The Dismutation of Formaldehyde by Liver Alcohol Dehydrogenase*

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Kendal and Ramanathan (1) reported that horse liver preparations catalyze the dismutation of formaldehyde to formic acid and methanol in the presence of diphosphopyridine nucleotide. The following reaction scheme was proposed:

$$CH_2O + DPN \rightarrow HCOOH + DPNH$$ (1)

$$CH_2O + DPNH \rightarrow CH_2OH + DPN$$ (2)

$$2 CH_2O \rightarrow HCOOH + CH_2OH$$ (3)

They also noted that when methanol was initially present the dismutation reactions of formaldehyde, Reactions 3 and 5, were formed by the oxidation of formaldehyde-methyl-hemiacetal by way of the following reaction sequence:

$$CH_2OCH_2CH \rightarrow HCOOCH_2CH \rightarrow HCOOCH_2CH + CH_2OH$$ (4)

The results obtained led the authors to the suggestion that Reactions 2 and 4 are catalyzed by liver alcohol dehydrogenase but that Reaction 1 is due to an aldehyde dehydrogenase present in their liver preparation. Because of the inhomogeneity of the enzyme preparation employed, a reinvestigation of this problem with more highly purified enzyme was suggested. With the use of crystalline horse liver alcohol dehydrogenase which was approximately 200 times as active as the preparations previously used, we were able to carry out the reactions described by Kendal and Ramanathan. We confirmed their conclusions concerning the nature of the reaction products and the stoichiometry of the reaction. The results obtained with the crystalline enzyme were in accord with the suggestion of Kendal and Ramanathan (1) that alcohol dehydrogenase itself probably catalyzes Reaction 4 as well as Reaction 2. We could not, however, confirm those observations which had led to the conclusion that different enzymes were required to catalyze Reactions 1 and 4. On the contrary, our findings are consistent with the view that alcohol dehydrogenase is the only enzyme necessary for catalysis of both dismutation reactions of formaldehyde, Reactions 3 and 5.

MATERIALS AND METHODS

Enzymes and Substrates—Alcohol dehydrogenase and lactic dehydrogenase were purchased from Worthington Biochemical Corporation. All alcohol dehydrogenase preparations were dialyzed from 12 to 20 hours against 0.01 M potassium phosphate buffer, pH 7.0, at 0-5°C in order to remove all ethanol. We also prepared alcohol dehydrogenase from horse liver (2). DPN and DPNH were purchased from Nutritional Biochemicals. Formaldehyde solutions were prepared by distilling aqueous solutions of hexamethylenamine (Merck) which had been acidified with an equivalent amount of H_2SO_4. The resulting distillate was neutralized and redistilled. The formalddehyde content of the solution was determined gravimetrically by dimedon precipitation (3). Pyruvaldehyde was kindly donated by Professor Westheimer and redistilled before use. All other reagents were purchased from Eastman Kodak and redistilled or recrystallized.

Enzyme Assays—Alcohol dehydrogenase activity was determined as described by Bonnichsen and Brink (4) by measuring the rate of reduction of DPN in the presence of enzyme and excess ethanol. The amount of enzyme which gave an increase in DPN concentration of 7.2 × 10^{-3} μmole per ml in 3 minutes was designated as 1 unit. Protein concentration was determined by measuring optical density at 280 μm. One-centimeter cells were used in all determinations. Assays were carried out at room temperature. The rate of formaldehyde disappearance in the presence of enzyme and added DPN was used as a measure of Reactions 3 and 5. Assays for Reactions 3 and 5 were carried out at 37°C.

Analytical Procedures—Formaldehyde was quantitatively determined by the chromotropic acid method (5) which is highly specific for formaldehyde. In our hands, the ester determination (1) previously employed did not give quantitative results and we therefore used the following procedure for ester determination: 1 ml of unbuffered reaction mixture was added to 1 ml of 0.030 N NaOH at 0°C. The mixture was maintained at this temperature for 5 minutes. The excess NaOH was then titrated. This procedure gave excellent recoveries when known amounts of ester were added to our reaction mixture. Lactic acid was determined enzymically (6).

