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VOLUME OF REACTION BY THE ARCHIBALD ULTRACENTRIFUGE METHOD (LOBSTER HEMOCYANIN)

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Samples of lobster hemocyanin (*Homarus americanus*) under conditions of reversible reaction between whole (25 S) and half (17 S) molecules have been subjected to accurately known nitrogen pressures in analytical ultracentrifuge cells. A modified pressurization chamber of the type developed by Schumaker and colleagues has been constructed for this purpose. The molecular weight was then determined at the top (liquid-gas) meniscus, by means of the Archibald method. The logarithmic dependence upon pressure of the derived equilibrium constant then gave directly the volume of reaction. Experiments were performed in veronal-citrate buffers at pH 8, where the molar volume of formation of whole (dodecameric) molecules from half molecules appears to be negative, and at pH 8.46 in veronal-citrate buffer in the presence of 0.003 molar free calcium ion, where the molar volume of formation was estimated to be +390 cm^3/mole. In glycine-sodium hydroxide buffer at pH 9.6 containing 0.0047 molar free calcium, the molar volume of formation of whole molecules was estimated to be +120 ± 70 cm^3, corresponding to an estimated difference in partial specific volume between whole molecules and half molecules of only 1.3 $(10)^{-4}$ cm^3/gram. The correctness of the sign of this value in glycine buffer has been verified by pressure-jump light-scattering experiments.

1. Introduction

The sensitivity of macromolecular reactions to hydrostatic pressure is magnified because of the high molecular weights of the reacting species, since the *molar* volume of reaction governs [1–3]. It is therefore often feasible to detect and even measure volumes of reaction in ultracentrifuge experiments [4,5], although corresponding direct measurements of changes in partial specific volume might be demanding or nearly impossible [6]. The experiments of Josephs and Harrington on myosin fibrils [3,4] utilized the hydrostatic pressure developed in the centrifugal field under a layer of oil. Schumaker et al. [7] developed a pressurization chamber in which a filled analytical ultracentrifuge cell can be sealed under a controlled nitrogen pressure and removed, already internally pressurized, prior to the ultracentrifuge experiment. These authors verified in this way that the phenomena described for sedimentation velocity experiments on myosin fibrils by Josephs and Harrington [3,4] are indeed wholly attributable to effects of hydrostatic pressure. In this report, we have applied their pressurization technique to make direct Archibald molecular weight determinations at the liquid-gas meniscus [8,9], as a function of pressure.

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2. Experimental

For the present study, we have constructed a modified nitrogen pressurization chamber based on the development of Schumaker et al. [7]. The chamber is made from a 3-inch diameter brass cylindrical rod, bored out with a blind hole slightly larger than the diameter of an ultracentrifuge cell. An adjustable key at the end of the hole permits correct positioning of either single-sector or double-sector cells for screwdriver access to the filling hole screw. A screwdriver tip was ground at the end of the shaft of a high pressure valve. The shaft in its valve seat was fastened through the curved side of the brass cylinder. The screwdriver tip is positioned over the slot of the filling hole screw before closing the pressure chamber with a

