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Simultaneous Binding of Competitive Ligands to Horse Liver Alcohol Dehydrogenase†

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ABSTRACT: Chloride ion binds to at least two different types of sites on horse liver alcohol dehydrogenase. In initial velocity experiments with coenzyme as the varied component at saturating alcohol or aldehyde levels, chloride was found to be competitive toward coenzyme with a K_I of 30–60 mM. Similarly, chloride was a competitive inhibitor against ethanol at saturating oxidized nicotinamide adenine dinucleotide (NAD^+) levels (K_I = 100 mM). However, chloride was a noncompetitive inhibitor against either acetaldehyde reduction or isobutyramide binding at saturating NADH levels. The inhibition of acetaldehyde is mainly due to chloride binding to the enzyme–NAD^+ complex formed from the reduction of aldehyde, thus inhibiting the release of NAD^+ from the enzyme. Some enzyme–NADH–aldehyde–Cl complex can also form which accounts for the inhibition being noncompetitive rather than uncompetitive. Chloride appears to bind to free enzyme excluding coenzyme and also simultaneously with coenzyme. Thus, there have to be at least two different types of chloride binding sites. The fact that the anion is competitive toward ethanol but binds simultaneously with isobutyramide (a specific aldehyde inhibitor) suggests that there may even be additional specific anion binding sites on the enzyme.

Bromide ion (Plane and Theorell, 1961) and chloride ion (Theorell *et al.*, 1955) are competitive inhibitors for NADH[1] and NAD^+ with horse liver alcohol dehydrogenase (EC 1.1.1.1). Since we have found by static methods that chloride can bind to enzyme concurrently with NADH (Coleman and Weiner, 1973), we have now performed kinetic experiments in the presence and absence of chloride ion in order to resolve

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[1] Abbreviations used are: enzyme, one subunit of alcohol dehydrogenase; K_D, dissociation constant; NAD^+ and NADH, oxidized and reduced nicotinamide adenine dinucleotide.

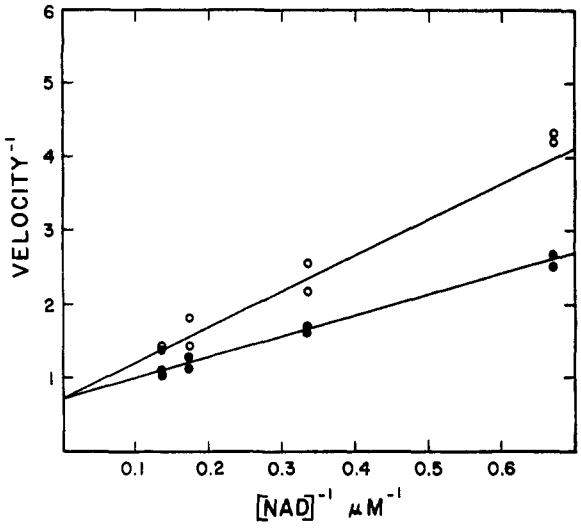


FIGURE 1: The effect of chloride ion on the initial velocity with NAD^+ as the varied component. Presented is an example of the inhibition of NAD^+ reduction by 50 mM chloride ion (○). The reaction was initiated by the addition of enzyme to the premixed solution of NAD^+ (1.3–8.0 μM), ethanol (10 mM), and chloride ion (0–125 mM) at 25° at pH 7.0 phosphate, $\mu = 0.1$. The K_I calculated for this example was 62 μM; the average K_I was 60 ± 5 mM.

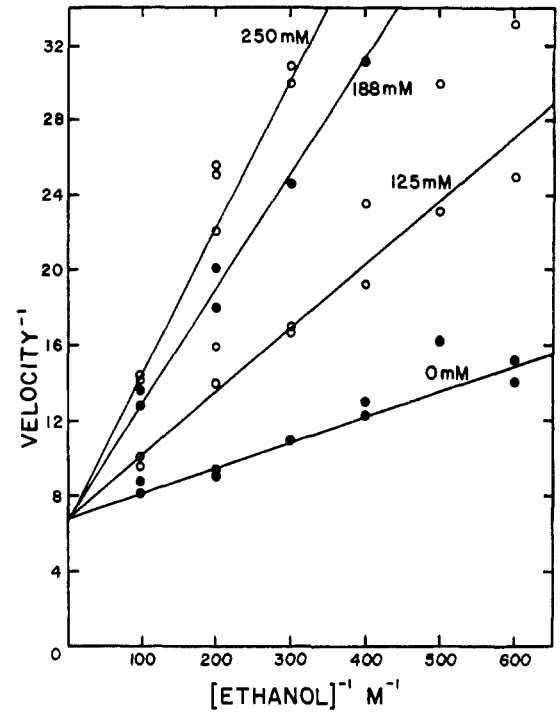


FIGURE 3: The effect of chloride ion on ethanol oxidaton. The ethanol concentration was varied from 1.6 to 10 mM, and chloride ion concentration was varied from 0 to 250 mM as indicated at a NAD^+ concentration of 100 mM. The K_I calculated was 100 ± 20 mM. Other conditions are the same as in Figure 1.

the apparent paradox of mutually competitive molecules being simultaneously bound to the same protein molecule.

