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FLOW KINETICS OF YEAST ALCOHOL DEHYDROGENASE ATTACHED TO NYLON TUBING

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Summary

Yeast alcohol dehydrogenase (alcohol:NAD^+ oxidoreductase, EC 1.1.1.1) was attached covalently to the inner surface of nylon tubing, and the immobilized enzyme retained its activity over a period of months. A study was made of the flow kinetics for the reaction between ethanol and NAD. With the ethanol held at saturating concentrations there was partial diffusion control, the extent decreasing with increasing flow rate and increasing NAD concentration. With the NAD at saturating concentrations there was no appreciable diffusion control. The apparent Michaelis constants varied with flow rate v_f, being linear in $v_f^{-1/3}$, and extrapolation to infinite flow rate ($v_f^{-1/3} = 0$) gave the intrinsic Michaelis constants.

The inhibition by products was also studied. The results for both NADH and acetaldehyde showed mixed competitive and non-competitive inhibition, with a preponderance of the former. Acetaldehyde is the stronger inhibitor, and this is consistent with the lack of diffusion control with variable ethanol. Inhibition by acetaldehyde is not affected by flow rate, but inhibition by NADH is affected, presumably because of the greater degree of diffusion control with variable NAD.

Introduction

Tubes with enzyme attached to their inner surfaces are useful as open tubular heterogeneous enzyme reactors in industrial preparations [1—5]. They are also being used in clinical medicine for the automated analysis of metabolites [6—9] and in extracorporeal shunts to remove undesirable substances from the body [10]. Kinetic investigations of such systems are useful because of these practical applications, and also because they lead to models for under-

standing the function of enzymes in vivo, particularly of enzymes attached to the interior walls of blood vessels [11].

A number of experimental investigations of flow systems have been carried out in this laboratory [12—15] on the basis of a theoretical treatment developed by Kobayashi and Laidler [16] for single-substrate systems. Engasser and Horvath [17] have also developed a theory of single-substrate systems, and Engasser et al. [18] considered an extension to two-substrate systems with special reference to aspartate aminotransferase (L-aspartate:2-oxoglutarate aminotransferase, EC 2.6.1.1). They found that diffusional limitations are entirely responsible for the differences between the behaviour of free enzyme and enzyme bound to collagen. A complete theoretical treatment of two-substrate systems presents considerable difficulty and has not yet been developed. Daka and Laidler [19] carried out experimental work on the flow kinetics of lactate dehydrogenase (L-lactate:NAD^+ oxidoreductase, EC 1.1.1.27) chemically attached to nylon tubing. The substrates were pyruvate and NADH (the reduced form of NAD) and the experiments dealt with the two limiting cases: one substrate present in excess and the concentration of the other varied. These conditions permit interpretation on the basis of the Kobayashi-Laidler one-substrate treatment.

The present paper describes a similar investigation with yeast alcohol dehydrogenase (alcohol:NAD^+ oxidoreductase, EC 1.1.1.1), the substrates being ethanol and NAD. Alcohol dehydrogenase is of considerable interest in medicine, especially in relation to ethanol metabolism and alcoholism, but its physiological role is not clear [20]. The enzyme has been used in forensic science [21—24] for the determining ethanol in blood samples and organs, and in synthetic organic chemistry. In particular, the liver enzyme has found application for the stereospecific oxidoreduction of cyclic ketones and alcohols [25] and for the preparation of a pure enantiomorph of ethanol-1-d [26]. Yeast alcohol dehydrogenase was, however, the first enzyme showing such specificity [27]. Hornby et al. [28] described a method for immobilizing dehydrogenases, including alcohol dehydrogenase, and discussed applications to automated analysis. Other studies of immobilized alcohol dehydrogenase have been reported [29,30] but no detailed kinetic studies have previously been made for flow systems.

