Subunit Structure of Dynein

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Contents

Part I

Twenty-one S Dynein from Sea Urchin Sperm Flagella

Introduction

Biological motion dependent on ATP hydrolysis is generated by two distinct systems: one is actin filament and myosin ATPase, and the other is microtubule and dynein ATPase. The motion associated with either of these two systems is based on a sliding filament mechanism. Muscle contraction represents the motion by actin-myosin system. Since Huxley and Hanson (1954) and Huxley and Niedergerke (1954) proposed the sliding filament mechanism for contraction of striated muscle, actin-myosin interaction has been studied for many years from physiological, morphological and biochemical standpoints. It has been widely accepted that myosin molecules hydrolyse ATP in cyclic manner and produce force by forming cross-bridges.

The bending motion of cilia and flagella is also based on active sliding movement between adjacent doublet microtubules of the axonemes (Satir ,1968; Summers & Gibbons, 1971). There is abundant evidence that this active sliding between microtubules occurs as a result of dynein-microtubule interaction (Gibbons & Gibbons, 1972; Summers & Gibbons, 1973; Gibbons & Gibbons, 1973; Gibbons & Gibbons, 1976; Sale & Satir, 1977; Gibbons et al., 1978). Although basic sliding mechanism is similar in both systems, dynein seems to be very different from myosin in its structure and enzymology. Myosin is far well characterized as compared to dynein. Myosin is a molecule which has two globular heads and long tail (Slayter & Lowey, 1967) and the molecular weight of 460,000 (Godfrey & Harrington,

1970), and forms thick filaments. It is established that dynein resides in two rows of arms along each doublet microtubule, but the molecular weight and the shape are still now controversial.

To elucidate the dynein-microtubule interaction, it is important to study molecular structure of dynein arms. However, there are some difficulties in dynein research. First, dynein shows diversity and multiplicity among a wide variety of sources, even within one species, or one organelle. There are two kinds of dynein arms in cilia and flagella, i.e., outer and inner arms, which appeared different in shape (Allen, 1968), in salt extractability (Gibbons & Gibbons, 1973), and immunologically (Ogawa et al., 1977). Physical heterogeneity is displayed by the existence of two extractable forms of dynein, one sedimenting at 18-30S, and the other sedimenting at 10-14S (Gibbons & Rowe, 1965; Mohri et al., 1969; Gibbons & Fronk, 1972; Mabuchi & Shimizu, 1974; Mabuchi et al., 1976; Piperno & Luck, 1979). Second, dynein is a very large molecule containing multiple high molecular weight polypeptide chains. The molecular weight determination by SDS gel electrophoresis has been difficult owing to the lack of adequate molecular weight markers. Large difference between the reported values (300,000 to 600,000; Kincaid et al., 1973; Linck, 1973; Mooseker & Tilney, 1973; Mabuchi & Shimizu, 1974; Ogawa et al., 1975; Mabuchi et al., 1976; Mabuchi, 1978; Piperno & Luck, 1979; Bell et al., 1979), however, would be partly attributed to the diversity of dynein from different sources and difference

in buffer system used in electrophoresis. Third, it is not easy to obtain sufficient amount of cilia and flagella, and the method for purification of sufficient amount of dynein other than sucrose density gradient method, which provides the means for only partial purification, has not yet been established. Fourth, extracted dynein tends to aggregate rapidly and no adequate method for storage has been developed. These features have limited the biochemical characterization of dynein. Only recently it was found that sea urchin 21S dynein preparation showed two heavy chain bands termed $A\alpha$ and $A\beta$ on SDS gels (Bell et al., 1979). However, a question arise as to whether these two heavy chain bands represent two distinct species or the smaller one is a cleaved product of the larger one.

In Part I of this thesis, I describe the biochemical characterization and elctron microscopic observation of sea urchin 21S dynein and its dissociated products. Most effort was made to clarify the similarity and difference between two heavy chains. I developed a SDS gel electrophoresis system containing 6 M urea, which allows us better separation of $A\alpha$ and $A\beta$ heavy chain than the Laemmli system (1971). Retrieval of these heavy chains separately from gel slices was possible and the biochemical analysis could be performed with least contamination of other polypeptide chains in the dynein preparation. These analyses indicated that there are several differences between these heavy chains.

Materials and Methods

1. Preparation of axonemes and isolation of 21S dynein

Sperm from the sea urchins Pseudocentrotus depressus and Hemicentrotus pulcherrimus were obtained by injecting the animals with 0.5 M KCl and centrifuging the released sperm at 4,000 x g for 5 min. The collected sperm was kept at 0°C and used within 2 days.

Axonemes were prepared by the method of Yano & Miki-Noumura (1980) with some modifications. The sperm was diluted with equal volume of sea water and then suspended in 20 times the volume of an axoneme isolation medium (0.25% Triton X-100, 0.1 M NaCl, 4 mM $MgCl_2$, 1 mM $CaCl_2$, 0.1 mM EDTA, 0.2 mM dithiothreitol (DTT), 0.1 mM phenylmethylsulfonyl fluoride (PMSF), 10 mM Tris-HCl, pH 8.0. The sperm suspension was stirred gently for 10 min on ice to solubilize the membranes and to separate tails from sperm heads. The suspension was centrifuged first at 1,500 x g for 10 min at 4°C. However, the separation was incomplete and the precipitate contained both isolated heads and intact sperm. Therefore, the pellet was resuspended in the 20 volume of fresh isolation medium and centrifuged again for 10 min. The supernatants from these centrifugations contained mainly isolated and demembranated tails, and were centrifuged at 10,000 x g for 10 min to collect axonemes.

The resultant pellet was washed with the axoneme isolation medium without Triton X-100. "High salt extracts" were obtained by suspending the washed pellet in ice bath in 0.6 M NaCl, 4 mM $MgSO_4$, 1 mM $CaCl_2$, 0.1 mM EDTA, 1 mM DTT, 0.1 mM PMSF, and 10 mM Tris-HCl, pH 8.0 at a protein concentration of around 10 mg/ml for 10 min. The suspension was centrifuged at 12,000 x g for 10 min, and the supernatant was clarified by centrifugation at 100,000 x g for 15 min. The extract was concentrated to a protein concentration of around 5 mg/ml by ultrafiltration under N_2 using Amicon XM 100A membrane.

Sucrose density gradient centrifugation of the concentrated extract was carried out on a 5 to 20% (w/w) sucrose gradient in a solution of 0.1 M NaCl, 4 mM $MgSO_4$, 1 mM $CaCl_2$, 1 mM DTT, 0.1 mM PMSF, 10 mM Tris-HCl, pH 8.0. After sedimentation at 26,000 rpm for 19 to 20 hr in a RPS 27-2 rotor of a Hitachi 55P ultracentrifuge, each gradient was separated into 20 to 22 fractions of equal volume by carefully lowering a capillary tube to the bottom of the centrifuge tube and removing the contents with a peristaltic pump. The fractions were numbered 1 to 22 according to their position from bottom to top of the gradient. The first peak of protein with ATPase activity (Fig. 1) was pooled and used as 21S dynein.

2. Preparation of subunits of 21S dynein

Subunits of 21S dynein was obtained by dialyzing the 21S dynein prepartion exhaustively against low salt solution (0.1 mM EDTA, 0.1 mM DTT, 0.1 mM PMSF, 1 mM Tris-HCl, pH 8.0) for 18

hr, followed by the concentration to 1-2 mg/ml using an Amicon XM 100A membrane. Centrifugation was done on a 5 to 20% sucrose density gradient in the law salt buffer and fractionated in the same manner as in the preparation of 21S dynein.

3. Preparation of two heavy chain polypeptides

As 21S dynein showed two heavy chains termed Aα and Aβ in SDS-urea polyacrylamide gel electrophoresis (Fig. 2), these two heavy chains could be separately retrieved from gel slices. To prepare the specimen for amino acid analysis, Tris-borate buffer system (Mornet et al., 1982) was used to suppress the intrusion of glycine from electrophresis buffer; otherwise, Tris-glycine system was used. To distinguish heavy chain bands without staining, 21S dynein preparation was treated with a fluorescent reagent N-(7-dimethylamino-4-methyl coumarinyl) maleimide (DACM, Yamamoto et al., 1977) prior to electrophoresis. Fluorescent bands corresponding to Aα and Aβ heavy chains were cut out from disc gels under UV lamp, and each heavy chain polypeptide was separately eluted from the gel slices by electrophoresis (Stephens, 1975). These recovered heavy chains had sufficient concentration for subsequent chemical analyses.

4. Proteolytic digestion of Aα and Aβ fractions and of denatured heavy chains

To analyze chemical composition of Aα and Aβ heavy chains, the Aα and Aβ fractions, which contain Aα and Aβ heavy chains,

respectively, and formed two peaks on sucrose denstity gradient (Fig. 3), were digested with trypsin at the weight ratio of 1/1000 or with χ-chymotrypsin at the weight ratio of 1/20 for 1 to 20 min at 37 °C. Alternatively, recovered heavy chains from SDS-urea gels were digested with trypsin at the weight ratio of 1/1000 for 1 to 10 min at 30 °C in the presence of 0.1% SDS. Digested specimens were examined by SDS gel-electrophoresis using the Laemmli system (1970).

5. Amino acid analysis

Amino acid analysis of $A\alpha$ and $A\beta$ heavy chains was performed on a hand-made analyzer (at laboratory of Prof. M. Yamazaki) using o-phtalaldehyde system (Benson & Hare, 1975). To prevent tryptophan digestion, the heavy chains recovered from SDS gels were hydrolyzed in 4 N methane sulfonate at 115°C for 24 hr (Simpson et al., 1976).

6. Determination of the number of thiol groups

The number of SH groups of 21S and its subunits was determined by the method of Ellman (1959) with a slight modification. The specimen was dialyzed against the buffer containing 10 mM Tris-HCl, pH 8.0 and 0.5 mM EDTA with or without 1% SDS. One-tenth volume of 10 mM 5,5'-dithiobis (2-nitrobenzoic acid) (DTNB) dissolved in 0.1 M phosphate buffer, pH 7.0 was added to the specimen at 25°C, and the absorbance at 412 nm was recorded for 90 min. The reaction

was completed within 30 min when SDS was contained. Under the non-denaturing conditions, the absorbance increased even at 90 min. A molar extinction coefficient of 14,000 M^{-1} cm^{-1} (Riddles et al., 1983) was assumed.

7. Determination of ATPase activity

For routine assay of ATPase activity of dynein extracts and of the gradient fractions, enzyme was added to 1 ml of an assay medium containing 0.1 M NaCl, 4 mM $MgCl_2$, 1mM $CaCl_2$, 10 mM Tris-HCl, pH 8.0 and 1 mM ATP, and incubated for 10 min at 25°C. The liberated inorganic phosphate was measured by the method of Murphy & Riley (1962). For steady state kinetic analysis, an ATP-regenerating system was used. Around 0.01 mg/ml of enzyme, 1 mM phosphoenolpyruvate and 0.05 mg/ml pyruvate kinase in the assay medium were preincubated for 1 min at 25°C. ATPase reaction was started by adding various concentrations of ATP, and the reaction was terminated after 10 min by adding 2,4-dinitrophenyl hydrazine to the final concentration of 0.1 mM. The mixture was incubated for 10 min at 37°C, and the concentration of hydrazine formed by liberated pyruvate and 2,4-dinitrophnyl hydrazine was measured according to the method of Raynard et al. (1961).

8. Determination of protein concentration

Protein concentration was determined by measuring the absorbance at 280 nm assuming that the extinction coefficient

of 1.0 cm^2/mg, or by the Bradford method (Bradford, 1976). At the later stage, an improved Bradford method (Read & Northcote, 1981) was employed. When 0.1% Serva Blue G was used, final concentration of 10 μg/ml of dynein gave the optical density of 0.61 at 595 nm. All of these values were based on the protein concentration measured by the biuret method.

9. Sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis

To study the composition of dynein molecules, SDS polyacrylamide gel-electrophoresis using the discontinuous buffer system (Laemmli, 1970) was performed on 6% slab gels.

To obtain highly resolved gel patterns in the heavy chain region (ca. 500,000 daltons), polyacrylamide gel containig 6 M urea was used. The gel contained 2.68% acrylamide, 0.11% N,N'-methylene-bis-acrylamide, 6 M urea, 25 mM Tris-glycine, pH 8.3, 0.05% SDS, 5% (v/v) glycerol, 0.13% ammonium persulfate and 0.1% N,N,N',N'-tetraethylmethylenediamine (TEMED). The gel was cured at least 10 hr at room temperature. Samples were boiled in a solution containing 8 M urea, 0.5% SDS, 5 mM EDTA, 20 mM Tris-HCl, pH 8.4, for 2 min. Care was taken to avoid excess boiling which would cause the degradation of the heavy chains (Kowit & Malony, 1982). The electrode buffer was 25 mM Tris-glycine, pH 8.3, containing 0.05% SDS.

Gels were run in constant current mode either at 15 mA for

6 hr or at 6 mA for 14 hr at 4°C, and stained with 0.01% Coomassie brilliant blue R-250 (Fairbanks et al., 1971) or with silver using BioRad silver staining kit (Merril et al., 1981).

To estimate the molecular weight of proteins, gel electrophoresis was performed using phosphate buffer system (Weber & Osborn, 1969) on the gels of 7 or 12 cm long containing 3% acrylamide and 0.1% N,N'-methylene-bis-acrylamide. For the molecular weight markers, bovine serum albumin (M_r = 68,000, Tanford et al., 1967; 66,000, Sigma Chemical Co.) oligomers cross-linked with glutaraldehyde (Payne, 1973) and commercially available from Sigma Chemical Co., bovine thyroglobulin (M_r = 335,000), cross-linked hemocyanin (M_r = 70,000, Sigma) and rabbit skeletal muscle myosin heavy chain (M_r = 194,000, Gazith et al., 1970; 212,000, Gershman et al., 1966) oligomers cross-linked with p-phenylene-N,N'-bis(maleimide) were used. Cross-linked myosin was the gift from Prof. K. Maruyama of Chiba University. Gels were run at constant current of 8 mA for 7 cm long gels and 5 mA for 12 cm long gels, respectively.

10. Isoelectric focusing

Isoelectric forcusing was performed using agarose IEF (Pharmacia) according to the method of Stromska (1982). The agarose gels containing 1% agarose IEF, 12% sorbitol, 2.4% Ampholine (1.2% pH 3.5-10 and 1.2% pH 5-7, LKB) and 6M urea, were cast flat on glass plates of 5 x 12 x 0.1 cm. Samples were applied into the wells (1.5 x 5 mm) made at the distance

from 3.5 cm from cathode. Collex-sponge electrodes (Pharmacia) were used. Electrolyte was 1 M NaOH and 0.05 M H_2SO_4 for cathode and anode, respectively. Electrophoresis was done at 4°C at the constant voltage of 100 V for 14 hr and 200 V for 1 hr.

After focusing, the gel was fixed in 10% trichloroacetic acid, 5% 5-sulfosalicylic acid for 1 hr, and washed with 30% methanol, 10% acetic acid for 3 hr with two or three changes. The gel was pressed flat by placing four or five layers of filter paper (Whatman, No.1), a glass plate and a 500 g weight. After 20 min, the flattened gel was dried in air. The dried gel was stained with 0.2% Coomassie brilliant blue R-250, 30% methanol, 10% acetic acid for 10 min, and was destained in 30% methanol and 10% acetic acid.

