suggest that the purine and phenoxazone rings stack in the 1 : 2 actinomycin D–d-pG complex. The stacking geometry in the complex has been evaluated based on the ring currents of the phenoxazone ring on the purine carbon chemical shifts of d-pG. Compared to a unique orientation of stacked phenoxazone and purine rings in the structure of the complex in the crystal (Sobell and Jain, 1972), a range of geometries has been suggested in aqueous solution.

A strong nucleotide–peptide intermolecular hydrogen bond involving the 2-amino proton of guanine (G-NH_2) was observed in the structure of the complex in the crystal (Jain and Sobell, 1972). The presence of this intermolecular hydrogen bond in solution was verified by (i) the downfield shift in the G-NH_2 resonance of d-pG on complexation with actinomycin D, (ii) a temperature coefficient of 2.4×10^{-3} ppm/°C for the G-NH_2 proton in the complex, and (iii) restricted rotation of the NH_2 group about the carbon–nitrogen bond in the complex as manifested in the temperature dependent line widths for this resonance.

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The Dissociation of Myosin in Acid†

Sara Szuchet* and C. Richard Zobel

ABSTRACT: Myosin has been dissociated into its constituent polypeptide chains by aqueous solutions of acetic acid (1–10 M). High-speed sedimentation equilibrium has been used to characterize the system. By using a very high speed (52,000 rpm), the molecular weights of the so-called "light subunits" could be measured in the presence of the "heavy" polypeptide chains. The system has proven to be complex; nevertheless, a rationale for the complexity has been given with the aid of information obtained from sodium dodecyl sulfate acrylamide gel electrophoresis. "Heavy" polypeptide chains free of the "light" counterpart have been obtained by fractionation of dissociated myosin on Sephadex G-200 using 1 M acetic acid. In the latter solvent "heavy" polypeptide chains exhibited molecular weight heterogeneity. This heterogeneity was greatly reduced and occasionally even disappeared (*e.g.*, at low concentrations), when the solutions were transferred to 10 M acetic acid. The apparent specific volume (ϕ') of "heavy" chains in 10 M acetic acid has been determined to be 0.704 ± 0.007 ml/g. Using this parameter we have obtained a weight average molecular weight of $[M_w]_{c=0}$ = 197,000 ± 2000. Our experiments suggest that (within experimental error) the two "heavy" polypeptide chains which make up the myosin molecule do not differ in molecular weight.

The quaternary structure of myosin has been the object of intense studies during the last 2 decades. As the techniques are more refined and the theory underlying them is better understood, so is the model for the molecule being refined too. Thus, according to the current model, the molecule is made up of two large polypeptide chains ("heavy" chains) and a number, yet to be exactly determined, of smaller polypeptide chains ("light" chains).

The dissociation of the "light" chains from the rest of the molecule is easily achieved, and a variety of physical and chemical means have been used for that purpose (Tsao, 1953; Kominz *et al.*, 1959; Wetlaufer and Edsall, 1960; Dreizen

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et al., 1966; Oppenheimer *et al.*, 1966; Locker and Hagyard, 1967a,b; Szuchet and Zobel, 1969; Gershman and Dreizen, 1970); the dissociation of the two "heavy" chains from each other, on the other hand, has been obtained only with high concentrations of guanidine·HCl or urea. Alkaline pH (*ca.* pH ~11), as used by Gershman *et al.* (1966) and others, fails to dissociate these chains. Acid pH was used by Tsao (1953), but under his experimental conditions hydrolysis occurred.

The molecular weight of the "heavy" chains in guanidine·HCl has been measured by Gershman *et al.* (1966) who estimated a molecular weight of 210,000 ± 5000. Unfortunately, these authors failed to report the partial specific volume used in arriving at this value. A significantly lower value, namely, 194,000, has been obtained by Gazith *et al.* (1970) using the appropriate apparent specific volume.

To date, apart from molecular weight, relatively little progress has been made in our knowledge of the "heavy" polypeptide chains; the main obstacle to progress has been the extreme difficulties in handling these chains (*e.g.*, they have a great tendency to aggregate even in dissociating solvents).

In contrast, there has been a great deal of research and progress in our knowledge of the "light" chains. The early discrepancy, among different laboratories, on the molecular weight of the so-called "alkaline" subunits (*i.e.*, low molecular weight component(s) dissociated by pH ≅11; see Kominz *et al.*, 1959; Gershman *et al.*, 1966; Frederiksen and Holtzer, 1968) has now been resolved with the aid of sodium dodecyl sulfate acrylamide gel electrophoresis (Shapiro *et al.*, 1967). With this technique, Weber and Osborn (1969) were the first to note molecular weight heterogeneity among the "light" chains. This has now been amply confirmed (Paterson and Strohman, 1970; Lowey *et al.*, 1971; Sreter *et al.*, 1971; Szuchet and Zobel, 1971). Furthermore, it has also been established that at least part (~50%) of these "light" chains can be removed with 5,5′-dithiobis(2-nitrobenzoic acid) without any apparent effect on the ATPase activity of myosin (Gazith *et al.*, 1970). However, reports are not conclusive as to the selectivity of this removal (*cf.* Paterson and Strohman, 1970; Lowey and Risby, 1971).

It has been shown that "light" chains from different types of muscle (*e.g.*, fast, slow, cardiac) differ slightly in their molecular weights (Sarkar *et al.*, 1971; Lowey and Risby, 1971). This observation, however, does not account for all the molecular weight heterogeneity, since both size and charge dispersity are still found in a single type of muscle. For instance, myosin from white muscle has been reported to have three "light" chains with molecular weights of 16,000, 18,000, and 25,000, while myosin from red muscle has only two "light" chains (20,000 and 27,000; see above references). The physiological significance of this heterogeneity remains to be elucidated.

Szuchet and Yphantis (1968, 1973) have used aqueous solutions of acetic acid without any added electrolyte to dissociate aldolase. Two considerations went into the choice of these experimental conditions. First, minimize the effect of preferential interaction with solvent components by using a dissociating agent whose $(1 - \bar{v}\rho)$ term is small (*e.g.*, acetic acid). Second, reduce aggregation (an undesirable complication found in most dissociating solutions) by working at very low ionic strength. For aldolase, these conditions proved successful and homogeneous solutions of subunits were obtained (*cf.* Szuchet and Yphantis, 1973). The system (*i.e.*, aqueous acetic acid without added electrolyte) leads to highly nonideal solutions. Nevertheless, the authors showed that reliable values for the molecular weight at infinite dilution are obtained.

The method of Szuchet and Yphantis appeared to offer interesting possibilities for solving some of the uncertainties (at the time) regarding the quaternary structure of myosin. In particular, the possibility of obtaining subunits free from aggregates appealed to us, since myosin's tendency to aggregate has been a major obstacle in structural studies. We have, therefore, used the method of Szuchet and Yphantis (*loc. cit.*) to dissociate myosin. We have found that aqueous acetic acid dissociates myosin in a manner similar to that of guanidine·HCl and quite distinct from alkaline pH. For instance, the two "heavy" polypeptide chains are dissociated from each other in acetic acid (Szuchet and Zobel, 1969). We have also found that under properly chosen experimental conditions (see text), 10 M acetic acid will yield a solution of "heavy" chains essentially free from irreversible aggregates. The present paper describes these experimental conditions, as well as the sedimenting properties of myosin in acetic acid.

