[CONTRIBUTION FROM THE MCCOLLUM-PRATT INSTITUTE, THE JOHNS HOPKINS UNIVERSITY]

Yeast Alcohol Dehydrogenase. III. Relation of Alcohol Structure to Activity[1]

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The specificity of yeast alcohol dehydrogenase toward alcohols has been investigated. It was concluded that three factors determine specificity: (1) nucleophilic character of the alcohol, (2) molecular dimensions of the alcohol, and (3) orientation of the alcohol molecule. The known stereospecificity of the enzyme is also apparent in the oxidation of truly asymmetric substrates. The absolute configuration of the suitable substrates is that of the L-series. The enzyme will serve as a lactic dehydrogenase, with ADP as an activator; this oxidation is specific for D(−)-lactic acid. The steric limitations of the alcohol oxidation are at least partially determined by the coenzyme used. The implications of these findings are discussed.

Introduction

The alcohol specificity of yeast ADH[2] has been studied by previous investigators.[3,4] However, no general conclusion was drawn from the structure of those alcohols which would function as a substrate for the enzyme. The only conclusion reached was that the enzyme is not specific for ethanol, but rather that many primary and secondary alcohols will be oxidized. It has recently been demonstrated that the oxidation of alcohol by yeast ADH is stereospecific with regard to ethanol.[5,6]

Previous work in this laboratory has shown that only one ethanol appears to be oxidized at a time,[7] even though four coenzyme molecules are bound.[7,8] In a search for explanations of this lack of stoichiometry an attempt was made to correlate the structure of the alcohol with its ability to serve as a substrate for yeast ADH. It was found that three factors govern the effectiveness of a given substrate: (1) the molecular dimensions of the substrate in the vicinity of the carbinol grouping; (2) the nucleophilic character of the alcohol; and (3) the orientation of the substrate with respect to the enzyme. In addition, data will be presented which indicate that the absolute configuration of the alcohol as oxidized is of the L-series.

Experimental

Enzymes.—Twice recrystallized yeast ADH was obtained commercially.[9] Crystalline beef heart LDH[9] was used as the L(+)-lactic acid specific lactic dehydrogenase; an ammonium sulfate cut between 50 and 75% saturation from a sonicate of *Leuconostoc mesenteroides* cells served as a source of D(−)-lactic acid specific LDH.[10]

Coenzymes.—DPN and ADP were obtained from the Pabst Laboratories. The coenzyme analogs were obtained by the action of pig brain DPNase on DPN in the presence of the appropriate pyridine base as described for the isonicotinic acid hydrazide[11] and acetylpyridine[12] analogs of DPN. DPNH was prepared enzymatically from DPN as described[13] previously.

Substrates.—All alcohols, when obtained as reagent grade, were used without prior purification. Those which were obtained in practical grade were purified by fractional distillation. Calcium D(−)-lactate and calcium L(+)-lactate were obtained from the California Foundation for Biochemical Research. Their purity was checked with the appropriate LDH, using acetylpyridine-DPN as coenzyme.[12] D(−)-Lactate contained less than 0.1% of the other isomer, but L(+)-lactic acid was contaminated with at least 5% of its enantiomorph. 2-Octanol was resolved *via* the brucine salt of the monohydrogenphthalates.[14]

Rate Determinations.—Rates were determined in a Beckman spectrophotometer, model DU, at the appropriate absorption maximum of the coenzyme used.[12,15] Rates were defined as the increase in optical density at the absorption maximum between the 15 and 30 second interval after starting the reaction with enzyme.

Results

Substrate Specificity

The Effect of Increasing Chain Length.—All primary alcohols tested will serve as a substrate for yeast ADH. In the straight chain homologous series, the reactivity decreases with increasing chain length. The only exception is methanol, which will be discussed below. The decrease in activity is not greater than can be expected from the decreased electronegativity of the carbinol grouping. Hine and Hine[16] have determined that the "acidity" of alcohols in a homologous series is approximately linearly related to the pK_a of the corresponding fatty acids. Thus, if one plots the logarithm of the relative rate of the straight chain alcohols *versus* the chain length, a straight line relationship should be obtained if the relative activity is due solely to the apparent dissociation ability of the alcohol. This indeed appears to be the case (Fig. 1). Variations of the concentration of the alcohols did not change the order of activity of the substrates.

