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An Improved Purification Method for Cytoplasmic Dynein

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An improved method has been devised for the purification of cytoplasmic dynein from sea urchin eggs (Strongylocentrotus droebachiensis and S purpuratus). This protocol introduces three changes over a previously published procedure (Hisanaga and Sakai: J Biochem 93:87, 1983)—the substitution of diethylaminoethyl (DEAE)-cellulose for hydroxylapatite chromatography, the elimination of sucrose density gradient centrifugation, and the use of phosphocellulose chromatography. These changes reduce the time and increase the efficiency of the purification procedure. The purified egg cytoplasmic dynein has enzymatic properties in common with axonemal dynein, including ionic specificity (Ca^{++}ATPase/Mg^{++}ATPase = 0.8) and inhibition by sodium vanadate and erythro-9-2,3-hydroxynonyl adenine (EHNA). As assayed by silver staining of polyacrylamide gels, the cytoplasmic dynein is composed of two high molecular weight polypeptides (> 300 kilodaltons) that comigrate with flagellar dynein heavy chains, and lesser amounts of three lower molecular weight bands. None of these polypeptides appears to contain bound carbohydrate. The purification procedure can be modified slightly to allow the preparation of cytoplasmic dynein in only 2 days from as little as 3–5 ml of packed eggs, a 20-fold reduction over the previous method. This more rapid and efficient method will facilitate the investigation of cytoplasmic dynein in other systems where starting material is limited, including tissue culture cells and nerve axoplasm.

Key words: dynein, cytoplasmic dynein, ATPase, sea urchin eggs

The mechanochemical-force-transducing protein of cilia and flagella is an enzyme called "dynein," or force (dyne) protein (in) [1]. Dynein is a Mg^{++}ATPase

Abbreviations used: ATP, adenosine triphosphate; DTT, dithiothreitol; EDTA, ethylenediamine tetraacetic acid; EGTA, ethyleneglycolbis-(β-aminoethyl ether) N,N′-tetraacetic acid; GTP, guanosine triphosphate; MES, 2-(N-morphilino) ethanesulfonic acid; PIPES, piperazine-N,N′bis (2-ethanesulfonic acid); PMSF, phenylmethyl-sulfonyl fluoride; Tris, tris (hydroxymethyl) aminomethane.

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that interacts with adjacent microtubule doublets in the axoneme to effect sliding [2]. There is a growing body of evidence that a similar enzyme can be found in the cytoplasm and that this cytoplasmic dynein may generate force in nonaxonemal microtubule-based motile systems.

In 1968, Weisenberg and Taylor [3] identified an enzymatic activity in unfertilized sea urchin eggs that had some properties in common with ciliary dynein. Subsequently, a partially purified preparation of the enzyme, called egg dynein or cytoplasmic dynein, was shown to have ionic and nucleotide specificities, inhibition by vanadate anion (+5), S value, and polypeptide composition (high molecular weight polypeptides, >300 kilodal [Kd]) in common with axonemal dynein [4]. In addition, cytoplasmic dynein was seen to associate with the fibrous components of the isolated sea urchin mitotic apparatus, suggesting that one possible function of the enzyme was as a force generator for chromosome movement [5].

Physiological studies have also suggested a functional role for dyneinlike ATPase in cytoplasmic microtubule-associated motility. Cande and Wolniak (1978) demonstrated that chromosome motion in lysed cell models (PtK_1) could be reversibly inhibited by vanadate. This dynein inhibitor also reversibly stops pigment granule transport in intact [6] or lysed melanophores [7,8], and vesicles and organelle transport in permeabilized lobster axons [9].

Hisanaga and Sakai [10] reported the successful purification of sea urchin egg (Hemicentrotus pulcherrimus) cytoplasmic dynein by a method employing Sepharose 4B, hydroxylapatite, and calmodulin (CaM) affinity chromatography, along with sucrose density gradient centrifugation (SDGC). The enzyme was purified to near homogeneity and is composed of a single high molecular weight polypeptide that comigrates with flagellar dynein 1 (polypeptide A [11]) on polyacrylamide gels. The use of CaM-4B affinity chromatography was a major advance in the purification of cytoplasmic dynein, but the overall procedure was hampered by two problems. First, large quantities of starting material were necessary (75–100 ml of packed, dejellied sea urchin eggs), and second, the procedure required 5 to 6 days, over which time it was suspected that some enzymatic activity was lost.

In this report, we present an improved method for cytoplasmic dynein purification from the eggs of Strongylocentrotus droebachiensis and S purpuratus. The major changes in the procedure are the substitution of DEAE-cellulose chromatography for hydroxylapatite, and the introduction of phosphocellulose chromatography as a final step, eliminating time-consuming SDGC. In addition, using slight modifications of this procedure, cytoplasmic dynein can be purified in only two days, starting with as little as 3–5 ml of packed, dejellied eggs. The purified enzyme has a slightly more complex polypeptide composition than that reported for H pulcherrimus [10] but is nearly identical in terms of specific activity, activation by calmodulin (CaM), and sensitivity to inhibitors. The results with the two species, S droebachiensis and S purpuratus, are virtually identical. The protocol introduced here now makes it feasible to attempt the biochemical and enzymatic identification of cytoplasmic dynein in other biological systems.

MATERIALS AND METHODS

Preparation of Egg Homogenate From S Droebachiensis and S Purpuratus

Sea urchin eggs, obtained by 0.5 M KCl injection, were dejellied by lowering the pH to 5.0, with 0.1 N HCl, for 1 min. The pH was raised to 8.0 by adding 2 M

Na_2CO_3, the eggs were washed twice by settling in artificial seawater (ASW) [12]. They were then washed twice in 5 volumes of calcium-free seawater (CFSW) [13]. The washed eggs were resuspended in 3 to 5 volumes of homogenization buffer (HB) consisting of 20 mM Tris-HCl (pH 7.5), 0.1 mM EDTA, 1 mM EGTA, 0.2 mM DTT, 10 μg/ml leupeptin, 0.5 mM PMSF or 10 μg/ml soybean trypsin inhibitor (SBTI), and 0.1 M KCl (modified from Hisanaga and Sakai [10]). The eggs were broken using five to eight strokes of a tight pestle in a Dounce homogenizer. The supernatant, after centrifugation at 35,000g for 30 min, was further clarified at 100,000g for 1 hr.

Preparation of Phosphocellulose

Phosphocellulose (PC) was precycled with 0.5 N NaOH and 0.5 N HCl according to the manufacturer's instructions (Whatman, Inc.). The resin was then saturated with $MgSO_4$ according to the method of Williams and Detrich [14].

