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STIMULATION OF ALCOHOL DEHYDROGENASE BY DIMETHYLDITHIOCARBAMATE

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RESEARCH DIRECTORATE

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On an investigation of the mechanisms of epoxytrichothecene inhibition of yeast alcohol dehydrogenase (YADH) reaction, I have shown that DMDTC was an effective activator of the enzyme system. Our kinetic data indicated that DMDTC was a competitive activator with respect to NAD but a "mixed type" activator with respect to ethanol. When the enzyme reaction was monitored by NADH formation at 340 nm, stimulation of YADH reaction by DMDTC resulted in the increase of both V_{max} and K_m values of the YADH reaction to about the same extent. The presence of 5×10^{-4} of DMDTC increased $K_{m, EtOH}$ values from 1.9×10^{-2} M to 2.2×10^{-2} M, and the corresponding V_{max} values from 0.12 A/min to 0.25 A/min for the control and plus activator experiments, respectively. However, the double reciprocal plots of the plus activator data gave biphasic curves that were concave downward. When the reaction was monitored with NAD concentration varied, increasing DMDTC concentrations from 0.5×10^{-4} M to 2.5×10^{-4} M resulted in the reduction of the $K_{m, NAD}$ values from 4.7×10^{-4} M to 1.8×10^{-4} M, but DMDTC concentrations had little effect on the corresponding

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19. ABSTRACT (Continuation)

V_{max} values, which were equal to 0.11 A/min. The plot of the 1/slope vs [DMDTC] of the kinetic data yielded a linear curve and K_A value with respect to NAD was estimated to be in the range of 5.0×10^{-7} M. The estimated K_A values of DMDTC with respect to EtOH varied from 4.3×10^{-5} to 5.9×10^{-4} M, calculated from the low and High [S] parts of the biphasic curves. The competitive activation mechanism by DMDTC with respect to NAD suggests an ordered bireactant mechanism in which DMDTC and NAD binding to the enzyme molecules is in an obligate order. The mixed-type activation kinetics with respect to substrate suggest the formation of abortive complexes of YADH-EtOH and YADH-DMDTC-EtOH with enhanced enzyme activity.

PREFACE

The work described in this report was authorized under Project No. 1L161102A71A, Research in Chemical & Biological Defense, Biotechnology. This work was started and completed in May 1984. The experimental data are recorded in Laboratory Notebook 830080.

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STIMULATION OF ALCOHOL DEHYDROGENASE BY DIMETHYLDITHIOCARBAMATE

1. INTRODUCTION

On an investigation of the mechanism of epoxytrichothecene inhibition of yeast alcohol dehydrogenase (YADH) reaction, we have accidentally found that dimethyldithiocarbamate (DMDTC) was an effective activator of the enzyme. DMDTC is a vulcanizing accelerator used extensively in manufacturing of rubber products such as stoppers. The compound is an analog of diethyldithiocarbamate (DEDTC), a metabolite of disulfiram (tetraethylthiuram disulfide, antabuse).[1] Disulfiram is a well known drug for treating alcoholism because of its inhibitory effect on aldehyde dehydrogenases.[2] In vivo, DEDTC is as effective an inhibitor of aldehyde dehydrogenase as disulfiram, but has little effect on the enzyme activity in vitro.[3,4] Similarly, we can not demonstrate any significant effect of DMDTC on aldehyde dehydrogenase activity in vitro. However, as a result of our recent in-house efforts, we have positively demonstrated the stimulation of the yeast alcohol dehydrogenase by this compound. The kinetic data obtained from our present investigation indicated that the DMDTC stimulation reaction followed an ordered bireactant mechanism in which DMDTC and NAD binding to the enzyme molecules is in an obligate order, i.e., DMDTC binding followed by NAD binding. On the other hand, evidence of formation of a substrate-YADH-DMDTC transitory complex with enhanced enzyme activity can also be shown from the kinetic data that we obtained. The results of our present investigation on the kinetics of the DMDTC stimulation of the YADH reaction will be presented in this paper.

2. MATERIALS AND METHODS

2.1 Chemicals.

DMDTC sodium salt was obtained from Aldrich Chemical Co., Inc., Milwaukee, WI. The oxidized form of nicontinamide adenine dinucleotide (NAD) and YEDH were obtained from Sigma Chemical Co., St. Louis, MO. All other chemicals and reagents were reagent grade or the highest purity and were used without further purification.

2.2 Kinetics of YADH Reaction.

The initial rate of the YADH reaction was measured in a Varian spectrophotometer by determination of NADH formation at 340 nm and assuming an absorption coefficient of 6.22 mM^{-1} cm^{-1} for NADH.[5] All kinetic studies were carried out with a 0.1 M sodium phosphate buffer, at pH 7.4, with the temperature of the reaction mixtures maintained at 25 °C. Reaction was initiated by addition of 2 µg of the enzyme in 20 µl of the buffer per cuvette of total volume of 2.0 ml. All kinetic data were obtained in duplicate and the kinetic parameters calculated by the least square method.

3. RESULTS

3.1 Effect of DMDTC Concentrations on the Initial Rate of the YADH Reaction.

The effect of DMDTC concentrations on the initial rate of YADH reaction is shown in Figures 1 and 2. The velocity curves of Figure 1 were obtained by varying DMDTC concentrations from 0.5 x 10^{-6} to 2.5 x 10^{-6} M, with initial substrate and NAD concentrations fixed at 8.5 x 10^{-2} and 3.8 x 10^{-4} M, respectively. As may be seen, the initial rate of the YADH-catalyzed formation of NADH increased with increasing concentrations of DMDTC. Even beyond the initial linear rate, the presence of DMDTC at different concentrations continued to increase the enzyme reaction profoundly, but in its absence the reaction leveled off rapidly in about 10 minutes. These results clearly indicate the profound stimulatory effect of DMDTC on the YADH reaction.

