Physico-chemical Properties of Lactic Dehydrogenase*

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Although the mechanism of the enzymatic reaction catalyzed by lactic dehydrogenase has been investigated by several workers, little work has been reported on the physical chemistry of this protein. In particular, a precise value for the molecular weight has not been reported. Meister (1) has estimated the molecular weight to be of the order of 100,000 to 150,000. Neelands (2) has reported a value of 135,000 to 150,000. In previous reports from this laboratory (3) the number of moles of coenzyme bound per mole of protein, with 135,000 as the molecular weight of the protein, has been found to be approximately 3.6. From similar measurements which yielded the same nonintegral ratio of moles bound per mole protein, Velick (4) has suggested that the correct molecular weight is 150,000.

The present paper presents results of measurements which suggest that beef heart lactic dehydrogenase has a molecular weight of approximately 72,000 and that the protein associates in solution as protein concentration is increased.

EXPERIMENTAL PROCEDURE

Materials—Fraction A of crystalline beef heart lactic dehydrogenase was prepared by the method developed in this laboratory (5). NAD and NADH, purchased from Pabst Laboratories, were used without further purification. Sodium p-chloromercuribenzoate was a product of the Sigma Chemical Company.

Methods—Protein concentration and enzymatic activity determinations were carried out as previously described (5). The apparent partial specific volume, calculated from the amino acid content of the protein (6), was found to be 0.747 ml per gm (see below).

Sedimentation studies were carried out in the Spinco Model E analytical ultracentrifuge. All sedimentation velocity determinations were made at 59,780 r.p.m. The temperature of the rotor was maintained by use of the “rotor temperature indicating and control unit” supplied with the instrument. The increase in radius of the rotor at the rotational rate used for measurements, determined by the method of Kegeles and Gutter (7), was found to be 0.03 cm. Sedimentation velocities, estimated from the position of the maximal ordinate of the gradient curve, were converted to $s_{20,w}$ values by the method previously used in this laboratory (8). A double sector cell was employed in sedimentation experiments in which the method of Baldwin (9) was used to estimate diffusion coefficients.

“Approach to sedimentation equilibrium” experiments were performed at rotor speeds of 2,095 to 10,589 r.p.m. In each run, 0.1 ml of FC-43, a fluorocarbon supplied by Spinco, was added to the 12-mm cell to create a transparent cell bottom (10). No interaction between the protein and the fluorocarbon was observed. A synthetic boundary cell was used to determine the initial protein concentration in optical units (11). The protein concentration at the meniscus and cell bottom was calculated from enlarged tracings of photographic plates (12).

Diffusion rates were estimated by use of the Rayleigh interference optical system of the Spinco Model H electrophoresis-diffusion apparatus. Boundaries were sharpened by the technique of Kahn and Polson (13). The photographic plates were measured with microcomparators1 and the data were treated by the procedure of Longsworth (14). Zero time corrections were estimated by the method of Longsworth (15).

RESULTS

Variation of Sedimentation Constant with Protein Concentration—The results of sedimentation velocity determinations at varying protein concentrations are shown in Fig. 1. It is evident that extrapolation to zero protein concentration, with the use of points obtained at protein concentrations greater than 2 g per liter, yields a value for $s_{20,w}$ of 7.71 S. Also, the curve exhibits a maximum at a concentration of approximately 1.5 g per liter. Qualitatively similar behavior has been observed with chymotrypsin (16) and hemoglobin (17). In these cases, this type of variation of sedimentation constant with concentration was taken to indicate association of the protein with increasing concentration.

Alternatively, however, the low sedimentation velocities at low protein concentrations might have been caused by boundary instability. This explanation is considered unlikely for the following reasons. (a) There is good agreement between values obtained in the 12-mm cell, indicated by open symbols in Fig. 1, and in the 30-mm cell, indicated by filled symbols. Boundary instability would be expected to be greater in the longer solution column. (b) Sedimentation constants of bovine serum albumin, measured at several concentrations less than 3 g per liter, increased in a linear manner with decreasing protein concentration.