Determination of Protein and ATPase Activity

Protein concentration was determined by the method of Lowry, et al [15], using bovine serum albumin as a standard.

Adenosine triphosphatase (ATPase) activity was determined at 25°C in an assay mixture (0.2 ml) containing 30 mM Tris-HCl, pH 8.0, 0.3 M KCl, 2 mM $MgCl_2$, 0.2 mM EGTA, and 1 mM ATP. When CaM stimulation was assayed, the reaction mixture contained 30 mM MES-KOH (pH 6.8), 2 mM $MgCl_2$, 0.2 mM EGTA or 0.2 mM $CaCl_2$, 100 μg/ml CaM (6 μM), and 1 mM ATP. Some assays were conducted according to the method of Hisanaga and Sakai [10], using the inorganic phosphate assay of Fiske and Subbarow [18]. Alternatively, the assays (0.2 ml) were started by adding ATP and stopped, usually after 20 min, by adding 0.1 ml of 1% sodium dodecyl sulfate (SDS) (or 5% SDS if the protein concentration exceeded 0.25 mg/ml). The inorganic phosphate (P_i) released was measured colorimetrically by adding 0.7 ml of developing solution (6 parts 0.24% ammonium molybdate in 1 N H_2SO_4 plus one part fresh 10% ascorbic acid), and incubating at 45°C for 20 min [17,18; Lloyd Waxman, personal communication]. Background phosphate levels for each sample were determined by stopping the reaction immediately after the addition of ATP and processing as above (zero time).

Polyacrylamide Gel Electrophoresis and Staining

Polyacrylamide gel electrophoresis (PAGE) was carried out in the presence of SDS by the method of Laemmli [19], using a polyacrylamide gradient (4–16% or 6.5–10%) in a slab gel of 0.75-mm thickness. Two to ten micrograms of protein were typically loaded in each lane, and the gels were stained by a modification of the procedure of Morrissey et al [20]. The gels were fixed in 50% methanol, 0.037% formaldehyde (optional) for a minimum of 6 hr. They were then incubated in 2.5 μg/ml DTT for 30 min and subsequently in 1 mg/ml silver nitrate for 30 min. The gels were then briefly washed twice in approximately 200 ml of deionized H_2O and then rinsed two times quickly in 100 ml of developer (3% Na_2CO_3, 0.018% formaldehyde). The bands were visualized by incubating the gel in 200 ml of developer for 2–5 min, usually over a light box. Development was stopped by adding 20 ml of 2.3 M citric acid. After 10 min, the gels were washed with deionized water. They were stored in deionized H_2O, in the dark, for 1–2 days before photographing.

RESULTS

Sepharose 4B Chromatography

When cytoplasmic dynein is prepared in large quantities (starting material 75–100 ml eggs) it is useful to fractionate the 100,000g supernatant on Sepharose 4B as outlined by Hisanaga and Sakai [10]. In this case, the proteins are first concentrated by precipitation with ammonium sulfate at 55% saturation and, after dialysis against 0.2 M KCl, 20 mM Tris-HCl (pH 8.0), 0.1 mM EDTA, 1 μg/ml leupeptin, and SBTI (0.2 KTE), are loaded onto a 5 × 100-cm column (1.8 liters) of Sepharose 4B, equilibrated, and eluted with the same buffer. This buffer is slightly modified from that of Hisanaga and Sakai [10], and includes a lower salt concentration, necessary for the DEAE-cellulose column which follows. We have found that cytoplasmic dynein tends to aggregate in low salt (<0.1 M) once exposed to 0.5 M KCl or NaCl. For good resolution, up to 1 g of protein can be loaded on the column in a maximum of 70 ml. The elution profile of such a column is shown in Figure 1. The ATPase activity is eluted in an early included fraction and the column is particularly useful for removing small vesicles and lipoproteins that are found in the void volume (data not shown). When cytoplasmic dynein is prepared from smaller quantities of starting material, the 100,000g spin is more effective at removing vesicular contamination, and those that remain are eliminated during DEAE-cellulose and CaM-affinity chromatography (see below).

DEAE-cellulose Chromatography

Cytoplasmic dynein can be further purified by DEAE-cellulose ion exchange chromatography (DE52, Whatman). This method has two advantages over hydroxyapatite chromatography: (1) the 1.5-cm diameter column can be run at up to 1.5 ml/

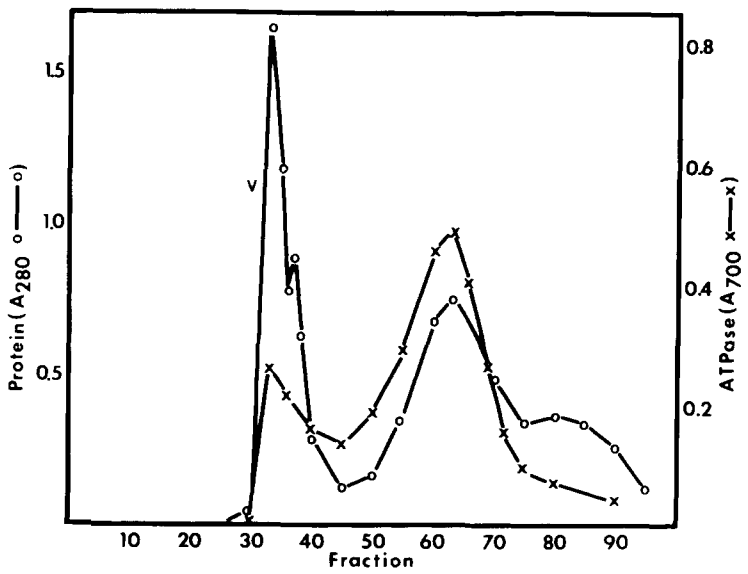


Fig. 1. Sepharose 4B column chromatography. Total column volume is 1.8 liters. Ninety-five fractions of 17.5 ml were collected. Protein (○) and ATPase activity (relative P_i released/20 min: ×) of fractions eluted. V marks void volume. (For details, see text).

min using a peristaltic pump, faster than hydroxyapatite; and (2) using a phosphate-free buffer obviates the need for radioactive phosphate (^{32}P-ATP) in the ATPase assay.

