ALDEHYDE DEHYDROGENASE, A DIPHOSPHOPYRIDINE NUCLEOTIDE-LINKED ENZYME*

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The dismutation of acetaldehyde to ethyl alcohol and acetic acid has been shown to occur in animal tissues (1, 2). The dependence of this reaction on diphosphopyridine nucleotide was demonstrated by von Euler and Brunius (3). Partial purification of the responsible enzyme system was achieved by Dixon and Lutwak-Mann (4). These authors clearly demonstrated that the mutase activity was independent of aldehyde (xanthine) oxidase activity and proposed that aldehyde mutase should be considered as a separate enzyme. This view has been accepted by recent text-books of enzymology in which the mutases are classified as separate enzymes (5, 6).

It is the purpose of this paper to show that the dismutation of aldehydes in the liver tissue is catalyzed by two separable enzymes. One is the known alcohol dehydrogenase recently obtained in crystalline form from liver tissue (7). The other is an aldehyde dehydrogenase which will be described in this paper. Both of these enzymes have been found to be present in considerable quantities in aldehyde mutase preparations purified according to Dixon and Lutwak-Mann (4).

EXPERIMENTAL

Methods

Spectrophotometric Test for Determination of Aldehyde Dehydrogenase Activity—The method is based on the reduction of diphosphopyridine nucleotide (DPN). This process, which is measured by the increase of absorption at 340 m\(\mu\), is catalyzed by the enzyme according to the following reaction:

\[
\text{Aldehyde} + \text{DPN} \rightarrow \text{acid} + \text{reduced DPN}
\]

In the presence of an excess of aldehyde and DPN the rate of reduction of DPN is a function of enzyme concentration (see Fig. 1) and can be used to measure enzymatic activity.

A Beckman quartz spectrophotometer was used for activity measurements. The solutions were pipetted into quartz cells. The control cell

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contained all the solutions with the exception of the substrate. The determination was made at pH 9.3, which is the optimal pH for the aldehyde enzyme and also serves to reduce interference by oxidation of reduced DPN by other enzymes such as alcohol dehydrogenase.

The test was carried out in a final volume of 3 ml. in 0.01 M pyrophosphate buffer. Because of the kinetics of the reaction, which will be discussed later, the enzyme can be measured with the use of relatively small quantities of DPN and substrates. For routine testing of the enzyme activity, 200 γ of DPN (65 per cent purity) and 500 γ of acetaldehyde were used.

Quantitative Determination of Acetic Acid—When acetaldehyde is oxidized to acetic acid by aldehyde dehydrogenase, diphosphopyridine nucleotide acts as the only hydrogen acceptor. DPN, which can be used

![Graph](http://www.jbc.org/Downloadedfrom)

**Fig. 1.** Quantitative determination of aldehyde dehydrogenase. Relation of protein concentration to activity.

in catalytic amounts in the mutase system, must therefore be added in stoichiometric amounts for aldehyde oxidation in the absence of alcohol dehydrogenase. To determine the end-product of the reaction it was desirable to use a microtechnique.

Recently Hutchens and Kass developed a quantitative colorimetric method for acetate determination based on the lanthanum color test. This method was found to be quite satisfactory with acetate solutions but could not be used in the presence of various impurities, as had already been pointed out by the authors.

It was possible to obtain reasonably good recoveries of acetate added to liver extracts by a combination of a micro diffusion technique with the colorimetric method of Hutchens and Kass. The procedure of the micro

diffusion technique was based on the method of Black (8) but had to be modified in several respects for the above purpose. Deproteinization and evaporation of the sample to dryness were omitted and mercuric oxide and magnesium sulfate had to be added in order to obtain quantitative recoveries. The details of the test will be described later.

Protein Determination—Protein concentrations were determined spectrophotometrically at 280 μm. Corrections for nucleic acid impurities were made according to Warburg and Christian (9).

Materials

DPN of 65 per cent enzymatic purity was prepared from bakers' yeast by the method of Williamson and Green (10) as modified by Ochoa. TPN of 45 per cent purity was kindly supplied by Dr. S. Ochoa.

Glycolaldehyde was synthesized (11) and crystallized from the distillate (12). The other aldehydes used were commercially obtained preparations.

Alcohol dehydrogenase was crystallized from yeast according to Negelein and Wulff (13).

