Hemerythrin: Molecular Weight and Dissociation into Subunits

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The molecular weight of methemerythrin has been determined from osmotic pressure, light scattering, and viscosity measurements, as well as with several sedimentation techniques. A best value of 107,000 has been obtained. Hydrodynamic properties indicate that the macromolecule is nearly spherical. It has also been demonstrated that the macromolecule can be dissociated into subunits (*merohemerythrins*) of molecular weight 13,500. Since the large unit is very symmetrical, it is likely that the eight subunits are arranged in a compact form, perhaps a cubic array.

The marine oxygen-carrying pigment, hemerythrin, is an attractive system for investigations of structure and function in metalloproteins. This protein is readily crystallized and its biological activity, oxygen uptake, is easy to follow by spectrophotometric techniques. Furthermore, in hemerythrin, which is devoid of heme groups, iron must be attached directly to the macromolecule, and hence one can study the direct effect of the protein environment on the chemical behavior of the metal.

In the course of studies of the oxygen equilibria of this protein we have encountered large discrepancies between our analytical data (for example for % Fe) and those in the early literature. It seems clear now (Klotz *et al.*, 1957; Holleman and Biserte, 1958) that the discrepancies arise from the inaccuracies of early analytical techniques, rather than from biological species differences.

Similar uncertainties have arisen in regard to the molecular weight of hemerythrin. Osmotic pressure measurements in the 1930's (Roche and Roche, 1935) led to a value of 66,000. Some exploratory sedimentation studies (Resnik and Klotz, 1951), however, have indicated a sedimentation coefficient, s, of 6.2 Svedberg units, surprisingly high for a protein of 66,000 molecular weight. Furthermore, combination of this s with a later measurement (Brill and Olson, 1953) of the diffusion coefficient, D, leads to a molecular weight near 100,000, if we assume a typical partial specific volume of 0.73–0.75 cm^3/g for hemerythrin. Recently a molecular weight of 120,000 ($\pm$1000) has also been reported (Love, 1957), based on x-ray diffraction photographs for the determination of unit cell parameters and microscopic micrometric measurements of the linear dimensions (and hence volume) of a single crystal. The reliability of the direct micrometric measurement of the volume of a single crystal has not been checked by application to the determination of molecular weight of a protein of established size. Furthermore in this unit of 120,000 purported molecular weight are reported to be 19 iron atoms; since 19 is a prime number, it is surprising that hemerythrin is readily dissociated into subunits, as has been discovered recently (Klotz and Keresztes-Nagy, 1962). In any event, the discrepancies in reported molecular weight necessitate a thorough examination of this property if the molecular characterization of hemerythrin is to proceed further.

Preparation of Protein

Crystalline oxyhemerythrin was prepared from the coelomic fluid of the marine worm *Golfingia gouldii* (or *Phascolosoma gouldii*) by procedures described previously (Klotz *et al.*, 1957). Oxyhemerythrin is relatively unstable, being converted slowly to methemerythrin, but crystals can be maintained if stored wet in the cold.

For most measurements methemerythrin, being stable, is the preferred form of this protein. It can be obtained conveniently by controlled oxidation of oxyhemerythrin with potassium ferricyanide. The conversion was carried out as follows. Oxyhemerythrin was dissolved in tris(hydroxymethyl)aminomethane buffer of *p*H 7.7 and its concentration computed from the observed optical density at 500 mμ. A slight stoichiometric excess of $K_3Fe(CN)_6$ was added slowly at room temperature. When color conversion was complete, the solution was passed through an IRA 400 anion exchange column, or Sephadex G-25 or G-75, which removed ferrocyanide and any excess ferricyanide. Tests of the effluent revealed no detectable quantity of these complex ions. Methemerythrin was crystallized out of solution by dialysis against 20% ethanol in the cold.

Reduction of oxyhemerythrin to the deoxy form can be achieved if air above the solution is replaced repeatedly with N_2 or H_2 or by enzymatic methods utilizing glucose oxidase-catalase mixtures and glucose. Chemical methods also are effective, and are essential for the reduction of methemerythrin to deoxyhemerythrin. Sodium hydrosulfite, $Na_2S_2O_4$, is particularly effective as a reducing agent but it usually also causes protein denaturation and precipitation. We have found that a combination of $NaHSO_3$ and $NaBH_4$ is as effective as hydrosulfite with much less danger of protein denaturation. (It has been reported recently (Panson and Weill, 1960) that a mixture of $NaHSO_3$ and $NaBH_4$ produces low concentrations of $Na_2S_2O_4$.) Nevertheless we have not been able to achieve complete conversion of the met to the deoxy form.

Reduced hemerythrin can be converted to the oxy form by air or by oxygen.

Although our crystalline samples of hemerythrin (oxy and met) sedimented as single peaks and showed single boundaries during electrophoresis (at *p*H 7.8), more critical investigation always revealed some polydispersity. Sedimentation velocity experiments in a double-sector cell, one side containing solvent alone and the other protein in solution, were particularly

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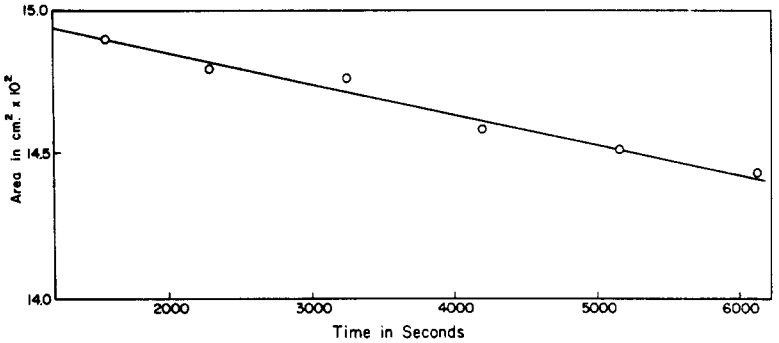


FIG. 1.—Decrease of area under Schlieren curve with time in a typical low-speed synthetic-boundary cell sedimentation experiment with hemerythrin.

