Electronic Substituent Effects during the Liver Alcohol Dehydrogenase Catalyzed Reduction of Aromatic Aldehydes†

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ABSTRACT: The electronic substituent effect during liver alcohol dehydrogenase catalyzed reduction of para-substituted benzaldehydes by NADH is very small (rate ratio for *p*-chloro- and *p*-methoxybenzaldehyde is ~2). This is in contrast to the substituent effect during reduction of these aldehydes with sodium borohydride (rate ratio for *p*-chloro- and *p*-methoxybenzaldehyde is ~100). There is also a large substituent effect on the rate of redution of ortho-substituted *p*-benzoquinones by NADH; this process is determined to occur by hydride transfer. One explanation consistent with the unexpectedly low substituent effect for the enzyme-catalyzed reaction is that polarization of the carbonyl function by zinc on the enzyme surface results in Lewis acid catalysis of hydride transfer from NADH.

Liver alcohol dehydrogenase catalyzes the following reaction

$$H^+ + RCH(=O) + NADH \rightleftharpoons RCH_2OH + NAD^+ \quad (1)$$

by an ordered mechanism (eq 2) (Theorell and Chance, 1951; Wratten and Cleland, 1963). The rate-limiting step in the direction of aldehyde reduction is k_4 or k_5 depending on the substrate; therefore, steady-state kinetics does not provide information concerning the catalytic step, k_3. Transient kinetics where enzyme is in excess of substrate or pre-steady-state kinetics reveal the rates associated with buildup of the steady-state condition, but it is difficult to study substrate dependence of kinetic processes under these experimental conditions. A new type of transient kinetics has been applied to liver alcohol dehydrogenase. This consists of adding pyrazole to the NAD^+–enzyme complex after alcohol desorption to form a stable unreactive ternary complex (eq 2). The rate of this addition is much faster than the rate-limiting step for enzyme turnover; therefore, the reaction is stopped at a single turnover. Transient kinetics using this new method consist of two steps; the first has a primary deuterium isotope effect of 2.5 indicating it is associated with hydride transfer, while the second has no primary isotope effect (Bernhard *et al.*, 1970; McFarland and Bernhard, 1972). It is possible to vary the concentration of substrate in excess of enzyme using this new method; we wish to report the electronic substituent effects on hydride transfer in liver alcohol dehydrogenase (equine) using this new kinetic method.

$$E + NADH \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} E\text{–}NADH + RCH(=O) \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} E\begin{matrix}NADH\\O\\RCH\end{matrix} \overset{k_3}{\underset{k_{-3}}{\rightleftharpoons}}$$

$$E\begin{matrix}NAD^+\\RCH_2OH\end{matrix} \overset{k_4}{\underset{k_{-4}}{\rightleftharpoons}} E\text{–}NAD^+ + RCH_2OH \overset{k_5}{\underset{k_{-5}}{\rightleftharpoons}} E + NAD^+ \quad (2)$$

Several proposals have been made regarding the mechanism of hydride transfer in dehydrogenase enzymes. The simplest is that the enzyme facilitates binding of NADH and aldehyde in close proximity to each other but that hydride transfer involves simple uncatalyzed transfer of a hydrogen with its electrons to the aldehyde acceptor. Blomquist (1966) reports that steady-state studies with substituted benzaldehydes show a large substituent effect on a kinetic step; such a large substituent effect would be expected for bimolecular reaction between NADH and aldehyde. A second proposal suggests that zinc may act as a Lewis acid to polarize the carbonyl of the aldehyde making it a better acceptor for hydride ion (Mahler and Douglas, 1957). A third proposal is that enzyme is reduced by transfer of hydride to tryptophan at the enzyme active site; this reduced enzyme then reduces the aldehyde substrate (Schellenberg, 1965).

Several model systems are of interest also. Abeles *et al.* (1957) investigated the reduction of substituted thioketones with benzyldihydropyridine. This process seems to involve an ionic mechanism in which hydride is transferred from the nicotinamide donor to thioketone acceptor. Evidence for the ionic mechanism consists of large isotope and solvent effects. Recently the first case of direct transfer of hydride from *N*-propyl-1,4-dihydronicotinamide to the zinc complex of 2,20-phenanthroline-2-carboxaldehyde has been observed (Creighton and Sigman, 1971). Transfer does not take place in the absence of zinc.

