Horse Liver Aldehyde Dehydrogenase

I. PURIFICATION AND CHARACTERIZATION*

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SUMMARY

Horse liver aldehyde:NAD oxidoreductase (EC 1.2.1.3) has been purified to homogeneity by a procedure consisting of salt fractionation, ion exchange chromatography, and isoelectric focusing. The purified material has a turnover number of 1.85 μmoles of NADH per min per mg of protein when assayed at pH 9.0 with propionaldehyde as substrate.

Values obtained for the molecular weight of the native enzyme by sucrose density centrifugation, sedimentation equilibrium, and multiple porosity disc gel electrophoresis were 220,000, 260,000, and 252,000, respectively. Sodium dodecyl sulfate polyacrylamide gel electrophoresis indicated a subunit molecular weight of 57,000, suggesting a tetrameric structure for the native enzyme.

Specificity studies indicated that both aromatic and aliphatic aldehydes were oxidized. For most aldehydes tested, the Michaelis constants were between 0.1 to 1 μM when corrected for equilibrium concentrations of inactive hydrated aldehyde. Chloral, which is completely hydrated, was an inhibitor of the dehydrogenase reaction.

Despite the broad aldehyde specificity, substrate analogues in which the aldehydic hydrogen of RCHO was replaced by NH₂, CH₂, or even OH were not found to be inhibitors.

Purification to homogeneity has been reported for two non-mammalian aldehyde dehydrogenases. The purified enzymes isolated from Pseudomonas aeruginosa (8) and from yeast (9) have properties that differ significantly from those found in mammalian systems with respect to substrate, coenzyme, and effector requirements, and hence cannot serve as models for the mammalian enzyme.

Here we report the purification to homogeneity of an aldehyde dehydrogenase from horse liver and a partial characterization of the enzyme. The following paper (10) contains studies pertinent to the kinetic mechanism of the reaction.

EXPERIMENTAL PROCEDURE

Materials—Benzaldehyde and isobutyraldehyde were obtained from Matheson, Coleman and Bell; 2-chloroacetaldehyde from Dow; furfural and propionaldehyde from Eastman Organic Chemicals; phenylacetaldehyde from Aldrich; D,L-glyceraldehyde from Sigma; and o-nitrobenzaldehyde from DuJae Laboratories. NAD and NADH were obtained from Boehringer; NADP, from Calbiochem, and ADP-ribose from Sigma. Enzyme grade ammonium sulfate was procured from Mann. CM-cellulose (CM 23) and DEAE-cellulose were obtained from Whatman and Sigma, respectively. Ampholytes for isoelectric focusing were obtained from LKB. Preliminary focusing of the (pH 4 to 6) ampholytes was performed to obtain the pH 4.4 to 5.5 ampholytes which were used in narrow gradient isoelectric focusing runs. Buffers were prepared from the sodium salt.

Activity Assay—The production of approximately 1 n mole of NADH with time was followed at 25°C with a thermostated Amino microfluorometer equipped with an American Instrument Co. filter No. 4-7113 (Corning 760) for the exciting light and a No. 4-7116 filter (Wratten 2A) for the emitted light. The standard velocity assay used measured the increase in fluorescence which occurred upon addition of enzyme to 1 ml of 0.1 M pyrophosphate buffer, pH 9.0, containing 0.3 mg of NAD and 0.0005% (v/v) propionaldehyde.

Assay of Protein Concentration—The concentration of protein throughout the course of the purification was determined by the procedure of Lowry et al. (11) with crystalline bovine serum albumin (Sigma) as the standard.

