Mechanistic Studies on Horse Liver Alcohol Dehydrogenase. The Influence of the Different Premixings on the Transient Kinetics of Aldehyde Reductions†

Pier Luigi Luisi* and Roberto Favilla

ABSTRACT: The transient kinetics of the reduction of benzaldehyde and acetaldehyde catalyzed by horse liver alcohol dehydrogenase is investigated with the stopped-flow technique in a wide pH range and under different premixing conditions. It is found that for both substrates the rate of the transient process is much accelerated when enzyme and coenzyme are premixed, with respect to the other sets of premixing conditions (enzyme unpremixed or premixed with aldehyde). This is taken as an indication that the coenzyme produces an activation of the enzyme probably *via* a slow isomerization of the ternary complex. Direct evidence is presented that, for benzaldehyde in the lower pH range (pH <8), under moderate excess of coenzyme over the enzyme, recycling of active enzyme is rate limited by the activation induced by the coenzyme binding. In the case of the reduction of benzaldehyde, the kinetic nonequivalence of the enzyme subunits and the cooperative subunit interaction in the ternary complexes of the enzyme, are found to be general characteristics of the enzyme, independent of pH, buffer, temperature, and concentration of reagents. Similarities and differences in the transient kinetics of benzaldehyde and acetaldehyde are discussed. An attempt is made to accommodate in a unique kinetic scheme the transient data obtained for benzaldehyde and acetaldehyde, as well as the Michaelian character of the enzyme, the nonequivalence of subunits, and the activation process brought about by the coenzyme binding. It is shown that a reliable possibility is a reciprocating subunit model, which allows for a half- or a full burst, according to whether the process regulating the subunit reciprocation is slower or faster than the reduction of substrate bound to the functional subunit.

Bernhard and coworkers recently investigated the reduction of some aromatic aldehydes catalyzed by horse liver alcohol dehydrogenase (EC 1.1.1.1) with stopped-flow techniques (Bernhard *et al.*, 1970). The results, obtained both under conditions of limiting enzyme concentration ([E] < [NADH], [S]), and under conditions of limiting substrate concentration ([NADH], [E] > [S]), led to the conclusion that the two subunits constituting the enzyme were kinetically nonequivalent. Later, the reduction of an aromatic nitroso compound, investigated with the same technique, was shown to be consistent with this picture (Dunn and Bernhard, 1971).

The claimed nonequivalence of the two enzyme active sites seems to be, however, conflicting with all the kinetic data which had been accumulated through the years with classical steady-state analysis (Theorell and Chance, 1951; Wratten and Cleland, 1965; Shore and Theorell, 1966). In fact, horse liver alcohol dehydrogenase obeys Michaelian kinetics, and coenzyme binding studies (Ehrenberg and Dalziel, 1958; Theorell and Winer, 1959; Geraci and Gibson, 1967; Luisi and Favilla, 1970) failed to reveal any cooperativity or any other source of induced or preexisting dissymmetry between the two subunits. Furthermore, the transient data obtained by Dutler (1970) for the reduction of cyclohexanone and by Shore and Gutfreund (1970) for the reduction of acetaldehyde could be explained assuming that the two enzyme subunits were kinetically identical.

As the data of Bernhard and coworkers refer to a relatively small alkaline pH range and to a single kind of buffer (most of the data refer to pH 8.75 pyrophosphate buffer, 0.05 M), it could be thought that the corresponding data were valid only under that particular set of conditions and therefore not of general validity.

In this paper it will be shown, however, that benzaldehyde behaves according to the model described by Bernhard and coworkers in a wide pH range and in a variety of different conditions. The aim of this paper is also to elucidate the influence of the different premixings on the transient kinetics, and to discuss the possible models which can accommodate both the transient data obtained for aromatic and aliphatic aldehydes and the classical steady-state analyses.

Materials and Methods

Enzyme Preparation. The enzyme was purchased from Boehringer and Söhne and prepared as previously reported (Luisi and Favilla, 1970). Most of the experiments were repeated with enzyme purified on a carboxymethylcellulose column under conditions that, according to recent literature data (Bush *et al.*, 1971), give the homogeneous "EE isoenzyme" (Pietruszko *et al.*, 1969). The same results were obtained with the different enzyme preparations. The enzyme activity was measured according to Theorell and Yonetani (Theorell and Yonetani, 1963), and found to range between 85 and 100% on the basis of an extinction coefficient of 3.53×10^4 cm^{-1} M^{-1} at 280 nm (Dalziel, 1957). The ratio of the 280- to 260-nm absorbancies in our preparation ranged between 1.40 and 1.44.

Coenzyme. NADH was purchased from Boehringer and Söhne (purity degree 1) and used without further purification. The amount of coenzyme enzymatically oxidable was checked each time before the experiments, using a catalytic amount of enzyme and large excess of acetaldehyde at pH 7.0. A molar extinction coefficient of 6.22×10^3 cm^{-1} M^{-1}

† From the Technisch-Chemisches Laboratorium der ETH, Zurich, Switzerland. *Received October 20, 1971.* This is the fourth of a series of manuscripts. See Bernhard *et al.* (1970); Dunn and Bernhard (1971); McFarland and Bernhard (1972).