1 The author is pleased to take this opportunity to acknowledge the generosity of Doctors Gerson Kegeles and John F. Taylor in allowing him to use their microcomparators for these measurements.
The sedimenting boundary was found to be highly symmetrical. Calculations of the first and second moments of the gradient curves indicate that these moments are identical with the position of the maximal ordinate over the range of protein concentration down to 2.3 g per liter. At lower concentrations the gradient curves are too small to allow precise measurement. Further, enzymatic assays indicated that the protein retained its full enzymatic activity during sedimentation. These results argue against the view that the anomalous sedimentation behavior, shown in Fig. 1, is caused by denaturation of the protein in the course of a sedimentation experiment. It thus appears that the data shown in Fig. 1 can be accounted for on the basis that the protein dissociates upon dilution.

Variation of Sedimentation Constant with pH and Ionic Strength—Since the value of $s_{20,w}$ for the monomeric form of the protein cannot be estimated with precision from the data shown in Fig. 1, a complete study of the variation of sedimentation coefficient with protein concentration was carried out at six pH values between 6.0 and 9.0. Plots of these results bore a general resemblance to Fig. 1 with the exception that the slopes of the lines at protein concentrations greater than approximately 2 g per liter varied with pH. Precise estimation of the sedimentation coefficient of the monomeric form of the protein was not possible from any of these data. At a constant protein concentration of 1 g per liter the sedimentation coefficient was found to increase from a value of 7.40 S at pH 6.0 to 7.55 S at pH 7.4 in 0.2 ionic strength phosphate buffers. At more alkaline pH values, the sedimentation coefficient again decreases, the value at pH 9.0 being 7.40 S.

The effect of added NaCl on the sedimentation coefficient at pH 6.0 is shown in Fig. 2. Although large corrections must be made for the viscosity and density of the medium, the limiting value of the sedimentation coefficient at high ionic strength and at infinite dilution is approximately 5.5 S. This value is presumed to characterize the protein monomer.

The sedimentation of the protein was not altered by the presence of a 100-fold molar excess of either NADH or NAD. Further, protein, which was allowed to react with an excess of mercuribenzoate until no further increase in absorbancy at 250 mp occurred (3), sedimented at the same rate as active enzyme.

Variation of Molecular Weight with Protein Concentration and Temperature—The Archibald "approach to sedimentation equilibrium technique" (18) was used to estimate the variation of weight average molecular weight with protein concentration and temperature. Fig. 3 shows the results of determinations carried out at 5° and at 20°. In this plot the values of protein concentration are those calculated at the meniscus or cell bottom rather than the initial concentration at the start of sedimentation. Since large changes in protein concentration did not take place under the conditions of the experiment, the average values of molecular weight and concentration at the meniscus or cell bottom, calculated from five gradient photographs taken during each run, are plotted in Fig. 3. Although the values obtained at low protein concentrations are less reliable than those obtained at high protein concentrations, extrapolation of the 20° data to a molecular weight of approximately 75,000 at infinite dilution appears reasonable. The results shown in Fig. 3 also indicate...

![Fig. 1](http://www.jbc.org/)  
**Fig. 1.** The dependence of the sedimentation coefficient upon protein concentration in ionic strength 0.2 phosphate buffer, pH 6.65. The protein concentration is the concentration before sedimentation began. Open circles represent results obtained with the 12-mm cell; filled circles represent results obtained with the 30-mm cell.

![Fig. 2](http://www.jbc.org/)  
**Fig. 2.** The dependence of the sedimentation coefficient upon ionic strength. The protein concentration was 2.6 g per liter; pH 6.0 phosphate buffer was present in each solution at an ionic strength of 0.2. The pH was maintained at 6.0 by the addition of NaOH when required.

