Chromatography of Myosin*

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Received December 5, 1962

The chromatography of rabbit myosin on DEAE-cellulose has been studied. The starting solvent used was 5 mM ATP and 5 mM Tris-HCl (*p*H 7.5), because it was found that myosin is soluble in this solvent and is completely adsorbed to the cellulose. The chromatogram of myosin eluted by a salt gradient to 0.5 M KCl was unsymmetrical, and the specific ATPase activity of the main component is constant and higher than that of the original myosin. The molecular weight of myosin in the main component is that of the monomer, and the myosin in the tail component is aggregated. A low-molecular-weight protein without ATPase activity was sometimes resolved from myosin as a leading component.

The chromatography of myosin on columns of DEAE-cellulose was initiated in our laboratory by Brahms (1959); later and independently, a similar study was reported by Perry (1960). In both instances, the initial solvent was *ca.* 0.2 M KCl, the *p*H was *ca.* 7.5, and adsorption was followed by elution in a linear gradient of KCl. In both instances, heterogeneity was evident. Brahms reported an "α" component (with all the properties of very pure myosin) followed by a gradient-eluted "β" component. Perry felt that his chromatograms demonstrated considerable microheterogeneity, sometimes as many as five or six "peaks." Brahms realized that his "α" peak might well represent *nonadsorbed* fraction of the system, since the 0.2 M KCl then thought to be necessary to keep myosin dissolved also rendered the column very ineffective. In an effort to overcome this difficulty, Brahms and Brezner (1961) investigated the solubility of myosin in the neutral *p*H range. They concluded that the resolubilization of myosin beyond the well-known minimum in electrolyte concentration was due to anion binding, which increases with increasing electrolyte concentration. Thus it became possible for the first time to obtain solutions of myosin at very low ionic strength, simply by using in the solvent a very strongly bound anion (*e.g.*, ATP^{4-}). Brahms reasoned that myosin might be well adsorbed from such solutions. Unfortunately, Brahms was unable to continue in our laboratory, and it has been left to the present author to examine his idea. The general result is that, from ATP or pyrophosphate solution in which the ionic strength is low, myosin can be truly adsorbed and then eluted like a typical protein (Peterson and Sober, 1956). In the chromatograms there is no indication of *micro*-heterogeneity, but it is clear that considerable purification is achieved.

Experimental

DEAE-cellulose purchased from BIO-RAD Laboratories (exchange capacity 0.69 mM/g, control No. B-1131) was washed several times with 0.5 M KOH, the *p*H was adjusted to 4.3, and the cellulose was exhaustively rinsed with redistilled water. Finally, this purified cellulose adjusted to *p*H 7.5 was packed into a column. DEAE-Sephadex (A-50, exchange capacity 2.8 mM/g, Lot No. To 7874 C) from Pharmacia Co. was washed in the same way as cellulose.

ATP and tris(hydroxymethyl)aminomethane (Tris) were obtained from Sigma Chemical Co. Other chemicals used were J. T. Baker Co. reagent grade.

Myosin A from rabbit back muscle was prepared by the standard method in this laboratory: minced muscle was extracted for 10 minutes in three times its volume with a solution of 0.3 M KCl, 0.05 M histidine-HCl (*p*H 6.8), 0.2 mM ATP. Myosin A and myosin B insoluble in 0.25 M KCl were eliminated by centrifugation in each stage of three purification cycles. We detected no change in light scattering intensity of the myosin on addition of ATP, *i.e.*, there was no myosin B contamination in the preparations. Purification and chromatography of myosin was always performed at 3–4°, and chromatography was always completed within 5 days of sacrificing the animal.

The column was equilibrated with 5 mM ATP and 5 mM Tris-maleate at *p*H 7.5 (at this *p*H myosin is stable; also, at this *p*H the degree of ionization of the column would be small at low salt concentration and large at high salt concentration). The same solvent was introduced into the original myosin preparation by dialysis, and prior to use the preparation was centrifuged at 7×10^4 *g* for 2 hours.

Column dimensions, flow rates, and individual tube volumes are reported with specific experiments. In the presence of competing 5 mM ATP anions, the adsorption capacity of the column for the protein is rather low, *ca.* 1.3 g myosin/100 g dry DEAE-cellulose.[1] The column was gravity fed. Linear elution gradients were imposed with the aid of a Technicon Auto-Grad. Fractions were collected in the tubes of a Gilson automatic constant-volume collector. The imposition of salt elution gradients was confirmed by measuring the electrical conductivity of the fractions with a Jones (Leeds and Northrup) impedance bridge.