To determine the pH gradient of the developed gel, a longitudinal strip of the gel was cut into 5 mm slices, and each slice was put in a glass test tube. Two ml of degassed water was added to each tube, and the tubes were vortexed periodically during a 1 hr equilibration period. The pH was measured at 4°C with a pH meter.

Two-dimensional gel electrophoresis was done according to the method described by O'Farrel (1975) with some modifications. The longitudinal strip of agarose gel was applied on the stacking gel of the Laemmli's system (1970). One % agar solution containing 0.5% SDS and 0.125 M Tris-HCl, pH 6.8, was warmed to 40°C and poured onto the agarose gel. Immediately

after the agar was set, electrophoresis of the second dimension on 6% acrylamide gel was started at the constant current of 25 mA at 4°C.

12. Electron microscopy

One drop of specimen containing 20 to 50 μg/ml of proteins was applied to a thin carbon film supported by carbon microgrids, and stained with 1% uranyl acetate (unbuffered) or 1% uranyl oxalate (pH 6.0) (Haschemeyer & Meyers, 1970). On occasion, 10 μg/ml bacitracin was included in the stain solution as wetting reagent (Gregory & Pirie, 1973).

To reduce the background noise, the thin carbon film was made by indirect deposition onto a freshly cleaved surface of mica and rendered hydrophilic by ultraviolet irradiation just prior to use (Johansen, 1977). Plastic microgrids were prepared according to the method of Fukami & Adachi (1965), and Triafol film was removed to avoid specimen drift (Toyoshima & Wakabayashi, 1984).

Specimens were examined on JEOL JEM 100CX operated at 80 kV, Hitachi H300 at 75 kV and H700 at 100 kV at the nominal magnification of 50,000 times. Magnification was calibrated by measuring 395 Å striation of tropomyosin tactoids (Casper et al., 1969).

Results

1. Preparation of 21S dynein and its subunits

The high salt extract of axonemes contained 10-15% in weight of proteins in axonemes and retained 50-55% of Mg^{2+}-ATPase activity of the whole axonemes which were able to show the sliding of microtubules in the presence of ATP and trypsin. These values were consistent with those reported by Gibbons and Fronk (1979). The sedimentation pattern of protein and ATPase activity on 5-20% sucrose density gradient is shown in Fig. 1. The first and the second peaks correspond to 21S and 13S dynein, respectively. The third peak of protein near the top of the gradient contains tubulin and other small polypeptides.

SDS polyacrylamide gel electrophoresis of 21S dynein in the Laemmli system showed a band of heavy chain corresponding to the A band dynein (Gibbons et al., 1976), three bands of intermediate chains termed IC1, IC2 and IC3 (Kincaid et al., 1973), and several bands of light chains (Fig. 2a). On SDS-urea gels, 21S dynein showed two bands of heavy chains (Fig. 2b), which would correspond to A_α and A_β heavy chains described by Bell et al. (1979) using the system of Laemmli. In my hands, SDS-urea gels brought forth much clear separation and constant results.

The 21S dynein could be dissociated to smaller components by dialyzing it against the low salt buffer (10 mM Tris-

HCl, 0.5 mM EDTA and 0.5 mM DTT) for 18 to 22 hr. These dissociated products were partially fractionated by sucrose density gradient centrifugation. As illustrated in Fig. 3, the sedimentation profile had two peaks. SDS-urea gel electrophoresis showed that the major components of fractions 15 and 18 were Aα and Aβ heavy chains, respectively. Fraction 18 also contained three intermediate chains. The profile of ATPase activity almost coincided with that of Aβ heavy chains, and a shoulder was present at the peak of Aα heavy chain. One or two fractions around the peaks were used for further studies. Hereafter, these fractions will be called Aα and Aβ fractions.

2. Molecular weight of 21S dynein

Various values from 300,000 to 600,000 have been reported for molecular weight of 21S dynein (Kincaid et al., 1973; Gibbons et al., 1976; Mabuchi, 1978; Bell et al., 1979). This large discrepancy would be attributed to the lack of good markers in high molecular region and to different electrophoretic buffer systems. As the buffer system, continuous phosphate buffer system (Weber & Osborn, 1968) should be adopted. Even in this buffer system, good linear relationship between the logarithm of molecular weight and the relative mobility persists only in the middle region of the gels. When 3% acrylamide gels were used, relative mobility of heavy chains was

about 0.2, within a linear region. As the molecular weight markers, cross-linked bovine serum albumin (BSA), hemocyanin, and myosin heavy chain were used.

As can be seen from Fig. 4, BSA oligomers cross-linked with glutaraldehyde according to the method of Peyne (1973) and hemocyanin oligomers obtained from Sigma showed good linearity. BSA oligomers available from Sigma had the same mobility with glutaraldehyde cross-linked BSA up to dimers but showed slower mobility when larger oligomers were concerned. Myosin monomer and dimer had the same mobility with commercial BSA oligomers; trimer and larger oligomers showed higher mobility, and there was a bend in the curve. Since the glutaraldehyde cross-linked BSA oligomers had good linear relationship and showed an intermediate mobility between commercial BSA and hemocyanin oligomers, and since thyroglobulin also lies on this line, the line obtained with glutaraldehyde cross-linked BSA oligomers was used as the standard for estimation of molecular weight.

This electrophoretic system could not resolve $A\alpha$ and $A\beta$ heavy chains and formed a single band of 500k daltons. The molecular weights of IC1, IC2 and IC3 intermediate chains were 125k, 94k and 73k, respectively, and roughly the same with the values published by Bell et al. (1979).

3. Kinetic properties of 21S dynein and its subunits

As shown in Fig. 3, both $A\alpha$ and $A\beta$ fractions had ATPase activity, and $A\beta$ fraction showed higher specific activity than

Aα fraction. To know the kinetic properties, steady state ATPase activity of 21S dynein and these two fractions were measured at various concentrations of Mg^{2+}-ATP. Fig. 5 shows double-reciprocal plots: the one for 21S dynein is a convex curve, while the ones for Aα and Aβ fractions are linear lines. Km values of these two lines were about 17 μM and 110 μM, respectively. These values and Vmax are listed in Table I.

These results indicate that Aα and Aβ fractions are different ATPase, and that the ATPase activity of 21S can be considered as the sum of these two ATPases.

4. Proteolytic digestion of Aα and Aβ heavy chains

To examine the similarity of Aα and Aβ heavy chain polypeptides, trypsin and chymotrypsin digestion patterns both under physiological and denaturing conditions were examined on SDS gels.

Fig. 6a and 6b show the time course of the trypsin and chymotrypsin digestions, respectively. When treated with trypsin, Aα heavy chain disappeared shortly and no distinct bands appeared on high molecular weight region, whereas three large polypeptides were produced from Aβ heavy chain and remained constant in 20 min digestion. When digested with chymotrypsin, fragments larger than the one produced by trypsin were brought forth from both heavy chains. The one from Aα heavy chain was disintegrated while that from Aβ heavy chain persisted in 20 min digestion.

Fig. 6c shows the digestion pattern with trypsin in the presence of SDS. Many bands from these two heavy chains do not coincide with each other.

5. Amino acid composition of Aα and Aβ heavy chains

To examine the amino-acid composition of Aα and Aβ heavy chains without contributions from other polypeptides such as intermediate chains, and from electrophoresis buffer, heavy chain bands were cut out from SDS-urea gels run using the Tris-borate buffer system.

Although the heavy chains were hydrolyzed in 4 N methane sulfonate to prevent the degradation of tryptophan residue, the peak of tryptophan could not be resolved. Proline could not be detected in this o-phthalaldehyde system.

The results were summarized in Fig. 7 and Table II, together with some published data. In summary, amino acid compositions of the Aα and Aβ heavy chains were almost the same. Comparison with the published data and other proteins will be described in "Discussion".

6. Number of SH groups

To determine the numbers of free and total SH groups, the measurements were performed in the presence and absence of 1% SDS. The time-course in the presence of SDS (Fig. 8) showed that 90% of the total SH groups were reacted within 10

min and the reaction reached plateau around 30 min with all three specimens. The numbers of total SH groups per 10^5g protein thus determined were 12.9, 9.3 and 10.6 for 21S dynein, A_α and A_β fractions, respectively. These values are listed in Table III.

Under non-denaturing conditions, apparently two phases were observed. In the first phase (within 10 min) the reaction proceeded rapidly. The number of SH groups reacted in this phase is considered to represent the number of free thiols. As can be seen from Table III, the numbers correspond to 50 to 60% of those of total SH groups. In the second phase, the time course was different among 21S dynein and its dissociated products. Although 21S dynein reached the plateau level at around 60 min, A_α and A_β fractions showed substantial increase still at 120 min. These results suggest that the conformations of A_α and A_β fractions change considerably by the modification of SH groups, and thus expose the SH groups initially located inside and make them accessible to DTNB.

7. Isoelectric focusing of 21S dynein and its subunits

Isoelectric points of the polypeptide chains constituting 21S dynein could be determined only in the presence of urea. The addition of Triton X-100, Nonidet P-40, SDS, sucrose or urea to the samples to be electrophoresed did not give good results unless the gels also contained 6 M urea. As shown in Fig. 9, inclusion of 3 M urea was insufficient and the

presence of 6 M urea was indispensable to obtain well resolved patterns. Addition of 10% sucrose to the specimen improved the separation.

Isoelectric focusing of 21S dynein showed two dense bands (Fig. 9): the basic one was diffuse while the acidic one was well defined. The gels of separated Aα and Aβ fractions showed only one of the two bands: the diffuse one corresponded to Aα heavy chain and the well-defined one corresponded to Aβ heavy chain. There were two other bands in the acidic region of the gels of 21S dynein and Aβ fraction. These two bands were identified as IC1 and IC3 by two-dimensional gel electrophoresis (Fig. 10). The spot of IC2 was diffuse and could be recognized only in the two-dimensional gel. Its isoelectric point was almost the same as that of Aα heavy chain. Isoelectric points of these polypeptides were estimated by the pH gradient curve measured with pH electrode (Fig. 11). The values were 6.1 for Aα heavy chain, 5.6 for Aβ heavy chain, 4.7 for IC1, 6.0 for IC2 and 5.3 for IC3 (Table IV).

8. Electron microscopy of 21S dynein and its dissociated products

Twenty-one S dynein and its dissociated products partially purified by sucrose density gradient centrifugation were negatively stained and examined in an electron microscope. Although electron microscopy was performed in the same manner as in the electron microscopy of Tetrahymena 30S dynein

described in Part II of this thesis, it was far more difficult to obtain good images. This difficulty mainly arose from the tendency of sea urchin dynein to make small size aggregates, and to dissociate into smaller particles.

Fig. 12 shows a large field of view of 21S dynein preparation. Particles in this field show various shapes and dimensions. Most of them appear as rounded triangles with the dimension of about 16 nm, which are similar to the images obtained in our earlier studies (Yano & Miki-Noumura, 1981; Yano et al., 1982, 1983). There are stain pits or line around the center of particles, and the substructure occasionally seen is symmetrical across the center line (Yano et al., 1982, 1983). Two particles in each circle in Fig. 12 appear to be connected by long filamentous structure and constitute a two-headed particle, which might be an analog to the three-headed "flower-bouquet" structure of 30S dynein from Tetrahymena cilia (Johnson & Wall,1983; Part II of this thesis), and two-headed 18S dynein from Chlamydomonas flagella (Witman et al., 1983). The dimension and the shape of the head portion are indistinguishable from other apparently isolated particles. Since the proportion of these two-headed particles was small, it is not possible to decide whether this two-headed structure is the genuine one.

Fig. 13a and 13b show electron micrographs of $A\alpha$ fraction and $A\beta$ fraction, respectively. $A\alpha$ fraction tended to form

aggregates of various sizes, and was thus the most difficult object. The size of the particles could not be well specified. Some of them have similar to or even larger dimension than that of 21S dynein, but evidently smaller particles (about 13 nm) also can be seen. Aβ fraction had weaker tendency to form aggregates. Nevertheless, dimension of the particles are different from particle to particle in Fig 13b. Major population has smaller dimension (13 nm) than that of 21S dynein, and there is no distinct stain pits or lines in the images. However, much more smaller particles (around 8 nm) are also seen in this figure. It is not yet possible to specify how Aα and Aβ subunits are integrated in 21S dynein.

Discussion

1. Preparation of 21S dynein

Gibbons & Fronk (1972) proposed the method for preparation of the latent form of 21S dynein (LAD-1) by extracting dynein at pH 7.0 from isolated axonemes in sea urchin Tripneustes gratilla. They noted that the specific activity of LAD-1 was 0.25 μmole Pi/mg/min, and that the preparation at pH 8.0 resulted in partial activation of 21S dynein. Terashita et al. (1983) prepared 21S dynein accroding to the method of Gibbons & Fronk from Japanese sea urchin Pseudocentrotus depressus, which was also used in the present study, and reported the value of 0.63 μmole Pi/mg/min for the specific activity. They also noted that the double reciprocal plot for the ATPase activity of dynein activated by the treatment at 40°C consisted of two straight lines, while that of non-activated 21S dynein fitted a single straight line.

In this study, 21S dynein was prepared from Pseudocentrotus depressus at pH 8.0. The specific activity was 1.05 μmole Pi/mg/min, and the double-reciprocal plot showed the convex profile. Therefore, the 21S dynein preparation used in this study might be partially activated.

Dissociation products of 21S dynein were obtained by a slightly different method from that of Tang et al. (1982). Nevertheless, the profile of protein concentration on sucrose density gradient had two peaks termed A_α and A_β fraction, where A_α and A_β heavy chains are major components and intermediate

chains were associated with Aβ fraction, providing the same results as those obtained by Tang et al. However, the parameters for kinetic properties of Aα and Aβ chains are different. Km of Aα and Aβ fractions reported by them were 7.3 and 2.8 μM, while the values presented here were 16.7 and 110 μM, respectively, showing large discrepancy. Vmax of Aα and Aβ fractions reported by them were 0.24 and 0.7 μmole Pi/mg/min, and the values obtained here were 0.32 and 1.10 μmole Pi/mg/min. Since the specific activities reported by Gibbons & Fronk (1972) and Terashita et al. (1983) were different from each other, at least a part of these large discrepancies would be attributed to the difference in species of sea urchins used.

The non-linear profile of the ATPase activity presented in double reciprocal plots could be accounted by simple summation of two ATPases, namely, Aα and Aβ fractions. Gibbons (1966) and Shimizu & Kimura (1974) reported the linear profile of ATPase activity of Tetrahymena 30S dynein in double-reciprocal plots. The range of ATP concentration in their experiments, however, was not so large as examined in this study. Indeed, the measurement of ATPase activity at wide range of ATP concentration revealed the presence of two ATPases with the Km of 1 μM and 20 to 30 μM (M. Takahashi, personal communication).

