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The Molecular Weight of Horse Liver Alcohol Dehydrogenase

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The molecular weight of highly purified horse liver alcohol dehydrogenase has been determined to be 83 300. This value is derived from a sedimentation coefficient of 5.11 S at infinite dilution, a diffusion coefficient of 5.96 F at a low protein concentration and a partial specific volume of 0.750. Titration with DPNH confirms that two molecules of coenzyme are bound per molecule of apoprotein.

Crystalline alcohol dehydrogenase (ADH) from horse liver has recently been prepared in an apparently purer and more active form than previously [1,2]. Since the kinetics and mechanism of the reaction catalyzed by this enzyme have been extensively studied, it was of interest to redetermine its molecular weight and its binding capacity for the coenzyme.

MATERIAL AND METHODS

The enzyme was prepared as described by Dalziel in the preceding article [1], including the purification by chromatography. It was precipitated by ethanol, dissolved in the proper buffer and dialyzed against the same buffer overnight immediately before the measurements. The buffer was 0.044 M phosphate of pH 7.0 with 1 % sodium chloride added for the diffusion and sedimentation experiments. The partial specific volume was determined on a sample dissolved in 0.022 M phosphate of pH 7.0. It was not possible to use a more dilute buffer because of the lability of the enzyme at lower ionic strengths. The protein concentration of this sample, determined as the difference between the dry weight of the sample and the buffer, was 9.76 mg/ml. The specific absorbancy of the protein at 280 mμ in a 1 cm layer was determined to be 0.42 for a concentration of 1 mg/ml. This value has been used for the concentration determination of the other samples.

The equipment and technique used for the sedimentation and diffusion studies and for the determination of the partial specific volume have been described elsewhere [2]. The temperature of the rotor in the Spinco ultracentrifuge was kept close to 20°C, while the diffusion experiment was made at 34°C. Both S and D were corrected to 20°C and water as solvent. The pycnometer used for the determination of the partial specific volume was equilibrated at 21.68°C. The absorbancy measurements were made in a Beckman spectrophotometer, model DU.

The DPNH used for the titration was obtained from Sigma chem. Co. The DPNH solution was assayed by measurement of the decrease of extinction at 340 mμ on complete enzymic oxidation to DPN with aldehyde and a catalytic amount of ADH, taking [3] $E_{mM} = 6.25$. The residual extinction after complete oxidation was 5 % of the initial value. The assay showed that the solid sample contained 71 % coenzymically active DPNH.

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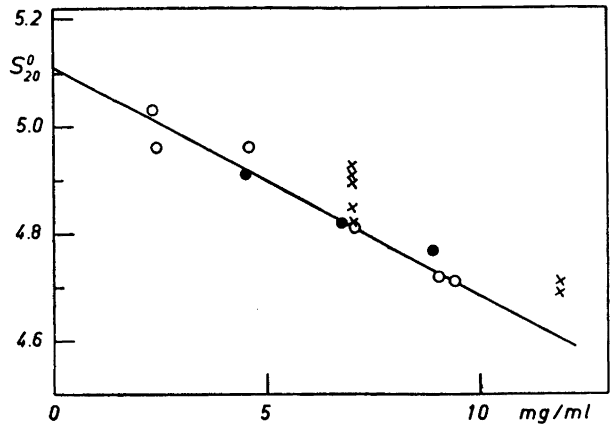

Fig. 1. Concentration dependence of the sedimentation coefficient of ADH. Abscissa: mean protein concentration during observation in the ultracentrifuge. Ordinate: Sedimentation coefficients of pure ADH, O, of a slightly less active material, ●, and the data published in an earlier investigation on salt and pH effects [4], ×.

RESULTS

Sedimentation coefficient. The sedimentation velocity was measured in six experiments at different concentrations. In Fig. 1 the corrected sedimentation coefficients, $S_{20}°$, have been plotted *versus* the mean protein concentration during the experiment. The best fitting line has been calculated by means of the least square method and is drawn in the figure. Its equation is

$$S_{20}° = 5.11 - 0.0425\ a$$

where a is the concentration of ADH in mg/ml and 5.11 is the sedimentation coefficient extrapolated to infinite dilution and measured in Svedberg units.

For comparison three values obtained on another fresh but somewhat less pure preparation (indicated by lower specific activity) have been included in Fig. 1. They do not deviate to any appreciable extent from the calculated line. Earlier data obtained in an investigation on pH and salt effects on the sedimentation [4] are also shown in the figure. This preparation was even less pure, the data are slightly higher than in the present investigation but extrapolate to about the same value at infinite dilution [4].