Experimental Section

Materials. Rabbit skeletal myosin was prepared by minor modifications of the Szent-Györgyi (1951) procedure. All operations were carried out in the cold (5°) with precooled solutions.

Prior to treatment with acid, the freshly prepared protein solution (*ca.* 20 mg/ml in 0.5 M KCl–0.05 M phosphate buffer, pH ~7) was centrifuged at 50,000 rpm for 90 min. After removal of any lipid contaminants, the top $^1/_3$–$^1/_2$ of solution was withdrawn for use. The remainder was discarded.

Transference of the protein into acid was achieved in two steps: a dilution step, followed by dialysis. The protein solution was first diluted to approximately 4–5 mg/ml either with the same solvent (*e.g.*, 0.5 M KCl–0.05 M phosphate buffer) or directly with acid. In the latter case, care had to be taken to add the protein solution to the acid very slowly and with constant stirring, otherwise gelling occurred. The diluted protein was then dialyzed against the desired acid solution.

When time was an important factor, dilution with acid was preferred. No significant difference was noticed with either way of handling the samples.

Union Carbide dialysis tubings were used. The tubings were first boiled with 5% $NaHCO_3$, then washed with glass-distilled water and boiled in water until all the $NaHCO_3$ was removed. Finally, they were left in a 0.01 M EDTA solution (pH ~7) in the cold room. Prior to use, the tubing was thoroughly rinsed with glass-distilled water. If an organic solvent was being used, the tubing was soaked in that solvent for a couple of hours.

All chemicals used were the best available on the market.

Equipment and Methods. The Spinco Model E analytical ultracentrifuge used was equipped with electronic speed control, schlieren and Rayleigh interference optics, and a temperature control unit. This unit was calibrated against a National Bureau of Standards thermometer. The optics were aligned as described by Yphantis (1960, 1964). Focal positions for the camera lens were found for both quartz and sapphire windows. Photographs were recorded on Kodak spectrographic plates, emulsion type IIG. A high contrast developer H.R.P. (Kodak), was used for the development of plates.

High-speed sedimentation equilibrium experiments were carried out as described by Yphantis (1964) using externally loaded six channel "Rexolite" centerpieces (Ansevin *et al.*,

1970), with sapphire windows. This type of cell allows three concentrations to be run simultaneously. Routinely, concentrations were chosen so that $C_H/C_L \simeq 10$, where C_H = highest concentration and C_L = lowest concentration. Cells were loaded with concentrations increasing in the direction of the centrifugal field; channels A, B, and C, respectively.

The standard loading for channels A and B of a 12-mm cell was: 0.01 ml of FC-43[1] + 0.11 ml of solvent or solution, respectively. Channel C was either loaded as above or simply with 0.12 ml of solvent or solution, respectively. For a 30-mm cell, the volumes were 0.05 ml of FC-43 + 0.28 ml of solvent or solution. Alternatively, for channel C 0.33 ml of solvent or solution was used. Experiments were performed at 20°.

All solutions contained 0.1–1% sucrose. The function of sucrose is to provide a density gradient to stabilize the meniscus area against convective disturbances (Yphantis, 1964; Szuchet and Yphantis, 1973).

Photographic records of each experiment were made at 12 hr, 48 min, and/or 4 hr, 16 min intervals, depending on the total length of the experiment. After equilibrium had been achieved, a series of pictures with different exposures (usually 10–25 min) was taken, with the camera lens at the 2/3 focal position.

To correct for optical distortion, which is invariably present at the high speeds used, water blanks were run at the beginning and end of each experiment. The cell was not disassembled throughout the whole experiment; *i.e.*, the two blanks and the actual run. Washing of the assembled cell was done as described by Ansevin *et al.* (1970).

To improve blank reproducibility the "blank" run was left for 0.5 hr at each speed before photographs were taken. Nevertheless, blank reproducibility was not always satisfactory. A thorough discussion of all the factors which can contribute to a "poor" blank and suggestions on how to improve many were given by Ansevin *et al.* (1970).

For the measurements of plates, a Nikon Model 6C microcomparator was used. Procedural details for the alignment and measurements of plates have been given by Szuchet and Yphantis (1973).

Data were analyzed with a computer program developed by D. E. Roark and D. A. Yphantis (to be published; also Roark, 1971). The program calculates four of the standard apparent molecular weight moments: number, weight, Z, and $Z + 1$ as experimental observables, "σ," where $\sigma \equiv M(1 - \bar{v}\rho)\omega^2/RT$ (Yphantis, 1964). Here M is the molecular weight of the solute, $\bar{v}$ its partial specific volume; ρ, the solution density; ω, the angular velocity in radians/sec, and RT, the gas constant and the absolute temperature, respectively. The σ's (or apparent reduced molecular weights) are calculated as a succession of local (point) averages for each radial position. Thus, from the manner in which the local averages vary with position in the cell, or with concentration, information about the system can be obtained. Higher average moments are calculated only if the amount and quality of data ensures reliable values.

Another interesting feature of the program is its capability to calculate c_m, the concentration at the meniscus; this relaxes the previous requirement when manual calculations were used, to have $c_m \simeq 0$ (Yphantis, 1964).

Measure of Molecular Weights in Aqueous Solutions of Acetic Acid. We are concerned with a three-component system, with the macromolecule having a net charge, Z. The molecular weight in the limit, as $c \to 0$, of a monodisperse solute in such a system is given by

$$\frac{1}{M_{2,a}} = \frac{1}{M^*}\left(1 + \frac{z^2}{2}\frac{C_2}{M_2 m_3}\right) \quad (1)$$

where M^* is the effective molecular weight of the solute observed at infinite dilution, M_2 is the molecular weight, C_2 the concentration (in g/kg of solvent) of the solute, and m_3 is the molality of the third component (*e.g.*, supporting electrolyte; *cf.* Johnson *et al.*, 1954; Williams *et al.*, 1958).

The value of M^* is affected by the observation technique used but is independent of the definition of components. As pointed out by Szuchet and Yphantis (1973), for high-speed sedimentation equilibrium experiments, and if the assumption is made that the only interaction between the solute (component 3) and BX, the supporting electrolyte (component 3) is that imposed by the requirement of electroneutrality, then M^* is given by

$$M^* = M_{PX_z}\left(1 - \frac{z}{2}\frac{M_{BX}}{M_{PX_z}}\frac{(1 - \bar{v}_{BX}\rho)}{(1 - \bar{v}_{PX_z}\rho)}\right) \quad (2)$$

where the symbols are assigned their usual meaning. Equation 2 is formally identical with eq 3[2] with Γ (the preferential

$$\lim_{c_2 \to 0} M_{2,a} = M_2[1 + \Gamma(1 - \bar{v}_3\rho)/(1 - \bar{v}_2\rho)] \quad (3)$$

binding parameter) equal to: $\Gamma = -(z/2)(M_3/M_2)$ if component 2 is defined as PX_z or $\Gamma = 0$, if Scatchard's definition of components is adopted: $PX_{z/2}B_{-z/2}$ (Scatchard, 1946).