Results

Purification of Aldehyde Dehydrogenase—Frozen beef liver, kept in a dry ice box, was used as starting material. An acetone-dried powder was prepared as follows: The thawed liver was mixed with 2 to 3 volumes of ice-cold acetone in a Waring blender, and the mixture poured into 8 volumes of acetone and then rapidly centrifuged in the cold room. The precipitate was washed once with 8 volumes of acetone, then pressed out between paper towels and crumbled into a fine powder which dried rapidly when distributed on large filter papers.

This powder was extracted with 8 volumes of distilled water at room temperature for 45 minutes under continuous mechanical stirring. From this point on all operations were carried out in the cold room. The mixture was centrifuged, and to each 100 ml. of the supernatant 70 ml. of cold 95 per cent ethyl alcohol were added in the course of 10 minutes. The temperature was allowed to rise to 12–14° during this procedure. After standing for an additional 20 minutes, the preparation was centrifuged, and to the clear supernatant 40 ml. of cold ethyl alcohol for each 100 ml. of the original extract were added slowly. After standing for 10 minutes at 0°, the precipitate was centrifuged off and dissolved in distilled water. The solution was dialyzed for 2 hours with mechanical stirring against large volumes of distilled water. The resulting precipitate was centrifuged off and discarded. For each gm. of protein present in the supernatant, 2 ml. of a neutralized 5 per cent solution of nucleic acid

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1 Ochoa, S., personal communication.
(Merck) were added, and the mixture was carefully adjusted to pH 5.2 with 0.1 m acetic acid. The precipitate formed was discarded even though considerable quantities of the aldehyde enzyme are present in this fraction. To the clear supernatant, half the amount of nucleic acid used in the above step was added, the pH readjusted to 5.2, and the mixture centrifuged. The precipitate was dissolved in dilute alkali and protamine sulfate (Squibb) was added in small fractions at pH 6.5 until the absorption ratio of 280 μ: 260 μ indicated the removal of nucleic acid (9).

This preparation can be purified further by ammonium sulfate fractionation. However, most experiments were carried out with preparations purified to the extent described above, since no detectable alcohol dehydrogenase was found in this fraction.

The degree of purification obtained by this method was usually from 20- to 30-fold, with a yield of 20 to 40 per cent. A typical protocol of purification and yields achieved is given in Table I. The enzyme thus purified is free of alcohol dehydrogenase and xanthine oxidase activity.

**Table I**

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Volume of solution</th>
<th>Units*</th>
<th>Protein</th>
<th>Specific activity</th>
<th>Yield</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aqueous extract</td>
<td>300</td>
<td>360,000</td>
<td>12,000</td>
<td>30</td>
<td>100</td>
</tr>
<tr>
<td>2nd alcohol ppt.</td>
<td>100</td>
<td>300,000</td>
<td>2,000</td>
<td>150</td>
<td>83</td>
</tr>
<tr>
<td>2nd nucleic acid ppt. (after nucleic acid removal)</td>
<td>10</td>
<td>140,000</td>
<td>200</td>
<td>700</td>
<td>38</td>
</tr>
</tbody>
</table>

* A unit has been defined as the amount of enzyme activity causing an increase of optical density of 0.001 per minute.

Dismutation Reaction of Acetaldehyde---As can be seen from Fig. 2, oxidation of acetaldehyde to acetic acid by the aldehyde dehydrogenase thus purified goes to completion in the absence of alcohol dehydrogenase. In the presence of alcohol dehydrogenase and an excess of acetaldehyde, the reduced DPN is reoxidized with simultaneous reduction of acetaldehyde to ethyl alcohol (Fig. 2). The result of the over-all reaction is a dismutation of acetaldehyde to acetic acid and alcohol, while DPN acting as a coenzyme to both dehydrogenases undergoes oxidation and reduction continuously. Considerable amounts of both dehydrogenases were found to be present in the preparations of mutase prepared according to Dixon and Lutwak-Mann (4).

All previous studies on the properties of the "mutase enzyme" were re-examined and interpreted in the light of the above findings. The data on
pH optimum, heat stability, and substrate specificity of the aldehyde enzyme were found to be at variance with those reported on mutase activity but the discrepancies are readily accounted for by the participation of alcohol dehydrogenase in the activity of the "mutase enzyme."

