ACTA CHEMICA SCANDINAVICA 6 (1952) 411–420

The Molecular Weight of Leghemoglobin

NILS ELLFOLK and ARTTURI I. VIRTANEN

Laboratory of the Foundation for Chemical Research, Biochemical Institute, Helsinki, Finland

Hemoglobin present in the root nodules of leguminous plants, called in brief leghemoglobin *, oxidizes so rapidly when isolated from the nodules that unless oxidation-preventing substances are added the hemoprotein appears in the form of hemiglobin. We have, therefore, made determinations of the molecular weight of leghemiglobin.

During electrophoresis leghemiglobin purified by ammonium sulphate precipitations separates into two components [1]. In the present paper an attempt is made to characterize these components more closely and to determine the molecular weight of the one which probably represents pure leghemiglobin.

Leghemiglobin preparations

Leghemiglobin was prepared in a similar way as before [2]. The nodules of soya bean were crushed in an equal weight of saturated ammonium sulphate solution (58 % saturation) centrifuged and the ammonium sulphate saturation adjusted to 58 %. After standing overnight at 0° C the precipitate was separated and the ammonium sulphate saturation was raised to 75 %. After again setting aside overnight at 0° C, the precipitate was separated and dissolved in a small quantity of distilled water, dialyzed against running tap water, and finally against distilled water. Precipitation and dialysis were repeated several times.

The preparation was then subjected to lyophilization. This gives leghemiglobin in the form of a pink amorphous powder, which dissolves immediately and completely in distilled water. In the dry state the leghemiglobin is quite stable. The material obtained was used for preparing concentrated solutions for osmotic investigations and preparative electrophoretic work.

---

* Keilin and Hartree (*Nature* **168** (1951) 266) have expressed the opinion that the prefix "leg" is inadequate, and furthermore that it is ambiguous in English. However, it would appear to be a logical abbreviation of the Latin word "legumen" from the verb "legere", and we shall continue to use the term "leghemoglobin" rather than the unwieldy "hemoglobin of leguminous root nodules". A. I. Virtanen.

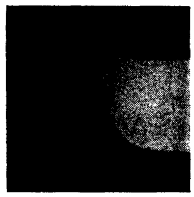

Fig. 1. Ascending limb, pH 6.40, u = 0.1, t = 120 min. Phosphate buffer.

The material had the following characteristics.

Spectrum. The spectrophotometric (Beckman) investigation showed the trivalent form of hemin iron, *i.e.*, leghemiglobin.

Electrophoresis. This showed the existence of two components as shown in Fig. 1. The apparatus used was Perkin-Elmer.

Iron content. Iron was determined photometrically by the *o*-phenanthroline method [3]. Combustion of the material was performed with sulphuric acid with addition of hydrogen peroxide, and 1 % hydroxylamine was used as reducing agent. The intensity of the colour of the ferrous *o*-phenanthroline complex was estimated using a Klett-Summerson photometer with filter 50, and the amount of iron was read from a calibration curve constructed with known concentrations. The iron content was 0.26—0.27 % calculated on the dry protein basis.

Separation of the two electrophoretically separable components was carried out by Klett electrophoresis apparatus during 7 hours in a 15 ml cell with 1 % leghemiglobin solution in a phosphate buffer (pH 7.10, $\mu = 0.1$). The potential gradient F was 8.9 volt/cm.

Two determinations of the iron content of the faster component gave the values 0.345 and 0.360 % of the dry matter. The former determination was made with a larger quantity of material and is probably the more accurate. The slower component was used for a diffusion measurement.

Diffusion measurements

Diffusion measurements were carried out at 20° C in an electrophoresis apparatus (Klett), a 10 ml preparative electrophoresis cell serving as the diffusion cell. To get evenly exposed photographs, we used a red filter, and a tungsten lamp as light source. The diffusion was followed by the Philpot-Svensson slit method.

The leghemiglobin preparation used in the diffusion measurements contained the two electrophoretically separable components (0.26—0.27 % iron). The solution was 0.8—0.9 %, and had been dialyzed against 1 molal NaCl ($\eta/\eta_0 = 1.087$) which was used to fill the upper compartments of the cell into which leghemiglobin was diffusing. The pH of the protein solution was $\sim$ 5.6.