sensitive in this respect and almost always showed a small amount of low-molecular-weight material, which was essentially nondetectable in a corresponding experiment with the standard ultracentrifuge cell. Low-speed ultracentrifuge experiments with a synthetic boundary cell also pointed to heterogeneity. In such a cell, the area under the refractive index gradient curve is proportional to concentration, and when corrected for radial dilution it should remain constant for a homogeneous solution (Trautman and Schumaker, 1954). In a heterogeneous system the maximum ordinate of the refractive index gradient curve and the square root of the second moment do not coincide. Consequently when the observed areas are corrected for radial dilution by the use of the simple equation valid for homogeneous systems, the corrected areas should decrease with time. As Figure 1 shows, the areas in a typical experiment with hemerythrin decrease linearly with time, dropping about 3% in 5000 seconds and thus providing additional evidence of heterogeneity. Analysis in terms of Fujita's (1956) equation, which takes self-sharpening of the boundary into account, gave graphs with upward curvature (Fig. 2), again a definite indication of some heterogeneity.

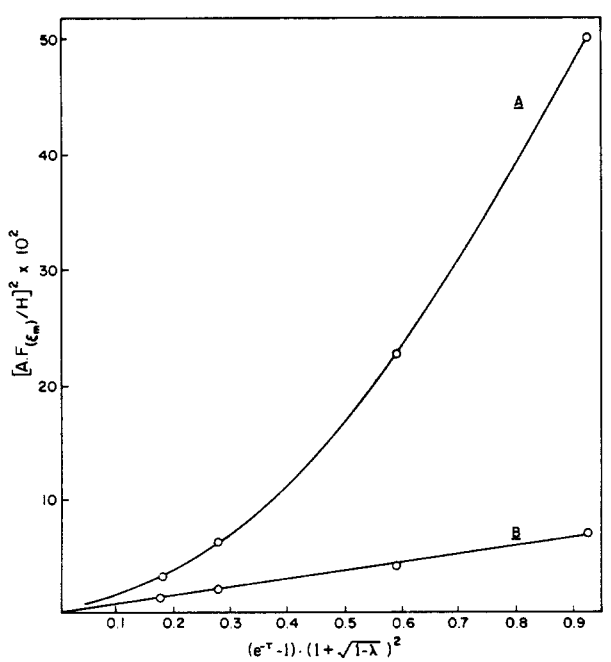


FIG. 2.—Application of the Fujita theory to methemerythrin. Curve *A* shows the results obtained with Fujita's equation, *B* shows calculations without correction for self-sharpening. In the ordinate and abscissa axes, A is the area and H the maximum ordinate of the gradient curve, $F(\xi m)$, τ, and λ are complicated functions of the sedimentation coefficient, diffusion coefficient, concentration dependence of s, and time elapsed from the start of the experiment. For details see Fujita (1956) or dissertation mentioned in footnote 1.

As will be described later, we know now that hemerythrin is composed of subunits. It is now apparent, therefore, that most of our preparations had a small quantity of dissociated hemerythrin mixed in with the normal macromolecule, and this component was the source of the slowly sedimenting material described above. Some attempts were made to remove this "light" material by filtration through Sephadex G-75. This objective was actually accomplished but at the expense of production of a small amount of a new aggregated component of higher sedimentation coefficient than the normal macromolecule. The net result was no gain, therefore.

Thus it is likely that all of our preparations contained a small amount of lighter molecules mixed in with the "native" macromolecules. This variable small degree of polydispersity has been the cause of an appreciable scatter in the molecular weights observed.

IRON CONTENT OF METHEMERYTHRIN

Previous detailed studies of the iron content of oxyhemerythrin gave a value of 0.81% (Klotz *et al.*, 1957). Since methemerythrin was prepared by exposure of the oxy form to ferricyanide it seemed prudent to analyze the product. Four determinations with the colorimetric *o*-phenanthroline procedure gave 0.82 ± 0.02% Fe. These results are not widely different from the value of 0.87% found recently by Holleman and Biserte (1958) in hemerythrin from *Sipunculus nudus*.

From the iron content one can compute a minimum molecular weight of 6800 for the protein. Furthermore, assuming that two atoms hold one O_2 molecule (Klotz and Klotz, 1955; Boeri and Ghiretti-Magaldi, 1957), then one active site corresponds to a unit weight of 13,600. Presumably, therefore, the actual molecular weight should be a multiple of 13,600. A series of such multiples is listed in Table I for comparison with experimental observations to be described.

PARTIAL SPECIFIC VOLUME

The partial specific volume, $\bar{v}$, of the protein is needed for the determination of molecular weight from sedimentation measurements, as well as for estimates of hydration and shape from hydrodynamic properties. The conventional procedure for measuring $\bar{v}$, determination of densities, was not convenient for hemerythrin, since this protein is soluble only in the presence of appreciable amounts of salt. Furthermore, concentration measurements based on colorimetric iron

TABLE I
POSSIBLE MOLECULAR WEIGHTS CONSISTENT WITH ANALYTICAL DATA

No. of Sites	Weight
1	13,600
2	27,200
3	40,800
4	54,400
5	68,000
6	81,600
7	95,200
8	108,800
9	122,400

analyses cannot be carried out with accuracies better than 1–2%. Neither of these limitations is a drawback if one uses the zero sedimentation method of Katz and Schachman (1955). In this procedure sedimentation coefficients are measured in D_2O-H_2O mixtures of different composition and density and a suitable function of s is extrapolated to zero sedimentation rate.

For these measurements 0.48% solutions of hemerythrin were prepared in 0.10 M phosphate buffer at *p*H 7.0 in aqueous solvents containing variable proportions of D_2O-H_2O. Sedimentation velocities were measured in a Spinco Model E Ultracentrifuge operating at 59,780 rpm and 24.9°. Densities of the protein solutions were obtained with a 5-ml. pycnometer and viscosities with an Ostwald-type viscometer. Pertinent information is summarized in Table II.