Figure 2A shows the velocity versus DMDTC concentration plot, and the corresponding Lineweaver-Burk plot[6] is shown in Figure 2B. Under the reaction conditions employed, the apparent value of $K_{A,DMDTC}$ was found to be 8.7 x 10^{-7} M. The corresponding V_{max} value of the reaction system was 10 µmoles/min of NADH formed.

3.2 Effect of DMDTC on Kinetic Parameters as NAD Concentration Varied.

Figure 3A shows the effect of DMDTC concentrations on the apparent $K_{m,NAD}$ and the corresponding V_{max} values of the YADH reaction as NAD concentrations varied. As a result, a family of linear Lineweaver-Burk plots was obtained. The corresponding kinetic constants as calculated from these reciprocal plots are listed in Table 1. As can be seen, when the sustrate concentration was kept constant at 8.4 x 10^{-2} M, the change of the fixed levels of DMDTC from 0.75 x 10-6 to 2.5 x 10^{-6} M resulted in the decreasing of the apparent $K_{m,NAD}$ values from 5.0 x 10^{-4} to 1.46 x 10^{-4} M. The reciprocal plots intersect at a common point on 1/v-axis, indicating that the apparent V_{max} values of the YADH reaction were independent of DMDTC concentrations present. The V_{max} value of the reaction system was thus obtained by averaging the apparent V_{max} values shown in Table 1 and found to be about equal to 15.5 µmoles of NADH/min.

Figures 3B and C show the replot of the apparent $K_{m,NAD}$ versus 1/DMDTC and that of slope versus 1/[DMDTC], respectively. The $K_{A,DMDTC}$ and $K_{m,NAD}$ values of the reaction system were then calculated to be in the range of 2.6 x 10^{-6} M and 1 2 x 10^{-4} M, respectively. V_{max} value, calculated from the intercept on the slope-axis of Figure 3B, was found to be 15.9 µmoles of NADH/min, which agrees well with the value calculated directly from Table 1.

3.3 Effect of NAD Concentrations on Kinetic Parameters as DMDTC Concentration Varied.

The kinetic data shown in Figure 3 can also be expressed using DMDTC as the varied ligand, and a series of 1/v versus 1/DMDTC plots is shown in Figure 4A. It can be seen that as NAD concentration increased, the reciprocal plots intersect at a common point, where 1/[DMDTC] = $-1/K_A$

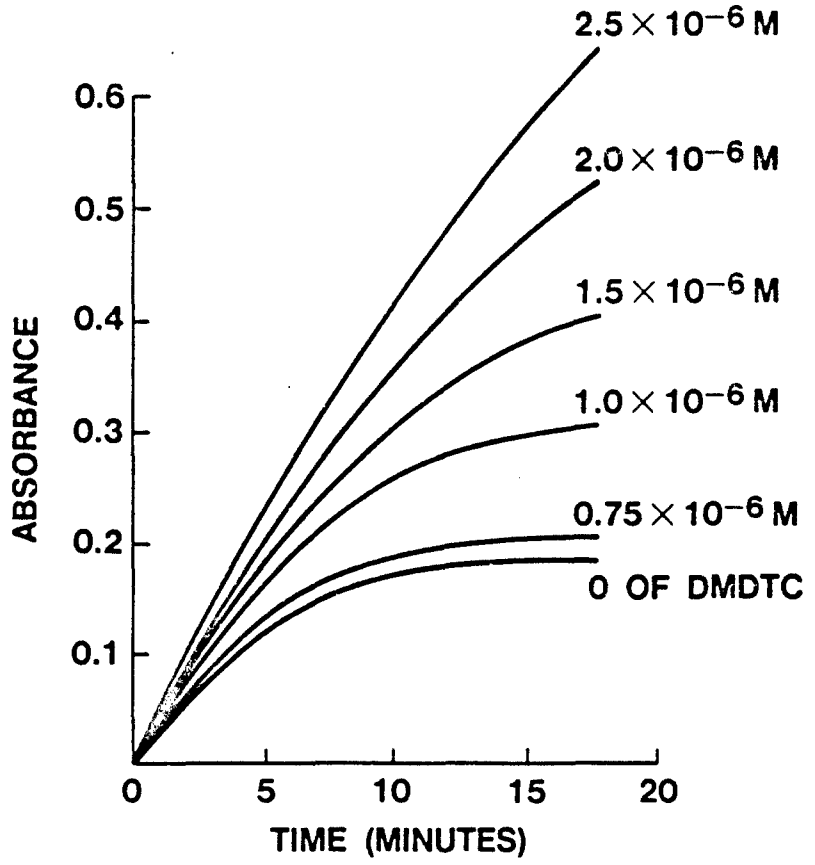


Figure 1. Effect of DMDTC Concentrations on the Initial Velocity of YADH Reaction

Reaction was carried out in 0.1 M sodium phosphate buffer, pH 7.4, with DMDTC concentrations varied from 0 to 2.5 x 10^{-6} M and the initial substrate and NAD concentrations fixed at 8.5 x 10^{-2} M and 3.8 x 10^{-4} M, respectively. Curves 0, 1, 2, 3, 4, and 5 were obtained in the presence of 0, 0.75 x 10^{-6}, 1.0 x 10^{-6}, 1.5 x 10^{-6}, 2.0 x 10^{-6}, and 2.5 x 10^{-6} M of DMDTC, respectively.