TABLE 1: Transient Kinetics of the Reduction of Benzaldehyde.

Expt No.	pH and Buffer	Reagent Concn E	NADH	S	Premixing Condn	Fast-Step Amplitude (μM)[a]	Observations	Rate of the Transient Processes[a,b] (sec^{-1})	Steady-State Parameters V_{max}[c]	K_m^S (μM)	K_m^R (μM)
1	pH 7.0 phosphate, 0.05 M	19	40	200	E + R	9	A first-order process follows the hidden half-burst	60	100	130	28
2		19	40	200		9		80			
3		19	22	2000		11		95			
4 (t = 3°)		19	22	2000		8		60			
5		19	14	200	E alone[f]	None	Single first-order exponential	65			
6		27	28	200				70			
7		17	20	2000				100			
8		19	50	2000			No burst observed	—			
9	pH 7.4 phosphate, 0.1 M	19	16	200	E + S	None	As in expt 5–7	55	65	N.d.	N.d.
10	pH 10, glycine–NaOH, 0.05 M	25	25[d]	2000	E + S	12	Biphasic pattern after the hidden half-burst	1.6[g]	8	N.d.	N.d.
11	pH 8.8, glycine–NaOH, 0.1 M	10	10[d]	200	E + R	5		10[g]	14	66	25
12	pH 8.0, Tris buffer, 0.1 M	15	12	200	E + R	6	As in expt 1–4	40	20	N.d.	N.d.
13	pH 8.8 pyrophosphate buffer, 0.1 M	18	100	200	E + R	9	Steady-state following the burst	—	14	66	26
14		50	80	36[d]	E + S	18	No hidden step[e]	80, 3.7[g]			
15		50	80	20[d]		10		80, 1.1[g]			
16		50	80	9[d]		~5		50, 0.7[g]			
17	pH 7.0 phosphate, 0.1 M	24	7	200	E alone[f]	None	As in expt 5–7	100	100	130	28

[a] ±10%. [b] Relative to the resolved first-order process(es). [c] Expressed in (moles of substrate/equiv of enzyme)sec^{-1}, obtained with saturating amount of NADH and benzaldehyde and catalytic amount of enzyme. [d] Concentration of limiting reagent converted into product. The initial concentration is somewhat larger. [e] The data have been elaborated as described in the legend of Figure 2. [f] E "alone" means that the enzyme has been not premixed with substrate or coenzyme. [g] Referred to the slowest step.

(Horecker and Kornberg, 1948) was used to determine the coenzyme concentration from the optical density change obtained at 340 nm.

As previously reported (Bernhard *et al.*, 1970) the addition of NADH to the enzyme produces a partial consumption of coenzyme. The origin of this very slow blank reaction is still under investigation, and it is not understood to date. All NADH concentrations, reported in this work for the experiments in which enzyme is premixed with coenzyme, refer to concentrations measured with the stopped-flow apparatus (with buffer in the second syringe) at the beginning of the experiment, a few minutes after the mixing of coenzyme with the enzyme. The optical density of the enzyme–coenzyme was remeasured at the end of each series of the experiments and was found to be constant during the short time (a few minutes) necessary to take a series of pictures (three–four).

Substrates. The aldehydes were purchased from Fluka at the highest degree of purity commercially available and freshly distilled before use. The concentration was determined on the basis of the following molar extinction coefficients (cm^{-1} M^{-1}): 8 at 277 nm for acetaldehyde; 1.41×10^3 at 279 nm for benzaldehyde. Some characteristic steady-state parameters for these two substrates are reported in Tables I and II.

Stopped-Flow Measurements. Stopped-flow measurements were performed with an Amico-Bowman rapid-mixing spectrophotometer. A Sorensen lamp power supply (Model QSB 28-8) was used to drive a 150-W iodide–tungsten lamp, and a high-voltage Fluka power supply (Model 415 B) was used to drive a highly sensitive EMI phototube (Model 9750 QB). The insertion of a Teledyne-Phylbrick logarithmic module (Model TP 4351) between the scope and the phototube allowed for the option of optical density readings. A similar device has recently been described by other authors (Di Franco and Iwatsubo, 1971). The dead time of the instrument was found to be 3–4 msec. In order to extrapolate back with more accuracy, it was found convenient to check the convergence point of two traces differing only in the time scale in the same picture (see, for instance, Figure 1b and Figure 2).

All measurements were made at 328 nm, the isosbestic point between NADH free and NADH bound to the enzyme (Theorell and Bonnichsen, 1951), and at room temperature (23 ± 1°), unless otherwise specified. All the concentrations of the reagents refer to concentrations after mixing in the stopped-flow mixing chamber.

Results

Transient Kinetics of Benzaldehyde Reduction. The first problem we will consider is whether the subunit nonequivalence observed in the reduction of aromatic aldehydes in pyrophosphate buffer (Bernhard *et al.*, 1970) holds for benzaldehyde in various buffers and pH values. We will deal now only with the case in which the enzyme is premixed with coenzyme in one of the two stopped-flow syringes. The influence of the different premixing conditions[1] will be considered later on in this paper.