Polyacrylamide Disc Gel Electrophoresis—Disc gel electrophoresis was performed in an apparatus similar to that described by Davis (12). The procedure followed was that outlined by

The metabolism of ethanol proceeds by two separate oxidation steps to yield acetic acid. The first step is catalyzed by alcohol dehydrogenase while the second is catalyzed primarily by the pyridine nucleotide-dependent aldehyde dehydrogenase (2). Mammalian alcohol dehydrogenase, especially that from the liver, has been well studied (3), whereas the mammalian aldehyde dehydrogenase has not been as thoroughly investigated. The reason is that the mammalian aldehyde dehydrogenase has not been purified to homogeneity although several partial purifications from different animals have been reported (4-7).

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‡ The experimental work is taken from the dissertation submitted by R. I. F. in partial fulfillment of the requirements for the degree of Doctor of Philosophy, Purdue University, 1971.

† The abbreviation used is: CM-cellulose, carboxymethylcellulose.
Heat Denaturation Studies—Heat denaturation studies were performed in a water bath at an uncorrected temperature of 54°. Protein solutions in 0.1 M phosphate buffer, pH 6.0, were heated in stoppered test tubes and aliquots withdrawn at various time intervals. The dehydrogenase activity was measured by the standard assay, defining the activity measured before heating as 100%. Extinction Coefficient—The dry weight of an aliquot of aldehyde dehydrogenase which had been extensively dialyzed against 0.1 M ammonium acetate buffer, pH 7.0, was determined by first drying the protein to constant weight. Scanning of the ultraviolet absorption spectrum and a Lowry protein determination were performed on an aliquot from the same dialysate.

Molecular Weight—Sucrose density gradient centrifugation, sedimentation equilibrium ultracentrifugation, and multiple porosity disc gel electrophoresis were used to determine the molecular weight of aldehyde dehydrogenase.

Sucrose density gradient centrifugation was performed with linear 5 to 20% sucrose gradients prepared in 0.1 M phosphate buffer, pH 6.0, containing 0.1% 2-mercaptoethanol. After equilibrating the 4.5-ml sample for 6 hours at 2°, 0.1 ml of a protein solution containing yeast alcohol dehydrogenase (Worthington) and horse liver aldehyde dehydrogenase was added to each tube. Centrifugation was performed at 35,000 rpm in a SW 39 rotor of a Spino model L centrifuge for 12 hours at 2°. After centrifugation, the tubes were punctured and 10-drop fractions were collected and assayed for alcohol and aldehyde dehydrogenase activities. Alcohol dehydrogenase activity was determined fluorometrically by measuring the rate of production of NADH in 0.1 M glycine buffer, pH 10, containing 0.3 mM NAD and 17.5 mM ethanol. Aldehyde dehydrogenase activity was determined by the standard fluorometric assay described above. The molecular weight of horse liver aldehyde dehydrogenase was calculated by the method of Martin and Ames (13). The molecular weight of 150,000 was assumed for yeast alcohol dehydrogenase (14).

Sedimentation equilibrium studies utilized a Beckman model E ultracentrifuge equipped with an ultraviolet scanner which was directly connected to a Digital Equipment Corp. LINC-8 computer. The scanner was designed to move across the cell at a uniform rate. At a time chosen by the operator, the voltage, which was proportional to the optical density of the protein solution, was determined. Independent voltage determinations (256), one every 300 msec, were made during the course of one scan. The distance from the center of rotation (z) and the relative optical density (O.D.) of each data point were then calculated by the computer with reference points indicated by the operator, and the results were obtained on a printout. The values of ln O.D. as a function of z² calculated by the computer were plotted manually and the molecular weight calculated by standard procedure (15, 16) with an estimated value of 0.74 for 6 and 1.0 for p. Centrifugation was performed at 4° and 6500 rpm for 2 to 4 days with protein concentrations of 0.14, 0.19, and 0.24 mg per ml. The 0.1 M phosphate buffer, pH 6.0, was supplemented with 0.1% 2-mercaptoethanol to help stabilize the protein during the time period required for equilibrium to be reached.