In the experiment shown in Figure 2, the included peak of ATPase activity (fractions 50–70, Fig. 1A) from a Sepharose 4B column were pooled and dialyzed against 50 mM KCl, 20 mM Tris-HCl, pH 7.5, 2 mM $MgCl_2$, 0.1 mM EDTA, 0.5 mM DTT, 10% glycerol (TDMK). The protein was loaded onto the DEAE-cellulose column (1.5 × 35 cm, 75 ml) equilibrated, and then washed with the same buffer. Under these conditions, up to 250 mg of protein could be loaded in any volume, and over 90% was bound to the column. The column was then eluted with a gradient of 0.05–0.35 M KCl in the same buffer, and finally washed with 0.5 M or 1 M KCl in the same buffer.

Figure 2A shows the elution profile of such a column. The peak of ATPase activity is eluted at a conductivity of 8.0 millimhos, which corresponds to 0.15 M KCl. Polyacrylamide gel electrophoresis reveals a large number of polypeptides coeluting with the ATPase activity, but it should be noted that the high molecular weight (HMW) bands comigrating with flagellar dynein are found exclusively in these fractions (Fig. 2B).

Once the fractionation pattern of the ATPase on DE52 was established, a typical column was eluted with a single step of 0.2 M KCl in TDMK. This served to concentrate the ATPase activity, and reduced even further the time required for this procedure. The rest of the protein elutes from the column at 1.0 M KCl.

Calmodulin-Sepharose 4B Affinity Chromatography

The ATPase containing fractions from the DE52 column were purified further by CaM-4B affinity chromatography according to Hisanaga and Sakai [10] with slight modifications. The published procedure calls for a buffer containing 0.5 M KCl to reduce aggregation of the cytoplasmic dynein originally extracted with high salt. We have found, however, that if the original homogenate contains lower salt (50 mM–0.2 M KCl), aggregation can be significantly reduced. Thus, the CaM-4B column can be run at 0.2 M KCl and the peak of ATPase activity eluted from DE52 can be loaded directly on the affinity column after diluting the sample to reduce the glycerol concentration to 5% (optional) and bringing the mixture to 2 mM $CaCl_2$. The column contains approximately 60 mg CaM/35 ml Sepharose 4B, and up to 100 mg of protein from the DE52 column can be efficiently fractionated.

Figure 3 shows that over 75% of the protein loaded on the CaM-4B column flows through at 2 mM $CaCl_2$ (see also Table I). A single sharp peak of protein is eluted with 2 mM EGTA, and this peak contains the ATPase activity and is enriched in the high molecular weight polypeptides (Fig. 3B). Since the specific activity increase at this step is not as high as would be expected, some loss of activity may be occurring. This cause of this loss, also reported by Hisanaga and Sakai [10], is unknown.

Phosphocellulose Chromatography

Since microtubule-associated proteins (MAPs) and a variety of phosphates and kinases can be purified by phosphocellulose (PC) chromatography, we examined the possibility of using this method to prepare cytoplasmic dynein. A variation of a tubulin purification buffer was used which contained 50 mM PIPES (pH 6.8), 2 mM $MgCl_2$, 0.1 mM EDTA, 0.3 mM DTT, 5% glycerol, 1 μg/ml leupeptin and SBTI,

