upon inactivation are analogous to the processes occurring in myosin upon covering of certain of its —SH groups (Kominz, 1961), except that in myosin the alterations are reversible. It appears as though the Ca-ATP prosthetic group is holding two parts of the native G-actin molecule in a metastable configuration, as suggested by Strohman and Samorodin (1962). It is not clear whether the role of the Ca-ATP prosthetic group in giving direction to the polymerization of native actin is simply to hold the molecular structure intact in this way, or whether it is to participate directly in bond formation, as inavailability to exchange of ADP in F-actin suggests (Martonosi *et al.*, 1960).

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The Molecular Weight of Rabbit Myosin A by Light Scattering

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A light scattering determination of the molecular weight of myosin was undertaken with particular attention to the measurement of auxiliary parameters, especially specific refractive index increment (dn/dc). A dn/dc, on a dry weight basis, of 0.192 ml/g was obtained. This was in good agreement with values found for other proteins but at variance with some values which have been used for myosin. A molecular weight of 5.2×10^5 g, including a 4% contribution due to bound salt, was obtained.

The recent proposal by Kielley and Harrington (1960) of a three-stranded model for the myosin molecule has stimulated new interest in the varying figures reported for the molecular weight of myosin.

Some recent studies using the Archibald analysis of sedimentation data (von Hippel *et al.*, 1958; Mommaerts and Aldrich, 1958) and light scattering (Brahms, 1959) have yielded molecular weights near 420,000. Two other light scattering determinations (Holtzer and Lowey, 1959; Holtzer *et al.*, 1962) gave values near 500,000 g/mole though widely differing numbers for the specific refractive index increment were employed. Molecular weights calculated from sedimentation (von Hippel *et al.*, 1958; Holtzer and Lowey, 1959; Parrish and Mommaerts, 1954; Johnson and Rowe, 1960) and diffusion (Parrish and Mommaerts, 1954; Johnson and Rowe, 1960) measurements cluster around 550,000 g. Kielley and Harrington's extensive studies using the Archibald method gave 619,000 g/mole.

The range of these figures, combined with a molecular weight of 206,000 g for the myosin subunit in guanidine·HCl (Kielley and Harrington, 1960) (the only value so far reported) is sufficient to include two-stranded as well as three-stranded models for native myosin.

We have undertaken yet another light scattering

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TABLE I

Prep. No.	$D^{1\%}_{279}$	*dn/dc* (ml/g)[a]	M_w (g)	R_g (A)[b]	*B*
1	5.04	0.186			
2	5.54	0.190			
3	5.58	0.196			
4	-	0.198			
5	5.43	0.189	5.2×10^5	483	0.72×10^{-4}
6a	5.49	0.192	5.1	496	1.25
6b			5.5	489	0.31
7a	5.47	0.192	5.0	453	0.63
7b			5.0	451	0.63
Mean	5.43	0.192	5.2×10^5	474	0.71×10^{-4}
Standard error of mean	0.08	0.002	0.1	9	0.15

[a] 3.0°. [b] 11°; see text.

determination of the molecular weight of myosin because we felt that, profiting from the experience of earlier authors, we might obtain a reliable value by careful attention to certain experimental details. In particular, we intended to resolve the widely varying values of the specific refractive index increment (*dn/dc*) used in previous studies (Brahms, 1959; Holtzer and Lowey, 1959; Holtzer *et al.*, 1962; Rupp and Mommaerts, 1957; Kay, 1960). Since this quantity enters quadratically into the molecular weight calculation, the 15% variation in reported values of *dn/dc* leads to a range of uncertainty of more than 30% in molecular weight.

EXPERIMENTAL

Materials.—Rabbit skeletal myosin A was prepared by the method of Kielley and Bradley (1956) and stored in the cold in 0.5 M KCl. Most experiments were done with preparations less than 10 days old. Light scattering measurements were performed with preparations between 2 and 6 days old. For dry weight, refractive index increment, and light scattering determinations, the myosin was diluted to roughly 10 mg/ml and dialyzed against several changes of 0.5 M KCl. The final dialysis was continued for at least 15 hours to permit complete equilibration. During dialysis, the *p*H of the dialysate was monitored and adjusted upward to 6.8–7.0 with KOH solution when necessary.