Experimental Section

The enzyme preparation has been described previously (Bernhard *et al.*, 1970; McFarland and Bernhard, 1972). NADH (Chromatopure) was obtained from P-L Biochemicals and used without further purification. β-Naphthaldehyde and *p*-chlorobenzaldehyde were obtained from Aldrich Chemical

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TABLE I: Substituent Effect Summary.[a]

Aldehyde	K_m	$K_{app}(H)$[b]	$K_{app}(D)$[b]	$k_{1,max}(H)$[b] (sec^{-1})	$k_{1,max}(D)$[b] (sec^{-1})	k_2-($NaBH_4$) (sec^{-1} M^{-1})
Benzaldehyde	2.0×10^{-5} [c]	$2.1 \pm 0.1 \times 10^{-4}$	$2.0 \pm 0.1 \times 10^{-4}$	339 ± 9	134 ± 7	2.2
p-Chlorobenzaldehyde	5.9×10^{-6}	$7.2 \pm 0.1 \times 10^{-5}$	$4.5 \pm 0.5 \times 10^{-5}$	410 ± 42	180 ± 23	19.0
p-Methoxybenzaldehyde	4.7×10^{-5}	$4.1 \pm 0.1 \times 10^{-4}$	$1.2 \pm 0.1 \times 10^{-4}$	253 ± 5	86 ± 6	0.20
β-Naphthaldehyde	1.5×10^{-6} [c]	$3.0 \pm 0.2 \times 10^{-5}$	$3.3 \pm 0.1 \times 10^{-5}$	422 ± 21	300 ± 40	8.1

[a] [Liver alcohol dehydrogenase] = 1.0×10^{-5} N; [NADH] = 5.0×10^{-5} M for D, 1.0×10^{-4} M for H; [pyrazole] = 0.02 M. [b] H denotes parameters determined from transient reaction with NADH; D denotes [4-^{2}H]NADH. [c] Bernhard *et al.*, 1970.

and purified by sublimation. *p*-Methoxybenzaldehyde and benzaldehyde (Aldrich and Allied Chemical) were distilled at reduced pressure under nitrogen. 1,4-Benzoquinone was obtained from Aldrich Chemical. 2-Methyl-1,4-benzoquinone was prepared by the method of Clark (1892). 2-Chloro-1,4-benzoquinone was prepared by the method of Snell and Weissberger (1939).

The following spectral maxima and extinction coefficients were used to determine aldehyde concentration: β-naphthaldehyde, λ_{max} 280 (ϵ 1.2×10^4); benzaldehyde, λ_{max} 255 (1.0×10^4); *p*-chlorobenzaldehyde, λ_{max} 254 (1.6×10^4); *p*-methoxybenzaldehyde, λ_{max} 280 nm (1.6×10^4). Sodium borohydride was obtained from Alfa Inorganics and used without further purification. [4-^{2}H]NADH was prepared by the method of Rafter and Colowick as described previously (McFarland and Bernhard, 1972).

Aldehyde Reduction by Borohydride. A saturated solution of sodium borohydride was prepared by adding an excess of borohydride to isopropyl alcohol (Mallinckrodt, analytical grade). The excess sodium borohydride was removed by centrifugation and the concentration of sodium borohydride in the solution was measured by reacting borohydride solution (1 ml) with 10 ml of 0.5 N hydrochloric acid and measuring the hydrogen evolved. A four- to fivefold excess of sodium borohydride was added to an isopropyl alcohol solution of the alcohol under study. The pseudo-first-order reaction rate was followed spectrophotometrically at the λ_{max} for the aldehyde. Rate studies were carried out on a Cary 14 double beam spectrophotometer.

NADH Oxidation by Quinones. Quinones react readily to oxidize NADH at pH 7.0. The stoichiometry of the reaction appears to be 1:1 as established by the optical density change at 340 nm during oxidation of excess NADH by a limiting amount of quinone. Reduction potentials are consistent with a quantitative reaction; this reaction has been reported previously for benzyldihydropyridines (Wallenfels and Gerlich, 1959).