_Coenzyme and Substrate Specificity_ Dixon and Lutwak-Mann (4) have demonstrated the specificity of coenzyme I (DPN) for mutase activity. Triphosphopyridine nucleotide (TPN) was inactive. This
specificity of DPN was fully confirmed for the aldehyde dehydrogenase. This is of particular interest in the light of recent findings of Ochoa and his collaborators (14), indicating that some of the dehydrogenases previously believed to be specific for DPN also react with TPN, although at a slower rate. In its specificity for DPN the aldehyde dehydrogenase is similar to glyceraldehyde phosphate dehydrogenase (14).

A number of aldehydes tested were found to be readily oxidized by the aldehyde dehydrogenase. These were formaldehyde, acetaldehyde, glycolaldehyde, propionaldehyde, butylaldehyde, isovaleraldehyde. The rate of formaldehyde oxidation, which was the least active of these substrates, was approximately half the rate at which the most rapidly attacked acetaldehyde was oxidized. Of special interest is the oxidation of salicylaldehyde, since this substance was found to be inactive when tested with the mutase preparation by Dixon and Lutwak-Mann (4). In fact, these authors listed the inability of the mutase preparation to oxidize aromatic aldehydes as one of the principal characteristics of mutase, differentiating it from aldehyde (xanthine) oxidase, which reacts with aromatic aldehydes as well. With the use of the spectrophotometric method it was possible to show that in the case of salicylaldehyde the difference in the substrate specificity is not due to the failure on the part of the oxidizing enzyme but rather due to the sluggish rate at which the reducing enzyme (alcohol dehydrogenase) attacks the substrate.

Benzaldehyde was also tested and was found to be inactive as a substrate. However, some interaction of this aldehyde with aldehyde dehydrogenase was suggested by a very marked inhibition of acetaldehyde oxidation in the presence of benzaldehyde. Purines which are oxidized by aldehyde (xanthine) oxidase are not attacked by the liver aldehyde dehydrogenase.

**Stability, pH Optimum, and Substrate Affinity**—After the purification outlined above, the enzyme becomes unstable and loses activity quite rapidly. In order to preserve it for analytical purposes (the quantitative determination of aldehydes), it was most convenient to store it in 0.02 M pyrophosphate buffer at pH 8.0, well stoppered and frozen in a dry ice box. A preparation thus stored had retained approximately 30 per cent of its original activity after 8 months. Stability is also improved if it is kept in 50 per cent ammonium sulfate at pH 8.0.

The aldehyde dehydrogenase enzyme is quite heat-labile. Heating to temperatures above 55° causes rapid loss of activity. Exposure to a temperature of 58° for 2 minutes results in 80 per cent inactivation.

The enzyme tested in 0.01 M pyrophosphate buffer shows a sharp optimum around pH 9.3 (see Fig. 3) with acetaldehyde as substrate. From Fig. 2 it can be seen that the enzyme has a very high affinity for the substrate. In fact, it was impossible to determine accurately the
Michaelis constant under these experimental conditions, since $K_m$ is less than $10^{-6}$ M, a concentration below which absorption changes of reduced DPN are too small to be read with accuracy. This high affinity for the substrate makes the enzyme a suitable tool for the microdetermination of aldehydes. 0.03 to 0.5 micromole can be measured accurately by this method (see Fig. 2). Due to the reactivity of the enzyme toward a number of aldehydes the method lacks specificity.

Identification of Oxidation Product—For the identification of the oxidation product it was necessary to use stoichiometric amounts of DPN, since no alcohol dehydrogenase is present in the purified enzyme preparation.

![Graph](image)

