ACTA CHEMICA SCANDINAVICA 14 (1960) 1819–1827

Crystalline Leghemoglobin

II. The Molecular Weights and Shapes of the two Main Components

NILS ELLFOLK*

Medicinska Nobelinstitutet, Biokemiska avdelningen, Stockholm, Sweden

The molecular weights of the two main components of leghemoglobin have been determined, on the basis of sedimentation and diffusion studies, to be 16 800 for the electrophoretically faster component, and 15 400 for the slower one. The sedimentation constants, extrapolated to infinite dilution, were 2.01 S for the electrophoretically faster component, and 2.04 S for the slower one, whereas the diffusion constant was 11.18 F for the faster component and 12.35 F for the slower one. The partial specific volume of the unresolved material was 0.740 ml/g. The molar frictional ratio was 1.118 for the electrophoretically faster component and 1.043 for the slower one. The results of this study show that the two main components of leghemoglobin differ slightly from each other in molecular size and shape.

Studies on the molecular weight of leghemoglobin (Lhb) have not been made until quite recently. In 1952 Ellfolk and Virtanen [1], on the basis of their osmotic and sedimentation and diffusion studies, reported that the unresolved chromoprotein had an average molecular weight of 20 000. Difficulties in resolving the chromoprotein into its components in good yield limited the studies of the individual components. The electrophoretically faster component was found to have an iron content of 0.34 % [1]. This caused the authors to assume that the electrophoretically faster component represented pure Lhb and that the electrophoretically slower component was an inhomogeneous protein. After the recent development of a procedure for preparing the two main components in good yield in a crystalline state [2], it was decided to reinvestigate the molecular sizes and shapes of the two individual main components.

EXPERIMENTAL

Material. Pure fractions of the electrophoretically faster and slower components of Lhb were prepared by a chromatographic procedure that was published earlier [2]. For the sedimentation and diffusion measurements, the protein solutions were dialyzed for

* Permanent address: Research laboratories of the State Alcohol Monopoly, Helsinki, Finland

48 h at + 3°C against a 0.283 μ buffer of pH 7 that contained 0.05 M sodium phosphate and 0.171 M sodium chloride.

Sedimentation. The sedimentation analyses were made in a Spinco analytical ultracentrifuge model E. All analyses were performed at 59 780 r.p.m. Because of the low molecular weight of the two chromoproteins studied, only the synthetic boundary cell was used. Inasmuch as the distances moved by the boundaries were rather small, ten photographs of the sedimenting boundary position were obtained in each experiment. The sedimentation coefficients were evaluated from enlarged images of the photographs projected onto millimeter graph paper. By inserting a scale into the rotor in place of the cell, a reference plate, which can be used to combine the magnification of the camera and enlarger into one, is obtained. The sedimentation constants were reduced to pure water at 20°C by applying the usual corrections for the density and viscosity of the medium [3]. The results are given in Svedberg units (1 S = 10^{-13} cm sec^{-1} $dyne^{-1}$).

Diffusion. The diffusion experiments were conducted in a Spinco diffusion and electrophoresis instrument at a temperature of 0.6°C. Boundary sharpening was obtained by withdrawing a small amount of solution from the vicinity of the boundary with a long hypodermic needle, and then allowing the diffusion to occur. By repeating this procedure, a sharp boundary was obtained. The optics were adjusted for studying the Rayleigh-Calvet-Philpot interferogram at 644 mμ. The number of fringes, and the position of the maxima and minima were measured visually by means of the optical system of a Hilger densitometer. The cell magnification was 1.0105. The diffusion constants were evaluated according to the "height-area" method described by Svensson [4].

$$D_A = \frac{(n_1 - n_2)^2}{4\,\pi\,t\,n'^2_{max\,(x)}}$$

where n_1 and n_2 are the refractive indices of the two solutions, t is the time, and $n'_{max(x)}$ is the maximum value for the derivative of the refractive index function with respect to position in the cell. Some exposures were evaluated according to the "moment" method using the equation by Svensson [5] as slightly modified by Ehrenberg and Agner [6]

$$D_m = \frac{\int_{n_o}^{n_c} x^2\,dn}{2(t-t_o)\,(n_c-n_o)} = \frac{a}{t-t_o}$$

where n denotes the refractive index increment measured at a particular fringe number, x is the fringe coordinate measured from the center of the boundary, and t is the length of time of diffusion. The diffusion constants were reduced to pure water at 20°C by applying corrections for the density and viscosity of the medium. The results are given in Fick's units (F = 10^{-7} cm^2 sec^{-1}).