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(2) Abbreviations used in this paper are: ADH, alcohol dehydrogenase; D-LDH, D(−)-lactic acid specific lactic dehydrogenase; L-LDH, L(+)-lactic acid specific lactic dehydrogenase; ADP, adenosine 5′-pyrophosphate; *DPN* and *DPNH*, oxidized and reduced diphosphopyridine dinucleotide, resp.; acetylpyridine-DPN and pyridine-3-aldehyde-DPN, the 3-acetylpyridine and pyridine-3-aldehyde analogs of DPN, resp.; and tris, trihydroxymethylaminomethane.

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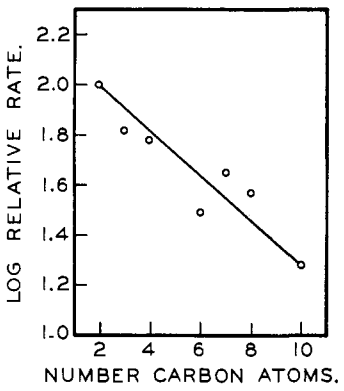

Fig. 1.—The effect of increasing chain length on the rate of oxidation: alcohol concentration 2.5×10^{-3} *M*, tris buffer, 0.05 *M*, *p*H 9.3, DPN 3.5×10^{-4} *M*. The value for amyl alcohol is not given, since the alcohol contained an inhibitor, which even on repeated fractional distillation could not be removed completely.

Effect of Steric Hindrance.—If the dissociation ability of the alcohol were the only determining step, compounds like chloroethanol or other alcohols with an electron attracting group should be better substrates than the corresponding primary alcohols. But this is not the case, chloroethanol is a poorer substrate than ethanol. The influence of substitution is indicated in Table I. It is clear that any substituent regardless of its electron donating or attracting ability, decreases the rate. That this effect is due to steric hindrance is best illustrated by the phenyl-substituted alcohols. This is shown in Fig. 2. Whereas benzyl alcohol and 2-phenylethanol are relatively poor substrates, 3-phenyl-1-propanol is oxidized at a quite rapid rate.

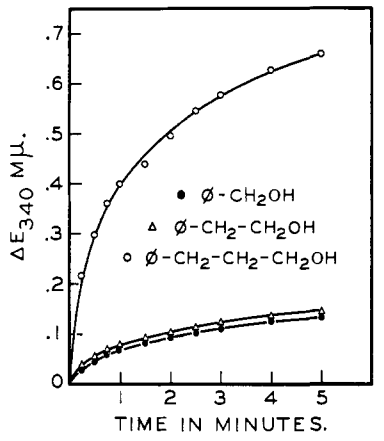

Fig. 2.—The effect of phenyl substitution on the rate of alcohol oxidation: substrate concentration 5×10^{-3} *M*, in tris buffer, *p*H 9.3, 0.05 *M*, DPN concentration 1.5×10^{-4} *M*, yeast ADH 250 mcg.

Effect of Orientation.—A third factor was found to play a role in the alcohol specificity. If steric hindrance were, together with nucleophilic character, the solely decisive factor, a large molecule would at all times be oxidized more slowly than a small molecule. However, *n*-decanol is not slower, relative to ethanol, than can be expected from the inductive effect of the sidechain. Therefore, the "size" of ethanol and *n*-decanol appears to be the same for the enzyme. This can only be the case if both are oriented in such a way that only the carbinol moiety will attack the enzyme. This is even more clearly illustrated in the methyl-*n*-alkylcarbinol series. Even though isopropyl alcohol is relatively slowly oxidized, methylpropylcarbinol reacts faster although the dimensions of the latter are greater and the acidity of the carbinol is less. If one plots the logarithm of the relative rate of these secondary carbinols *versus* the chain length, a maximum is observed at 5 carbons (Fig. 3). The expected rate, assuming no

TABLE I

THE INFLUENCE OF SUBSTITUTION ON THE ABILITY OF ALCOHOLS TO SERVE AS SUBSTRATE FOR YEAST ALCOHOL DEHYDROGENASE

Alcohol[a]	Relative rate	Alcohol[a]	Relative rate
Methanol	8	Diethylamino-ethanol	0
Ethanol[b]	1000	2-Chloroethanol	8
n-Alkylcarbinols	[c]	Isobutyl alcohol	4
Methyl-*n*-alkyl carbinols	[d]	Isoamyl alcohol	4
Ethylene glycol	1.2	3-Hexanol	0
Glycerol	7	*t*-Butyl alcohol	0
Erythritol	2	D(−)-Lactic acid	3
L-Arabitol	1.5	Glycolic acid	17
2-Aminoethanol	22	DL-β-Hydroxy-butyric acid[e]	1
Dimethylamino-ethanol	0		