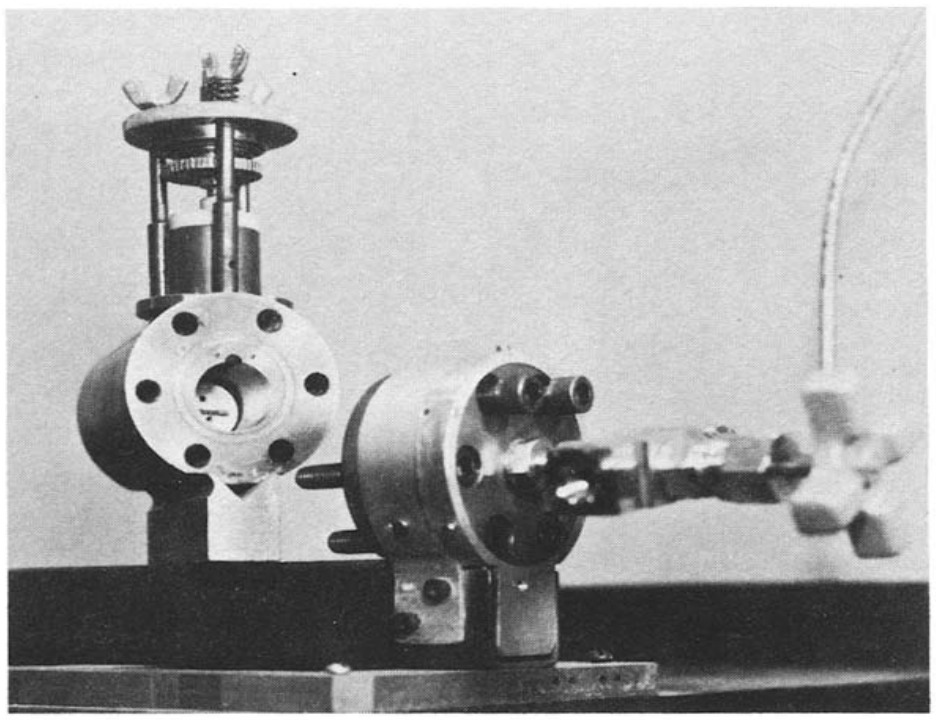

Fig. 1. Modified Schumaker-type pressurization chamber. Nitrogen pressure forces screwdriver shaft up out of contact with filling hole screw. Wing nuts and springs drive pressure plate (upper disc) against screwdriver handle (knurled disc), returning screwdriver tip into slot of filling hole screw. Ball-thrust-bearing occupies space between pressure plate and screwdriver handle.

heavy cylindrical cap. One or two of the standard ultracentrifuge cell filling hole polyethylene washers suffice to seal the cell. The filling hole screw is left in a nearly fully tightened position before closing the chamber: in this position, the pressurizing nitrogen quickly passes into the cell through the threads and washers, and only a fraction of a turn of the screwdriver then tightens the cell. The pressurization chamber is shown in fig. 1.

Schumaker et al. [7] have shown how the Schlieren optical system of the ultracentrifuge can be used to determine the gas pressure at the top liquid surface, during an experiment. Their method is based on the ideal gas law and the proportionality between gas pressure and refractive index. We have used their procedure, but have also noted that with a gas bubble of customary size in a standard 4° sector angle 12 mm thick cell centerpiece, it is very easy to weight the nitrogen in a cell pressurized to 50–100 atmospheres. Once the volume of gas is estimated from the known cell thickness and sector angle, and the Schlieren photograph of the gas-liquid column, the pressure is directly obtainable from the properties of nitrogen, or from the ideal gas law. The weighing method has given results in good agreement with that of Schumaker et al. [7]. The use of the analytical balance before and after ultracentrifugation also permits a check on the possibility of slow gas pressure leaks.

Our earlier ultracentrifuge studies of lobster hemocyanin [10] have indicated a small reversible pressure dependence of sedimentation patterns on hydrostatic pressure, and have also reported molecular weights, non-ideality coefficients and equilibrium constants for the hexamer–dodecamer reaction at one atmosphere pressure. The present direct measurement of molecular weights under controlled pressure in two different buffer systems provides estimates for the volume of reaction at pH 8.46 and pH 9.6.

3. Results

In table 1 are shown a series of weight-average molecular weight determinations by the Archibald method in 0.1 ionic strength pH 8 veronal buffer containing 0.00667 M citric acid. Table 2 shows a corresponding series of values in a similar buffer system at pH 8.46, containing 0.003 molar free calcium ion. These weight-average molecular weights are true values, corrected for thermodynamic non-ideality by measuring the apparent weight-average molecular weight of non-reacting whole (dodecameric) molecules in the presence of excess calcium ion as a function of protein concentration, and assuming the identical non-ideality factor for hexameric molecules [10]. From the weight-average molecular weight and the assumption that only two species, reacting whole molecules and reacting half molecules, are present, the formation constant of dodecameric whole molecules is directly calculated [10,11].

The volume of reaction ΔV is then computed from the thermodynamic relationship in integrated form

$$\Delta V = -(RT \ln K_2/K_1)/(p_2 - p_1). \qquad (1)$$

Here K_1 and K_2 are values of the formation constant at the pressures p_1 and p_2, R is the molar gas constant and T is the absolute temperature. Usually K_1 has been taken at the reference pressure $p_1 = 1$ atmosphere. In table 1, which represents one example from many similar experiments, it is noticed that at both pressures there is a steady downward drift with time from the start of sedimentation, not only of correct-

Table 1
0.619% hemocyanon, pH 8 veronal-citrate, 0.1 ionic strength

4059 RPM 25°C Time (min)	p = 1 at.			p = 100 at.		
	c, meniscus	M_w	$K^{a)}$	c, meniscus	M_w	$K^{a)}$
25	0.512	756 000	7.8	0.493	838 000	34
30	0.504	693 000	3.4	0.488	807 000	18
35	0.506	668 000	2.5	0.479	747 000	7.3

a) K = (whole molecules)/(half molecules)^2.

