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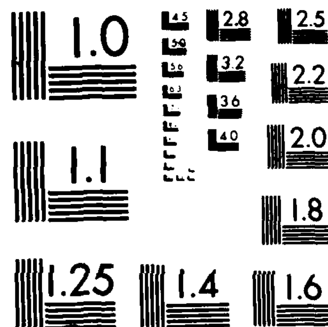

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UNCOMPETITIVE INHIBITION OF YEAST ALCOHOL DEHYDROGENASE BY DIACETOXYSCIRPENOL

by Homer R. Yeh, Ph. D.
RESEARCH DIRECTORATE

October 1986

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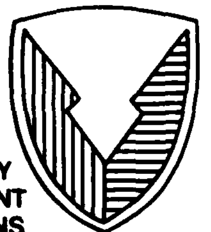

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A yeast alcohol dehydrogenase (YADH)-fusarium X complex was reported to have a molecular ratio of 1:4. The kinetics of the inhibitory effects of diacetoxyscirpenol (ADS) on YADH are presented here. Our kinetic data indicate that, at fixed levels of ADS, a series of parallel Lineweaver-Burk plots were obtained as concentrations of either ethanol or NAD varied. In the presence of 2.1×10^{-3} M of ADS there was approximately a 6-fold decrease in $K_{m,EtOH}$ and V_{max} values. The respective $K_{m,EtOH}$ and V_{max} values decreased about 15- to 13-fold when ADS levels were increased to 5.3×10^{-3} M. In the presence of 2.1×10^{-3} M of the inhibitor, $K_{m,NAD}$ and the corresponding V_{max} values decreased 2- to 3-fold, respectively, while in the presence of 5.3×10^{-3} M of ADS, the corresponding kinetic constants reduced about 5-fold. These results indicate that ADS is either an uncompetitive or a mixed-type inhibitor with respect to either ethanol or NAD. However, replots of $1/V_{max}$ and $1/K_{m,app}$ versus inhibitor concentrations gave linear curves, which indicates a

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pure uncompetitive inhibition with respect to both ethanol and NAD. As estimated from the replots, $K_{i,EtOH}$ and $K_{i,NAD}$ values of ADS were found to be in the range of 4×10^{-4} M and 1.8×10^{-3} M, respectively. The results suggest the formation of dead-end complexes of E-I-substrate and E-I-cofactor.

PREFACE

The work described in this report was authorized under Project No. 1L161102A71A, Research in Chemical & Biological Defense, Biotechnology. This work was started in December 1984 and completed in April 1985. The experimental data are recorded in laboratory notebook 84-0108.

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UNCOMPETITIVE INHIBITION OF YEAST ALCOHOL DEHYDROGENASE BY DIACETOXYSCIRPENOL

1. INTRODUCTION

Epoxytrichothecenes are the major components of the Fusarium mycotoxins identified as the causative agents for the epidemic outbreak of the alimentary toxic aleukia during the war years of 1941-1945 involving the districts of western Siberia of the USSR.[1] However, Fusarium mycotoxicosis is not a regional disease. It has been a world-wide health problem associated with the consumption of foodstuffs heavily contaminated with toxic fungi.[1,2,3]

The primary mechanism of the biological activity of trichothecenes was considered to be the disruption of protein synthesis.[4] The effect of trichothecenes on nucleic acid synthesis may be secondary to the inhibition of protein synthesis.[5]

It is possible that trichothecenes do not require metabolic activation to exert biological effects, but it is known that the epoxy ring of trichothecenes is essential for biological activity.[6] The hydrolytic or reductive opening of the 12,13-epoxy ring eliminates the cytotoxicity of the epoxytrichothecenes.[7] The epoxy ring can also spontaneously interact with the thiol groups of a variety of SH-containing enzymes, resulting in inhibition of the enzyme activity. Ueno and Matsumoto[8] reported the isolation of a molecular complex by interaction of ^{3}H-Fusarenon-X with yeast alcohol dehydrogenase (YADH). The Fusarium X-YADH complex was isolated and reported to contain four molecules of bound toxin per mole of enzyme. This finding agrees well with the fact that YADH contains four reactive SH-groups per enzyme molecule.[9] However, the kinetic behavior of epoxytrichothecenes on these thiol-containing enzymes is still largely unknown. Here we report the results of our study on the kinetics and mechanism of the inhibitory effect of diacetoxyscirpenol (ADS) on the YADH reaction.