Materials and Methods

Chemicals

The enzyme, alcohol dehydrogenase from yeast, lot 107C-8500, having 233 U/mg protein was obtained from Sigma Chemical Company, U.S.A. The coenzyme, NAD from yeast (type III) lot 106C-7140, and the disodium salt of β-NADH also from yeast (grade III) lot 117C-74801, were also obtained from Sigma. The nylon tubing (0.206 cm internal diameter) for the attachment of the enzyme was obtained from Canus Plastics Company, Ottawa. The other substrate, ethanol, was a product of Consolidated Alcohol, Toronto, and was more than 99.9% pure. Benzidine used in the coupling processes was obtained from Sigma Chemical Company, while glutaraldehyde was obtained from Eastman Kodak Company, Rochester, NY. Coupling buffers containing EDTA

and β-mercaptoethanol were made by using sodium phosphates obtained from J.T. Baker Chemical Co., Phillipsburg, NJ. Sodium borate and boric acid, used for another buffer, were analytical grade, obtained from Anachemia Chemicals Ltd. and British Drug Houses Ltd., respectively. Dicyclohexylcarbodiimide and acetaldehyde were procured from Matheson Coleman and Bell, Manufacturing Chemists, OH.

Attachment procedure

The procedure follows essentially that described by Daka and Laidler [19], which is a modification of the methods used by Hornby and co-workers [8,28, 31,32] and by Allison et al. [33], and of those used previously in this laboratory [12—15]. It consists of partial hydrolysis and cleavage of amide bonds, followed by coupling of the carboxyl groups with benzidine in the presence of dicyclohexylcarbodiimide and of amino groups with glutaraldehyde. The enzyme is then attached to the surface through its polar groups. This method leaves an electrically neutral surface, thereby improving the stability of the preparation as discussed by Daka and Laidler [19].

A 1 m portion of the tubing was used as a tightly wound coil. The tube was first filled with a mixture of 18% (w/v) $CaCl_2$ in a solution of 18% (v/v) water in methanol, and was incubated for 30 min at 40°C. The available surface area was increased as a result of the removal of amorphous nylon by rinsing with water at a flow rate of 5 $cm^3 \cdot min^{-1}$ for about 20 min. Partial hydrolysis of the inside of the tube was then accomplished at 40°C by pumping approx. 4 M HCl for 20 min at a flow rate of 5 $cm^3 \cdot min^{-1}$. The hydrolysis was stopped by washing the tube for 30 min with ice-cold distilled water at 5 $cm^3 \cdot min^{-1}$.

A mixture of 1% (w/v) benzidine with 1% (w/v) dicyclohexylcarbodiimide in methylene chloride was used at 1 $cm^3 \cdot min^{-1}$ for 4 h at 10°C to couple with the carboxyl groups liberated in the previous step. Free benzidine was then washed away with 50 cm^3 methylene chloride, 50 cm^3 acetone, and 50 cm^3 ice-cold distilled water, successively, at a flow rate of 1 $cm^3 \cdot min^{-1}$. The free amino groups were then blocked by reaction with a solution of 12.5% (v/v) glutaraldehyde in 0.2 M borate buffer (pH 8.5) perfused at 1 $cm^3 \cdot min^{-1}$ for 1 h and at 0—4°C. The excess of the bifunctional reagent was removed afterwards by washing at 5 $cm^3 \cdot min^{-1}$ with 0.1 M phosphate buffer (pH 7.5) containing 1 mM EDTA and 1 mM β-mercaptoethanol.

The same phosphate buffer was used for making the enzyme solution (1 mg · cm^{-3}), which was allowed to circulate through the tube at 0.5 $cm^3 \cdot min^{-1}$ overnight to ensure complete reaction, the temperature being maintained within 0—5°C by the use of crushed ice. Immediately after the circulation, the absorbance at 280 nm due to enzyme protein was measured; a difference of 0.54 was noted and the initial absorbance showed that a considerable amount of the enzyme was bound to the tube. Any non-covalently attached enzyme was removed by washing the tube with 1 dm^3 of 0.5 M NaCl solution in 0.1 M phosphate buffer (pH 7.0) at 5 $cm^3 \cdot min^{-1}$. The tube was then filled with the assay buffer (pH 7.5) and stored below 5°C.