[a] The alcohols were tested at a concentration of 5.0×10^{-3} *M*, in tris buffer, 0.05 *M*, *p*H 9.3, DPN 1.5×10^{-4} *M*. Slow rates were checked with ADP as cofactor at a concentration of 7.0×10^{-3} *M*. [b] Arbitrarily assigned 1000. [c] See Fig. 1. [d] See Fig. 3. [e] Tested at double concentration.

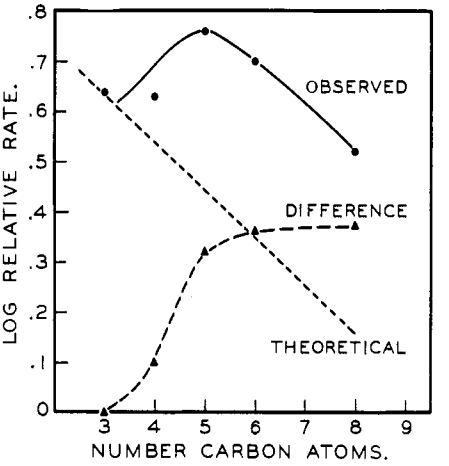

Fig. 3.—The effect of increasing chain length on the rate of oxidation of methyl-*n*-alkyl-carbinols: the concentration of alcohols was 2.5×10^{-3} *M* of the active isomer. The DL-isomers were employed for the alcohols of chain length of four carbons or more. Tris buffer, *p*H 9.3, 0.05 *M*, DPN 3.5×10^{-4} *M*. The theoretical rate was determined from the equation

$$\log \text{relative rate} = -0.09 (\text{no. carbon atoms}) + 0.88$$

This relationship was derived from the data in Fig. 1. The difference curve describes the deviation of the observed values from the theoretical relationship, and is proposed to predict the effect of orientation.

steric hindrance and a decrease in nucleophilic character similar to the *n*-alkylcarbinols, would give a decrease of the rate with increasing chain length. The difference between expected and observed rates shows that an increase occurs up to about six carbon chain length (Fig. 3). This can be interpreted as an indication of orientation of the molecule. Similarly, lactic acid is oxidized stereospecifically.[17] Yet, if steric hindrance is taken into account, both the carboxy group and the methyl group could attack the enzyme. It may be mentioned here, that even though compounds like lactic acid are very poor substrates, significant and even rapid rates can be observed using about 7×10^{-3} *M* ADP as an activator[18] (Fig. 4).

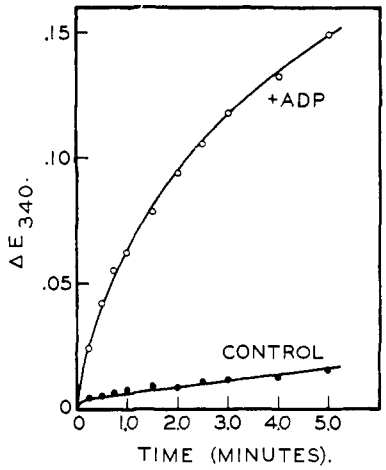

Fig. 4.—The effect of ADP on the rate of lactate oxidation: D(−)-lactic acid 7×10^{-3} *M*, DPN 1.5×10^{-4} *M*, in tris buffer, *p*H 9.3, 0.05 *M*, ADP concentration 8×10^{-4} *M*. Yeast ADH added 25 mcg.

