

## Determination of Molecular Weights and Diffusion Coefficients in the Ultracentrifuge

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A simple method of using the boundary condition at the meniscus in the sector shaped centrifuge cell for the determination of the ratio  $S/D$  or  $M$  is described. Some pure proteins are studied and the results compared to other data, especially analytical ones. The diffusion coefficients are also computed separately. The homogeneity of the sample is investigated by following the time dependence of  $M$  at the meniscus. Information about inhomogeneous samples is thus gained, and reversible dimerizations or polymerizations can be studied.

The possibility of obtaining a value of the ratio of the sedimentation coefficient and the diffusion coefficient from the boundary condition at the meniscus in the centrifuge cell was demonstrated by Archibald<sup>1</sup>. Its value as a purity test has been emphasized by Pedersen<sup>2</sup> and the importance of simplifying and standardizing the method has been discussed<sup>2</sup>. The method seems to have been experimentally applied only in a few cases, first by Li *et al.*<sup>3</sup> and by Porath<sup>4</sup> using an oil turbin centrifuge, and more recently by Brown *et al.*<sup>5</sup> and by Klainer and Kegeles<sup>6,7</sup> using an electrically driven Spinco centrifuge. In these cases the methods of evaluation appears to be rather involved and time consuming. In the present work Archibald's method has been applied in a fashion rather similar to that used by Klainer and Kegeles, but the mode of evaluation has been simplified considerably.

### TECHNICAL

A Spinco analytical ultracentrifuge, model E, was used. The diaphragm of the schlieren optics had been replaced by a phaseplate<sup>8,9</sup>, and a Wratten light filter, 77A, was used in all the experiments, where it was possible to work with the green monochromatic light of 546  $m\mu$  wavelength. A 12 mm synthetic boundary cell with a rubber valve was employed throughout the work. An automatic temperature control, also supplied by the centrifuge manufacturer, was an important additional facility, since the sedimentation and diffusion coefficients depend differently on the temperature and since any convection in the cell would have decreased the accuracy of the method. All the experiments were conducted at temperatures between 18 and 23°C. The surface measurements were made by means of a mechanical planimeter.

DETERMINATION OF THE RATIO  $S/D$  AND THE MOLECULAR WEIGHT

Let us consider a sector shaped cell in a rotor revolving at the angular velocity  $\omega$ . The amount of sedimenting and diffusing solute transported per second through a concentric cylindrical surface in the cell at a distance  $r$  from the axes of rotation is  $\Phi(r)$ .

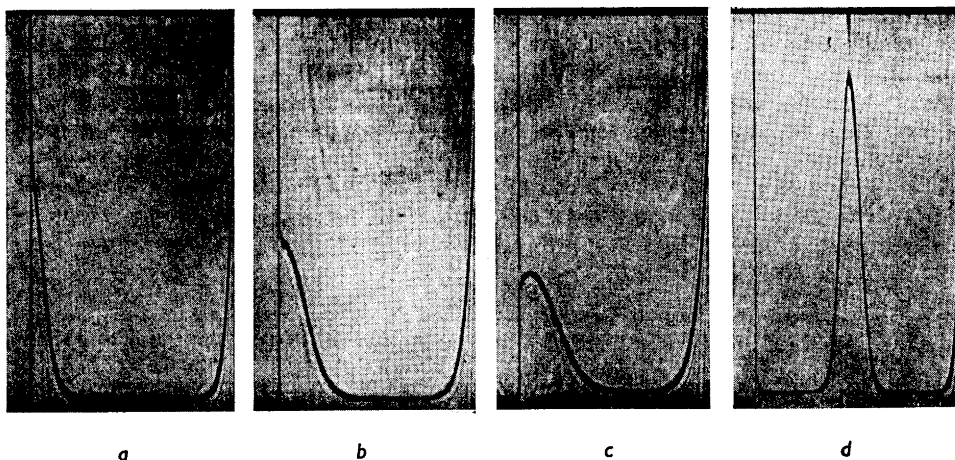
$$\Phi(r) = \Theta rh \left( \omega^2 rcS - D \frac{\partial c}{\partial r} \right) = D \omega^2 rc \Theta h \left( \frac{S}{D} - \frac{1}{\omega^2 rc} \cdot \frac{\partial c}{\partial r} \right) \quad (1)$$

Here  $\Theta$  denotes the sector angle and  $h$  the cell height;  $c$  is the concentration,  $S$  the sedimentation coefficient and  $D$  the diffusion coefficient of the solute. At equilibrium  $\Phi(r) = 0$  and

$$\frac{S}{D} = \frac{1}{\omega^2 rc} \cdot \frac{\partial c}{\partial r} \quad (2)$$

at every distance  $r$  in the cell. The same condition (2) is valid during the whole experiment at the meniscus and at the bottom of the cell as both are impervious to the substance. The ratio  $\frac{\partial c}{\partial r} / c$ , between the concentration gradient and the concentration, at these boundaries the whole time adjusts itself to fulfill eqn. (2). How this boundary condition is utilized for the determination of the quantity  $S/D$  will be made clear from the description of an actual experiment.