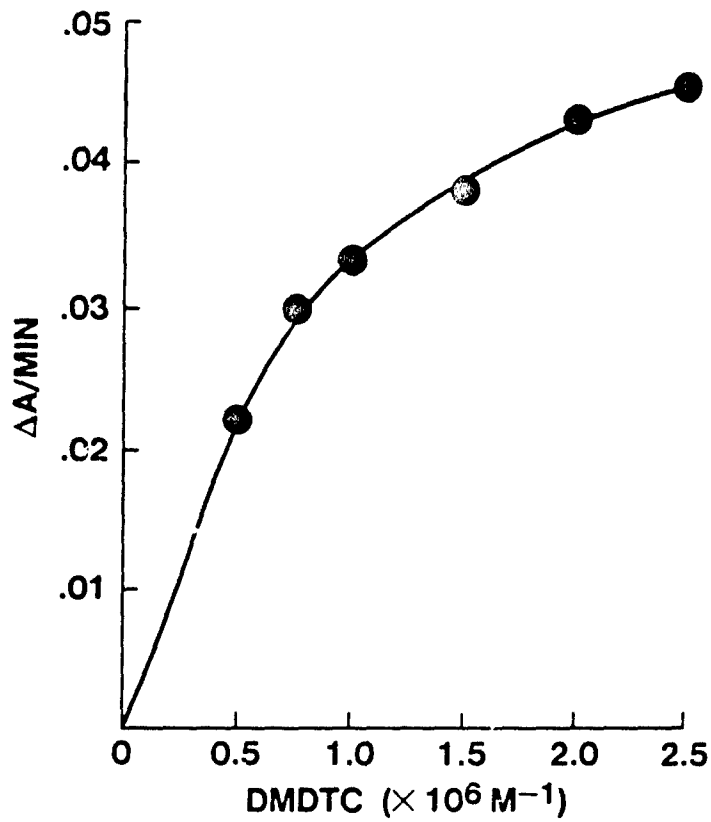


Figure 2A. The Velocity Versus DMDTC Concentration Plot

A is the velocity versus DMDTC concentration plot and B is the corresponding double reciprocal plot of the kinetic data shown in A. Data were taken from Figure 1.