Concentration Measurements.—Protein concentrations of all myosin preparations were determined by a dry weight method. Samples were weighed into flat weighing bottles and dried, first at 40° under partial vacuum to remove most of the water, finally at 105° under full vacuum over P_2O_5 (kept at room temperature) for several days. Initial volumes were computed from known solution densities. Samples were dried until constant weight, to 0.1 mg, was maintained for at least a day. Parallel samples of the final KCl dialysate were also weighed and dried, and the appropriate weight of KCl was subtracted from the protein dry weights, taking into account the exclusion of salt from the volume occupied by the protein molecules. Measurements were made in triplicate with sample volumes of 4 to 10 ml (40 mg to 100 mg of myosin). The triplicate samples were deliberately taken of varying size as a check against systematic errors in drying and weighing. Since myosin solutions tend to form a hard crust and then dry very slowly, the expedient of adding 10 μl of a 1% trypsin solution to each sample was adopted. This greatly speeded the drying process. Dry weights were corrected for water of hydrolysis introduced by the trypsin-catalyzed proteolysis (~50 moles $H_2O/10^5$ g myosin [Mihalyi and Harrington, 1959], or roughly 1% of the dry weight). Concentrations are accurate to ± 2%.

Kjeldahl nitrogen analyses were also performed on three of the preparations. A nitrogen factor of 5.99 (Bailey, 1948) was used. The Kjeldahl results were lower than the dry weights, presumably owing to a bound salt contribution to the dry weight (see discussion).

Specific Refractive Index Increment.—This parameter was measured in a wedge cell (Svensson and Odengrim, 1952, modified by H. A. Saroff) with myosin solution in one compartment and its dialysate in the other. The interference optical system of an Aminco Model B electrophoresis apparatus was used at a wave length of 436 mμ. The temperature was 3.0°. The calibration of the cell was known from geometrical considerations alone but was also checked with several KCl solutions of known concentrations, at 25°, using the data of Stamm (1950). The agreement was better than 0.2%.

In this system a myosin solution of 1% concentration produces roughly 40 fringes on the photographic plate. The displacement was measurable to less than a tenth of a fringe. The error is well below that in the protein concentration, which then limits the accuracy to which *dn/dc* is known. All *dn/dc* values were calculated on the basis of dry weight concentration, as were the other figures given in Table I.

Light Scattering.—Measurements were made in a Brice Phoenix Light Scattering Photometer at 436 mμ. Cylindrical cells with flat entrance and exit windows were used; the customary reflection correction (Sheffer, and Hyde, 1952) was applied to the data. The cells were calibrated with Ludox suspensions which were fractionated by differential centrifugation and then passed through a Millipore filter (type HA). The optical density of the Ludox suspensions was measured in a 10-cm cell in a Cary model 14 spectrometer. Several Ludox concentrations were used, and the calibration constant was extrapolated to zero concentration (Maron and Lou, 1954). The optical quality of the Ludox was checked by its adherence to the fourth-power scattering law, and by its small dissymmetry (~ 1.03).

The angular distribution of light from the cells was checked with dilute fluorescein solutions, a green filter being used before the photomultiplier to remove the scattered light.

For the myosin work a cooled cell table was used, and a stream of cold dry air was led into the photometer through the light trap. By these means, the temperature of the solution was held at 10–12°. (In our experience, letting myosin solutions stand at room temperature for 30–60 minutes caused a barely perceptible change in the scattering envelope.)

For light scattering, stock myosin solutions of known concentration were diluted gravimetrically with dialysate. Diluted samples were centrifuged under refrigeration for 2 hours at 24,000 rpm in the No. 30 rotor of a Spinco model L preparative ultracentrifuge (50,000 × *g*) using plastic tubes and plastic-coated metal caps. The solutions were then transferred with a pipet to the scattering cells, care being taken to avoid the sedimented material and surface dust in the centrifuge tubes. Pipets and cells were pre-rinsed with Millipore-filtered solvent. All glassware used in these experiments was silicone coated ("Siliclad," Clay-Adams Co., N. Y.). Sample concentrations, calculated from the gravimetric dilution, were corrected for the fraction of protein lost in the centrifuge by measuring the optical density, at 279 mμ, of the diluted solutions before and after centrifugation. Samples uniformly lost 6% to 7% of their optical density during centrifugation.