TABLE II
SEDIMENTATION OF HEMERYTHRIN IN D_2O-H_2O MIXTURES

Proportion of D_2O (%)	Density of Solution ρ (g/ml)	Viscosity η (centipoise)	$s \times 10^{13}$ Observed (sec.)	k
99.9	1.1156	1.1740	4.228	1.0150
85	1.0997	1.1415	4.555	1.0127
73	1.0868	1.1157	4.842	1.0110
63.7	1.0768	1.0955	5.024	1.0095
52.5	1.0651	1.0705	5.316	1.0079
42.5	1.0546	1.0500	5.646	1.0064
0.0	1.0095	0.9595	6.821	1.0000

When the protein is dissolved in D_2O-H_2O mixtures, deuterium-hydrogen exchange will increase its molecular weight and hence its density and v. This change may be expressed conveniently (Martin *et al.*, 1959) in terms of a factor k, the ratio of the molecular weight of the protein in the D_2O-H_2O mixture to that in pure H_2O. For many proteins this ratio has been observed (Martin *et al.*, 1959) to be 1.0150 in pure D_2O. For intermediate mixtures k may be computed by linear interpolation (Table II).

A graph of $\eta s/k$ *vs.* ρ/k using the data of Table II fitted a linear relation well. By the method of least squares the following equation was found to fit the results:

$$\frac{\eta s}{k} = 25.449 - 18.714\,\frac{\rho}{k}$$

From this equation it follows that at zero sedimentation rate, *i.e.*, at $\eta s = 0$,

$$\rho/k = 1.360 \text{ g/ml}$$

and hence

$$\bar{v} = k/\rho = 0.735 \text{ ml/g}$$

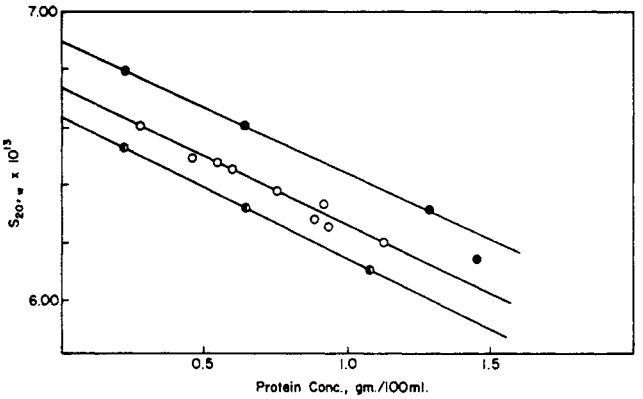


FIG. 3.—Concentration dependence of the sedimentation coefficient of hemerythrin in various buffers and at different *p*H levels: ●, methemerythrin in 0.1 M phosphate, *p*H 7.0; ○, methemerythrin in 0.5 μ borate, *p*H 8.0; ◐, oxyhemerythrin in 0.5 μ borate, *p*H 8.0.

A partial specific volume was also computed from preliminary rough amino acid analyses, and a value of 0.730 was obtained, in good agreement with the experimental value given above.

MOLECULAR WEIGHT FROM SEDIMENTATION VELOCITY AND DIFFUSION

1. *Sedimentation Experiments.*—Svedberg and Hedenius (1934) in their early survey of the sedimentation coefficients of a variety of blood proteins reported a (low) s for hemerythrin (*Phascolosoma vulgare*), of the order of that of hemoglobin, but did not state any specific value. More recently Resnik and Klotz (1951) gave an s_{20}^0 of 6.2 S for hemerythrin in phosphate buffer. Corrected for the presence of buffer a value of $s_{20,w}$ of 6.75 S is obtained. Much more extensive studies of s have now been carried out for both oxy and methemerythrin at *p*H 7 and *p*H 8. In all experiments the Spinco Model E Ultracentrifuge was used, at a speed of 59,780 rpm. Sedimentation coefficients at the temperature of operation were computed by the method recommended by Schachman (1957) and corrected to values in pure water and 20° by standard procedures. In one instance s was calculated using the square root of second moment of the gradient curve (Schachman, 1959) instead of the maximum ordinate, but the sedimentation coefficient obtained was not significantly different by this laborious method. Densities and viscosities needed for corrections to 20° were measured for the borate-NaCl buffer and computed from tables (Svedberg and Pedersen, 1940) for the phosphate buffer.

Figure 3 shows the dependence of s on concentration for oxy- and for methemerythrin at two different *p*H values. A single experiment in 0.1 M NaCl at *p*H 6.6 gave an $s_{20,w}$ of 6.68 S at 0.83% concentration of hemerythrin.

The sedimentation coefficient of oxyhemerythrin is slightly but significantly lower than that for the met form in the same solution. It seems, therefore, that there is a small change in shape or hydration as the protein is oxygenated.

Also there is a small increase in $s_{20,w}$ as the *p*H of the solution is lowered from 8 to 7. This might be a reflection of a slightly higher degree of dissociation at the higher *p*H, as indeed may be the changes in s with oxygenation. On the other hand, the concentration dependence of s on c (Fig. 3) shows no indication of such dissociation.

2. *Diffusion Measurements.*—Brill and Olson (1953)

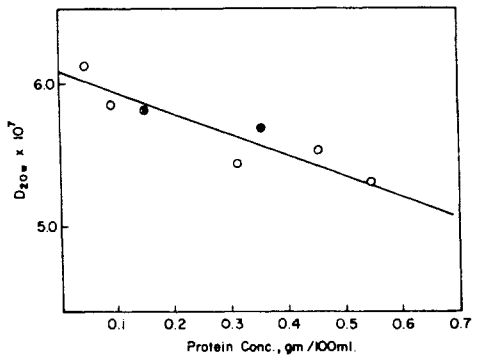


FIG. 4.—Concentration dependence of the diffusion coefficient of methemerythrin in 0.5 μ borate buffer at *p*H 8.0, O; also shown are the result of Brill and Olson (1953), ◑; and that from one experiment with a synthetic boundary cell in the ultracentrifuge at low speed, ●.

made a few rough measurements of the diffusion coefficient, D, at a single concentration of hemerythrin using a Perkin-Elmer electrophoresis apparatus. In view of the importance of D for the computation of the molecular weight, a detailed study was carried out with much better equipment, a Model H Spinco diffusion and electrophoresis apparatus. Both the Schlieren and interferometric optical systems were used.

