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THE EFFECT OF PREMIXING ON THE OXIDATION OF ETHANOL BY LIVER ALCOHOL DEHYDROGENASE

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SUMMARY

Contrary to our predictions, premixing of enzyme with NAD has little effect on the transient phase of the oxidation of ethanol by liver alcohol dehydrogenase. This observation has led us to re-examine the theory of premixing effects and to consider the consequences of this theory for the reduction of aldehydes by alcohol dehydrogenase.

Hijazi and Laidler [1, 2] have recently carried out a theoretical analysis of the transient kinetics of two substrate enzyme systems, including the effects of premixing. We have used this analysis [2], with the published rate constants [3–5], to predict the effect of premixing on the oxidation of ethanol by horse liver alcohol dehydrogenase (alcohol:NAD oxidoreductase, EC 1.1.1.1) at alkaline pH. This prediction was tested experimentally on a stopped-flow spectrophotometer.

Our prediction was based on the Theorell–Chance mechanism:

$$E \underset{k_{-1}}{\overset{k_1[A]}{\rightleftharpoons}} EA \xrightarrow{k_2[B]} \underset{+X}{EY} \xrightarrow{k_3} E + Y \qquad (1)$$

where A, B, X and Y represent NAD, ethanol, acetaldehyde and NADH, respectively. The transient reaction could perhaps be more accurately represented by the ordered ternary complex mechanism because the hydrogen transfer step is the slowest step of the transient phase [3, 6]. However treatment according to this mechanism is more difficult and the effect of premixing is the same [2].

The values of the rate constants at pH 9.0, defined in terms of Eqn 1, are given in Table I. The Theorell–Chance approximation requires that the rate constant for the hydrogen transfer step [3] is used for $k_2[B]$. These values were substituted into the equations [1, 2] for the formation of the first product for the case with enzyme concentration limiting. When enzyme is mixed at zero time with ethanol and NAD, the resulting equation is:

$$[X] = 4.83\,[E]_0\,t - 0.10\,[E]_0\,(1 - e^{-1722t}) + 1.04\,[E]_0\,(1 - e^{-163t}) \qquad (2)$$

TABLE I

INDIVIDUAL RATE CONSTANTS FOR THE OXIDATION OF ETHANOL BY ALCOHOL DEHYDROGENASE

Values of the rate constants of Eqn 1 used in this work. At pH 9.0, 25 ± 2 °C, ionic strength 0.1, [NAD] = 2 mM.

Rate constant	Value	Unit	Reference
k_1	0.85	$\mu M^{-1} \cdot s^{-1}$	4
$k_1]A]_0$	1700	s^{-1}	
k_{-1}	20	s^{-1}	4
$k_2[B]_0$	160	s^{-1}	3
k_3	5*	s^{-1}	5

* At pH 8.0. Dalziel [4] has given a value of 4 s^{-1} at pH 9.0 but this small difference has little effect on the transient phase.

If enzyme is premixed with NAD, the rate equation becomes:

$$[X] = 4.83\,[E]_0\,t + 8.60\,[E]_0\,(1 - e^{-1722t}) - 7.42\,[E]_0\,(1 - e^{-163t}) \quad (3)$$

These equations predict that the two transient phases, with rate constants of 1722 s^{-1} and 163 s^{-1}, should differ in both sign and magnitude between the two premixing conditions. Since the stopped-flow instrument has a dead-time of about 2–3 ms, the faster phase will be complete before observation commences and therefore the observed transient will correspond only to the slower phase. This transient should have a rate constant of 163 s^{-1} and show a change in magnitude of 7.1-fold on premixing*. A similar treatment using the published data for pH 8.0 [3–5] again leads to the prediction that the sign of the observed transient will change on premixing although the change in magnitude should only be about 2-fold.

The observation of a negative burst on the stopped-flow, as predicted by Eqn 3, would provide a dramatic confirmation of premixing effects. We therefore studied the pre-steady state phase of the oxidation of ethanol by alcohol dehydrogenase. The reaction was followed at 328 nm, the isosbestic point for free and bound NADH [7]. Hence the observed absorbance was a measure of the total concentration of Y and EY; it can easily be shown that this is always equal to the concentration of the first product, X. Reactions were carried out on a Durrum-Gibson stopped-flow spectrophotometer using horse liver alcohol dehydrogenase from Worthington Biochemical Corp. (lot HLADHL 2JA, 2.9 units/mg) and NAD from Sigma Chemical Corp. (Grade III) in phosphate–glycine buffer [4] (pH 9.1, ionic strength 0.1) or phosphate buffer (pH 7.9, ionic strength 0.1).