Diffusion coefficient: Two experiments were performed, one on "100 %" pure ADH and one on "85 %" pure ADH. The purity was judged from activity tests. In both cases the protein concentration was close to 1.54 mg/ml, and the total fringe number was found to be 24.95 and 24.78, respectively. A fringe interval, Δn, of 5 was hence chosen in the calculation of the maximal fringe density, n'_{max}.

When applying the "height-area" method the rate of increase of $\left(\frac{1}{n'_{max}}\right)^2$ with time has to be determined. The corresponding plot of data derived from six exposures on the pure ADH is given in Fig. 2. The equation of the line

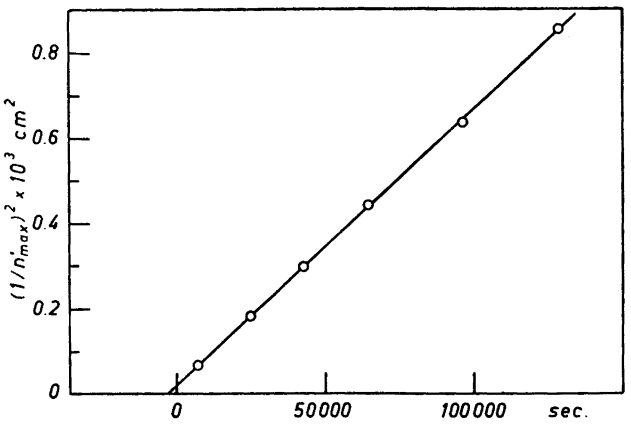

Fig. 2. Graph from which the diffusion coefficient of pure ADH has been calculated according to the "height-area" method. Abscissa: time measured from end of sharpening procedure. Ordinate: inverted square of maximal fringe density.

has been calculated by means of the least square method. The apparent starting time is at —3 100 sec. The diffusion coefficient $D_{1.31} = 3.15\ F$ which after correction becomes $D_{20^\circ} = 5.97\ F$. The fit to the laws of ideal diffusion was investigated by plotting fringe data of the last exposure, taken after 36 h, on a normal distribution graph [2], Fig. 3. The same exposure was also evaluated according to the moment method [2,5], since any small error in the zero time correction in this case can be neglected. A value of 5.93 F was found for D_{20°. $D_{20^\circ} = 5.95\ F$ is obtained as a mean value for the two methods of calculation.

The sample of 85 % active ADH gave for four exposures $D_{20^\circ} = 5.60\ F$ and $t_0 = -7\,300$ sec with the "height-area" method. The moment method applied to the last exposure, taken after 40 h, gave $D_{20^\circ} = 5.86\ F$. The same exposure has been tested on the normal distribution graph, Fig. 3. A close analysis shows that the curve is slightly S-shaped.

The partial specific volume was found to be 0.750 ($\pm$ 0.004) ml/g in a single determination.

DPNH titration. DPNH forms a compound with liver ADH in which the absorption maximum is displaced from 340 mμ to 325 mμ. This spectral shift enabled Theorell and Bonnichsen [6] to demonstrate that at pH 7 one molecule of ADH binds two molecules of DPNH. A similar spectrophotometric titration was made with the enzyme preparation used in the present investigation.

To 3 ml enzyme solution, 5.2 mg/ml in phosphate buffer of pH 7 and μ = 0.1, in a cuvette 5.1 μl portions of 16.1 mM DPNH were added on a stirrer. After each addition the absorbance was measured at 310 and 350 mμ, the wavelengths at which the specific absorptions of free and bound DPNH differ most. The titration was carried out as quickly as possible, but it was necessary to correct the absorbance differences for a slow decline of absorp-

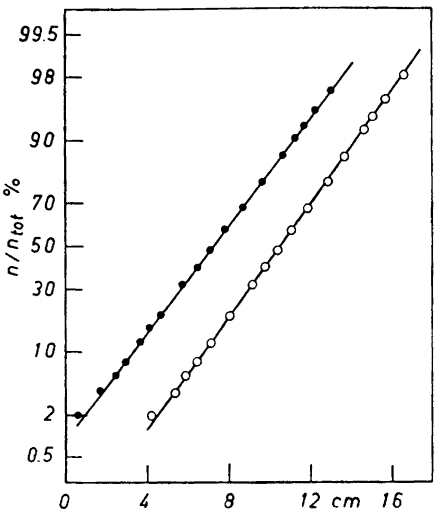

Fig. 3. Normal distribution plots for testing fit of boundary shape to that required by the ideal laws of diffusion. Abscissa: fringe coordinates. Ordinate: relative fringe numbers of exposures on "100 %" pure ADH taken after 36 h, O, and on "85 %" pure ADH taken after 40 h, ●.