All experiments were carried out with methemerythrin, at various concentrations, in 0.5 μ borate buffer at *p*H 8. Diffusion proceeded for 2–4 days at 1°, and both fringe and Schlieren patterns were photographed.

Calculation of diffusion coefficients from Rayleigh interference patterns for monodisperse ideal systems have been described by Schachman (1957). Such a calculation could not be carried out with our data for hemerythrin, presumably because of the polydispersity inherent in our preparations due to the presence of some subunits of the macromolecule as described above as well as perhaps because of the concentration dependence of D. On the other hand an average diffusion coefficient could be evaluated (from either fringes or Schlieren pattern) at each concentration by the conventional height-area (H-A) method, a zero-time correction being obtained from the intercept on the abscissa of a graph of $1/H^2$ *versus* time, t. Diffusion coefficients so calculated were converted to standard conditions (Schachman, 1957), $D_{20,w}$, and the results as a function of the relative mean concentration are shown in Figure 4.

A diffusion coefficient can also be estimated from the spreading of the boundary with time during an ultracentrifugation experiment with a synthetic boundary cell. One such result is shown in Figure 4 and agrees well with the values obtained with the standard diffusion apparatus.

Extrapolation of the diffusion data to zero concentration leads to a value of 6.10×10^{-7} cm²/second for $D_{20,w}^0$.

3. *Molecular Weight.*—Utilizing the Svedberg equation for the molecular weight, M, (where R is the gas

$$M = \frac{s}{D} \frac{RT}{(1 - \bar{v}\rho)}$$

constant and T, the absolute temperature; all other quantities have been defined previously) and inserting $s_{20,w}^0$ and $D_{20,w}^0$ for hemerythrin in borate buffer at *p*H 8 we compute a molecular weight of 102,000.

Molecular Weight from Approach to Equilibrium

A series of sedimentation experiments was carried out at 6730 rpm for analysis by the Archibald (1947) principle. Since it was recognized that the presence of some dissociated subunits might make hemerythrin behave as a polydisperse system, in which the molecular weight at the meniscus decreases with time, it seemed advisable to use some extrapolation to zero time. For this purpose we followed the "short-cut" procedure of Yphantis (1959) and plotted the intercepts on the Schlieren pattern at the meniscus, $(\partial c/\partial x)_m$, as a function of the square root of the time of sedimentation, t. The values of $(\partial c/\partial x)_m$ were extrapolated to $t = 0$. The molecular weight was then calculated from the equation

$$M = \frac{RT}{(1 - \bar{v}\rho)} \frac{1}{\omega^2 x_m c_0} \left(\frac{\partial c}{\partial x}\right)_{m,t=0}$$

where ω is the angular velocity, x_m the distance from the center of rotation to the meniscus, and c_0 the initial concentration of hemerythrin. In practice c_0 is obtained in terms of areas of the Schlieren diagrams by means of a separate experiment with a synthetic boundary cell.

A series of molecular weights computed in this manner for a range of hemerythrin concentrations is summarized in Figure 5. The reciprocal of M has been plotted *versus* concentration by analogy to graphical expressions used in osmotic pressure and light-scattering measurements. Extrapolation to zero concentration gives a molecular weight of 115,000.

No calculations were made from patterns at the bottom of the cell because of obvious denaturation effects at the oil-water interface.

Molecular Weight from Sedimentation Equilibrium

Most experiments were carried out with solutions of 0.5-cm column height and at speeds of 7,000 rpm. Equilibrium was usually reached in 3–4 days. Although methemerythrin is stable in solution for weeks under normal circumstances, there was evidence of denaturation at the bottom of the ultracentrifuge cell, where the protein was in contact with a silicone (Dow-Corning-No. 555) or fluorocarbon (Minnesota Mining and Manufacturing FC 43) interface. For this reason we could not be certain that the concentration of hemerythrin was the same at the end of the experiment as at the outset.

Graphs of log $(1/x)(\partial c/\partial x)$ *versus* x^2 were made for all runs. A slight curvature was observed in most of the curves; nevertheless the best straight line was drawn with emphasis on the points at the extremities. The values obtained in this way have been assembled in Table III. For reasons mentioned in this and the preceding paragraph, the results are somewhat unreliable. Nevertheless it seems evident that the molecular weight of hemerythrin is near 100,000. There is also some suggestion of aggregation as the solution is cooled to near 0°.

Osmotic Pressure Measurements

For this purpose a slightly modified form of the Scatchard (1952, 1954) version of the Hepp osmometer was used. In essence, in this apparatus circular filter paper containing protein-free solution rests on a lucite disc (with a hole in the center permitting contact of the solution of the filter paper with that in a capillary U-tube below), a circular cellophane membrane is held in place on top of the filter paper by means of a lucite collar pressing down along the rim, and protein solution is placed above the cellophane. Flow of

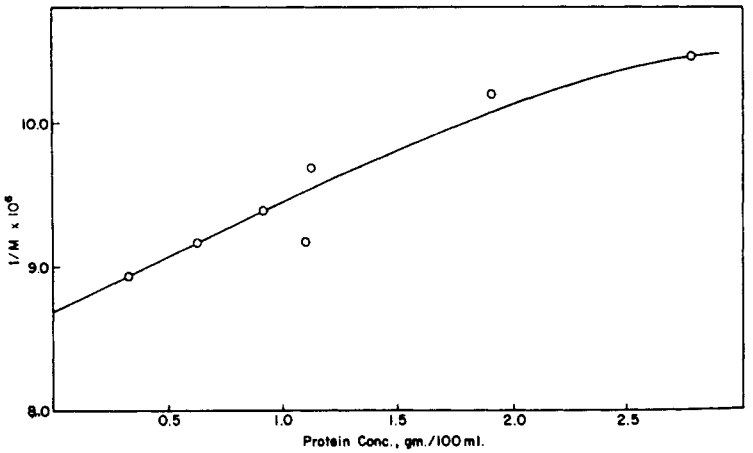


FIG. 5.—Reciprocal of apparent molecular weight, obtained by the Archibald method, plotted as a function of the concentration of methemerythrin in 0.5 μ borate buffer, pH 8.0.