Reaction products were identified as NAD^+ and hydroquinone as follows. Reaction of hydroquinone and NAD^+ was allowed to proceed for 1 half-life. At this time ethyl alcohol and liver alcohol dehydrogenase were added to the reaction mixture; there was immediate production of NADH (observed at 340 nm). The nucleotide product of the reaction of hydroquinone and NADH is most probably NAD^+ since the enzyme-catalyzed reaction is quite specific for NAD^+. Hydroquinone was identified by its characteristic absorption at 288 nm (ϵ 2800); neither NAD^+ (λ_{max} 260 nm (ϵ 18,000)) nor quinone (λ_{max} 245 nm (ϵ 9700)) shows any absorption at this wavelength. NADH absorbs slightly at 288 nm ($\epsilon \sim 1500$). Spectra were taken during reaction of NADH (8.5×10^{-6} M) and benzoquinone (1×10^{-4} M). As NADH was oxidized, the absorbance at 288 nm increased indicating formation of hydroquinone. Reaction was allowed to proceed for 4 half-lives and the spectrum of NAD^+ produced during reaction as well as the remaining excess quinone were subtracted from the observed spectrum. This difference spectrum, which should be characteristic of the quinone product, showed λ_{max} 285 nm; the spectrum of hydroquinone is nearly identical with the difference spectrum.

Mechanistic studies were performed in pH 7.0 phosphate buffer (0.1 M) by watching oxidation of NADH at 340 nm. Various substituted quinones were allowed to react with NADH at 25°; kinetics were followed using a Beckman DB spectrophotometer with a Heath Model EU-20V recorder. All kinetic runs were second order and analysis was performed by linear regression analysis.

Enzyme Substituent Effects. Kinetics of the hydride transfer step (the first of the two steps in the reaction with enzyme; see McFarland and Bernhard, 1972) was followed at 330 nm using both NADH and [^{2}H]NADH.

All enzyme kinetic studies with [^{2}H]NADH were carried out on a Durrum stopped-flow spectrophotometer using a tungsten lamp as a light source and air actuated pushing device. NADH studies were carried out as reported previously (McFarland and Bernhard, 1972). In all cases the rates of the first and second kinetic processes were sufficiently separated to allow visual determination of the infinity point for the first reaction. All rate constants reported are averages of at least two kinetic determinations; each kinetic rate constant was calculated using least-squares procedures, and errors in individual rates are ranges in the rate constants of the determinations. Double reciprocal plots of individual rate constants as a function of aldehyde concentrations were prepared; errors reported in slope and intercept of these plots are standard deviations from the linear regression procedure.

Results

Model Systems. In order to decide what a normal electronic substituent effect might be, we studied two model systems. The first was a typical organic hydride transfer reaction, reduction of substituted aldehydes by sodium borohydride. The results for four aromatic aldehydes are shown in Table I. There is a substituent effect of 100 on going from the slowest, *p*-methoxybenzaldehyde, to the fastest, *p*-chlorobenzaldehyde. This corresponds to a Hammett reaction constant $\rho = 4.7$.

In order to establish whether this large substitutent effect would also be apparent with NADH as a donor, and if reaction

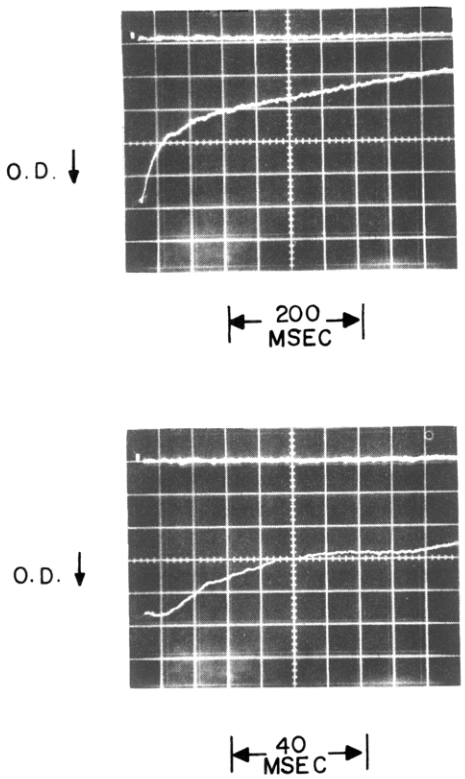


FIGURE 1: Optical density changes during transient kinetic oxidation of [^{2}H]NADH by *p*-methoxybenzaldehyde: [liver alcohol dehydrogenase] = 1×10^{-5} N; [[^{2}H]NADH] = 5×10^{-5} M; [pyrazole] = 0.02 M; [*p*-methoxybenzaldehyde] = 2.6×10^{-4} M.

of an electron acceptor with NADH would be an ionic (hydride transfer) rather than a radical process, we investigated the reaction of NADH with several benzoquinones. The results are shown in Table II. There is a large substituent effect indicative of an ionic reaction; also change to a less polar solvent slows the reaction (also indicative of an ionic process). The isotope effect data require some explanation; the uncorrected isotope effects are multiplied by two since the deuterated NADH is specifically deuterated while the model reaction would have equal probability of removing deuterium or protium depending on the side of attack on the coenzyme. Corrected isotope effects are included in Table II.

