MOLECULAR WEIGHT AND ELECTROPHORESIS OF CRYSTALLINE RIBONUCLEASE

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The present paper describes the results of ultracentrifugation, diffusion, and electrophoresis of crystalline ribonuclease whose methods of preparation and properties have been reported by Kunitz (1). 1

Electrophoresis Studies.—Experiments were carried out from pH 4 to pH 10 in a Tiselius apparatus at 0° using the Longsworth scanning method for the observation of the boundaries (2). 25 to 30 ml. of solutions containing 1 per cent of three times crystallized ribonuclease were made up in appropriate buffers and dialyzed in collodion bags for several days at 5°C. against 2 liters of the corresponding buffer solution. The outside buffer solutions were used to fill the upper compartments of the Tiselius apparatus.

The results have been summarized in Fig. 1 which represents the mobilities of the sharper boundaries as a function of pH. Phosphate buffers were used from pH 6.8 to pH 8.8 and acetate buffer at pH 4.1, the ionic strength being \( \approx 0.055 \). Two experiments were made in borate buffer at pH 8.9 and pH 10.1, ionic strength \( \approx 0.03 \).

The mobilities observed in the two latter cases were considerably displaced. The following values were found: \( u = +1.4 \times 10^{-5} \) at pH 8.9 and \( u = -1.4 \times 10^{-5} \) at pH 10.1. It is apparent from Fig. 1 that ribonuclease has a very high isoelectric point at about pH 7.8.

The electrophoretic patterns indicated the presence of only one moving component. The boundaries showed the phenomenon of reversible boundary spreading to a considerable extent. The boundaries moving towards the cathode were much sharper than the boundaries moving towards the anode. A few patterns have been reproduced in Fig. 2. The boundaries at pH 4.1, 7.8, and 8.9 can be seen in Fig. 2 a, 2 b, and 2 c respectively.

Diffusion Measurements.—Diffusion measurements were carried out at 25° in the apparatus used for the electrophoresis experiments. A 1 per

1 I am greatly indebted to Dr. Kunitz who kindly prepared the solutions of ribonuclease used in these investigations.
cent solution of ribonuclease in 0.5 M (NH₄)₂ SO₄, pH 5.8 was used. This solution had been dialyzed against a large volume of 0.5 M (NH₄)₂SO₄ which was used to fill the upper compartments of the cell into which the ribonuclease was diffusing. The results can be seen in Fig. 3 which represents the appearance of the boundary 53, 193, 373, 663, 1337, and 1842 minutes after its formation. The following formula was used for the calculation of the diffusion constant:

\[
D = \frac{S^3}{4\pi H^2 \text{max}}.
\]

where \(D\) is the diffusion constant in cm²/sec., \(S\) the diffusion area in cm², \(t\) time in seconds and \(H_{\text{max}}\) the maximum height of the curve in cm. (3). Since the magnification factor of the camera was unity, and since the terms \(S\) and \(H\) are both squared, the absolute magnitude of the deflection of the rays depending on the geometry of the apparatus has no importance. The only assumption is that the refractive index varies linearly with the concentration (within the range 0 to 1 per cent). The diffusion area should stay constant during the entire experiment. As can be seen from Table I, the value found for the areas is reasonably constant.²

From these data the value of the diffusion constant was computed to be \(D^{ex} = 1.36 \times 10^{-6}\) (in 0.5 M (NH₄)₂ SO₄).

The diffusing areas after 53' and 193' were too large. This increase in area might be due partly to spherical aberration of the camera objective which might play a part when the extreme rim of the lens was used in the focusing of the extreme deviated rays.
Ultracentrifuge Studies.—The apparatus used was an air-driven centrifuge of the turret type described by Bauer and Pickels (4). Three methods were used to follow the course of sedimentation: the light absorption method for which a resonance mercury arc lamp served as source of light, the scale method of Lamm, and the "schlieren method" as used by Svensson in electrophoretic studies (5). The speed of the centrifuge was measured by a 631 A Strobotac built by the General Radio Company.

Rate of Sedimentation.—On account of the relatively low molecular weight of this enzyme, long time intervals were needed to obtain a sufficient displacement of the boundary. During that time a considerable amount of diffusion took place, making it difficult to determine accurately the maxima of the displacement curves obtained by the scale or schlieren methods as well as the position of 50 per cent concentration range when the absorption method was used. Only one moving component was observed, and the symmetry of the curves (absorption, schlieren, or "scale" curves) indicated the fair homogeneity of the material. A schlieren diagram of the last experiment reported in Table II can be seen in Fig. 4.