Equation 3 predicts that when $(1 - \bar{v}_3\rho) = 0$, the measured molecular weight will not be affected by preferential interactions with solvent components. For acetic acid, $(1 - \bar{v}_3\rho) = 0.133$ for a 1 M solution at 20° and 0.084 for a 10 M solution. Hence, errors introduced by preferential binding of acetic acid should not be too large. Szuchet and Yphantis (1973) have calculated the magnitude of the fractional error, ϵ [$\epsilon \equiv (M_{p,a} - M_p)/M_p$], incurred by using the partial specific volume for the native protein in obtaining molecular weights. For aldolase subunits in 1 M acetic acid, this error fell within the experimental uncertainties of molecular weight measurements, but for 10 M acetic acid the error was significantly higher (~6%).

A more rigorous approach, whenever experimentally feasible, is to measure ϕ' (the apparent specific volume), defined operationally by Casassa and Eisenberg (1964) as

$$\left(\frac{\partial\rho}{\partial c_2}\right)_\mu = 1 - \phi'\rho^0 \quad (4)$$

where ρ and ρ^0 are the solution and solvent densities, respectively; c_2 is the concentration of component 2 and the subscript μ indicates constancy of the chemical potential of all components diffusible through a semipermeable membrane (*i.e.*, solution densities are measured relative to the dialysates with which they were equilibrated).

Molecular weights for the "light" chains were calculated using the partial specific volume for the native protein. For the "heavy" chains, an apparent specific volume (ϕ') was measured, both in 1 and 10 M acetic acid.[3] Molecular weights

[1] Perfluorotributylamine, Minnesota, Mining and Mfg. Co.

[2] Equation 3 gives the molecular weight at infinite dilution of a monodisperse neutral solute in a three-component system (*cf.* Williams *et al.*, 1958).

[3] Apparent specific volumes were actually measured using dissociated myosin instead of purified "heavy" chains. The latter are obtained as sufficiently dilute solutions to preclude reliable density measurements with the equipment available.

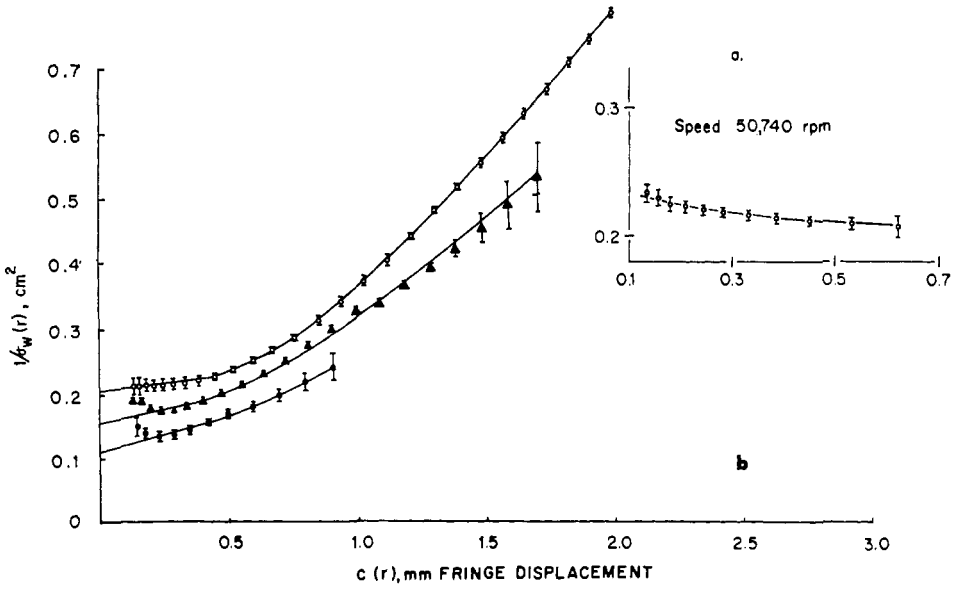

FIGURE 1: Plots of $1/\sigma_{w,a}(r)$ *vs.* $c(r)$ from a high-speed sedimentation equilibrium experiment of myosin in 10 M acetic acid + 0.1% sucrose, 12-mm cell, 20°: (a) 50,740 rpm, (○) channel C, $C_0 \simeq 0.106\%$; (b) 21,740 rpm, (●) channel A, $C_0 \simeq 0.015\%$; (▲) channel B, $C_0 \simeq 0.036\%$; (○) channel C, $C_0 \simeq 0.106\%$.

were, therefore, computed using ϕ', and compared to the values obtained when $\bar{v}_2$ (the partial specific volume for the native protein) was used instead.

Apparent Specific Volume. A stock solution of myosin was dialyzed thoroughly (48–72 hr), with constant stirring, against the desired solvent. The solution and dialysate were then filtered through 0.8-μ Millipore filters. Appropriate dilutions (with dialysate) were then made and densities of solutions and dialysate measured. Densities were measured in a 5 or 10 ml bulb-type pycnometer at 20 ± 0.01°, as described by Szuchet and Johnson (1966). At least four concentrations were used for every set of density measurements (ranging from 2.5 to 10 mg/ml); apparent specific volumes were then calculated from an average of at least three such sets.

Partial Specific Volume. A value of 0.720 ml/g was assumed for native myosin (Kay, 1960).

Densities. Solvent densities were estimated from data in the International Critical Tables, or measured as described above.

Concentration Measurements. Concentration of protein solutions were determined with a Gilford spectrophotometer using an extinction coefficient, $\epsilon_{280}^{1\%} = 5.50$ cm^{-1} (Godfrey and Harrington, 1970). No corrections were made for change of the extinction coefficient in acid.

Column Chromatography. Chromatographic media were swollen as recommended by the manufacturer. The swelling was always performed at room temperature. Prior to the packing of a column, time was allowed for the slurries to be equilibrated to the desired temperature. For gel filtration, 1–2 m long, 1 cm diameter columns were used. Routinely between 1 and 2.5 ml of solution was applied on such columns. For other types of chromatogrpahy, usually shorter and wider columns were preferred.

Acrylamide Gel Electrophoresis with Sodium Dodecyl Sulfate. The technique of Weber and Osborn (1969) with minor modification was followed. Thus, 5% sucrose, rather than glycerol, was used for applying the protein. Approximately 0.05 mg of protein in a volume not exceeding 100 μl was applied. Proteins were stained with 0.5% Amido Black dissolved in a mixed solvent (50 vol of CH_3OH + 50 vol of H_2O + 10 vol of CH_3COOH). The same solvent was used for destaining gels. Destaining was achieved either by electrophoresis or by simple diffusion. In the former case 1 M acetic acid was used for the chambers.

Results

When a high-speed sedimentation equilibrium experiment is performed on a solution of myosin in 1–10 M acetic acid, the following results are obtained. (1) At 52,000 rpm the bulk of the protein sediments to the bottom of the cell, leaving behind material of low molecular weight (Figure 1a). (2) When the speed is reduced to 22,000 rpm, the previously sedimented protein is redistributed, giving a much higher molecular weight (Figure 1b).