Partial specific volume. The partial specific volume was determined for solutions of Lhb that were extensively dialyzed against distilled water. The densities of the solutions and of the solvent were measured at 24.45°C using a pycnometer of the Sprengel-Ostwald type having a capacity of about 10 ml.

RESULTS

Sedimentation coefficients. The two main components of Lhb, as far as could be judged from the photographs obtained in the ultracentifuge experiments, appeared to be homogeneous throughout the concentration ratios studied (Fig. 1). All the sedimentation patterns consisted of single, symmetrical peaks showing no indications of more than one component. The sedimentation constants for the two components were concentration dependent, increasing in value with dilution. Values of the sedimentation constants at different

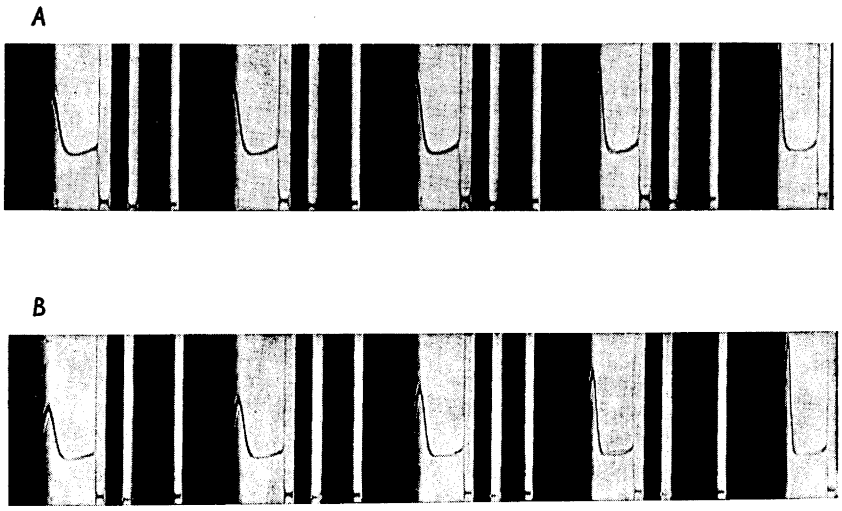

Fig. 1. Representative photographs obtained during the sedimentation analyses of the two main components of leghemoglobin. The pictures at the right represent the beginning of the runs.

A) The electrophoretically faster component at a protein concentration of 8.23 mg/ml (in sodium phosphate-NaCl buffer as described in the text) measured at an average temperature of 19.83°C and at a rotor speed of 59 780 rpm. Photographs were taken at intervals of 8 min. The five first photographs are reproduced.

B) The electrophoretically slower component at a concentration of 6.40 mg/ml measured at an average temperature of 18.78°C. The other conditions are the same as in A.

protein concentrations are shown in Fig. 2. The lines drawn through these data were calculated by the method of least squares, and fit the equation

$$S^{\circ}_{20} = S_{\infty} - \beta a$$

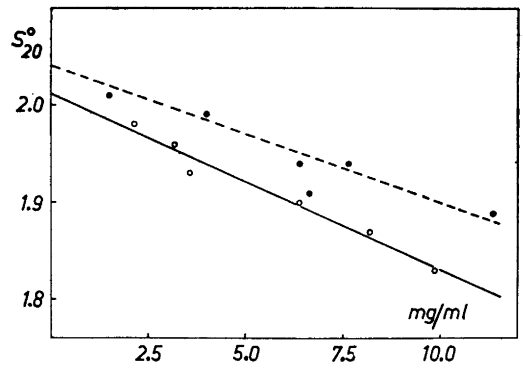

Fig. 2. Concentration dependence of the sedimentation constants of the electrophoretically faster (O) and slower (●) components of leghemoglobin in sodium phosphate-NaCl buffer (pH 7.0, ionic strength 0.283).