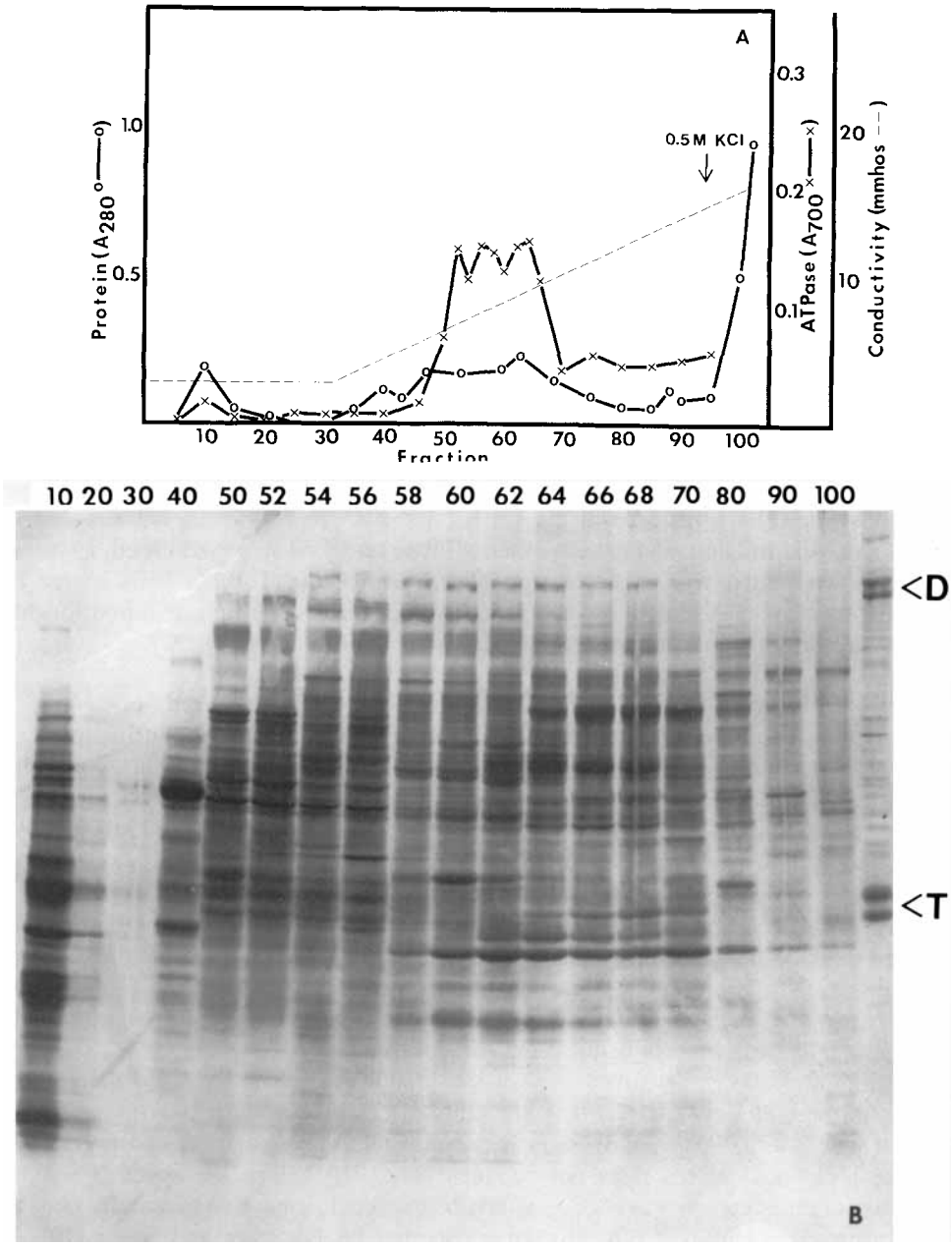


Fig. 2. DEAE-cellulose (DE52) column chromatography. Total column volume is 75 ml. One hundred fractions of 10 ml each were collected. A gradient of KCl (0.05 M to 0.35 M) was started at the first arrow, and 0.5 M KCl was started at the second arrow. (For details, see text). A) Protein (○) and ATPase activity (relative P_i released/20 min: ×) of fractions eluted. Conductivity of each fraction is also recorded (---). B) Polyacrylamide gel electrophoresis of indicated fractions from the column. The gel contains a 4–15% gradient of acrylamide and is stained with silver nitrate as indicated in the Materials and Methods. Arrows mark the dynein region (D), and tubulin is also indicated (T) on a lane containing flagellar axonemes.

and 0.1 M KCl (PMEK). When the EGTA-eluted peak from the CaM column is dialyzed against the buffer, a fibrous precipitate forms. This precipitate, which can be removed by centrifugation at 35,000g for 30 min, contains approximately 40% of the protein in the CaM pool, but none of the ATPase activity. Thus, the dialysis and centrifugation alone effect some purification of the enzyme.

The clarified dialysate can then be fractionated on a phosphocellulose column equilibrated with PMEK. Up to 1 mg of protein can be loaded per ml of packed PC, but maximum resolution is obtained with 0.7–0.8 mg protein per ml PC. As shown in Figure 4, virtually all of the ATPase activity flows through the column, along with a subset of two high molecular weight polypeptides and some lower molecular weight bands (Fig. 4B). Other proteins from the CaM pool can be eluted with a gradient of KCl.

Since the cytoplasmic dynein appears in the flow-through fraction, dilution is a problem. Typically, the CaM pool is concentrated before dialysis by Amicon high pressure filtration using a YM30 filter, or by precipitation with ammonium sulfate at 55% saturation, to reduce this problem. Another problem observed at first with this procedure was that the dynein seemed to lose activity quickly (within 1 day) after this procedure. We suspected that this was due to loss of Mg^{++} ions, as reported when tubulin is purified using phosphocellulose [14]. This problem was eliminated by saturating the PC with Mg^{++} ions before preparing the column [14].

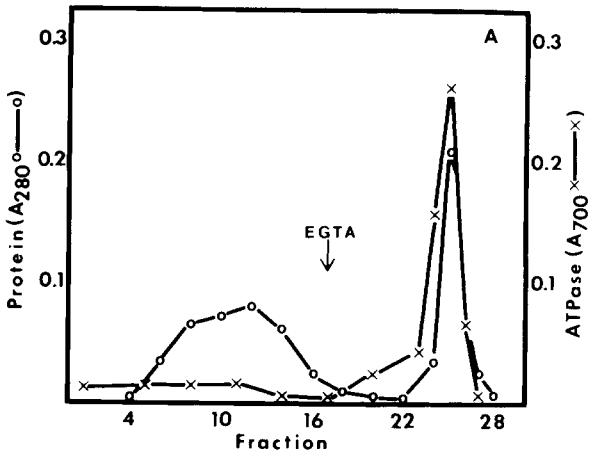
Rapid Purification of Cytoplasmic Dynein

For rapid purification of cytoplasmic dynein from smaller quantities of starting material (5 to 20 ml packed, dejellied eggs), the 100,000g supernatant of the egg homogenate (see Material and Methods) was brought to 20 mM Tris-HCl (pH 7.5), 50 mM KCl, 2 mM $MgCl_2$, 10% glycerol, and 0.5 mM DTT, by dilution with an appropriate buffer. The protein mixture was loaded directly onto the DE52 column, equilibrated with TDMK, without fractionation over Sepharose 4B. After washing the column with 5 volumes of TDMK, the ATPase activity was eluted in a single step at 0.2 M KCl in TDMK.

The buffer conditions in the DE52 pool were then adjusted so that the pool could be loaded directly onto the CaM-Separose 4B column. Typically, the sample was diluted to reduce the glycerol concentration to 5%, and the buffer was brought to 0.2 M KCl, 20 mM Tris-HCl (pH 7.5), 0.1 mM EDTA, 2 mM $MgCl_2$, 2 mM $CaCl_2$, 0.25 mM DTT, and 1 μg/ml leupeptin and SBTI. The protein was then fractionated by CaM-4B chromatography as described above.

The EGTA-eluted pool was then concentrated fourfold by Amicon filtration or ammonium sulfate precipitation, and dialyzed against 100 volumes of PMEK overnight. Alternatively, the CaM-4B pool could be dialyzed against PMEK overnight, and then concentrated the next morning. The precipitation of contaminating proteins in this buffer (see above) was, however, more efficient if the sample was concentrated before dialysis. The concentrated dialyzed CaM-4B pool was clarified by centrifugation at 35,000g for 30 min, and the supernatant was fractionated by PC chromatography as described above.

Table I outlines the extent of purification and recovery at each step during one large and one small volume preparation of egg dynein from S droebachiensis. Figure 5 shows the polypeptide composition of the ATPase containing fractions from each of the columns, comparing the long, large-volume procedure and the rapid, small-


A
0.3
0.2
0.1
Protein (A_{280} o—o)
ATPase (A_{700} ×—×)
EGTA
4
10
16
22
28
Fraction

