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AN EXAMINATION OF OCTANOL AND OCTANAL METABOLISM TO OCTANOIC ACID BY HORSE LIVER ALCOHOL DEHYDROGENASE

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Summary

The kinetics of the horse liver alcohol dehydrogenase (alcohol: NAD^+ oxidoreductase EC 1.1.1.1) catalyzed metabolism of octanol and octanal to octanoic acid have been examined. On incubation of octanol with horse liver alcohol dehydrogenase in the presence of NAD^+, NADH as well as octanal and octanoic acid were seen as the initial products. However, on continued incubation, the octanal concentration progressively decreased to where only negligible quantities were present in the incubation after 10 min. The production of NADH was biphasic. An initial phase was followed in about 2 min with a slower but linear rate of NADH production. The production of octanoic acid was approximately linear throughout the 10 min incubation period. Since octanal is an intermediate in the oxidation of octanol to octanoic acid, the ability of semicarbazide to inhibit the metabolism of octanol to octanoic acid was examined. At a concentration of semicarbazide which was 63 times the concentration of octanol in the incubation media, the rate of formation of octanoic acid was inhibited by only 30%. The results of these experiments suggest that in the oxidation of octanol to octanoic acid a portion of the octanal formed from octanol is not released from the enzyme but, in the presence of NAD^+, is oxidized to octanoic acid.

Introduction

Horse liver alcohol dehydrogenase has recently been reported to oxidize the aliphatic aldehydes acetaldehyde, butanal, hexanal and octanal to the cor-

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responding acids [1]. The oxidation of aldehydes by horse liver alcohol dehydrogenase has been reported previously [2,3,4], but this was the first report of a net reduction of NAD^+ with the oxidation of aldehydes by this enzyme. The K_m values for the oxidation of this series of aldehydes were found to be similar ($\approx 10^{-5}$ M), but there was a significant increase in the maximal velocities for the oxidation of these aliphatic aldehydes with increase in chain length. The oxidation of octanal to octanoic acid was found to have a maximal velocity which was approximately one-tenth that of ethanol.

When horse liver dehydrogenase was incubated with octanol for periods of time greater than 10 min in the presence of high concentrations of NAD^+, the only products detected were NADH and octanoic acid [1]. An examination of the stoichiometry of this reaction revealed that 2 mol of NAD^+ were reduced per mole of octanol oxidized to octanoic acid.

In the present report we have examined various aspects of the kinetics of the metabolism of the alcohol octanol to octanoic acid by horse liver alcohol dehydrogenase. The ability of horse liver alcohol dehydrogenase to oxidize a wide variety of alcohols and aldehydes to the corresponding acids has also been examined.

Materials and Methods

Horse liver alcohol dehydrogenase (2.5 units/mg lyophilized) was purchased from Worthington Biochemical Corporation, Freehold, New Jersey. The purity of these preparations of alcohol dehydrogenase was examined by subjecting aliquots to starch gel electrophoresis [5] followed by staining of the gel for protein and for activity [6]. The sample of enzyme used in these studies was found to be essentially free of protein components that would not exhibit activity staining using ethanol as the substrate. The pyridine nucleotides NAD^+ and NADH were obtained from Boehringer Mannheim, New York, New York. Octanal was purchased from the Aldrich Chemical Company, Atlanta, Georgia, and purified from contaminating octanoic acid and possible contamination with octanol by vacuum distillation. Octanoic acid was a product of Eastman Chemical, Kingsport, Tennessee. Octanol was obtained from Fisher Chemical Company, Englewood Cliffs, New Jersey. The purity of the octanol, octanal and octanoic acid was verified using gas chromatography [1]. Vitamin A aldehyde and chloral hydrate were purchased from the Sigma Chemical Company, St. Louis, Missouri. [^{14}C]cetyl alcohol was obtained from Nuclear Chicago, Des Plaines, Illinois. [^{14}C]benzyl alcohol and [^{14}C]benzaldehyde were purchased from Amersham Searle, Atlanta, Georgia. Tryptophol and 5-hydroxytriptophol were purchased from K and K Laboratories, Plainview, New York. Thiamine was a product of Merck and Company, Incorporated, Rahway, New Jersey. The compound 5-(2-hydroxyethyl)-4-methylthiazole was prepared by cleavage of thiamine according to the method of Williams et al. [7]. The purity of these compounds was examined by thin layer chromatography. The gas chromatographic column packing, C6 diethylene glycol succinate on Anakrom ABS 110/120 mesh P, was a product of Analabs, Incorporated, Hamden, Connecticut.