where S_∞ is the sedimentation coefficient extrapolated to infinite dilution, and a is the concentration of protein in mg/ml. S_∞ was found to be 2.012 S for the faster component and 2.041 S for the slower one. The equations for the sedimentation coefficients of the two components were:

$$S^\circ_{20} = 2.012 - 0.0180\ a \quad \text{(fast component)}$$
$$S^\circ_{20} = 2.041 - 0.0138\ a \quad \text{(slow component)}$$

The close similarity in the sedimentation data for the two components indicates that these proteins cannot be distinguished from each other by their behavior in the ultracentrifuge.

Diffusion coefficient. Several experiments were performed on each of the two components prepared from different batches. The experiments usually were performed in the standard cell. The protein concentrations for the two

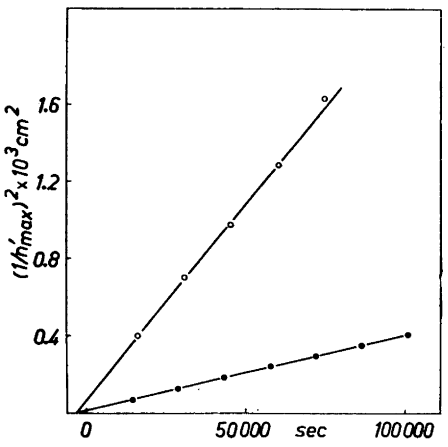

Fig. 3. A plot of inverted squares of the maximum fringe densities *versus* the length of time of diffusion. This plot was used for the calculation of the diffusion constants for the electrophoretically faster component according to the "height-area" method. Protein concentrations were 1.35 mg/ml (O) and 3.20 mg/ml (●) in sodium phosphate-NalC buffer (pH 7.0, ionic strength 0.283).

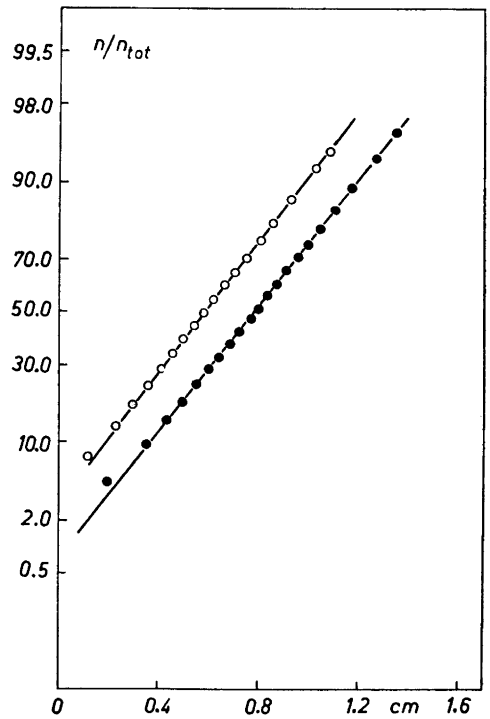

Fig. 4. Plots of relative fringe numbers *versus* fringe coordinates on a normal distribution graph in order to test the fit of the experimental diffusion curve of the electrophoretically faster component. The exposures are for protein concentratoins of 1.35 mg/ml (after 73 980 sec. of diffusion) (O), and for 3.20 mg/ml (after 86 400 sec.) (●).

Table 1. Diffusion data for the two main components of leghemoglobin obtained by the "height-area" method.