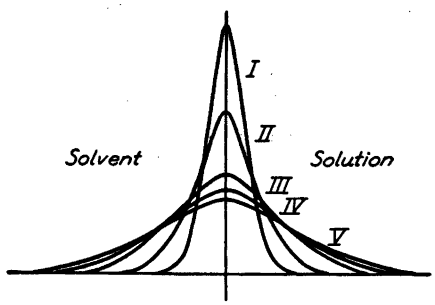

Fig. 2. Diffusion diagrams I, II, III, IV, and V refer to exposures taken 171, 388, 1 200, 1 586, and 1 836 minutes after the beginning of the diffusion process, and show the concentration gradient as a function of the distance from the initially sharp boundary between solvent and solution.

The curves in Fig. 3 characterize the course and momentary state of diffusion at five different times. The diffusion constant D has been calculated from these curves using the "area method" and is defined in the following way

$$D_A = \frac{A^2}{4 \cdot \pi \cdot t \cdot H_{max}^2}$$

where A denotes the area between the curve and x-axis, H_{max} is the maximum height of the curve, and t is the time after the formation of the boundary.

In our experiment the angle of inclination of the diagonal slit $\Theta = 30°$. The magnification factor of the camera was 0.95. From the curves in Fig. 2 we obtained the following values of D_A given in units of 10^{-7} c.g.s.

Diffusion time, mins.	171	388	1 200	1 586	1 836
D_A	8.50	8.92	7.90	8.40	8.84
D_A, corrected for water	9.23	9.70	8.58	9.12	9.60

After graphical levelling of the corrected values

$$D_A = 9.08$$

Another diffusion measurement was carried out with a more dilute solution of leghemiglobin (ca. 0.3 %) in phosphate buffer (0.01 M Na_2HPO_4, 0.023 M NaH_2PO_4 and 0.047 M NaCl, pH 6.4). The conditions were the same as before. $\Theta = 75°$. We obtained the following values of D_A in units of 10^{-7} c.g.s.

Diffusion time, mins.	1 705	2 650	3 810
D_A (in phosphate buffer)	8.88	8.65	9.15

after graphical levelling of the values

$$D_A = 8.25 \text{ (in phosphate buffer)}$$

A diffusion measurement was also carried out with the electrophoretically slower component [1], using the same method. The diffusion cell was a 2 ml

analytical electrophoresis cell. $\Theta = 81°$. Protein concentration: 0.1—0.2 %. Medium: phosphate buffer (0.02 *M* NaH_2PO_4, 0.046 *M* Na_2HPO_4, 0.094 *M* NaCl, pH 6.6). The values of D_A in units of 10^{-7} c.g.s. obtained were as follows:

Diffusion time, mins.	492	1 140	1 557
D_A (in phosphate buffer)	4.8	3.7	3.6

after graphical levelling of the values

$$D_A = 4.1 \text{ (in phosphate buffer)}$$

Unfortunately we could not make any diffusion measurements in lack of material.

Ultracentrifuge studies

The apparatus employed was an air-driven ultracentrifuge (Phywe) and the "schlieren" method was used to follow the course of the sedimentation. The speed used was 50.000 r.p.m, and the distance from the axis of rotation to the meniscus of the liquid was 5.75 cm. Because of the relatively low molecular weight of this protein, a long time was required to obtain an adequate displacement of the boundary and considerable diffusion took place thus making it difficult to determine accurately the maxima of the displacement curves obtained by the schlieren method.

Solutions of 0.3—0.4 % leghemiglobin in phosphate buffer (0.01 *M* Na_2HPO_4, 0.023 *M* NaH_2PO_4 and 0.047 *M* NaCl, pH 6.40) were used. Our preparations indicate a considerable homogeneity in the ultracentrifuge field, although some separation into components was detectable. However, in contrast to the electrophoretic behaviour, two components of equal concentration could not be distinguished. The predominating component showed a sedimentation constant *s* in Svedberg units (1 S = 10^{-13} c.g.s. units)

$$s_{20} = \sim 2 \text{ S (in phosphate buffer)}$$

The other component had a greater molecular weight than the predominating one but its concentration was very much less. It could only be observed in the early stages of ultracentrifugation, and disappeared during the run. The sedimentation constant of this component seemed to be between 5 S and 6 S (the actual value obtained was 5.3 S).

Molecular weight from the sedimentation rate

The molecular weight *(M)* was calculated by means of the equation

$$M = \frac{RT \cdot s_{20}}{D\ (1 - V\varrho)}$$

where R = gas constant, T = absolute temperature, ϱ = density of the solvent and V = partial specific volume of the protein.

Taking $s_{20} = 1.90 \cdot 10^{-13}$ (the value of Pedersen, see below)

$D_{20} = 9.08 \cdot 10^{-7}$

$V = 0.749$ (the partial specific volume of blood hemoglobin) we obtain the molecular weight 20 300.