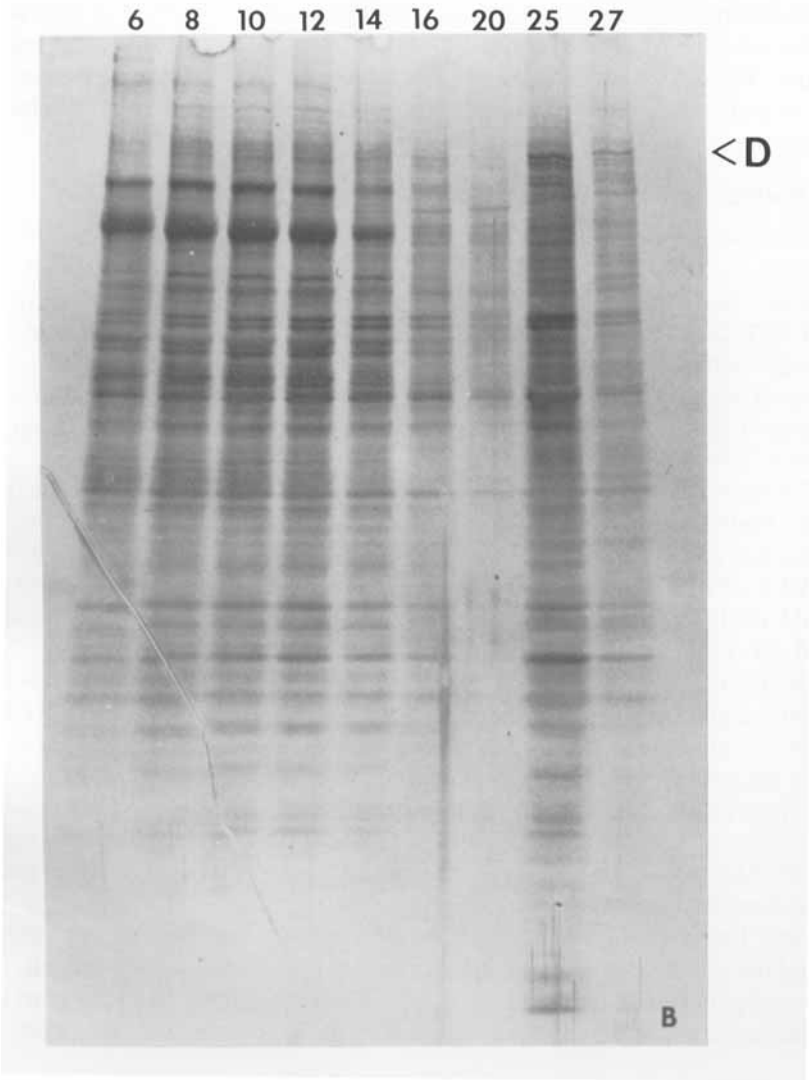

6 8 10 12 14 16 20 25 27
D
B

Figure 3

volume procedure. The recovery of ATPase units is greater with the rapid preparation, but the longer method usually provides a purer enzyme, as judged by SDS-PAGE.

Characteristics of the Purified Cytoplasmic Dynein

Purified cytoplasmic dynein has unique enzymatic properties characteristic of dynein ATPase (Table II). The ATPase is activated by either Mg^{++} or Ca^{++}, with a ratio of Ca^{++} ATPase/Mg^{++} ATPase of 0.8, and has no activity in 0.3 M KCl and 2 mM EDTA. The enzyme is also 100% inhibited by 100 μM sodium vanadate, a potent inhibitor of dynein ATPase [21–23]. Erythro-9-2, 3-hydroxynonyl adenine (EHNA) has also been shown to specifically, though less potently, inhibit dynein ATPase [24,25]. The extent of inhibition is dependent on the ratio of EHNA concentration to ATP concentration, and is greatest when the ratio is two or more. At equimolar EHNA and ATP (1 mM), purified cytoplasmic dynein is 30% inhibited. Both oligomycin (20 μg/ml) and ouabain (50 μM), inhibitors of mitochondrial ATPase and Na^+K^+ATPase, respectively, effect a slight stimulation of cytoplasmic dynein ATPase, as has been reported previously [4]. Sodium azide (1 mM NaN_3), an

TABLE I. Protein Content and ATPase Activity in Egg Fractions During Cytoplasmic Dynein Purification*

Sample	mg	Units ATPase[a]	Specific activity[b]	Purification (fold)[c]	Recovery (%)[d]
Preparation 1					
55AS	684.4	4.20	.006		
4B Pool	143.0	1.65	.012	2.0	39.0
DE52 Pool	30.2	.628	.021	3.5	15.0
CaM Pool	3.77	.135	.036	6.0	3.2
PC Pool	0.09	.017	.188	31.3	0.4
Preparation 2					
Egg Supernatants	104.0	.889	.009		
DE52 Pool	17.6	.400	.023	2.5	44.0
CaM Pool	.72	.073	.101	11.2	8.2
PC fraction 4	.05	.013	.260	29.0	1.4

*Samples are whole egg supernatants, 55% saturated ammonium sulfate fraction (55AS), and pools of ATPase-containing fractions from the columns. Both preparations are from S droebachiensis eggs. Preparation 1 started with 70 ml of packed dejellied eggs using the longer procedure. Preparation 2 started with 5 ml of packed dejellied eggs, using the rapid procedure.

[a] μmol P_i/min.

[b] μmol P_i/min × mg.

[c] Increase in specific activity over starting egg supernatant or 55% ammonium sulfate fraction: the specific activity of a whole egg homogenate is at least three times lower than that of the supernatant. Thus, the final enzyme preparation is approximately 100-fold purified over the egg homogenate.

[d] % of total ATPase units in the starting supernatant or ammonium sulfate fraction.

Fig. 3. Calmodulin-Sepharose 4B (CaM-4B) column chromatography. Total column volume is 35 ml. Twenty-eight fractions of 5 ml each were collected. The load and wash buffer contained 2 mM $CaCl_2$, and at fraction 17, this was replaced with a buffer containing 2 mM EGTA. (For details, see text). A. Protein (○) and ATPase activity (relative P_i released/20 min: ×) of fractions eluted. B. Polyacrylamide gel electrophoresis of indicated fractions, as described in Figure 2. The dynein region is marked (D).