Component	Conc. mg/ml	t_o sec.	$D_{0.6}$ F	D^o_{20} F
Fast	1.35	−3 769	5.787	11.29
»	3.20	−3 874	5.620	10.96
		Average value		11.13
Slow	2.40	−3 111	6.174	12.04
»	7.40	−1 491	6.143	11.98
		Average value		12.01

experiments with the faster component were 1.35 mg/ml and 3.20 mg/ml, and the total fringe numbers were 19.10 and 42.62 respectively. Fringe intervals, Δn, of 4 and 9, respectively, were chosen for the calculation of the maximum fringe density, n'_{max}[4]. When applying the "height-area" method, the rate of increase of $(1/n'_{max})^2$ with time was determined. Five and seven exposures were evaluated in the first and second experiments, respectively. The corresponding plots of the data for the two experiments are shown in Fig. 3. The equations of the lines drawn through the points obtained in the two experiments have been calculated by the least squares method. The numerical data are given in Table 1. Four exposures from the higher concentration were evaluated according to the "moment method", and the results are given in Table 2. The zero-time correction, as determined by the "height-area" method, has been used.

The diffusion curve of a homogeneous, ideal-diffusing substance has the form of the normal probability curve, and for such a substance D_A and D_m should have the same value. Curves of mixtures have sharper peaks than the normal curve, and hence $D_m > D_A$. The close agreement between the values obtained

Table 2. Diffusion data for the two main components of leghemoglobin obtained by the "moment" method.

Component	Conc. mg/ml	t sec.	$t-t_o$ sec.	$D_{0.6}$ F	D^o_{20} F
Fast	3.20	43 200	47 074	5.784	11.28
		57 600	61 474	5.769	11.25
		86 400	90 274	5.746	11.20
		100 800	104 674	5.720	11.15
			Average value		11.22
Slow	7.40	66 600	68 091	6.575	12.82
		81 000	82 491	6.488	12.65
		95 400	96 891	6.464	12.61
			Average value		12.69

by the two different methods for the faster component is an indication of the essentially homogeneous character of the protein.

A more sensitive test of the diffusion behaviour, however, is to compare the experimental curve with a normal probability curve drawn on graph paper [6]. In Fig. 4, the relative fringe numbers, n/n_{tot}, have been plotted against the fringe coordinates determined from two exposures, one for each concentration. The line drawn for the lower concentration (19.10 fringes) corresponds to $D^{\circ}_{20} = 11.29$ F, whereas the line for the higher concentration (42.62 fringes) corresponds to $D^{\circ}_{20} = 10.98$ F. The zero-time correction obtained by the "height-area" method was applied in these calculations.

A value of 11.18 F is obtained as an average value for the two methods of calculation in the concentration range studied. This value has been used for the calculations of the molecular weight and the asymmetry of the faster component.

In a preliminary study, a diffusion constant equal to 8.5 F was reported for the slower component [2]. In more recent studies, this value has not been confirmed. If precaution were taken to avoid all possibility of denaturation during

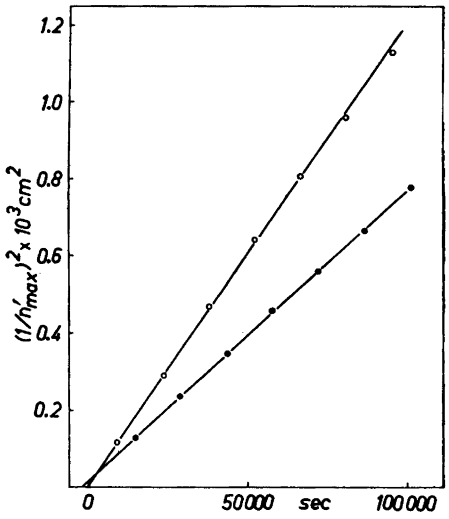

Fig. 5. A plot of inverted squares of the maximum fringe densities *versus* the length of time of diffusion. This plot was used for the calculation of the diffusion constants for the electrophoretically slower component according to the "height-area" method. Protein concentrations were 2.40 mg/ml (O) and 7.40 mg/ml (●) in sodium phosphate-NaCl buffer (pH 7.0, ionic strength 0.283).