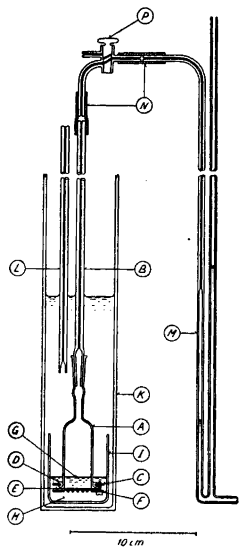

Fig. 3. The osmometer.

OSMOTIC MEASUREMENTS

The osmotic measurements were made because the ultracentrifuge studies indicated that our preparations precipitated with ammonium sulphate consisted of a molecular mixture the chief part of which contained homogeneous protein of low molecular weight.

Apparatus. The osmometer used for these experiments described by Güntelberg and Linderstrøm-Lang [4] and illustrated by Fig. 3 was placed in a thermostat at a temperature of 20 ± 0.002° C.

Experimental procedure.

The membranes used in our investigations were prepared according to the principle of Bjerrum and Manegold [5]. An iron ring was placed in a large Petri dish containing mercury, and collodion was poured on to the surface of the mercury inside the ring. The dish was covered with a glass plate until the collodion had spread evenly, then the plate was raised enough to allow the collodion to dry under a free access of air. After 3 hours most of alcohol and ether had evaporated and the iron ring with the wet membrane was removed from the mercury and allowed to dry in the air. To get the right permeability the iron ring with the membrane was put into water. If this was done too early or too late the permeability became accordingly too great or too small. This is naturally a question of experience. The permeability of our membranes was, expressed in terms of Bjerrum and Manegold [6]. $D \sim 10^{-7}$. It appeared that the permeability decreased during the osmosis.

Small membranes cut off from the large one were stored in 1 molal NaCl solution for half an hour, dried between filter papers, and placed on the bottom of a steel plate F. The glass vessel A, the flange of which was greased with vaseline, was pressed against F and fixed to the bottom plate with 3 screws.

4.5 ml of the test solution, freed from air, were put into the chamber which was then filled with toluene. The capillary tube B was greased with a special grease [7] insoluble in toluene, filled with toluene and put in position. The chamber was then placed in a beaker containing 25 ml of the outer solution (1 molal NaCl) care being taken to ensure that there were no air bubbles in the holes of the steel plate. The beaker was then filled

with toluene and lowered into the glass cylinder K containing toluene. Since the room temperature was higher than the temperature of the thermostat, the toluene in B had to retract to the middle part of the tube so that the toluene column in B was often 2—5 cm higher or lower than in L. The two columns were approximately levelled by the application of a pressure higher or lower than the osmotic pressure assumed. In this way it is possible to find the protein concentration without analysing the test solution, provided the exact concentration of the stock solution and the capillary volume per cm are known so that the dilution or concentration of the solution can be determined.

The outer solutions were normally 1 molal with respect to NaCl. The stock solution for the inner solution — the test solution — was prepared by dissolving the freeze-dried preparation in a small quantity of distilled water. The pH of this solution was $\sim$ 5.7, and several more dilute solutions were prepared so that the concentration of NaCl became 1 molal.

To determine the concentration of the inner solutions we used the procedure described above and did not analyze the inner solutions at equilibrium. The quantities were calculated on the dry protein basis.

The osmometer was left overnight at 20° C to reach equilibrium. The osmotic pressure was measured as the pressure externally applied by means of the manometer M, at which the meniscus in B is stationary, corrected for the difference in height of the columns in B and L. This was done in the following way.

The rate at which the meniscus moved in periods of 5 minutes was measured with a cathetometer at four different pressures, *i. e.* about 2 and 4 cm above and below the pressure at which the meniscus was expected to be stationary, and the true value was then found by graphical interpolation. The same experiment was continued for several days to test for possible leakage of the cell.

Molecular weight from the osmotic measurements

The apparent molecular weight (M_{app}) was calculated from each measurement by the equation [4]

$$M_{app} = \frac{RT \cdot \gamma}{\omega' \cdot p}$$

where γ = protein g per 1 000 g water, p = osmotic pressure measured in cm H_2O, ω' = specific volume of water plus specific volume of NaCl in solution = 1.02 l, RT (20°) = 24 870 l, cm H_2O/Mol.

Table 1 shows the experimental results at different concentrations of protein.