Enzyme Assays

The rates of oxidation of octanol and octanal were determined either spectrophotometrically using a Gilford recording spectrophotometer or by gas chromatography. The enzyme activity was measured spectrophotometrically by adding horse liver alcohol dehydrogenase to a 10-mm cuvette containing 200 μmol of sodium bicarbonate/CO_2 buffer, pH 7.4 and NAD^+. This solution was temperature equilibrated for 5 min at 37° C. Following equilibration, substrate was quickly added and the initial rate of increase in optical density at 340 nm was measured and compared with the rate observed in a cuvette containing buffer, enzyme, and NAD^+ but no substrate. The final volume of all incubations was 2.0 ml.

The compounds benzyl alcohol, benzaldehyde, cetyl alcohol, tryptophol, 5-hydroxytryptophol, thiamine, and 5-(2-hydroxyethyl)-4-methylthiazole were incubated with horse liver alcohol dehydrogenase, NAD^+ and a NAD^+ regenerating system (lactate dehydrogenase and pyruvate) and the product of each incubation determined by thin layer chromatography. Incubations were carried out in a mixture which contained either 2 mM [^{14}C]benzyl alcohol, 0.05 mM [^{14}C]cetyl alcohol, or 40 mM [^{14}C]benzaldehyde in 0.05 M sodium bicarbonate/CO_2 buffer, pH 7.4, containing 0.25 mg horse liver alcohol dehydrogenase, 5 mM NAD^+, 40 mM sodium pyruvate and 10 μl (65 units) lactate dehydrogenase (Boehringer-Mannheim, New York, New York) in a total volume of 0.25 ml. Other incubations were carried out with either 10 mM tryptophol, 5-hydroxytryptophol, thiamine or 5-(2-hydroxyethyl)-4-methylthiazole in 0.1 M sodium bicarbonate/CO_2 buffer, pH 7.4, containing 7.5 mM NAD^+, 50 mM sodium pyruvate, 1 mg horse liver alcohol dehydrogenase, and 10 μl (65 units) lactic dehydrogenase in a total volume of 0.5 ml. All incubations were run a minimum of 6 h and the product of each reaction determined by thin layer chromatography. Appropriate control incubations containing the same concentrations of substrates and all other components except alcohol dehydrogenase were also carried out. None of these alcohols or aldehydes were found to be substrates for lactic dehydrogenase.

Solutions of NAD^+ and NADH were prepared fresh daily. The concentrations of NAD^+ and NADH were determined spectrophotometrically. The extinction coefficient of NAD^+ at 260 nm in distilled water was taken to be $1.8 \cdot 10^4$ 1 $mol^{-1} \cdot cm^{-1}$ while the extinction coefficient of NADH at 338 nm and pH 10.0 was taken to be $6.22 \cdot 10^3$ 1 $mol^{-1} \cdot cm^{-1}$. The concentration of horse liver alcohol dehydrogenase in a stock solution of this enzyme prepared fresh daily was detected at 280 nm with an absorbance of 0.455 equaling 1 mg/ml [8]. The same sample of horse liver alcohol dehydrogenase was used throughout the studies described.

Gas chromatographic analysis

The concentrations of octanol and octanoic acid in the incubations were determined by gas chromatographic analysis of a hexane extract of the acidified incubation mixture as previously described [1]. The concentration of octanol present in substrate solutions was determined by direct gas chromatographic analysis of 5 μl samples of the aqueous solutions. Since octanal could not be completely extracted from the incubations, its concentration resulting

from the enzymatic oxidation of octanol was estimated indirectly by subtracting two times the amount of octanoic acid formed in the incubation from the amount of NADH formed. The remainder was considered to be equal to the amount of octanal present in the incubation at the time of assay. This calculation can be performed since it had been shown that 2 mol of NADH are formed per mol of octanol oxidized to octanoic acid [1].