Oscilloscope traces of typical reactions at pH 9.1 for the two premixing conditions are shown in Fig. 1. The rate constant for the transient phase is 150 ± 20 s^{-1}, in agreement with the published data [3]. The sign of the observed transient is obviously unchanged by premixing and the magnitude decreases by about 15%. Accurate determination of the burst size is hindered by the fact that up to 40% of the burst is

* The total transient amplitude, including the hidden portion, will, however, be approximately the same for the two premixing conditions.

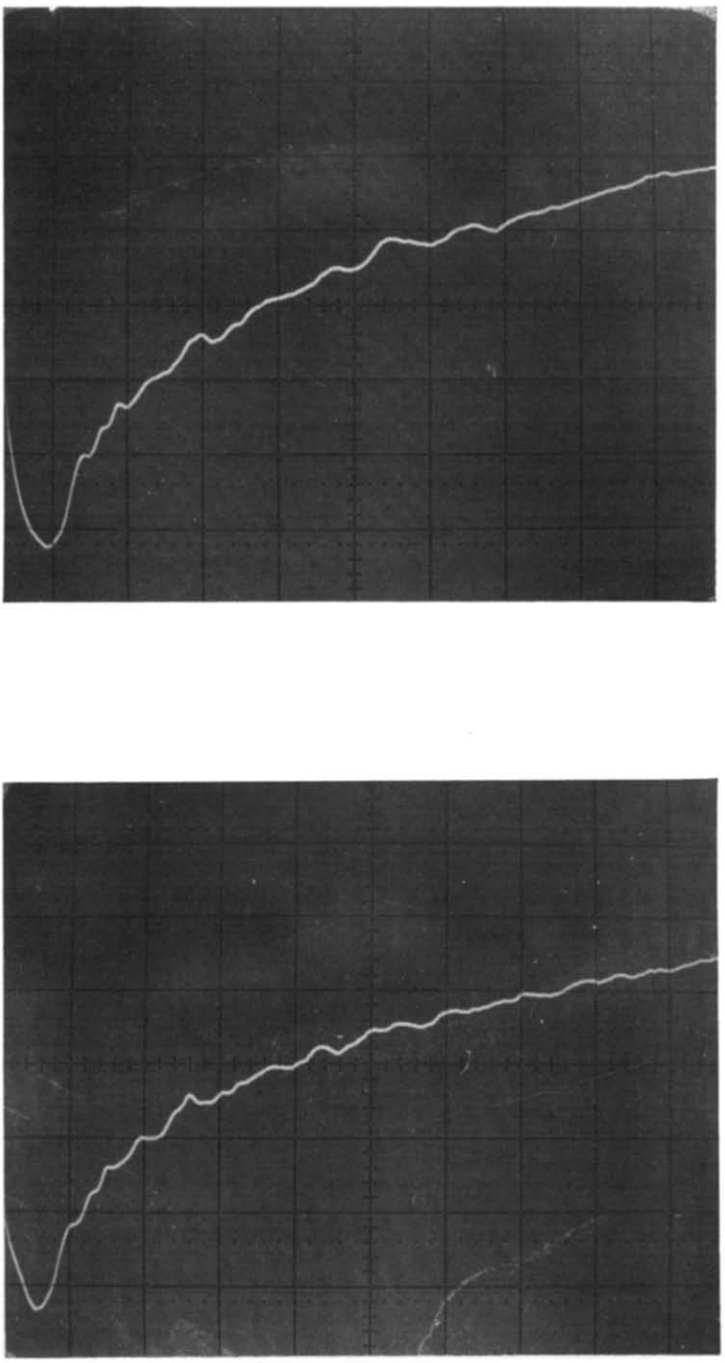

Fig. 1. Time course of the absorbance change at 328 nm during oxidation of ethanol by alcohol dehydrogenase at pH 9.1. Vertical scale: 0.01 absorbance unit per div, horizontal scale: 5 ms per div, time constant: 0.5 ms. (a) Syringe 1: 4 mM NAD and 0.2 M ethanol, syringe 2: enzyme (1.9 mg/ml). (b) Syringe 1: 0.2 M ethanol, syringe 2: enzyme (1.9 mg/ml) and 4 mM NAD.

complete within the dead time of the instrument. However it is clear that the observed behaviour is markedly different from that predicted by Eqns 2 and 3. The effect of premixing at pH 7.9 was found to be similar to that at pH 9.1. We conclude that this theoretical treatment does not predict the observed effect of premixing at either pH and we have therefore re-examined the derivation of the equations for the effect of premixing.