Disc gel electrophoresis was performed with 4, 4.8, 6, 8.4, and 12% polyacrylamide gels. Bovine serum albumin (Sigma), yeast hexokinase (Sigma), and apoferritin (Mann) were used as standards. Data were treated by the method of Hedin and Smith (17). Plots of log 100 X RF versus percentage of acrylamide were prepared for each of the protein standards. The slope of each group was then replotted against the molecular weight of each standard protein and the best straight line through the data was determined by linear regression.

Subunit Molecular Weight—Sodium dodecyl sulfate polyacrylamide disc gel electrophoresis was used to determine the subunit molecular weight of aldehyde dehydrogenase. The procedure of Weber and Osborn (18) was followed, except that only 10% acrylamide gels were used and the gel buffer was diluted 1:2 with water instead of 1:1. Mobility was calculated as the distance of protein migration relative to the tracking dye. The gels were stained with either Coomassie brilliant blue as described by these authors, or with a 1% solution of Amido Schwarz in 7% acetic acid as directed by Davis (12). Bovine serum albumin (Sigma), horse liver alcohol dehydrogenase (Worthington), glyceraldehyde 3-phosphate dehydrogenase (Worthington), myoglobin (Mann), human hemoglobin (Mann), and cytochrome c (Nutritional Biochemicals) were used as markers. Both aldehyde dehydrogenase and its carboxymethylated derivative were run.

Carboxymethylation was performed by reacting a sodium dodecyl sulfate-mercaptoethanol-treated protein with excess (0.32 M) iodoacetate for 3 to 4 hours. One of the standards, bovine serum albumin, was carboxymethylated in the same manner.

Isolation of Horse Liver Aldehyde Dehydrogenase—In a typical preparation, approximately 250 g of minced, semifrozen horse liver were homogenized in a Waring Blender in 50-g portions plus 2 ml of water, then combined. The combined thick syrup was extracted with 300 ml of distilled water containing 1 mM reduced glutathione. Insoluble material was removed from the extract by centrifugation at 45,000 X g for 40 min at 4°. The pellet was extracted with an additional 150 ml of the glutathione solution. After centrifugation, the second extract was combined with the first, the total supernatant fluid was between 450 and 500 ml/250 g of liver.

To the combined extracts was added 0.5 ml of 2-mercaptoethanol, followed by the addition of 170 g per liter of ammonium sulfate (30% saturation). The material was allowed to stand in the cold for at least 45 min after which it was centrifuged for 1 hour at 45,000 X g and the precipitate was discarded. Solid ammonium sulfate (127 g per liter) was added to the supernatant fluid to make the solution approximately 50% saturated with respect to ammonium sulfate. In addition 0.5 ml of 2-mercaptoethanol was added. After the preparation had been allowed to stand for 90 min at 4°, the precipitate was collected by centrifugation, suspended in sodium phosphate (T/2 = 0.0025) at pH 6.0 containing 0.025% mercaptoethanol, and then dialyzed against several 4-liter changes of the same buffer and finally recentrifuged for 15 min at 45,000 X g.

Fractionation of the dialyzed material was performed on a
CM-cellulose column, 30 × 4 cm, equilibrated with sodium phosphate (Γ/2 = 0.0025) at pH 6.0. The column eluate immediately following the void volume contained the aldehyde dehydrogenase.

The eluate was dialyzed for 24 hours against several 4-liter changes of sodium phosphate (Γ/2 = 0.004) at pH 6.8, containing 0.025% 2-mercaptoethanol, and then applied to a DEAE-cellulose column, 22 × 6 cm, which had been equilibrated with the same buffer except that 0.5 mM dithiothreitol was substituted for the mercaptoethanol. The column was first eluted with 500 ml of this buffer which removes a small quantity of an aldehyde dehydrogenase. A linear ionic strength gradient at pH 6.8 was used to remove the firmly bound aldehyde dehydrogenase which was used in this study. The pH 6.8 gradient was made from 1 liter of sodium phosphate, Γ/2 = 0.004 and 1 liter of sodium phosphate, Γ/2 = 0.02. Each buffer contained 0.5 mM dithiothreitol. Routinely, 500 ml of the high ionic strength buffer was passed through the column to insure complete removal of the enzyme.