We present results which resolve the above problem by accounting for more than one type of chloride ion binding site. The effect of chloride ion on the kinetics of the two substrate pairs as well as the binding to an enzyme–NADH–isobutyramide ternary complex[1] argue for a minimum of two types of chloride ion sites.

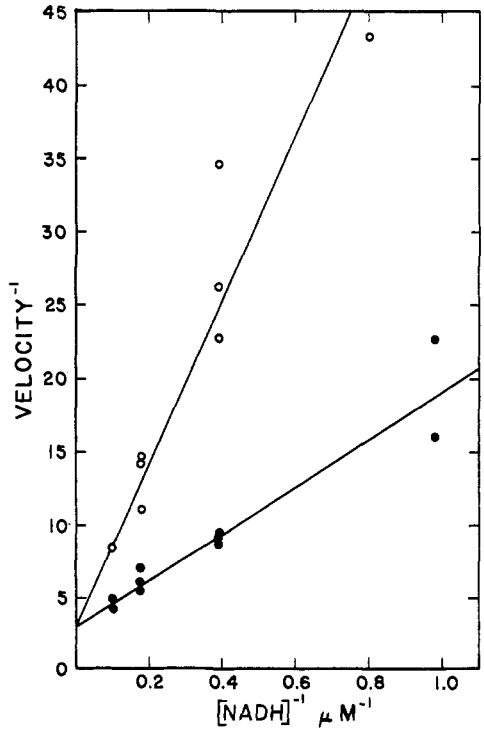


FIGURE 2: The effect of chloride ion on the initial velocity with NADH as the varied component. In this example of the inhibition of NADH oxidation by chloride (○), NADH was 1–10 μM, acetaldehyde was 1.0 mM, and chloride ion was 80 mM. The average K_I calculation for chloride ion concentrations ranging from 0 to 125 mM was 30 ± 5 mM. Other conditions are the same as in Figure 1.

Experimental Section

All compounds and experimental procedures are the same as described in the previous paper (Coleman and Weiner, 1973) unless otherwise noted.

Kinetic assays were performed at 25° with an Aminco filter fluoromicrophotometer fitted with excitation filter no. 4-7113 and emission filter no. 4-7116 (Wrattan 2A), and recorded on a Sargent-Welch recorder.

All calculations were performed on a Wang computer Model 360E/370/371 using a program for least-squares linear regression and correlation coefficient using nonweighted values in the computations. All experimental points were obtained at least in triplicate, and all individual experiments were performed twice and lines through data points are computer-determined least-squares fits.

Isobutyramide was added to preformed enzyme–NADH complexes in the presence of 0.0–1.0 M NaCl. Fluorescence was observed at 430 nm with an excitation wavelength of 340 nm using an Aminco-Bowman spectrofluorophotometer as described previously (Coleman and Weiner, 1973; Iweibo and Weiner, 1972).

Results

Kinetic Investigation in the Presence and Absence of Chloride. Double reciprocal plots of the initial velocities at various chloride concentrations with varying NAD^+, NADH, and ethanol are presented in Figures 1, 2, and 3, respectively. In each case the inhibition by chloride ion is competitive with

TABLE I: Dissociation Constants of Chloride Ion from Various Complexes with Horse Liver Alcohol Dehydrogenase.

Complex	K_I (mM)	Method of Measurement[a]
E–Cl	30	NADH kinetics
E–Cl	60	NAD^+ kinetics
E–NAD^+–Cl	100	Ethanol kinetics
E–NADH–Cl	200	Fluorescence titration[b]
E–NADH–Cl	1100	ORD titration[b]
E–NADH–acetaldehyde–Cl	150 (K_{II})	Acetaldehyde kinetics
	410 (K_{II})	Acetaldehyde kinetics
	650 (K_{IS})	Acetaldehyde kinetics
E–NADH–isobutyramide–Cl	750 (K_{IS})	Fluorescence titration

[a] Kinetics refer to the varied substrate. [b] Coleman and Weiner, 1973. The values of K_I were calculated using at least three chloride ion concentrations for each experiment. E is the enzyme subunit, K_{II} is the dissociation constant calculated from the intercept term, and K_{IS} is that calculated from the slope term (Cleland, 1970).