Stereochemistry of the Yeast ADH Reaction

Steric Specificity.—The substrates, which have an asymmetric center at the position of oxidation, are oxidized asymmetrically. This holds even for ethanol,[5,6] which has a *meso* carbon.[19] The substrates which show this effect clearly are lactic acid and the methyl-*n*-alkylcarbinols. If the racemic mixture is used, only one isomer is oxidized (Table II). Similarly, only the D(−)-lactate is used as a substrate. This can be shown by comparing the rates of pure D(−)- and L(+)-lactate. Even when all traces of L(+)-lactate are removed from the D-isomer with L-LDH and acetylpyridine-DPN, the compound still serves as a substrate. Conversely, pyruvate, DPNH and ADH give a product which can be reoxidized with D-LDH and acetylpyridine-DPN, but not with L-LDH and the coenzyme. The use of acetylpyridine-DPN in these experiments is indicated by the more favorable potential for lactate oxidation of this analog as compared to DPN.[12,20] The effectiveness of the analog with many dehydrogenases recently has been demonstrated.[20]

TABLE II

THE STEREOSPECIFICITY OF YEAST ALCOHOL DEHYDROGENASE FOR SECONDARY ALCOHOLS

Alcohol oxidized[a]	μ Moles added	μ Moles DPN[b] reduced
Isopropyl alcohol	0.25	0.22
DL-2-Butanol	.25	.11
DL-2-Octanol	.25	.12
L(+)-Octanol-2[c]	.25	.25

[a] The substrates were tested in tris buffer, *p*H 9.3, 0.05 *M*, containing DPN 1.8×10^{-3} *M*, in a final volume of 3 ml. [b] Molar extinction coefficient of DPNH used: 6.22×10^{3}. [c] Monohydrogenphthalate $[\alpha]^{21}D$ +47.2° (*c* 0.65, ethanol); reported[14] +48.4°.

Influence of Orientation on Stereospecificity.—When 2-octanol was resolved, it became apparent that the isomer utilized was L(+)-octanol-2.

It was made likely above that *n*-alkyl carbinols are oriented in such a way that only the carbinol moiety will attack the enzyme. Since 3-hexanol cannot serve as a substrate, the octanol molecule appears to be oriented with the methyl group toward the enzyme. The correlation of configuration of L-(+)-octanol-2 and D(−)-lactate, as indicated in Fig. 5, has been demonstrated by Levene and Haller.[21] Therefore, since octanol is oriented with the methyl group toward the enzyme, lactate must be oriented with its carboxyl group toward the enzyme in order to explain the observed stereospecificity. Thus it becomes evident that the substrate molecule is directed with the negative grouping toward the enzyme. This orientation is sufficiently strong to allow stereospecificity even when the molecular dimensions are such that another position would be possible within the limitations imposed by the enzyme, as is the case with lactic acid.

Since the orientation of lactate and octanol appears to be strong, it is highly likely that ethanol will be similarly oriented. If we assume ethanol labeled with deuterium as in Fig. 5, the action of yeast ADH on this molecule would give rise to labeled acetaldehyde. The enantiomorph of the ethanol-*d* represented would give rise to deuterolabeled DPN.

(17) The activity of enzymes on uncommon substrates requires proof that the enzyme is indeed homogeneous and does not contain traces of a contaminating protein catalyzing this reaction. Yeast ADH as used is homogeneous by the physical criterion of ultracentrifugation, and the chemical criterion of number of coenzymes bound per mole of enzyme, both by ultracentrifugational separation techniques (ref. 8), and spectral shift due to the hydroxylamine addition reaction (ref. 7). Furthermore, yeast does not contain a lactic dehydrogenase, but rather a lactic acid oxidase. This latter enzyme is L(+)-lactic acid specific. All activity of whole yeast toward D(−)-lactic acid can be accounted for as due to ADH. This was shown by following the purification procedure of yeast ADH as described by Racker (E. Racker, *J. Biol. Chem.*, **194**, 313 (1950)), and measuring activity toward ethanol and D(−)-lactic acid thoughout the purification. The ratio of activity toward both substrates remained constant. The activity toward D(−)-lactic acid was also not due to trace contaminations with alcohols. This was shown by the following points: first, crystalline horse liver alcohol dehydrogenase failed to attack our D(−)-lactic acid preparation; secondly, D(−)-lactic acid can be oxidized stoichiometrically by the action of DPN and yeast ADH, and thirdly, pyruvate will serve as a substrate for yeast ADH. The product of this reaction will serve as a substrate for D-LDH.

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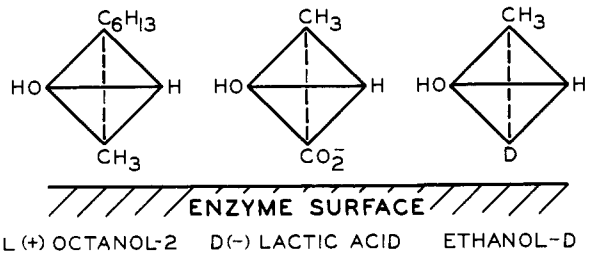

Fig. 5.—The configuration of substrates for yeast ADH.

