. Analysis of the peptides showed that Tyr 92, Tyr 97, and Tyr 25 were uniodinated. Structural implications, based on the results of this and the accompanying paper, are presented in the following paper [Li, L.-K., Riehm, J. P., and Scheraga, H. A. (1966), *Biochemistry 5*, 2043].

As an important step in the study of the folding of protein molecules in solution, it is desirable to determine which amino acid side chains are exposed to the solvent and which are buried. In the case of bovine pancreatic ribonuclease (RNAase), an abundance of physical evidence indicates that three of the six tyrosines behave normally while the other three show anomalous titration behavior and are presumably buried (Shugar, 1952; Tanford *et al.*, 1955). The three anomalous tyrosines are not all equivalent as indicated by differences in the spectral shifts occurring under various denaturation conditions (Bigelow, 1961). On the basis of the work of Hermans and Scheraga (1961) and Scott and Scheraga (1963), it appears that the buried tyrosyl residues may be near carboxyl groups and surrounded by nonpolar groups.

Tyrosine residues can be iodinated under relatively mild conditions in aqueous solution, and the iodination of RNAase has been studied by Cha and Scheraga (1963) and by Donovan (1963). They were able to locate, respectively, two and one of the buried tyrosyls in the amino acid sequence. Recently, Riehm *et al.* (1965) have located the three abnormal carboxyl groups of RNAase and have suggested possible pair relationships between these carboxyls and the known buried tyrosyls. Fujioka and Scheraga (1965) have located the single buried tyrosine which remains in pepsin-inactivated RNAase. To complete the picture, it is necessary to locate the remaining buried tyrosine in native RNAase.

The present paper describes the iodination of RNAase under somewhat milder conditions than those used by Cha and Scheraga (1963) and by Donovan (1963). These workers used the conditions recommended by Hughes and Straessle (1952), *i.e.*, pH 9.5 and 0°, to minimize side reactions. We have carried out our iodinations at neutral pH and 4°. At this lower pH, the reaction is much slower, but no evidence of oxidative side reactions was observed.

The product from iodination was fractionated, and the major component was characterized by spectrophotometric titration, ultraviolet difference spectra, and amino acid analysis following performic acid oxidation and acid hydrolysis. Three tyrosyl groups were found to be uniodinated. The positions of these uniodinated groups in the amino acid sequence were de-

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