After the volume of protein-free effluent far exceeded the volume of the column (*i.e.*, after the adsorption of the protein was certain) a linearly increasing elution gradient of KCl was imposed, while the ATP and buffer concentrations were kept constant. The concentration of KCl was allowed to rise to 0.5 M, by which time the adsorbed protein was largely recovered.

Various tests were made on the contents of the collection tubes. Protein concentrations were measured by the method of Lowry *et al.* (1951). An aliquot of 0.5 ml myosin was drawn for determination of

* This research was supported by grants from the National Science Foundation (G-19442), the American Heart Association (CI 8), and the Muscular Dystrophy Associations of America.

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[1] If 2 mM ATP is used instead of 5 mM ATP, the adsorption capacity of the cellulose for the protein is increased to 3–4 g myosin/100 g dry DEAE-cellulose. Chromatography can also be done under this condition, but the solubility of the myosin is correspondingly decreased.

[2] Approximately this interpretation of Brahms' results was communicated to our laboratory by Professor P. H. von Hippel, then our colleague at Dartmouth Medical School.

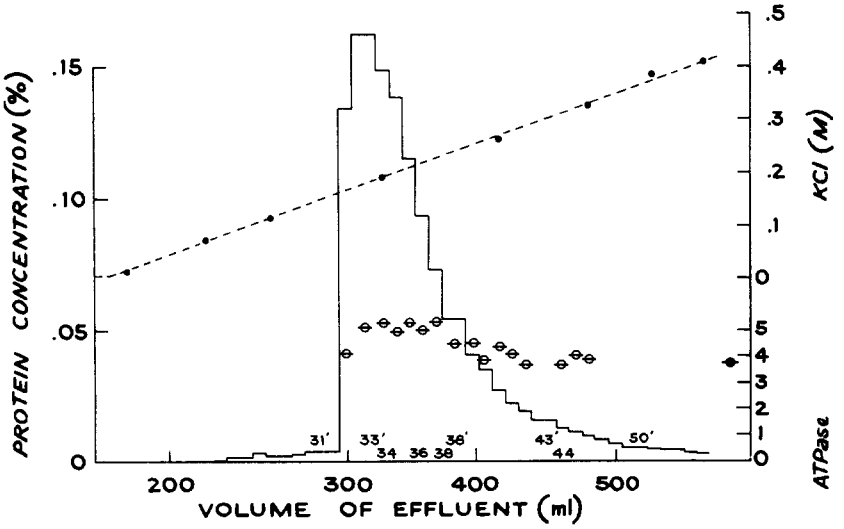

FIG. 1.—Elution profile and specific ATPase of myosin chromatographed on DEAE-cellulose. Fifteen ml of 0.89% myosin was dialyzed against 5 mM ATP and 5 mM Tris-maleate, *p*H 7.5, and was then applied to a 2.2 × 30 cm DEAE-cellulose column equilibrated against the same solvent. After complete adsorption elution was conducted at 15 ml/hr by imposition on this solvent of a linearly increasing gradient in KCl concentration to 0.5 M. Collecting tubes contained 8.8 ml each. Specific ATPase activity was measured in 0.6 M KCl, 0.05 M Tris-HCl, 10 mM $CaCl_2$, and 1 mM ATP, at *p*H 8.0 and 25°. Dashed lines, KCl in effluent; solid circles, ATPase before chromatography; open circles, ATPase activity in μmoles Pi g^{-1} sec^{-1}. Numbers in graph designate tube numbers.

ATPase activity, which was measured by the Fiske-Subbarow method at 25°, *p*H 8.0, in a medium of 0.6 M KCl, 0.05 M Tris-HCl, 10 mM $CaCl_2$, and 1 mM ATP. Under these conditions it was established that ADP at maximally expected concentrations did not affect the ATPase activity. After the nucleotide was dialyzed out, deaminase activity was measured by following the decrease in OD_{260} on addition of AMP; the medium was 0.6 M NaCl and 0.05 M Tris-HCl (*p*H 8.0). By assuming that the second virial coefficient of myosin is zero in the solvent used as well as in 0.6 M KCl (Blum *et al.*, 1953; Holtzer and Lowey, 1959), weight average molecular weights were calculated from light-scattering measurements made at room temperature with a Sofica apparatus; green light (546 mμ) was used. The instrument was calibrated for absolute scattering intensity by measuring the scattering of benzene (Carr and Zimm, 1950). A value of 0.208 ml/g (Holtzer and Lowey, 1959) was adopted for the dn/dc of myosin. As already noted by Brahms, passage through a DEAE column removes dust, so the light-scattering cell could be filled directly from the column. For most further uses of the chromatographed myosin, elimination of 5 mM ATP-ADP is necessary. This elimination can be accomplished by prolonged dialysis or, more easily, by passage through a column (*e.g.*, 2 ml into a column of 1.0 × 27 cm) of DEAE-Sephadex which has been equilibrated with 0.2 M KCl and 5 mM tris-HCl (*p*H 8.0).