The enzyme was thoroughly dialyzed against a Γ/2 = 0.004 phosphate-0.5 mM dithiothreitol, pH 6.8, and applied to a second DEAE-cellulose column, 13 × 4.3 cm. The column was equilibrated with Γ/2 = 0.004 sodium phosphate-0.5 mM dithiothreitol, pH 6.8, buffer and the enzyme eluted from the column with an ionic strength gradient. The gradient consisted of 750 ml of this buffer and 750 ml of a Γ/2 = 0.10 phosphate-0.5 mM dithiothreitol at pH 6.8.

The aldehyde dehydrogenase eluted from the column was concentrated under nitrogen with the appropriate size Diaflo cells and PM-30 membranes supplied by Amicon. After dialyzing the concentrated enzyme against 4 liters of sodium phosphate (Γ/2 = 0.0025) containing 0.025% mercaptoethanol at pH 6.0, narrow gradient isolectric focusing was performed at 4° with the preliminary focusing technique suggested in a LKB 8100 Ampholine Instruction Manual (19). A minor change was made in the procedure by substituting an aqueous solution of 0.05% 2-mercaptoethanol for the water used in making up the heavy and light gradient solutions. Focusing was generally performed at 500 volts and was allowed to proceed until no further decrease in amperage was noted. Usually 48 hours were required.

When focusing was complete, the column effluent was collected in 2-ml fractions which were assayed for aldehyde dehydrogenase activity, 280 nm absorbance, and pH. The aldehyde dehydrogenase-containing fractions having constant specific activity were pooled and dialyzed 6 to 10 hours against 0.1 mM sodium phosphate at pH 6.0, containing 0.1% 2-mercaptoethanol, in order to partially remove the ampholytes and sugar. The resultant homogeneous enzyme preparation was stored at 4°. Before use, remaining ampholytes were removed by extensive dialysis against four changes of the desired buffer.

RESULTS

Isolation of Aldehyde Dehydrogenase—The isolation of aldehyde dehydrogenase from a typical preparation is summarized in Table I. Approximately 17 mg of homogeneous enzyme can be isolated from 250 g of liver.

As seen in Fig. 1, the aldehyde dehydrogenase activity present in the CM-cellulose eluate is resolved by DEAE-cellulose chromatography into at least two components. The material eluting from the first DEAE-cellulose column between 2.47 and 3.00 liters was pooled and applied to the second DEAE-cellulose column. The aldehyde dehydrogenase, eluted from the second DEAE-cellulose column was subjected to isoelectric focusing for final purification. The isoelectric point of aldehyde dehydrogenase as determined by isoelectric focusing is 5.05 (Fig. 2).

Heterogeneity and Criteria of Purity—Two types of heterogeneity were noted. The first, an activity peak immediately following the void volume from the first DEAE-cellulose column, is probably indicative of a distinct enzyme species. No attempt was made to purify or characterize this enzyme. The second form of heterogeneity was observed during two preparations after the second DEAE-cellulose chromatography step. In

| Procedure | Total protein mg | Total activity μmoles/min | Specific activity μmoles/mg |
|-----------|-----------------|--------------------------|-----------------------------|
| 1. Extraction | 15,040 | | |
| 2. (NH₄)₂SO₄ (176 g per liter) | 15,250 | 81.3 | 7.0 |
| 3. (NH₄)₂SO₄ (127 g per liter) | 11,760 | 69.5 | 8.2 |
| 4. CM-cellulose chromatography | 8,450 | 59.7 | 37.2 |
| 5. DEAE-cellulose chromatography I | 1,040 | 39.7 | 37.2 |
| 6. DEAE-cellulose chromatography II | 295 | 35.4 | 120 |
| 7. Isoelectric focusing | 17 | 12.2 | 720 |