It is interesting that Aα fraction shows higher ATPase activity than Aβ fraction at lower (5 to 10 μM) ATP concentration range. This feature might have physiological meanings,

since the Km value of beat frequency of the Triton model of sperm flagella is 100 μM or so (Gibbons & Gibbons, 1972), and the recovery of rigor wave takes place at 1 μM ATP (Gibbons & Gibbons, 1974).

2. Molecular weight of 21S dynein heavy chains

The values reported for the molecular weight of dynein heavy chains from sea urchin sperm flagella have widely varied between 300,000 and 600,000 (Table V). When the Tris-glycine buffer system is used, the apparent molecular weight of heavy chains is about 350k (380k, Gibbons et al., 1976; 330k, Bell et al., 1979) on SDS gels. When the phosphate buffer system is used, the molecular weight appears much larger (520k, Kincaid et al., 1973; 600k, Mabuchi et al., 1976). The value obained in this study is 500k.

Non-linearity of the logarithm of the molecular weight to relative mobility in the discontinuous buffer system has been pointed out (Neville, 1971), whereas good linearity has been reported when the continuous phosphate buffer system was used (Weber & Osborn, 1966). Thus, the phosphate buffer system was employed for molecular weight estimation in this study. Difficulties were encountered, however, as to the difference in apparent molecular weights of several marker proteins. It should be noted that cross-linked molecules do not necessarily show the same mobility as that of a single molecule with the

same molecular weight, and that the difference in charge of the molecule may result in different mobility. BSA oligomers cross-linked with glutaraldehyde (Payne, 1973) was chosen as the standards, since they showed good linearity and intermediate mobility, higher than BSA oligomers and lower than hemocyanin oligomers both obtained from Sigma, and since the presence of octamer and the use of 3% gels have eliminated the necessity of extraporation. All the values reported by other workers listed in Table 5 have been obtained using extraporation.

Thus, I believe that the value of 500k obtained here is the most plausible estimation by SDS gel electrophoresis. From the study of guanidine hydrochloride-denatured 21S dynein heavy chains, Bell reported 500k as the molecular weight of one heavy chain (Bell, 1983). Sedimentation equilibrium and sedimentation diffusion study (Gibbons & Fronk, 1979) provided the value of 1,250k for whole molecule, which also agrees with the present value, considering that the sum of the weights of intermediate chains (290k) and the presence of two heavy chains.

3. Biochemical characterization of A_α and A_β subunits

As described in previous sections, A_α and A_β fractions have different ATPase properties. Nevertheless, their molecular weights were very close to each other, and SDS polyacrylamide gel electrophoresis in the phosphate buffer system

could not resolve them as two heavy chains as in the SDS-urea gel electrophoresis. This finding suggests the difference in charge between two heavy chains, as confirmed by isoelectric focusing.

Limited proteolytic digestion clearly revealed the difference between two heavy chains. On the other hand, amino acid analysis showed that the amino acid compositions are almost the same. These compositions can be compared with published data. Amino acid composition of dynein reported by Ogawa et al. (1975), which would be the mixture of 21S and 13S dynein, shows smaller amount of serine, glycine and tyrosine, and larger amount of alanine than the present data. Tetrahymena 30S dynein (Kaji, 1973) contained larger amount of isoleucine and lysine and smaller amount of glycine. As a whole, the difference in amino acid composition among the various species of dynein was smaller than that between myosin and its subfragments (Lowey et al., 1971).

As to the amount of SH groups in dynein, Gibbons & Fronk (1972) reported that 6 mole/10^5g of SH reacted within 3 min under native conditions, and 11 mole/10^5g of SH reacted with DTNB in the presence of 8 M urea. These values are in good accordance with the present results. Shimizu et al. (1979) reported that 30S dynein from Tetrahymena cilia contain SH groups of 3.5 mole at the native state and 10.2 mole in the presence of 2.5% SDS. Total number of SH was estimated to be 12.9 mole from the time course. Accurate comparison between

Aα and Aβ heavy chains was impossible, since Aβ fraction contained other polypeptides. To estimate the weight of Aβ heavy chains and intermediate chains in the Aβ fraction, densitometry of the gels loaded with DACM labeled specimen was performed, but definite results have not been obtained so far.

4. Isoelectric focusing of 21S dynein components

This is the first report about the isoelectric point of dynein. Isoelectric focusing is a sensitve method of resolving proteins on the basis of their charges. Difference in isoelectric point may be utilized in separating the components of 21S dynein. The difficulty related to the large weights of polypeptide chains in 21S dynein has been overcome by the use of agarose gels instead of polyacrylamide gels as the supporting material. Large pore size of agarose gels reduced the sieving effect which would affect the ability of large proteins to reach their isoelectric points, and the new form of charge-balanced agarose greatly decreased the electroosmosis. Nevertheless, whole molecule of 21S dynein is still too large, and the isoelectric points of the components of 21S dynein could be measured only in the presence of 6 M urea.

The interaction of proteins with urea may result in larger values for isoelectric point. That is, the presence of urea causes the basic shift. Moreover, the presence of urea would decrease the activity coefficient of hydrogen ion, which

necessitates the correction of -0.42 for pH value in the presence of 6 M urea (Ui, 1971). Thus, the actual isoelectric points may be more acidic than those presented here.

The isoelectric point of tubulin measured under similar conditions was about 5.5 (Stromska, 1982). The isoelectric point of Aβ heavy chain would be almost the same as that of tubulin, and that of Aα heavy chain would be more basic. The difference in pI between Aα and Aβ heavy chains was 0.5, which suggests the possibility of developing a new method for separating Aα and Aβ chains using ion-exchange chromatography.

5. Electron microscopy of 21S dynein and its dissociated products

Electron microscopy of sea urchin 21S dynein and its dissociated products was found to be much more difficult than that of 30S dynein from Tetrahymena cilia. They appear to form aggregates of various sizes and to collapse on specimen grids. Thus it was not possible to describe their dimensions or shapes exactly. Of three specimens examined, the size of the globular part of 21S dynein was the most uniform. It is of interest to know how Aα and Aβ heavy chains constitute 21S dynein. STEM analysis of 30S dynein from Tetrahymena cilia Johnson & Wall, 1983) and 18S dynein from Chlamydomonas flagella (Witman et al., 1983) showed three-headed and two-headed particles, respectively. It was also noted that the size of dynein arms on microtubules is much smaller than the simple sum

of the dimensions of multiple heads. Negatively stained images of these dynein preparations also showed the characteristic stain pit in each globular head. Sale (1983) reported that clusters of 3 to 4 particles were seen when 21S dynein was dried from glycerol on mica substrate and rotary shadowed. The diameter of the globular particles found in the electron micrographs of 21S dynein (Fig. 12) was almost the same as that of 30S dynein (15 nm, Fig. 16a) and that of dynein arms on doublet microtubules (Yano et al., 1982). Recombined arms with microtubules again showed the stain pit in each arm (Yano et al., 1983). Thus, the globular particles may represent whole dynein arms or their components.

In contrast to 30S dynein from Tetrahymena, 21S dynein can be dissociated into smaller components by low salt treatment. Electron micrographs published so far (Mohri et al., 1968; Tsuprun & Shanina, 1980) showed globular particles of low salt extracted dynein, the dimension of which was roughly 10 nm and similar to the smaller particles found in dissociated products studied here ("Results", 8.). Although prepared as 21S dynein, dilution of the specimen with a buffer of not so high ionic strength for electron microscopic examination might cause such a dissociation. In addition, 21S dynein was usually prepared at pH 8.0 and this condition might cause partial activation of 21S dynein (Gibbons & Fronk, 1972), which was also observed when 21S dynein was dissociated with Triton X-100. The tendency of 21S dynein to dissociate into smaller

fragments might explain the small number of apparently two-headed particles in the images of 21S dynein (Fig. 12). Thin rod protruding from globular part (Goodenough & Heuser, 1982; Yano et al., 1982; W.S. Sale, personal communication; Johnson & Wall, 1983; Part II of this thesis) was observed only with two-headed particles, consistent with the idea that large proportion of 21S dynein was dissociated when observed in an electron microscope under the present conditions. However, the thin rods protruding from globular heads are less than 3 nm in diameter in sea urchin sperm and Chlamydomonas flagellar axonemes (W.S.Sale, perosnal communication; Goodenough & Heuser, 1982), and may not be readily seen in the negatively stained specimen. In fact, it is not impossible to interprete that the major proportion of the particles in Fig. 12 are paired. Preliminary experiments on LAD-1 prepared without Triton X-100 according to the method of Bell et al. (1982) showed an increase in two-headed particles, but the extent is different from preparation to preparation. Protein concentration on speicmen grids seems to be critical. More direct methods, such as labelling with antibodies, will be required to settle this problem.

Part II

Thirty S Dynein from *Tetrahymena* Cilia

Introduction

Tetrahymena cilia is the oldest (Gibbons, 1963) and still a major source of dynein. Tetrahymena cilia contain two classes of dynein termed 30S and 14S dynein by Gibbons & Rowe (1965). Although recent measurements of sedimentation coefficient have provided the value of 21S for 30S dynein (Mitchell & Warner, 1981; Johnson & Wall, 1983), the term "30S dynein" has still been widely used and will be also used here.

Many structural investigations have been done on dynein arms using disintegrated cilia or isolated doublet microtubules from Tetrahymena cilia. Warner and co-workers analyzed ATP-disintegrated cilia and isolated dynein, and claimed that dynein arms on outer microtubule consisted of lineary arranged three globules (Warner et al., 1977). However, since negatively stained images provide the projections of structures, overlap of two rows of dynein arms should be taken into account (Yano et al., 1982). Recent structural studies using rapid-freezing and metal replication techniques proposed the different architecutre of dynein arms (Goodenough & Heuser, 1982; Tsukita et al., 1983; W.S. Sale, personal communication). Scanning transmission electron microscope (STEM) analysis of freeze-dried or negatively stained isolated 30S dynein showed that 30S dynein molecules are composed of three globules and thin rods constituting a "flower-bouquet", and provided a measurement of molecular weight which was 1.9 million (Johnson & Wall, 1983). Our electron microscopic analysis of 30S dynein

negatively stained at more neutral pH than formerly employed, confirmed the three-headed "flower-bouquet" structure. Further, SDS-urea gel electrophoresis, successfully used in resolving two heavy chains of sea urchin 21S dynein as described in Part I of this thesis, revealed 30S dynein comprises three heavy chains. Molecular weight estimation on SDS gels provided the value of 500k for all these three heavy chains.

To characterize the heavy chains and to examine the configuration of three globules in the dynein arms on microtubules, it is necessary to identify or isolate each of three heavy chains. I found that chymotryptic digestion could provide useful means when combined with SDS-urea gel elctrophoresis. Chymotrypsin could produce large fragments of 370k to 430k daltons; time course of the digestion was quite different among heavy chains; digestion pattern was different depending on the presence or absence of magnesium ion. Electron microscopy showed one-headed and two-headed particles in the digested products. Thus, I could isolate at least one of three heads in almost intact form, and these observations suggest that one head corrseponds to one heavy chain, and each of three heads is different from one another.

Materials and Methods

1. Preparation of 30S dynein

Tetrahymena pyriformis (strain W) and *Tetrahymena thermophilla* (SB-255, gift from Dr. E. Orias, University of California, Santa Barbara, CA, U.S.A) were grown in a medium containing 1% proteose peptone, 0.5% yeast extract (both from Difco Co.), 0.87% glucose and a small amount of anti-foaming agent (Nakarai Chem. Co.) with aeration at 25°C. After collecting cells by low speed centrifugation, cilia were detached and isolated according to the method of Porter and Johnson (1983) with a slight modification. The collected cells were resuspended in a fresh culture medium without anti-form agent, and dibucaine hydrochloride was added to a final concentration of 5 mM. After observing that the cilia were detached and the cells began to round up under a phase contrast microscope, an equal volume of fresh medium was added and centrifuged twice at 1,500 g for 5 min at 4°C. The supernatant was then centrifuged again at 10,000 g, 15 min, 4°C to collect the cilia.

The cilia were then washed with 0.1 M NaCl, 10 mM HEPES, pH 7.4, 4mM $MgCl_2$, 0.1 mM EGTA, 1 mM dithiothreitol (DTT) and 1 mM phenylmethylsulfonyl fluoride (PMSF) by differential centrifugation at 1,000 g, 2 min , 4°C to remove cell bodies and 10,000 g, 10 min, 4°C to pellet cilia. The ciliary membrane was extracted for 20 min at 4°C with 0.25% Nonidet-P-40 in the washing solution. The demembranated axonemes were

then washed three times with the washing solution to remove traces of the detergent. Finally, the washed axonemes were resuspended in 0.6 M NaCl, 10 mM HEPES, pH 7.4, 4 mM $MgCl_2$, 0.1 mM EGTA, 1 mM DTT, 1 mM PMSF, left to stand for 20 min at 0°C, and centrifuged at 40,000 g for 20 min.

The extract was fractionated on a 5 to 25% (w/w) sucrose density gradient in a solution of 0.1 M NaCl, 4 mM $MgCl_2$, 0.1 mM EGTA, 1 mM DTT, 0.1 mM PMSF and 10 mM HEPES, pH 7.4. After the sedimentation at 26,000 rpm for 19 to 20 hr at 4°C in a RPS 27-2 rotor of a Hitachi 55P ultracentrifuge, the content of the centrifuge tube was separated into 20 or 22 fractions of equal volume. The fractions of the first and the second peaks of protein with ATPase activity (Fig. 14) were pooled and used as 30S and 14S dynein preparation, respectively.

2. Chymotryptic digestion of 30S dynein

The 30S dynein prepartion was exhaustively dialyzed against 0.1 M NaCl, 0.1 mM EDTA, 1 mM DTT, 10 mM HEPES, pH 7.4, to remove sucrose. Protein concentration was adjusted to 0.5 mg/ml. When concentration was necessary, Amicon XM 100A ultrafiltration membrane was used. To study the effect of magnesium ion, 1 M $MgCl_2$ was added to one portion of the prepartion to the final concentration of 4 mM, and 100 mM EDTA was added to another portion to the final concentraion of 0.5 mM. After the preincubation at 25°C, γ-chymotrypsin (Sigma) was added to the final concentration of 0.02 mg/ml and the reaction was terminated by adding PMSF to the final concentration of 1 mM.

The dynein digested with γ-chymotrypsin for 7 to 60 min was mostly fractionated on a 5 to 20% (w/w) sucrose density gradient in a solution of 0.1 M NaCl, 4 mM $MgCl_2$, 0.1 mM EGTA, 1 mM DTT, 0.1 mM PMSF and 10 mM HEPES, pH 7.4. The centrifugaion and fractionation were done in the same manner as in the preparation of 30S dynein.

In some cases, digested products were fractionated by DEAE-Sephacel column chromatography. Two ml of digested products (about 0.5 mg/ml) was applied to 2.5 ml of DEAE-Sephacel (Pharmacia), packed in a glass pipette (5 ml) and equilibrated with 10 mM HEPES, pH 7.4, 4 mM $MgCl_2$, 0.5 mM EGTA, 1 mM DTT and 0.1 mM PMSF. Material was eluted from the column with 30 ml of a 0-0.5 M NaCl linear gradient in column buffer, and collected in 0.5 ml fractions.