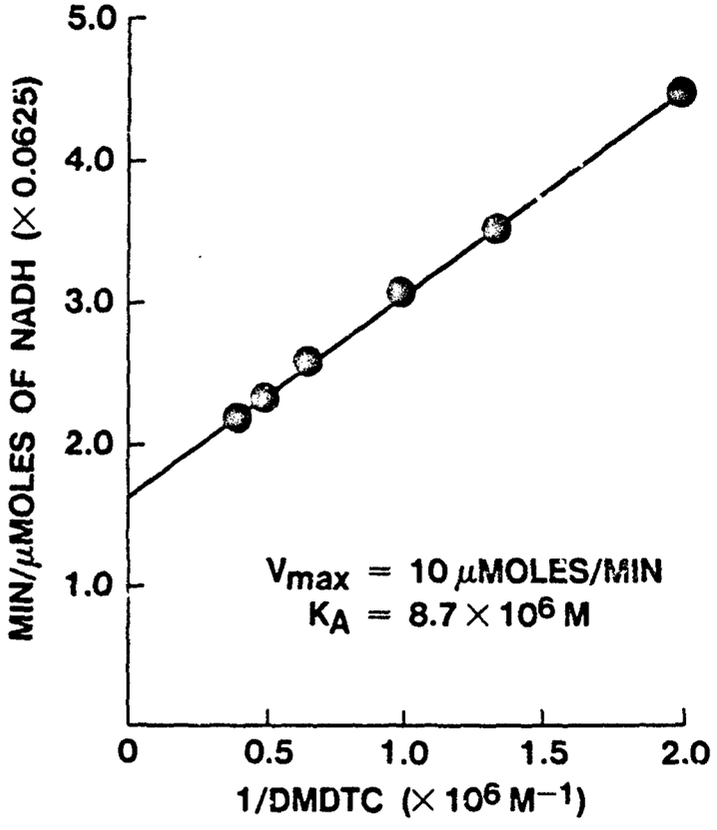


Figure 2B. Double Reciprocal Plot of 1/v Versus 1/DMDTC Concentration

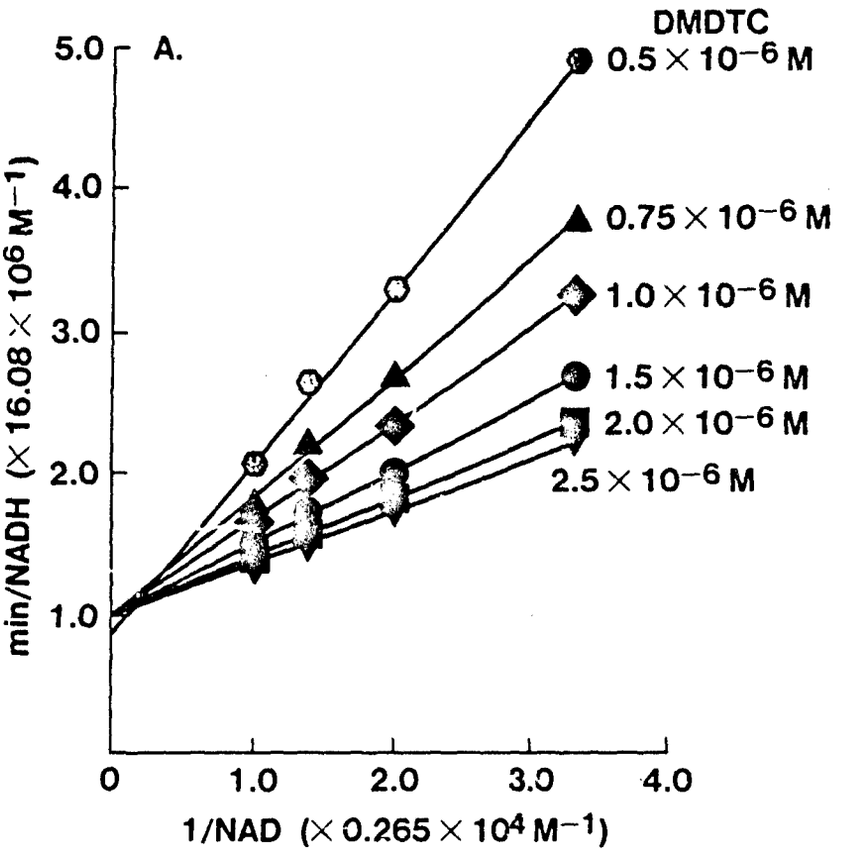


Figure 3A. Effect of DMDTC Concentrations on the Apparent $K_{m,NAD}$ and V_{max} Values

A, reciprocal plot of 1/v versus 1/NAD; B, replot of $K_{NAD,app}$ versus 1/DMDTC; and C, replot of slope versus 1/DMDTC. NAD concentrations varied from 1.13 x 10^{-4} M to 3.77 x 10^{-4} M, as substrate concentrations were kept constant at 8.4 x 10^{-2} M. DMDTC concentrations were fixed at levels shown in Figure 3A.

Table 1. Effect of DMDTC Concentrations on the Apparent Values of $K_{m,NAD}$ and V_{max}

DMDTC (x10-6 M)	$k_{m,NAD}$ (M)	(A/min)	V_{max} NADH (μmoles/min)
0.5	5.0×10^{-4}	0.11	17.7
0.75	2.9×10^{-4}	0.09	14.15
1.0	2.4×10^{-4}	0.091	14.63
1.5	1.91×10^{-4}	0.094	15.11
2.0	1.64×10^{-4}	0.097	15.59
2.5	1.46×10^{-4}	0.098	15.76