A solution of the substance under investigation is prepared in a suitable buffer and dialyzed against the same buffer for at least 12 h. After the dialysis some of the buffer is saved. In experiment I a centrifuge run is made at



*Fig. 1.* Some of the photographs taken in the experiments on the old yellow enzyme. Experiment I a) 3 700 s, b) 11 400 s, and c) 19 050 s. Experiment II d) 3 700 s after that 12 590 rpm was attained.

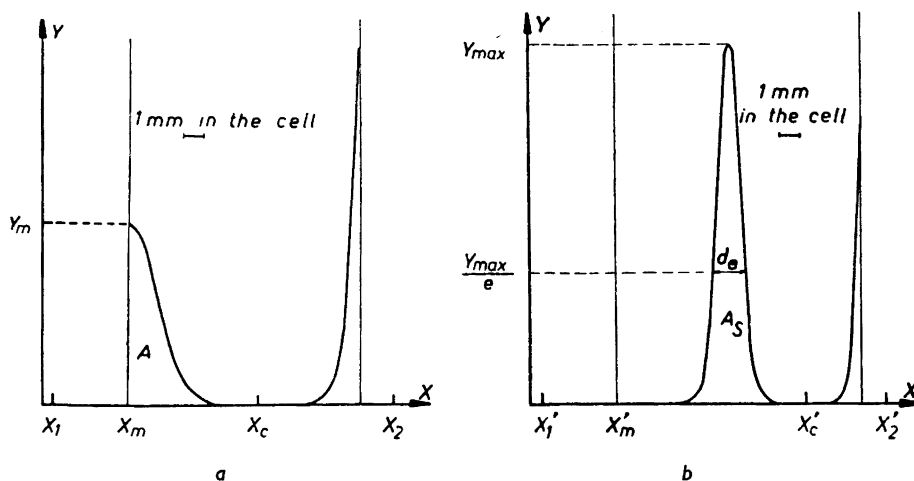


Fig. 2. Reproductions of drawings, with inlaid coordinate axes, made from photographs corresponding to a) Fig. 1 b and b) Fig. 1 d. 1 cm on the original drawings corresponds to 1 mm in the cell. For further explanation see text.

a low speed in the 12 mm synthetic boundary cell but without using the layering technique. Fig. 1 a — c shows three of the exposures taken, one in the beginning, one in the middle and one at the end of the experiment. The pictures are placed in a magnifying apparatus and drawings made of the refractive index gradient curves in a convenient scale (1 cm on the paper corresponded to 1 mm in the cell). Fig. 2 a shows such a drawing with inlaid coordinate axes. The  $y$ -coordinate of the refractive index gradient is proportional to the concentration gradient of the substance. At the meniscus we thus have

$$\left(\frac{\partial c}{\partial r}\right)_m = k_1 y_m \quad (3)$$

where the subscript  $m$  refers to the meniscus and  $k_1$  is the constant of proportion. Because of optical distortion near the meniscus the curve has to be extrapolated the last few millimeters on the paper. The most accurate values of  $y_m$  are hence obtained from the exposures in the middle period of the experiment for which the refractive index gradient curves are horizontal or nearly so at the meniscus\*. A relation similar to (3) is also valid at the bottom of the cell, but because of the piling up of material the curves are usually steep there and the  $y$ -coordinate of the intercept at the bottom is difficult to determine with the precision needed. Because of these reasons we have so far only treated the data obtained at the meniscus.

\* Note added in proof: This eliminates to a large extent the influence of the error in the meniscus image, recently discussed by P. J. Cheng, *J. Phys. Chem.* **61** (1957) 695.

It is essential that a region in the middle of the cell remains with a flat baseline and with the concentration varying according to the formula given by Pedersen<sup>10</sup>

$$c = c_0 e^{-2\omega^2 S t} \quad (4)$$

Here  $c$  is the concentration of solute at time  $t$  and  $c_0$  the initial concentration. The concentration at the meniscus at time  $t$  is thus obtained by the equation

$$c_m = c - \int_{r_m}^{r_c} \frac{\partial c}{\partial r} \alpha r \quad (5)$$

or

$$c_m = c_0 e^{-2\omega^2 S t} - k_1 k_2 \int_{x_m}^{x_c} y dx \quad (6)$$

$x_m$  is the  $x$ -coordinate of the meniscus,  $x_c$  that of a point in the part of the cell where eqn. (4) is valid.  $k_2$  is the inverse of the enlargement in the  $x$ -direction.  $y$  corresponds to an exposure taken at time  $t$ . The integral of eqn. (6) is the surface  $A$  between the curve, the meniscus and the  $x$ -axes of Fig. 2a:

$$A = \int_{x_m}^{x_c} y dx \quad (7)$$

Insertion of expressions (3), (6) and (7) in eqn. (2) gives

$$\frac{S}{D} = \frac{k_1 y_m}{\omega^2 r_m (c_0 e^{-2\omega^2 S t} - k_1 k_2 A)} \quad (8)$$