TABLE III
MOLECULAR WEIGHTS FROM SEDIMENTATION EQUILIBRIUM

Form of Hemerythrin	Concentration (%)	Buffer	pH	Temperature	Molecular Weight
Met	1.91	0.5 μ Borate	8.0	Room	97,500
Met	1.86	0.5 μ Borate	8.0	Room	95,000
Met	1.82	0.5 μ Borate	8.0	Room	93,500
Met	1.70	0.5 μ Borate	8.0	Room	93,500
Met	1.30	0.5 μ Borate	8.0	Room	92,700
Met	1.10	0.5 μ Borate	8.0	Room	100,000
Met	1.24	0.5 μ Borate	8.0	3°	106,000
Met	1.45	0.1 M Phosphate	7.0	Room	106,000
Met	0.72	0.1 M Phosphate	7.0	Room	111,000
Oxy	1.37	0.1 M Phosphate	7.0	2°	119,500
Oxy	1.16	0.1 M Phosphate	7.0	3°	115,000
Oxy	0.62	0.1 M Phosphate	7.0	2°	124,000
Oxy	0.58	0.1 M Phosphate	7.0	2°	122,500

solvent across the membrane is registered by changes in height of liquid in the capillary in contact with the filter paper. Once the equilibrium point has been approximated, its position can be determined precisely by increasing or decreasing the pressure on the protein solution and following the return to a plateau. This osmometer ought to attain equilibrium in a period of an hour or so.

In practice great difficulty was encountered owing to minor mechanical motions of the large flat membrane fixed only at its rim and the sponge-like behavior of the filter paper in response to changes in pressure. Similar difficulties have been encountered by others (Meschia, 1954). As a first attempt to prevent ballooning a small glass container with mercury was placed as a weight on top of the membrane. The weight was insufficient to effect a major improvement; 12–24 hours was required for unequivocal equilibrium.

A marked improvement in performance was obtained when a perforated thin lucite plate was placed on top of the cellophane membrane and the lucite collar then used to fasten the thin plate rigidly. The perforations in the plate consisted of circular holes of slightly over 5 mm diameter and constituted about two thirds of the area of the plate. With this plate and a membrane of cellophane 300, recovery from a displacement from an equilibrium position took about half an hour.

Osmotic pressure measurements were made at 25.00 ± 0.01°. The results, assembled in Table IV, vary appreciably from sample to sample, depending particularly on the history of the preparation. As we became aware of the possible presence of subunits in the preparations and attempted to remove these, particularly by passage through Sephadex G-75, the molecular weights observed tended to be higher. The average value of the observations listed in Table IV is 100,300.

TABLE IV
MOLECULAR WEIGHTS FROM OSMOTIC PRESSURE MEASUREMENTS

Form of Hemerythrin	Concentration (%)	Buffer	pH	Molecular Weight
Met	2.46	Borate	8.0	90,000
Met	2.42	Borate	8.0	89,000
Met	2.07	Borate	8.0	84,000
Met	1.74	Borate	8.0	82,000
Met	1.27	Borate	8.0	80,000
Met	1.26	Borate	8.0	104,000
Met	1.22	Borate	8.0	113,000
Met	1.10	Borate	8.0	116,000
Met	1.01	Borate	8.0	77,000
Met	0.91	Borate	8.0	116,000
Met	0.87	Borate	8.0	91,000
Met	0.86	Borate	8.0	106,500
Met	0.76	Borate	8.0	85,000
Met	0.64	Borate	8.0	115,000
Met	0.61	Borate	8.0	114,000
Met	0.58	Borate	8.0	95,000
Met	0.56	Borate	8.0	112,000
Met	0.45	Borate	8.0	115,000
Oxy	2.10	Phosphate	7.0	105,500
Oxy	0.53	Phosphate	7.0	106,500
Laked blood	1.24	None	—	116,000
Laked blood	0.80	None	—	95,000

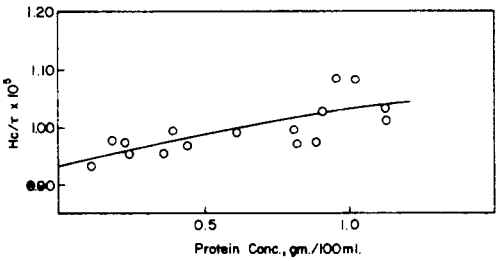


FIG. 6.—Variation of Hc/τ with concentration. Curve fits equation: $\frac{Hc}{\tau} = 0.9326 \times 10^{-5} + 0.0120 \times 10^{-5}c - 0.0003 \times 10^{-5}c^2$.

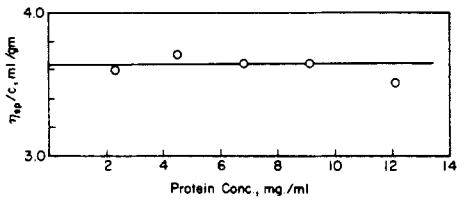


FIG. 7.—Reduced viscosity as a function of concentration for hemerythrin in borate buffer *p*H 8.0.

LIGHT-SCATTERING MEASUREMENTS

For these studies a Brice-Phoenix Universal 500 light-scattering photometer was used at a wave length of 546 mμ. Methemerythrin solutions in 0.5 μ borate buffer were centrifuged at 25,000 rpm and then filtered through a sintered glass ultrafine filter directly into the light-scattering cell; in this way the dissymmetry factor could always be kept between 1.02 and 1.10. Methemerythrin solutions are yellow, and therefore the scattering was corrected (Brice *et al.*, 1953) for absorption, even though it is small at 546 mμ. The refractive index increment of the protein solution, measured with a Brice-Phoenix differential refractometer at 546 mμ, was 0.190 $(g/100\ ml)^{-1}$.