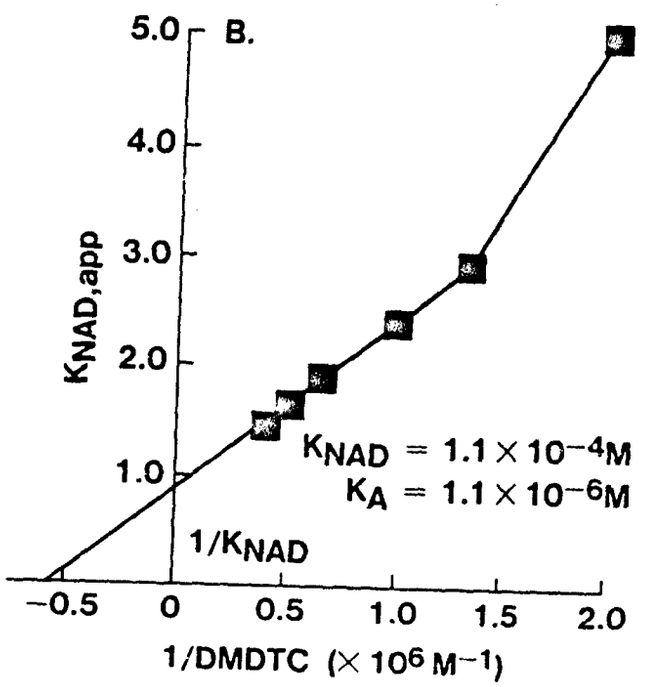


Figure 3B. Replot of $K_{NAD,app}$ Versus 1/DMDTC

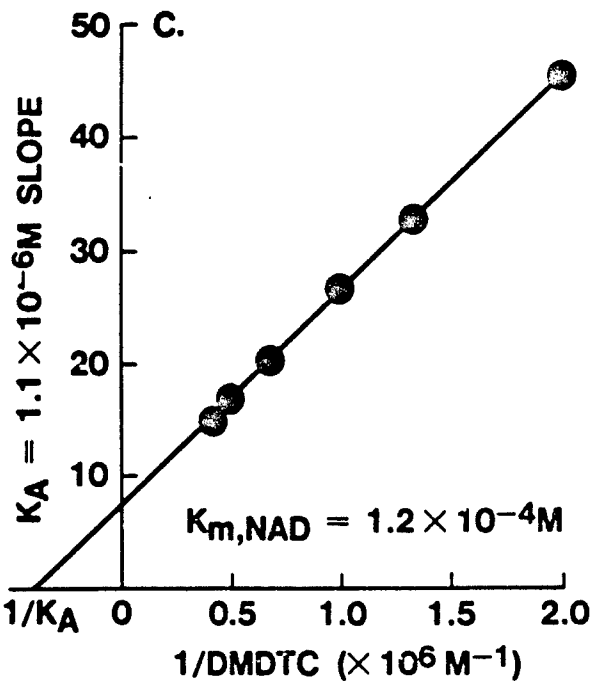


Figure 3C. Replot of Slope Versus 1/DMDTC

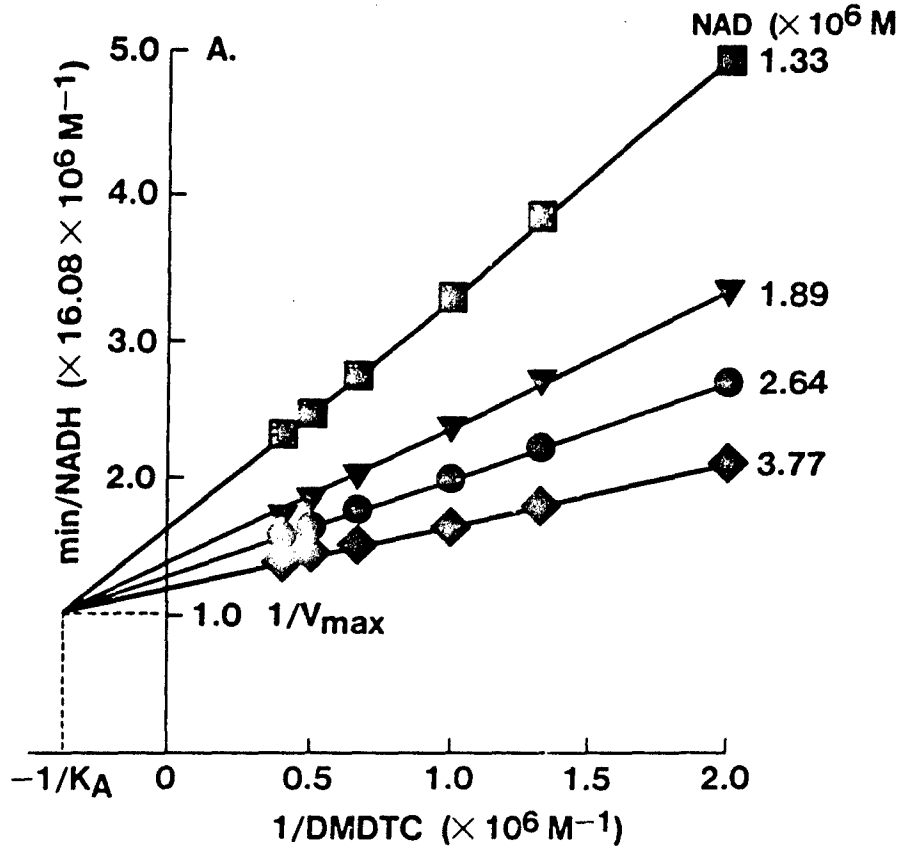


Figure 4A. Effect of NAD Concentrations on Kinetic Parameters as DMDTC Concentration Varied

Kinetic data shown in Figure 3 are expressed using DMDTC as the varied ligand. A, 1/v versus 1/DMDTC plots. B, replot of $1/V_{max,app}$ versus 1/NAD. C, replot of $1/K_{A,DMDTC}$ versus NAD concentrations. D, replot of slope versus 1/NAD.

and $1/v = 1/V_{max}$. The kinetic parameters may be calculated directly from this intercept point, but it is preferred to determine these values from the replots. Figures 4B, C, and D were obtained by replots of $1/V_{max,app}$ versus 1/[NAD], $1/K_{A,app}$ versus [NAD], and slope versus 1/[NAD], respectively. The stimulatory constant (K_A) of DMDTC determined from these plots and replots was found to be in the range of 2.5×10^{-6} M, and the corresponding V_{max} value was about 15.8 μmoles/min of NADH formed. These values agreed well with those obtained from Figure 3. Table 2 lists the apparent values of V_{max} and K_A, as determined from these plots. The results indicated that both the apparent V_{max} and K_A values varied with NAD concentrations, but in opposite directions. As NAD concentrations increased, the apparent values of V_{max} increased and approached V_{max} of the enzymatic reaction (also see Figure 4B). As the [NAD] increased the $K_{A,app}$ decreased and approached K_A as a limit (Figure 4C). The results shown in Figure 4D indicated that the slope of the family of reciprocal plots decreased as NAD concentrations increased and approached the limit of zero. Again, it may be seen from Figure 4D that as [NAD] became very large, the velocity of the reaction became independent of DMDTC concentrations. In addition, the family of reciprocal plots shown in Figure 4A bears no symmetry to that of Figure 3A. These results may indicate that the stimulatory effect of DMDTC on the YADH reaction followed an ordered bireactant mechanism under the reaction conditions employed.

3.4 Effect of DMDTC on the Kinetic Parameters as Substrate Concentrations Varied.

The reciprocal plots of 1/v versus 1/EtOH is shown in Figure 5. The reactions were carried out with substrate concentrations varied from 1.7×10^{-2} to 1.7×10^{-1} M in the presence or absence of 2.5×10^{-4} M of DMDTC. NAD concentrations were fixed at 3.8×10^{-4} M. The reciprocal plots of both control and plus activator experiments yielded biphasic curves, which were concave downward. These results may indicate the presence of substrate activation reaction. The data shown here indicated significant effect of DMDTC on both the apparent values of $K_{m,EtOH}$ and V_{max} of the reaction. The presence of 2.5×10^{-4} M of DMDTC reduced $K_{m,EtOH}$ values from 4.7×10^{-2} to 3.0×10^{-2} M and increased the corresponding V_{max} values from 57.9 to 64.3 μmoles/min of NADH formed for the control and plus activator experiments, respectively. The DMDTC-stimulatory effect depended on the substrate concentrations present. At lower substrate concentrations, the $K_{m,EtOH}$ values were estimated to be in the range of 2.2×10^{-2} M and 2.4×10^{-2} M and the corresponding V_{max} values were 27.3 and 38.6 μmoles/min obtained in the absence and presence of DMDTC, respectively. The kinetics of the stimulatory reaction resembled the mixed-type nonessential activation mechanism. Accordingly, the K_A values of DMDTC with respect to EtOH were estimated to vary from 2.1×10^{-5} M to 1.3×10^{-4} M, calculated from the low and high (S) parts of the biphasic curves.