Figure 1 shows experiments carried out with benzaldehyde at pH 7.0 phosphate buffer, 0.1 M. The fast step is so fast that it cannot be resolved with our present instrumentation. It is,

TABLE II: Transient Kinetics of the Reduction of Acetaldehyde.

Expt No.	pH and Buffer	Reagent Concn[a]		Premixing Condns	Fast-Step Amplitude (μM)[b]	Observations	Rate of the Transient Process (sec^{-1})[b,c]	Steady-State Parameter		
		E	NADH					V_{max}[d]	K_m^{Se} (μM)	K_m^{Re} (μM)
1	pH 8.0 phosphate, 0.1 M	14	8	E alone[f]	Negligible	Single first-order exponential	50	80	270	5.4
2		14	15				35			
3		15.5	18	E + R	12	Hidden burst followed by a first-order process	75			
4		15.5	30		16		65			
5	pH 10 glycine–NaOH, 0.1 M	16	7.5	E alone[f]	~3	Biphasic, no hidden step[g]	40; 4.2	8	800	2.1
6	pH 9.3 pyrophosphate, 0.1 M	16	23	E + R	12	As in expt 3–4	11	10	500	2.7
7		16	38		15		—			
8	pH 8.4 phosphate, 0.1 M	16	7	E + S	Negligible	As in expt 1–2	70	N.d.	330	4.5
9		16	14				50			
10[a]	pH 7.0 phosphate, 0.05 M	20	50	E alone[f]	8	As in expt 5[g]	40; 0.05	200	150	8
11[a]		20	50	E + R	8					

[a] In all these experiments the concentration of CH_3CHO is 3.5 mM, except for expt 10 and 11, where it is 17 μM (acetaldehyde limiting). [b] ±10%. [c] Relative to the resolved first-order process(es). [d] Expressed in (moles of substrate/equiv of enzyme)sec^{-1} obtained with saturating amount of acetaldehyde and NADH and catalytic amount of enzyme. [e] Extrapolated values from Sund and Theorell (1963). [f] "E alone" means that the enzyme is not premixed with substrate or coenzyme. [g] The data have been elaborated as indicated in the legend of Figure 2.

[1] Since in our stopped-flow instrumentation we have only two syringes, which bring two reagent solutions in the mixing chamber, and since with dehydrogenases we are dealing with three reagents, it is unavoidable that two of them are "premixed" in one of the syringes.

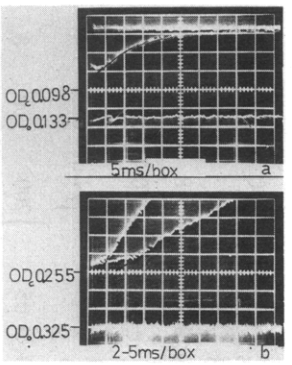


FIGURE 1: Reduction of benzaldehyde, pH 7.0 phosphate buffer, 0.1 M. Enzyme and coenzyme are premixed. [E] 19 μN, [S] 200 μN, [NADH] 20 μN (a), and 54 μN (b). In this picture, $OD_0 = (OD_{sub} + OD_{NADH} + OD_{enz})_{t=0}$, and OD_c is the optical density corresponding to the central line of the screen. The upper line corresponds to the optical density of the reacted mixture at equilibrium. Each box in the ordinate scale corresponds to 0.0232 ΔOD. Note that the amplitude of the (hidden) burst is independent of coenzyme concentration and corresponds to 0.052 $\pm$ 0.006 ΔOD (9 $\pm$ 1 μM, to be compared with a total enzyme concentration of 19 μN. Time on abscissa.

however, relatively easy to measure the amplitude of this "burst." This can be done by comparing the optical density at $t = 0$, (OD_0), with the optical density from which the trace of each picture actually starts, (OD_s). The difference $OD_0 - OD_s$ provides a measure of the amplitude of the burst. With the procedure of comparing OD_0 to OD_s we could obtain reliable and reproducible burst amplitude values up to a $[E]^2/[NADH]$ ratio of *ca.* 0.2. Under conditions of limiting enzyme (*i.e.*, [E] < [NADH], [S]), the amplitude of the burst is in every case independent of NADH concentration and corresponds to one half of the total enzyme active-site concentration.

This feature holds for benzaldehyde in a variety of buffers and pH values as shown in Table I. Furthermore, it is not affected by lowering the temperature. We carried out experiments at $T = 3°$, obtaining the same quantitative relationship in different buffers and pH values (see, for instance, experiment 4 in Table I).

Some experiments reported in Table I refer to conditions of excess coenzyme and substrate over the enzyme. Table I also reports a series of experiments carried out for benzaldehyde under conditions of enzyme premixed with coenzyme and [E] $\geq$ [NADH]. This set of conditions corresponds to "single transient experiments," *i.e.*, conditions in which the enzyme should not need to recycle in order to convert the limiting coenzyme into product. The importance of this kind of experiment stems from the fact that a single exponential is expected for the time course of the reaction if the enzyme behaves in the simplest possible way.