Figure 1 is a plot of the reciprocals of the apparent point average reduced molecular weights $[1/\sigma_{w,a}(r)]$ *vs.* concentration $[c(r)]$ from such an experiment. In Figure 1a results from the highest speed used (52,000 rpm) are presented. Only data from channel C (the highest loading concentration) are included. The two other concentrations used were too low (initially) to yield meaningful data at this high speed. In Figure 1b the effect of lowering the speed to 22,000 rpm is seen. In contrast to the case mentioned above, here data from the three loading concentrations are available, thus providing more information on the system. The fact that we have obtained an independent curve for each initial concentration is a good indication of heterogeneity (Yphantis, 1964).

To ascertain the absence of low molecular weight impurities, two control experiments were performed. In one, we tested whether further purification of native myosin (fractionation on a 2 m long Sephadex G-150 column) would result in a significant change of the low molecular weight fraction. In the other, we searched for low molecular weight material under otherwise identical conditions, but in a neutral solvent (*e.g.*, 0.5 M KCl–0.05 M phosphate buffer (pH ~7)). Both experiments led to the conclusion that no low molecular weight contaminants were present.

The Low Molecular Weight Component(s). To obtain more information on the low molecular weight component(s), we performed a high-speed sedimentation equilibrium experiment using more than twice the initial concentration used in

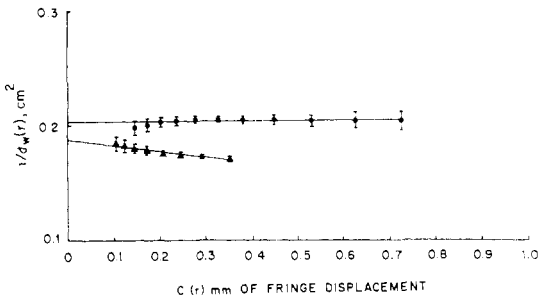

FIGURE 2: Plots of $1/\sigma_{w,a}(r)$ *vs.* $c(r)$ from a high-speed sedimentation equilibrium experiment of myosin at 52,640 rpm: solvent, 10 M acetic acid + 0.1% sucrose; 12-mm cell, 20°; (▲) channel B, $C_0 \simeq 0.082\%$; (●) channel C, $C_0 \simeq 0.237\%$.

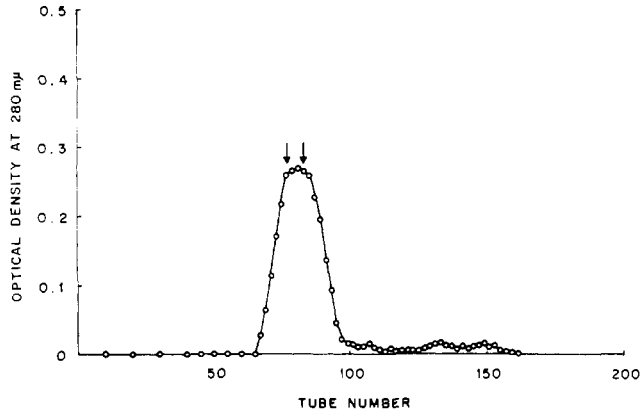

FIGURE 3: Fractionation of myosin in 1 M acetic acid. Elution profile from a (200 × 1) cm Sephadex G-200 column equilibrated with the same solvent.

the previous experiments. The purpose of this approach was to gather data from channels other than channel C, the highest initial concentration (see Figure 1a). Such data are indispensable if reliable information on the homogeneity of the low molecular weight material is to be obtained. The result of this experiment is shown in Figure 2 in the form of a plot of $1/\sigma_{w,a}(r)$ *vs.* $c(r)$. Only data from channels B and C are included. Channel A, the lowest initial (loading) concentration, still did not yield enough data to allow the calculation of point average molecular weights.

Examination of Figure 2 shows that the system is clearly heterogeneous. This heterogeneity is expressed in several ways. Thus, a direct manifestation of heterogeneity is the lack of superposition between the experimental point average reduced molecular weights, obtained at the two different loading concentrations (channels B and C), when plotted *vs.* their respective concentrations.

Heterogeneity can also be inferred from the peculiar behavior of data from the experiment at the highest loading concentration (channel C). Thus, the plot of $1/\sigma_{w,a}(r)$ *vs.* $c(r)$ (Figure 2) shows no concentration dependence. The solvent used, 10 N acetic acid without any added electrolytes, gives rise to a highly nonideal solution, with apparent reduced molecular weights decreasing rapidly as the concentration is increased (see Figure 1b). For the range of concentrations covered in Figure 2, a straight line with a positive slope (second virial coefficient, B) would be expected. A heterogeneous solution should give a negative virial coefficient. Since $B \cong 0$ (Figure 2), this must be taken as resulting from the fortuitous cancellation of heterogeneity and nonideality.

Extrapolation of data from channel C to $c = 0$ (meniscus) gives $[\sigma_w]_{c=0} = 4.90$ cm^{-2}; a similar extrapolation of data from channel B gives a slightly higher value ($[\sigma_w]_{c=0} = 5.32$ cm^{-2}). Assuming a partial specific volume of $\bar{v} = 0.725$ ml/g, and taking an average value for $[\sigma_w]_{c=0}$ from six independent experiments (data from the highest loading concentration), we obtain a weight average molecular weight of $[M_w]_{c=0} = 17{,}800 \pm 400$.

As mentioned above, insufficient data precluded the calculation of point average molecular weights from channel A (the lowest loading concentration). However, a plot of ln c *vs.* X (x-coordinate measurements) was linear and the slope of this line gave a weight average molecular weight of $M_w(r) = 27{,}500$.

Thus, the results of the experiment at the high loading concentrations gave a clear indication that the low molecular weight material was a complex system. Further evidence to support this conclusion came from an independent observation: purified "heavy" chains (*vide infra*) were frequently found contaminated with a variable, but, in general, very small amount of low molecular weight material. The weight average molecular weight of this fraction ranged from 25,000 to 30,000.

To facilitate comparison with the "alkaline" subunits, the experiment at high loading concentrations and high speed was repeated, using 0.4 M KCl–0.1 M Na_2CO_3 (pH 11) instead of acetic acid. Heterogeneity was evident in this system too. Thus, plots of $1/\sigma_{w,a}(r)$ *vs.* $c(r)$ gave three independent lines, one for each loading concentration (Yphantis, 1964). The weight average molecular weight at zero concentration obtained, $[M_w]_{c=0} = 17{,}900$, using $\bar{v} = 0.725$ ml/g is in excellent agreement with the value obtained from the analogous experiment in acetic acid.

In high-speed sedimentation equilibrium, a molecular weight average for the original solution can only be obtained by extrapolation to the base of the cell (see Yphantis, 1964). Such an average cannot be obtained from the experiments in acetic acid. Indeed, the nonideality of these solutions precludes extrapolation to the base of the cell.