The abscissa represents the length of the cell, the material sedimenting from right to left. The ordinate is the displacement measuring the refraction gradient. The possibility of superimposing on a single plate the patterns obtained during the course of sedimentation is a distinct advantage of the method since it permits an easier computation of the rate of sedimentation. The interval between the first and second, as well as between the second and third curves was 1 hour, whereas between the third and fourth curves it was 2 hours.
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The microphotometer curves obtained by the absorption method for run 2 of Table II are reproduced in Fig. 5. The time of the first curve

![Fig. 3. Diffusion patterns of ribonuclease](image)

**Table I**

<table>
<thead>
<tr>
<th>Time, min.</th>
<th>373</th>
<th>653</th>
<th>1337</th>
<th>1842</th>
</tr>
</thead>
<tbody>
<tr>
<td>Area</td>
<td>2.23</td>
<td>2.23</td>
<td>2.22</td>
<td>2.22</td>
</tr>
<tr>
<td>Maximum height</td>
<td>3.40</td>
<td>2.65</td>
<td>1.89</td>
<td>1.61</td>
</tr>
</tbody>
</table>

**Table II**

<table>
<thead>
<tr>
<th>$a_2$</th>
<th>$n^{20}$</th>
<th>Thickness cell</th>
<th>Speed</th>
<th>Total time</th>
<th>Mean temperature</th>
<th>Absorption</th>
<th>Scale</th>
<th>Schlieren Absorption</th>
<th>Scale</th>
<th>Schlieren</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.1160</td>
<td>1.527</td>
<td>3</td>
<td>54000</td>
<td>5⅔</td>
<td>26</td>
<td>1.12</td>
<td>1.01</td>
<td>—</td>
<td>2.35</td>
<td>2.12</td>
</tr>
<tr>
<td>1.0366</td>
<td>1.145</td>
<td>3</td>
<td>54000</td>
<td>5</td>
<td>27.2</td>
<td>1.91</td>
<td>1.88</td>
<td>—</td>
<td>2.41</td>
<td>2.37</td>
</tr>
<tr>
<td>1.0366</td>
<td>1.145</td>
<td>6</td>
<td>57600</td>
<td>4</td>
<td>27.8</td>
<td>—</td>
<td>1.79</td>
<td>1.87</td>
<td>—</td>
<td>2.26</td>
</tr>
<tr>
<td>1.0366</td>
<td>1.145</td>
<td>6</td>
<td>57600</td>
<td>4⅔</td>
<td>26.5</td>
<td>—</td>
<td>1.85</td>
<td>1.86</td>
<td>—</td>
<td>2.33</td>
</tr>
</tbody>
</table>

* $n^{20}$ is the viscosity referred to that of water at 25° as unity.

being zero, that of the second, third, fourth, fifth, sixth, and seventh curves is 1, 2, 2⅔, 3, 4, and 5 hours, respectively.
Different preparations of crystalline ribonuclease were used. No difference in the rate of sedimentation could be detected in material recrystallized from ammonium sulfate solution or from dilute alcohol. Solutions of 1 per cent ribonuclease and cells 3 and 6 mm. thick and 15 mm. long were used. The distance from the center of rotation to the middle of the cell was 6.5 cm. The results have been summarized in Table II. The medium was a solution of 0.4 saturated ammonium sulfate in 0.1 m acetate buffer pH 4.1 for the first run and a solution of 0.5 m \((\text{NH}_4)_2\text{SO}_4\) for the others. The constants of sedimentation, \(s_0^2\), included in the last column, have been calculated from the usual correction formula

\[
{s_0^2 = \frac{s_1 \eta_0}{\eta_t} \left(1 - \frac{1}{V_t \rho_t}\right) - \frac{V_0 \rho_0}{\rho_t}}
\]

where \(\eta_0\) is the viscosity and \(\rho_0\) the density of water at 25° and \(\eta_t\) and \(\rho_t\) the same constants for the medium used in the experiment. \(V\) is the specific volume of the protein as determined below. The observed rate of sedimentation of the first experiment reported in Table II is small on
account of high viscosity and high density. However, this value reduced to water agrees as well as can be expected with the values calculated from the other experiments. As can be seen, no systematic deviation could be observed between the three methods of observation of the boundaries.

At the highest speed (57,600 R.P.M.) an increase of temperature of the rotor of \( \sim 1.2^\circ \) per hour was observed when the vacuum was as good as possible; i.e., \( 10^{-5} \) mm. Hg. This increase was mainly due to heat generated by friction of the residual air on the rotor. When computing the rate of sedimentation, the mean temperature between each successive position of the boundary was taken into account.

**Specific Volume.**—The specific volume was calculated from accurate determination of densities of solutions of known concentration. The following determinations were made:

Concentration 4.89 per cent in water, \( d_{25}^4 = 1.0114,* \) specific volume \( V_{25} = 0.709 \)

" 4.89 " " " " \( d_{20}^0 = 1.01276 " " V_{25} = 0.704 \)

" 2.443 " " " " \( d_{25}^0 = 1.0043 " " V_{25} = 0.707 \)

" 2.375 " " " " 0.5 \( \text{m (NH}_4\text{)}_2\text{SO}_4 \), \( d_{25}^0 = 1.0405 \)

* Specific volume \( V_{25} = 0.710 \)

* Densities are based on weights in vacuo.