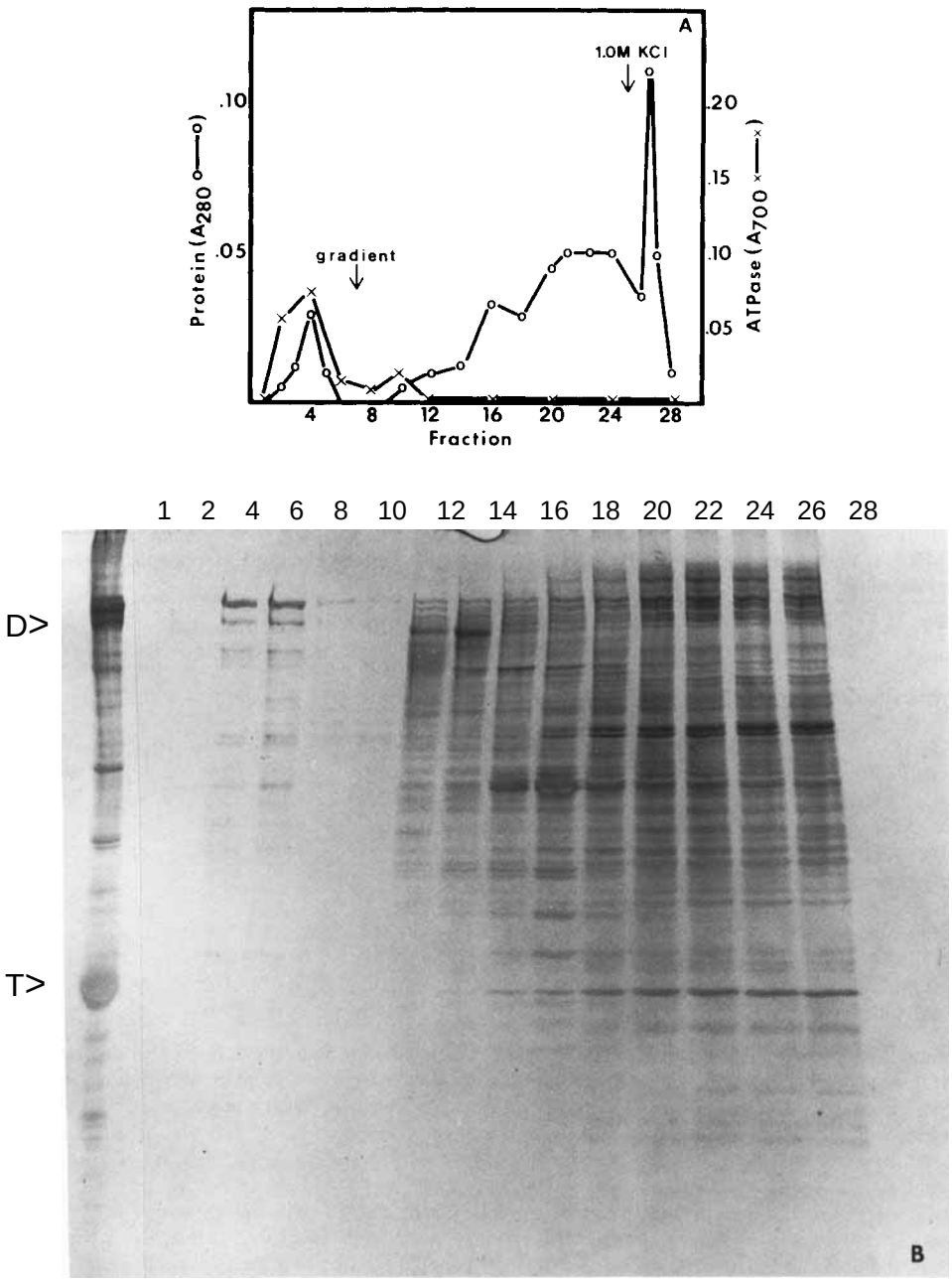


Fig. 4. Phosphocellulose (PC) column chromatography. Total column volume is 4 ml. Twenty-eight fractions of 0.75 ml each were collected. A gradient of 0.1–0.6 M KCl in PMEK was started at the first arrow, and this was replaced with PMEK containing 1 M KCl at fraction 24. (For details, see text). A) Protein (○) and ATP activity (relative P_i released/20 min: ×) of fraction eluted. B) Polyacrylamide gel electrophoresis of indicated fractions, as described in Figure 2.

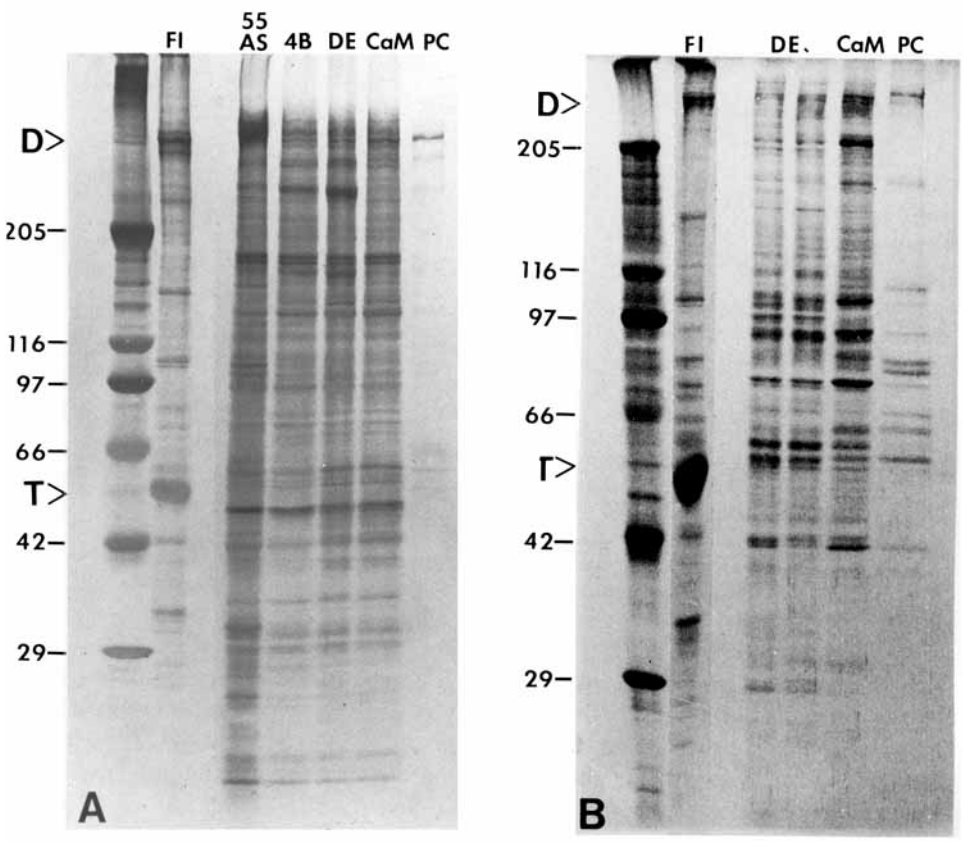


Fig. 5. SDS-polyacrylamide gel electrophoresis of cytoplasmic dynein samples during purification. A) For this preparation, 80 ml of packed eggs were fractionated by the longer procedure. The gel contains a 4–15% gradient of polyacrylamide. Standard proteins are labelled according to their molecular weights (Kd). Dynein (D) and tubulin (T) are also marked on a lane containing flagellar axonemes (Fl). Samples are as follows: 55AS, egg proteins precipitated at 55% ammonium sulfate; 4B, the ATPase activity pool from the Sepharose 4B column; DE, the ATPase activity pool from the DE52 column; CaM, the ATPase activity pool from the CaM-4B column; PC, the peak ATPase fraction from the phosphocellulose column. B) For this preparation, 3 ml of packed eggs were fractionated by the rapid procedure. The gel contains a 6.5–10% gradient of polyacrylamide. Standard proteins are labeled according to their molecular weights, and the dynein region is marked (D). The sample designations are as in Figure 5A.

inhibitor of soluble mitochondrial F_1 ATPase, has a slight inhibitory effect on the enzyme (13%).