![Fig. 1. Elution profile of aldehyde dehydrogenase activity from the first DEAE-cellulose column. The material eluting immediately following the void volume from the CM-cellulose column was chromatographed on a DEAE-cellulose column (22 × 6 cm) by a linear gradient consisting of 1 liter of Γ/2 = 0.004 and 0.07 sodium phosphate-5 × 10⁻⁴ M dithiothreitol, pH 6.8. The 280-nm absorbance (○) and aldehyde dehydrogenase activity (□) were determined.](http://www.jbc.org/issue)
these preparations a biphasic heat denaturation profile at 54° was observed when the log of the percentage of activity remaining was plotted as a function of time. A mixture consisting of 30 to 40% relatively short-lived material (t 1/2 = 20 min) and 60 to 70% more heat-stable material (t 1/2 = 60 min) was indicated. When one of these preparations was subjected to isoelectric focusing, two distinct species, one isoelectric at pH 4.8 and the other at pH 5.05, were isolated. The enzyme species with the lower isoelectric point had a half-life of 20 min at 54°, while the one with an isoelectric point of 5.05 had the 60-min half-life. The two enzyme species were similar to each other in molecular weight as judged by sucrose density centrifugation, and migrated together as a single band on disc gel electrophoresis. Dithiothreitol (1 mM) did not convert one species into the other, indicating that the oxidation of sulfhydryls was not the cause of the heterogeneity. In subsequent preparations the heat denaturation profile was determined immediately after CM-cellulose chromatography. At this stage, only the more heat-stable form of the enzyme was present. It is this form of the enzyme (isoelectric point, pI = 5.05) which was used for all the experiments reported in this paper.

Enzyme preparations were considered homogeneous and suitable for characterization and mechanistic studies if (a) the specific activity after isoelectric focusing was constant across the activity peak; (b) the pooled isoelectric-focused material gave one band when subjected to electrophoresis on polyacrylamide gels and stained with Amido schwarz; and (c) a monophasic heat denaturation profile was obtained when the log of the percentage of activity remaining was plotted against time. The enzyme obtained after careful isoelectric focusing met these three criteria.

Stability—The enzyme was most stable when stored at 4° in 0.1 M phosphate buffer, pH 6.0, supplemented with approximately 0.1% 2-mercaptoethanol and an undetermined amount of sugar and electrophoretic ampholytes remaining from the isoelectric focusing step. Under these conditions the enzyme underwent only a slow loss of activity less than 4% per week over a period of several months. No change in heat denaturation pattern or in the K values for substrate has been observed with homogeneous enzyme preparations stored as long as 5 months.

However, despite the enzyme's apparent stability, the specific activity of all preparations was not the same. The highest specific activity ever obtained was 1.8 μmoles per min per mg of protein. However, the specific activity decreased to the more representative level of 0.7 to 1.0 μmole per min per mg upon final dialysis of the isoelectric-focused material in those preparations. Once this specific activity was reached, the preparations assumed the apparent stability of the other preparations. The variability in specific activity noted among preparations did not represent individual variation due to the species, age, or condition of the horse donor, as all the preparations used in the study were prepared from a single liver.

Various other methods of storage were attempted. The enzyme was stable to storage in liquid nitrogen after rapid freezing, but rapidly lost activity if stored at −10°. It was not stable in low freezing or pH values below 4 or above 10.

Absorption Spectrum—The absorption spectrum of 0.5 mg per ml of aldehyde dehydrogenase was measured between 250 to 600 nm. Only a single absorption peak which occurred at 280 nm could be found. The lack of a visible spectrum indicates that the aldehyde dehydrogenase isolated is not a flavoprotein, nor does it contain bound NAD as doos glyceraldehyde 3-phosphate dehydrogenase.