Kinetic measurements

All solutions were made up in 0.1 M sodium phosphate buffer, at pH 7.50 ±

0.05, with 1 mM EDTA and 1 mM β-mercaptoethanol. The kinetic runs were carried out at 25.5 ± 0.1°C, and were followed spectrophotometrically by measuring the absorbance of NADH at 340 nm with a Pye Unicam SP 1800 UV Spectrophotometer. The enzyme-tube was kept immersed in a temperature-controlled water bath, one end being connected by means of tygon tubing to the temperature-controlled feed solution through a LKB Varioperpex II peristaltic pump. The product was delivered to a flow cell (1 cm path length) in the spectrophotometer. The rates were calculated using $\epsilon_{340}^{1\,cm} = 6.22\ mM \cdot cm^{-1}$ [34]. A steady state was maintained throughout each kinetic run, and this was always confirmed by the fact that the amount of product remained practically constant at various time intervals for any run. For the present purpose, however, the data used were the average of several such measurements.

Theoretical

Immobilized enzymes behave differently from those in free solution as a result of conformational, environmental, partitional and diffusional effects [35,36]. A general equation for two-substrate systems which applies to several mechanisms is [37]

$$v = \frac{V[A][B]}{K'_{mA}K_{mB} + K_{mB}[A] + K_{mA}[B] + [A][B]} \tag{1}$$

where in the present application A and B refer to the substrates ethanol and NAD respectively. If one substrate, such as A, is in excess, the equation becomes of the Michaelis form for a single substrate:

$$v = \frac{V[B]}{K_{mB} + [B]} \tag{2}$$

The theory [16,36] indicates that when the enzyme is immobilized the rate of a single-substrate reaction is closely approximated by an equation of the Michaelis form, with the Michaelis constant modified by conformational, environmental, partitional and diffusion effects. The same is true for a two-substrate system when one substrate is in excess. We will write the modified Michaelis constants as $K_{mA,app}$ and $K_{mB,app}$.

The theory [16] leads to the result that $K_{mA,app}$ and $K_{mB,app}$ will increase with increasing diffusional control according to the equation

$$K_{m,app} = K'_m + \frac{V'}{2.58}\left(\frac{rL}{D^2}\right)^{1/3} v_f^{-1/3} \tag{3}$$

where r (cm) and L (cm) are the radius and length of the tube, D ($cm^2 \cdot s^{-1}$) is the diffusion coefficient for the substrate in the solution, v_f ($cm \cdot s^{-1}$) is the flow rate, V' is the maximal rate for the immobilized enzyme, and K'_m is the value of the Michaelis constant in the absence of diffusion. $K_{m,app}$ thus approaches K'_m in the limit of very high flow rates and K'_m can be determined from the $K_{m,app}$ values by plotting them against $v_f^{-1/3}$.

At low substrate concentrations and low flow rates, when the diffusion layer is thick and there is full diffusion control, the concentration of product at the

exit is predicted to be

$$[P]_e^D = 2.56\left(\frac{DL}{r^2 v_f}\right)^{2/3}[S] \tag{4}$$

At high substrate concentrations with no diffusion control, the predicted exit concentration is

$$[P]_e^0 = \frac{2L}{r v_f} v_r \tag{5}$$

where v_r is the inherent rate of the enzyme-substrate reaction. Double-logarithmic plots of $[P]_e$ against v_f therefore provide information about the extent of diffusion control.

The Kobayashi-Laidler treatment [16] defines two-dimensionless parameters as follows:

$$\phi = \frac{[P]_e}{[S]}\left(\frac{v_f r^2}{DL}\right)^{2/3} \tag{6}$$

and

$$\rho = \frac{K_{m,app}}{[S]} \tag{7}$$

A plot of ϕ against ρ (an example is to be found in Fig. 2) can be divided into regions according to the extent of diffusion control, and allows this to be determined for a particular experiment. A parameter η, the utilization factor, is the ratio of the actual rate to that in the absence of diffusional effects. In the case of competitive inhibition, found in the present system, the ρ values are modified by the factor $1 + [I]/K_i$ were [I] is the inhibitor concentration and K_i the inhibition constant.

Results and Discussion

Stability

The stability of the enzyme attached to the tube and stored at about 4°C was checked by periodic assays. During the first 2 weeks after attachment the rate decreased by about 20%, presumably because of the detachment from the surface of loosely-bound enzyme. After that period the activity remained constant within ±4% over a period of 10 months.