Thin-layer chromatography

The enzymatic oxidation products of the substrates benzyl alcohol, benzaldehyde, cetyl alcohol, tryptophol, and 5-hydroxytryptophol were determined by thin layer chromatography on Baker-flex silica gel IB-F plates (J.T. Baker, Phillipsburg, New Jersey) comparing the R_F values to those of authentic standards. Using the solvent system hexane/ether/acetic acid (80 : 20 : 2, v/v) benzoic acid was found to have an R_F of 0.38. In the solvent system hexane/chloroform/methanol/acetic acid (50 : 50 : 10 : 1, v/v) indole-3-acetic acid had an R_F of 0.43. The products of the metabolism of thiamine and 5-(2-hydroxyethyl)-4-methylthiazole by horse liver alcohol dehydrogenase (thiamine acetic acid [9,10] and 4-methylthiazole-5-acetic acid, respectively) were determined by thin layer chromatography on thin layers of microcrystalline cellulose using the solvent systems previously described [9,10].

Determination of kinetic constants

The Michaelis constants (K_m) and maximal velocity constants (V) were determined mathematically by a least squares method using a computer program written for this purpose [11]. Prior to making the computer calculations, a Lineweaver-Burk plot of the raw data was made to insure a linear relationship existed between substrate or co-factor concentration and the initial velocity of the reaction.

Results

Kinetics of oxidation and reduction of octanol and octanal

The results of studies of the kinetics of the metabolism of octanol and octanal by horse liver alcohol dehydrogenase are shown in Tables I and II. As can be seen in Table I, the apparent K_m values for the oxidation and reduction of octanal and the oxidation of octanol are very similar. The maximal velocities are, however, quite different. The rate of reduction of octanal is approximately 750 times greater than the rate of oxidation of this compound and the initial rate of oxidation of octanol is 14 times greater than the initial rate of oxidation of octanal. Under similar conditions the apparent K_m values for the oxidation of ethanol and acetaldehyde are 0.36 and 0.06 mM, respectively. The maximal velocities for oxidation of ethanol and acetaldehyde under these conditions are 1.86 and 0.002 μmol of NADH formed per min per mg of enzyme, respectively.

The data in Table II shows the K_m value of NADH for reduction of octanal to octanol is considerably lower than the K_m value of NAD^+ for oxidation of octanal to octanoic acid. In addition, in the oxidation of octanol by horse liver alcohol dehydrogenase two very different K_m values for NAD^+ were

TABLE I

APPARENT K_m AND V VALUES OF THE OXIDATION AND REDUCTION OF OCTANAL AND THE OXIDATION OF OCTANOL BY HORSE LIVER ALCOHOL DEHYDROGENASE

The rates of oxidation of varying concentrations of octanol and octanal were determined spectrophotometrically in a Gilford recording spectrophotometer. The enzyme activity was measured by adding 100 μg horse liver alcohol dehydrogenase to a 10-mm cuvette containing 200 μmol sodium bicarbonate/CO_2 buffer, pH 7.4 and 16.6 μmol NAD^+. This solution was temperature equilibrated for 5 min at 37° C. Following equilibration substrate was added and the initial rate of increase in absorbances at 340 nm was measured and compared with the rate observed in a cuvette containing buffer, enzyme, and NAD^+ but no substrate. The final volume of all incubations was 2.0 ml. The rate of reduction of octanal was determined similarly except the incubations contained 10 μg of enzyme and 0.32 μmol of NADH in place of NAD^+. The initial rate of decrease in absorbance at 340 nm was measured and compared with the rate observed in a cuvette containing buffer, enzyme, and NADH but no substrate.

Substrate	V (μmol NAD reduced/min/ mg enzyme)	V (μmol NADH oxidized/min/ mg enzyme)	Apparent K_m (M)
Octanol	1.44	—	$0.014 \cdot 10^{-3}$
Octanal	0.10	—	$0.029 \cdot 10^{-3}$
Octanal	—	74.80	$0.046 \cdot 10^{-3}$

found. For the oxidation of octanol as measured by the initial rate of formation of NADH, the K_m value of NAD^+ was found to be $0.12 \cdot 10^{-3}$ M. When the K_m value of NAD^+ was calculated on the basis of the rate of octanoic acid production from octanol it was found to be $1.71 \cdot 10^{-3}$ M. This K_m value of NAD^+ for the production of octanoic acid from octanol is very similar to the K_m value of NAD^+ for the oxidation of octanal to octanoic acid as measured spectrophotometrically ($1.88 \cdot 10^{-3}$ M).