Extinction Coefficient—A 0.1% solution of aldehyde dehydrogenase in ammonium acetate at pH 7 was found to have an absorbance of 2.08 cm⁻¹ at 280 nm.

Molecular Weight—From the three independent methods used to determine the molecular weight of horse liver aldehyde dehydrogenase an average of 245,000 was obtained. Sucrose density centrifugation, involving the simple comparison method of Martin and Ames (13), yielded a molecular weight for aldehyde dehydrogenase of 220,000. From sedimentation equilibrium an average molecular weight of 264,000 was calculated from six independent determinations representing three different protein concentrations. A representative plot of d ln c/dx² is presented in Fig. 3. Lastly from the polyacrylamide gel electrophoretic technique of Hedrick and Smith (17) a molecular weight of 252,000 was obtained. For the latter technique it was necessary to first prepare a calibration curve of increase in the log Ry versus gel concentration for many proteins. From this data it was possible to estimate the molecular weight of aldehyde dehydrogenase by measuring the slope of the log Ry versus gel concentration and comparing it to the standard curve.

Subunit Molecular Weight—A subunit molecular weight of 57,000 ± 6,000 was determined for horse liver aldehyde dehydrogenase by sodium dodecyl sulfate polyacrylamide gel electrophoresis (Fig. 4). In order to verify that the molecular weight of 57,000 did not represent the aggregation of two subunits, a sample of aldehyde dehydrogenase treated first with mercaptoethanol, and then carboxymethylated with excess iodoacetate, was subjected to electrophoresis under the same conditions as those in the previous molecular weight determin-
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0.42 - 0.48 - 0.49 - 0.50 - 0.51 - 0.52 (CM)

Fig. 3. Sedimentation equilibrium data for the molecular weight determination of aldehyde dehydrogenase. Sedimentation equilibrium data were obtained at 6800 rpm. The linearity of the data is an indication of the homogeneity of the enzyme.

0.3 - 0.35 - 0.4 - 0.45 - 0.5 - 0.55 - 0.6 - 0.65 - 0.7 - 0.75 - 0.8 - 0.85 - 0.9

MOBILITY

MOL. WGT. (x 10^4)

0.1 - 0.2 - 0.3 - 0.4 - 0.5 - 0.6 - 0.7 - 0.8 - 0.9

Fig. 4. Estimation of the subunit molecular weight of aldehyde dehydrogenase as determined by sodium dodecyl sulfate gel electrophoresis. The subunit molecular weight of aldehyde dehydrogenase (AldH) and of its carboxymethylated derivative (C-AldH) was determined by sodium dodecyl sulfate polyacrylamide gel electrophoresis using bovine serum albumin (BSA), carboxymethylated bovine serum albumin (C-BSA), horse liver alcohol dehydrogenase (ALDH), rabbit muscle glyceraldehyde-3-phosphate dehydrogenase (GDH), myoglobin, hemoglobin, and cytochrome c as standards.

### Table II

| Aldehyde               | Apparent $K_m$ | Corrected $K_m$ | Relative $V_{max}$ |
|------------------------|----------------|-----------------|--------------------|
| Propionaldehyde        | 0.4 $\mu$M    | 0.2 $\mu$M      | 100                |
| 2-Chloroacetaldehyde   | 5.9 $\mu$M    | 0.1 $\mu$M      | 260                |
| d,L-Glyceraldehyde     | 200 $\mu$M    |                 |                    |
| Phenylacetaldelyde     | 0.4 $\mu$M    |                 | 110                |
| Isobutyraldehyde       | 0.4 $\mu$M    | 0.1 $\mu$M      | 100                |
| Benzaldehyde           | <0.3 $\mu$M   | <0.3 $\mu$M     | 20                 |
| Furfural               | 0.5 $\mu$M    |                 | 11                 |
| o-Nitrobenzaldehyde    | 0.5 $\mu$M    |                 | 3                  |

Corrected $K_m$ values are based on the percentage of free carbonyl calculated for each substrate from the gem-diol dissociation constants given in Bell (21).