The first term in the denominator is determined in a separate experiment, II, a principle that was also used by Klainer and Kegeles<sup>6</sup>. The same synthetic boundary cell is used as in the previous experiment, and the equilibrated buffer is layered over the sample at a speed of 5 000—7 000 rpm. In all other details, such as acceleration time, rotational speed, temperature, phaseplate angle, exposure times and, if possible, exposure intervals, this experiment is an exact duplicate of the previous one. Fig. 1 d shows a typical exposure and Fig. 2 b the corresponding enlarged diagram. This time it is essential that there are two flat regions of the curve, one between the meniscus and the peak where the concentration is zero and one between the peak and the bottom with the concentration varying according to eqn. (4). In analogy with the previous discussion we now get the relation

$$c = c_0 e^{-2\omega^2 S t} = k_1 k_2 \int_{x'_m}^{x'_c} y dx = k_1 k_2 A_s \quad (9)$$

where  $A_s$  is the surface under the curve in Fig. 2 b. If expression (9) is inserted in (8) we obtain the simple relation

$$\frac{S}{D} = \frac{y_m}{\omega^2 r_m k_2 (A_s - A)} \quad (10)$$

$y_m$  is the distance from the baseline to the intersection of the curve and the meniscus of the enlarged drawing of an exposure of experiment I and can be determined to better than  $\pm 1\%$ .  $A$  is the surface between curve, baseline and meniscus of the same exposure,  $A_s$  the surface between the curve and the baseline in the corresponding exposure of experiment II. These surfaces are easily determined with a planimeter and the error in their difference is usually smaller than  $\pm 1\%$ . The factor  $k_2$  is suitably adjusted to  $0.1000 \pm 0.0002$ . The radius of the meniscus,  $r_m$ , is determined from the position of  $x_m$  relative to the two indices  $x_1$  and  $x_2$  on the drawing, the factor  $k_2$ , and the radius of the center of the cell. The latter is known to be better than  $\pm 0.1\%$  at the moderate speeds used; at higher speeds, say exceeding 40 000 rpm, the accuracy is somewhat less due to elastic distortion of the rotor. This figure might be taken as an estimate of the error in  $r_m$ .  $\omega$  is determined from the selected rotor speed which is kept constant to better than  $\pm 0.5\%$  over any period of time. In  $\omega^2$ , which enters in the expression (10), this means an error of  $\pm 1\%$ . The temperature is kept constant within  $\pm 0.1^\circ\text{C}$ . As the viscosity effects  $S$  and  $D$  identically, the temperature error enters in  $S/D$  only because  $D$  is proportional to the absolute temperature. The error due to the temperature is thus about  $\pm 0.04\%$ . All these contributions added together give a maximum error in a single determination of  $S/D$  of  $3.4\%$ .

By means of a blank experiment with the buffer it is possible to evaluate a correction for gradients due to sedimenting salt and for curvature of the baseline. This correction was found to be negligible for rotor speeds below 20 000 rpm. Whenever such a correction is needed the maximum error is greater than  $3.4\%$ . Other possible optical distortions or imperfections have not been considered.

Klainer and Kegeles<sup>6</sup> did not use the same cell in their experiments corresponding to I and II. This technique has been tried but found to be inferior since the two cells usually have slightly different depths and since the properties of the quartz windows might be slightly different.

The simple eqn. (10) can be used as long as the time schedules of experiments I and II are identical. If  $A_s$  can be determined only in an early period of experiment II, because of the diffusion which after some time violates the flat regions at both sides of the peak (Fig. 2 b),  $A_s$  in (10) has to be replaced by  $A_{s_0} \cdot e^{-2\omega^2 St_I} \approx A_{s_0}(1 - 2\omega^2 St_I)$ , where the time  $t_I$  refers to experiment I.  $A_{s_0}$  is determined from experiment II as a mean of the values  $A_s \cdot e^{2\omega^2 St_{II}} \approx A_s(1 + 2\omega^2 St_{II})$ . This means that a knowledge of  $S$  under the actual experimental conditions is necessary. If it is not known beforehand, it may be determined accurately enough at the end of experiment II. The rotor is then accelerated to a higher speed, and a few exposures are taken from which  $S$  can be calculated in the usual way. Because of the adiabatic cooling of the rotor this  $S$ -value corresponds to a slightly lower temperature than the original

and a proper correction has to be made before it is used in the calculation of  $S/D$ . If necessary a separate experiment has to be made in the synthetic boundary cell for the determination of  $S$ .