The light-scattering results are summarized in Figure 6 in terms of the usual parameter H, the concentration, *c*, in grams per 100 ml, and the turbidity, τ. The line through the points was obtained with a quadratic least-squares equation. The intercept on the ordinate axis leads to a molecular weight of 107,000.

INTRINSIC VISCOSITY OF HEMERYTHRIN

Viscosity measurements of methemerythrin in 0.5 μ borate buffer at 25.00 ± 0.01° were made with a capillary viscometer, of the type described by Schachman (1957), having a length of 70 cm and a diameter of 0.76 mm. With 4 ml. of liquid and an average pressure head of 16.5 cm, the outflow time was 85 seconds. Density measurements were obtained with a special pyconometer.

Reduced viscosities are plotted in Figure 7. The intrinsic viscosity $[\eta]$ obtained by extrapolation to zero concentration is 3.64 ml/*g*.

Such a small value of $[\eta]$ indicates that methemerythrin must be a compact, symmetrical particle. It seems worthwhile therefore to compute a molecular weight by using the Scheraga-Mandelkern (1953) β function

$$\beta = \frac{Ns[\eta]^{1/3}\eta}{M^{2/3}(1 - v_t)}$$

and assigning a value of 2.16×10^6 to β. By this procedure we obtain a molecular weight of 115,000. Osmotic pressure measurements on the same preparation led to a molecular weight of 114,000. The preparation had been freed of subunits of methemerythrin by passage through a column of Sephadex G-75; however, sedimentation at 59,780 rpm showed the presence of a small amount of dimeric methemerythrin. We conclude, therefore, that the viscosity measurements are essentially in agreement with osmotic pressure results and that both indicate a true molecular weight somewhat below 115,000.

BEST VALUE OF MOLECULAR WEIGHT

The molecular weights obtained from the different experimental techniques involved are summarized in Table V. We have no strong grounds for assigning different weightings toward an average and hence we have obtained the average value 107,000 by simple arithmetic averaging. We know that small amounts of subunits of disaggregated hemerythrin were present in several samples used in osmotic pressure and sedimentation equilibrium measurements. On the other hand we also know that the intrinsic viscosity measurements were on a sample containing a small quantity of dimeric aggregate of hemerythrin. These two deviations probably balance each other in the averaging process.

TABLE V
SUMMARY OF MOLECULAR WEIGHTS

Method	Molecular Weight
s and *D*	102,000
Archibald	115,000
Sedimentation equilibrium	100,000
Osmotic pressure	100,000
Light scattering	107,000
$[\eta]$, *s* and β	115,000
Av.	107,000

If we compare the values in Table V with those permissible from iron analyses, Table I, we conclude that the molecular weight must be near 108,800. Even the lowest values of Table V are appreciably above the possible molecular weight of 95,000 listed in Table I, and the highest values are distinctly below the listing of 122,000 permitted by the iron stoichiometry. On the other hand a molecular weight of 108,800 from iron analyses is consistent with all of the measured particle weights. Furthermore, this molecular weight implies 8 oxygen-binding sites on each macromolecule, whereas the other choices imply 7 or 9. A preference for the even number might be considered merely "number mysticism." However, we now know, from observations described below, that hemerythrin is composed of subunits each with a weight corresponding to one O_2 site (or two Fe atoms), and it seems more likely that 8 of these would pack together well than would 7 or 9.

In summary, then, it seems that the native hemerythrin molecule has a molecular weight near 107,000.

ASYMMETRY OF HEMERYTHRIN MOLECULE

Frictional ratios, f/f_0, can be evaluated with various combinations of *M*, *s*, and *D* from the equations of Svedberg and Pedersen (1940). For hemerythrin f/f_0 is 1.13. This is a remarkably low value. From the contour diagrams of Oncley (1941) one finds that an assumption of only 40% hydration is compatible with a spherical shape and even an unhydrated molecule would have an axial ratio, if approximated by a prolate ellipsoid, of only 3.5.

Similarly the intrinsic viscosity of 3.6 ml/g indicates a compact symmetrical molecule. It is of interest to note that bushy stunt virus, a definitely spherical macromolecule, has an $[\eta]$ of 4 (Schachman, 1960).

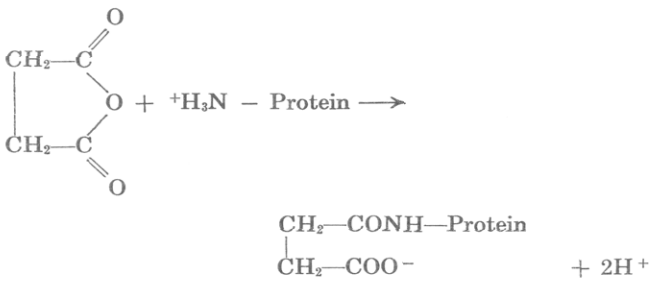
Subunits of Hemerythrin

As Table I indicates, a unit of molecular weight 13,600 contains two Fe atoms, in other words the iron needed to hold one O_2 molecule. The question arises, therefore, whether the native molecule of 107,000 weight might not be composed of subunits held together by non-covalent bonds.

A common procedure for disrupting non-covalent bonds is the addition of high concentrations of urea. Since we wished to determine the molecular weight of the subunit we avoided the urea method in view of attendant uncertainties due to the presence of high concentrations of this third component in the solutions.

Such uncertainties are much less serious in solutions of the detergent sodium dodecyl sulfate (Schachman, 1960). Dissociation of hemerythrin into subunits of 2.1 *S* was indeed achieved with 0.5–2% detergent (Fig. 8). On the other hand complete dissociation was not attained even after 24 hours' standing. Dissociation could also be achieved with 0.1 M Na_2CO_3 at *p*H values above 11 but again only incompletely unless drastic conditions (~100°) were used.