A molecular weight of 57,000 was observed for the carboxymethylated aldehyde dehydrogenase.

**General Kinetic Properties**—The rate of formation of product in the normal assay was found to be directly proportional to the amount of enzyme present, usually $10^{-2}$ to $10^{-2}$ mg per ml. Plots of initial velocity versus substrate concentration gave no indication of sigmoidicity at low concentration and Lineweaver-Burke plots were linear with the exception that substrate inhibition was noted in the initial velocity studies of the dehydrogenase reaction at very high (>1 mm) aldehyde concentrations.

**pH Velocity Profile**—The velocity of the dehydrogenase reaction, determined with the standard assay in pyrophosphate buffer adjusted to the required pH with HCl or NaOH, was found to increase continuously from pH 7.0 to approximately pH 10, where irreversible denaturation of the enzyme occurred within the time period of the activity assay. Compared to pH 9, the velocity at pH 7 was 26%, at pH 8 it was 51%, and at pH 10 it was 165%.

**Coenzyme Specificity**—The $K_m$ for NAD in the standard assay at pH 9 was 57 $\mu$M and 100 $\mu$M at pH 8 in 10 mM barbital buffer (10). No activity with NADP at concentrations as high as 0.1 mM was observed at either pH.

**Aldehyde Specificity**—As is found with many of the mammalian aldehyde dehydrogenases studied, the horse liver enzyme is capable of oxidizing a great variety of aldehydes, including both aromatic and aliphatic compounds. Both the $K_m$ and $V_{max}$ differ with the type of aldehyde. As seen from the compounds in Table II, those aldehydes having electron-withdrawing groups adjacent to the carbonyl carbon give greater maximal velocities than those which do not.

The $K_m$ values shown in Column 2 of Table II represent apparent Michaelis constants in that they are not corrected for the percentage of aldehyde present in the nonreactive (20) hydrated form. This percentage differs for each aldehyde and depends upon the nature of the substituents next to the carbonyl. The corrected $K_m$ values shown in Column 3 lie in the range from 0.1 to 0.3 $\mu$M. Even these extremely low values may prove to be upper estimates of the true Michaelis constants, as they were calculated strictly from the gem-diol dissociation constants and do not take into consideration possible competitive inhibition by the hydrated form of the substrate.
Inhibition Studies—Trichloroacetaldehyde, chloral hydrate, was found to be a potent inhibitor of horse liver aldehyde dehydrogenase. A concentration of 1 mM was sufficient to give 60% inhibition when assayed in the presence of 5 mM (>10,000 \times K_i) propionaldehyde.

Assuming by analogy with the human enzyme (20) that the substrate for the horse liver enzyme is RCHO and not RCH(OH)_2 compounds with substitutions for aldehydic hydrogen were investigated as possible inhibitors of the dehydrogenase reaction. An amide, an acid, and a ketone were examined. No inhibition of the dehydrogenase activity was noted in the presence of 10 mM isobutyramide, 2.5 mM furoic acid, 10 mM acetic acid, or 2 mM acetone. When the ester p-nitrophenyl acetate was tested as an inhibitor of the dehydrogenase reaction, inhibition did occur. However p-nitrophenyl acetate is also a substrate for the homogenous enzyme. Details of the esterase activity of aldehyde dehydrogenase will be discussed in the following paper (10).

A variety of small molecules bearing no resemblance to substrates or products were found to inhibit the dehydrogenase reaction. These include recrystallized ammonium sulfate and Tris. The latter was inhibitory only at low aldehyde concentrations, suggesting that the complexing of substrate rather than a direct effect on the enzyme may be responsible for the inhibition.