The value 0.709 was used for calculation. It should be noted that it is a very low value compared to that found for most proteins with the exception of cytochrome.

**Molecular Weight from Rate of Sedimentation.**—Taking \( s_{25} = 1.85 \times 10^{-13} \) (in 0.5 \( \text{m (NH}_4\text{)}_2\text{SO}_4 \)) and \( D = 1.36 \times 10^{-6} \) (in 0.5 \( \text{m (NH}_4\text{)}_2\text{SO}_4 \)), the molecular weight according to the expression \( M = \frac{RTs}{D(1 - Vp)} \) becomes \( M = 12,700 \).

**Molecular Weight from Sedimentation Equilibrium.**—A sedimentation equilibrium run was made in 0.5 \( \text{m (NH}_4\text{)}_2\text{SO}_4 \) solution in a cell 6 mm. thick and 15 mm. long. The speed was 14,400 R.P.M. and the temperature 21.8\(^\circ\). The scale method was used for the determination of the distribution of the refraction gradients with a scale distance of 10 cm. Total time of centrifugation was 66 hours.

Fig. 6 summarizes the results. The curve represents the scale displacement as a function of the distance from the center of rotation from 6.466 cm. to 7.047 cm.\(^3\) Open circles represent measurements after 59\(\frac{1}{2}\) hours;

\(^3\) The difference 7.047 - 6.466 = 0.581 corresponds to 6 divisions of the diagram because the latter are a measure of the comparator readings which have to be reduced to cell distances by the factor 0.968, hence \( \frac{0.581}{0.968} = 0.6 \).
solid triangles, measurements after 66 hours. No systematic differences can be observed between the two sets of values and it can be concluded that equilibrium had been reached.

![Graph](image)

Fig. 6. Sedimentation equilibrium curve by the scale method

The following formula was used for the calculation of molecular weight:

\[ M = \frac{2RT \ln \left( \frac{Z_a x_a}{Z_b x_b} \right)}{(1 - \nu) (x_a^2 - x_b^2)} \]

where \( Z_a \) is the scale displacement at distance \( x \) from the center of rotation. From four pairs of values of \( Z_a \) and corresponding \( x \) read on the curves, the following values for \( M \) were obtained: 12,000, 12,900, 13,400,
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13,700, with a mean value $M = 13,000$ in good agreement with the value obtained from rate of sedimentation and diffusion data. Kunitz (1) found $M = 15,000 \pm 1000$ from osmotic pressure measurements.

There was observed a regular shift in the molecular weight depending on which pair of values was chosen for the calculation. Values corresponding to positions near the bottom of the cell gave higher values than those near the meniscus.

This low value found for the molecular weight of a protein molecule shows how much caution should be exerted before accepting the idea of a universal protein building-stone of a weight of 17,600 (6).

Dissymmetry Factor.—Since the molecular weight has been determined from parameters which do not include the assumption of Stokes' law (equilibrium measurements or sedimentation plus diffusion measurements) it is possible to calculate the dissymmetry factor $f/f_0$ from the formula

$$
\frac{f}{f_0} = \frac{RT}{6\pi\eta ND} \left[ \frac{4\pi ND(1 - V\rho)}{3VRT_s} \right]^{\frac{1}{2}}
$$

where all symbols have their usual significance, $\eta$ is the viscosity in poises of the medium (not the solution) and $N$ the Avogadro number. Introducing the numerical values, one finds $\frac{f}{f_0} = 1.04$. This low value shows the high degree of symmetry of the shape of the molecule of ribonuclease, the most symmetric of all proteins investigated. With the aid of the formula of Herzog, Illig, and Kudar and of Perrin, the ratio of the two main axes of the molecules is calculated to be about 2.

SUMMARY

Electrophoretic studies on purified crystalline ribonuclease showed the absence of any impurities differing in mobility from the bulk of material.

The isoelectric point of ribonuclease was found by electrophoresis to be at about pH 7.8.

Ultracentrifuge studies indicated fair homogeneity of ribonuclease in solution. Only one moving component has been observed.

The molecular weight of ribonuclease was found to be 12,700 from rate of sedimentation ($s^{20} = 1.85 \times 10^{-13}$ in 0.5 M (NH₄)₂SO₄) and diffusion measurement ($D = 1.36 \times 10^{-4}$ in 0.5 M (NH₄)₂SO₄), in good agreement with the average value of 13,000 found from equilibrium measurements. This low value for the molecular weight of a protein would seem to discredit the value 17,600 as representing a universal unit weight for proteins in general.
The specific volume was found to be 0.709.

The dissymmetry factor $\frac{f}{f_0}$ was found equal to 1.04.

REFERENCES