The system at alkaline pH contains a high concentration of neutral salts and, therefore, does not exhibit nonideality effects to the same extent as are encountered in our systems with acid. Consequently, extrapolations to the base of the cell are permissible. From an extrapolation of $M_w(r)$ (data from channel A, see Yphantis, 1964) to the base of the liquid column, a Z-average molecular weight ($\bar{M}_z$) for the "initial"[4] solution of $\bar{M}_z = 27{,}400$ is obtained, revealing again, the presence of higher molecular weight species.

In summary, qualitatively the same results were obtained with alkali as with acid.

It was clear that the ultracentrifuge could not yield any further information on the low molecular weight components, unless an attempt was made to isolate the various molecular weight species, apparently present in this fraction.

Myosin dissociated in acetic acid can be fractionated on Sephadex equilibrated with the same solvent. Figure 3 shows the result of such a fractionation on Sephadex G-200. As can be seen in Figure 3, the main peak (corresponding to the "heavy" chains, see later) is well separated from the lower molecular weight species. Moreover, the latter are resolved in a series of peaks. We have found this method very useful for the preparation of "heavy" chains (see later);

[4] "Initial" refers to a hypothetical solution containing only the low molecular weight components. At the speed used, the "heavy" chains should be packed at the bottom of the cell and the assumption is made here that they do not contribute to the solute distribution.

but it is quite impractical for obtaining significant amounts of the lower molecular weight material. The main difficulty arises from the high viscosity of solutions of myosin in acetic acid, which limits considerably the range of concentrations which can be fractionated successfully.

Another approach was also possible. Shapiro *et al.* (1967) were the first to show that proteins denatured with sodium dodecyl sulfate migrated in acrylamide gel electrophoresis with rates proportional to their particle size. Furthermore, it became apparent from the work of Weber and Osborn (1969), that myosin "light" chains could be resolved by this method. Since these authors have also shown that reliable values for the molecular weight of protein subunits can be obtained from sodium dodecyl sulfate acrylamide gel electrophoresis, we have used this technique, primarily, as a guide for the interpretation of our data from the ultracentrifuge (Szuchet and Zobel, 1971).

The composition of myosin "light" chains, as obtained from sodium dodecyl sulfate acrylamide gel electrophoresis, has now been elucidated. Several papers have appeared over the last 2 years, bearing on this point (see introduction). The results presented here are, essentially, in agreement with those published.

In Figure 4 the electrophoretic patterns of a preparation of myosin, with different degrees of purification, are compared. Figure 4a corresponds to a solution of myosin prepared by the standard procedure (see Methods); Figure 4b shows the same myosin after $(NH_4)_2SO_4$ precipitation (45% saturation); and Figure 4c shows the latter solution after chromatography on DEAE-Sephadex A-50 according to the procedure of Richards *et al.* (1967).

Analysis of Figure 4 reveals that during the process of purification only four bands remained unaltered: the slowest band at the top of the gel (1) and the three fastest bands (2, 3, and 4). To correlate these bands to the components we have detected with the ultracentrifuge, migration in the gels were calibrated in terms of molecular weights, by using proteins of known molecular weight. Thus, band 1 which migrates in the gel with an apparent molecular weight of $(2\text{–}2.2) \times 10^5$, must correspond to the species we detect at 22,000 rpm ("heavy" chains). Bands 2, 3, and 4, with apparent molecular weights (in the gel) of 27,500, 17,800, and 15,400, respectively, must correspond to the components "seen" in the ultracentrifuge at 52,000 rpm.

If we assume that acetic acid and sodium dodecyl sulfate dissociate myosin in a similar manner (*i.e.*, they yield the same products of dissociation) and if we further assume that our molecular weights from sodium dodecyl sulfate acrylamide gels are accurate within $\pm 10\%$,[5] then the molecular weight obtained from high-speed sedimentation equilibrium (*i.e.*, 17,800) must represent a weight average molecular weight of components 3 and 4 (Figure 4). In effect, under the experimental conditions used, the difference in the apparent reduced molecular weights (σ) of the two components concerned would only be 0.6 (*i.e.*, $\sigma_3 - \sigma_4 \cong 0.6$). Such difference would not be expected to give rise to a significant fractionation in the meniscus area. Moreover, if we accept the stoichiometry recently published by Lowey and Risby (1971), the ratio of these proteins on a per cent basis is 1:3. This, together with the molecular weights obtained from acrylamide gels (15,400 and 17,800, respectively), gives a weight average molecular weight of 17,200, in good agreement with the value obtained.

[5] The values reported here are in good agreement with those given by other workers (see introduction).

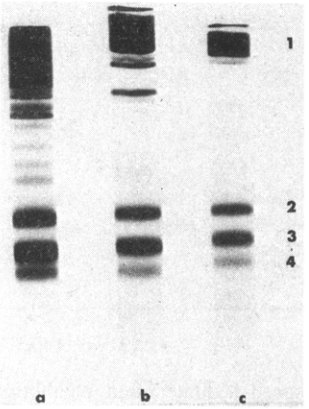

FIGURE 4: Acrylamide gel electrophoresis with sodium dodecyl sulfate. Effect of different purification procedures for native myosin on the patterns obtained: (a) myosin obtained by Szent-Györgyi procedure; (b) the same myosin after precipitation with $(NH_4)_2SO_4$; (c) myosin from (b) after fractionation on DEAE-Sephadex G-50.

A significantly different molecular weight, *viz.*, 27,500, was obtained from data of channel A (*vide supra*). Taken in isolation, not too much weight can be attached to this value. However, taken in conjunction with data from acrylamide gel electrophoresis, we can conclude that it must represent an apparent average of the three proteins concerned (bands 2, 3, and 4).

During the work with sodium dodecyl sulfate, one of us (S. S.) made the following observations: when a solution of myosin at high concentration (~20 mg/ml) is dialyzed against a phosphate buffer containing 0.05–0.1% sodium dodecyl sulfate, the solution gels. The liquid trapped within the gel contains all the fast moving components. Based on this observation, a technique for the preparation of the low molecular weight fraction has been developed. A full account of this technique, as well as further characterization of the low molecular weight fraction, will be given separately (S. Szuchet, paper in preparation).

"*Heavy*" *Polypeptide Chains.* As mentioned earlier, when the speed of a high-speed sedimentation equilibrium experiment of myosin in acetic acid is reduced from 52,000 to 22,000 rpm, the distribution shown in Figure 1b is obtained. Notice the high nonideality of the system and also the heterogeneity (Figure 1b). Part of the observed heterogeneity can be accounted for by the low molecular weight fraction described above.

Solutions of "heavy" chains, virtually uncontaminated with light material, can be obtained by fractionating dissociated myosin on Sephadex G-200 columns (2 m long) using 1 M acetic acid (Figure 3). The success of such a fractionation is highly dependent upon the total concentration of the sample to be applied. The lower the concentration the better the resolution. However, starting with a too low concentration has the disadvantage that the individual fractions do not contain enough material for direct characterization, thus requiring further handling of the samples.

Based on the observation that during dialysis against 10 M acetic acid there is an effective twofold increase in concentration, we have developed a procedure which allows us to use solutions of low concentration for the fractionation step and still obtain individual fractions sufficiently concentrated for equilibrium runs. Briefly, the procedure consists in fractionating myosin in 1 M acetic acid at concentrations of ~2–4 mg/ml and dialyzing the desired fractions, generally those with the highest optical density at 280 nm, against 10 M acetic acid.