If the sedimentation constant is not known from the beginning and the wrong speed of rotation is chosen initially in experiment I, the rotor might be stopped, the cell taken out and shaken a little to make the solution homogeneous again. The experiment may then be started again and a more suitable speed of rotation selected. The two experiments I and II are usually performed within 6—8 h. All the calculations are made in 3 or 4 h by one person. If the plates instead were evaluated in a microcomparator and numerical integrations were made, the calculations were much more timeconsuming without much gain in accuracy.

The value of  $S/D$  thus obtained is used for the calculation of the molecular weight by insertion in the well known equation

$$M = \frac{S}{D} \cdot \frac{RT}{1 - V\rho} \quad (11)$$

Here  $R$  is the gas constant and equal to  $8.313 \times 10^7$  erg.mole<sup>-1</sup>. degree<sup>-1</sup>,  $T$  is the absolute temperature at which the experiment was performed,  $\rho$  is the density of the buffer at that temperature and  $V$  is the partial specific volume of the solute at the same temperature.

The behaviour of a sample with several components of different  $S/D$  is easy to predict if it is permitted to superpose the systems. Each component,  $n$ , alone would give

$$\frac{S_n}{D_n} = K \frac{y_{mn}}{A_{sn} - A_n}$$

The observed ratio is

$$\frac{S}{D} = K \frac{y_m}{A_s - A}$$

$$\text{where } y_m = \Sigma y_{mn} \text{ and } A_s - A = \Sigma (A_{sn} - A_n)$$

Hence we obtain

$$\frac{S}{D} = \frac{\Sigma \frac{S_n}{D_n} (A_{sn} - A_n)}{\Sigma (A_{sn} - A_n)} \quad (12)$$

At time zero all the  $A_n$  are zero. If the specific refractive index increments are equal for all the components the value of  $S/D$  extrapolated to zero time is the mean of the different  $S_n/D_n$  weighted with their corresponding relative concentrations (the weight-average value). As the experiment goes on all the  $A_n$  values increase. Those corresponding to the higher  $S_n/D_n$  increase faster than the others and hence the contribution of these  $S_n/D_n$  in  $S/D$  will be relatively smaller and the observed  $S/D$  will decrease towards the lowest value  $S_n/D_n$ . The speed of this decrease depends on the ratio between the different values of  $S_n/D_n$  and on the rotor velocity. Only a solute that is

homogeneous with respect to the ratio  $S/D$  and for which this ratio is independent of the concentration will exhibit a value of  $S/D$  that does not change with time during the experiment.

A sample where the solute molecules are in a monomer-dimer equilibrium will also show a decrease with time in the ratio  $S/D$  or the proportional  $M$ . This decrease is due to the dilution of the solute close to the meniscus which favours the monomer.

#### DETERMINATION OF DIFFUSION COEFFICIENTS

The diffusion coefficients were determined from the exposures of the type II experiments in which a boundary between solution and solvent was formed in the centre of the cell. The data were evaluated in two different ways which both are easily derived from the solution of the differential equation of diffusion.

In the "maximum ordinate — area" method the following relationship is used:

$$\frac{A_s^2}{y_{\max}^2} = 4 k_2^2 \pi D (t - t_0) \quad (13)$$

where  $A_s$  is the area between the gradient curve and the baseline at time  $t$ , measured from the instance when the synthetic boundary was formed.  $y_{\max}$  is the maximum height of that curve (Fig. 2 b),  $k_2$  is the magnification along the baseline and  $D$  is the diffusion coefficient.

According to the "maximum ordinate" method the relation

$$d_e^2 = 16 k_2^2 D (t - t_0) \quad (14)$$

is used. Here  $d_e$  is the distance along the baseline between the points on the gradient curve having  $y = y_{\max}/e$ .

$D$  is obtained from the slope of the line obtained by plotting either  $A_s^2/y_{\max}^2$  or  $d_e^2$  versus  $t$ . Agreement between the two methods in combination with slightly negative values of  $t_0$  has been taken as evidence of a successful experiment. Positive values of  $t_0$  indicate that the boundary formation had been disturbed and made the results less reliable.

The right hand sides of eqns. (13) and (14) also should contain the factor  $[1 + \frac{1}{2} \omega^2 S(t - t_0)]$  corresponding to the correction factor given by Lamm<sup>11</sup>. The correction term, however, never caused a deviation of more than 0.5 % from the straight line.