4. DISCUSSION

For a rapid equilibrium ordered bireactant systems, the reaction may be described in the following equilibria.

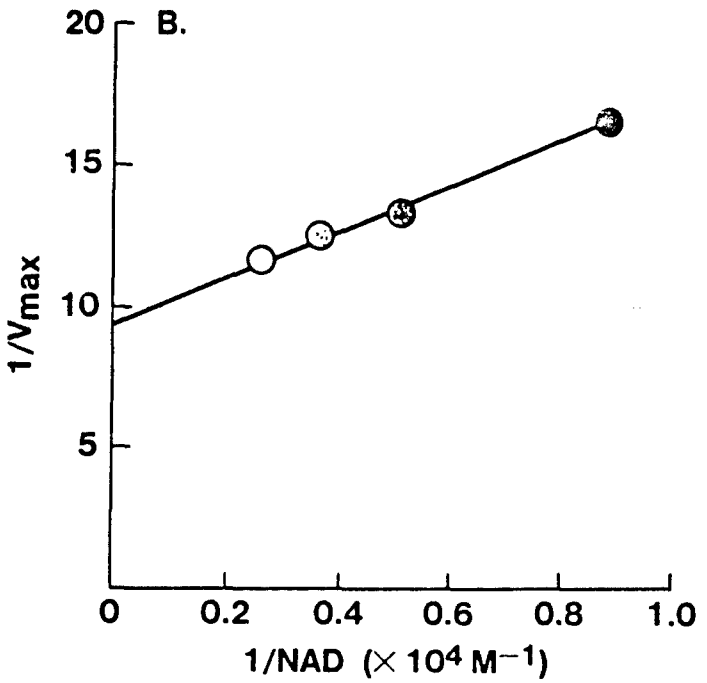


Figure 4B. Replot of $1/V_{max,app}$ Versus 1/NAD

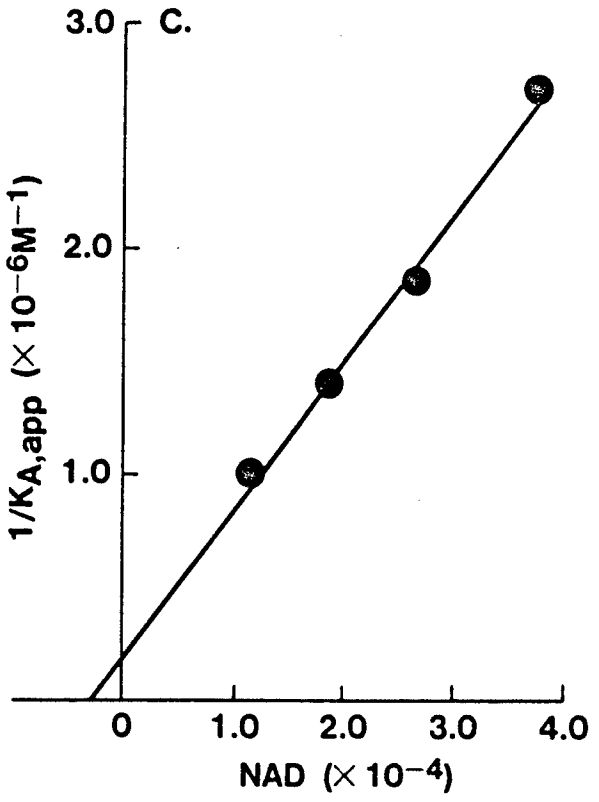


Figure 4C. Replot of $1/K_{A,DMDTC}$ Versus NAD Concentrations

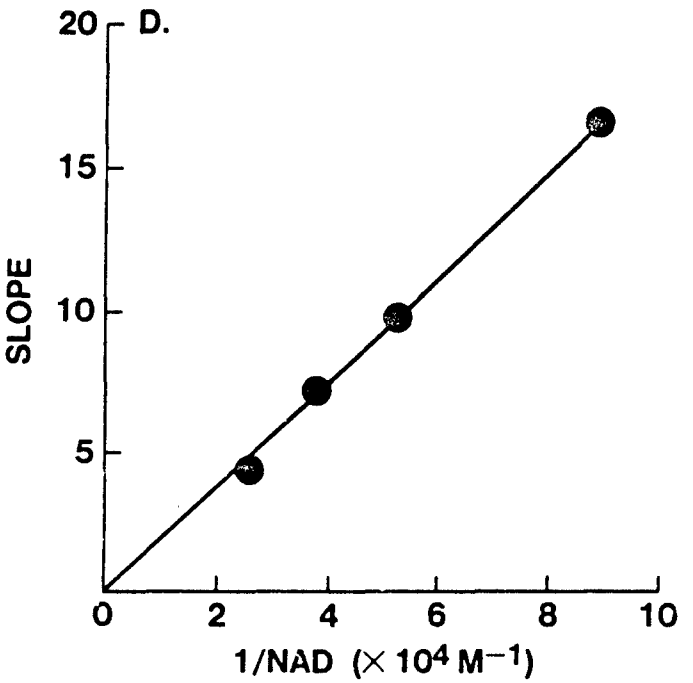


Figure 4D. Replot of Slope Versus 1/NAD

Table 2. Effect of NAD Concentrations on the Apparent Values of $K_{A,DMDTC}$ and the Corresponding V_{max}