![Fig. 3](http://www.jbc.org/)  
**Fig. 3.** The variation of the weight average molecular weight with protein concentration and temperature. Filled triangles represent results obtained at 5°, filled circles show results at 20°, and open circles the results of the equilibrium test experiment conducted at 20° (see text). The lines were calculated with the equilibrium constants presented in Table II for an equilibrium between monomers, dimers, and trimers.
that the molecular weight approached at higher protein concentrations is of the order of 150,000.

Rao and Kegeles (19) have emphasized in their study of the polymerization of chymotrypsin that it must be demonstrated that a system is at equilibrium before the mass law can be applied. To establish whether the lactic dehydrogenase system is a reversible equilibrium, two experiments were performed with rotational rates and times such that the concentrations at the meniscus and cell bottom underwent large changes. If the system were readily reversible, it would be expected that the concentrations of monomer and polymer would be readjusted to equilibrium values as polymer is removed from the meniscus and concentrated at the cell bottom under the influence of the centrifugal field. The results of these experiments, shown by open circles in Fig. 3, indicate that the same weight average molecular weights are obtained when the differences in concentration between meniscus and cell bottom are approximately 2 g per liter as when the concentration differences are of the order of 0.3 g per liter.

It is also apparent from Fig. 3 that the weight average molecular weight is higher at 5° than at 20° at the same protein concentration. A single experiment, carried out under conditions such that the concentration difference between meniscus and cell bottom was 3 g per liter, indicated that the weight average molecular weight at the meniscus was lower and that at the cell bottom higher than the values shown on the curve of 5° data in Fig. 3. This result is taken to indicate that the rates of association and dissociation of protein particles are sufficiently slow at 5° that equilibrium conditions did not prevail in this experiment.

**Concentration and Temperature Dependence of Diffusion Coefficient**—Diffusion coefficients, measured at 1° and at 20°, are shown in Fig. 4. The small concentration dependence of diffusion coefficients at 1° might indicate that the protein exists in only one state of aggregation throughout the concentration range studied at this temperature. However, Rayleigh fringe deviation graphs, constructed by the method of Longsworth (20) and shown in Fig. 5, are parabolic in shape. Since these graphs should be straight lines for a single pure solute, this result is taken to indicate the presence of protein species whose diffusion coefficients differ from that of the principal component (21).

At 20° the concentration dependence of the diffusion coefficient is more striking and resembles that shown by insulin (22) and chymotrypsin (23). Rectilinear extrapolation from the points obtained at concentrations of less than 4 g per liter yields a value for the diffusion coefficient at infinite dilution of \(7.39 \times 10^{-7}\) cm² per sec. This value is presumed to be that of the protein monomer.

**Diffusion Coefficients Estimated from Sedimentation Velocity Experiments**—Baldwin’s method of estimating diffusion coefficients from sedimentation diagrams (9), although not derived from systems which exhibit pronounced dependence of the diffusion coefficient upon concentration, has been applied by Ellenberger et al. (24) to sedimentation diagrams of myosin and has been found to yield results that agree well with those obtained in free diffusion experiments. It was of interest to determine whether the Baldwin method would yield diffusion coefficients for the lactic dehydrogenase system which are consistent with those obtained in free diffusion measurements and also to determine whether this treatment of the data would reveal heterogeneity which is not apparent in the sedimentation diagrams themselves.

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**Fig. 4.** The dependence of the diffusion coefficient upon protein concentration and temperature. Open circles represent results obtained at 1.0°, filled circles, results at 20.0°. Filled triangles are diffusion coefficients estimated from sedimentation velocity diagrams (see text). The protein concentration here is that of the bulk of the solution. The medium in all cases was phosphate buffer, pH 6.65, ionic strength 0.2.