#### MATERIALS

The old yellow enzyme, myoglobin I and apomyoglobin I were prepared by Theorell and Åkeson as they have recently described<sup>12,13</sup>. The rhodanese was prepared by Sörbo<sup>14</sup>, the myeloperoxidase by Agner<sup>15</sup>, and the liver alcohol dehydrogenase by Dalziel<sup>16</sup>. The growth hormone samples have been described elsewhere<sup>17</sup>. The bovine serum albumin was obtained from Armour Laboratories, lot No. J 10603.

The cytochrome c was prepared in the crystalline form from beef heart by Paléus and Theorell, and two different samples were used. One sample was prepared according to Hagihara *et al.*<sup>18</sup>, had an iron content of 0.42 %, and was used for the centrifuge experi-

ments. The other sample was prepared according to Paléus and Theorell<sup>19</sup> and had an iron content of 0.46 %. It was used for the determination of the partial specific volume.

The pituitary growth hormones were dissolved in a 0.05 M borate buffer of pH 9.9. In all other cases a 0.05 M phosphate buffer of pH 7.0 was used. All the solutions contained 1 % NaCl.

In all cases the partial specific volume was assumed to depend on temperature in the same way as that of haemoglobin<sup>10</sup>.

## RESULTS AND DISCUSSION

A few proteins considered to be essentially pure and homogeneous have been investigated. The substances were chosen because of availability and because of possibility to compare with other data, especially analytical values. The main results have been collected in Table 1 and Fig. 3 a.

In spite of the fact that *bovine serum albumin* is known not to be completely homogeneous<sup>20</sup>, it was subjected to several experiments because it has been so thoroughly investigated by many investigators and by means of many methods. The sedimentation coefficient of the sample was determined at a few concentrations and it was found to depend on the concentration according to the formula

$$S_{20}^{\circ} = 4.44 (1 - 0.0060 \cdot a) S \quad (15)$$

where  $a$  is the mean concentration in mg/ml. This concentration dependence agrees well with the data reported by Schulman<sup>21</sup>. A small fraction amounting to less than 5 % could be seen to move somewhat faster in the centrifuge.

In three experiments at a protein-concentration of 20 mg/ml the calculated  $M$  was found to increase with about 0.18 % in every period of 1 000 sec. No such increase could be identified in one experiment at half that protein

Table 1.

Protein	$V_{sp}$	$S_{20}^{\circ}$ ( $S$ -units)	$D_{20}^{\circ}$ ( $F$ -units)		$M$			
			in a diffusion cell	in the centri- fuge	from $S$ and $D$ (diffu- sion cell)	from $S$ and $D$ (centri- fuge)	from $S/D$ at the meniscus	from analysis
Cytochrome $c^{18,19}$ (beef heart)	0.728	1.71 *	—	11.4	—	13 300	13 300	13 300
Apomyoglobin I <sup>13</sup>	0.743 <sup>28</sup>	1.89	—	10.4	—	17 200	18 800	18 200 <sup>13</sup>
CO-myoglobin I <sup>13</sup>	0.743 <sup>24</sup>	2.01	—	10.3	—	18 500	18 300	18 800 <sup>13</sup>
Rhodanese <sup>14</sup>	0.742 <sup>14</sup>	3.03 * <sup>14</sup>	7.5 * <sup>14</sup>	8.2	38 000	35 600	36 300	—
Serum albumin (bovine)	0.7434 <sup>35</sup>	4.44	5.805 <sup>20</sup>	5.7	72 200	73 500	73 200	—
Alcohol dehydrogenase <sup>16</sup> (liver)	0.750 <sup>27</sup>	5.11 <sup>27</sup>	5.95 * <sup>27</sup>	5.9	83 300 <sup>27</sup>	84 000	84 400	88 000 <sup>27</sup>
Old yellow enzyme <sup>12</sup>	0.753 <sup>12</sup>	5.82	—	5.54	—	102 000	106 000	104 000 <sup>12</sup>
Myeloperoxidase <sup>15</sup>	0.731 <sup>29</sup>	7.93 <sup>29</sup>	4.81 * <sup>29</sup>	4.9	149 000 <sup>29</sup>	145 000	157 000	151 000 <sup>29</sup>

1  $S$  (Svedberg unit) =  $10^{-13}$  s

1  $F$  (Fick unit) =  $10^{-7}$  cm<sup>2</sup> s<sup>-1</sup>

\* indicates that the value has not been extrapolated to infinite dilution. None of the diffusion coefficients determined in the centrifuge has been extrapolated in this way.