A sharp biphasicity, as that observed in the case of benzaldehyde, in the experiments in which enzyme and coenzyme are premixed (see Figure 1a) is in general diagnostic of some complexity of the enzyme mechanism. In our case we have to consider the important fact that the amplitudes of the fast and slow step, under conditions of [E] $\geq$ [NADH] and enzyme premixed with coenzyme, are equal. Though some experiments give a relatively high discrepancy (up to $\pm$20% with

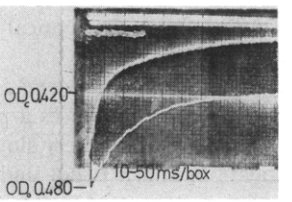


FIGURE 2: Single transient experiment for benzaldehyde reduction. Enzyme and benzaldehyde are premixed, pH 8.8 pyrophosphate buffer, 0.05 M, [E] 50 μN, [NADH] 80 μM, [S] converted (total) 19 μM. OD_0 and OD_c defined as in Figure 1. The rate of the slowest step is obtained reporting the whole course of the reaction as a first-order process, and measuring the ΔOD changes with time with respect to the upper line (equilibrium). The rate of the faster process has been obtained measuring the ΔOD changes with time with respect to the tangent to the final part of the 10 msec/box trace. The intersection of this tangent with the ordinate (ΔOD axis) gives a measure for the amplitude of the (first) faster step.

respect to the theoretical half-burst), the average burst amplitude among a very large number of experiments clearly indicates the reliability of the 1:1 relationship. This stoichiometric feature is independent of buffer, pH, and concentration of the three reagents, which convalidates the original hypothesis of Bernhard and coworkers (1970) that the biphasicity has to be ascribed to a nonequivalence of the enzyme subunits. Note from Table I that, in the experiments at pH 7.0 in which [E] $\geq$ [NADH] and enzyme and coenzyme are premixed, the slower step after the hidden half-burst is a single first-order exponential. This observation, however, does not hold in the whole pH range and for all sets of concentration. Under certain conditions, in fact, the resolved step following the hidden half-burst shows a marked biphasicity (pH 6.0 phosphate buffer or pH 8.8 pyrophosphate buffer with [E] $\simeq$ [NADH] $\simeq$ 15 μN are examples).

In the first paper of this series, some experiments were carried out under conditions of excess enzyme with respect to the limiting substrate, *i.e.*, [NADH] > [E] > [S]. It was was found that, in the case of aromatic aldehydes in pyrophosphate buffer (usually pH 8.75, 0.05 M), the limiting aldehyde was converted to product *via* two steps well separated in rate and having the same amplitude. The extensive investigations carried out in the present work for a wide range of pH and buffers confirms the complexity of the transient kinetics under conditions of substrate limiting only in the higher pH range. A typical experiment is reported in Figure 2 (in Table I a few more experiments are illustrated). Note that the relative amplitude of the fast and slow step are about equal, independent of the substrate-limiting concentration. The fact that benzaldehyde is not at saturating levels cannot be responsible for this complexity, as the characteristic (at least) biphasic pattern does not disappear by changing the concentration of benzaldehyde (Table I, experiments 13–16).

In the lower pH range, when the coenzyme is not premixed with enzyme, the time course of the reaction under conditions of benzaldehyde limiting obeys a single first-order exponential (Table I experiment 17).

Influence of the Different Premixing Conditions on the Transient Kinetics.[1] In the case of benzaldehyde in the lower pH range,[3] part of the transient kinetics is very much accelerated when enzyme is premixed with coenzyme, as opposed to the

[2] R = NADH, O = NAD, S = aldehyde, P = alcohol, E_2 indicates the enzyme dimeric molecule.

[3] We use the term "lower pH range" and "higher pH range" to indicate the pH range approximately below and above pH 8 for benzaldehyde and below and above pH 9 for acetaldehyde.

other two sets of premixing conditions. In fact, when enzyme is premixed with coenzyme, there is a fast (hidden) step that corresponds to one-half of the total equivalents of enzyme (half-burst, as discussed in the preceding section), whereas no biphasicity is apparent with the other two sets of premixings (see Table I). A first relevant question is whether the slower rates observed in the transient kinetics when enzyme and coenzyme are not premixed can be ascribed to the slow rate of coenzyme binding, when enzyme and coenzyme are not premixed. We can answer negatively to this question, on the basis of the following experimental observations.

(1) The time course of the reaction, when enzyme is not premixed with coenzyme, proceeds without a "burst" even at a coenzyme concentration as high as 50 μM. This indicates that the transient kinetics under the different premixing conditions does not tend to equalize simply by increasing the coenzyme concentration (Table I, experiment 8).

(2) When enzyme and coenzyme are not premixed under condition of [E] $\geq$ [NADH], the reaction follows a single first-order exponential. The rate of this process does not change by doubling the enzyme concentration with respect to the coenzyme, or by doubling the concentration of either enzyme or coenzyme (see Table I, experiments 5 and 6).

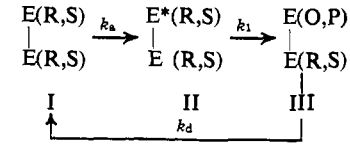
Actually, in the experiments under conditions of enzyme unpremixed with coenzyme, care was taken to use reagent concentrations such that the rate of coenzyme binding (Geraci and Gibson, 1967) would not interfere with the transient kinetics.