Influence of Coenzyme on Magnitude of Steric Hindrance.—The enzyme appears to operate according to the reaction sequence[7,22]

Enzyme ⟶ enzyme–coenzyme ⟶
enzyme–coenzyme–substrate

The specificity of the reaction is therefore determined by the enzyme–coenzyme complex, and not by the enzyme alone. The steric limitations are largely governed by the coenzyme used. This can be shown by several lines of evidence. Previously described observations[15] show that some analogs of DPN will not catalyze ethanol oxidation even though the reduced form will reduce acetaldehyde.

Methanol is a poor substrate, since it is not oriented, and since it is too small to afford a fit on the enzyme. However, if one compares the rate of methanol oxidation relative to that of ethanol using different coenzyme analogs, a great variation in methanol effectiveness can be observed (Table III). It is seen that pyridine-3-aldehyde-DPN makes methanol become a reasonable substrate, while with acetylpyridine-DPN the rate with methanol is negligible. For isopropyl alcohol as substrate similar relationships hold. Thus one can assume that the "fit" of the substrate is afforded by the coenzymes and that relatively speaking pyridine-3-aldehyde-DPN gives a better "fit" for methanol than does DPN. The slow rates with acetylpyridine-DPN are not the result of its potential, since there seems to be very little potential difference between acetylpyridine-DPN and pyridine-3-aldehyde-DPN.[20] Furthermore the affinity of the enzyme for the three coenzymes is similar.[7]

TABLE III

THE INFLUENCE OF COENZYME ON THE SPECIFICITY OF YEAST ADH

Coenzyme	Relative rate of coenzyme reduction[a] Ethanol	Methanol
DPN	100	0.8
Acetylpyridine-DPN	22.3	.017
Pyridine-3-aldehyde-DPN	7.3	.86

[a] All rates were performed in tris buffer, 0.05 *M*, *p*H 9.3. Coenzyme concentration, 1.5×10^{-4} *M*, substrate concentration, 5.0×10^{-3} *M*. Rate with DPN and ethanol assigned arbitrarily 100.

The Effect of Reduced DPN on the Rate of a Sterically Hindered Substrate.—The geometry of reduced DPN is markedly different from that of the oxidized form.[15] If the four DPN molecules on the enzyme are responsible for the magnitude of steric hindrance, the enzyme can be made less specific by keeping one of the coenzymes in the reduced state. Thus it can be shown that the rate with D(−)-lactic acid in the presence of ethanol is greater than the sum of the rates of the two substrates tested separately (Fig. 6A). One can postulate the following sequence to be operative

Enzyme-DPN_4 + ethanol ⟶
Enzyme-DPN_3-DPNH + acetaldehyde + H^+

Enzyme-DPN_3-DPNH + lactate ⟶
Enzyme-DPN_2-$DPNH_2$ + pyruvate + H^+

The same phenomenon is also observed with isopropyl alcohol, though the magnitude of the stimulation is not as marked as it is for lactate.

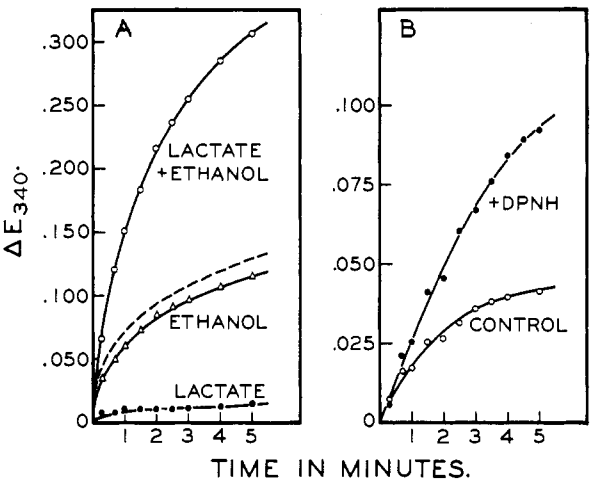

Fig. 6.—The effect of reduced coenzyme on the rate of lactate oxidation: A: Ethanol 5×10^{-3} *M*, D(−)-lactate 7×10^{-3} *M*, DPN 1.5×10^{-4} *M*, in tris buffer, *p*H 9.3, 0.05 *M*. The dashed curve represents the summation of the rates of lactate and ethanol taken separately. B: D(−)-lactate 7×10^{-3} *M*, DPN 1.5×10^{-4} *M*, in tris buffer, *p*H 9.3, 0.05 *M*; DPNH concentration 1.3×10^{-5} *M*.