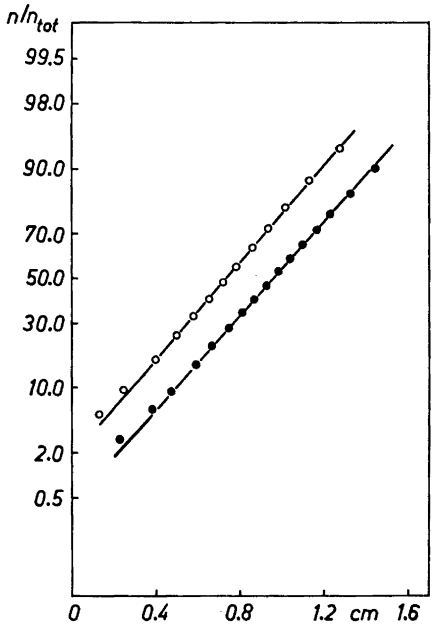

Fig. 6. Plots of relative fringe numbers *versus* fringe coordinates on a normal distribution graph in order to test the fit of the experimental diffusion curve of the electrophoretically slower component. The exposures are for protein concentrations of 2.40 mg/ml (after 95 400 sec. of diffusion) (O) and for 7.40 mg/ml (after 100 800 sec.) (●).

preparation, the value obtained was higher. Several experiments were run, two of which are reported here. One experiment in which the protein concentration was 2.40 mg/ml was conducted in the standard cell, whereas another experiment in which the protein concentration was 7.40 mg/ml was conducted in the micro cell. (The optical path in the micro cell is 1/4 of that in the standard cell). The total fringe numbers were 32.20 for the lower concentration and 25.60 for the higher one. A fringe interval, Δn, of 5 and 7 was used in the calculation of the maximum fringe density, n'_{max}, with 7 exposures evaluated at both concentrations. The corresponding plots of the data for the two experiments as calculated by the "height-area" method are given in Fig. 5, whereas the numerical data are recorded in Table 1.

Three exposures from the higher concentration were evaluated according to the "moment method", and the results are recorded in Table 2. The zero-time correction from the "height-area" method was used.

The closeness of fit to the requirements of ideal diffusion was checked by plotting the data from the two experiments on a normal distribution graph (Fig. 6). The line drawn for the lower concentration (32.60 fringes) corresponds to $D^{\circ}_{20} = 12.21$ F, whereas for the higher concentration, the line corresponds to 12.35 F.

An average value of 12.35 F was obtained by the two methods of calculation. This value has been used for the calculation of the molecular weight and asymmetry of the slower component.

Partial specific volume. The partial specific volume was determined in three experiments. The unresolved material was used because as high a protein concentration as possible was desired. A solution of 5 mg/ml gave the following values: 0.740, 0.740 and 0.739 ml/g. A value of 0.740 ml/g was used for the calculations that follow.

Molecular weight. The values of the molecular weights of the two components were calculated by means of Svedberg's formula [3]

$$M = \frac{RTs}{D(1-V\varrho)}.$$

From the determined values of s, D and V, the electrophoretically faster component was found to have a molecular weight of 16 800, and the electrophoretically slower one a molecular weight of 15 400.

Molecular shape. The molecular frictional ratio f/f was calculated according to the following formula [3]:

$$f/f_o = \left[\frac{1-V}{D^2_{20}s_{20}V}\right]^{1/6} 10^{-8}$$

The molar frictional ratio was found to be 1.118 for the faster component and 1.043 for the slower one. Following the procedure of Oncley [3], f/f_o can be represented as a product of two factors, f/f_e and f_e/f_o. The first factor, f/f_e, represents the effect of hydration, while the second factor, f_e/f_o, represents the influence of the asymmetry of the molecule. Kramer [3] has shown that the hydration factor is related to the grams of water per gram of protein, w, by the formula

$$f/f_e = \left[1 + \frac{w}{V\varrho}\right]^{1/3}$$

in which ϱ is the density of the water solvating one gram of pure solute of the partial specific bolume V. The maximum possible degree of hydration for the electrophoretically faster component would make f/f_e equal to 1.118 and would lead to a value of 0.29 for w. On the other hand, if the entire molar frictional ratio is due to asymmetry, f_e/f_o would be equal to 1.118. In the equations of Perrin [3], this value would represent axial ratios of 3.1 and 3.2 for prolate and oblate ellipsoids, respectively. Assuming an elongated ellipsoid and zero hydration, the faster component would have a minor axis of 23 Å and major axis of 72 Å.