Since the rate of the coenzyme binding *per se* is not responsible for the observed difference in the transient kinetics with different premixings in the lower pH range, one has to assume that there is an enzyme activation brought about by the coenzyme binding, which limits the rate of the transient when enzyme and coenzyme are not premixed. This process is likely to be an intramolecular isomerization of the ternary complex. However, since the precise nature of this event is still far from clarified from a chemical viewpoint, we will refer to it with the more general expression "enzyme activation."

The fact mentioned under point 1, namely that no burst is observed when enzyme is not premixed with coenzyme under condition of excess coenzyme and substrate, is of some mechanistic meaning. Since, under those conditions, several enzyme recyclings are needed to complete the reaction, the absence of a burst indicates that the rate-limiting step for the enzyme turnover must be contained in the series of steps occurring during the single transient. The consequence of this is that the product dissociation cannot be the rate-limiting step for the enzyme turnover. The rate-limiting step for the enzyme recycling has to be the aforementioned slow enzyme activation brought about by the coenzyme binding. Note, however, that in these experiments the investigated range of enzyme and coenzyme concentration (enzyme between 10 and 30 μN and coenzyme between 10 and 50 μM) is not very large.

Scheme I,[4] in our opinion, is able to satisfy all the data thus far obtained for benzaldehyde at pH 7.0. In Scheme I, k_a represents the process of enzyme activation brought about by the coenzyme binding, and the asterisk (*) indicates the activated subunit; k_1 indicates the rate of reaction of this subunit (the burst) and k_d corresponds to the process of product dissociation.

SCHEME I



Basic assumptions of the model are that $k_1 > k_a$ and that the species I and III are "inactive." With this we mean that the species I should undergo the process of activation in order to react, and that III does not have the time to produce $E_2(O,P)_2$, as the competitive processes (dissocation of product and formation of I, and the process leading from I to II) are faster. The species I is formed when enzyme and coenzyme are not premixed, whereas when enzyme and coenzyme are premixed, we have already the species II when we start our experiments. In the latter case, a burst corresponding to one-half of the enzyme-active site is expected and further reaction will be regulated by k_a and no burst is observed.

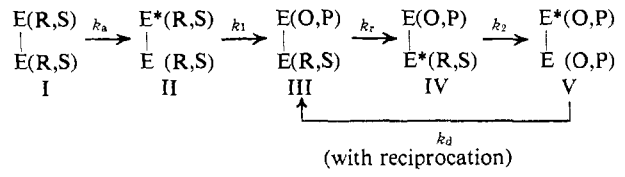
In the case of benzaldehyde in the higher pH range,[3] dramatic differences in the transient kinetics are also observed with the different premixing conditions. Again, when enzyme and coenzyme are premixed, part of the transient process is much accelerated with respect to the other two sets of premixing conditions. Two basic differences are found with respect to the analogous data obtained in the lower pH range. (1) In the single transient experiments, the time course of the reaction shows a marked complexity also when the enzyme is not preincubated with coenzyme (see Figure 2 and Table I, experiments (13–16). This complexity cannot be ascribed to a bimolecular binding process. (2) Under conditions of enzyme limiting, a fast step prior to the steady-state cycle is obtained also when enzyme and coenzyme are not premixed. This fast step can be resolved by our instrumentation when moderate concentration of reagents are used (enzyme and coenzyme around 10–15 μN). The apparent rate of this process increases by increasing the reagent concentration, and eventually it becomes so large that it cannot be resolved by our instrumentation (hidden half-burst).

In order to explain point 1 in terms of Scheme I, we can assume that above pH 8 the dissociation of product from III becomes the slowest step. In this case, when $k_1 > k_a > k_d$, a biphasic (or even a more complex) behavior can be obtained in the single transient experiments either starting from I or from II.

Case of Acetaldehyde. The data of transient kinetics for acetaldehyde obtained in the present work can be summarized as follows. (1) Under conditions of [E] $\geq$ [NADH] in the lower pH range,[3] a first-order exponential is obtained when moderate concentrations (up to 20 μN) of *unpremixed* enzyme and coenzyme are used (Table II, experiments 1, 2, 8, and 9). For the same sets of concentration, but with enzyme and coenzyme premixed, a very fast step (not resolved by our instrumentation) is obtained (Table II, experiments 3, 4, 6, 7). This difference in the transient kinetics with different premixing conditions cannot be ascribed to the rate of binding of coenzyme. It is instead indicative, as in the case of benzaldehyde, of some enzyme activation brought about by the coenzyme binding. (2) When moderate concentration of

[4] In this and the following scheme we will neglect, for the sake of simplicity, the reverse reactions, the presence of [H+], and the binding steps necessary for the formation of the ternary complexes. The unsaturated enzyme species have been neglected, under the assumption that the formation of I from enzyme, substrate, and coenzyme is a very fast process under the concentration conditions used in our experiments.