2. MATERIALS AND METHODS

The oxidized form of nicotinamide adenine dinucleotide (NAD), YADH, and ADS 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. When ethanol (EtOH) concentrations were varied, YADH reaction mixtures contained 1.3×10^{-4} M of NAD, and from 1.18×10^{-2} to 3.0×10^{-2} M of EtOH, in the presence and in the absence of 2.1×10^{-3} M or 5.2×10^{-3} M of ADS. When NAD concentrations were varied, EtOH concentrations were fixed at 3.0×10^{-2} M, and NAD concentrations were varied from 5.6×10^{-6} to $1\ 3 \times 10^{-5}$ M, in the presence and in the absence of 2.1×10^{-3} M or 5.2×10^{-2} M of ADS.

All reaction mixtures were prepared in 0.1 M of Na_2HPO_4 buffer, pH 7.4, in a total volume of 1.15 ml per cuvette. ADS was initially dissolved in 0.05 ml of dimethyl sulfoxide (DMSO) and an equivalent amount of DMSO was also present in all control reaction mixtures to compensate for the DMSO solvent effect. Enzyme reaction was initiated by addition of 6 μg of YADH per cuvette and followed at 340 nm in a Cary 210 spectrophotometer maintained at 25 °C.

3. RESULTS

3.1 Effect of ADS on K_m and V_{max} Values as EtOH Concentrations Varied.

Figure 1 shows the effect of ADS concentrations on the Lineweaver-Burk[10] plots of the activity vs ethanol concentration assays. The corresponding kinetic constants, as calculated from the same data shown in Figure 1, are listed in Table 1. As a result of the ADS inhibitory effect, a series of linear, parallel, double reciprocal plots were obtained. The data indicate that, in the presence of 2.1×10^{-3} M of ADS, there was approximately a 6-fold decrease in $K_{m,EtOH}$ and V_{max} values of the enzyme reaction as substrate concentrations varied. When ADS concentrations were increased to 5.2×10^{-3} M, the respective $K_{m,EtOH}$ and V_{max} values decreased about 15- to 13-fold. These observations may indicate that ADS is either an uncompetitive or a mixed type inhibitor with respect to ethanol, since the presence of ADS decreased both $K_{m,EtOH}$ and the corresponding V_{max} values to about the same extent.[11] However, replots of $1/V_{max}$ and $1/K_{m,app}$ versus inhibitor concentrations gave linear curves (Figure 2A and B). The replots will be nonlinear if ADS is a mixed type inhibitor.[11] This clearly indicates that ADS is a pure uncompetitive inhibitor with respect to the substrate, EtOH.[11] As evaluated from the replots, the $K_{i,EtOH}$ value of the ADS inhibitory effect was found to be in the range of 4×10^{-4} M.

3.2 Effect of ADS on K_m and V_{max} Values as NAD Concentrations Varied.

Figure 3 shows the effect of ADS concentrations on $K_{m,NAD}$ and the corresponding V_{max} values as NAD concentrations varied. As a result, a series of linear, parallel Lineweaver-Burk plots was obtained. The corresponding kinetic constants are listed in Table 2. As can be seen, in the presence of 2.1×10^{-3} M of ADS, the $K_{m,NAD}$ and the corresponding V_{max} values decreased about 2- to 3-fold, respectively; whereas, in the presence of 5.2×10^{-3} M of ADS, the corresponding kinetic constants decreased about 5-fold. Similar to what was observed for ethanol, when NAD concentrations were varied, the inhibitory effect of ADS resulted in the reduction of the $K_{m,NAD}$ and the corresponding V_{max} values of the enzyme reaction to about the same extent. This may also indicate that ADS is either an uncompetitive or a mixed type inhibitor with respect to NAD.[11] The linear $1/V_{max}$ and $1/K_{m,app}$ versus ADS concentration replots (Figure 4A and B) confirm that ADS is also an uncompetitive inhibitor with respect to NAD under the experimental conditions employed here.[11] As estimated from the replots, $K_{i,NAD}$ of the ADS inhibitory effect was found to be in the range of 1.8×10^{-3} M.[11]

The uncompetitive inhibition of YADH reaction by ADS with respect to both the substrate and cofactor indicates the possibility of formation of the dead-end ternary complexes of E-EtOH-ADS and E-NAD-ADS.[11]