To obtain the correct value for M from the above equation, the usual procedure is to determine the osmotic pressure over a series of protein concentrations and to plot M_{app} against γ. The method of the least squares can also be used by applying the equation: $M_{app} = a + b\,\gamma$ where

$$a = \frac{\Sigma\gamma\,\Sigma M_{app}\gamma - \Sigma\gamma^2\,\Sigma M_{app}}{\Sigma\gamma\,\Sigma\gamma - n\Sigma\gamma^2};\quad b = \frac{\Sigma\gamma\,\Sigma M_{app} - n\Sigma\gamma\,\Sigma M_{app}}{\Sigma\gamma\,\Sigma\gamma - n\Sigma\gamma^2}$$

(n = number of experiment)

Table 1. Osmotic pressures and apparent molecular weights of leghemiglobin preparations at 20° C in 1 molal NaCl, pH 5.7.

No.	γ (protein, g/1 000 g H_2O)	p pressure in cm H_2O	M_{app}
1	4.6	5.7	19 700
2	5.6	6.6	21 000
3	7.1	8.7	19 950
4	8.8	10.4	20 650
5	11.3	13.1	21 000
6	12.7	13.3	23 600
7	19.6	22.3	21 400
8	32.0	33.7	23 200

Calculating a and b from the values in Table 1, excluding experiment 6, we get

$$a = (M) = 19\,617;\ b = 107.7$$

If, however, we determine the values a and b, neglecting experiments 1, 6, and 8, we obtain

$$a = (M) = 20\,132;\ b = 63.8$$

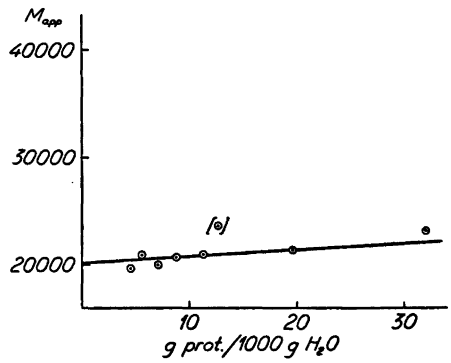

Fig. 4. Correlation between M_{app} and γ. The straight line has been constructed on the basis of the last values of a and b.

Earlier studies

In 1945—1947, Dr. K. O. Pedersen kindly carried out ultracentrifuge studies on three hemiglobin preparations sent by one of us (V.). The preparations had not been examined electrophoretically and hence their homogeneity was not established. These three preparations gave different values in diffusion measurements but approximately the same s_{20} (= 1.90 S) for the red-brown chief component. In addition, the preparations contained components with s_{20} within the region 5—12 S.

In regard to the first preparation, Pedersen found that "the great difference between D_A and D_m indicates that the preparation is non-homogeneous". He was very uncertain about the diffusion values but if they were used the most likely value for M was $\sim$ 34 000[2].

Pedersen made a number of diffusion experiments using the third sample and obtained D_A values between 8.2×10^{-7} and 10.8×10^{-7}. After a critical examination of the values he came to the conclusion that the molecular weight of leghemiglobin probably does not exceed 19 000 [8].

Because of the heterogeneity and differences between the samples no definite conclusions could be made of the mol.wt. of the pure leghemiglobin.

DISCUSSION

On the basis of the results recorded in the previous paper [1] and in the present one, certain conclusions can be drawn about the composition of the leghemiglobin preparations isolated from the root nodules, and the molecular weight of pure leghemiglobin. The molecular weights obtained for our leghemiglobin preparations, by the osmotic method, have been about 20 000.

Svedberg and Eriksson-Quensel [9] have found in some lower animals erythrocruorins with molecular weights of the same order, *i.e.* between 17 000 and 34 000.

	M	s_{20}
Erythrocruorin, *Thyone briareus*	23 600	2.6 S
» *Myxine glutinosa*	23 100	2.3 S

As the simplest explanation of the behaviour of these erythrocruorins, the authors advanced the idea "that they represent mixtures of the molecules 34 500 and 17 250, that is, mixtures of molecules with two and one hemin group per molecule, respectively".

The applicability of this explanation to our leghemiglobin preparations with molecular weights of $\sim$ 20 000 seems unlikely. The electrophoresis studies have revealed two components of equal concentration. The hemin content of the faster one is twice that of the slower one. If the faster component is hemin protein with a molecular weight of about 17 000, then the slower one must have a molecular weight considerably higher than 34 000 since it gave a diffusion constant $D_A = 4.1 \cdot 10^{-7}$. True, this value must be taken with some reserve, because the determination was made at a very low concentration, but it justifies the assumption of a large molecule or of a highly asymmetric molecule (unless hydration is considered). Calculation of the molecular weight of the slower component gives a value of $\sim$ 45 000, when $s_{20} = 1.9$ S and $D_A = 4.1 \cdot 10^{-7}$. The suggestion that the leghemiglobin preparations are a mixture of hemin proteins with mol. weights of $\sim$ 17 000 and $\sim$ 34 000 is thus unjustified.