**Fig. 5.** Rayleigh fringe deviation graphs for lactic dehydrogenase diffusing at 1.0°. The coordinates for this plot are defined (20). Filled circles show results obtained with an initial protein concentration of 7.0 g per liter. Filled triangles show results obtained with an initial protein concentration of 2.4 g per liter. The solvent was phosphate buffer, pH 6.65, ionic strength 0.2.
The extrapolated value of $s_0$ from Fig. 1, 7.71 S, was corrected for the viscosity and density of the buffer to obtain $s_0$. The value of the diffusion coefficient calculated in this way is shown by triangles in Fig. 4. The agreement with diffusion coefficients obtained by the free diffusion technique is satisfactory.

Amino Acid Content of Lactic Dehydrogenase—Amino acid analyses were performed on three chromatographically homogeneous preparations of Fraction A by the Analytica Corporation, New York, following the procedures of Moore, Spackman, and Stein (25). Cysteine and cystine were determined as cysteic acid by the method of Schram, Moore, and Bigwood (26) and reported in Table I as cystine. Table 1 shows the mean results of five separate analyses.

The minimal molecular weight of the enzyme was estimated from the values shown in Table I by the procedure outlined by Brand et al. (27). Cystine was chosen as the base of calculation since it is present in small amount, and the analytical error of determination was of the order of 2%. The mean minimal molecular weight calculated is 71,700 ± 700. This value is in fair agreement with that determined from measurements made in the ultracentrifuge.

### DISCUSSION

Analysis of the molecular weight versus concentration curves was made by the methods of Rao and Kegeles (19) and Steiner (28). Both treatments indicated that the association proceeds beyond the dimer stage to the trimer. In Table II are shown the equilibrium constants obtained by Steiner's method.

Klee and Cantoni (29) have recently made use of the Stockmayer theory (30) in their study of the polymerization of methylothinin pheromone. Theoretical distributions of polymeric species were found to be in reasonable accord with those experimentally determined when the functionality of the monomer was assumed to be three. In similar calculations made with lactic dehydrogenase, it was found that, for all possible values of the fraction of functional groups reacted, a monomer functionality of two best fitted the distribution of $n$-mers calculated from data obtained from approach to equilibrium sedimentation experiments. The inference drawn from these calculations is that the association reaction produces a linear polymer (30). It is possible, however, that the linear polymer forms a closed triangle as was proposed for insulin by Oncley et al. (31).

The apparent dissociation of lactic dehydrogenase in solutions of high ionic strength, illustrated in Fig. 2, suggests that association depends at least in part upon electrostatic forces. However, since alterations in pH between pH 6.0 and pH 9.0, the pH range of stability of the protein, have only small effects upon the sedimentation behavior of the protein, the imidazolium group is excluded as one of the charged groups involved in the electrostatic interaction. Similarly, the lack of effect of $p$-chloromercuribenzoate in changing the sedimentation rate of the protein also excluded sulfhydryl groups from playing a role in the association reaction.

If the limiting value of the sedimentation coefficient at high salt concentrations, 5.5 S, is employed together with the diffu-