Effect of Phosphate on Dehydrogenase Reaction—The kinetic effect of 10 mM phosphate on the dehydrogenase reaction was evaluated at pH 8.0 in 10 mM sodium barbital buffer containing 0.5 mM NAD and 0.002% (v/v) propionaldehyde as coenzyme and substrate, respectively. No stimulation of the reaction rate was found. The possibility of the formation of an acylphosphate reaction product in a nonrate-determining step was tested by the procedure of Harting and Velick (22) except that propionaldehyde rather than acetaldehyde was used as substrate, cysteine was absent, and the concentration of phosphate was 5 mM. No acylphosphate product was detected. Tests with commercial acylphosphate indicated that any propynylphosphate formed during the course of the reaction should have been stable to the incubation conditions.

DISCUSSION

Aldehyde dehydrogenases have been isolated from many sources and purified to various degrees of homogeneity (4–7, 23–25). While the yeast (23) and P. aeruginosa (24) enzymes are considered to be homogeneous, none of the mammalian enzymes discussed in the literature have been isolated in pure form. The procedures used by Erwin and Deitrich (5), Kraemer and Deitrich (6), Blair and Bodley (25), and Sheppard et al. (7) for the isolation of various mammalian aldehyde dehydrogenases are similar to those which were used to obtain the enzyme from horse liver. The additional isoelectric focusing step allowed us to obtain a homogeneous preparation of NAD-dependent aldehyde dehydrogenase from horse liver as assessed by disc gel electrophoresis, isoelectric focusing, and heat denaturation studies.

During the course of purification two types of heterogeneity of aldehyde dehydrogenase activity were noted. The aldehyde dehydrogenase activity present in the CM-cellulose eluate was resolved by DEAE-cellulose chromatography into at least two components. The presence of two aldehyde dehydrogenase activity species, separable by DEAE-cellulose chromatography, has also been reported as for the human liver (25) and yeast (26) enzymes.

The second type of heterogeneity, represented by the isolation of a species isoelectric at pH 4.8 in addition to the normally isolated species (pI = 5.05), may possibly indicate a modification of the enzyme during the course of purification. Clark and Jakoby have recently reported extensive investigations of the causes of microheterogeneity in preparations of purified yeast aldehyde dehydrogenase (26). Microheterogeneity was caused by proteolysis and could be prevented by inclusion of diisopropyl fluorophosphate as protease inhibitor. The three enzymes obtained from yeast when diisopropyl fluorophosphate was not included, had the same pI optimum and K_m values for substrate and coenzyme. The cause of the enzyme's heterogeneity was not investigated.

Molecular weight estimates for aldehyde dehydrogenases from various species ranged between 250,000 calculated for the P. aeruginosa enzyme (8) to the 90,000 found by Kraemer and Deitrich (6) for a human liver aldehyde dehydrogenase. Even among the mammalian enzymes, the variation in reported molecular weights has been wide. The highest molecular weight estimate reported for a mammalian aldehyde dehydrogenase is the 200,000 found by Blair and Bodley (25) for an enzyme isolated from human liver. The data presented in this paper, including those on subunit molecular weight studies on the enzyme and its carboxymethylated derivative, are consistent with a species of molecular weight approximately 245,000 and of individual subunit weight of approximately 57,000. Clark and Jakoby (27) have reported for the yeast enzyme subunit molecular weights of 56,000 also determined by sodium dodecyl sulfate polyacrylamide gel electrophoresis and 51,000 determined by sedimentation equilibrium experiments in 6 M guanidine hydrochloride-0.1 M 2-mercaptoethanol, and have suggested that the yeast enzyme is probably tetrameric in structure.

Although aldehyde dehydrogenases capable of using NADP as the coenzyme are found in some nonmammalian species (28–30), no activity was observed with NADP in the case of the horse liver enzyme. Analogues of NAD have been shown to substitute for NAD with beef liver (31) and beef brain (32) enzymes.

In the accompanying paper some kinetic and mechanistic properties of the enzyme are discussed (10).

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