These measurements also give a fairly precise $D^{1\%}_{279}$ for mysoin (Table I) which differs significantly from some values previously used for extinction coefficient.

Scattering envelopes were recorded over the angular range 27° to 135° for each of four concentrations. The weight average molecular weight (M_w), radius of gyration (R_g), and second virial coefficient (B), were obtained by the usual Zimm (1948) double extrapolation method.

One of our five Zimm diagrams is shown in Figure 1. Most weight is assigned to the points taken at intermediate angles. The points at very low angles are included to indicate the relative freedom of our preparations from contaminant large particles. The arcing over of the curves at higher angles has been previously discussed in the literature (Holtzer *et al.*, 1962).

Results and Discussion

Table I summarizes our results, obtained with seven myosin preparations. The final value of 0.192 ml/g for dn/dc at 436 mμ falls well within the normal range for proteins (Perlmann and Longsworth, 1948; Halwer *et al.*, 1951) and agrees closely with some previous figures for myosin (Rupp and Mommaerts, 1957; Kay, 1960).

The light scattering studies give a weight-average molecular weight of 5.2 × 10^5 g, based on five experiments. We may note that, after the first successful experiment, no results were discarded. Since our result lies squarely between those expected for two and for three strands (taking the subunit weight as 2.06 × 10^5 g), it gives no help in deciding between these alternatives. Several attempts at light scattering measurements of myosin in 5 M guanidine·HCl, with the goal of seeing whether this discrepancy might be accounted for by an internal calibration factor, have been uniformly unsuccessful owing to extreme curvature of the angular scattering envelope, probably caused by large aggregates.

In disagreement with some previous light-scattering studies (Brahms, 1959; Holtzer and Lowey, 1959) we find a non-zero second virial coefficient (B = 0.71 × 10^{-4} moles ml/g²), though admittedly with poor precision. Our result is in better agreement with the results of Portzehl (1950) and Kielley and Harrington (1960) (0.90 × 10^{-4} moles ml/g²) than our data warrant.

The radius of gyration, 470 A, is in good agreement with previous values (Holtzer *et al.*, 1962; Parrish and Mommaerts, 1954; Brahms, 1959). The close agreement in the values for R_g found by light scattering is not surprising since R_g is independent of errors in instrument calibration, in refractive index increment, and in concentration scale.

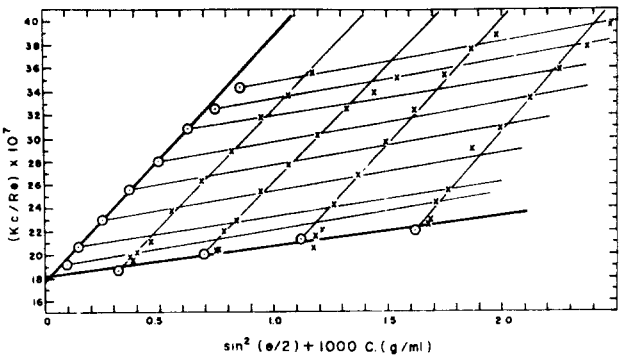

FIG. 1.—Zimm plot for myosin preparation 6b, 0.5 M KCl, *p*H 7, 11°.

A discussion of molecular weights of proteins in aqueous salt solutions must take account of the analysis by Casassa and Eisenberg (1960, 1961) of multicomponent effects in such systems. They have shown that the correct molecular weight for the protein is obtained by making all measurements, save that of protein concentration, on the component defined by dialysis equilibrium. Concentration must be measured by a method that sees the protein alone (for example, nitrogen determination) and not salt bound, or linked by the Donnan effect, to the protein. If concentration is also measured relative to the dialyzed component, then the molecular weight of that component (protein + bound salt) is obtained.

In the present study, concentrations were obtained by dry weight determinations relative to the salt dialysate. Thus the molecular weight in Table I will be too high in proportion to the amount of salt bound to the protein. According to the work of Lewis and Saroff (1957) myosin, under conditions similar to those used here, binds roughly 4% of its weight of KCl. A similar figure was indicated by Kjeldahl determination of three of our myosin samples. These averaged 2–5% lower than the dry weight concentrations. A 4% correction yields a final molecular weight of 5.0 × 10^5 g.