The above postulation is strengthened by the observation that the addition of DPNH will activate the oxidation of lactate by DPN. This addition can be expected to yield the enzymatic species enzyme–DHN_3–DPNH, which is more efficient as a lactic dehydrogenase than is the species enzyme–DPN_4 (Fig. 6B). The effect is optimal by adding DPN and DPNH in a ratio of about 40 to 1. This is in accord with the stronger binding of DPNH by yeast ADH.[8] The addition of DPNH to a mixture oxidizing ethanol decreases the rate of ethanol oxidation as expected.

Discussion

The picture for alcohol specificity thus requires a model where the enzyme truly gives a place for the substrate to fit in. The easiest picture to explain this phenomenon is given by postulating the four DPN molecules close together and but one substrate molecule fitting in between. The size of the "hole" would be determined by the height of the DPN molecule and the distance between the four coenzyme molecules. A rigid geometry between

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the enzyme–coenzyme complex and substrate is adequate to explain stereospecificity toward the substrate, even when no formal bond between enzyme and substrate exists. Similarly a rigid geometry implies equally a stereospecificity toward the coenzyme. These forms of stereospecificity indeed do exist.[5,6,23]

This picture of enzyme–coenzyme–substrate complex is supported by the fact that the enzyme binds four moles of coenzyme,[7,8] but appears to bind only one substrate molecule at a time.[7] Additional evidence is given by the fact that modification of the structure of the DPN-tetrad changes the specificity of the enzyme. This can be done both by changing from DPN to a DPN analog in the reaction, or by converting one DPN to DPNH.

The dependence of the rate on the "acidity" of the alcohol can be explained by the mode of binding of the substrate to the protein–DPN complex. It has been made likely[7,24] that the actual binding reaction of alcohols to DPN linked dehydrogenases is an addition of the substrate to the *para*-position of the DPN molecule through the hydroxyl group. Thus the affinity would be a direct function of the nucleophilic nature of the alcohol. The exact mechanism of hydrogen transfer will be the subject of future publications.

The activity of yeast ADH can only be described by Michaelis-Menten kinetics under very specific conditions. The K_m for substrate can vary from zero to infinity, depending on the concentration of the coenzyme.[25] Similarly, the maximal velocity of the enzyme at substrate saturation is a meaningless entity, since the enzyme shows at least two levels of saturation.[25,26] A detailed description of the kinetics of yeast ADH will be forthcoming.[27] However, the decreased activity of less nucleophilic alcohols is proportional with a similar decrease in the ability of homologous *n*-alkyl mercaptans to form ternary complexes with the enzyme and coenzyme, or to form addition compounds with the coenzyme alone.[27] Thus it is felt that the decreased affinity for the *para*-position of the DPN molecule rather than a decreased rate of hydrogen transfer (*i.e.*, a lower k_3) is responsible for the lower rates with unnatural substrates.

It is not possible to translate the stereospecificity of yeast ADH to lactate and LDH. It is necessary for the assignment of absolute configurational specificity to know both specificity and orientation. There is also no *a priori* indication so far which side of the DPN molecule is reduced. In general, it is necessary to point out that the data solely apply to yeast ADH and are not be generalized to other dehydrogenases until further information is available.

The configuration of the secondary alcohols which are substrates is in agreement with the old observations of Neuberg and Nord[28] that fermenting yeast acts upon 2-octanone, yielding dextrorotatory 2-octanol. It is highly probable that the present studies relate to the ADH inside the yeast cell. Thus it appears that the stereospecificity observed is not an artifact, but truly a property of the enzyme *in vivo*. The significance of activation by nucleoside-polyphosphates[18] within the intact cell remains to be seen. However, since the activation by ADP is quite large, the yeast ADH in the presence of this nucleotide becomes a very unspecific enzyme. It must be pointed out again, however, that no substrate becomes oxidized because of the presence of ADP; the nucleotide merely serves as a tool to give rates rapid enough for convenient comparison with other substrates.

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