The molecular weight ~ 20 000 found by osmotic measurements for the leghem*i*globin preparation containing 0.26 % Fe, the iron content of the electrophoretically faster component, 0.35 % (corresponding to 1 atom of iron per mol. weight ~ 17 000), and the occurrence of a slower component with a higher mol. weight, can be explained by assuming that the faster component is pure leghem*i*globin with mol. weight ~ 17 000 and the slower one a heavier protein.

The ultracentrifuge studies have shown that our preparations of leghem*i*globin containing 0.26 % Fe contain a predominating component with a sedimentation constant of about 1.9 S and a heavier one, which, in our investigations, gave a sedimentation constant of 5—6 S. This component seems to form a part of the electrophoretically slower component with low iron content. We assume it to be some kind of specific, relatively stable, adsorption compound of a heavy iron-free protein and leghem*i*globin, possibly a globulin surrounded by leghem*i*globin molecules. However, it must be admitted that in this case it is somewhat surprising that the ratio of the low-hemin and high-hemin (leghem*i*globin) components has been the same in our electrophoresis experiments with different preparations.

On the basis of the above, leghem*i*globin should be analogous to myoglobin and erythrocruorin from the species *Petromyzon* with the following properties:

	M	s_{20}	I. P.
Myoglobin	17 600 [10]	2.04 S [10]	6.99 [11]
Erythrocruorin, *Petromyzon*	19 100 [9]	1.9 S [9]	5.6 [9]
Leghem*i*globin	~17 000	1.9 S	4.4

Leghem*i*globin has the lowest I.P. of all the known hemoglobins and similar oxygen carriers. Its histidine content is very low hardly 1/4 of that of myoglobin. In this respect as well as in respect of the I. P., the erythrocruorins are between leghem*i*globin and myoglobin.

SUMMARY

Ultracentrifuge studies of our leghem*i*globin preparations containing 0.26—0.27 % Fe indicate a predominating component with a sedimentation constant 1.9 S, and a smaller heavier component.

The molecular weight of our preparations, estimated by the osmotic method, were found to be about 20 000. This is in good agreement with the values obtained from the rate of sedimentation ($s_{20} = 1.9$ S) and from diffusion measurements ($D_{20} = 9.08 \cdot 10^{-7}$). The iron content of the electrophor-

etically faster component was ~ 0.35 % indicating one hemin group per mol. weight ~ 17 000.

The molecular weights of the two components, which can be separated elctrophoretically have been discussed. The results suggest that the faster component with I. P. 4.4 has a molecular weight of ~ 17 000, and this component is assumed to be pure leghemiglobin.

We are deeply indebted to Dr. E. Uroma, Director of the State Serum Institute, for allowing us to use the equipment for lyophilization, diffusion, electrophoretic separation, and ultracentrifugation. We wish to express our gratitude to Phil. Mag. Antti Louhivuori, Head of the Chemical Department of the same Institute, for his valuable assistance in the course of the work, and to Dr. Korsgaard-Christensen of the Carlsberg Laboratory, Copenhagen, for his valuable advice on the preparation of the osmosis membrane.

REFERENCES

1. Ellfolk, N., and Virtanen, A. I. *Acta Chem. Scand.* **4** (1950) 1014.
2. Virtanen, A. I., Jorma, J., Linkola, H., and Linnasalmi, A. *Acta Chem. Scand.* **1** (1947) 90.
3. Schaefer, K. H. *Biochem. Z.* **304** (1940) 417.
4. Güntelberg, A. V., and Linderstrøm-Lang, K. *Compt. rend. trav. lab. Carlsberg. Sér. chim.* **27** (1949) 1.
5. Bjerrum, N., and Manegold, E. *Kolloid-Z.* **42** (1927) 97.
6. Bjerrum, N., and Manegold, E. *Kolloid-Z.* **43** (1927) 5.
7. Johansen, G. *Compt. rend. trav. lab. Carlsberg. Sér. chim.* **26** (1948) 399.
8. Pedersen, K. O. Personal communication.
9. Svedberg, The, and Eriksson-Quensel, I.-B. *J. Am. Chem. Soc.* **56** (1934) 1700.
10. Svedberg, The, and Pedersen, K. O. *The Ultracentrifuge* (1940). Oxford.
11. Theorell, A. H. T. *Biochem. Z.* **252** (1932) 1.

Received November 15, 1951.