**Fig. 3.** Effect of pH on oxidation of acetaldehyde by aldehyde dehydrogenase in 0.01 M pyrophosphate buffer.

A typical experiment was set up as follows: 0.5 ml. of purified enzyme, 0.3 ml. of 0.1 M pyrophosphate buffer, 15 micromoles of enzymatically active DPN, and 10 micromoles of acetaldehyde were present in a final volume of 3 ml. By the addition of small amounts of 0.2 N sodium hydroxide, to neutralize the acetic acid formed, the mixture was kept at a pH of approximately 9.0, measured with the glass electrode. At 20 minute intervals, 0.03 ml. samples were withdrawn and the formed DPNH₂ measured by the increased absorption at 340 mμ. In the above experiment close to 10 micromoles of reduced DPN (calculated according to Ohlmeyer's (15) molecular absorption coefficient) were found after 40 minutes, and additional incubation resulted in no further increase in absorption at 340
The entire sample was transferred to a screw-capped glass bottle, 4 cm. in diameter and about 5 cm. high. 2 gm. of magnesium sulfate and approximately 500 mg. of mercuric oxide were added. A center cup of about 2 cm. in diameter and 1 cm. in height was then placed in the bottle on a support of glass raised well above the fluid level. Into the center cup was pipetted 0.11 ml. of a 15 per cent potassium iodide solution in 0.1 N sodium hydroxide. Finally, 1 ml. of 15 N H₂SO₄ was rapidly pipetted into the main compartment of the bottle, any spilling on the center cup being avoided. The opening of the bottle was quickly covered with aluminum foil and the cap tightly screwed on. The samples were then placed in an air oven at 105° for 24 hours.

After this period the center cup was washed with small amounts of water, the washings pooled, and the acetate determined on an aliquot containing about 3 micromoles of acetic acid. Known amounts of acetic acid between 80 to 200 γ, neutralized with the potassium iodide-sodium hydroxide solution, were run at the same time.

The amount of alkali used in the center cup in the above experiment was adjusted to an amount only in slight excess of that necessary to neutralize the expected acid, since a larger excess of alkali interferes with the colorimetric test. In case of unknown quantities of acetic acid, the excess alkali can be neutralized with nitric acid to pH 8.2, with phenolphthalein as external indicator.

Control vessels, either without DPN or with boiled enzyme solutions, were run simultaneously with the samples containing all active components. Recovery experiments in the presence of all reagents were performed by adding 10 micromoles of acetic acid instead of acetaldehyde.

In the above experiment 9.3 micromoles of acetic acid formed from acetaldehyde were obtained. No acetic acid was found in a control experiment in which DPN was omitted. In another control in which 10 micromoles of acetic acid were added instead of acetaldehyde, 10.3 micromoles of acetic acid were recovered.

Attempts to Test Reversibility of Reaction—With the end-product thus established as acetic acid, attempts were made to test the reversibility of this reaction. Sodium acetate with reduced DPN failed to react with the enzyme. Likewise, a reversal of the reaction was not obtained with acetyl phosphate or with acetate and adenosine triphosphate. The latter experiments were performed with active purified preparations of aldehyde dehydrogenase with and without addition of a crude liver extract or of a highly purified preparation of the enzyme catalyzing the phosphorylation of 3-phosphoglyceric acid (16).

These experiments were carried out because of certain similarities between the above reaction and the oxidation of glyceraldehyde phosphate...
by triose phosphate dehydrogenase. In addition, recently Stadtman and Barker (17) showed a participation of phosphate in the oxidation of acetaldehyde by Clostridium kluyveri. However, no evidence was found for the participation of phosphate in the oxidation of acetaldehyde by the liver dehydrogenase. The reaction catalyzed by purified, well dialyzed preparations proceeds in the absence of added phosphates.

**DISCUSSION**

A conclusive demonstration of the existence of a single enzyme catalyzing a Cannizzaro reaction is not available. The evidence presented in this paper reveals that aldehyde mutase, the last of the enzymes still classified as mutases in text-books, consists of two separable enzymes, at least in the case of liver preparations.

Differences in the properties of the aldehyde dehydrogenase and the "mutase" are readily explained by the participation of alcohol dehydrogenase in the dismutation reaction. The absence of dismutation of some aromatic aldehydes is due to the sluggish reaction of the reducing enzyme with this substrate. The rate of oxidation of salicylaldehyde is of the same order as that of aliphatic aldehydes.

The reported absence of alcohol dehydrogenase in the mutase preparation of Dixon and Lutwak-Mann (4) can be explained by the accidental removal during the purification process of diaphorase, which is required in their test system. This possibility was later suggested by Dixon (18).

**SUMMARY**

1. Purified aldehyde mutase preparations contain two separable enzymes, alcohol dehydrogenase and aldehyde dehydrogenase, which together catalyze the dismutation of aldehydes.

2. The purification of a diphosphopyridine nucleotide-linked aldehyde dehydrogenase from beef liver is reported.

3. The results presented in this paper suggest that mutases catalyzing the Cannizzaro reaction may not exist as single enzymes.

**BIBLIOGRAPHY**

ALDEHYDE DEHYDROGENASE, A DIPHOSPHOPYRIDINE NUCLEOTIDE-LINKED ENZYME

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