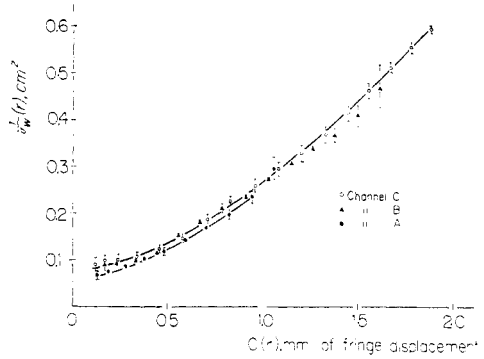

FIGURE 5: High-speed sedimentation equilibrium experiment on purified "heavy" chains: solvent, 10 M acetic acid + 0.1% sucrose; speed, 22,000 rpm, 20°; (●) channel A, $C_0 \simeq 0.015$; (▲) channel B, $C_0 \simeq 0.033$; (○) channel C, $C_0 \simeq 0.079\%$.

Equilibrium runs were, therefore, performed in this solvent.

The effect of reducing the concentration for the fractionation step is manifested in a much sharper elution pattern. But, more important, the major component appears to be virtually free of lower molecular weight contaminants.

Contents of tubes 78–83 inclusive (marked with an arrow in Figure 3) were pooled, dialyzed against 10 M acetic acid, and studied by high-speed sedimentation equilibrium. For a more critical examination, three speeds were used in this experiment: 52,000, 22,000, and 15,000 rpm. The speeds were chosen so as to detect the low molecular weight fraction, the "heavy" chains and undissociated myosin.

Measurements of the photographic plates from the experiment at 52,000 rpm after blank correction (see Methods) revealed a slight gradient (0.044 mm, 0.065 mm, and 0.120 mm of fringe displacement at the bottom of channels A, B, and C, respectively). We believe that at least part, and probably all, of these gradients have arisen from uncorrected optical distortions (Ansevin *et al.*, 1970). Support for this hypothesis can be surmised from the lack of blank reproducibility and the fact that the blank at the end of the experiment gave the smaller gradient (see Ansevin *et al.*, 1970). This was particularly significant for channel C. Furthermore, a series of consecutive experiments, in the same ultracentrifuge cell without disassembling, showed a progressively increasing gradient related solely to the number of hours the cell had been in use.

The arguments outlined above lend strong support to our contention that the fraction eluted from Sephadex G-200 ("heavy" chains) is free from low molecular weight contaminants. Moreover, even if these slight gradients (see above) do, indeed, represent protein contaminants, their amount is truly insignificant.

In Figure 5 the solute distribution at 22,000 rpm is presented. Comparison of Figure 5 with Figure 1b shows that the purity of the fraction has improved considerably. In effect, judging from the superposition of the experimental points arising from the three different loading concentrations, the system appears reasonably homogeneous. Nevertheless, a small amount of irreversible (within the time required to reach equilibrium) aggregates still appear to be present in this solution as evidenced by a slight separation (almost within error bars) of data from channel A,[6] from the line determined by the remaining experimental points (channels B and C).

[6] Channel A corresponds to the lowest initial concentration; here observations to the base of the cell can be made and, therefore, aggregates are mainly detected in this channel.

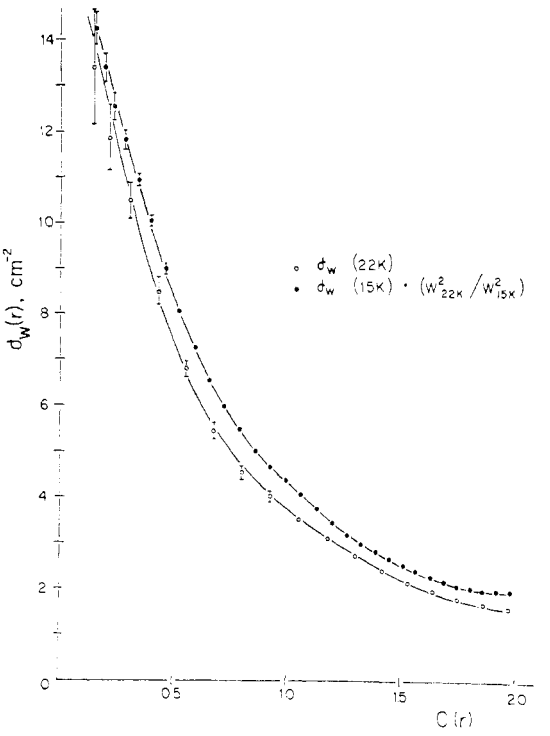

FIGURE 6: Purified "heavy" chains. Comparison of the effect of speed on the apparent reduced weight average molecular weight ($\sigma_{w,a}$) distribution: (○) 22,000 rpm; (●) 15,000 rpm; solvent; 10 M acetic acid + 0.1% sucrose, 20°, $C_0 \simeq 0.079\%$.

The presence in this solution of species with molecular weights higher than that of "heavy" chains is revealed more fully at 15,000 rpm. This is illustrated in Figure 6 where the reduced molecular weights (σ's) obtained at two different speeds, 22,000 and 15,000 rpm, are compared. Since σ is a function of the square of the angular velocity (w^2), data at the lower speed were corrected by multiplying them by the ratio, w_H^2/w_L^2, where the subscripts H and L refer to high and low speed, respectively. Notice that throughout the whole molecular weight distribution, the apparent reduced molecular weights [$\sigma_w(r)$] obtained at the lower speed (15K) are higher than the corresponding σ's at the higher speed (22K) (Figure 6). The curves, however, do converge at lower concentrations, and can be extrapolated to yield the same molecular weight at infinite dilution; admittedly, within greater than usual experimental uncertainty.

By reducing the speed from 22,000 to 15,000 rpm, we have effectively reduced the centrifugal field by a factor of 2. Thus, high molecular weight particles which had sedimented to the bottom of the solution at 22,000 rpm are redistributed at the lower speed and thereby contribute to the molecular weight distribution.

Figure 6 also shows very clearly the extreme nonideality of solutions of "heavy" chains in 10 M acetic acid (S. Szuchet, paper in preparation).

The extent of heterogeneity found in 10 M acetic acid varied for the different preparations. A few solutions appeared virtually homogeneous; however, the majority did show a discernible heterogeneity when subjected to a critical analysis.

In the experiment described above, fractions from a Sephadex G-200 column in 1 M acetic acid were first dialyzed against 10 M acetic acid and then studied by high-speed sedimentation equilibrium. If a similar study is made on the fractions without prior dialysis, *i.e.*, in 1 M acetic acid, the results shown in Figure 7 are obtained. One important point becomes apparent

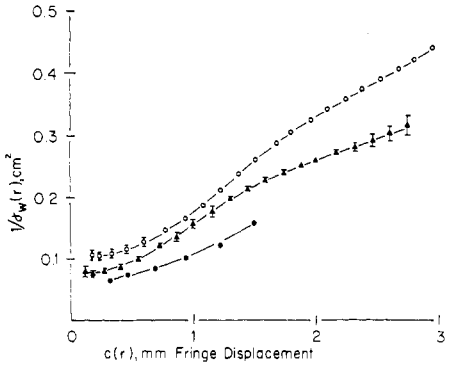

FIGURE 7: High-speed sedimentation equilibrium experiment of purified "heavy" chains in 1 M acetic acid + 0.1% sucrose: speed, 21,740 rpm, 20°; (●) channel A, $C_0 \simeq 0.013\%$; (▲) channel B, $C_0 \simeq 0.038\%$; (○) channel C, $C_0 \simeq 0.073\%$.