3. Measurement of ATPase activity

For the assay of ATPase activity, sample was added to 1 ml of an assay medium containing 0.1 M NaCl, 4 mM $MgCl_2$, 10 mM Tris-HCl, pH 7.5, and 1 mM ATP, and incubated for 10 min at 25°C. The liberated inorganic phosphate was measured by the method of Murphy & Riley (1962).

4. Determination of protein concentration

Protein concentration was formerly determined by measuring the absorbance at 280 nm using an extinction coefficient of 1.0 cm^2/mg based on the protein concentration determined by the

biuret method. However, it was found that unknown material, which was not protein and contributed to the absorbance at 280 nm, always existed in the dynein preparation. When 30S dynein and its cleavage products were applied to DEAE-Sephacel, this unknown material eluted slightly faster than proteins and formed a large peak when monitored at 280 or 260 nm. Thus, at the later stage, protein concentration was measured by the Bradford method (Bradford, 1976; Read & Northcote, 1981).

5. Sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis

SDS polyacrylamide gel electrophoresis using the discontinous Tris-glycine buffer system (Laemmli, 1970) and the continuous phosphate buffer system (Weber & Osborn, 1968), and SDS-urea polyacrylamide gel electrophoresis were performed as described in Part I, with an exception of polyacrylamide concentration of the slab gels (3.2% instead of 2.8%) used for SDS-urea gel electrophoresis.

Native gel electrophoresis was carried out by the method of Davis (1965) on 3% polyacrylamide slab and disc gels.

6. One-dimensional peptide mapping

Specimens were treated with DACM prior to electrophoresis on SDS-urea gels as described in Part I. Heavy chain bands were cut out and the resultant gel slices (1.5 x 4 mm) were put into the sample slots of stacking gels of the Laemmli system

(1970). When detailed comparsion of two heavy chains was intended, two cut out heavy chain bands were put into the same slot. Concentrated solution of γ-chymotrypsin was diluted with various amounts of SDS sample buffer and added to the gel slices in the slots, and electrophoresed for 14 hr at the constant current of 8 mA (Cleveland et al., 1977). Gels were stained with silver.

7. Electron microscopy

Electron microscopy was performed as described in the previous part of this thesis.

Results

1. SDS gel electrophoresis

High salt extract of axonemes from Tetrahymena cilia was partially purified by sucrose density gradient centrifugation. Fig. 14 shows the profiles of protein concentraion and ATPase activity on 5 to 25% sucrose density gradient. The peak at the higher densitiy has been identified as 30S dynein, and the one at the lower density as 14S dynein (Gibbons & Rowe, 1965). The protein composition of these two peaks was examined by SDS gel electrophoresis using the discontinuous Tris-glycine buffer system (Laemmli, 1970). Fig. 15a shows the gel patterns of 30S and 14S dynein fractions on a 6% slab gel. Thirty S fraction shows a major dense band at high molecular weight region (heavy chain) and rather faint bands around the midway of the gel (intermediate chains). The gel of 14S fraction shows a heavy chain band with slightly higher mobilitiy than that of 30S fraction, several intermediate chains and a contamination of tubulin. According to Mabuchi and Shimizu (1973) heavy chains of 30S and 14S dynein fractions have been termed A-band and B-band dynein, respectively.

Fig. 15b shows the SDS gel electrophoresis on 3% gel using the continuous Tris-glycine buffer system containing 6 M urea. Heavy chains of 30S dynein now shows three bands with apparently equal density, which is the constant feature through the fractions of 30S peak. These three bands are tentatively

termed A1, A2 and A3 bands in the ascending order of mobility. A1 band shows almost the same mobility with the A-band heavy chain of 21S dynein from sea urchin sperm flagella, and other two heavy chains show higher mobility than the sea urchin A-band heavy chains.

Two strains of Tetrahymena, that is, T. thermophilla SB 255 and T. pyriformis W, were used in this work. As shown in Fig. 15b, there was no difference in SDS-urea gel pattern between them. SDS gel patterns with the Tris-glycine system and the phosphate buffer system also did not show any difference (Fig. 15a, c).

To estimate the molecular weight of these heavy chains, SDS gel electrophoresis with the phosphate buffer system (Weber & Osborn, 1969) was performed on 3% disk gels (Fig. 15c). With this buffer system, three A band heavy chains formed a single band. This band shows slightly higher mobility than that of A-band heavy chain of sea urchin 21S dynein. The molecular weight of 30S and 14S dynein heavy chains were estimated to be 500,000 and 470,000, respectively, using BSA oligomers as molecular weight markers. As described in Part I, BSA oligomers cross-linked with glutaraldehyde (Payne, 1973) show very good linearity and intermediate mobility among various proteins, and thus are considered to be the good molecular weight markers.

2. Electron microscopy of 30S and 14S dynein

Morphology of 30S and 14S dynein preparations were examined by staining the specimens negatively with uranyl acetate or uranyl oxalate. Other staining reagents, namely, phosphotungstate, silicotungstate and ammonium molybdate were tried but did not provide good images comparable to uranyl stains. Inclusion of bacitracin improved the "wetness" of the support film to some extent, but increased the background noise even under low salt conditions (Malech & Albert, 1979). With uranyl acetate, relatively thin staining and high contrast was realized. Although large aggregates were formed sometimes, uranyl acetate gave constant results. Uranyl oxalate was used at higher pH (6.0). When the good support film was provided, this stain formed a thicker uniform sheet around the particles and produced the images with apparently superior quality. However, there were no essential difference in the morphology of dynein molecules between these two stains.

Fig. 16 shows large field of views of 30S and 14S dynein preparations stained with uranyl oxalate (pH 6.0). In accordance with Johnson & Wall (1983), 30S dynein molecules constantly had three globular heads and three stalks which were bound together at the roots. These pictures show that the "flower bouquet" structure (Johnson & Wall, 1983) is not an artefact resulted from extremely low salt conditions required for STEM mass analysis or low pH (3.8) of the negative staining reagent (2% aqueous uranyl sulfate solution).

These three globular heads appeared as rounded triangle in projection and were similar in shape and size (about 15 nm in diameter). In the images which clearly resolved three stalks, two stalks were bound together near but not at the root, and bound to the third one at the distal end. This third one was slightly longer and thicker than the others (Fig. 16a). All of the three heads had stain accumulation or stain pits around the center. To see whether this stain accumulation corresponds to the hole in the particle, specimen grids were rotated in a microscope by $\pm 25°$ (Fig. 17). No such obvious change in the size and shape of this stain accumulation as reported in the negatively stained specimen of adenovirus (Nermut & Perkins, 1979) was recognized. Nevertheless this range of tilting might be insufficient to reveal the difference. Tilted images also provide a rough estimate of the dimension of the particles perpendicular to the plane of support film. The dimension across the tilt axis in 25° rotated images was decreased to 94.5% of that in 0° images, which gave the ellipticity of 0.63 (McEwen et al., 1980, corrected by Toyoshima, 1982).

Images of 14S dynein preparation indicated that the shape was similar to one head of 30S dynein. The diameter was about 18 nm and larger than that of 30S dynein. No connecting structure between the particles was observed. The stain accumulation around the center of particle was larger, and some particles appeared C-shaped (indicated by arrows in Fig. 16b) suggesting an opened structure of 14S dynein particles.

All of the micrographs presented here and those published by Johnson & Wall (1983) were obtained with the preparations from T. thermophilla SB 255. To examine whether the "flower bouquet" structure is peculiar to this strain, 30S dynein prepared from T. pyriformis W was also examined. Again the three-headed particles were observed. Thus, neither gel electrophoresis nor electron microscopy revealed any difference between dyneins from these two sources. Since much larger amount of dynein could be obtained, T. thermophilla SB 255 was exclusively used for further analyses.

3. Biochemical characterization of chymotryptic digestion of 30S dynein

i) General

Electron microscopic images showed that the 30S dynein molecule consists of three globular heads and connecting thin rods, and thereby suggested the possibility of isolating each globular head or decomposing the molecule into several components. In contrast to the 21S dynein from sea urchin sperm flagella, which can be dissociated into two subunits by dialyzing aginst a low salt buffer (Bell et al., 1982; Part I of this thesis), 30S dynein persists under high salt (0.5 M NaCl) and low salt (1 mM Tris-HCl and 0.1 mM EDTA) conditions. More severe treatments with 5 M NaCl, 3 M LiCl, or 8 M urea prior to sucrose density gradient centrifugation failed to separate the components.

Three proteases, namely, trypsin, chymotrypsin and V8 protease from Staphylococcus aureus were tried in this study to isolate heavy chains. Their performance was monitored by SDS gel electrophoresis and native gel electrophoresis. As shown in Fig. 18, trypsin cleaved the heavy chains too extensively, whereas V8 protease too slightly. Chymotrypsin digested the heavy chains into several bands as revealed by SDS gels (Fig. 18a), and formed large fragments which were able to enter into the matrix of 3% acrylamide gels under the non-denaturing conditions (Fig. 18b).

After examining the time course of chymotrypsin digestion with several different concentrations of dynein and enzyme-to-substrate ratio, routine digestion conditions were determined so that 0.5 mg/ml of 30S dynein was digested by 0.02 mg/ml of chymotrypsin at 25°C, with either 4 mM $MgCl_2$ or 0.5 mM EDTA. In the course of the chymotryptic digestion, seven major bands derived from three heavy chains were resolved on SDS-urea gels. Although the time course and the stable digestion products were different depending on the presence or absence of magnesium ion, these seven bands occupied apparently the same positions on SDS-urea gels in both cases. They were identified and numbered as indicated in Fig. 19.

ATPase activity of 30S dynein was measured in the course of chymotryptic digestion. As shown in Fig. 20, Mg-ATPase activity increased to 2 fold and 2.5 fold of the native one, when the digestion was performed in the presence of Mg^{2+} and EDTA, respectively. In both cases, no more activation of

the ATPase activity was observed after 40 min.

ii) Chymotryptic digestion of 30S dynein in the presence of magnesium ion

In the presence of Mg^{2+}, band-1 completely disappeared and two bands (5, 5') with higher mobility than other two native bands emerged after 1 min (Fig. 19a). Band-5' appeared to be further degraded and could not be recognized after 5 min. Band-6 was recognized after 5 min and its density increased slowly, apparently concomitant with the disappearance of band-5. Band-6 was one of the stable products by the chymotryptic digestion. It was degraded further only slightly by the prolonged digestion.

Within 5 min of digestion, substantial decrease of band-2 and band-3 heavy chains was observed. The density of band-2 decreased further and that of band-4 increased apparently in parallel. After the treatment for 30 min, band-2 nearly vanished and the density of band-4 reached the maximum and remained constant up to 90 min. Although the density of band-3 was diminished very slowly during 20 min digestion, this band was rather resistant to chymotrypsin cleavage, and still remained even when the digestion was prolonged to 210 min.

Band-7 could be recognized after 20 min digestion, and the density increased slowly. The intensity of this band appeared to be different depending on the stain. When the gels were stained with silver, this band was intense and one of the

major bands after 60 min (Fig. 21), while it was barely recognized in the gels stained with Coomassie brilliant blue at 90 min (Fig. 19a).

After 40 min digestion no substantial change in the gel pattern was observed: band-3, -4, -6 (and -7) were the stable final products, although band-3 and -4 decreased slowly in the prolonged digestion (Fig. 21).

To clarify the relationship among the cleavage products, the specimens digested for various time were fractionated on 5-20% sucrose denstity gradient or DEAE-Sephacel. Fig. 22 shows typical profile of the protein concentration and ATPase activity of the 60 min digested products on sucrose density gradient. These two peaks associated with ATPase activity were termed peak I and II, which comprised band-3 and -6 heavy chains, and band-4 heavy chain, respectively (Fig. 22a and b). The positions of peaks on sucrose density gradient were similar to those of sea urchin 21S and 13S dynein, respectively, and was evidently shifted to lighter position than 30S dynein on the density gradient.

When the specimen digested for shorter period (20 to 30 min) was fractionated on sucrose density gradient, there were two peaks again at similar positions, and the peak I contained band-2, -3, -5, -6 heavy chains, while the peak II contained mainly band-4 heavy chain. The amounts of band-5 and band-7 heavy chains were smaller than that of band-3 heavy chain.

Fractionation of chymotryptic fragments obtained by 40 to 60 min digestion with DEAE-Sephacel column at pH 7.5 showed that band-4 heavy chain was eluted first, and band-3 and band-6 heavy chains were eluted together later from the column (Fig. 23). This finding suggests that the isoelectric point of band-4 heavy chain is higher than those of other two heavy chains. Fractionation on Sephacryl S-400 was also tried and provided the consistent results that the band-4 heavy chain was isolated from other components, and band-3 and band-6 heavy chains moved as an entity. As described in the next section, electron microscopic visualization of the particles in these two peaks also supported this idea.

These results could be easily explained by a model in which band-5 and -6 heavy chains were the products of band-1 heavy chain still connected with band-3 heavy chain, and band-4 heavy chain originated from band-2 heavy chain. However, the situation has been revealed to be much more complex.

When 30S dynein was digested for shorter time (7 to 10 min) and the digestion products were fractionated on sucrose density gradient, peak I shifted to the denser position and contained band-2, -3, -5, -5' and -6 heavy chains, whereas peak II fractions contained band-2, -3, -3', -4, and -5 heavy chains (Fig. 24). These features were also observed when digested with larger amount of chymotrypsin (1 : 10 in weight, digested for 10 min). The presence of heavy chains other than band-4 heavy chain indicates that all of native heavy chains,

at least a portion of them or at the initial stage of the digestion, could be isolated. Whether the presence of these heavy chains can be explained simply by the presence of much larger amount of native heavy chains at the initial stage, or only by the presence of other conformations susceptible to chymotrypsin could not be answered at this stage. Quantitative study is necessary.

Molecular weights of cleaved heavy chains were estimated by SDS gel electrophoresis using the phosphate buffer system and BSA oligomers as molecular weight markers. Fig. 25 shows the relative mobility of BSA oligomers and the cleaved heavy chains. From this chart, apparent molecular weights of band-3, -4, -5, -7 heavy chains were estimated to be 500k, 430k, 370k and 320k, respectively.

iii) Relationship among heavy chain bands on SDS-urea gels

Further chymotryptic digestion of digestion products fractionated on density gradient and of heavy chains retrieved from SDS-urea gels was done to clarify the relationship among heavy chain bands.

Each fraction of both peak I and peak II digestion products was dialyzed to remove sucrose, and subjected to second chymotryptic digestion. Fig. 26 presents the resultant gel patterns. The gel of peak I fraction indicated that band-4 heavy chain was produced from band-3 heavy chain, while the gel of peak II fraction showed essentially the same pattern throughout the digestion.

When heavy chains recovered from SDS-urea gels were digested on SDS gels (Cleveland et al., 1977) and electrophoresed on the same slot with another heavy chain to be compared, detailed comparison was possible. In this experiment, the discontinuous Tris-glycine buffer system (Laemmli, 1970) was used. In this buffer system three heavy chains of 30S dynein could be hardly resolved, and band-4 and band-5 heavy chains showed almost the same mobility and could not be distinguished from each other (Fig. 27a).