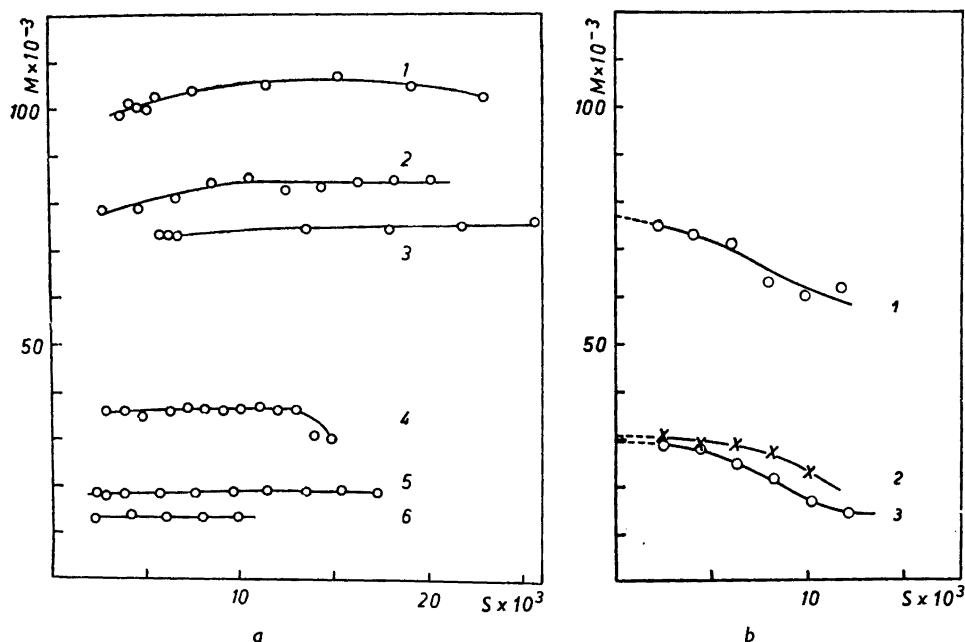


Fig. 3. Time dependence of the molecular weights of different proteins as evaluated from the boundary condition at the meniscus. The time is measured from the instance when the rotor attained equilibrium at the selected speed. a) Curve 1) old yellow enzyme (12 590 rpm); 2) liver alcohol dehydrogenase (12 590 rpm); 3) bovine serum albumin (12 590 rpm); 4) rhodanese (17 980 rpm); 5) apomyoglobin (23 150 rpm) and 6) cytochrome c (23 150 rpm). b) Curve 1) ox pituitary growth hormone (14 290 rpm); 2) human pituitary growth hormone (17 250 rpm) and 3) human pituitary growth hormone (23 150 rpm).

concentration. In the parts of these experiments later than 10 000 sec after the rotor had attained equilibrium speed the following mean molecular weights were obtained: 75 200, 69 900 and 72 700. One experiment at 10 mg/ml gave a value of 75 000. At concentrations of 5 mg/ml or lower the experiments were more difficult to evaluate and the results were no longer consistent.

The *Old yellow enzyme* was investigated because it was uncertain whether the molecule contained one or two prosthetic groups. The new analytical data<sup>12</sup> required a molecular weight of  $n \times 52\,000$ , while earlier studies<sup>22</sup> on a less pure preparation had given a value of 82 000, or 76 000 when corrected for the slightly elevated rotor temperature in the centrifuge used<sup>2</sup>. The partial specific volume of the crystallized enzyme was redetermined<sup>12</sup> and was found to be 0.753 at 25°C as compared with the previous value of 0.731 at 20°C. The difference accounts for an increase in  $M$  of 9 % which would bring it up to 83 000 again. Also the sedimentation coefficient has been redetermined. The concentration dependence was investigated and the following formula was found to be valid:

$$S_{20}^{\circ} = 5.82 (1 - 0.0046 \cdot a) S \quad (16)$$

where  $a$  is the concentration in mg/ml. The previously reported value<sup>22</sup> was 5.76  $S$ , or 5.33  $S$ , when corrected for the rotor temperature. The protein concentration in these experiments was not reported but must have been of the order of 10–20 mg/ml. Correction for this according to the formula (16) yields 5.5–5.7  $S$ , which is only slightly lower than the present value.  $S_{20}^{\circ} = 5.82 S$  instead of 5.33  $S$ , however, adds another 8.5 % to  $M$  and brings it to 90 500.

The molecular weight has now been determined in one experiment at a protein concentration of 18.4 mg/ml. The calculated  $M$  appeared to increase in the first part of the experiment with about 0.45 %/1 000 s and to decrease in the later part having a maximum about 15 000 s after that the full speed had been reached. The maximum of 106 000 is taken as the best value of  $M$ .

The diffusion coefficient has been determined from two experiments with undisturbed synthetic boundary formation, one at 18.4 mg/ml and the other at 7.8 mg/ml. It was found to be 5.54 ( $\pm 0.05$ )  $F$ , which is appreciably lower than the old value<sup>13</sup> of 6.3  $F$ . Together with  $S_{20}^{\circ}$  and  $V$  reported here it gives  $M = 102\ 000$ .