Kinetic Measurements—All rate measurements, except assay for alcohol dehydrogenase activity, were carried out at 37°C. Determinations in which only the formaldehyde disappearance was measured were carried out in buffered solutions. The detailed composition of the reaction mixture is given with each experiment. Experiments in which formic acid formation was measured were carried out in unbuffered solutions. The pH was maintained at the desired value by addition of base from a micro burette. While the solution was agitated with a magnetic stirrer the amount of base added was recorded at 1-minute intervals. A plot of acid formation against time was linear for the first 5 minutes. The average amount of base added per minute during this period was taken as the initial velocity. The reaction was stopped by the addition of 2 drops of H_2SO_4. Aliquots were used...
TABLE I

Stoichiometry of formaldehyde dismutation

The reaction mixture contained: alcohol dehydrogenase 300 units; DPN 5 mg; CH₃O 394 μmoles; CH₃OH 500 μmoles. Total volume: 10 ml; pH maintained at 7.4; reaction time: 15 minutes.

<table>
<thead>
<tr>
<th>Additions</th>
<th>Decrease of CH₃O</th>
<th>Acid formed</th>
<th>Ester formed</th>
</tr>
</thead>
<tbody>
<tr>
<td>CH₃OH</td>
<td>44</td>
<td>21.8</td>
<td>0</td>
</tr>
<tr>
<td>CH₃OH + CH₃OH</td>
<td>124</td>
<td>13.9</td>
<td>51.3</td>
</tr>
</tbody>
</table>

TABLE II

Effect of methanol and ethanol on formaldehyde dismutation

The reaction mixture contained: alcohol dehydrogenase 400 units; DPN 5 mg; CH₃OH 398 μmoles, total volume: 10 ml; pH maintained at 7.4; reaction time: 15 minutes.

<table>
<thead>
<tr>
<th>Additions</th>
<th>Decrease of CH₃O</th>
<th>Acid formed</th>
<th>Ester formed</th>
</tr>
</thead>
<tbody>
<tr>
<td>CH₃OH</td>
<td>0</td>
<td>33.2</td>
<td>0</td>
</tr>
<tr>
<td>200</td>
<td>94</td>
<td>28.6</td>
<td>18.4</td>
</tr>
<tr>
<td>500</td>
<td>116</td>
<td>20.4</td>
<td>37.6</td>
</tr>
<tr>
<td>1,000</td>
<td>201</td>
<td>18.8</td>
<td>81.7</td>
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<tr>
<td>10,000</td>
<td>145</td>
<td>2.9</td>
<td>69.6</td>
</tr>
<tr>
<td>CH₂H₂O</td>
<td>1</td>
<td>25.0</td>
<td>0</td>
</tr>
<tr>
<td>4</td>
<td>66</td>
<td>22.2</td>
<td>9.8</td>
</tr>
<tr>
<td>10</td>
<td>62</td>
<td>10.2</td>
<td>29.8</td>
</tr>
</tbody>
</table>

* Calculated from CH₃O decrease and amount of acid formed.

TABLE III

Aldehyde mutase activity during alcohol dehydrogenase purification

Composition of reaction mixture for assay of Reaction 3: potassium phosphate buffer, pH 8.0, 80 μmoles; DPN 2 mg; CH₃O 16 μmoles; enzyme 0.2 ml. Total volume: 2 ml; reaction time: 10 minutes; temperature: 37°C. For assay of Reaction 5, 200 μmoles of CH₂OH were added to the above reaction mixture.

<table>
<thead>
<tr>
<th>Purity of alcohol dehydrogenases</th>
<th>Activity ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>units/mg protein*</td>
<td>Reaction 3 ADH activity</td>
</tr>
<tr>
<td>3.7</td>
<td>11</td>
</tr>
<tr>
<td>57</td>
<td>8.7</td>
</tr>
<tr>
<td>690</td>
<td>11</td>
</tr>
</tbody>
</table>

* For definition of units used see "Experimental."
† Activity ratios were obtained by dividing the activities represented by Reactions 3 and 5, by alcohol dehydrogenase (ADH) activity.

for formaldehyde analysis. When the ester concentration was also determined the reaction was carried out in a completely enclosed system so that loss of the volatile ester could be eliminated. At the end of the reaction the mixture was rapidly cooled to 0°C and aliquots were then used for ester and formaldehyde analysis. Although maximal velocities were obtained at higher pH values, most experiments were carried out at pH 7.4 in order to prevent ester hydrolysis.