The digestion pattern of three native heavy chains are evidently different from one another (Fig. 27a). However, digestion patterns of band-2 and band-3 heavy chains were remarkably close to each other, and a dense band was produced at the position of band-4 or -5 in the lanes where minute amount of chymotrypsin (0.1 μg) was applied (Fig. 27a). Since the major bands were varied depending on the amount of chymotrypsin, digestion patterns at various concentrations of chymotrypsin should be compared.

Fig. 27b presents several SDS gels showing the digestion patterns of band-2, -4, and band-3, -4 heavy chains in the same lanes. Since band-2 and band-3 heavy chains were close to each other, many of the bands derived from band-4 heavy chain could be related to the ones from both band-2 and band-3 heavy chains. However, the two bands indicated by arrows could be related to only those from band-3 heavy chain, although the corresponding bands from band-3 heavy chain were not very clear. These data suggest the contribution of band-3

heavy chain to band-4 heavy chain, although the one from band-2 heavy chain is also plausible.

As noted in ii), both peak I and peak II fractions were associated with ATPase activity. Since peak I contained band-3 heavy chain and band-5, -6 heavy chains derived from band-1 heavy chain, and since peak II contained only band-4 heavy chain, originated from band-3 (and/or band-2) heavy chain, the profiles shown in Fig. 22 indicate that band-3 heavy chain has ATPase activity or both band-1 and band-2 heavy chains, at least, have ATPase activity.

iv) Chymotryptic digestion of 30S dynein in the presence of EDTA

To examine the effect of magnesium ion, chymotrypsin digestion in the presence of EDTA was performed. Note that EGTA was also present when the digestion was done in the presence of Mg^{2+}. Fig. 19b shows the time course of the degradation. Here also band-1 heavy chain disappeared and band-5, -6, -7 appeared very shortly, but band-6 was denser than other two bands from the beginning and band-5 also increased in density up to 20 min. Band-2 diminished and band-4 emerged in due course as in the presence of magneisum ion, but band-3 heavy chain decreased to a trace after 90 min digestion. When 90 min passed by, band-7 was the major cleavage product. Band-4 and -6 were recognized, but their amounts were much less than those in the presence of Mg^{2+}.

Fig. 28 shows the profiles of the protein concentration and ATPase activity on 5-20% sucrose density gradient. Again the profiles showed two peaks: peak I (heavier one) contained band-5 and -6 heavy chains, and peak II (lighter one) contained band-4 heavy chain as the major components (Fig. 28a, b). Only trace of band-3 heavy chain was found on the gel.

Molecular weights of these cleaved heavy chains were the same as those for cleaved heavy chains obtained in the presence of Mg^{2+}.

4. Electron microscopy of the chymotryptic digestion products of 30S dynein

Chymotryptic digestion products of 30S dynein were fractionated by sucrose density gradient centrifugation, negatively stained with uranyl acetate or uranyl oxalate, and examined in an electron microscope. Figs. 29 and 30 show the general views of peak I and peak II fractions obtained in the presence of magnesium ion and EDTA, respectively. Almost all of the particles from peak II fraction were isolated globular particles and there was no indication of the presence of stalk. The particles from peak I fraction showed various features: major proportion of the globular particles were apparently paired, having thin rod connecting two globules, but a measurable fraction of particles were isolated and not paired. To estimate the proportion of paired particles, arbitrarily chosen 223 particles from 5 micrographs were examined and 172 particles (77.1%) were judged to be paired. These data support

the idea that the peak II consists of isolated particles, whereas the peak I consists of mainly two-headed particles.

The sizes of the particles both in peak I and II were similar to those of one head of 30S dynein (15 nm).

Discussion

1) Molecular weight of 30S dynein heavy chain

Molecular weight of the 30S dynein heavy chain has been estimated by many workers (see Bell, 1982). Warner et al. (1977) and Porter and Johnson (1983) reported the values around 350k judged by the relative mobility on the gel using the Tris-glycine buffer system. The value obtained in this work using the phosphate buffer system is 500k. This large discrepancy originated from the difference in buffer system and moleucular weight markers. Due to the lack of suitable molecular weight markers at this molecular weight range and/or inadequate concentration of acrylamide, extraporation has been used, which would result in underestimation of molecular weight. BSA oligomers cross-linked with glutaraldehyde (Payne, 1973) showed very good linearity in the phosphate buffer system and made the extraporation unnecessary. Although the molecular weight measured by SDS gel electrophoresis is no more than a rough estimate (see Part I of this thesis), it is noteworthy that STEM mass measurements provided the value of 2000k daltons for an integral molecule and 400k to 550k daltons per globular head (Johnson & Wall, 1983). Since 30S dynein contained three heavy chains and a small amount of intermediate chains, these values give 500k to 600k daltons as the weight of single heavy chain, in accordance with the present estimate.

Comparison with the heavy chain of 21S dynein from sea urchin sperm flagella showed that there was only slight difference in the weight of heavy chains between 21S and 30S dynein, as pointed out by Mabuchi et al. (1976).

2. Subunit structure of 30S dynein

SDS gel electrophoresis using the Tris-glycine-urea system revealed the presence of three heavy chains with different mobility in the 30S dynein preparation. Since SDS gel-electrophoresis using the phosphate buffer system showed only one band at 500k daltons even in the gel of as low as 2% acrylamide, these three heavy chains appear to have almost the same molecular weight: presumably, slight difference in charge provides the separation in SDS-urea gel, suggested by the chromatography on DEAE-Sephacel.

Nishino & Watanabe (1974) reported that there were three immunologically different heavy chains in 30S dynein fraction. Although their SDS gel pattern (Fig. 2 of Nishino & Watanabe, 1974) showed two bands of heavy chain, they described that the SDS-urea gel system similar to the present one was used in the immunological assay. Therefore, three immunologically different heavy chains termed α, β and γ would be identical to A1, A2 and A3 heavy chains. As a consequence, A2 and A3 heavy chains would not be the proteolytic fragments of A1 heavy chain.

STEM mass analysis by Johnson & Wall (1983) indicated that the molecular weight of 30S dynein was about 2000 k. Thus, three or four heavy chains would constitute a 30S dynein

molecule. Further, since chymotryptic digestion released one heavy chain which corresponds to one globular head as revealed by electron microscopy, three heavy chains resolved on SDS-urea gel do not seem to be isomers which compose three different types of molecules. They would correspond to three globular heads and thin rods which constitute a single 30S dynein molecule.

3. Chymotryptic digestion of 30S dynein

Chymotryptic digestion of 30S dynein revealed that the conformation of 30S dynein varies depending on the presence or absence of magnesium ion, and provided the means to isolate at least one of three heads of 30S dynein in almost intact form. Chymotryptic digestion caused more than twofold increase in ATPase activity in the course of the digestion, which was also reported by Hoshino (1977) for tryptic digestion.

It is not possible to describe the precise pathway of the degradation of heavy chains. The most important point would be whether band-4 heavy chain was derived from band-2 heavy chain or from band-3 heavy chain. If band-4 heavy chain is a digestion product of band-2 heavy chain, this implies the presence of multiple ATPase sites, as suggested by Shimizu & Johnson (1983), since both peak I and peak II fractions had ATPase activity and, in this case, they have no parent heavy chains in common. Limited chymotryptic digestion of heavy chains in the presence of SDS (Fig. 27) indicated that band-3

heavy chain would be (one of) the origin of band-4 heavy chain. It also showed that band-2 heavy chain could potentially produce band-4 heavy chain as the major digestion product (Fig. 27), and the time course (Fig. 19) suggested the concomitant loss of band-2 heavy chain with the emergence of band-4 heavy chain. Thus, the question arises as to whether band-2 heavy chain contributed to band-4 heavy chain.

In relation to this question, three other problems arise. First, if band-3 is the origin of band-4 heavy chain, how the apparently constant densities of band-3 and band-4 heavy chains between 20 min and 90 min of digestion (Fig. 19) is explained ? Second, why isolated band-5 or band-6 heavy chain was not contained in the peak II fractions (Fig. 22), if band-3 heavy chain was released from the complex of band-3 and -5 (or -6) heavy chains and was contained in peak II fractions as band-4 heavy chain ? Third, since band-2 heavy chain disappeared in the course of digestion, it should have been digested and formed several distinct bands or too numerous bands which could not be detected as separate bands: if band-2 heavy chain did not contribute to band-4 heavy chain, the latter case should have been realized, since no other distinct bands appeared to be related to band-4 heavy chain: then, why band-4 heavy chain was a major digestion product of band-2 heavy chain in the presence of SDS and not in the absence of SDS ?

There were two bands in the peptide map of band-4 heavy chain which could be related to band-3 heavy chain but could not to band-2 heavy chain. Nevertheless, there was no band

exclusively related to band-2 heavy chain. Most bands derived from band-2 and band-3 heavy chains were, however, very similar to one another (Fig. 27), so it is impossible to conclude wherther band-2 heavy chain contributed to band-4 heavy chain. Two-dimensional peptide mapping would be necessary.

Apparently constant density of band-3 heavy chain after 20 min of digestion could be explained at least in the following two ways. One is the conversion of band-2 heavy chain to band-3 heavy chain, and the other is the presence of two confor mations of band-3 heavy chain susceptible and insusceptible to chymotryptic cleavage. First possibility could be excluded, however, since the peptide mapping of band-3 heavy chain recoverd from the gel loaded with the specimen digested for 40 min, showed the bands which was not related to band-2 heavy chain. If only somewhat denatured band-3 heavy chain is susceptible to chymotryptic digestion in the absence of SDS, the initial decrease of band-3 heavy chain and the emergence of band-4 heavy chain from peak I fractions could be explained. Almost complete disappearance of band-3 heavy chain at 40 min in the digestion of peak I fractions (Fig. 26) is suggestive from this standpoint, since the concentration of chymotrypsin was the same as in the experiment illustrated in Fig. 22.

Possible pathway of the degradation of 30S dynein heavy chains by chymotryptic digestion is illustrated in Fig. 31. Solid lines represent the paths confirmed in this study. Quantitative work is required to answer the remaining problems.

Immunochemistry and two-dimensional peptide mapping would allow us to determine the relationship among heavy chain bands.

In this study, I have developed the means to study substructure of 30S dynein. Binding experiments, proteolytic digestion in the presence of ATP and microtubules, immunochemistry of each heavy chain band or each head, etc., would provide new important knowledge about the dynein structure and dynein-microtubule interaction.

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Table I

ATPase properties of 21S dynein and its dissociated products

Specimen	Km (μM)	Vmax (μmol Pi/min/mg)
21S	——	1.05
A_α[a]	16.7	0.32
A_β	110.0	1.10

a. A_α and A_β represent the fractions of sucrose density gradient containing A_α and A_β heavy chains as the major component, respectively.

Table II

Amino acid composition of dynein heavy chains

	A_α	A_β
Asp	106.4	109.4
Thr	48.1	46.4
Ser	95.3	97.2
Glu	142.2	142.6
Pro	-	-
Gly	93.3	98.8
Ala	39.2	38.7
Cys	33.8	21.9
Val	57.7	59.4
Met	21.3	21.3
Ile	50.1	51.4
Leu	92.1	95.5
Tyr	43.1	39.2
Phe	41.4	40.4
Lys	61.6	62.4
His	17.3	16.4
Arg	58.1	58.9
Trp	-	-

(residues of amino acids per 1,000 residues)

Table III

Number of SH groups in 21S dynein and its dissociated products

Specimen	Reaction time		
	10 min	30 min	60 min
21S native[a]	6.6[b] (51%)[c]	8.0 (62%)	8.6 (67%)
SDS	11.7 (91%)	12.7 (98%)	12.9 (100%)
A_α[d] native	5.6 (60%)	7.0 (75%)	8.1 (87%)
SDS	8.3 (89%)	9.0 (97%)	9.3 (100%)
A_β native	6.1 (60%)	7.6 (72%)	8.4 (79%)
SDS	9.6 (91%)	10.5 (99%)	10.6 (100%)

a. "Native" and "SDS" indicate that the reaction with Elman's reagent was done under non-denaturing conditions and in the presence of 1% SDS, respectively.

b. Numbers are given in moles per 10^5g protein.

c. Numbers in parentheses represent the fraction of SH groups reacted within specified reaction time.

d. A_α and A_β represent the fractions of sucrose density gradient containing A_α and A_β heavy chains as the major component, respectively. Note that A_β fraction also contains three intermediate chains.

Table IV

Molecular weights (M_r) and isoelectric points (pI) of 21S dynein components

	M_r	pI
HC A_α[a]	500k[b]	6.1[c]
HC A_β	500k	5.6
IC 1	125k	4.7
IC 2	94k	6.0
IC 3	73k	5.3

a. HC, heavy chain; IC, intermediate chain.
b. Molecular weight was estimated by SDS-polyacrylamide gel electrophoresis (Weber & Osborn, 1969) using BSA oligomers (Payne, 1973) as molecular weight markers.
c. Isoelecric point was measured in the presence of 6 M urea.

Table V

Molecular weights of dynein heavy chains from sea urchin sperm flagella reported by various authors

Authors	A-band	B-band	Buffer system
Kincaid *et al.* (1973)	520k	460k	Pi[a]
Gibbons *et al.* (1976)	380k	340k	TG
Mabuchi *et al.* (1976)	600k	--	Pi
Bell *et al.* (1979)	330k (A_α)		TG
	320k (A_β)		

a. Pi, continuous phosphate buffer system (Weber & Osborn, 1969); TG, discontinuous Tris-glycine buffer system (Laemmli, 1970).