SCHEME II



enzyme and coenzyme are used, a profound influence of the premixing conditions on the transient kinetics is present also in the higher pH range. Here, however, when the enzyme is not premixed with coenzyme, the resolved time course of the reaction is biphasic (Table II, experiment 5). (3) The amplitude of the fast step when enzyme and coenzyme are premixed varies depending on the reagent concentration and pH, and a maximal "full burst" can be obtained (fast hidden step with an amplitude corresponding to the total enzyme active-site concentration) (Table II, experiments 4, 7). (4) When acetaldehyde is limiting a biphasic behavior is obtained (see Table II, experiments 10, 11). As it is apparent from this survey of data, some analogies, and also some differences, are found with respect to the case of benzaldehyde. The above-mentioned points 1, 2, and 4 hold for both substrates. However, the fact that the amplitude of the fast step is not constant under conditions of coenzyme limiting and premixed with the enzyme, and the fact that a full burst can be obtained, reflect characteristic and meaningful differences between the two substrates. The finding that acetaldehyde gives a full burst, and other features of the transient kinetics of acetaldehyde reduction under conditions of enzyme and coenzyme premixed, have been reported by other authors (Shore and Gutfreund, 1970).

Discussion

Proposed Model. The incongruity between the transient kinetics of acetaldehyde and benzaldehyde (for which respectively a full burst and a half-burst are obtained) is in our opinion the starting point for drawing a comprehensive kinetic pattern for horse liver alcohol dehydrogenase. We think that the mechanism of action of the enzyme must be the same for the different substrates, and that the difference observed in the transient for various aldehydes can only be ascribed to differences in the rate constants of the same chemical steps. Certain substrates, just because of the particular numerical relationship between the rates involved in the transient and in the enzyme recycling, are more suitable than others to elucidate the mechanism of action of enzymes. In this sense, the mechanistic peculiarities of horse liver alcohol dehydrogenase can be better defined in terms of some aromatic aldehydes, which act as the kinetic indicators of the nonequivalence of the enzyme subunits and of the cooperative subunit interaction.

The basic characteristic of Scheme I previously proposed for benzaldehyde is that the species III is not reactive, *i.e.*, the dissociation of product is faster than the process leading to the species $E_2(OP)_2$. If this last process were faster than all the other steps involved in the transient, a full burst could be obtained, even though the two subunits are kinetically nonequivalent.

In order to propose that this is indeed the case for acetaldehyde, one should provide a reasonable rationalization for the different behavior between benzaldehyde and acetaldehyde. At the same time, any proposed model should be consistent with the Michaelian character of the enzyme, and possibly show analogies with other enzyme mechanisms known in literature.

Lazdunski and coworkers recently wrote an interesting paper on alkaline phosphatase, proposing for this enzyme what they call "Flip–Flop Mechanism" (Lazdunski *et al.*, 1971). According to this model, the dimeric enzyme would work with an alternation of function of the two subunits. A "reciprocating subunit model" for another dimeric enzyme, pig heart malate dehydrogenase, already had been proposed by Wolfe and coworkers (Harada and Wolfe, 1968a,b). Bernhard and associates (Bernhard *et al.*, 1970) already alluded to the possibility that a reciprocating subunit model could be the mechanism of action of horse liver alcohol dehydrogenase. The relevant point for us about this kind of model is that the classical steady-state kinetic analysis would then fit with our transient kinetic data. In fact, as extensively discussed by Lazdunski and associates, a reciprocating subunit may obey Michaelis–Menten kinetics.

The characteristic feature of a subunit reciprocation model is that it contains a chemical step which separates the reaction at the two subunits. In particular, this chemical step allows for reaction at the "second" subunit once the "first" subunit has already undergone reaction. We will indicate with k_r the rate of this reciprocation process. There are actually several possible mechanisms that one can propose, once one has accepted this main hypothesis. One possibility is the following (Scheme II). According to this scheme, species III undergoes further reaction *via* activation in the "second" subunit. This activation is triggered by product formation in the "first" one. In turn, formation of product in the "second" subunit triggers dissociation of product from the "first" one. Under conditions of excess R and S, species III can be formed again, but the subunit that now contains (O,P) is the one that in the previous cycle contained (R,S) and *vice versa* (reciprocation). On the basis of Scheme II, the data for acetaldehyde would be explained assuming that $k_2 \geq k_1$ and $k_r > k_2$. A possible way for the enzyme recycling is V → III → IV → V, etc.

It seems reasonable to us that for the "natural" substrate (acetaldehyde), the process regulated by k_r (which is the most characteristic feature for the proposed enzyme mechanism), is faster than for aromatic aldehydes. This corresponds, in fact, to the assumption that the process of subunit reciprocation is blocked when aromatic pseudosubstrates are used. In this sense, aromatic aldehydes would be the reagents able to reveal an important mechanistic feature, that in the case of aliphatic aldehydes, is hidden because of the large k_r. Such a feature, furthermore, can be revealed only through transient kinetics, due to the Michaelian character of the steady-state kinetics of both aromatic and aliphatic substrates.