On the basis of these trials it seemed that dissociation was favored by the introduction of negative charge on the protein. A procedure for attaching negative charges through a covalent linkage was tried, therefore. As has been shown by others (Maurer and Lebovitz, 1956; Habeeb *et al.*, 1958) succinic anhydride reacts readily with proteins, under mild conditions, as shown in the reaction scheme:



Methemerythrin, dissolved in dilute salt solution, was succinylated, by the addition of successive increments of anhydride, at room temperature and *p*H 7–8. The *p*H was maintained in the range indicated by addition of dilute NH_4OH. After each increment of anhydride had reacted, as judged by attainment of a constant *p*H, a sample of solution was removed and examined in the analytical ultracentrifuge (Fig. 8). The native peak decreased in area and the subunit peak increased until, with sufficient anhydride, the former disappeared completely. The final protein solution was then transferred to 0.5 μ borate buffer by passage through Sephadex G-25 previously equilibrated with this buffer. The effluent from the Sephadex column was also dialyzed against 0.5 μ borate for 24–48 hours; the subunit did not leak out of the cellophane casing.

The spectrum of succinylated hemerythrin was indistinguishable in appearance from that of the original methemerythrin (Klotz *et al.*, 1957). Furthermore, if the concentration of subunit was determined from an iron analysis and a computation based on 0.81% iron content for the original nonsuccinylated material, the observed optical density at 280 mμ led to a molecular extinction coefficient equal to that of the original methemerythrin. Clearly the dissociation of

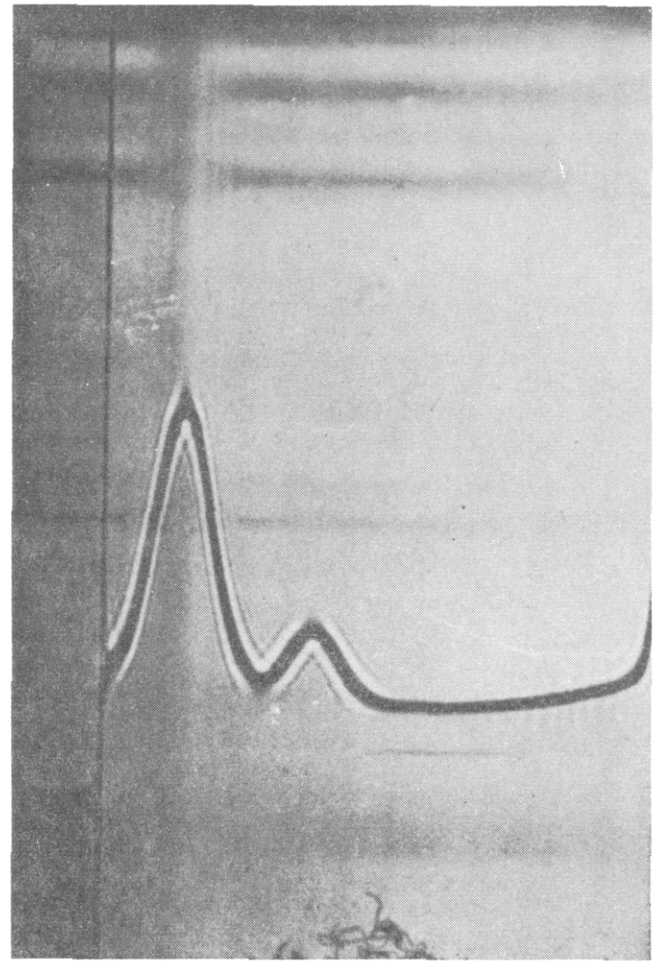


Fig. 8.—Schlieren photograph of methemerythrin dissolved in sodium dodecyl sulfate and sedimented at 59,780 rpm. Change of shading near middle of slower-sedimenting peak corresponds to yellow color to right of peak which is associated with subunit as well as the large macromolecule. Similar patterns were obtained in succinylation, relative areas of peaks changing in parallel with increasing addition of anhydride.

macromolecule had not been accompanied by a loss of iron. On the other hand, the succinylated subunit was no longer able to hold O_2, even after reduction of the Fe to ferrous iron.

*Oxy*hemerythrin was also succinylated and was also dissociated completely into subunits.

Succinylated subunits in 0.5 μ borate were examined by the sedimentation velocity method. Corresponding experiments were carried out also at lower ionic strength, in 0.2 μ borate. The observed sedimentation rates, corrected to standard conditions, were indistinguishable. Consequently 0.2 μ is an adequate ionic strength for damping out any electrostatic effect on the sedimentation coefficient. In this buffer *s* was also found to be independent of protein concentration. From experiments using a synthetic boundary cell, $s^0_{20,w}$ was found to be 1.95 ± 0.05 *S*. The diffusion coefficient was also determined by the height-area method during high-speed centrifugation, and a value of 12.3×10^{-7} cm²/second obtained. Assuming $\bar{v}$ to be 0.735 as in the 107,000 unit, these parameters lead to a molecular weight of 14,500 for the succinylated subunit. Although no analyses for bound succinyl groups were carried out, the quantities of anhydride used were high enough to form amides with all the (ten) lysine groups (Klotz and Heiney, 1962). A correction for the contribution of the succinyl groups

to the weight would thus be about 1000. Hence the weight of a single nonsuccinylated subunit, which we shall call *mero*hemerythrin (by analogy with *mero*myosin), is about 13,500.

A sedimentation equilibrium experiment, with a column height of 3 mm, was also run. A plot of log $(1/x)(\partial c/\partial x)$ *vs.* x^2 showed deviations from linearity at the bottom of the cell, indicative of the presence of some aggregates. Nevertheless, the linear portion covered almost 80% of the distance from the top to bottom and was used to compute the molecular weight of merohemerythrin. The computation in this situation is more complicated than usual because the sedimenting unit includes n small cations (Na^+) associated with each strongly anionic succinylated subunit. Usually the cation contribution is trivial. In this case, however, each merohemerythrin has 10 anionic succinyl groups attached to the 10 original lysine residues as well as about 10 uncompensated glutamyl and aspartyl negative charges. Thus approximately 20 Na^+ ions form part of the sedimenting unit.