These data definitely prove that there are two flavin mononucleotide molecules bound per molecule of the old yellow enzyme, for which case the  $M$ -value derived from analysis becomes 104 000. The great difference between the value of  $M$  determined now and the old value appears to be an unfortunate combination of errors in all the three quantities  $S$ ,  $D$  and  $V$ .

The purified *myoglobin I*<sup>13</sup> has been investigated both in the form of apomyoglobin and in the form of CO-myoglobin. In both cases a partial specific volume of 0.743 has been used. This value has been determined<sup>24</sup> for ferri Mb and the same can be calculated for apo Mb on the basis of the amino acid analysis<sup>25,26</sup>. The sedimentation coefficient determined earlier<sup>13</sup> in the same centrifuge have been used. At that time the automatic temperature control had not yet been installed and no correction was made for the adiabatic cooling of the rotor. In a set of experiments this cooling has been found to be 0.87°C| when the rotor is accelerated up to 59 780 rpm. A correction of 2.15 % had thus to be added to the previous data. Another small correction of 0.5 % has also been added according to Lamm's formula<sup>11</sup>. The data thus become:

$$S_{20}^{\circ} = 1.89 (1 - 0.00682 \cdot a) S \text{ for apoMb I} \quad (17)$$

and

$$S_{20}^{\circ} = 2.01 (1 - 0.00642 \cdot a) S \text{ for Mb I} \quad (18)$$

The colourless apo Mb I has been investigated in one experiment at a protein concentration of 12.5 mg/ml. Because of the high rotor speed of 23 150 rpm a minor correction had to be explored in a separate run on the buffer. Again  $M$  was found to increase slightly in the first part of the experiment (0.70 %/1 000 s). An asymptotic level of  $M = 18\ 800$  was reached.

A much less reliable experiment was conducted on a less concentrated sample of the carbon monoxide compound of Mb I. This derivative was used because of its low absorption above 600 m $\mu$ , and a red filter was used cutting off below this wavelength. The phaseplate, however, is manufactured for the green mercury line of 546 m $\mu$ , and long exposure times had to be used because

of the low efficiency of the lamp in the red region. Hence the pictures were not very sharp and the calculated molecular weight of 18 300 cannot be considered to be accurate to better than  $\pm 4\%$ .

The diffusion coefficients of 10.4 and 10.3  $F$ , respectively, are, as would be expected, rather smaller than the value of 11.25  $F$ , that was earlier determined by Polsen<sup>23</sup> on an old preparation of Mb, which had an iron content 0.34 % as compared to 0.297 % for Mb I<sup>13</sup>.

The  $M$ -values, as calculated from  $S/D$  or  $S$  and  $D$ , do not agree completely with the data obtained from iron-analysis of Mb I (Table 1). It is obvious that there might be an error in the determination of either  $S_{20}^{\circ}$  or  $D_{20}^{\circ}$  of apo Mb I. It is probable that  $S_{20}^{\circ}$  should be greater than 1.89  $S$ , since the drop from 2.01  $S$  for Mb I appears to be rather big, if not the removal of the haem is accompanied by a rather drastic change of molecular shape.

One experiment on *liver alcohol dehydrogenase* was performed at a protein concentration of 10 mg/ml. The  $M$ -value increased in the early part of the experiment but a level of 84 400 was reached before 10 000 s. This value is in good agreement with the other data discussed elsewhere<sup>27</sup> and referred to in Table 1, but it is considerably higher than the value of 67 500 (corrected for the rotor temperature<sup>2</sup>) that was determined on an earlier and less pure preparation of the enzyme<sup>28</sup>.

*Rhodanese* was investigated at a protein concentration of 10 mg/ml. The  $M$ -value appeared to be practically constant until the decrease at the end due to back diffusion. The sedimentation constant of 2.97  $S$  given by Sörbo<sup>14</sup> has in the table been corrected for the adiabatic cooling of the rotor. The present values of  $M$  are lower than the old one, which is also reflected in the greater diffusion constant obtained now. The absence of an initial increase in the calculated  $M$ -values might indicate the presence of some smaller molecular fragments, which would have caused an increase in  $D$ . The previous diffusion experiments<sup>14</sup> were made, however, under rather different conditions; the protein concentrations were low, the temperature was low and the observations were made over time periods of more than 30 h. The most likely explanation of the low diffusion coefficients obtained seems to be that some denaturation and aggregation occurred in the samples.

The partial specific volume of the *crystallized cytochrome c* was determined in a 10 ml pycnometer at 21.7°C as described elsewhere<sup>29</sup>. The protein concentration of the electrolyte free solution was 18.73 mg/ml. Two experiments gave  $V = 0.728$  (mean of 0.7273 and 0.7282), which is appreciably higher than the old value 0.702—0.707<sup>30,31</sup>. The new value implies that the sterical configuration of the helices in cytochrome c does not need to be as closely packed as was recently suggested<sup>26</sup>.