NAD (x 10-4 M)	$K_{A,DMDTC}$ (M)	(A/min)	V_{max} NADH (moles/min)
1.13	0.99 x 10-6	0.061	9.8
1.89	0.68 x 10-6	0.073	11.7
2.64	0.54 x 10-6	0.078	12.5
3.77	0.37 x 10-6	0.084	13.5

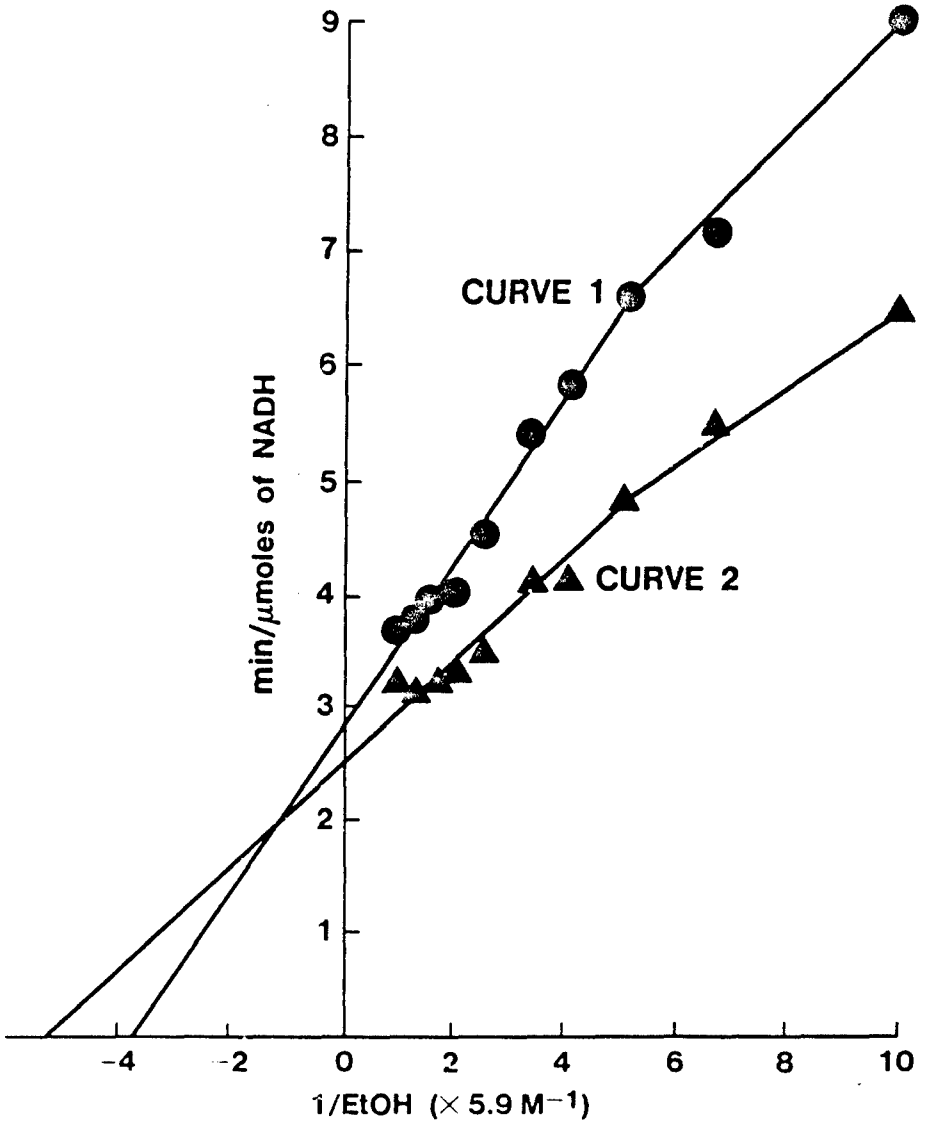
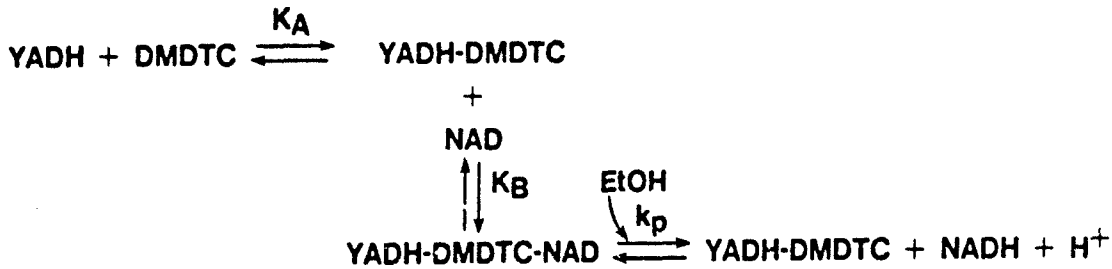


Figure 5. Effect of DMDTC Concentrations on Kinetic Parameters as Ethanol Concentration Varied

The reaction was carried out with ethanol concentrations varied from 1.7 x 10^{-2} to 1.7 x 10^{-2} M in the presence (Curve 2) and absence (Curve 1) of 2.5 x 10^{-4} M of DMDTC. NAD concentrations were kept constant at 3.8 x 10^{-4} M.