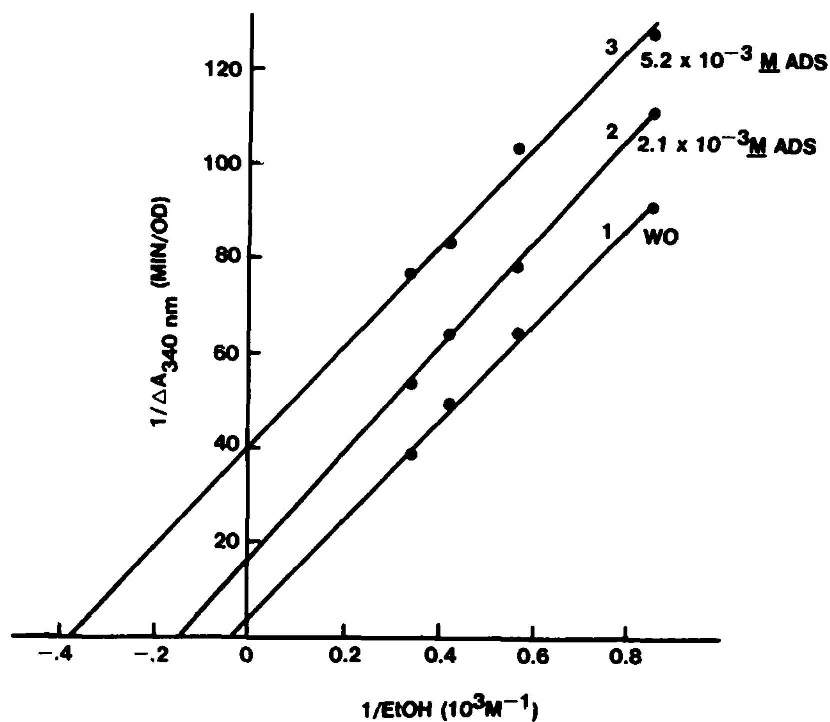


Figure 1. Effect of Diacetoxyscirpenol (ADS) on Km and V_{max} of YADH Oxidation of EtOH.

The reaction mixtures contained 1.3 x 10^{-4} M of NAD, from 1.2 x 10^{-2} to 3.0 x 10^{-3} M of ethanol, and 0.1 M of sodium phosphate buffer, pH 7.4, in a total volume of 1.15 ml per cuvette. Curve 1 was obtained in the absence of ADS, and Curves 3 and 4 were obtained in the presence of 2.1 x 10^{-3} M and 5.2 x 10^{-3} M of ADS, respectively. Reaction was initiated by addition of 6 μg of YADH and followed at 340 nm in a Cary 210 spectrophotometer.

Table 1. Effect of Diacetoxyscirpenol on Kinetic Constants of Ethanol Oxidation by YADH

DIACETOXYSCIRPENOL CONCENTRATIONS ($\times 10^{-3}$ M)	$K_{m,EtOH}$ ($\times 10^{-3}$ M)	V_{max} (OD/MIN)
WO	40.0	0.33
2.1	6.7	0.058
5.3	2.6	0.025

Note: Data were taken from Figure 1.