In this situation the sedimentation equilibrium equation should be written (Katz and Schachman, 1955)

$$\frac{2RT}{\omega^2}\frac{\partial \ln c}{\partial x^2} = M_p(1 - \bar{v}_p\rho) + 20M_{Na}(1 - \bar{v}_{Na}\rho)$$

where M_p is the molecular weight of succinylated protein and M_{Na} that of a sodium ion and the appropriate $\bar{v}$'s are indicated by subscripts. To evaluate M_p we must estimate $\bar{v}$ of Na^+ ion. An estimate near zero seems reasonable from a survey of the literature (Monk, 1961). With this value and assuming $\bar{v}_p$ of succinylated merohemerythrin is 0.735, we find M_p to be 14,800. Corrected for the contribution of ten succinyl groups, merohemerythrin thus shows a molecular weight of 13,800. The presence of the charge on the protein and the density gradient created by the electrolyte, nevertheless, still leave a residual uncertainty in this calculated molecular weight. This error can be estimated from equations provided by Williams *et al.* (1958). Under the conditions of our experiments (0.3% protein, 3% salt) this error should not exceed 8%. The actual error is probably less, since the molecular weight obtained from sedimentation equilibrium studies agrees well with our other results.

Finally a molecular weight was also determined from osmotic pressure measurements, since previous experiments had shown that the subunit would not diffuse through the membrane. Equilibrium was attained more slowly in these experiments than in those with the 107,000 particle, perhaps because of the high charge on the succinylated merohemerythrin. Nevertheless equilibrium could be approached from either side. A number average molecular weight of 13,500 was computed for merohemerythrin from experiments with 0.16 and 0.32% protein.

An average weight from the three methods used is thus 13,600. Eight subunits should give a particle of 109,000 molecular weight, a result closely consistent with the average value obtained experimentally for hemerythrin (Table V).

In view of the compact symmetrical nature of native methemerythrin, it is clear that the merohemerythrins cannot be arranged in a linear array but rather must be aggregated into a compact form. The simplest configuration presumably would be at the eight corners of a cube. However details as to arrangement of subunits, as well as questions of interparticle forces and of identity or nonidentity of subunits, will require other methods of examination besides hydrodynamic and thermodynamic techniques.

References

Archibald, W. J. (1947), *J. Phys. Chem. 51*, 1204.

Boeri, E., and Ghiretti-Magaldi, A. (1957), *Biochim. Biophys. Acta 23*, 489.

Brice, B. A., Nutting, G. C., and Halwer, M. (1953), *J. Am. Chem. Soc. 75*, 824.

Brill, A. S., and Olson, J. M. (1953), *Biol. Bull. 105*, 371.

Fujita, H. (1956), *J. Chem. Phys. 24*, 1084.

Habeeb, A. F. S. A., Cassidy, H. G., and Singer, S. J. (1958), *Biochim. Biophys. Acta 29*, 587.

Holleman, J. W., and Biserte, G. (1958), *Bull. Soc. Chim. Biol. 40*, 1417.

Katz, S., and Schachman, H. K. (1955), *Biochim. Biophys. Acta 18*, 28.

Klotz, I. M., and Heiney, R. E. (1962), *Arch. Biochem. Biophys. 96*, 605.

Klotz, I. M., and Keresztes-Nagy, S. (1962), *Nature 195*, 900.

Klotz, I. M., and Klotz, T. A. (1955), *Science 121*, 477.

Klotz, I. M., Klotz, T. A., and Fiess, H. A. (1957), *Arch. Biochem. Biophys. 68*, 284.

Love, W. E. (1957), *Biochim. Biophys. Acta 23*, 465.

Martin, W. S., Winkler, C. A., and Cook, W. H. (1959), *Can. J. Chem. 37*, 1662.

Maurer, P. H., and Lebovitz, H. (1956), *J. Immunol. 76*, 335.

Meschia, G. (1954), *Yale. J. Biol. Med. 27*, 206.

Monk, C. B. (1961), Electrolytic Dissociation, New York, Academic Press, Inc., p. 271.

Oncley, J. L. (1941), *Ann. N. Y. Acad. Sci. 41*, 121.

Panson, G. S., and Weill, C. E. (1960), *J. Inorg. Nucl. Chem. 15*, 184.

Resnik, R. A., and Klotz, I. M. (1951), *Biol. Bull. 101*, 227.

Roche, J., and Roche, A. (1935), *Bull. Soc. Chim. Biol. 17*, 1494.

Scatchard, G. (1952), *Am. Scientist 40*, 61.

Scatchard, G., Gee, A., and Weeks, J. (1954), *J. Phys. Chem. 58*, 783.

Schachman, H. K. (1957), *in* Methods in Enzymology, vol. IV, Colowick, S. P., and Kaplan, N. O., editors, New York, Academic Press, Inc., pp. 55, 88–95, 99.

Schachman, H. K. (1959), Ultracentrifugation in Biochemistry, New York, Academic Press, Inc., pp. 64–67.

Schachman, H. K. (1960), *Brookhaven Symp. Biol. 13*, 49.

Scheraga, H. A., and Mandelkern, L. (1953), *J. Am. Chem. Soc. 75*, 179.

Svedberg, T., and Hedenius, A. (1934), *Biol. Bull. 66*, 191.

Svedberg, T., and Pedersen, K. O. (1940), The Ultracentrifuge, London, Oxford University Press, p. 40, appendices II, III, IV.

Trautman, R., and Schumaker, V. (1954), *J. Chem. Phys. 22*, 551.

Williams, J. W., Van Holde, K. E., Baldwin, R. L., and Fujita, H. (1958), *Chem. Rev. 58*, 715.

Yphantis, D. A. (1959), *J. Phys. Chem. 63*, 1742.