RESULTS

The chromatographic behavior of myosin fully adsorbed from 5 mM ATP and 5 mM Tris-maleate, *p*H 7.5, and then eluted by superimposing a linear KCl concentration gradient is shown in Figure 1. Elution commences when the concentration of KCl has reached about 0.15 M. The elution peak is unsymmetrical, with a prolonged "tail" even at high KCl concentrations. Within normal experimental error, the specific ATPase activity is constant in the fraction eluted by 0.15–0.25 M KCl and then drops off gradually; the activity of the first (and major) fraction is higher than that in the original myosin. The chromatogram represents the recovery of over 90% of the protein initially adsorbed. In the experiment of Figure 2, the protein corresponding to the peak of the elution curve (tube 33′ in this chromatogram) has a weight average molecular weight of 0.47 × 10^6 g. This is not reported as an accurate value of the molecular weight of myosin, but in view of molecular weight studies (von Hippel *et al.*, 1958; Lowey and Cohen, 1962) the result shows that the material in this fraction is probably "mono-

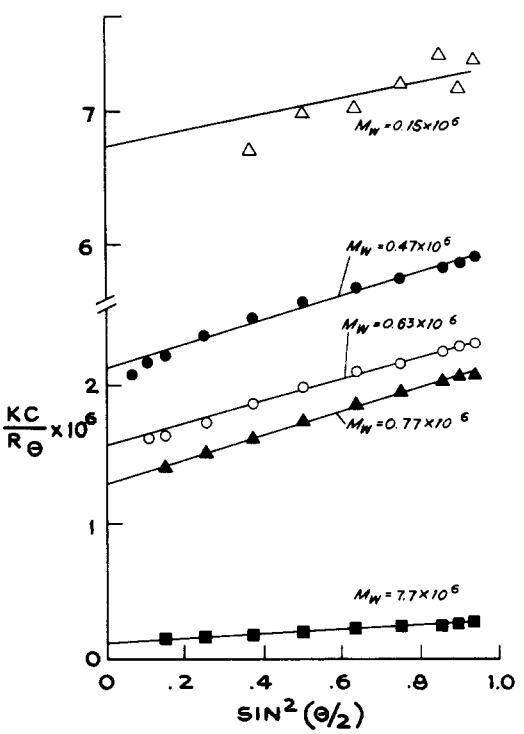

FIG. 2.—Zimm plot of light scattering data for zero-angle extrapolation at 546 mμ. For calculation procedure see text. Solutions were taken from the effluent of Fig. 1. ●, tube 33′; ○, tube 38′; ▲, tube 43′; ■, tube 50′; △, tube 31′).