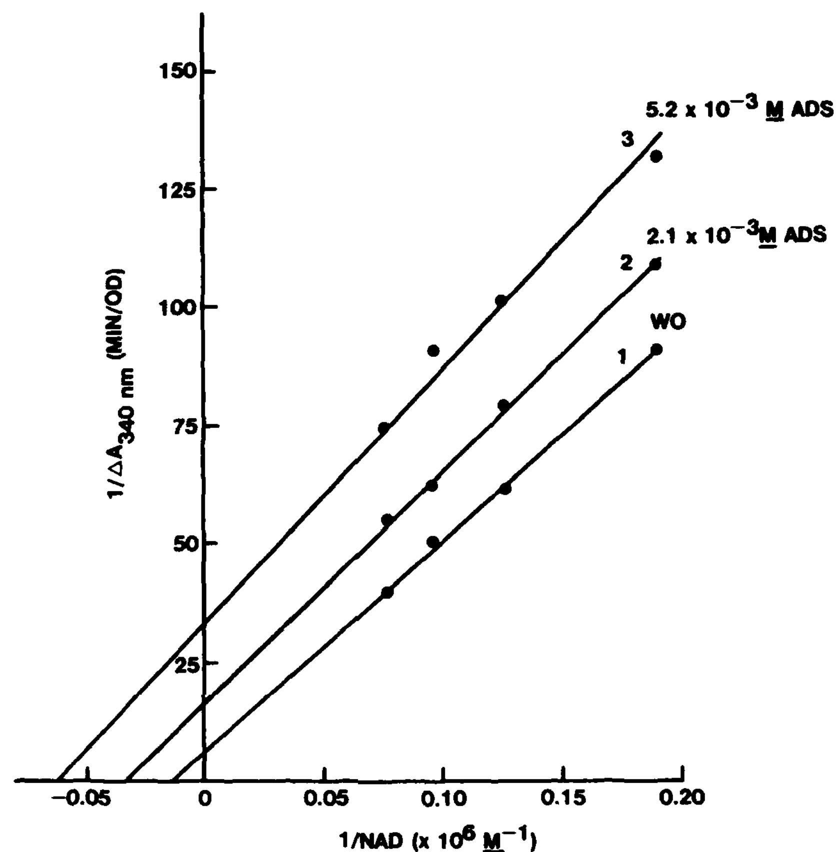


Figure 2. Effect of Diacetoxyscirpenol (ADS) on K_m and V_{max} of YADH Reduction of NAD.

The reaction mixtures contained 3.0 x 10-2 M of EtOH, from 5.6 x 10^{-6} to 1.3 x 10^{-5} M of NAD, and 0.1 M of sodium phosphate buffer, pH 7.4, in a total volume of 1.15 ml per cuvette. Curve 1 was obtained in the absence of ADS, and Curves 2 and 3 were obtained in the presence of 2.1 x 10^{-3} M and 5.2 x 10^{-3} M of ADS, respectively. Reaction was initiated by addition of 6 μg of YADH and followed at 340 nm in a Cary 210 spectrophotometer.

Table 2. Effect of Diacetoxyscirpenol on Kinetic Constants of NAD Reduction by YADH

DIACETOXYSCIRPENOL CONCENTRATIONS ($\times 10^{-3}$ M)	$K_{m,NAD}$ ($\times 10^{-3}$ M)	V_{max} (OD/MIN)
WO	66.7	0.189
2.1	30.3	0.063
5.3	16.7	0.037

Note: Data were taken from Figure 2.

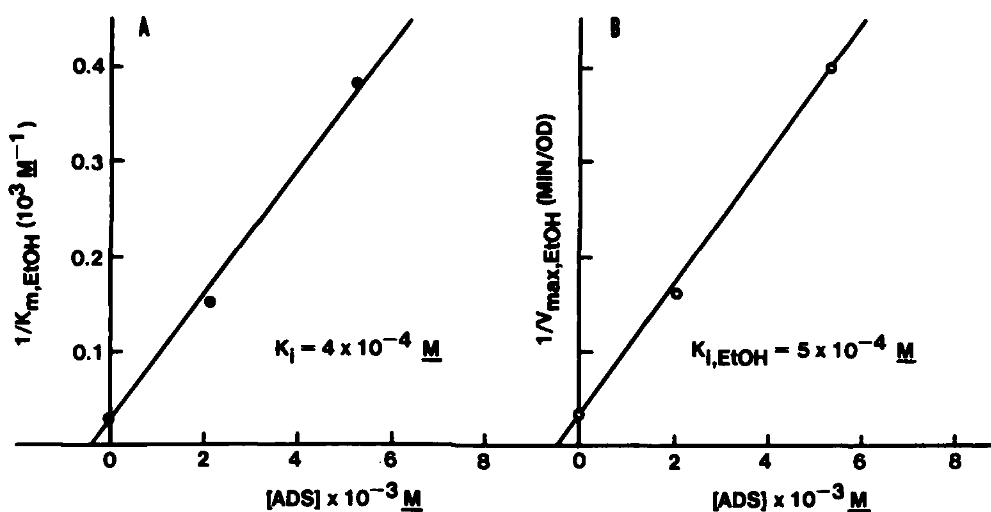


Figure 3. Replots of Data Taken from the Reciprocal Plot Shown in Figure 1 (A) $1/K_m$ versus (I). (B) $1/V_{max}$ versus (I).