EXPERIMENTAL RESULTS

The products resulting from the action of horse liver alcohol dehydrogenase and DPN upon formaldehyde were qualitatively identified by distilling an acidified reaction mixture consisting of 10 ml of 0.05 M potassium phosphate buffer pH 8.0, 400 μmoles of formaldehyde, 5 mg of DPN, and 400 units of enzyme, in the presence of phenylhydrazine. The distillate was divided into two parts. In one portion the presence of methanol was demonstrated (7) and in the other, formate (8). When formaldehyde and methanol were initially present, the formation of ester was demonstrated with the hydroxamic acid method (1).

Quantitative analysis of the reaction products led to the results shown in Table I. Two moles of formaldehyde yield 1 mole of formate, and in the presence of methanol a total of 1 mole of methyl formate and formate. This is in perfect agreement with the stoichiometric requirements of the reaction scheme proposed (1).

The results obtained when varying concentrations of methanol and ethanol were added to the reaction mixture are shown in Table II. Increasing the concentrations of methanol causes an increased disappearance of formaldehyde but a decreased rate of formate production. Methanol concentrations greater than 0.1 M are inhibitory. This observed decrease in formate formation differs from previous findings (1) which indicate that over a methanol concentration ranging from 0 to 0.25 M the acid formation remained essentially unchanged.

In order to determine whether the various activities (the alcohol dehydrogenase activity and the enzymes responsible for Reactions 1 and 4) could be separated, the ratios of these activities were measured during various stages in the isolation of alcohol dehydrogenase from horse liver. The first determination was made following the heat inactivation step. The results obtained are shown in Table III. The data in the last line represent a crystalline enzyme preparation. During the course of a 200-fold purification, no separation of activities was achieved. The activity ratio found in the crystalline enzyme prepared by us was identical to that found in the commercial preparation which we used. Numerous attempts to achieve a higher degree of purification by recrystallization were unsuccessful. During these purification attempts we assayed approximately 20 different enzyme preparations in various states of purity. In no case could we observe any significant fractionation of the activities assayed. Heat denaturation also did not lead to a separation. Alcohol dehydrogenase preparations were maintained at 37°C for 90 hours. At this point 40% of the original alcohol dehydrogenase activity had been lost. An equivalent decrease in the other activities was also found.

The effect of iodoacetate upon the formation of formate, Reaction 3, and methyl formate, Reaction 5, was examined. The results obtained are shown in Table IV. When iodoacetate and the appropriate substrate were simultaneously added to the enzyme and DPN, Reaction 3 as well as reaction 5 was...
inhibited. Fig. 1 shows that this inhibition, unlike the usual iodoacetate inhibition, appears to behave like a competitive inhibition under the experimental conditions used. If the enzyme is preincubated with iodoacetate in the absence of DPN, both Reactions 3 and 5 are inhibited. The degree of inhibition obtained depends upon the length of preincubation. Fifteen minutes of preincubation at 37° results in loss of more than 90% of the activity. The inhibition obtained under these conditions appears to be irreversible.

Incubation of liver alcohol dehydrogenase with iodoacetate in the absence of DPN results in nearly complete loss of alcohol dehydrogenase activity. The inhibitory effect of iodoacetate upon the dismutation reaction could therefore be due to failure of the alcohol dehydrogenase to reoxidize the DPNH formed in Reaction 1, or 4. We examined the inhibited reaction mixture spectrophotometrically, but found no evidence of DPNH accumulation.

From the data of Fig. 1, \( V_{max} = 7.5 \times 10^{-4} \text{ M min}^{-1} \) and \( K_{m} = 1.3 \times 10^{-1} \text{ M} \) can be calculated. Similar treatment of the data for pH 8 gives \( V_{max} = 1.2 \times 10^{-4} \text{ M min}^{-1} \) and \( K_{m} = 1.3 \times 10^{-1} \text{ M} \).