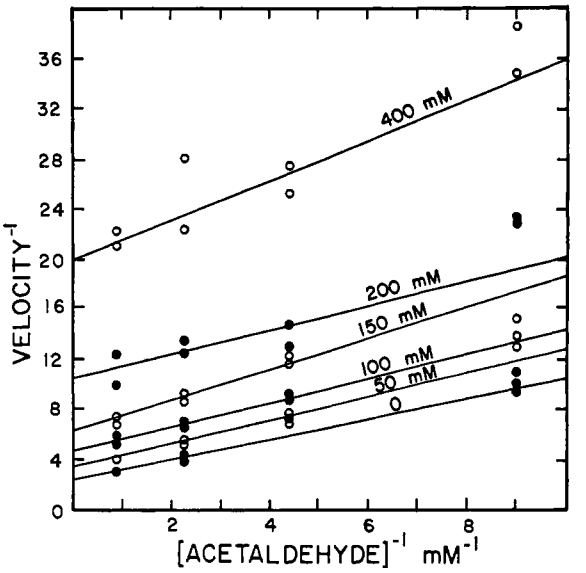


FIGURE 4: The effects of chloride ion on acetaldehyde reduction. The acetaldehyde concentration was varied from 0.11 to 1.0 mM. NADH was constant at 70 μM, and chloride ion was varied from 0 to 400 mM as indicated. Other conditions are the same as Figure 1.

the varied compound. The values of K_I were calculated from the equation $[I]/K_I = (K_m^{app}/K_m) - 1$ using the plot of $(K_m^{app}/K_m) - 1$ *vs.* [I]. [I] is the inhibitor (chloride) concentration, K_I is the inhibition constant, K_m is the calculated Michaelis constant in the absence of chloride, and K_m^{app} is the apparent K_m at some chloride ion concentration, [I]. The values of K_I for chloride ion for their respective varied compounds are presented in Table I.

The inhibition pattern with varied acetaldehyde (Figure 4) is noncompetitive since both the intercept and the slope are functions of the chloride ion concentration. Figure 5 presents a plot of the calculated intercept *vs.* [NaCl]. Zero- to fourth-order equations were fit by computer and the best line which fits the data is a parabola of the general formula $y = a(1 + bI + cI^2)$ where the b and c values are association constants for chloride ion to the protein and I represents the chloride ion concentration (Cleland, 1970). The values obtained for b^{-1} and c^{-1} as dissociation constants are 150 and 410 mM, respectively. From the linear subplot of slope *vs.* NaCl concentration, $K_I = 800 \pm 200$ mM (Figure 5).

Effect of Chloride Ion on the Binding of a Substrate Inhibitor, Isobutyramide. The coenzyme fluorescence of the ternary enzyme–NADH–isobutyramide complex is greatly enhanced over that of the binary complex and is used to determine the stoichiometry of coenzyme binding (Winer and Theorell, 1960). This technique was employed in the presence and absence of chloride ion (Figure 6). The break point shows that at 50 mM isobutyramide the stoichiometry of 2 mol of coenzyme binds per 80,000 g of protein in chloride solution, unchanged from the results in the absence of chloride. This result agrees with the observation of Lindman *et al.* (1972) that in the presence of isobutyramide, chloride had no effect on the final stoichiometry of NADH with alcohol dehydrogenase.

The above experiment was performed at a high isobutyramide concentration and therefore could not be used to determine whether or not chloride ion is an inhibitor of isobutyramide binding. This might be expected since the kinetic experiments showed that chloride did inhibit acetaldehyde binding. The titration was reversed by adding aliquots of isobutyramide to preformed enzyme–NADH complex in the presence of chloride while observing the fluorescence increase which is indicative of the formation of ternary complex. Chloride ion inhibited the formation of the ternary complex (Figure 7) and the mode of the inhibition was noncompetitive, the same as that observed with acetaldehyde kinetics.