$K_{m,app}$ values

The $K_{m,app}$ values for NAD are substantially less than 1.0 mM, and a concentration of 2.0 mM was used to give essentially complete saturation by NAD. Rates were measured at ethanol concentrations varying from 3.0 mM to 100.0 mM and with flow rates varying from 0.10 to 3.90 cm · s^{-1}. Fig. 1a shows some representative Lineweaver-Burk plots at 2.0 mM NAD, and the apparent Michaelis constants, denoted by K_m (ethanol), are shown in Table I.

Similar plots with alcohol in excess at 100.0 mM are shown in Fig. 1b. The results show deviations from linearity at the higher substrate concentrations, and this may be due to a change in the degree of diffusion control. Similar

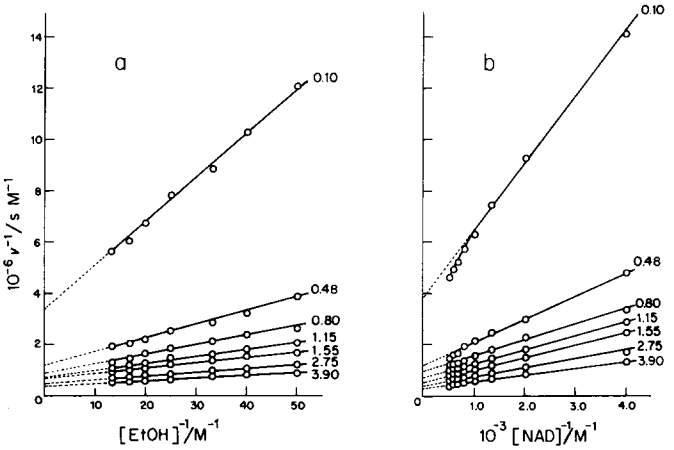

Fig. 1. Lineweaver-Burk plots (a) with excess NAD at 2.0 mM, and (b) with excess ethanol (EtOH) at 100.0 mM. Temperature 25.5° C, pH 7.5. Each flow rate is shown at the right-hand end of the appropriate line, expressed as cm · s^{-1}.

results were obtained with the alcohol at 500.0 mM and NAD concentrations varying from 0.10 mM to 2.0 mM. The $K_{m,NAD}$ values are also shown in Table I.

As predicted by Eqn. 3, the $K_{m,app}$ values varied linearly with $v_f^{-1/3}$, and led

TABLE I

VALUES OF $K_{m,app}$ AND K'_m FOR ETHANOL AND NAD WITH ALCOHOL DEHYDROGENASE

Type of enzyme	Matrix	pH	Temperature	Flow rate (cm · s^{-1})	$K_{m,app}$		Reference
					K_m (NAD) (μM)	K_m (ethanol) (mM)	
Yeast	Nylon	7.5	25.5	0.48	780	46.7	Present work
Yeast	Nylon	7.5	25.5	0.80	673	41.6	Present work
Yeast	Nylon	7.5	25.5	1.15	620	35.8	Present work
Yeast	Nylon	7.5	25.5	1.55	606	32.9	Present work
Yeast	Nylon	7.5	25.5	2.75	560	28.0	Present work
Yeast	Nylon	7.5	25.5	3.90	520	25.0	Present work
Yeast	Nylon	7.5	25.5	∞	250 (K'_m)	4.6 (K'_m)	Present work
Liver	Polyacrylamide	8.0	25.0	*	—	0.57	29
Liver	Copolymer of acrylamide and methyl acrylate	8.0	25.0	*	—	0.54	29
Liver	(free)	8.0	25.0	*	—	0.30	29
Yeast	(free)	8.2	room	*	—	14	38
Yeast	(free)	7.7	20.0	*	—	16	39
Liver	(free)	7.15	23.5	*	10	—	40
Liver	(free)	8.0	23.5	*	5	—	40

* Static system.

to extrapolated K'_m values of 4.6 mM for variable ethanol and 250 μM for variable NAD. Similar linear relationships have been found with other systems [12—14,19], and in those cases the K'_m values were not far from the K_m for the enzyme in free solution. In the present work, the K'_m (ethanol) value of 4.6 mM is comparable with the values found for the free enzyme and in static studies of the immobilized enzyme (see Table I). However, the K'_m (NAD) value is two orders of magnitude higher than that for free solution. As will be discussed, this is attributed to the substantial diffusion control found in this situation; the ethanol can not bind to the free enzyme but only to the enzyme-NAD complex.