A comparison of our results with those of other workers shows major disagreement to reside only in the value of dn/dc used to calculate molecular weights. If the light scattering molecular weights recorded in the more recent literature are recalculated using any specific value for dn/dc, close agreement among the light scattering measurements themselves are seen to obtain. If our figure for dn/dc is accepted, most values[1] (Brahms, 1959; Holtzer and Lowey, 1959; Holtzer *et al.*, 1962) fall within 6% of 5.0 × 10^5 g/mole.

We are unable to explain the clear disagreement between sedimentation equilibrium and light scattering determinations for the molecular weight of myosin. The method of protein preparation used by Kielley and Harrington (1960), which has recently been sharply criticized by Holtzer *et al.* (1962), cannot be at fault since the identical method was used in this investigation. It is possible to question whether the limiting slopes to Zimm diagrams for myosin should not properly be drawn to points at lower angles than have normally been used, a procedure which would probably yield a higher value for the molecular weight. Such a necessity might arise, for example, from polydispersity in the

[1] In the one exception (Rupp and Mommaerts, 1957), in which a weight of 6.5 × 10^5 g was found, the procedure, now considered questionable, of filtering through sintered glass was employed.

protein preparation and from non-uniform distribution of mass along the length of the myosin rod. It is also possible that as yet incompletely understood charge and salt effects (*e.g.*, Pedersen, 1958; Wallis and Record, 1962), which may affect sedimentation and light scattering measurements differently, play a role.

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Heterogeneity of Human Fibrinogen*

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Chromatographic fractionation of human fibrinogen on DEAE cellulose was carried out with use of a continuous salt and *p*H gradient. The elution patterns revealed two major peaks and, generally, a small third peak, all of which contained clottable protein. This heterogeneity was confirmed by rechromatography and was observed in Blombäck fraction I-4, in fibrinogen prepared by the method of Laki, in Cohn fraction I, in any of these preparations after lysine treatment to remove plasminogen, as well as in whole human plasma. The chromatographically separated components did not differ ultracentrifugally (all sedimenting as single boundaries of $s^0_{20,buffer} = 7.5$ *S*) or immunologically, nor were they significantly different with respect to solubility in ethanol solutions, clotting times, $A^{1\%}_{1cm}$ at 280 mμ, ultraviolet spectrum, or *N*-terminal amino acids. They did show small differences in electrophoretic mobility at *p*H 5.5 or 8.6, the component of lowest anionic binding capacity, which amounted to 85 ± 2% of the fibrinogen of various preparations, having the least negative charge.

With the availability of a method for the preparation of human fibrinogen free of plasminogen (Mosesson, 1962), it became important to learn whether the lysine treatment involved in this purification induced any permanent changes in the fibrinogen. Chromatographic studies revealed that, although the elution patterns of treated and untreated fibrinogens were identical, both contained two major peaks of clottable protein (Mosesson and Finlayson, 1962). The present work was carried out to confirm this apparent heterogeneity and was extended to include studies of the nature of the components.

MATERIALS AND METHODS

Materials.—Single donor or pooled human plasma was frozen within 24 hours after blood collection into ACD.[1] Fraction I was prepared according to method 6 of Cohn *et al.* (1946). From this, purified fibrinogen was prepared by the method of Laki (1951) or that of Blombäck (Blombäck and Blombäck, 1956) (fraction I-4). Plasminogen was removed from these preparations by precipitation of the fibrinogen thrice in the presence of 0.1 M lysine (*p*H 7.0, ionic strength 0.15) at an ethanol concentration of 7% (Mosesson, 1962).

For certain studies fibrinogen was freeze dried from 0.3 M NaCl and reconstituted with water to the original volume before use. Purified fibrinogen was usually kept as a 1–2% solution in 0.3 M NaCl. All samples were stored at −20° until used.

The clottability of Blombäck fraction I-4 was greater than 95%; that of fibrinogen prepared by the method

* A summary of these results has been presented at the Ninth Congress of The International Society of Hematology.

[1] Abbreviations: Tris, tris(hydroxymethyl)aminomethane; ACD, acid citrate dextrose anticoagulant, NIH solution A; DEAE, diethylaminoethyl; EDTA, ethylenediaminetetraacetic acid.