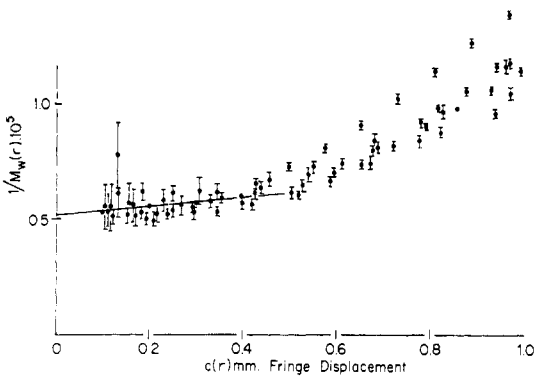

FIGURE 8: Purified "heavy" chains in 10 M acetic acid. Plots or reciprocals of the apparent point weight average molecular weights $[1/M_{w,a}(r)]$ *vs.* c(r). Experiments at 18,000 and 22,000 rpm. Straight line is a least-squares fit.

when Figure 7 is compared with Figure 5: heterogeneity is greatly reduced when the solvent is changed from 1 M acetic acid (Figure 7) to 10 M acetic acid (Figure 5), indicating 10 M acetic acid to be a much more powerful dissociating agent for myosin than 1 M acetic acid. This is in accord with a similar observation made by Szuchet and Yphantis (1973) in the case of aldolase.

The Molecular Weight of the "Heavy" Polypeptide Chains. It is a recognized fact that most macromolecular solutions exhibit nonideality (*i.e.*, concentration dependence). For the common type of nonidealities encountered in biological systems (*e.g.*, excluded volume effects; Donnan effects; reversible association), the behavior of the system can be represented by

$$\frac{1}{M_{w_2,a}} = \frac{1}{M_{w_2,i}} + 2Bc_2 \qquad (5)$$

where B is the colligative second virial coefficient (Tanford, 1961).

Inspection of Figures 5 and 7 shows that "heavy" chains in 1 or 10 M acetic acid exhibit a more complex concentration dependence, and only approach the behavior predicted by eq 4 in the limit as $c \rightarrow 0$; thus, from the intercept and limiting slope of a plot of $1/\sigma_{w,a}(r)$ *vs.* $c(r)$, the apparent reduced molecular weight at infinite dilution $[\sigma_{w,a}]_{c=0}$ and the second virial coefficient (B) can be obtained, respectively. This extrapolation is performed with data from the experiment at the highest initial concentration (channel C), to obtain information on the smallest species present (Yphantis, 1964).

From the experiments in 1 M acetic acid we have obtained a $(1/\sigma_{w,a})_{c=0} = 0.087 \pm 0.007$ cm^2. The experiments in 10 M acetic acid were performed at either 18,000 or 22,000 rpm; the corresponding values of $(1/\sigma_{w,app})_{c=0}$ are 0.138 ± 0.007 and 0.092 ± 0.006 cm^2, respectively. These σ's are readily converted into the respective molecular weights by using the appropriate factor: $RT/(1 - \phi'\rho^0)w^2$, where $(1 - \phi'\rho^0) = (\partial\rho/\partial c_2)_\mu$ (Casassa and Eisenberg, 1964).

Densities and concentrations of protein solutions were measured as described (see Methods). From the slope of a plot of ρ (solution density) *vs.* c_2 (protein concentration), the term $(\partial\rho/\partial c_2)_\mu$ was calculated. There are a number of uncertainties inherent in $(\partial\rho/\partial c_2)_\mu$ as obtained here: first, densities were measured on dissociated myosin and not on purified "heavy" chains (*i.e.*, low molecular weight proteins were also present in these solutions); second, although concentrations (optical densities at 280 nm) were measured on the same solutions, the exact extinction coefficient was unknown and, therefore, the extinction coefficient for the native protein had to be used instead. Undoubtedly, these uncertainties will introduce an error in the final value of the molecular weight, whose magnitude we shall attempt to assess.

The change in absorptivity for most proteins, upon denaturation by different reagents, is of the order of ±5–10%, with a shift in the spectral maxima of 1–2 nm (Herkovits, 1965). This appears to hold for myosin in 5 M guanidine·HCl, where a change of ~−7 to 10% has been reported (Woods *et al.*, 1963; Frederiksen and Holtzer, 1968). Moreover, the extinction coefficient of the "light" chains in 5 M guanidine·HCl does not differ significantly from that of the "heavy" chains in the same solvent (Gazith *et al.*, 1970).

In 10 M acetic acid (using the extinction coefficient for the native protein), we have obtained an apparent specific volume, $\phi' = 0.704 \pm 0.007$ ml/g. The apparent specific volume of dissociated myosin and purified "heavy" chains in 5 M guanidine·HCl was given as 0.710 ± 0.005 ml/g (Woods *et al.*, 1963; Gazith *et al.*, 1970). This latter value agrees rather well with our value. However, the agreement may be coincidental, since the apparent specific volume is a resultant of all the interaction parameters in a given solvent, and there are no *a priori* reasons for these to be the same in 5 M guanidine·HCl and 10 M acetic acid.

On the other hand, if we assume that our concentrations are in error by ~−7% and correct them accordingly, we calculate: $\phi' = 0.720$ ml/g, which is not too different from the apparent specific volume for the native protein (in 0.5 M KCl + 0.05 M phosphate buffer (pH 7), we have obtained $\phi' = 0.722 \pm 0.005$ ml/g). Thus, while there is a possibility that the measured value (0.704 ml/g) is in error, it is also equally possible that our assumed extinction coefficient leads to an overcorrection.[7] We have, therefore, adopted the "middle of the road" approach by taking an average of these two values.

When the apparent reduced molecular weights at infinite dilution, from experiments at 18,000 and 22,000 rpm (*vide supra*), were converted into the respective molecular weights, we obtained 204,000 and 205,000, respectively (if the uncorrected apparent specific volume would have been used instead, these values would have been 197,000 and 198,000, respectively). Each value represents an average of several independent experiments (2 at 18,000 rpm and 4 at 22,000 rpm). The combined data are presented in Figure 8, where we

[7] Gershman *et al.* (1966) reported a constancy (within ±2%) in the absorption at 280 nm of a solution of myosin, when its pH was varied from 6.5 to 12.5.

have plotted the apparent point average molecular weights $[1/M_w(r)]$ *vs.* their point concentrations $c(r)$. At low concentrations (*ca.* 0.5 g/l.) there is good agreement between the various experimental points and they can be fitted with a straight line. At higher concentrations (*i.e.*, as we move toward the base of the cell), the effect of the different degrees of heterogeneity in the individual experiments (solutions) becomes apparent and the experimental points are more scattered (Figure 8). In particular, it is interesting to note that one set of experimental points deviates more strikingly from the others (Figure 8). These experimental points were obtained with a virtually homogeneous solution of "heavy" chains, and what we are seeing is a full expression of the nonideality (S. Szuchet, in preparation). In all the other cases, this nonideality has been masked to different extents by the heterogeneity of the system.