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**TABLE I**

<table>
<thead>
<tr>
<th>Amino acid</th>
<th>g-residues/g protein</th>
<th>Molecular weight</th>
<th>Residues per 71,000 g</th>
<th>$W_iV_i$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aspartic...</td>
<td>10.77 ± 0.04</td>
<td>70,541</td>
<td>66</td>
<td>6.46</td>
</tr>
<tr>
<td>Threonine...</td>
<td>3.95 ± 0.09</td>
<td>71,680</td>
<td>28</td>
<td>2.77</td>
</tr>
<tr>
<td>Serine.....</td>
<td>6.55 ± 0.28</td>
<td>70,724</td>
<td>54</td>
<td>4.13</td>
</tr>
<tr>
<td>Glutamic...</td>
<td>11.50 ± 0.30</td>
<td>71,864</td>
<td>64</td>
<td>7.59</td>
</tr>
<tr>
<td>Proline....</td>
<td>2.80 ± 0.16</td>
<td>72,848</td>
<td>21</td>
<td>2.13</td>
</tr>
<tr>
<td>Glycine....</td>
<td>4.51 ± 0.22</td>
<td>70,860</td>
<td>56</td>
<td>2.89</td>
</tr>
<tr>
<td>Alanine....</td>
<td>4.06 ± 0.08</td>
<td>71,795</td>
<td>41</td>
<td>3.00</td>
</tr>
<tr>
<td>Cystine....</td>
<td>1.25 ± 0.03</td>
<td>71,133</td>
<td>4</td>
<td>0.76</td>
</tr>
<tr>
<td>Valine.....</td>
<td>9.62 ± 0.29</td>
<td>71,114</td>
<td>69</td>
<td>8.27</td>
</tr>
<tr>
<td>Methionine</td>
<td>3.30 ± 0.11</td>
<td>70,924</td>
<td>18</td>
<td>2.50</td>
</tr>
<tr>
<td>Isoleucine.</td>
<td>6.34 ± 0.38</td>
<td>71,403</td>
<td>40</td>
<td>5.71</td>
</tr>
<tr>
<td>Leucine....</td>
<td>11.61 ± 0.64</td>
<td>71,596</td>
<td>73</td>
<td>10.45</td>
</tr>
<tr>
<td>Tyrosine...</td>
<td>3.30 ± 0.18</td>
<td>72,006</td>
<td>15</td>
<td>2.41</td>
</tr>
<tr>
<td>Phenylal-.</td>
<td>1.99 ± 0.15</td>
<td>73,963</td>
<td>10</td>
<td>1.53</td>
</tr>
<tr>
<td>Lysine.....</td>
<td>8.62 ± 0.23</td>
<td>71,381</td>
<td>48</td>
<td>7.07</td>
</tr>
<tr>
<td>Histidine...</td>
<td>2.44 ± 0.06</td>
<td>73,075</td>
<td>13</td>
<td>1.63</td>
</tr>
<tr>
<td>Arginine...</td>
<td>3.66 ± 0.13</td>
<td>72,554</td>
<td>17</td>
<td>2.56</td>
</tr>
</tbody>
</table>

**Total** | 96.39† | Mean = 71,737 | $\sum W_iV_i = 71.86$‡ |

* The error given here is the mean deviation from the mean.
† Ammonia values were erratic and are not included.
‡ The partial specific volume of the protein is given by (6)

$$
\frac{\sum W_iV_i}{\sum W_i}
$$
sion coefficient obtained at 20°, $7.3 \times 10^{-7}$ cm² per sec, a molecular weight of approximately 72,000 is found. Since this molecular weight agrees well with the minimal molecular weight estimated from the amino acid content of the protein, it is presumed that these values of $S$ and $D$ are those of the monomer. The frictional ratio calculated with these values is 1.04. This low value argues for an almost spherical molecule. The deviation from the value for an anhydrous sphere may be accounted for by assuming an hydration of 0.084 g water per g protein.

**SUMMARY**

1. On the basis of data obtained from velocity ultracentrifugation, approach to equilibrium sedimentation, and diffusion studies, it is concluded that crystalline Fraction A of beef heart lactic dehydrogenase exhibits concentration-dependent association. The molecular weight of the monomer is 72,000. Association in concentrated solutions appears to involve dimers and trimers.

2. The minimal molecular weight, calculated from amino acid analysis, is also 72,000.

3. The diffusion coefficient of the monomer is estimated to be $7.39 \times 10^{-7}$ cm² per sec and the sedimentation coefficient to be approximately 5.5 S. The frictional ratio of the monomer is 1.04.

Acknowledgment—The author gratefully acknowledges the continual encouragement and support given by Dr. George W. Schwert.

**REFERENCES**


2 In a recent communication (22), Appella and Markert have indicated that treatment of beef heart lactic dehydrogenase with 5 m guanidine hydrochloride results in the dissociation of the protein into subunits of molecular weight 35,000.
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