Hisanaga and Pratt [26] have reported that cytoplasmic dynein ATPase is stimulated 4 to 8 fold by Ca^{++}-calmodulin. The pool of ATPase from the Sepharose 4B column is stimulated approximately 2.5-fold by 100 μg/ml of CaM (prepared from bovine brain), and 0.2 mM $CaCl_2$, at pH 6.8, and this increases to sixfold stimulation of the PC-purified enzyme. The purified dynein ATPase is also stimulated 4–5-fold by Triton X-100, as reported for both flagellar [27], and cytoplasmic dynein [26] (Table II).

TABLE II. Enzymatic Characteristics of Purified Cytoplasmic Dynein*

Conditions	ATPase[a]	% of Mg^{++}ATPase
Mg^{++}	14.3	
Ca^{++}	11.3	81
K^{+}EDTA	0	0
Mg^{++} plus—		
100 μM Na_3VO_4	0	0
1 mM EHNA	9.8	70
1 mM NaN_3	12.2	87
20 μg/ml oligomycin	18.1	129
50 μM ouabain	17.4	124

*Egg dynein was prepared by the rapid method from eggs of S droebachiensis. All assays contain 30 mM Tris-HCl (pH 8.0), 0.3 M KCl, 1 mM ATP, and 4.6 μg/ml egg dynein and the designated ions and/or inhibitors. All assays containing Mg^{++} also contain 0.2 mM EGTA. The specific activity of the preparation in 2 mM $MgCl_2$, 0.2 mM EGTA was approximately 0.3 μmol P_i/mg × min.
[a]Arbitrary units.

The purified cytoplasmic dynein (Fig. 5A, lane PC) is composed of two major polypeptides that comigrate on SDS-polyacrylamide gels with flagellar dynein heavy chains. They have estimated molecular weights of 385 Kd and 320 Kd, as previously reported for a less purified preparation of the enzyme [4]. In addition, two lower molecular weight components of 125–135 Kd and 65–75 Kd are seen along with a variable component around 200–240 Kd. These polypeptides are unreactive either with periodic acid-Schiff reagent or dansyl-hydrazine [28] stains, which reveal carbohydrates.

DISCUSSION

In this report, we present an improved method for the purification of cytoplasmic dynein. The major improvements in the protocol, over previous methods, are the use of DE52 and phosphocellulose chromatography and the elimination of three lengthy dialysis steps and 16 to 18 hr of sucrose density gradient centrifugation. In addition, the protocol can be adapted such that reasonable quantities of the enzyme can be prepared from as little as 3–5 ml of packed dejellied sea urchin eggs, a 20-fold reduction over previous methods [10], in only 2 days.

The use of phosphocellulose chromatography is particularly interesting in view of the successful purification of standard microtubule-associated proteins (MAPs) and tubulin by this method [30]. Although MAP purification provided the rationale for fractionating cytoplasmic dynein on phosphocellulose, the enzyme behaves quite differently from typical MAPs. While egg dynein flows through the columns at 0.1 M KCl and pH 6.8, MAPs elute only at much higher salt concentrations at neutral pH [14,31].

The instability of the dynein ATPase eluted from the phosphocellulose column could be completely eliminated by saturating the PC with Mg^{++} before use [14].

Williams and Detrich [14] have clearly shown that PC sequesters Mg^{++}, and that this affects the stability and polymerization characteristics of tubulin purified by this method. There is some indication that dynein is also more stable when Mg^{++} is bound to the enzyme. Axonemal dynein isolated in the presence of Mg^{++} has a higher specific activity than EDTA-isolated dynein [31,32], and Mg^{++} (or Ca^{++}) is required for ATPase activation. In addition, axonemal dynein rebinding to microtubules is enhanced by higher Mg^{++} ion concentrations (around 4–5 mM) [33–35]. Axonemal dynein, however, can be isolated and stored in an EDTA-containing buffer, lacking Mg^{++}, and retains activity; thus, it seems that cytoplasmic dynein is more sensitive to Mg^{++} depletion, losing substantial activity in less than 18 hr. With regard to this magnesium sensitivity, it is interesting to note that addition of magnesium to the homogenization or Sepharose-4B elution buffers does not increase the quantity or activity of purified enzyme. Presumably, the egg contains sufficient Mg^{++} to saturate the enzyme, and this bound magnesium is only removed by the strong phosphocellulose chelation.

One problem with the purification protocol is the low recovery of ATPase units. Part of the loss probably reflects the fact that not all of the ATPase activity in the original egg extract is cytoplasmic dynein. Other reasonable causes of enzyme loss are aggregation and adherance to surfaces, characteristics of dynein proteins that lead to denaturation. The rapid procedure eliminates some of this loss by decreasing the amount of dialysis and the number of transfers between vessels. In any case, the recovery is comparable to the 0.68% reported by Hisanaga and Sakai [10] for H pulcherrimus.

The purified egg cytoplasmic dynein is composed primarily of two high molecular weight polypeptides over 300 Kd. These are the same heavy chains originally identified by Pratt [4]; however, the earlier preparation was only partially purified and contained comigrating contaminating proteins. In contrast to a previous report [4], these heavy chains do not appear to contain bound carbohydrate. The purified enzyme preparation also contains lower molecular weight polypeptides. Two of these, at 125–135 Kd and 65–75 Kd, are similar in size to flagellar dynein intermediate chains IC_1 (125 Kd) and IC_2 (75 Kd) [36]. Variable polypeptides around 200 Kd may be a stubborn contaminant, possibly yolk protein. Polypeptides of similar molecular weight can sometimes be seen in flagellar dynein preparation [36,37], however, and may be breakdown products of one of the dynein heavy chains. Hisanaga and Sakai [10] reported that cytoplasmic dynein prepared from H pulcherrimus eggs was composed of a single high molecular weight polypeptide that comigrated with flagellar dynein A [11]. It may be that the more complicated composition reported here is a result of silver staining the gels which not only reveals small quantities of protein [38], but also stains a slightly different constellation of polypeptides than Coomassie blue (CB). When the Strongylocentrotus sp egg dynein is stained with CB, the low molecular weight proteins are not visualized, but at least two high molecular weight bands are always seen. This suggests that the different protein composition may reflect species differences between the two sea urchins.