TABLE II: Reaction of Substituted Quinone with NADH (pH 7.0, 0.1 M Phosphate Buffer).

	[Quinone] (M)	k_2 (l./(mol min))
2-Methyl-1,4-benzoquinone	1.7×10^{-5}	63.5
2-Chloro-1,4-benzoquinone	1.7×10^{-5}	2736
1,4-Benzoquinone	1.7×10^{-5}	268
[NADH] = 7.2×10^{-5}		

Isotope Effects

	[Quinone] (M)	k_H/k_D	Corrected
2-Methyl-1,4-benzoquinone	1.7×10^{-5}	1.67	3.4
2-Chloro-1,4-benzoquinone	1.7×10^{-5}	1.51	3.0
[NADH] = 7.2×10^{-5}			

Solvent Effects

	[Quinone] (M)	% Isopropyl Alcohol	k_2 (l./(mol min))
[1,4-Benzoquinone]	1.7×10^{-4}	0	203
[NADH]	6.3×10^{-5}	20	173
		40	135

Results of our transient kinetic experiment with enzyme are shown in Figure 1; the kinetics consist of two first-order processes. The first of these processes has a primary deuterium isotope effect and thus represents hydride transfer. It is the substituent effect on this first rate process which we have determined. The substituent effect for the enzyme reaction is shown in Table I for NADH oxidation. Equivalent substituent effects for the same substrates are shown in Table I and Figure 2 using [^{2}H]NADH as a coenzyme. The experiments were repeated with [^{2}H]NADH because the hydride transfer rates for NADH itself were too large to allow determination by stopped-flow techniques under conditions of substrate saturation. We have therefore taken advantage of the deuterium isotope effect of 2.5 on the hydride transfer rates to slow the saturated rates to easily observable values. Figure 3 illustrates the saturation behavior of the rate of hydride transfer as a function of benzaldehyde concentration. It is the saturated rates which are reported as $k_{1,max}$; these indicate only a very small substituent effect, unlike our model systems.

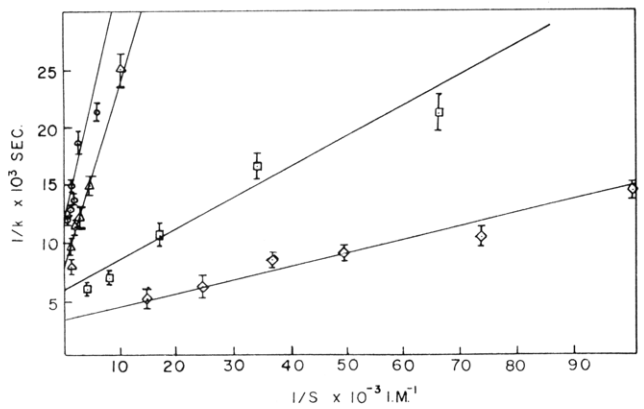


FIGURE 2: Substituent effect on hydride transfer: [liver alcohol dihydrogenase] = 1×10^{-5} N; [[^{2}H]NADH] = 5×10^{-5} M; [pyrazole] = 0.02 M; (⊙) *p*-methoxybenzaldehyde; (△) benzaldehyde; (⊡) *p*-chlorobenzaldehyde; (◇) β-naphthaldehyde.

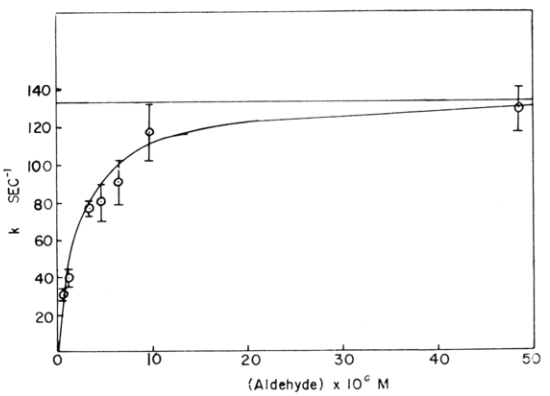


FIGURE 3: Dependence of hydride transfer rate on benzaldehyde concentration: [liver alcohol dehydrogenase] = 1×10^{-5} N; [[^{2}H]NADH] = 5×10^{-5} M; [pyrazole] = 0.02 M.