Although the data presented do not suggest the involvement of an enzyme other than alcohol dehydrogenase in the dismutation reactions of formaldehyde, this possibility must still be considered. If the oxidation of formaldehyde, Reaction 1, and the formation of methanol, Reaction 2, are the result of a coupled reaction between aldehyde dehydrogenase and alcohol dehydrogenase, then DPNH formed in Reaction 1 must diffuse to the alcohol dehydrogenase of Reaction 2. There is then present in solution a small amount of free DPNH which should be available to enzymes other than alcohol dehydrogenase. To test this possibility we added high concentrations of lactic dehydrogenase and sodium pyruvate to a reaction mixture in which the dismutation of formaldehyde, Reactions 1 and 2, was going on. The results of Line 2, Table V show that only 1 \( \mu \) mole of lactic acid was formed, although from the amount of formaldehyde which underwent dismutation, it can be calculated that 34 \( \mu \) moles of DPNH were produced during the course of the reaction. The experiment thus shows that the DPNH formed during the course of the dismutation cannot be utilized by the lactic dehydrogenase present in the reaction mixture. It might be argued that this is due to the fact that the affinity of alcohol dehydrogenase for DPNH is greater than that of lactic dehydrogenase. To test this possibility we added DPNH slowly to a reaction mixture in which formaldehyde dismutation proceeded and which also contained lactic dehydrogenase and pyruvate. Line 3 of Table V shows that 50% of the added DPNH was utilized for lactic acid formation. Thus, under our experimental conditions, lactic dehydrogenase can successfully compete with alcohol dehydrogenase for free DPNH. These experiments therefore indicate that the DPNH formed during the dismutation of formaldehyde is never free. This suggests that a system consisting of two enzymes and coupled through DPN is not involved. The alternate explanation that alcohol dehydrogenase catalyzes both Reactions 1 and 2 and that the dismutation of formaldehyde is catalyzed by alcohol dehydrogenase alone is consistent with the experimental results.

Several other aldehydes were incubated with alcohol dehydrogenase and DPN in order to determine whether the dismutation of aldehydes other than formaldehyde occurs. The following aldehydes listed in order of decreasing reactivity gave rise to acids: pyruvaldehyde, acetaldehyde, and acrolein. DL-Glyeraldehyde did not react. Acid production from pyruvaldehyde was slightly faster than from formaldehyde. In addition to acid, this aldehyde gave rise to a compound which yielded formaldehyde upon periodate oxidation. Hydroxyacetone, one of the expected products of pyruvaldehyde dismutation, will react in this manner. No further attempts were made to identify the products of these reactions. To establish whether alcohols other than methanol and ethanol could give rise to lactic acid formation, hydroxyacetic acid and mercaptoacetic acid were incor-
DISCUSSION

We have demonstrated that the dismutation of formaldehyde, Reactions 3 and 5, which was originally reported to occur in crude horse liver alcohol dehydrogenase preparations is catalyzed by highly purified horse liver enzyme. The rate of formaldehyde dismutation compares favorably to the rate of oxidation of ethanol. The $V_{max}$ for formate formation at pH 8 (37°C) is $3 \times 10^{-3} \mu$moles min$^{-1}$ µg$^{-1}$ of enzyme, whereas the oxidation of ethanol at pH 9.6 (25°C) proceeds at a rate of 2.5 $\times 10^{-3} \mu$moles min$^{-1}$ µg$^{-1}$ (4) of enzyme. We have presented evidence which suggests that alcohol dehydrogenase may be the only enzyme involved in this dismutation, and possibly also in the dismutation of other aldehydes.

Evidence was presented earlier (1) which indicated that more than one enzyme may be involved in the dismutation of formaldehyde. This was based upon the effects obtained with methanol and iodoacetate. It was observed that increasing the concentration of methanol increased the rate of methyl formate production, Reaction 5, but did not affect the rate of formate production, Reaction 3. This led to the conclusion that the two reactions proceeded independently of each other and involved two different enzymes. We found that the rate of formate production is strongly affected by the methanol concentration (Table II). This type of effect would be expected if both Reactions 1 and 4 were catalyzed by the same enzyme. Increasing concentrations of formaldehyde hemiacetal would compete with formaldehyde and thus reduce formate formation. Our results are consistent with the assumption that the same enzyme is involved in the oxidation of formaldehyde, Reaction 1, and formaldehyde hemiacetal, Reaction 3.

It was also reported (1) that iodoacetate inhibits the oxidation of formaldehyde but not that of ethanol and formaldehyde-methyl-hemiacetal. From this difference in iodoacetate sensitivity and the reported iodoacetate insensitivity (10) of alcohol dehydrogenase, it was concluded that at least one enzyme other than alcohol dehydrogenase is involved in these reactions. The precise experimental conditions were not given and we are therefore not certain that we have repeated the experiments exactly as previously carried out. Nevertheless, our iodoacetate experiments did not differentiate between the various activities involved. It will be noted from the results of Table IV that iodoacetate when added to the enzyme and DPN, inhibits formate as well as methyl formate formation. If the enzyme is preincubated with iodoacetate in the absence of DPN the formation of formate, Reaction 3, and methyl formate, Reaction 5, can be almost completely inhibited. Under these conditions an irreversible inhibition is obtained.