The measured apparent specific volume in 1 M acetic acid is 0.717 ± 0.005 ml/g. This value agrees, within experimental error, with the partial specific volume ($\bar{v}_2$ = 0.720 ml/g) obtained by Kay (1960) for the native protein. Although our measurements in 1 M acetic acid are subject to the same criticisms as those in 10 M acetic acid (*vide supra*), we do believe the data are not affected to the same extent. We base our belief on the results obtained by Szuchet and Yphantis (1973) with aldolase. The weight average molecular weight in 1 M acetic acid thus obtained is 195,000 (average of two experiments). Taking an average over all our measurements (1 and 10 M acetic acid) we obtain a weight average molecular weight at infinite dilution of $[M_w]_{c=0}$ = 202,000 ± 7000. Again, this value would be lowered to 197,000 ± 2000 were the uncorrected ϕ' used, instead.

Discussion

It is not our objective in this paper to discuss the mechanism whereby myosin is dissociated by acetic acid. It is, however, evident that dissociation is favored by an increase in the concentration of acid. In effect, when a solution of myosin in 1 M acetic acid (Figure 7) is transferred to 10 M acetic acid (Figure 5) most of the heterogeneity disappears. Since this heterogeneity has been shown to arise from the presence of species with high molecular weight (higher than "heavy" chains), these species (aggregates), be it undissociated myosin or products of association of dissociated myosin, must be reversible with higher concentration of acid. As previously pointed out by Szuchet and Yphantis (1973), it is likely that more than one factor from charge repulsion to hydrogen bond formation (see Tanford, 1970; Cann, 1971) is operative in the actual dissociation.

The uncertainties in our measurements of apparent specific volumes led to two possible values for the weight average molecular weight of the "heavy" chains in acetic acid; namely, 202,000 ± 7000 and 197,000 ± 2000. Although these two values are within experimental error of each other, we tend to favor the lower value as being more representative of the actual molecular weight of the "heavy" chains. Two considerations enter in this preference: first, the molecular weight obtained in 1 M acetic acid, 195,000 (ϕ' = 0.717 ml/g) or 197,000 ($\bar{v}_2$ = 0.720 ml/g), is in better agreement with the lower value;[8] second, the value given by Gazith *et al.* (1970), 194,000, would also suggest that the lower value should be preferred.

Reducing the speed from 22,000 to 18,000 rpm did not result in any change of the apparent molecular weight distribution and the two curves were extrapolated to the same molecular weight. Consequently, since myosin has two "heavy" chains, our results lead to the conclusion that the molecular weight of these chains cannot be too dissimilar (within the errors of our experiments). This conclusion is consistent with the results from acrylamide gel electrophoresis, where the "heavy" chains gave a single band (Figure 4c, band 1); admittedly, rather broad.

The findings (corroborated by different laboratories) that there is more than one type of low molecular weight protein strongly associated with myosin (irrespective of, whether or not they are essential) set a lower limit on the molecular weight of myosin.

There is, in general, good agreement in the literature on the molecular weight of the heavy chains. Assuming a value of 2×10^5 (this represents an approximate average of the values found in the literature, including our own), and using the stoichiometry for the low molecular weight proteins given by Lowey and Risby (1971), we obtain 4.77×10^5 as a tentative value for the minimum molecular weight of myosin. [Lowey and Risby (1971) assumed a molecular weight of 4.7×10^5 for their calculations. Lowering this value to 4.5×10^5 does not affect the stoichiometry.]

The molecular weight of myosin has been the object of detailed studies recently. Using high-speed sedimentation equilibrium, Godfrey and Harrington (1970) obtained a value of 458,000. A slightly higher value (468,000) was given by Dreizen and Gershman (1970). We have also reinvestigated the molecular weight of myosin, at different speeds and in different solvents (the results of these studies will be published separately); we obtain a value of 472,000.

Undoubtedly, the last word on myosin has not been said. As our knowledge on the composition and biological function of the low molecular weight proteins accumulates and as their relation to myosin is better understood, a redefinition of myosin molecule proper will be in order.

Acknowledgment

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[8] The molecular weight in 1 M acetic acid is not affected significantly whether ϕ' or $\bar{v}_2$ is used. Since, Szuchet and Yphantis (1973) have shown that use of $\bar{v}_2$ yields a correct molecular weight for aldolase, we are assuming that this also holds for myosin and, therefore, conclude that ϕ' cannot be greatly in error.

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Extraction and Purification of Calcium-Activated Photoproteins from the Ctenophores *Mnemiopsis* sp. and *Beroë ovata*†

William W. Ward‡ and Howard H. Seliger*

ABSTRACT: Calcium-activated bioluminescent "photoproteins" from the ctenophores *Mnemiopsis* sp. and *Beroë ovata* have been isolated by extraction with EDTA and purified 3800-fold and 7800-fold, respectively. The steps consisted of ammonium sulfate and protoamine sulfate treatments, followed by several steps of ion-exchange, gel filtration, and solubility chromatography. The purified photoproteins are virtually homogeneous by the criterion of sodium dodecyl sulfate polyacrylamide gel electrophoresis. Approximately 30,000 adult specimens of *Mnemiopsis*, totalling more than 600 kg wet wt, yielded 2 mg of purified photoprotein. *Mnemiopsis* photoprotein has been resolved into two functionally identical forms ("isoproteins"), termed mnemiopsin-1 and mnemiopsin-2, by chromatography on DEAE-cellulose. Berovin, the *Beroë* photoprotein, chromatographs as a single symmetrical peak under the same conditions.

Bioluminescent proteins, termed "photoproteins" (Shimomura and Johnson, 1966), were originally isolated from the hydromedusae, *Aequorea* (Shimomura *et al.*, 1962, 1963a; Shimomura and Johnson, 1969, 1970) and *Halistaura* (Shimomura *et al.*, 1963b). Similar photoproteins have been isolated from the colonial hydroid, *Obelia* (Morin and Hastings, 1971a,b), and the ctenophore, *Mnemiopsis* (Morin and Hastings, 1971a,b; Ward and Seliger, 1973a,b). These photoproteins differ from the classical luciferin–luciferase system in having no exogenous requirement for diffusible organic substrates or molecular oxygen. Bioluminescence is an intramolecular reaction triggered by the addition of Ca^{2+} or Sr^{2+} (Kohama *et al.*, 1971). There is no evidence to date that these photoproteins turn over and no system has been found *in vitro* that can restore bioluminescent activity to a "spent" reaction mixture.

The photoprotein aequorin, extracted from *Aequorea* with EDTA, has been purified to homogeneity by alternate chromatography on G-100 Sephadex and DEAE-cellulose (Shimomura and Johnson, 1969). The present paper describes a

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