The second kinetic step during reduction of aldehydes by NADH catalyzed by liver alcohol dehydrogenase also has a small substituent effect as shown in Table III. This rate process

TABLE III: Substituent Dependence of k_2.

Substrate	V_{max}/E_0[a] (sec^{-1})	k_2[b] (sec^{-1})
Benzaldehyde	3.3	2.0
p-Chlorobenzaldehyde	2.2	1.2
p-Methoxybenzaldehyde	3.1	2.5
β-Naphthaldehyde	0.5	0.6
Azoaldehyde	1.6	1.1

[a] From steady-state kinetic experiments. [b] k_2 is the first-order rate constant for the second kinetic step in aldehyde reduction.

has no primary isotope effect and is independent of substrate concentration. It is most probably a rate associated with the dissociation of substrate from the enzyme surface. The rate constants for the second kinetic step and steady-state turnover are quite similar (Table III); the steady-state rate for liver alcohol dehydrogenase is known to be limited by substrate or coenzyme dissociation (Wratten and Cleland, 1963). Furthermore, the rate of formation of inactive enzyme–NAD$^+$–pyrazole ternary complex in our single turnover experiments occurs at the rate of the second kinetic process even though the rate of formation of the complex in the absence of substrate is ~100 sec^{-1} (McFarland and Bernhard, 1972). This also serves to establish the second rate constant as dissociation of substrate from the enzyme surface.

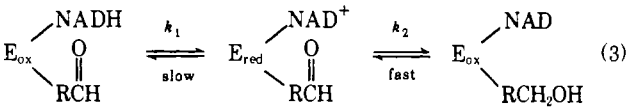
Discussion

As expected the model systems for hydride transfer show a large substituent effect. In the case of borohydride reduction the rate changes by ~100 in going from the least electron-deficient carbonyl carbon (*p*-methoxybenzaldehyde) to the most electron-deficient carbonyl carbon (*p*-chlorobenzaldehyde), Table I. Since reduction is attack by a nucleophile (hydride) on a positively charged carbonyl carbon, the rate of attack should depend on the positive charge at the carbonyl carbon, becoming faster as the positive charge increases (*p*-chlorobenzaldehyde) and slower as it decreases (*p*-methoxybenzaldehyde).

The oxidation of NADH by substituted quinones also appears to involve an ionic process (hydride transfer) rather than a radical reaction. Evidence suggesting an ionic transition state is the large substituent effect, the decrease in reaction rate with a decrease in solvent polarity, and the fact that a radical scavenger, hydroquinone, does not affect the reaction rate (Table II). Again, as in the case of borohydride reduction, the rate of hydride transfer from a nicotinamide coenzyme to an acceptor is sensitive to the degree of positive charge at the carbonyl carbon. Therefore, our model systems would lead us to predict that a bimolecular hydride transfer from NADH to aldehyde on the enzyme surface should be very sensitive to the degree of positive charge at the carbonyl carbon, *i.e.*, to electronic effect of substituents.

The substituent effect observed for the enzyme-catalyzed reactions of para-substituted benzaldehydes does not meet our expectation for bimolecular reaction, with the enzyme acting only to bind the substrate. The electronic substituent effect is no greater than twofold on going from *p*-methoxy- to *p*-chlorobenzaldehyde (Table I, Figure 2). That is, the reaction catalyzed by enzyme is much less sensitive to substituents adjacent to the carbonyl carbon atom than is expected for simple bimolecular reaction. On the other hand we believe that there is a measurable substituent effect, *i.e.*, the differences we observe in the rates of reaction between *p*-methoxybenzaldehyde and *p*-chlorobenzaldehyde are significant. One indication that this is indeed correct is that with the exception of the structurally different β-naphthaldehyde, the substituent effects, although much reduced, are in the direction of increased rates of hydride transfer with increasing positive charge at the carbonyl function.