The effect of iodoacetate upon the dismutation of formaldehyde exactly parallels its effect upon alcohol dehydrogenase activity. Thus, when iodoacetate is added without preincubation to the enzyme and DPN, a competitive inhibition (Fig. 1) appears to take place. This is reminiscent of the reported inhibition of alcohol dehydrogenase by various acid anions (11). When the enzyme is preincubated with iodoacetate in the absence of DPN, the dismutation reaction as well as the oxidation of ethanol is nearly completely inhibited.

Attempts to separate the formaldehyde dehydrogenase activity from alcohol dehydrogenase failed. As shown in Table III, the ratio of the activities assayed remained constant over a two hundredfold purification. During repeated recrystallization of the enzyme, no fractionation was achieved. Heat denaturation also gave no separation, but demonstrated unusual stability of the enzyme involved.

An attempt was made to demonstrate the occurrence of a coupled reaction in the dismutation of formaldehyde by adding lactic dehydrogenase and pyruvate as a trapping agent for the DPNH which couples Reaction 1 and Reaction 2. Only a small fraction of the DPNH formed during the dismutation could be trapped. It appears probable, therefore, that the dismutation of formaldehyde does not require the transfer of DPNH between two enzymes. If the reaction were carried out by one enzyme such a transfer would become unnecessary since the DPNH formed could be immediately reoxidized through Reaction 2 without leaving the enzyme. The fact that lactic dehydrogenase does not trap DPNH would thus be explained. We do not claim that an experiment of this type proves the involvement of only one enzyme in the dismutation of formaldehyde. The inability of lactic dehydrogenase to trap DPNH during the dismutation of formaldehyde could be due to other features of the system. This observation is, however, consistent with the assumption that alcohol dehydrogenase can catalyze the dismutation of formaldehyde. This conclusion is greatly strengthened by the fact that all other attempts to demonstrate the existence of a second enzyme failed.

It might at first seem improbable that one enzyme should oxidize two different functional groups such as alcohols and aldehydes. However, an aqueous solution of formaldehyde exists largely as the hydrated form (12) (Structure I). This, as well as formaldehyde-methyl-hemiacetal (Structure II), structurally resembles alcohols.

The order of reactivity of the various aldehydes agrees with the suggestion (1) that the hydrated structure undergoes oxidation. Formaldehyde which is completely hydrated as well as pyruvaldehyde, which is probably extensively hydrated, are oxidized more rapidly than acetaldehyde which is approximately 50% hydrated (12). No information is available concerning acrolein, but in view of its ultraviolet spectrum and loss of resonance energy which would result from hydration, it appears very probable that this compound is hydrated to a very slight extent. Its rate of oxidation is less than that of acetaldehyde. The reason for the unreactivity of glycerinaldehyde is not clear.

It is of interest to compare some of the characteristics of well established aldehyde dehydrogenase with the enzyme preparation employed here. Formaldehyde dehydrogenase (13) shows a definite requirement for glutathione and a high specificity for formaldehyde. No cofactor requirement other than DPN could be demonstrated for the dismutation reactions of formaldehyde. Its activity is maintained after prolonged dialysis with no precautions to protect SH groups. During the purification the preparation is exposed to 52°C for 15 minutes.
Most of the subsequent steps are then carried out at room temperature. In this respect, the enzyme is strikingly different from the formaldehyde dehydrogenase (13) as well as aldehyde dehydrogenase (14). The latter enzyme also gives no evidence of ester formation in the presence of methanol. It was the unusual stability of our preparations which first suggested the possibility that alcohol dehydrogenase may also possess aldehyde dehydrogenase activity. Our subsequent findings which are presented here are consistent with this view.

SUMMARY

Highly purified horse liver alcohol dehydrogenase preparations catalyze the formation of formate from formaldehyde, and the formation of methyl formate from formaldehyde and methanol. This reaction is identical with that previously observed with relatively impure horse liver preparations.

Kinetic results, inhibition with iodoacetate, and classical fractionation procedures suggest that alcohol dehydrogenase is responsible for the dismutation of formaldehyde.

It was also shown that other aldehydes were oxidized by the alcohol dehydrogenase used in these studies.

REFERENCES

The Dismutation of Formaldehyde by Liver Alcohol Dehydrogenase
Robert H. Abeles and Howard A. Lee, Jr.


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