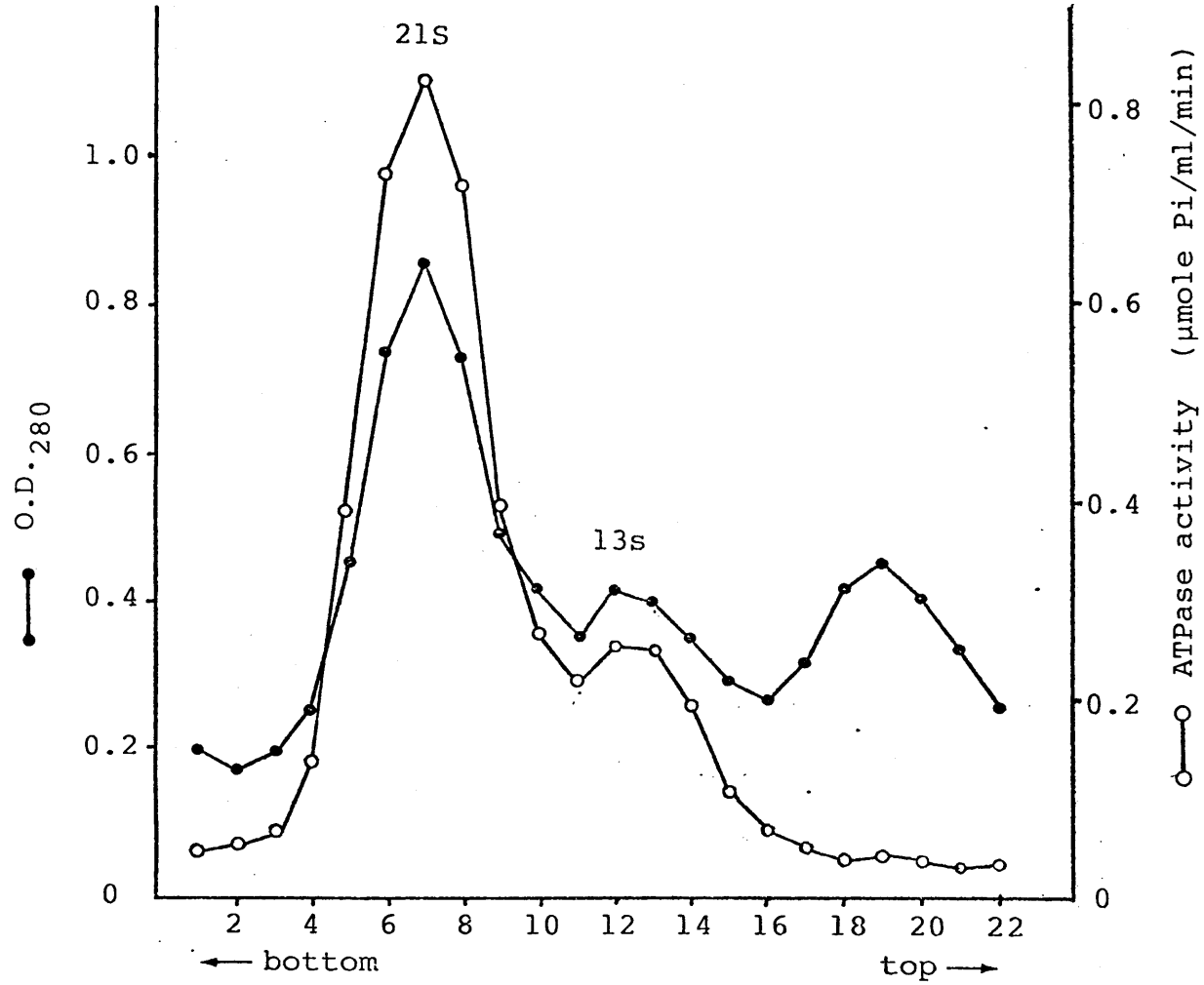


Fig. 1. Sedimentation profiles of protein concentration and ATPase activity of a high salt extract of sea urchin sperm flagellar axonemes on sucrose density gradient. One ml of the high salt extract (5 mg/ml) was applied on 15 ml of a 5-20% sucrose linear density gradient containing 10 mM Tris-HCl, pH 8.0, 0.1 M NaCl, 4 mM $MgCl_2$, 1 mM $CaCl_2$, 0.1 mM EDTA, 1 mM DTT, 0.1 mM PMSF. Centrifugation was carried out at 26,000 rpm for 20 hr at 4°C using a Hitachi 55P ultracentrifuge with a RPS 27-2 rotor.

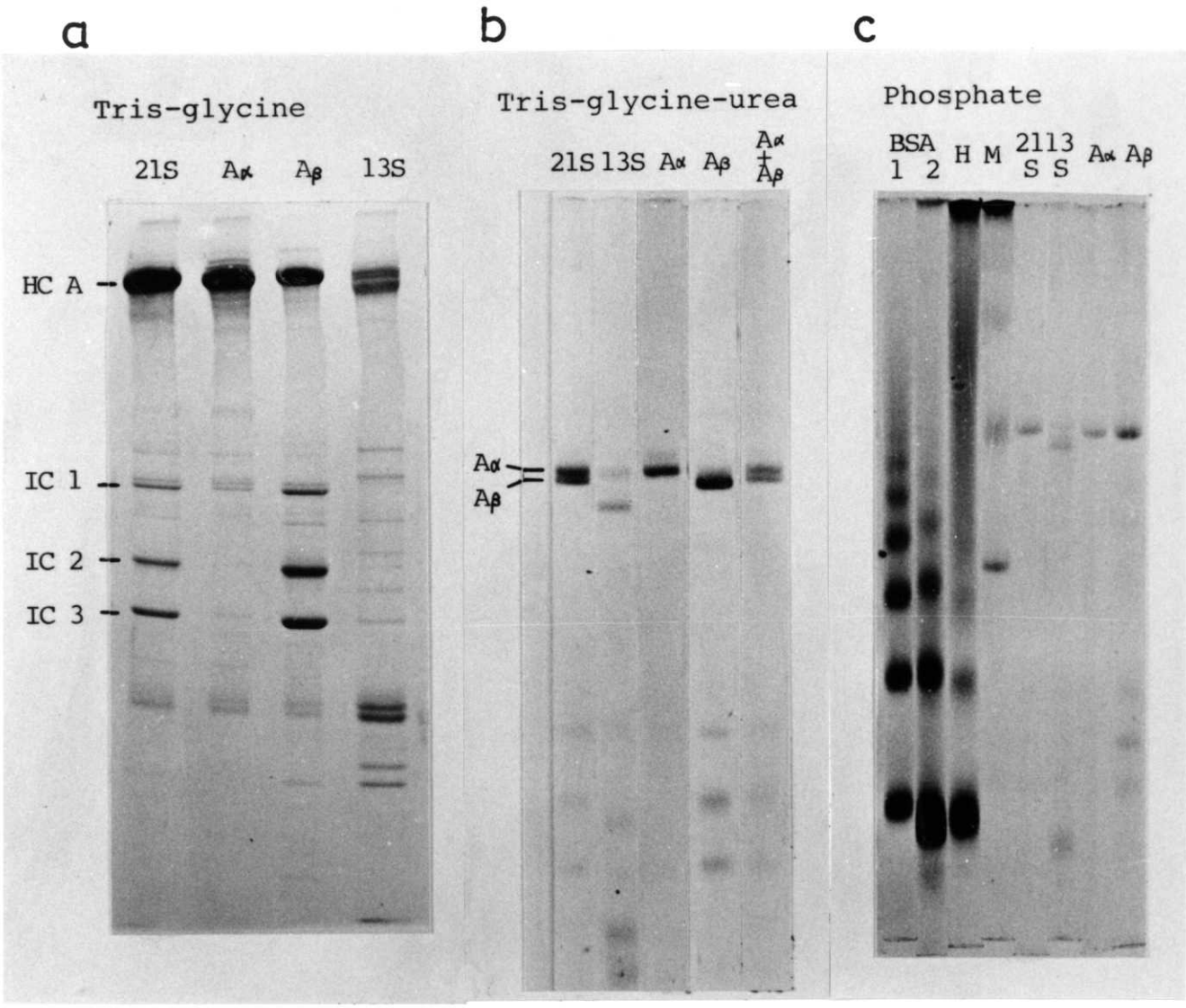


Fig. 2. SDS-polyacrylamide gel electrophoresis of 21S and 13S dynein, and the dissociated products ($A\alpha$ and $A\beta$) of 21S dynein. (a) Electrophoresis on a 6% polyacrylamide gel using the discontinuous Tris-glycine buffer system (Laemmli, 1970). (b) Electrophoresis on 2.8% polyacrylamide gels containing 6 M urea using the continuous Tris-glycine buffer system. Note that $A\alpha$ and $A\beta$ chains are well resolved. (c) Electrophoresis on 3% polyacrylamide gels using the phosphate buffer system (Weber & Osborn, 1969) to estimate molecular weights. BSA1, BSA oligomers cross-linked with glutaraldehyde; BSA2, BSA oligomers obtained from Sigma; H, hemocyanin oligomers prepared by Sigma; M, myosin heavy chain oligomers cross-linked with p-phenylene-N,N'bis-(maleimide); HC A, dynein A band heavy chain; IC, dynein intermediate chain.

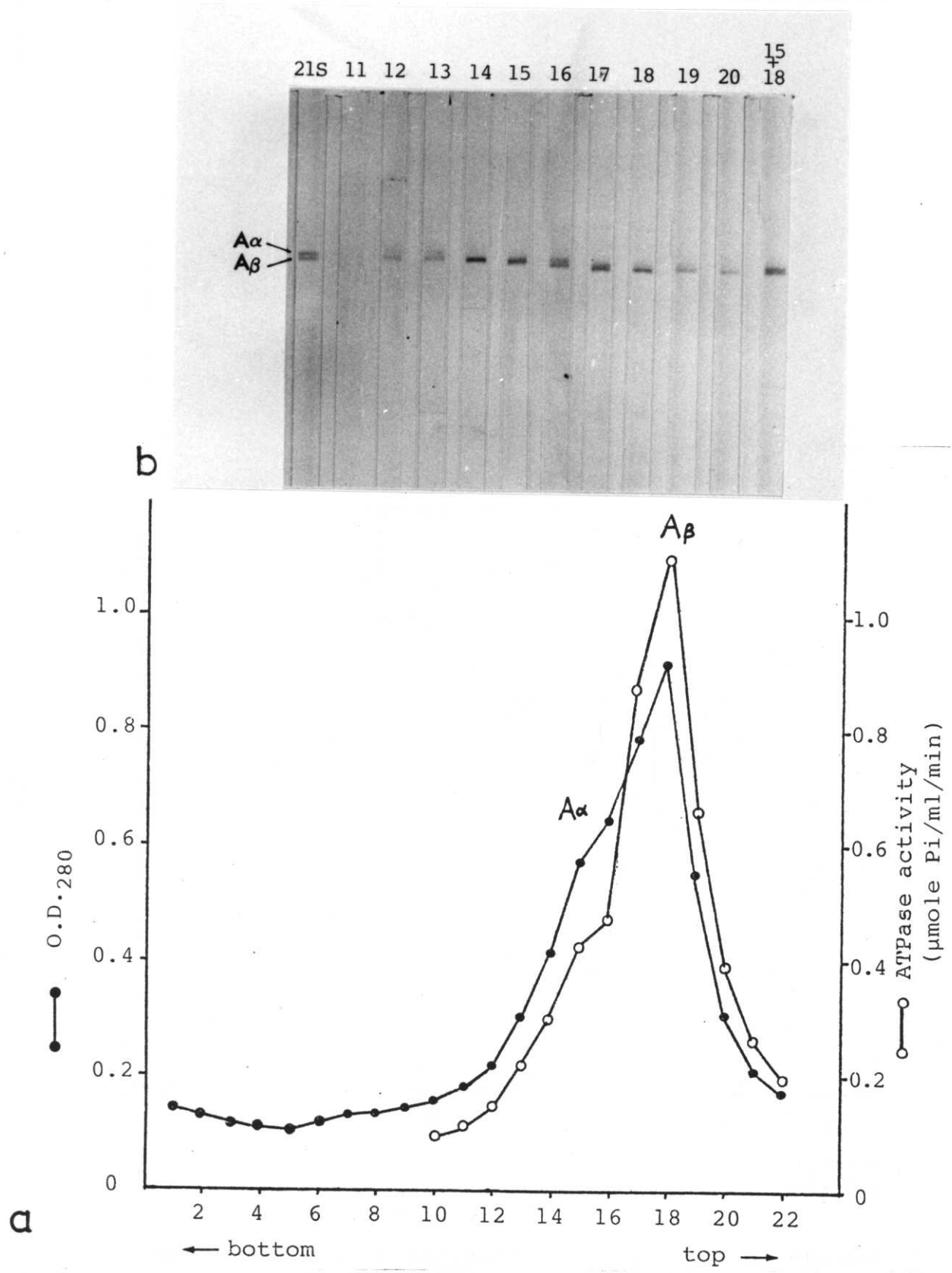


Fig. 3. (a) Sedimentation profiles of protein concentration and ATPase activity of dissociated products of 21S dynein on sucrose density gradient. One ml of dissociated products of 21S dynein (2 mg/ml) was applied on 15 ml of a 5-20% sucrose linear density gradient containing 1 mM Tris-HCl, pH 8.0, 0.1 mM EDTA, 0.1 mM DTT, 0.1 mM PMSF. Centrifuga- tion was carried out at 26,000 rpm for 20 hr at 4°C using a Hitachi 55P ultracentrifuge with a RPS 27-2 rotor. Note that there are two peaks labeled Aα and Aβ. (b) Upper region of a 3% SDS-urea gel showing the polypeptide chains contained in two peaks in (a). 21S, 21S dynein; 15 + 18, co-electrophoresis of fractions 15 and 18. Other numbers indicate fraction numbers in (a).

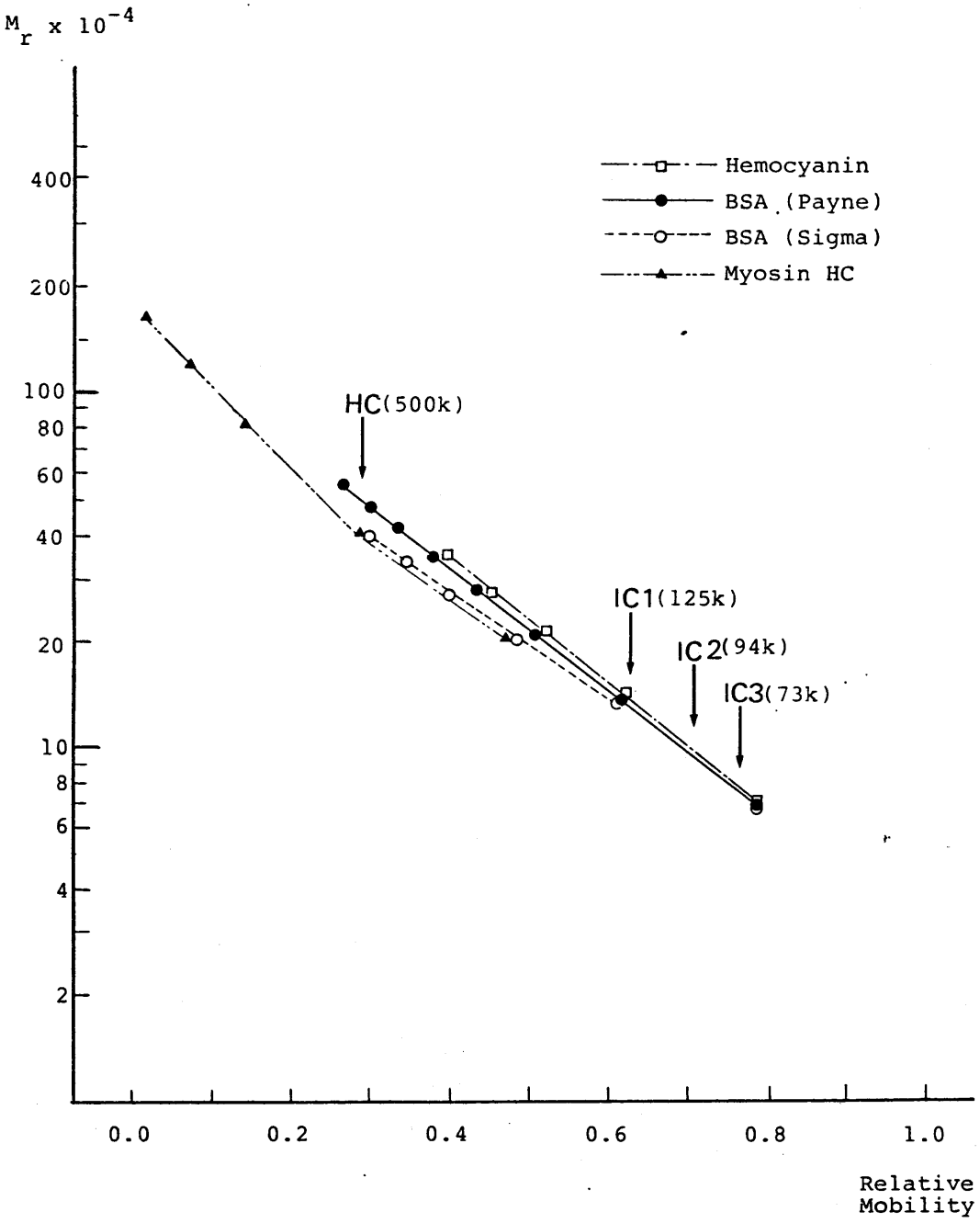


Fig. 4. Molecular weight estimation of polypeptide chains contained in 21S dynein fraction. Molecular weights versus relative mobilities of marker proteins on 3% polyacrylamide gels in phosphate buffer system (Weber & Osborn, 1969). Arrows indicate the relative mobility of the polypeptides in 21S dynein fraction. • , BSA oligomers cross-linked with glutaraldehyde (Payne, 1973); o , BSA oligomers obtained from Sigma; □ , hemocyanin oligomers from Sigma; ▲ , myosin heavy chain oligomers cross-linked with p-phenylene-N,N'-bis(maleimide); HC A, dynein A band heavy chain; IC, dynein intermediate chain.