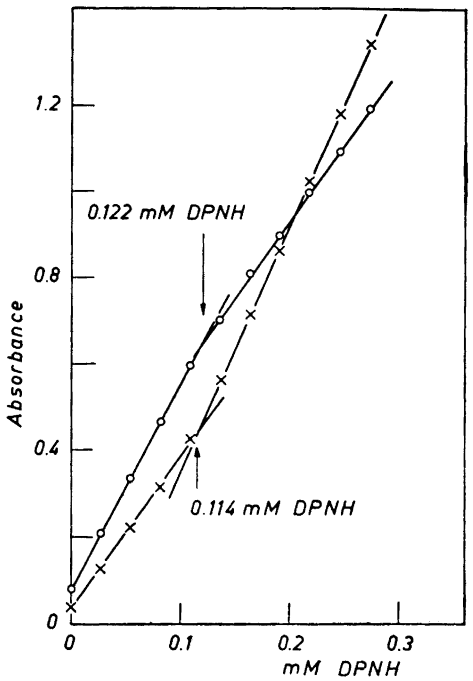

Fig. 4. Titration of ADH, 5.2 mg/ml, with DPNH. Abscissa: concentration of coenzymically active DPNH. Ordinate: Absorbance readings at 310 mμ, O, and 350 mμ, ×. The breaking points of the slopes are indicated.

tion at both wavelengths. The corrected data are plotted in Fig. 4 against the total DPNH concentration.

The equivalence point, indicated by sharp changes of slope, comes at a mean value of 0.118 mM DPNH. The titration indicates hence an equivalent weight of $5.2/118 \times 10^{-6} = 44\,000$ per binding site of the enzyme. The slopes give values of $E_{mM} = 4.8$ and 3.5 for the compound, and $E_{mM} = 3.6$ and 5.9 for free DPNH, at 310 and 350 mμ, respectively, in good agreement with previous values [6].

DISCUSSION

From the values, $S_{20}^{\circ} = 5.11\ S$, $D_{20}^{\circ} = 5.95\ F$ and $V_{sp} = 0.750$ ml/g, a molecular weight of 83 300 is calculated by means of the Svedberg formula. This is in good agreement with the value 84 400 obtained by the Archibald method [7], but deviates considerably from the value 67 500 [6,7] obtained for previous and less active preparations of ADH [8,9]. This discrepancy is due to differences mainly in the sedimentation and diffusion coefficients since the partial specific volume of the present material is 0.750 ml/g, as compared to

the previous value [6] 0.751 ml/g. The sedimentation and diffusion of the previous material was studied by Pedersen [6]. He obtained $S_{20}^{\circ} = 4.9\ S$ on a sample with a protein concentration of about 10 mg/ml when investigated in the oil driven ultracentrifuge. This becomes 4.5 S after correction for a probable error in the measured rotor temperature, as suggested by Pedersen [10]. The present material has a sedimentation coefficient of 4.7 at the same concentration. The main difference in S is thus due to the fact that our data have been extrapolated to infinite dilution. The small remaining difference indicates that the previous material contained some low molecular impurities. This conclusion is supported by the values of the high diffusion coefficient obtained by Pedersen [6], $D_{20}^{\circ} = 6.5\ F$ as calculated by the "height-area" method and $D_{20}^{\circ} = 7.3\ F$ as determined by the moment method.

The main source of error in the present determination of M is the uncertainty in the partial specific volume, which might introduce an error of about $\pm$ 2 % in M. The S and D values are more accurate than that and an error in the molecular weight 83 300 of about $\pm$ 2 000 might be estimated.

The equivalent weight of 44 000 obtained in the DPNH titration confirms [6] that two molecules of DPNH are attached to each molecule of enzyme, for which a molecular weight of 88 000 is indicated. Considering the big errors involved in the experiment this figure is much less reliable than the one given above.

The behaviour of the ADH in the sedimentation velocity experiments, the diffusion experiment and the titration experiment as well as in the molecular weight determination experiment according to Archibald [7] indicates that the present preparation [1] really has a high degree of purity. The best value of the molecular weight of horse liver ADH to be given at the present is 84 000, a mean of the figure reported here and the value obtained by Archibald's method [7].

The less active material exhibited a slightly lower diffusion coefficient, indicating some contamination of larger molecular species. It may be remarked that ADH stored in the buffer solutions for some time lost some activity and often gave higher sedimentation coefficients.

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