ed weight-average molecular weights, which might be explained at first sight by mass-action effects of dilution, but also of the computed formation constant of whole molecules, which implies something much more serious. This effect is consistent with a failure of the reacting hemocyanin molecules to remain at equilibrium during sedimentation. From light-scattering stopped-flow dilution experiments of the type previously reported for hemocyanin at pH 9.6 [12], we confirmed that the decay time for the hexamer–dodecamer reaction in this veronal–citrate buffer can be as long as 20–30 minutes. This slow re-equilibration rate would couple with separation and affect sedimentation velocity ultracentrifuge experiments [13], and might also have serious effects in low speed Archibald molecular weight experiments. At the very least, data would have to be available for a very careful extrapolation back to zero time, because of continuing partial accumulation of non-equilibrated hexameric molecules at the meniscus. While the data in table 1 appear to indicate a stabilization of whole molecules with increasing pressure in this buffer system, any estimate of a value for ΔV under these conditions would appear overoptimistic. Since the rate of re-equilibration of subunits to whole molecules increases with increasing calcium ion concentration [12], data such as those shown in table 2 were obtained in the presence of 0.003 molar free calcium ion. The consistent downward drift of apparent formation constant evident in table 1 is no longer characteristic of the results in table 2. If one takes the average listed values for the formation constant in the two experiments in table 2, one arrives from eq. (1) at an optimistic estimate $\Delta V = +390$ cm^3/mole of whole molecules, pressure favoring dissociation.

Another set of determinations was made for the volume of reaction in 0.6% hemocyanin dissolved in a buffer containing 0.0047 M free calcium ion in 0.1 ionic strength glycine-sodium hydroxide buffer at pH 9.6. In this buffer, the re-equilibration rate is fast enough [12] to make the approximation that hexamer and dodecamer are locally in chemical equilibrium throughout the sedimentation experiment [13]. The best average values of the weight-average molecular weights from a large number of determinations repeated at 4059 rev/min on this solution were 777 000 at one atmosphere and 743 000 at 100 atmospheres. Insertion of these values in eq. (1) leads to $\Delta V = +120 \pm 70$ cm^3/mole. The estimate of error ± 70 cm^3 corresponds to 1% error in weight-average molecular weight.

Table 2
0.619% hemocyanin, pH 8.46 veronal-citrate, 0.1 ionic strength, .003 M Ca^{2+}

4059 RPM 25°C Time (min)	p = 1 at.			p = 100 at.		
	c, meniscus	M_w	$K^{a)}$	c, meniscus	M_w	$K^{a)}$
25	0.492	947 000	–	0.497 b)	829 000 b)	28 b)
30	0.485	882 000	120	0.485	833 000	31
35	0.481	891 000	169	0.477	811 000	20

a) K = (whole molecules)/(half molecules)^2.
b) Values at 27 minutes of sedimentation.

These measurements are at the very limit of precision of the experimental method.

4. Discussion and summary

The experimental error of the ΔV determination appears very large, but the entire change in partial specific volume is estimated to be only 1.3 $(10)^{-4}$ cm^3/gram in pH 9.6 glycine buffer, a value so small as to be extremely difficult to determine at all by direct density measurements [6]. We have verified the correctness of the algebraic sign of this value in pH 9.6 glycine-sodium hydroxide buffer by pressure-jump light-scattering measurements [14]. The appearance of an actual change of sign of ΔV in shifting from pH 8 veronal-citrate buffer to pH 8.46 veronal-citrate buffer containing 0.003 molar free calcium ion suggests the complications which can arise in attributing changes in directly measured partial specific volume to shifts in population of macromolecular species, as judged from sedimentation velocity diagrams.

The technique reported here appears to be useful, in that the measurement of the volume change of a macromolecular reaction is sensitive to extremely small shifts in partial specific volume. Moreover, the direct computation of ΔV is based on fundamental thermodynamic principles. One must caution, however, that the calculation is no better than the identification of an equilibrium constant, or a corresponding volume of reaction, with a reaction between recognizable species, which identification must be at least partially extra-thermodynamic in nature.

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Note added at the conference

Because of a non-zero volume of ionization of the buffer system, there will generally be a small but finite shift of pH on pressurization. For the hemocyanin system, or for other interacting systems in which the protein–protein interaction is compled to protons, a correction would be required for the measured molecular weights at high pressure, because of this pH shift. This effect is discussed by Dr. James Ifft in a paper presented at this conference on Sedimentation Equilibrium of Proteins in Density Gradients.

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