Our observation of a small electronic substitutent effect on the reduction of substituted benzaldehydes eliminates several mechanistic possibilities. These results are not consistent with simple bimolecular transfer of hydride from NADH to aldehyde since our model studies suggest this process would show a much larger substituent effect. The results are also inconsistent with rate-limiting transfer of hydride from NADH to enzyme, k_1, followed by rapid reduction of aldehyde with reduced enzyme, k_2, eq 3. If this were the case, we would ex-



(3)

pect no substituent effect because aldehyde reduction would occur after the rate-limiting process. We cannot rule out a mechanism in which the first step, k_1, *i.e.*, reduction of enzyme, is fast and hydride transfer from enzyme to aldehyde, k_2, is rate limiting.

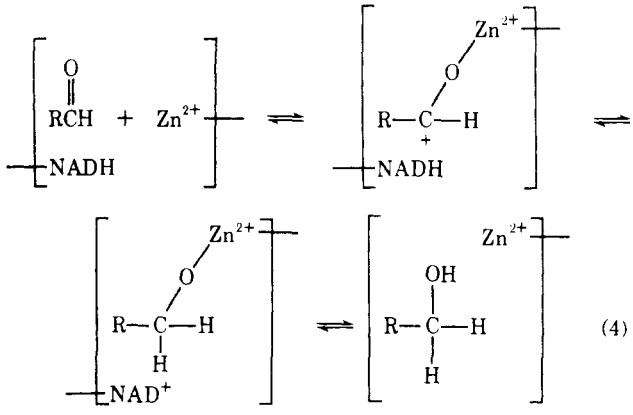
Ordinary aldehydes and ketones do not react with NADH in the absence of enzyme; however, thiobenzophenones react with dihydropyridine with a rate of 2.025 l. M^{-1} sec^{-1}. This may be the result of the increased positive charge at the thioketone carbon (Abeles *et al.*, 1957) as evidenced by the greater dipole moment (3.4) of thiobenzophenone (Hunter and Partington, 1933) compared to 2.95 for benzophenone (Donle and Volkert, 1930).

The substituent effect observed in the case of reaction of a dihydropyridine with substituted thiobenzophenones is 15 on going from a *p*-Cl to a *p*-OMe substituent; this is much reduced from the 100-fold substituent effect on borohydride reduction of substituted benzaldehydes and similar large substituent effects in other carbonyl addition reactions. It seems likely that this is the effect of the increase in positive charge at the thioketone carbon over that at the carbonyl carbon in simple aldehydes. Such an increase would be expected to enhance the reactivity of the carbonyl carbon reducing its sensitivity to electron-withdrawing or -releasing substituents.

Such a reduced substituent effect is consistent with arguments based on the Hammond postulate (Hammond, 1955). Increasing positive charge should reduce the activation energy of the reaction, shifting the transition state along the reaction coordinate toward starting material. Such an effect would reduce the amount of conjugation between the aromatic and carbonyl groups; since this conjugation is responsible for the substituent effect, such a change in transition state structure should lead to reduced substituent effects. Another system showing such behavior is the acid-catalyzed addition of semi-

carbazide to substituted benzaldehydes. The slope of a Hammett σ–ρ plot is $\rho = 0.9$ at pH 1.75, but a similar plot at pH 3.9 shows a much larger substituent effect with $\rho = 4.1$. Again this can be expected to result from the increased positive charge at the carbonyl carbon in the protonated species such as $RC^+(OH)H$ which are present in larger concentration at the lower pH (Jencks and Anderson, 1960). In general, then, anything which decreases electron density at the carbonyl carbon (such as acid catalysis) can be expected to reduce the effect of substituents on electrophilic reaction at the carbonyl carbon. The only observed case of reaction of an aldehyde with a dihydropyridine is the result of complexation of the aldehyde with Zn^{2+}. This complexation with a Lewis acid presumably increases the positive charge at the carbonyl carbon.

Accordingly, one mechanism consistent with our small substituent effects is one in which Zn^{2+} acts as a Lewis acid catalyst increasing the electrophilicity of the carbonyl carbon and decreasing its sensitivity to the electron-withdrawing or -donating substituent, eq 4.



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In conclusion we have observed an unexpectedly low substituent effect during reduction of para-substituted benzaldehydes by NADH catalyzed by liver alcohol dehydrogenase. One mechanism consistent with our findings is Zn^{2+}-catalyzed transfer of hydride to the positively charged carbonyl of the enzyme-bound zinc complex.

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