Dimensionless parameters

Fig. 2 shows a plot of the dimensionless parameters ϕ and ρ defined by Eqns. 6 and 7. In the calculation of ϕ, the diffusion coefficient was taken as $4 \cdot 10^{-6}$ cm$^2 \cdot$ s^{-1}, which is appropriate for molecules of the kind we are dealing with [41]. This plot has been divided into three theoretical regions, as explained in the caption. For constant ethanol and variable NAD the points lie in regions 2 and 3 corresponding to substantial diffusion control. With constant NAD and variable ethanol all of the results are well inside the diffusion-free region (region 1). This arises from the very low ϕ values, which result from the fact that the product concentrations are very much smaller than the ethanol concentrations, the latter being large because of the large $K_{m,app}$ values. In general the theory predicts diffusion-free behavior at high substrate concentrations. In our experiments with saturation by NAD and variable ethanol the concentrations of the latter are high enough so that there is no diffusion control at all of the flow rates employed.

The trends found with constant ethanol and variable NAD (Fig. 2) are also consistent with less diffusion control at the higher substrate concentrations. The points lying near region 1 (little diffusion control) correspond to these conditions, while those in region 3 (considerable diffusion control) are for low substrate concentrations.

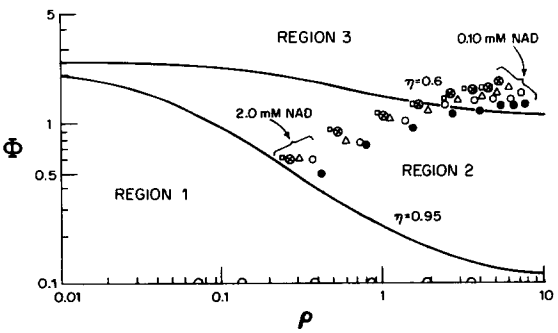

Fig. 2. Double-logarithmic plots of the dimensionless parameters ϕ and ρ. The half-circles are typical points for constant NAD at 5.0 mM and variable ethanol (3.0—100.0 mM). Other symbols are due to the results with excess ethanol at 500.0 mM and variable NAD (0.10—2.0 mM) for the flow rates 0.30 (●), 0.50 (○), 1.00 (△), 2.00 (⊗), and 3.30 (□) in cm $\cdot$ s^{-1}. The theoretical regions are region 1, little diffusional control (<5%); region 2, moderate diffusional control (5—60%); region 3, considerable diffusional control (>60%).

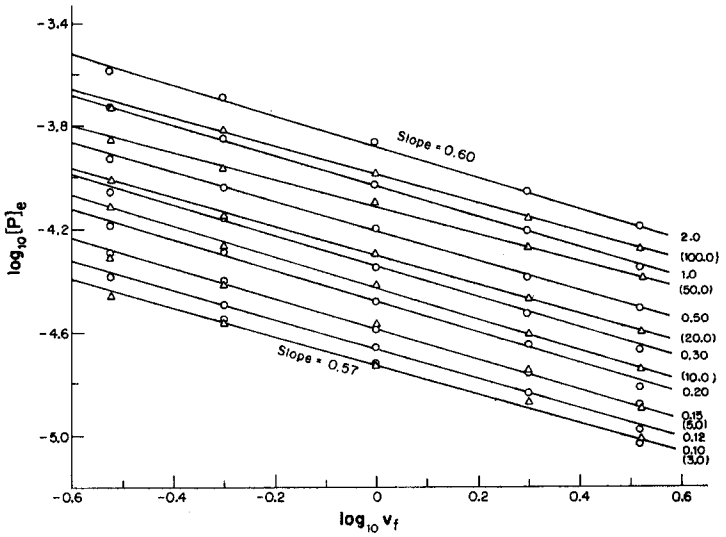

Fig. 3. Double-logarithmic plots of the product concentration at the exit $[P]_e$, against the flow rate v_f, for the results with ethanol in excess at 500.0 mM (○, circles) and for excess NAD at 5.0 mM (△, triangles). Variable NAD concentrations (0.10—2.0 mM) for the former and variable ethanol concentrations (3.0—100.0 mM) for the latter (within brackets) are shown (figures express mM).