One of the initial products of the metabolism of octanol by horse liver alcohol dehydrogenase should be octanal which is further metabolized to octanoic acid. Shown in Fig. 1 is a study of the kinetics of formation of

TABLE II

K_m VALUES OF NAD^+ AND NADH FOR OXIDATION AND REDUCTION OF OCTANAL AND FOR NAD^+ FOR OXIDATION OF OCTANOL BY HORSE LIVER ALCOHOL DEHYDROGENASE

Substrates	Reaction measured	K_m of NAD or NADH (M)
Octanal, NAD^+	NADH formation[a]	$1.88 \cdot 10^{-3}$
Octanol, NAD^+	Octanoic acid formation[b]	$1.71 \cdot 10^{-3}$
Octanol, NAD^+	NADH formation[a]	$0.12 \cdot 10^{-3}$
Octanal, NADH	NADH oxidation[a]	$0.03 \cdot 10^{-3}$

[a] The rates of oxidation of NADH and reduction of NAD^+ were determined as described in Table I using $0.15 \cdot 10^{-3}$M concentrations of octanol and octanal and varying concentrations of NADH and NAD^+

[b] The rate of formation of octanoic acid was determined using a $0.15 \cdot 10^{-3}$M concentration of octanol and varying concentrations of NAD^+. The incubations were carried out for 10 min and the concentration of octanoic acid in the incubations determined by gas chromatograhpy [1]. Preliminary studies indicated the rate of formation of octanoic acid was linear for greater than 10 min at the lowest concentration of NAD^+ used ($1 \cdot 10^{-3}$M).

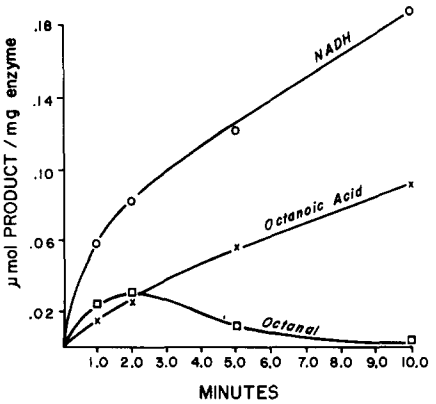

Fig. 1. Kinetics of formation of NADH, octanal and octanoic acid on incubation of octanol with horse liver alcohol dehydrogenase. The rate of formation of NADH was determined in a series of incubations containing horse liver alcohol dehydrogenase (1.1 mg), 200 µmol of sodium bicarbonate/CO_2 buffer, pH 7.4, and 20 µmol NAD^+. These solutions were temperature equilibrated for 5 min at 37°C. Following equilibration octanol (0.30 µmol) was added to give a final volume of 2.0 ml and the rate of increase in absorbance at 340 nm was followed and compared with the rate observed in an incubation containing buffer, enzyme, and NAD^+ but no substrate. At the end of each designated time period the amount of NADH formed in an incubation was recorded and the reaction stopped by addition of 50 µl concentrated HCl. The amount of octanoic acid and octanal formed in each incubation was determined as described in Materials and Methods. It was necessary to use large amounts of enzyme in these incubations in order to produce sufficient octanoic acid during these short periods of incubation to be measured accurately.

octanoic acid, NADH, and octanal on incubation of octanol with horse liver alcohol dehydrogenase. The formation of octanal was estimated as described in Materials and Methods. The slower rate of production of NADH in this experiment as compared to the *V* value determined in the experiment described in Table I, is a function of the use of larger amounts of enzyme and the fact that the period of linearity of the initial production of NADH using octanol as a substrate is very short (less than 10 s). The larger amounts of enzyme were required to produce sufficient octanoic acid during the short periods of incubation to be measured accurately. These same comments apply to the experiments in Table III.