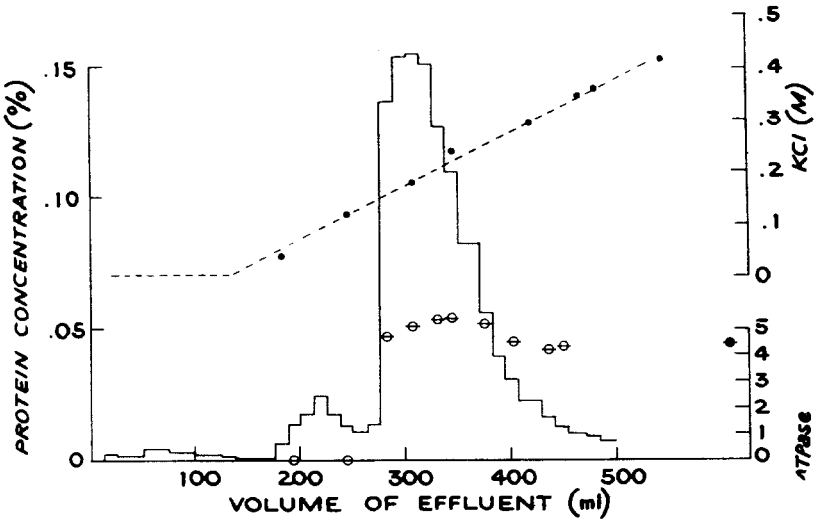

FIG. 3.—Elution profile and specific ATPase activity of myosin chromatographed on DEAE-cellulose. Thirty ml of 0.33% myosin was dialyzed against 5 mM ATP and 5 mM Tris-maleate, *p*H 7.5, and was then applied to a 2.2 × 28 cm DEAE-cellulose column equilibrated against the same solvent. After complete adsorption elution was conducted at 20 ml/hr by imposition on this solvent of a linearly increasing gradient in KCl concentration to 0.5 M. Collecting tubes contained 12.5 ml each. Conditions of measurement and symbols as in Fig. 1.

meric." The material corresponding to the "tail" of the chromatogram exhibits a higher m.w. (tubes 38′ and 43′ in this chromatogram), but when this material is freed of nucleotides by dialysis and ATP is added to it there is no turbidity drop; therefore, it is not myosin B but probably aggregated myosin A.

Chromatograms such as that shown in Figure 1 have been obtained most frequently, but occasionally we have detected a leading component (seen only as a trace in Fig. 1) eluted at concentrations of KCl well under 0.15 M. Such a case is illustrated in Figure 3. This leading component exhibits neither ATPase nor deaminase activity, and its m.w. is about 0.15 × 10^6 g; it is therefore probably unrelated to myosin.

The question arises whether the distribution of aggregates suggested by Figure 1 corresponds to an easily displaced equilibrium. To examine this question, we restored "peak" material and "tail" material to the original adsorbing solvent and 2 days later we chromatographed each separately. The resulting chromatograms are not dissimilar (Fig. 4*A* and *B*), suggesting that to some extent the "peak" material aggregated and the "tail" material dissociated; it is possible, however, that if the work were done more rapidly the "peak" material could be isolated.

Though not with the same rationale or result described here, chromatography of myosin from a pyro-

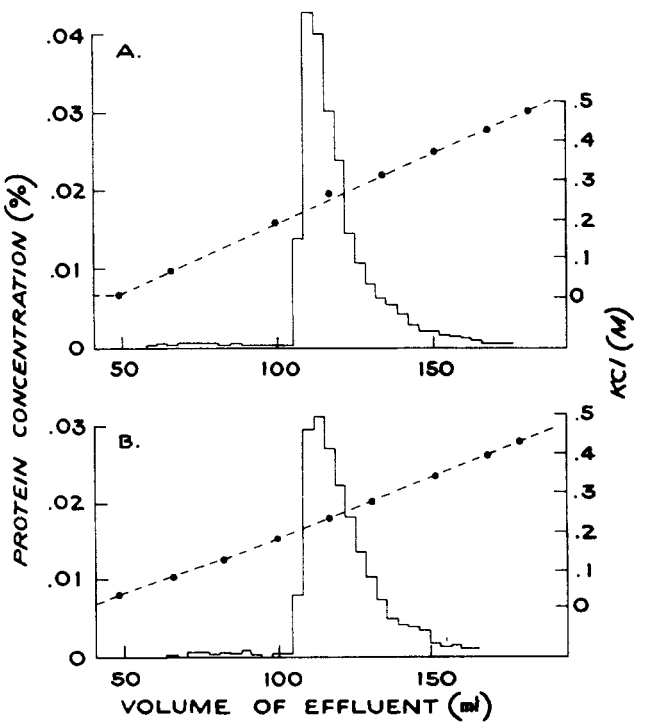

FIG. 4.—Rechromatography of myosin. *A*, Rechromatography of "peak component" of myosin (Fig. 1, tubes 34-36). *B*, Rechromatography of "tail component" of myosin (Fig. 1 tubes 38-42). In each case 9.6 mg of myosin was applied to a 1.0 × 26 cm column, and elution proceeded at 10 ml/hr. Procedures and symbols otherwise as in Fig. 1.