Product concentrations

Fig. 3 shows double-logarithmic plots of product concentration at the exit against flow rate, for experiments done with constant NAD and ethanol concentrations of 5.0 mM and 500.0 mM, respectively. The slopes vary from —0.57 to —0.60. Corresponding plots for the series at constant NAD of 2.0 mM gave slopes of about —0.40; those for constant ethanol at 100.0 mM gave slopes of —0.45.

The simple Kobayashi-Laidler treatment [16], for no complications such as inhibition by products, predicts that the slopes of such plots should be —1.0 with no diffusion control (Eqn. 5) and —0.67 with full diffusion control (Eqn. 4). The smaller dependence of product concentration on flow rate shows that the situation is not so simple. Bunting and Laidler [12] found a similar low dependence of product concentration on flow rate with L-asparaginase, and concluded that it was due to complete conversion into products before the substrate had reached the tube outlet. In the present work, however, the conversion was much smaller and this explanation is not applicable.

Another explanation is that there is inhibition by products, which remain attached to the enzyme and block further reaction. This will have a stronger effect at low flow rates, when the residence times are longer and allow more blockage of reaction by products. As the flow rates are reduced the product concentrations at the exit will therefore increase to a smaller extent than in the absence of inhibitor, and the negative slopes of the $\log_{10}[P]_e$ vs. $\log_{10} v_f$ plots will be smaller, as in Fig. 3. We therefore tested for inhibition by products and in the following section we show that this occurs.

Inhibition by products

Wratten and Cleland [42,43] found inhibition by products for liver and

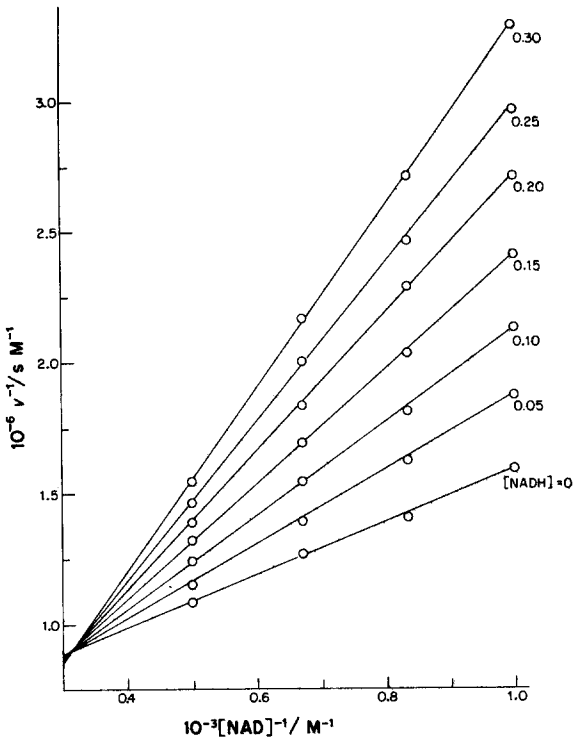

Fig. 4. Lineweaver-Burk plots for constant ethanol at 100.0 mM and variable NAD concentrations with NADH added as indicated (figures express mM) at 25.5°C, pH 7.5 and a flow rate of 1.15 cm · s^{-1}.

yeast alcohol dehydrogenase in free solution. To see whether this also is the case with the immobilized enzyme, we carried out a number of studies in which each product, in amounts considerably greater than produced in the reaction, are added.

Fig. 4 shows some Lineweaver-Burk plots at a fixed flow rate and variable NAD concentration, with various amounts of NADH added. The plots suggest mixed competitive and non-competitive inhibition, with a preponderance of the former. The inhibition constant K_i obtained from the secondary plot is 120 μM. Plots with variable ethanol and acetaldehyde added were similar, also indicating mixed competitive and non-competitive inhibition, and gave an inhibition constant of 62 μM. These results are somewhat similar to those for the free enzyme [42,43]. Our results indicated that acetaldehyde (K_i = 62 μM) is a stronger inhibitor than NADH (K_i = 120 μM), whereas Wratten and Cleland [42] with the free enzyme found NADH to have a K_i of 108 μM and acetaldehyde to be a much weaker inhibitor with a K_i of 780 μM. These differences are understandable in view of the fact that in our experiments diffusion control is much more important with variable NAD than with variable ethanol. The greater the amount of diffusional control, the less the inhibition, since inhibitors affect the chemical interaction at the enzyme but not the diffusion processes.