As can be seen from these data (Fig. 1), octanal is, in fact, one of the initial products of octanol oxidation by horse liver alcohol dehydrogenase. However, on continued incubation (>2 min) the computed octanal concentration progressively decreases to where only negligible quantities are present in the incubation after 10 min. NADH production appears to be biphasic. An initial phase is followed in about 2 min with a slower but linear rate of NADH production. The production of octanoic acid is approximately linear over the entire incubation period. These results show that after 10 min of incubation the major product of the oxidation of octanol by horse liver alcohol dehydrogenase is the corresponding acid, octanoic acid, and that approximately 2 mol of NADH are formed per mol of acid formed. These data support the previous observation, namely, that when octanol is incubated with horse liver alcohol dehydrogenase for long periods of time the only products detectable are NADH and octanoic acid [1].

TABLE III

EFFECT OF SEMICARBAZIDE ON THE HORSE LIVER ALCOHOL DEHYDROGENASE CATALYZED METABOLISM OF OCTANOL TO OCTANOIC ACID, NADH AND OCTANAL

The rate of formation of NADH and octanoic acid was measured in incubations containing horse liver alcohol dehydrogenase (1.1 mg); 200 μmoles sodium bicarbonate/CO_2 buffer, pH 7.4; 20 μmol NAD^+; and semicarbazide concentrations of 0, 5 or 10 mM. These solutions were temperature equilibrated for 5 min at 37°C. Following equilibration octanol (0.30 μmol) was quickly added to give a final volume of 2.0 ml and the rate of increase in absorbance at 340 nm was followed and compared to the rates observed in incubations containing buffer, enzyme, NAD^+, plus or minus semicarbazide but no substrate. Each of the three incubations was terminated after 0.161 μmol of NADH had been formed. The incubation which contained no semicarbazide was terminated after 6.0 min; the incubations containing 5 mM and 10 mM semicarbazide were terminated after 5.5 and 4.1 min incubation, respectively. The amount of octanoic acid and octanal formed in each incubation was determined as described in Materials and Methods. It was necessary to use a large amount of enzyme in these incubations in order to produce sufficient octanoic acid to measure accurately.

Semicarbazide concentration (mM)	A NADH formation (nmol/min/ mg enzyme)	B Octanoic acid formation (nmol/min/ mg enzyme)	C Octanol formation column A minus 2 x column B (nmol/min/ mg enzyme)
0	24.4	11.4	1.6
5	26.6	8.4	9.8
10	35.7	7.8	20.1

Effect of semicarbazide on the oxidation of octanol to octanoic acid

Octanol was incubated with horse liver alcohol dehydrogenase and NAD^+ in the presence and absence of semicarbazide to see if the presence of an aldehyde trapping agent would prevent the formation of octanoic acid. These results are shown in Table III. Since NADH could possibly inhibit the oxidation of octanol to octanoic acid by horse liver alcohol dehydrogenase by competing with NAD^+ for the enzyme, each reaction was terminated after a constant production of NADH (0.161 μmol).

These data show that semicarbazide at the highest concentration used increased the rate of formation of NADH by one and one-half times over then in an incubation not containing semicarbazide. The rate of formation of octanal was also increased in the presence of this same concentration of semicarbazide. The rate of octanoic acid formation, however, is decreased only to a rate two-thirds that of a control which contains no semicarbazide. These results show that even though octanoic acid production is inhibited at a concentration of semicarbazide which is 67 times greater than that of the substrate octanol, significant amounts of octanoic acid are still produced. The increase in the rate of formation of NADH in the presence of semicarbazide probably results from NADH not being oxidized back to NAD^+ as rapidly since the octanal formed and released from the enzyme is trapped as the semicarbazone.

Substrate specificity for the horse liver alcohol dehydrogenase catalyzed oxidation of alcohols and aldehydes to acids

A summary of the different alcohols and aldehydes which were examined

TABLE IV

SUBSTRATE SPECIFICITY FOR THE HORSE LIVER ALCOHOL DEHYDROGENASE CATALYZED METABOLISM OF ALCOHOLS AND ALDEHYDES TO ACIDS

TLC refers to thin layer chromatography; GC refers to gas chromatography.