The molecular weight was determined on cytochrome c in its reduced form at a concentration of 10 mg/ml, and the red filter cutting off below 600  $m\mu$  had to be used. The reduction was achieved by adding a small amount of ascorbic acid to the sample in the cellophane bag before starting the dialysis. This was made against a previously degassed buffer in a closed vessel filled with nitrogen. The calculated  $M$  did not change with time and it is in perfect agreement with the value obtained from the iron content.

The diffusion coefficient comes out rather close to the value  $11,1 F$  determined by Theorell<sup>31</sup> on an old preparation of cytochrome *c*.

A few experiments have been performed with the crystallized *myeloperoxidase*. In spite of its green colour, the light absorption at  $546 m\mu$  made it impossible to work with concentrations higher than 5 mg/ml. In combination with the high sedimentation velocity this circumstance made the results rather unreliable. The mean  $M$ -value of three experiments, however, came out as 157 000, in rough agreement with the other data listed for this enzyme in Table 1<sup>29</sup>.

The slow increase of  $M$  with time in the early parts of the experiments, that has been observed for several of the proteins now discussed, might to a large extent have been caused by the concentration dependence of  $S$ . From eqns. (15)—(18) and the speed of dilution at the meniscus this effect can be calculated for serum albumin, old yellow enzyme and myoglobin. It amounts to about 0.3 %/1 000 s in the early part of the experiments and decreases to less than 0.1 %/1 000 s after 15 000 s. Part of the increase might, however, be an artefact due to difficulties in making the extrapolation to the meniscus from the first few photographs.

Some experiments have been made at pH 9.9 on *growth hormones* obtained from human and ox pituitaries, and the main results have already been reported<sup>17</sup>. The time dependence of the calculated  $M$ -values is seen from Fig. 3 b. The  $M$ -values decrease from the very beginning of the experiment instead of increasing slightly or remaining practically constant as observed for substances that are homogeneous. It has been shown in eqn. (13) that such a decrease of  $M$  is due to inhomogeneity with respect to  $S/D$  or the proportional  $M$ . In sedimentation studies at pH 9.9 the hormones seem to be quite homogeneous, but the sedimentation coefficients increase with increasing concentration<sup>17,32</sup>. These facts show that the hormone is in a state of equilibrium between molecules of different sizes, presumably a monomer-dimer equilibrium<sup>17</sup>. The  $M$ -values extrapolated to the time when the rotor obtained its equilibrium speed can thus be taken as a weight-average  $M$  of the sample, valid for the concentration used:  $M_{\text{mean}} = 30\ 000$  for human hormone at 5—10 mg/ml and  $M_{\text{mean}} = 75\ 000$ —80 000 for ox hormone at 10 mg/ml. The molecular weights extrapolated in the later part of the experiment would correspond to the monomeric molecules (or a mean of the smallest molecules):  $M_{\text{monomer}}$  is hence approximately 15 000 and 50 000, respectively.

Similar more or less drastic decreases of  $M$  have been observed for impure protein preparations or for preparations known to be very inhomogeneous, e.g. cheese extracts<sup>33</sup> and stomach juice<sup>34</sup>. Also in these cases a mean value of  $M$  for the whole sample may be estimated as well as a mean  $M$  for the smaller molecules. If the components can be resolved by sedimentation analysis it is also possible to estimate  $M$  of the larger molecules.

## CONCLUSIONS

The determination of molecular weights of homogeneous substances from the boundary condition at the meniscus appears to be quite accurate. The data generally agree within 2—3 % with those obtained from separate determina-

tions of sedimentation and diffusion coefficients or from analysis. As in case of sedimentation equilibrium the method is based on the balance between sedimentation and diffusion of the molecules, but the time requirement is much smaller. The diffusion coefficients are determined separately and appear to be reliable within a few percent. Only a small amount of material is needed, 15 mg is usually sufficient, and the method described here is simple and rapid to evaluate.

It is possible to investigate a rather wide range of molecular sizes. With a low speed attachment that makes it possible to run the centrifuge at a few thousands rpm molecular weights up to several hundred thousands might be determined. At the highest speed, 59 780 rpm, molecular weights as low as a few hundreds can be determined, as has been shown by Klainer and Kegeles <sup>6</sup>.

By studying the time dependence of the ratio  $S/D$  or the proportional  $M$  the homogeneity of the sample might be explored. A constant or slightly increasing value indicates that the substance is homogeneous. A value that immediately starts to decrease shows that the sample is not monodisperse. Comparison with sedimentation analysis reveals the eventual presence of a reversible dimerization or polymerization.

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