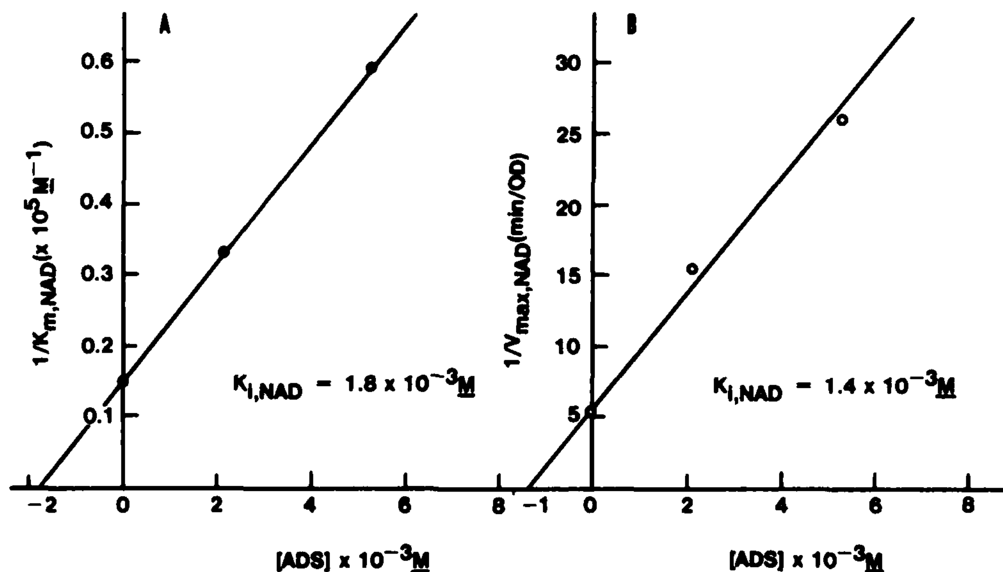
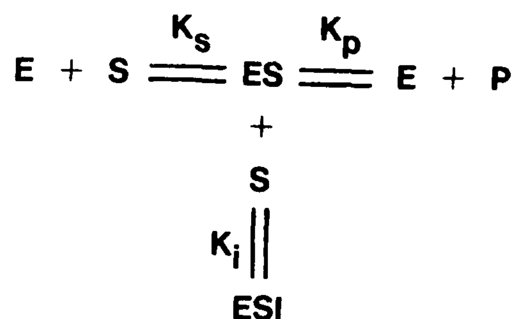


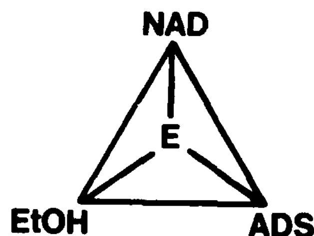
Figure 4. Replots of Data Taken from the Reciprocal Plot shown in Figure 2 (A) $1/K_m$ versus [I]. (B) $1/V_{max}$ versus [I].

4. DISCUSSION

In steady-state kinetics, uncompetitive inhibition may be described by the following equilibria.[11]



The equilibria indicate that, at any inhibitor concentration, an infinitely high substrate or cofactor concentration will not drive all of the enzyme to the active enzyme substrate (ES) form. In other words, some nonproductive dead-end enzyme substrate inhibitor (ESI) product will always be present. In consequence, the presence of ADS in the enzyme reaction mixtures decreases both K_m and V_{max} values of the YADH system to about the same extent because of the formation of the dead-end E-ADS-EtOH and E-ADS-NAD complexes that remove some active ES from reaction.[11] On the other hand, the equilibria also indicate that ADS, as an uncompetitive inhibitor, can bind to YADH only after the substrate or cofactor binds.[11] This appears to contradict the observation that epoxytrichothecenes can generate covalently linked YADH-ADS complexes with thiol-containing enzymes.[8] The protective effect of both the substrate and cofactor against the direct binding of ADS with YADH may explain the compulsorily ordered binding mechanism of the ADS inhibitory reaction as described in the above equilibria.[11] The uncompetitive reaction mechanism indicates that ADS inhibits because of its effect on the V_{max} of the YADH reaction. ADS is actually an activator with respect to K_m values of either ethanol or NAD. On the other hand, the present studies were carried out in the presence of relatively high concentrations of EtOH and NAD. If either substrate or cofactor concentration is low enough, the effect of ADS on V_{max} may be cancelled by its effect on K_m, and little or no inhibition will be observed.[11] Since ADS can form the dead-end product with either substrate and cofactor, this raises the possibility that a quaternary complex may be generated in the reaction mixture. The quaternary complex may have the structure such as



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