If the molecular frictional ratio, $f/f_o = 1.043$, of the slower component is due entirely to hydration, the value for w would be 0.032. If the frictional ratio is due only to asymmetry, the axial ratios would be equal to 2.0 for both prolate and oblate ellipoids. An unhydrated ellipsoid of the slower component would have a minor axis of 26 Å and a major axis of 52 Å.

DISCUSSION

Both of the main components of leghemoglobin appear to be single, homogeneous proteins in the ultracentrifuge. Earlier ultracentrifugal studies [1] distinguished two components of unequal concentration. The predominant component was slowly sedimenting (1.9 S), whereas the lesser component was a rapidly sedimenting fragment (5 to 6 S). The rapidly sedimenting fraction seemed to form a part of the electrophoretically slower component. The presence of such a small, heavy component could not be confirmed in the present studies of the individual components.

The molecular weight of 20 000 found earlier for the unresolved material [1] deviates considerably from the new molecular weights of 16 800 and 15 400 presently determined for the electrophoretically faster and slower components, respectively. This discrepancy is essentially due to differences in the diffusion constants of the different preparations investigated. The latter difference probably occurs because of the introduction of chromatography in the purification of the individual components [2]. The chromatographic procedure fractionates the unresolved material into at least four different components and removes some denaturated material which was not removed by ammonium sulphate fractionation [2]. The heavy component observed earlier in the unresolved material might consist of this denaturated material. Hence, the former assumption [1] that the electrophoretically slower component is an artefact of a heavy, iron-free protein surrounded by leghemoglobin molecules has no experimental basis.

The two main components are highly symmetrical in shape. The values for the frictional ratios calculated for both of the components are among the lowest that have been described. Our knowledge of the hydration of proteins is not in an advanced state, but undoubtedly water is immobilized on the surfaces of protein molecules. A value of 0.3 g of water per g of dry protein would

seem to be a reasonable figure and would lead to a value of 1.120 for the frictional ratio. This is evidently an upper limit of the possible contribution of hydration to the frictional ratio. If the frictional ratios of the two components were explained only on the basis of hydration a great difference in hydration would exist between the two components. This may be explained as being caused by differences in the amino-acid content of the components.

Leghemoglobin shows similarities to other hemin proteins in respect to molecular weights. The molecular weight of 16 800 for the faster component closely resembles the unit molecular weight of animal hemin proteins. Such a molecular weight has also been found for the hemoglobin of *Lampetra fluviatilis* [3]. The electrophoretically slower component, with its molecular weight of 15 400, falls in a class between the molecular weight of *Lampetra* hemoglobin and that of animal cytochrome c.

Acknowledgements. The author wishes to express his thanks to Professor Hugo Theorell and to Dr Anders Ehrenberg for valuable discussions and advice during the course of this work. Able technical assistance has been given by Miss Bodild von Sobeck in running the ultracentrifuge. The investigation has been supported by a grant from *Finlands State Commission of Natural Sciences* (*Statens Naturvetenskapliga Kommission*) which is gratefully acknowledged.

REFERENCES

1. Ellfolk, N. and Virtanen, A. I. *Acta Chem. Scand.* **6** (1952) 411.
2. Ellfolk, N. *Acta Chem. Scand.* **13** (1959) 596; **14** (1960). 609.
3. Svedberg, T. and Pedersen, K. O. *The Ultraceentrifuge.* Oxford University Press New York, N.Y. 1940.
4. Svensson, H. *Acta Chem. Scand.* **5** (1951) 72.
5. Svensson, H. *Acta Chem. Scand.* **5** (1951) 1410.
6. Ehrenberg, A. and Agner, K. *Acta Chem. Scand.* **12** (1958) 95.

Received June 2, 1960.