Discussion

Coenzyme binds prior to substrate in the ordered binding of ligands to horse liver alcohol dehydrogenase (Theorell and Chance, 1951). Since chloride is a competitive inhibitor for either coenzyme when the nonvaried substrate is used at

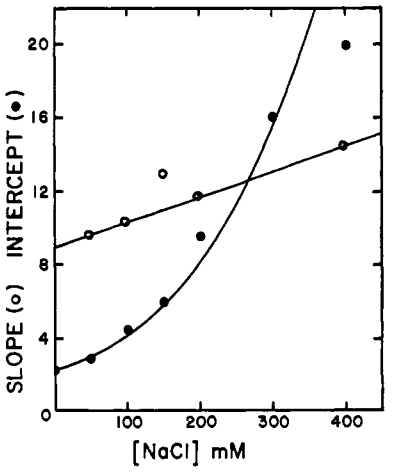


FIGURE 5: A secondary plot for chloride ion inhibition of the acetaldehyde reduction. The slopes of intercepts from Figure 4 are plotted against sodium chloride concentration (Cleland, 1970). The computer determined K_I values were equal to 150 and 410 mM from the intercept and 800 mM from the slope data.

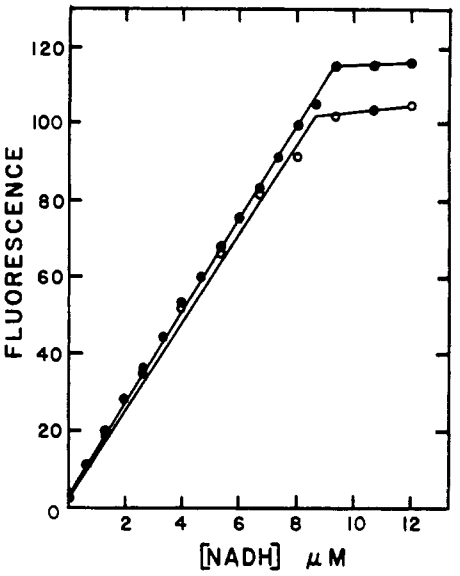


FIGURE 6: The effect of chloride ion on the active site titration of alcohol dehydrogenase. NADH was added to a solution of enzyme (8.5 μM) and isobutyramide (50 mM) in pH 7.0 phosphate, $\mu = 0.1$, in the presence (○) and absence (●) of chloride ion (0.5 M); excitation at 340 nm; emission at 430 nm.

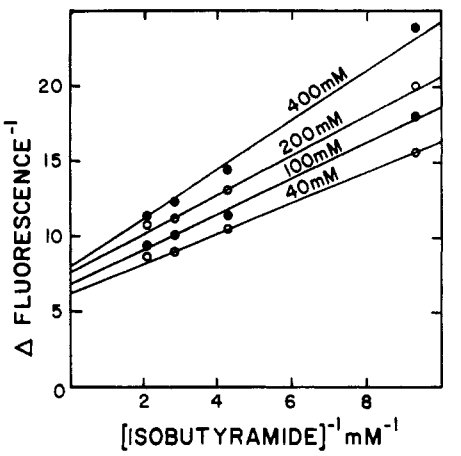


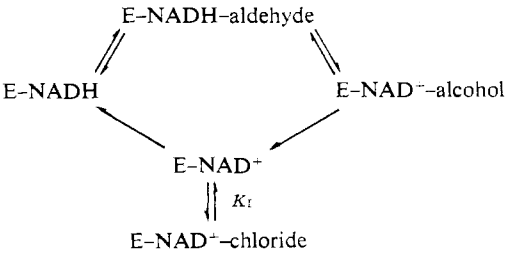
FIGURE 7: The effect of sodium chloride on the formation of a ternary complex of alcohol dehydrogenase. Isobutyramide was added to a solution of enzyme (8.5 μM), NADH (20 μM), and chloride ion which was excited at 340 nm and emission was measured at 430 nm. The NaCl concentrations used are indicated adjacent to their respective data lines.

saturating conditions, it can be concluded that the anion binds to the free enzyme. The nuclear magnetic resonance experiments of Ward and Happe (1971) as well as the results of Reynolds and McKinley-McKee (1969) and Young and Wang (1971) and the kinetic results of Plane and Theorell (1961) concur with this interpretation. Plane and Theorell (1961) observed that bromide ion binds to free alcohol dehydrogenase with dissociation constants of 23 and 69 mM. These values agree well with the values of 30 and 60 mM calculated here for chloride ion inhibition *via* kinetic competition.