The purified cytoplasmic dynein ATPase has a number of enzymatic properties in common with axonemal dynein [39]. The enzyme requires either Mg^{++} ions or Ca^{++} ions for activity, and the Mg^{++}ATPase activity is inhibited by two flagellar dynein ATPase inhibitors, but not by inhibitors of Na^+K^+ATPase or mitochondrial ATPase. In addition, the cytoplasmic Mg^{++}ATPase activity is stimulated 5–6-fold by

calmodulin, in a calcium-dependent manner, as has been previously reported for both cytoplasmic dynein and flagellar 21S dynein [10,26] from H pulcherrimus. It is particularly significant that the degree of stimulation by Ca^{++}-calmodulin increases with greater purification. These data support the hypothesis that CaM activation is the result of a direct interaction of cytoplasmic dynein and calmodulin, rather than an indirect effect modulated by a kinase which might phosphorylate the dynein [26]. The latter seems unlikely since the amount of a contaminating kinase should decrease rather than increase with greater purification.

Murphy and co-workers [40,41] have recently identified an ATPase isolated from brain tissue that had previously been tentatively identified as a dyneinlike enzyme [42]. This ATPase is associated with membrane vesicles and has properties in common with mitochondrial F_1 ATPase [40,41]. Cytoplasmic dynein can be clearly distinguished from this ATPase since it is not associated with vesicles and it is only slightly inhibited by sodium azide, a potent inhibitor of the brain vesicle ATPase.

The modified protocol that allows the rapid purification of cytoplasmic dynein from smaller quantities of eggs has several advantages. We find that freezing cytoplasmic dynein perturbs some of the enzyme's properties; thus it is useful to be able to prepare "fresh" enzyme once or twice a week. In addition, the rapid purification assures a more "native" enzyme that is suitable for structural studies, including scanning-transmission electron microscopy (STEM) [29]. The increased efficiency of the method now makes it feasible to investigate cytoplasmic dynein in other systems where starting material is limited, for example, isolated mitotic apparatus, tissue culture cells, and nerve axoplasm.

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REFERENCES

1. Gibbons IR, Rowe AJ: Science 149:424, 1965.
2. Summers KE, Gibbons IR: Proc Natl Acad Sci USA 68:3092, 1971.
3. Weisenberg R, Taylor EW: Exp Cell Res 53:372, 1968.
4. Pratt MM: Devel Biol 74:364, 1980.
5. Pratt MM, Otter T, Salmon ED: J Cell Biol 86:738, 1980.
6. Beckerle MC, Porter KR: Nature 295:701, 1982.

7. Clark TG, Rosenbaum JL: Proc Natl Acad Sci USA 79:4655, 1982.
8. Stearns ME, Ochs RL: J Cell Biol 94:727, 1982.
9. Forman DS, Brown KJ, Livengood DR: J Neurosci 3:1279, 1983
10. Hisanaga S, Sakai H: J Biochem 93:87, 1983.
11. Gibbons IR: Fronk E, Gibbons BH, Ogawa K: In Goldman R, Pollard T, Rosenbaum J (eds): "Cell Motility." New York: Cold Spring Harbor, p 915, 1976.
12. Emerson CP, Humphries T: Dev Biol 23:86, 1970.
13. Burgess DR, Schroeder TE: J Cell Biol 74:1032, 1977.
14. Williams RC, Detrich HW: Biochemistry 18:2499, 1979.
15. Lowry OH, Rosebrough N, Farr AL, Randall RJ: J Biol Chem 193:265, 1951.
16. Fiske CH, Subbarow Y: J Biol Chem 66:375, 1925.
17. Ames B: Methods Enzymol 8:115, 1966.
18. Waxman L, Goldberg AL: Proc Natl Acad Sci USA 79:4883, 1982.
19. Laemmei UK: Nature 227:680, 1970.
20. Morrisey JH: Anal Biochem 117:307, 1981.
21. Cande WZ, Wolniak SM: J Cell Biol 79:573, 1978.
22. Gibbons IR, Cosson M-P, Evans JA, Gibbons BH, Houck B, Martinson K, Sale W, Tang W-J: Proc Natl Acad Sci USA 75:2220, 1978.
23. Kobayashi T, Martensen T, Nath J, Flavin M: Biochem Biophys Res Commun 81:1313, 1978.
24. Bouchard P, Penningroth SM, Cheung A, Gagnon C, Bardin, CW: Proc Natl Sci USA 78:1033, 1981.
25. Penningroth SM, Cheung A, Bouchard P, Gagnon C, Bardin CW: Biochem Biophys Res Commun 104:234, 1982.
26. Hisanaga S, Pratt MM: Biochemistry, 23:3032, 1984.
27. Gibbons IR, Fronk E: J Biol Chem 254:187, 1979.
28. Eckhardt AE, Hayes CE, Goldstein IJ: Anal Biochem 93:192, 1976.
29. Johnson K, Wall J: J Cell Biol 96:669, 1983.
30. Weingarten MD, Lockwood AH, Hwo S-Y, Kirschner MW: Proc Natl Acad Sci USA 72:1858, 1975.
31. Gibbons IR: J Biol Chem 241:5590, 1966.
32. Gibbons IR, Gibbons BH: J Cell Biol 54:75, 1972.
33. Haimo LT, Telzer BR, Rosenbaum JL: Proc Natl Acad Sci USA 76:5759, 1979.
34. Mitchell DR, Warner FD: J Cell Biol 87:84, 1980.
35. Warner FD, McIlvain I: Cell Motility 2:329, 1982.
36. Bell CW, Fronk E, Gibbons IR: J Supramol Struct Cell Biochem 11:311, 1979.
37. Bell CW, Gibbons IR: J Biol Chem 257:516, 1982.
38. Merril CR, Dunau ML, Goldman D: Anal Biochem 110:201, 1981.
39. Gibbons IR, Fronk E: J Cell Biol 54:365, 1972.
40. Murphy DB, Hiebsch RR, Wallis KT: J Cell Biol 96:1298, 1983.
41. Murphy DB, Wallis KT, Hiebsch RR: J Cell Biol 96:1306, 1983.
42. Hiebsch RR, Hales DD, Murphy DB: J Cell Biol 83:345a, 1979.
43. Pratt MM, Hisanaga S, Begg DA: J Cell Biol 97:204a, 1983.