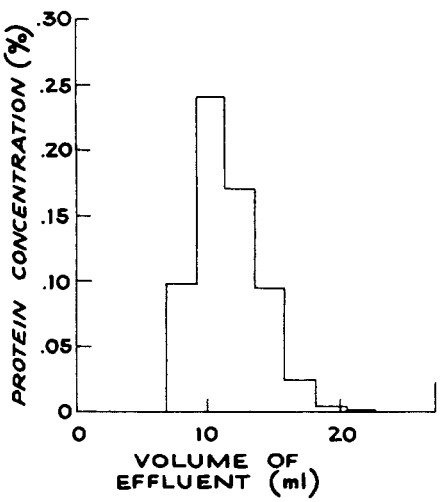

FIG. 5.—Elimination of nucleotide contaminants of myosin by passage through DEAE-Sephadex. Two ml of 0.89% myosin in 2 mM ATP, 0.2 M KCl, 5 mM Tris, *p*H 8.0, was applied to a 1.0 × 27 cm DEAE-Sephadex column equilibrated with 0.2 M KCl, 5 mM Tris-HCl (*p*H 8.0). Filtration proceeded at 18 ml/hr. The effluent was analyzed for material adsorbing at 260 mμ after deproteinization, but none was found until the column was made alkaline, *i.e.*, the column completely rids the myosin of nucleotide contaminants.

phosphate solvent was first reported by Love (1960). It has also been performed by Brahms (unpublished work). In our hands chromatography from pyrophosphate solution gives results similar to those depicted in Figure 1; however, the ATP ion is to be preferred because pyrophosphate binds tightly to myosin and is a powerful inhibitor of ATPase.

As already implied, the nucleotides contaminating myosin obtained in chromatography from an ATP-solvent were totally eliminated (see Fig. 5) by passing through a DEAE-Sephadex column equilibrated with 0.2 M KCl, 5 mM Tris-HCl, *p*H 8.0 (in which solvent myosin is soluble even in the absence of ATP). After a column's worth of fluid had filtered through and 83% of the nucleotide-free protein had been recovered, the nucleotides were recovered by eluting with alkaline solution.

Discussion

We feel that the experiments described establish a practical method for the chromatographic purification of myosin; the method is based on the Brahms idea that in the presence of a myosin-adsorbed polyanion (*e.g.*, ATP) myosin is solubilized at an ionic strength low enough to permit its adsorption on DEAE-cellulose. The present investigation shows that a standard preparation of myosin is probably a slowly associating system, so that chromatography reveals "monomers" and a distribution of "*n*-mers." It seems to us likely that the "monomers" in solutions of *ca.* 0.2 M KCl described by Brahms (1959) and Perry (1960) simply filtered through the column, whereas a fraction (perhaps the major fraction) of "*n*-mers" remained truly adsorbed until the KCl reached a sufficiently high concentration.[2] If so, the α-component of Brahms (1959) corresponds to the "peak material" in the present work, and the β-component of Brahms to a portion of the "tail material." Thus the combination of filtration and chromatography in the older work may effect approximately the same fractionation as the present true chromatography, but would fail to eliminate "leading" components detected in the present work.

In other ways, the present results do not agree with those of earlier workers. Our "tail material" does not exhibit an enhanced ATPase activity as suggested by Brahms, and nowhere have we found the microheterogeneity reported by Perry. We suggest that the latter might actually arise from errors in estimating the *myosin* concentration because OD_{280} is subject either to scattering errors or to errors arising from contaminating proteins.

Acknowledgments

The author wishes to express his gratitutde to Professor Manuel Morales in this laboratory for many valuable suggestions, continuous encouragement, and kind help in completing this work. The author is also indebted to Dr. J. Brahms for valuable advice. The excellent technical assistance of Miss J. Young and Mr. W. Reichert is gratefully acknowledged and appreciated.

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The Use of Gel Filtration in the Isolation and Purification of Beef Insulin

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Received December 21, 1962

A method employing gel filtration for the purification of beef insulin is described. A crude extract (prepared by acid-alcohol treatment of pancreatic tissue followed by ether-alcohol precipitation) is subjected to gel filtration on Sephadex G-50, with ammonium bicarbonate as the solvent. The insulin-containing fraction is lyophilized and crystallized twice. The protein which is obtained is free of nonincorporated radioactive amino acid contamination and has the correct amino acid composition. By performic acid oxidation and a simple countercurrent extraction, the A and B chains may be isolated in pure form.

A convenient procedure for the isolation of insulin from small quantities of pancreatic tissue is necessary for *in vitro* studies of insulin biosynthesis. Many methods for insulin purification have been described, nearly all of which rely upon the preparation of a crude extract by acid-alcohol treatment of the tissue followed by ether-alcohol precipitation. Further purification of the extract is then obtained by paper chromatography (Light and Simpson, 1956; Grodsky and Tarver, 1956; Fenton, 1959; Taylor *et al.*, 1961, 1962), immunochemical precipitation (Taylor *et al.*, 1962), fibril formation and crystallization (Pettinga, 1958; Voelker *et al.*, 1962), or column chromatography with urea-

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