Substrate	Product	Method of assay
Thiamine	Thiamine acetic acid[a]	TLC[b]
5-(2-hydroxyethyl)-4-methylthiazole	4-methylthiazole-5-acetic acid	TLC[b]
Benzyl alcohol	Benzoic acid	TLC[b]
Cetyl alcohol	Palmitic acid	TLC[b]
Tryptophol	Indole-3-acetic acid	TLC[b]
5-hydroxytryptophol	5-Hydroxyindole-3-acetic acid	TLC[b]
Benzaldehyde	Benzoic acid	TLC[b]
Octanol	Octanoic acid	GC
Octanal	Octanoic acid	GC

[a] 3-(2'-methyl-4'-amino 5'-pyrimidylmethyl)-4-methylthiazole-5-acetic acid.

[b] These substrates were incubated with horse liver alcohol dehydrogenase, NAD^+, pyruvate, and lactate dehydrogenase as described in Materials and Methods.

and found to be oxidized to the corresponding acids by horse liver alcohol dehydrogenase is shown in Table IV.

Additional aldehyde substrates which were shown to reduce NAD^+ in the presence of horse liver alcohol dehydrogenase are chloral hydrate, vitamin A aldehyde, tryptophal, acetaldehyde [1], butanal [1] and hexanal [1].

Discussion

Horse liver alcohol dehydrogenase has been previously shown to catalyze the reversible oxidation of alcohol to aldehydes [12]. Schematically, this mechanism is as follows where Alc represents octanol and Ald represents octanal and ADH represents alcohol dehydrogenase [13]:

$$ADH + NAD^+ \rightleftharpoons ADH\text{-}NAD^+ \quad (1)$$

$$ADH\text{-}NAD^+ + Alc \rightleftharpoons ADH\text{-}NAD^+\text{-}Alc \quad (2)$$

$$ADH\text{-}NAD^+\text{-}Alc \rightleftharpoons ADH\text{-}NADH\text{-}Ald + H^+ \quad (3)$$

$$ADH\text{-}NADH\text{-}Ald \rightleftharpoons ADH\text{-}NADH + Ald \quad (4)$$

$$ADH\text{-}NADH \rightleftharpoons ADH + NADH \quad (5)$$

This mechanism explains the detectable formation of octanal that is shown in Fig. 1.

Two mechanisms can be envisioned to explain the oxidation of octanol to octanoic acid. In the first, octanol would be oxidized to octanal by the Theorell-Chance mechanism shown above. Octanal free in the incubation media could then reassociate with the enzyme and, in the presence of NAD^+, be oxidized to octanoic acid. Table I shows that the K_m values of octanol and octanal for their oxidation by horse liver alcohol dehydrogenase are quite similar. Consequently, the oxidation of octanol to octanoic acid by this mechanism would not be expected to be linear with time during the initial period of

the incubation since the concentration of octanol would greatly exceed that of octanal and the predominant initial product would be octanal with little formation of octanoic acid. Since Fig. 1 shows that the oxidation of octanol to octanoic acid is essentially linear with time, at least for the first ten minutes, we believe this mechanism is inadequate in explaining the oxidation of octanol to octanoic acid.

A second mechanism which may better explain the oxidation of octanol to octanoic acid is one in which in some unknown percentage of the reactions involving the oxidation of octanol to octanal, the NADH rather than octanal is released first from the enzyme-NADH-octanal complex (Eqn 6). This is followed by binding of NAD^+ to be enzyme-octanal complex (Eqn 7) and oxidation of octanal to octanoic acid (Eqns 8 and 9). Schematically, this mechanism is as follows where the first 4 reactions are identical to the Theorell-Chance mechanism shown previously:

$$ADH + NAD^+ \rightleftharpoons ADH\text{-}NAD^+ \quad (1)$$

$$ADH\text{-}NAD^+ + Alc \rightleftharpoons ADH\text{-}NAD^+\text{-}Alc \quad (2)$$

$$ADH\text{-}NAD^+\text{-}Alc \rightleftharpoons ADH\text{-}NADH\text{-}Ald + H^+ \quad (3)$$

$$ADH\text{-}NADH\text{-}Ald \rightleftharpoons ADH\text{-}NADH + Ald \quad (4)$$