Two types of chloride binding sites have been found. One is presumably the coenzyme binding site (K_D = 30–60 mM) and the second type is the site which forms the enzyme–NADH–Cl complex (K_D = 200–1000 mM). Direct evidence for the existence of the latter is the fluorescence data presented in the accompanying paper (Coleman and Weiner, 1973). The competitive inhibition of chloride for ethanol (at saturating NAD^+ levels) shows that an enzyme–NAD^+–Cl complex can similarly form. The results of Shore and Gutfreund (1970) can be interpreted to mean that chloride ion binds to the enzyme–NADH complex in order to effect the more rapid release of coenzyme observed in their transient kinetic experiments.[2] In addition, results of Plane and Theorell (1961) allude to the formation of an enzyme–NADH–bromide complex.

The binding of chloride to the enzyme–NADH complex results in noncompetitive binding inhibition toward acetaldehyde or isobutyramide. Since isobutyramide and chloride ion do not mutually exclude one another, they bind simultaneously and, by analogy, the quaternary complex of enzyme–NADH–acetaldehyde–Cl may also then exist.

The rate limiting step in the reduction of aldehyde is release of NAD^+ from the enzyme (7 sec^{-1}), not interconversion of ternary complexes (>200 sec^{-1}) (Shore and Gutfreund, 1970). Since chloride in the presence of saturating NADH and acetaldehyde causes the enzyme to have a lower V_{max} the anion must be inhibiting the release of NAD^+ from the enzyme. Initial velocity experiments at saturating NADH concentrations would produce the following situation



which leads to an uncompetitive inhibition pattern for chloride if acetaldehyde is the varied substrate.

$$v = \frac{V_{max}}{1 + \frac{K_m}{[\text{acetaldehyde}]} + \frac{[Cl^-]}{K_I}}$$

The fact that both K_m for aldehyde as well as V_{max} changed in the presence of chloride at saturating NADH levels suggests then that in addition to chloride binding to E–NAD some must bind to E–NADH. The resulting complex of E–NADH–Cl then would have a higher K_m for aldehyde. The K_I for chloride calculated from the change in slope of Figure 4 is 800 mM which agrees well with the value of 750 mM found for the inhibition of isobutyramide binding.

Chloride ion appears to be able to bind to different types of sites on alcohol dehydrogenase. There is one site which is the coenzyme binding site and another may be the ethanol binding site. However, since we have shown that coenzyme can bind to regulatory sites which competitively prevent the binding of substrates (Iweibo and Weiner, 1973) it cannot yet be determined if the second binding site is the coenzyme regulatory binding site or the ethanol binding site.

The noncompetitive nature of chloride with respect to both acetaldehyde and isobutyramide suggests that chloride may not actually be binding to the coenzyme's regulatory sites. For if it were one would have expected chloride to be a

[2] We did not observe an enhanced rate in the steady state measurement.

competitive inhibitor for aldehyde or isobutyramide. The observation that chloride is a competitive inhibitor for alcohols but not for aldehydes is similar to what is found for fatty acids interacting with alcohol dehydrogenase (Winer and Theorell, 1960).

The binding of chloride to the nonactive site most likely produces a conformational change in the active site since the enhanced fluorescence emission of bound NADH is quenched and the extrinsic Cotton effect removed (Coleman and Weiner, 1973). However, in the absence of coenzyme no evidence for a conformationally altered enzyme was found in the presence of chloride. Even though the K_D for chloride is 30–60 mM from enzyme–Cl and only 100–300 mM from enzyme–coenzyme–Cl it is not possible to determine whether, in the absence of coenzyme, chloride binds to both its sites or that the second site is induced by coenzyme binding. It is possible that the binding to only one type of chloride binding site may not produce a spectroscopically measurable conformational change.

The complex interaction of chloride may be because the anion is mimicking some true regulator of the enzyme. Chloride ion is not found in high concentration in liver (Cantarow and Schapartz, 1962). However, having shown that anions, as well as coenzyme, are capable of modulating the *in vitro* activity of the enzyme, the question of the regulation of alcohol dehydrogenase in the liver is opened.

The *in vivo* reactions catalyzed by this enzyme have never been fully explained. The enzyme concentration in the liver (1 mg/ml) is high considering the relatively minor roles that the interconversion of acetaldehyde and ethanol or the anerobic regeneration of NAD^+ play in an active aerobic liver. The recent results from Iweibo (1972) and other laboratories (Landau and Merlevede, 1963; Hassinen, 1965) suggest that alcohol dehydrogenase is intimately involved in the reactions associated with fructose metabolism (glyceraldehyde metabolism). Since carbohydrate metabolism is a major function of the liver it would be expected that the enzymes involved would be present in larger amounts. Given that alcohol dehydrogenase may serve this function it would be expected that the enzyme would be regulated by various phosphate anions of carbohydrates or lipids or by organic salts, and perhaps chloride is mimicking one of these compounds.

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