Further Substantiation for the Proposed Model. A few features of the proposed model are in agreement with the present knowledge of the mechanism of action of the enzyme. For instance, the enzyme activation found in this work, as a consequence of the coenzyme binding, is in keeping with the Theorell–Chance mechanism. As is well known, one of the aspects of the Theorell–Chance mechanism, set up long ago for liver alcohol dehydrogenase, assumes that the binding site for the substrate (aldehyde or alcohol) is "ready" only after binding of coenzyme to the enzyme (Theorell and Chance, 1951; Theorell, 1964, 1968).

The postulated dissymetrization of the two subunits (the process regulated by k_n in Schemes I and II) is in agreement

with the crystallographic data so far collected by Bränden. As it is well known, this author showed that the crystallographic molecular symmetry of the enzyme dimeric molecule is lost in crystals when coenzyme molecules are bound to the enzyme (Bränden, 1970). Isomerization of the enzyme–NADH complexes has been advocated by Shore and Gutfreund by investigating the transient kinetics of acetaldehyde reduction (Shore and Gutfreund, 1970).

The fact that no cooperativity has been found thus far for the binding of coenzyme to the enzyme is not inconsistent with the cooperativity postulated in the model. In fact, the subunit cooperative interaction can be operative only at the level of the ternary complexes when both substrate and coenzyme are bound to the active site.

The proposed model seems also in keeping with the "allosterism" advocated by Theorell on the basis of the rate of formation of ternary complexes in microcrystalline suspensions of the enzyme, on the basis of the crystallographic data for the binary complexes, and on the basis of fluorescence investigations (Theorell, 1968).

A question which deserves some further comment is whether other models can explain the data thus far presented.

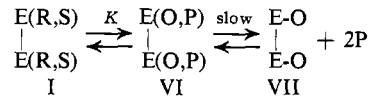
We wish to stress again that the equal amplitude of the fast and slow step, found in the experiments under condition of [E] $\geq$ [NADH] (enzyme and coenzyme premixed), is obtained independent of substrate and coenzyme concentration. Furthermore, this behavior is independent of temperature and pH. The same is true for the burst amplitude under conditions of enzyme limiting and premixture with coenzyme. These findings rule out, in our opinion, any model based on binary and ternary complexes of the enzyme in equilibrium with free ligands and/or H^+. Also any model, in which the stoichiometric features found for benzaldehyde are due to the casual interplay of rate constants, can be countered on the basis of the same argument: the amplitude of the burst and the relative amplitudes of the fast and slow step in the single transient experiments should in this case vary with the concentration of the reagents, at least at concentrations below saturation.

A model based on two forms of the enzyme in equilibrium (before or after the binding of reagents) regulated by an equilibrium constant near unity is also to be rejected. Aside from the lack of indications to this kind of phenomenon from electrophoretic or chromatographic measurements of binary and ternary complexes of the enzyme, this equilibrium constant should be insensitive to all the thermodynamic parameters that usually affect a chemical equilibrium, like temperature, pH, and structure of the substrates (consider in fact that β-naphthaldehyde, an azoaldehyde, and an aromatic nitroso compound are also characterized by a half-burst in the higher pH region, as illustrated in the first two papers of this series).

The following scheme,[5] in which the enzyme subunits behave as kinetically equivalent, could in principle explain our data.

If the equilibrium constant K between the transient species I and VI is near unity, and if the process leading to VI is faster than the process of disappearance of VI from equilibrium, a biphasic process is to be expected in the transient, with a fast step close to one-half of the concentration of species I initially present. The disappearance of VI from equilibrium could be due to product dissociation, as indicated in Scheme III. The lack of influence of pH in the amplitude of the fast step could be ascribed to the fact that the fast step is an intramolecular hydrogen transfer, for instance from the protein to the oxidized substrate. The lack of influence of the structure of the aldehydes could be explained assuming that K is controlled only by the NADH–NAD redox potential. The lack of influence of temperature on the relative amplitude of the fast and slow steps could be explained assuming that K varies only slightly in the investigated pH range. Alternatively, in order to smother the unreasonableness of the above assumption that the equilibrium constant between I and VI is several orders of magnitude different from the overall equilibrium constant, one could assume that an equilibrium constant near unity exists between the species VI and a successive fast-formed isomer VI .

SCHEME III



Though we accept the general concept that there is no reason to expect that an enzyme should behave according to the simplest possible mechanistic scheme, it seems to us that the series of assumptions that is needed in order to propose a model based on Scheme III, is by far less resaonable than the basic assumption of Scheme I (kinetic nonequivalence of the subunits due to cooperativity in the enzyme ternary complexes) in the reduction of benzaldehyde.

Our methodological assumption, that the feature found for benzaldehyde must be present also in the reduction of aliphatic aldehydes, is more questionable. We like to recall, however, the many analogies in the transient kinetics of acetaldehyde and benzaldehyde as pointed out in the preceding section; the fact that the proposed mechanism seems operative in other dimeric enzymes with their physiological substrates; and the fact that the proposed model seems to be the only one which is able to accommodate both the transient data obtained for benzaldehyde and acetaldehyde. A more detailed investigation of the transient kinetic of acetaldehyde however is certainly needed. The logical continuation of this work also requires a better understanding of the chemical meaning of the rate constants regulating the transient. In particular, the investigation of the saturation behavior of the rate of the fast and slow step with respect to the concentration of the three reagents, and a comparsion between the steady-state V_{max} and the rate of the transient processes should be of interest. Some indications could be inferred from the preliminary data reported in Tables I and II. We feel, however, that a more systematic investigation is needed in order to draw some reliable conclusions. For this reason we wish to delay discussion on the magnitude of rates to a subsequent paper.