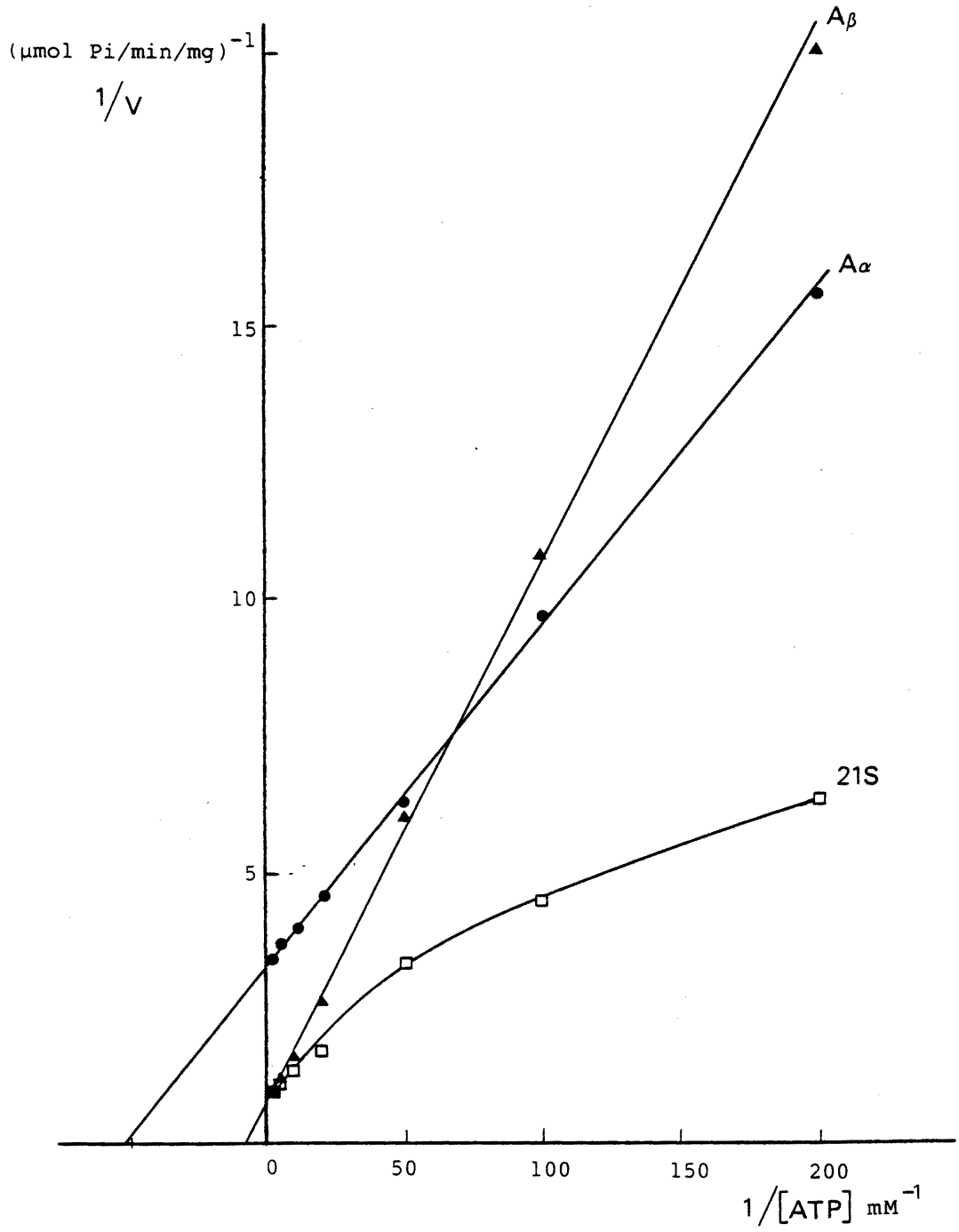


Fig. 5. Double reciprocal plots of ATPase activities of 21S (□), Aα(●) and Aβ(▲) fractions at various concentrations of ATP. The assay solution contained 0.1 M NaCl, 4 mM $MgCl_2$, 1 mM $CaCl_2$, 10 mM Tris-HCl, pH 8.0, 1 mM phosphoenolpyruvate and 0.05 mg/ml pyruvate kinase, various concentrations of ATP (5 to 1000 μM), and 4.8 (21S), 7.6 (Aα) or 6.9 (Aβ) μg/ml of dynein fractions.

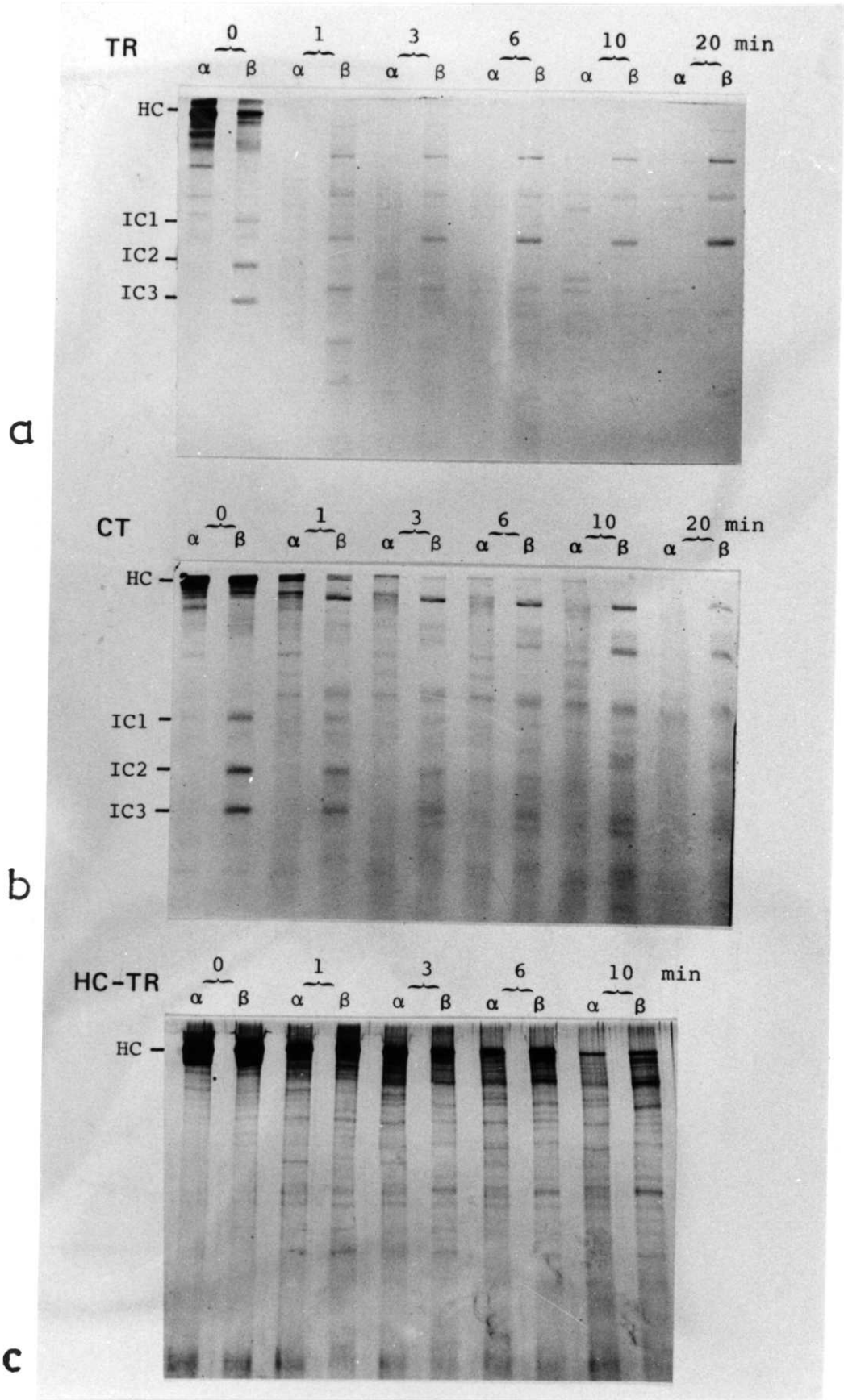


Fig. 6. SDS polyacrylamide gel patterns showing the time course of proteolytic digestion of Aα and Aβ heavy chains. 6% polyacrylamide gels in the Tris-glycine buffer system (Laemmli, 1970). (a) Trypsin digestion of Aα and Aβ fractions (0.4 mg/ml) at the weight ratio of 1:1000 (trypsin: dynein) for 1 to 20 min at 25°C. (b) Chymotrypsin digestion of Aα and Aβ fractions (0.4 mg/ml) at the weight ratio of 1:20 for 1 to 20 min at 25°C. (c) Trypsin digestion of denatured Aα and Aβ heavy chains eluted from SDS-urea gels, at the weight ratio of 1:1000 for 1 to 10 min at 30°C in the presence of 0.1% SDS. (a) and (b), Stained with Coomassie brilliant blue; (c), stained with silver.

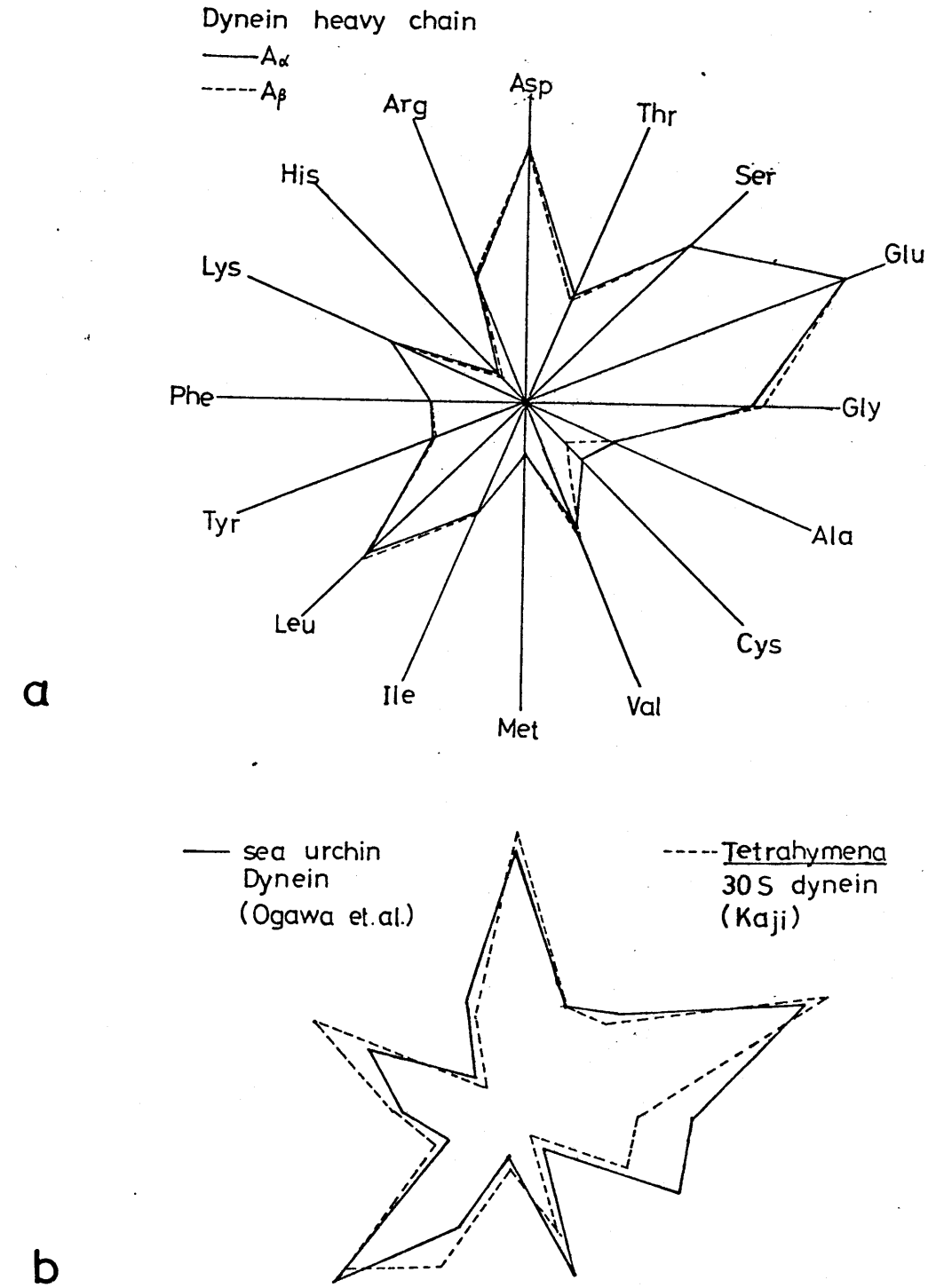


Fig. 7. Amino acid compositions of dynein. (a) Amino acid compositions of Aα and Aβ heavy chains. Solid line, Aα heavy chain; broken line, Aβ heavy chain. (b) Amino acid compositions of sea urchin dynein and 30S dynein from Tetrahymena cilia. Solid line, sea urchin dynein, published by Ogawa et al. (1975); broken line, 30S dynein from Tetrahymena cilia taken from Kaji (1973).