THEORY OF PREMIXING EFFECTS

The basis for the treatment of premixing effects is that [EA] at the time of mixing is not zero, as the equilibrium between E, A and EA is already established. Therefore the derivation must take into account the initial condition:

$$[EA] = \frac{k_1[E][A]}{k_{-1}} \tag{4}$$

Since our experiments were carried out with enzyme concentration limiting ($[A]_0 = 2$ mM, $[E]_0$ is approx. 15 μM) we have considered only this situation. In the published treatment [2] for this case the term [E] in Eqn 4 is treated as identical to the total enzyme concentration $[E]_0$ in the equation [1] for the formation of EA. This is incorrect since under these conditions the concentration of free enzyme at zero time [E] is less than the total enzyme concentration $[E]_0$ by [EA]. Since $[A] \approx [A]_0$, an initial condition in which the concentration of EA at the time of mixing, $[EA]_i$, is expressed in terms of $[E]_0$ and the total concentration of A, $[A]_0$, can be obtained from Eqn 4:

$$[EA]_i = \frac{k_1[A]_0[E]_0}{k_1[A]_0 + k_{-1}} \tag{5}$$

The use of Eqn 5 in the derivation of the equations for the formation of the products leads to an amplitude factor for premixing of

$$1 - \lambda_i/(k_1[A]_0 + k_{-1})$$

This factor should replace the factor $(1 - \lambda_i/k_{-1})$ in the published equations [2]. The rate equation for enzyme premixed with NAD then becomes

$$X = 4.83\,[E]_0\,t + 0.0001\,[E]_0\,(1 - e^{-1722t}) + 0.94\,[E]_0\,(1 - e^{-163t}) \tag{6}$$

A small decrease, about 10%, in the size of the slower transient is predicted by Eqns 2 and 6. This is in reasonable agreement with the observed behaviour.

As an additional check on the validity of our treatment, we have carried out digital analogue simulation, using an extended precision CSMP package on an IBM 1130 computer. Simulated progress curves are shown in Fig. 2 for the two premixing conditions. The curves are almost identical after the steady state is reached (about 35 ms) and therefore the total amplitudes of the transient phases are equal. When E and A are premixed, the transient is essentially composed of a single phase (cf. Eqn 6) but in the absence of premixing there is an additional lag phase for the first 2–3 ms, cor-

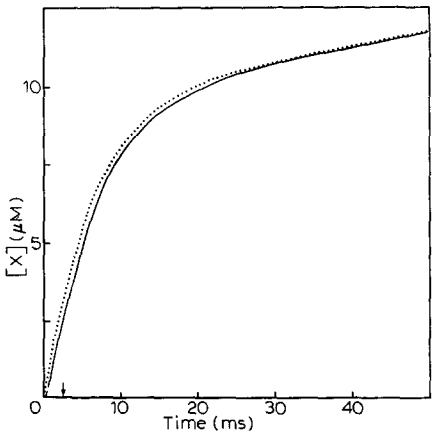

Fig. 2. Computer simulation of the effect of premixing on the oxidation of ethanol by alcohol dehydrogenase. The time course of formation of X was simulated for the Theorell–Chance mechanism, Eqn 1, with the rate constants in Table I. Integration interval = 0.1 ms. Initial conditions: ———, [E] = 10 μM, [EA] = 0; · · · · · ·, [E] = 0.12 μM, [EA] = 9.88 μM (calculated from the binding constant $k_{-1}/k_1[A]_0$). The arrow on the time axis indicates the point at which observations on a stopped-flow instrument commence.

responding to the faster transient in Eqn 2. Therefore the progress curves differ slightly until the steady state is reached. These simulated curves indicate that the burst size measured after the instrument dead time would be about 10% less when E and A were premixed. The lag phase would be complete within the dead time and not directly observable.

The simulation experiment is therefore consistent with the experimental evidence shown in Fig. 1 and with our modification of the theoretical treatment, as exemplified in Eqns 2 and 6.

THE REDUCTION OF ALDEHYDES BY ALCOHOL DEHYDROGENASE

Stopped-flow studies of the reduction of aromatic aldehydes by alcohol dehydrogenase have shown [8, 9] that the amplitude of the observed transient is reduced to a value corresponding to about one half the concentration of enzyme sites on premixing of enzyme and NADH. It has been suggested [2] that this behaviour is consistent with a simple Theorell–Chance mechanism.

In this report we have shown that the amplitude factor for the effect of premixing is $1 - \lambda_i/(k_1[A]_0 + k_{-1})$, where $[A]_0$ is the total concentration of NADH in this case. A reduction in amplitude of one half on premixing could only arise in an independent subunit model by a fortuitous combination of rate constants that gives a value of λ equal to $\frac{1}{2}(k_1/[A]_0 + k_{-1})$. Since a half burst was observed over the pH range 7.0–8.8 [9], where k_1 for NADH changes 2.5-fold [4], and over a 10-fold range of $[A]_0$ ($[A]_0 > [E]_0$), this explanation is clearly improbable*.

We therefore believe that treatment of premixing effects according to the

* The general argument against explanations of this type has been outlined by Luisi and Favilla 9].

Theorell–Chance mechanism does not explain the observed behaviour [8, 9] and that it may be necessary to assume kinetic non-equivalence of the subunits of alcohol dehydrogenase, as originally suggested [8, 9].

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