$$ADH\text{-}NADH\text{-}Ald \rightleftharpoons ADH\text{-}Ald + NADH \quad (6)$$

$$ADH\text{-}Ald + NAD^+ \rightleftharpoons ADH\text{-}Ald\text{-}NAD^+ \quad (7)$$

$$ADH\text{-}Ald\text{-}NAD^+ \rightleftharpoons ADH\text{-}Acid\text{-}NADH + H^+ \quad (8)$$

$$ADH\text{-}Acid\text{-}NADH \rightleftharpoons ADH + Acid + NADH \quad (9)$$

This mechanism would explain why the oxidation of octanol to octanoic acid, under the conditions of the experiments described here, is linear with time for at least the first ten minutes of the reaction. The dissociation of NADH from the enzyme-NADH-octanal complex shown in eqn 6 may be rationalized on the basis of the similarity of the K_m values of octanal and NADH for horse liver alcohol dehydrogenase (Tables I and II). This mechanism may also explain why semicarbazide at a concentration which was sixty-seven times that of the octanol did not substantially inhibit the oxidation of octanol to octanoic acid.

Fig. 1 shows that during the first two minutes of the incubation of octanol with horse liver alcohol dehydrogenase octanal accumulates in the incubation medium and then decreases until, after ten minutes, it is barely detectable. This may be explained on the basis of the accumulation of NADH in the incubation media. Thus, as the NADH concentration in the incubation mixture increases an increase in the enzyme catalyzed reduction of the octanal, which has accumulated in the reaction media, to octanol is also seen. Eventually, the rate of oxidation of octanol to octanal with release of a portion of the octanal from the enzyme and the rate of reduction of octanal to octanol are equal and the concentration of aldehyde does not increase further. As the NADH concentration continues to increase largely as a result of oxidation of octanol and, to some small extent, octanal to octanoic acid, the rate of binding and reduction

of octanal to octanol exceeds the rate at which octanal is released from the enzyme and the octanal concentration in the incubation media decreases to a very low level. With incubation times longer than 10 min, the net result of these various reactions is the oxidation of octanol to octanoic acid without the accumulation of octanal in the incubation media.

The K_m values of NAD^+ for the oxidation of octanol to octanoic acid and octanal to octanoic acid are much higher than the K_m value of NAD^+ for oxidation of octanol to octanal. On the other hand, the K_m value of NAD^+ for oxidation of octanol to octanal is not greatly different than the K_m value of NADH for reduction of octanal to octanol (Table II) or for NAD^+ for the oxidation of ethanol to acetaldehyde [1]. The reason for the marked difference in the K_m values of NAD^+ for oxidation of octanol to octanal and octanol to octanoic acid is probably related to the fact that the rate of reduction of octanal to octanol is approximately 750 times the rate of oxidation of octanol to octanoic acid (Table I). Thus, once an enzyme-octanal-NADH complex has formed, the probability that it will react to form products before either the octanal or NADH dissociates is more likely than is the case with the enzyme-octanal-NAD^+ complex. Therefore, high concentrations of NAD^+ are required in the incubation to force the reaction in the direction of octanal oxidation. No similar competitive reaction occurs in the oxidation of octanol to octanal.

The ability of NAD^+-requiring primary alcohol dehydrogenases to catalyze the two-step oxidation of primary alcohols to acids has been previously reported [14—17]. The two step oxidation of thiamine to thiamine acetic acid by a purified flavoprotein has also been reported [18].

Since the K_m of NAD^+ for the horse liver alcohol dehydrogenase catalyzed production of octanoic acid using the substrates octanol and octanal is quite high ($\approx$1.8 mM), the physiological significance of the oxidation of naturally occurring aldehydes and alcohols to acids by this enzyme is open to question. Bassham et al. [19] determined the concentrations of the various forms of the pyridine nucleotides in rat liver and found the NAD^+ and NADH concentrations to be approximately 0.5 and 0.3 mM, respectively. It seems more likely that the liver alcohol dehydrogenases may play a more predominant role in the oxidation of a number of naturally occurring alcohols to the corresponding aldehydes followed by oxidation of these aldehydes to acids by the numerous aldehyde dehydrogenases present in animal tissues.

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