We also studied the effect of flow rate on the product inhibition. In the case

TABLE II

EFFECT OF FLOW RATE ON PRODUCT INHIBITION BY ADDED ACETYLDEHYDE WITH CONSTANT [EtOH] = 20.0 mM AND EXCESS [NAD] = 5.0 mM AT 25.5°C AND pH 7.5

Concentration of added CH_3CHO (mM)	Degree of inhibition at flow rates (cm · s^{-1})			
	0.50	1.00	2.00	3.30
0.10	0.35	0.36	0.34	0.37
0.20	0.54	0.55	0.54	0.56
0.30	0.65	0.65	0.64	0.66
0.40	0.72	0.72	0.71	0.71

of inhibition by acetaldehyde, flow rate has no significant effect on the degree of inhibition, as shown by the results in Table II. With NADH, however, there was a decrease in degree of inhibition as the flow rate was increased (see Table III). This difference of behavior is consistent with the fact that with variable ethanol there is essentially no diffusion control, whereas there is significant diffusion control with variable NAD.

General discussion and conclusions

Of particular interest is the fact that the attachment procedure we used leads to a preparation of considerable stability. This result is to be contrasted with that reported by Kelly et al. [44], who used a different attachment procedure.

Our results have shown that when the NAD concentration is held constant and the ethanol concentration varied there is no appreciable diffusion control, but that the converse conditions lead to moderate diffusion control. The mechanisms involved with alcohol dehydrogenase in free solution are by no means clear and the yeast and liver enzymes behave differently. There is some evidence [42,43,45] that with both yeast and liver enzymes there is an ordered mechanism, the NAD adding first and the product NADH leaving last. Various investigations have shown that NAD and NADH occupy the same binding sites [46—49], NADH being bound more tightly [50], and Dunn [51] has

TABLE III

FLOW RATE DEPENDENCE OF PRODUCT INHIBITION BY ADDED NADH WITH CONSTANT [NAD] = 0.50 mM AND EXCESS ETHANOL AT 100.0 mM

Concentration of added NADH (mM)	Degree of inhibition at flow rates (cm · s^{-1})		
	0.48	1.15	2.75
0.05	0.20	0.18	0.15
0.10	0.34	0.29	0.24
0.15	0.43	0.39	0.33
0.20	0.50	0.46	0.40
0.25	0.56	0.52	0.46
0.30	0.60	0.56	0.50

concluded that under steady-state conditions the rate of substrate turnover is limited by the rate with which NADH leaves. However, Silverstein and Boyer [52] for both the horse liver and the yeast enzyme showed that a random mechanism is important, and for the yeast enzyme Dickinson and Monger [53] concluded that there is a partly random mechanism for addition of NAD and alcohol and a compulsory order of product dissociation. Dead-end complexes [54] and abortive enzyme-NADH-alcohol ternary complexes [52] have also been proposed.

The situation is therefore complicated even for the free enzyme. Our results are most satisfactorily explained if under our experimental conditions the mechanism is an ordered one, the NAD adding first and the alcohol reacting with the enzyme-NAD complex. Thus, in our experiments with NAD fixed at 2.0 mM and 5.0 mM and the alcohol concentration varied, the enzyme will be saturated with NAD and the process will involve the diffusion of alcohol to the enzyme-NAD at the surface and the subsequent reaction. This diffusion can occur readily, and there is no appreciable diffusion control. When, on the other hand, the alcohol concentration is in excess of the $K_{m,app}$ the enzyme will not be saturated by alcohol; the NAD will first have to diffuse to the surface and form an enzyme-NAD complex, after which the alcohol must diffuse to the enzyme-NAD at the surface and form the ternary complex. Diffusion is therefore much more heavily involved, and the larger NAD molecules will diffuse more slowly, the diffusion control is therefore understandable.

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