The reaction scheme indicates that the addition of DMDTC to YADH before NAD binding is essential for the stimulatory reaction. The experimental data that support the ordered bireactant mechanism may be summarized as follows. (1) The family of reciprocal plots of v versus [NAD](Figure 3A) bears no symmetry with that of v versus [DMDTC] plots (Figure 4A). If the reaction follows the random ordered bireactant reaction mechanism, Figure 3A will have a common intersecting point above 1/[NAD]-axis at $-1/K_{NAD}$. This was found not to be the case. (2) The results shown in Figure 3A and Table 1 indicate that, as [NAD] varied, the V_{MAX} values of the reaction system were independent of DMDTC concentrations. These data are inconsistent with the random ordered reaction mechanism. (3) The linear replot of slope versus 1/[NAD] of the experimental data shown in Figure 4A had intercept at the origin, which substantiates the independence of the reaction velocity on [DMDTC] as [NAD] became infinitely high. The competitive activation of NAD reduction by DMDTC is one of the major criteria to distinguish the ordered reaction mechanism from the random ordered rapid equilibrium mechanism. (4) The stimulation reaction was a result of increasing YADH affinity for NAD by DMDTC binding as seen by the reduction of $K_{m,NAD}$ in the presence of different fixed levels of DMDTC. (5) The magnitude of the kinetic constants is in the order of $K_{A,DMDTC} < K_{m,NAD} < k_{m,EtOH} = 2.5 \times 10^{-6}$ M $< 1.2 \times 10^{-4}$ M $< 3.0 \times 10^{-2}$ M.

If the assumption that DMDTC stimulation reaction follows the ordered bireactant mechanism with respect to NAD is correct, the rate equation may be expressed in the following form.

$$\frac{v}{V_{max}} = \frac{[DMDTC]\,[NAD]}{K_A\,K_{NAD} + K_{NAD}\,[DMDTC] + [DMDTC]\,[NAD]} \qquad (1)$$

When DMDTC is the varied ligand, the equation may be expressed:

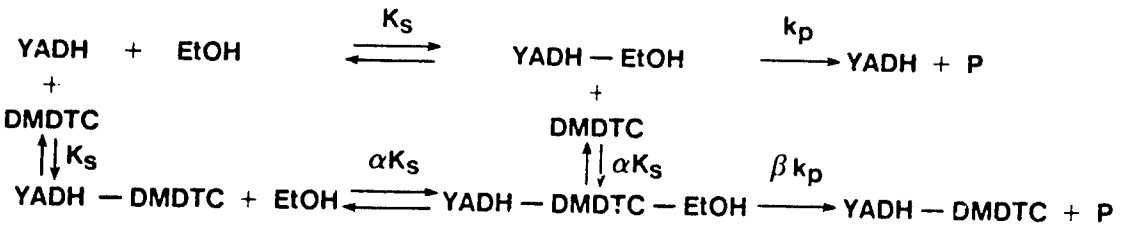
$$\frac{v}{V_{max}} = \frac{[DMDTC]}{K_A\left(\frac{K_{NAD}}{[NAD]}\right) + [DMDTC]\left(1 + \frac{K_{NAD}}{[NAD]}\right)} \qquad (2)$$

When NAD varied, the equation was expressed as:

$$\frac{v}{V_{max}} = \frac{[NAD]}{K_{NAD}\left(1 + \frac{K_A}{[DMDTC]}\right) + [NAD]} \qquad (3)$$

Equation 3 indicates clearly the competitive activation of NAD by DMDTC. Equation 2 indicates that at different fixed levels of DMDTC, the apparent values of V_{max} increase and those of $K_{A,DMDTC}$ decrease as NAD concentration varied. The experimental data obtained are consistent with the rate equations derived, based on the assumption that the stimulatory reaction follows the ordered bireactant mechanism. Since DMDTC is a mild chelating agent, binding of the activator with the zinc at the active center of the enzyme may change the conformation so that the YADH-DMDTC binary complex is more active than the free enzyme. Based on the initial rate and product inhibition studies, Wratten and Cleland[7] proposed an ordered mechanism for the yeast enzyme. However, a partly random mechanism was indicated by isotope exchange experiments[8,9] and steady-state kinetics with various secondary alcohols.[10,11] The general reaction mechanism may also include the formation of abortive complexes E-NADH-EtOH and E-EtOH.[10,12,13] Recently, the results obtained from the studies[14] of the deuterium isotope effect again indicated that the compulsory ordered mechanism originally proposed by Theorell and Chance[15] is correct. These discrepancies may be due to the ligand-induced protomer-protomer interactions.[16] The DMDTC-induced subunit interactions must facilitate either substrate binding or product release or hydride transfer.

The biphasic reciprocal points shown in Figure 5 indicate deviation from normal Michaelis-Menten behavior, which have been frequently observed in ternary complex systems having more than one reactant. The deviations from normal Michaelis-Menten kinetics with respect to one substrate are more pronounced at low concentrations of second substrate.[17,18] The relatively low NAD concentrations (3.8×10^{-4} M) employed for experiments shown in Figure 5 may have enhanced the randomness of the reaction. The mixed type activation kinetics with respect to substrate suggest the formation of YADH-DMDTC-EtOH complex in the reaction scheme shown below. Here α and β values were found to be 0.66 and 1.1, respectively.



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