Summary

(1) For both acetaldehyde and benzaldehyde, the coenzyme binding to the enzyme brings about an activation of the enzyme. (2) In the case of benzaldehyde reduction, product dissociation can be the rate-limiting step for the enzyme recycling only above pH 8. Below pH 8, the rate-limiting step for the enzyme recycling is the process of enzyme activation induced

[5] We would like to thank one of the Reviewers for pointing our attention to this type of model. This suggestion, together with other pieces of constructive criticism advanced by this Reviewer, have been extremely useful to the authors for designing new experiments and for clarifying some aspects of the problem.

by the coenzyme binding (at least when a moderate amount of coenzyme is used). (3) In the case of benzaldehyde reduction, the enzyme is characterized by subunit nonequivalence and by a high degree of cooperative subunit interaction in the active ternary complexes. (4) The necessity to postulate common mechanistic features for the enzymic reduction of different substrates bring us to the model represented in Scheme II. Characteristic of this model is the reciprocation of subunit function. The process regulating the reciprocation process is fast in the case of the natural substrate (acetaldehyde) and blocked in the case of aromatic pseudosubstrates.

Acknowledgments

This work has been initiated in the period that one of us (P. L. L.) spent in the laboratories of Dr. Sidney Bernhard in Eugene, Oregon. The guidance and suggestions of Dr. Bernhard have been of the utmost importance in carrying out this work. We would like to thank Dr. Michael Dunn (Riverside, Calif.) and Dr. Poul Schack (Lynbgy, Denmark) for their constructive criticism on this work. The technical assistance of K. Zeman of the Elecctronic Workshop of the Technisch-Chemisches Labor der ETH has been very useful in setting up our stopped-flow equipment. Finally, we would like to thank Professor P. Pino (ETH, Zurich) for his generous financial support and continuous interest in this work.

References

Bernhard, S. A., Dunn, M. F., Luisi, P. L., and Schack, P. (1970), *Biochemistry 9*, 185.

Bränden, C. J. (1970), *in* Pyridine Nucleotide-Dependent Dehydrogenases, Sund, H., Ed., Springer-Verlag, Berlin.

Bush, K., Mahler, H. R., and Shiner, V. J. (1971), *Science 172*, 478.

Cleland, W. W. (1964), *Biochemistry 3*, 480.

Dalziel, K. (1957), *Acta Chem. Scand. 11*, 396.

Di Franco, A., and Iwatsubo, M. (1971), *Biochemie 53*, 153

Dunn, M. F., and Bernhard, S. A. (1971), *Biochemistry 10*, 4569.

Dutler, H. (1970), *Proc. Plenary Sess. Int. Congr. Biochem., 8th*, 85.

Ehrenberg, A., and Dalziel, K. (1958), *Acta Chem. Scand. 12*, 465.

Geraci, G., and Gibson, Q. H. (1967), *J. Biol. Chem. 242*, 4275.

Harada, K., and Wolfe, R. G. (1968a), *J. Biol. Chem. 243*, 4123.

Harada, K., and Wolfe, R. G. (1968b), *J. Biol. Chem. 243*, 4131.

Horecker, B. L., and Kornberg, A. (1948), *J. Biol. Chem. 175*, 385.

Lazdunski, M., Petitclerc, C., Chappelet, D., and Lazdunski, C. (1971), *Eur. J. Biochem. 20*, 124.

Luisi, P. L., and Favilla, R. (1970), *Eur. J. Biochem. 17*, 91.

McFarland, J. T., and Bernhard, S. A. (1972), *Biochemistry 11*, 1486.

Pietruszko, R., Ringold, H. J., Li, T. K., Vallee, B. L., Akeson, A., and Theorell, H. (1969), *Nature (London) 221*, 440.

Shore, J. D., and Gutfreund, H. (1970), *Biochemistry 9*, 4655.

Shore, J. D., and Theorell, H. (1966), *Arch. Biochem. Biophys. 116*, 255.

Sund, H., and Theorell, H. (1963), *Enzymes 7*, 26.

Theorell, H. (1964), *Biochim. Biophys. Acta Lib. 4*, 147.

Theorell, H. (1968), *in* Molecular Association in Biology Pullman, B., Ed., New York, N. Y., Academic Press.

Theorell, H., and Bonnichsen, R. (1951), *Acta Chem. Scand. 5*, 1105.

Theorell, H., and Chance, B. (1951), *Acta Chem. Scand. 5*, 1127.

Theorell, H., and Winer, A. D. (1959), *Arch. Biochem. Biophys. 83*, 291.

Theorell, H., and Yonetani, T. (1963), *Biochem. Z. 338*, 537.

Wratten, C. C., and Cleland, W. W. (1965), *Biochemistry 4*, 2442.