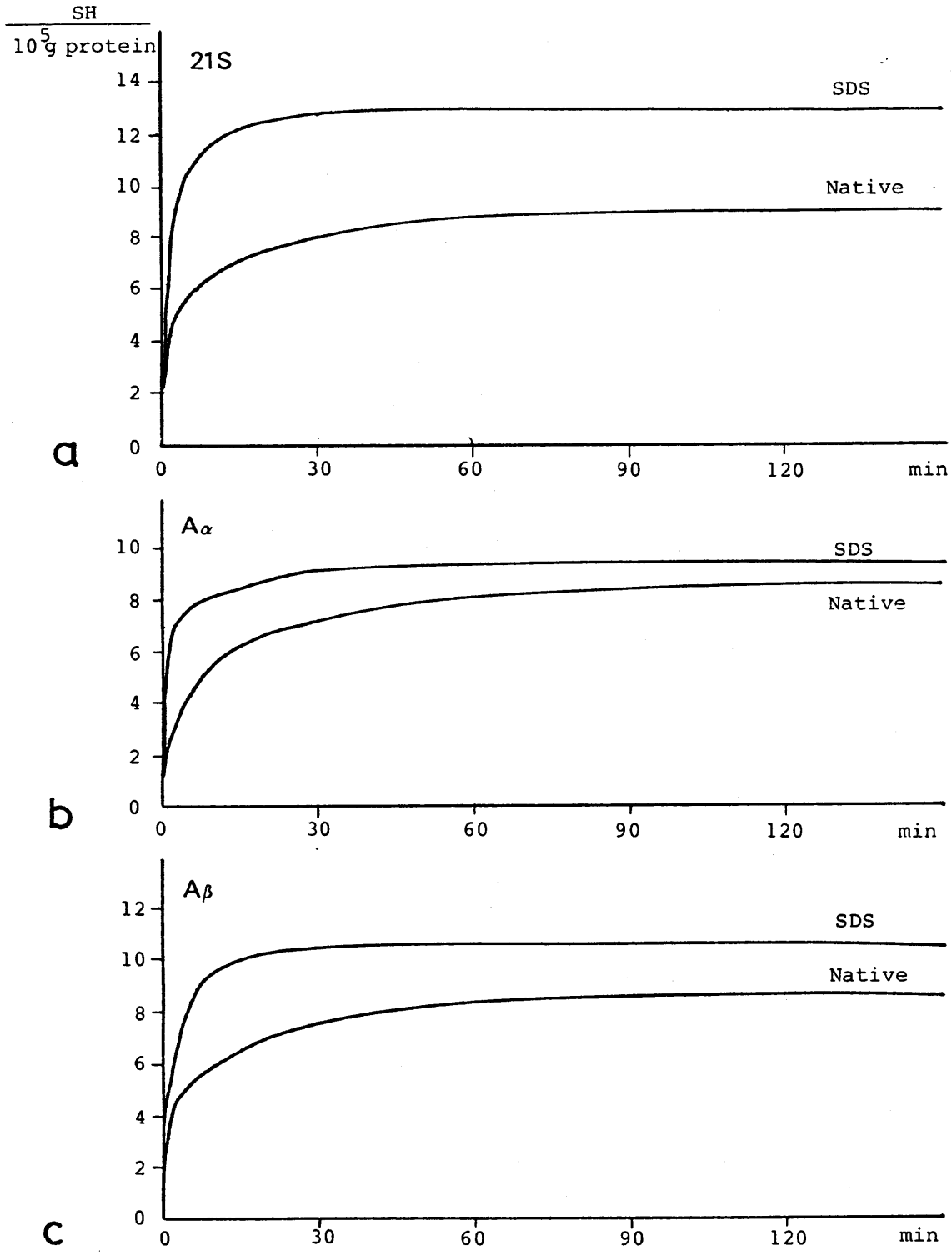


Fig. 8. Time course of the reaction of SH groups of dynein with DTNB in the presence or absence of 1% SDS. (a) 21S dynein (0.425 mg/ml), (b) Aα (0.320 mg/ml) and (c) Aβ (0.340 mg/ml) in 0.5 mM EDTA and 10 mM Tris-HCl, pH 8.0, with or without 1% SDS. Reaction was started by adding 1/10 volume of 10 mM DTNB in 0.1 M phosphate buffer, pH 7.0, at 25°C.

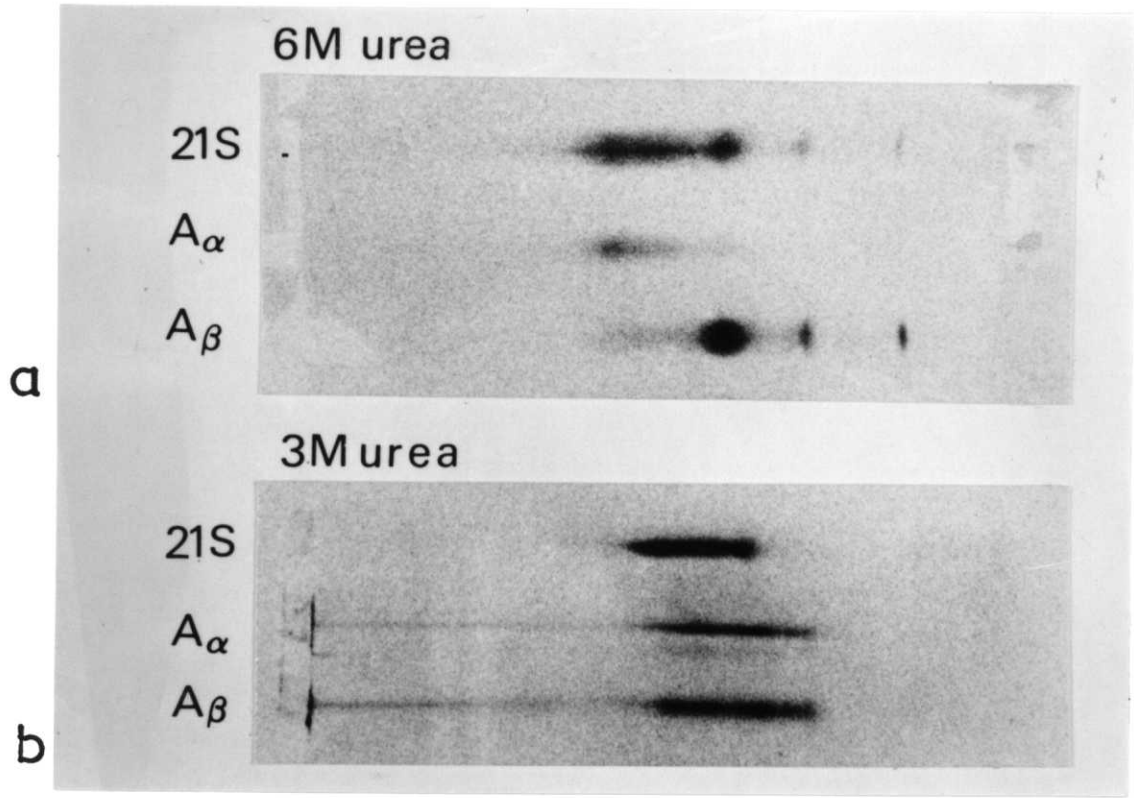


Fig. 9. Isoelectric focusing patterns of 21S dynein and its A_α and A_β fractions on agarose gels. The gels contained 1% Agarose IEF, 12% sorbitol, 2.4% Ampholine (pH 3.5-10 and pH 5-7, 1.2% each) and 6 M (a), or 3 M (b) urea.

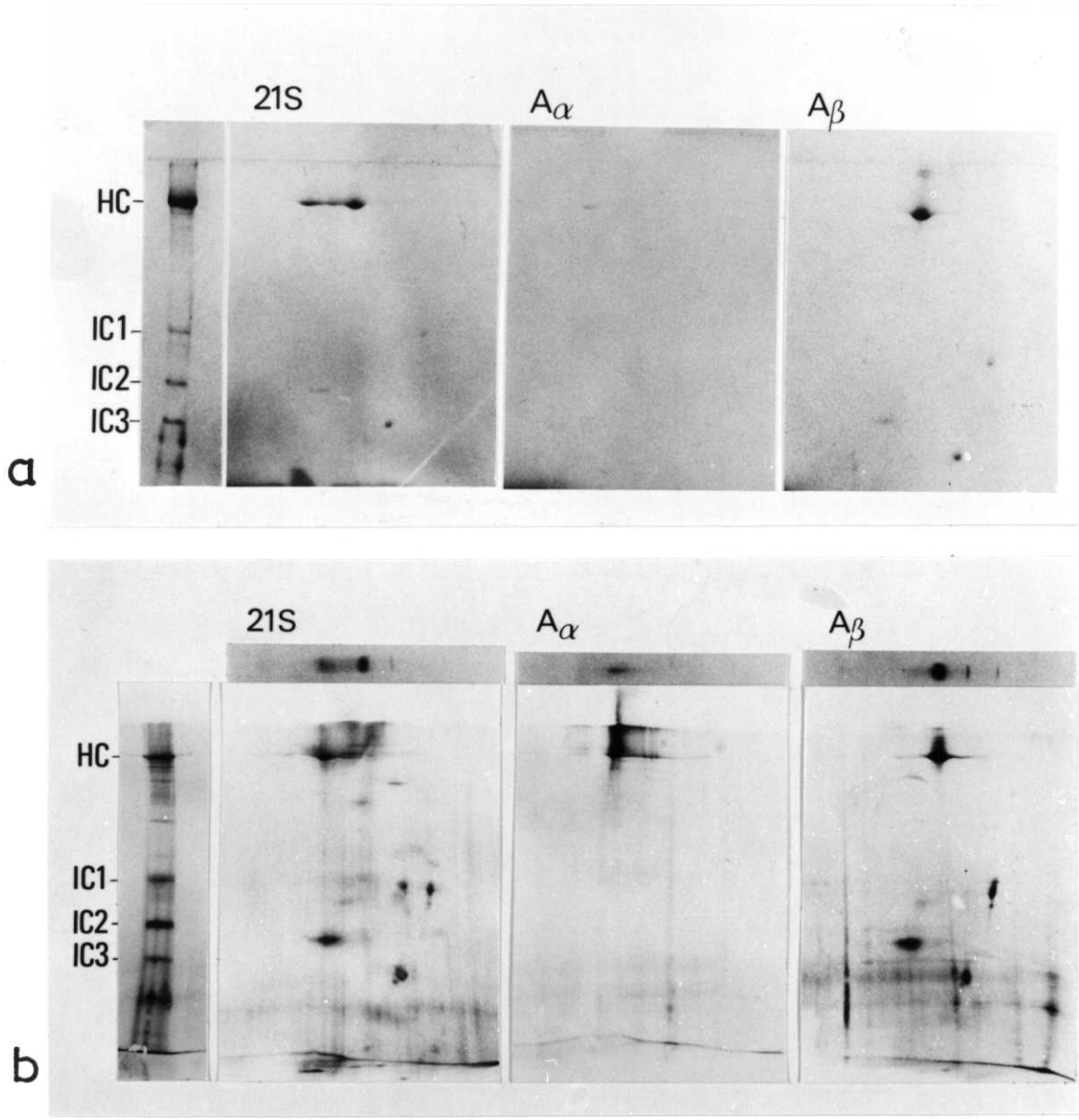


Fig. 10. Two-dimensional gel electrophoresis of 21S, Aα and Aβ fractions. Longitudinal strip of agarose gel of the fisrt dimension was applied on a stacking gel of the second dimension. As markers, SDS gel patterns of 21S dynein run on the same gels are shown at the left side. The gels were stained with Coomassie brilliant blue (a), and with silver (b).

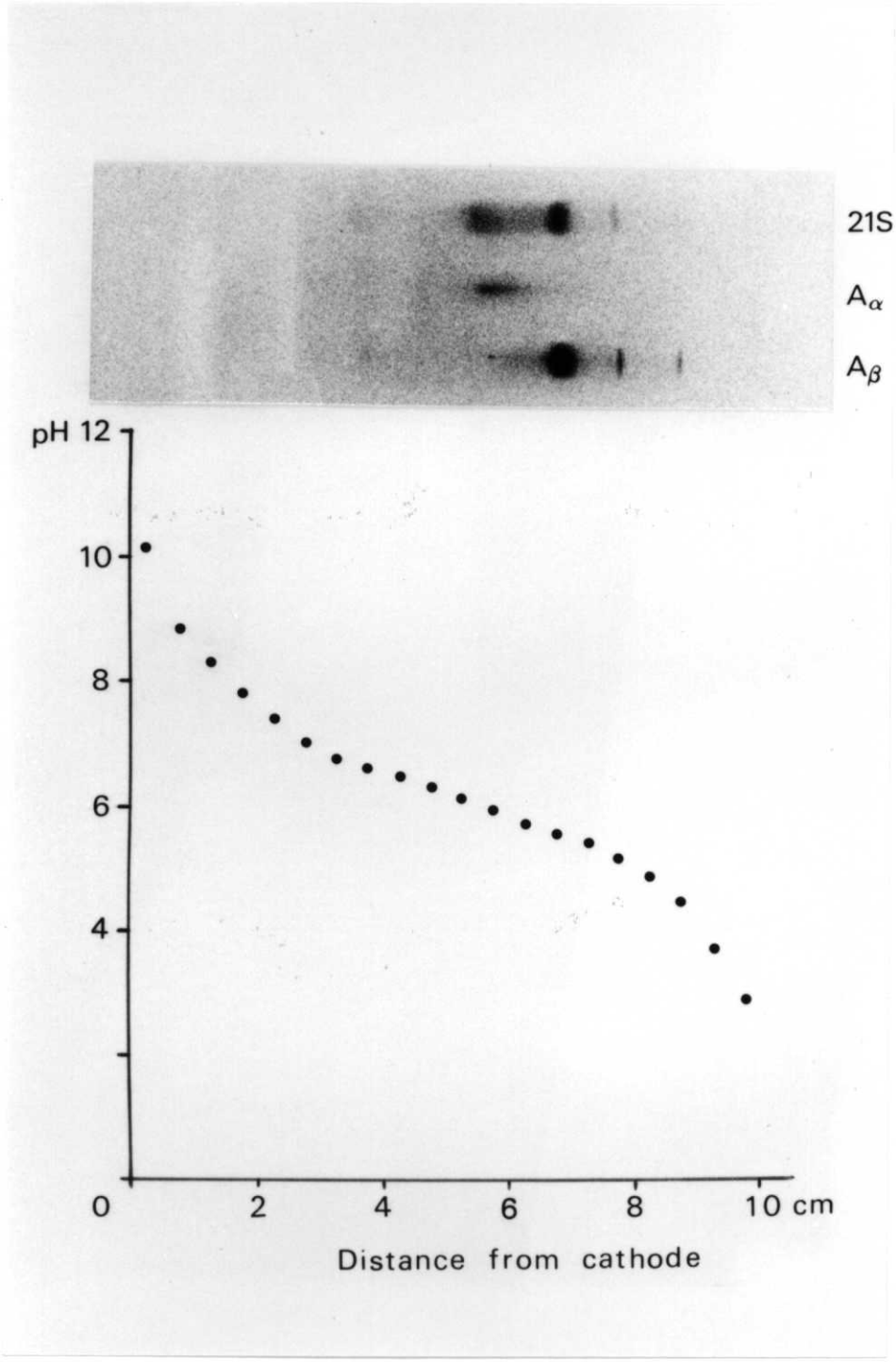


Fig. 11. PH gradient formed on agarose gels after electrofocusing. Longitudinal strip of the gel was cut into 5 mm slices, and each of them was put into 2 ml of degassed water. After the equilibration for 1 hr, pH was measured at 4°C with a pH meter. Upper photograph shows isoelectric focusing pattern of 21S dynein, and $A\alpha$ and $A\beta$ fractions.

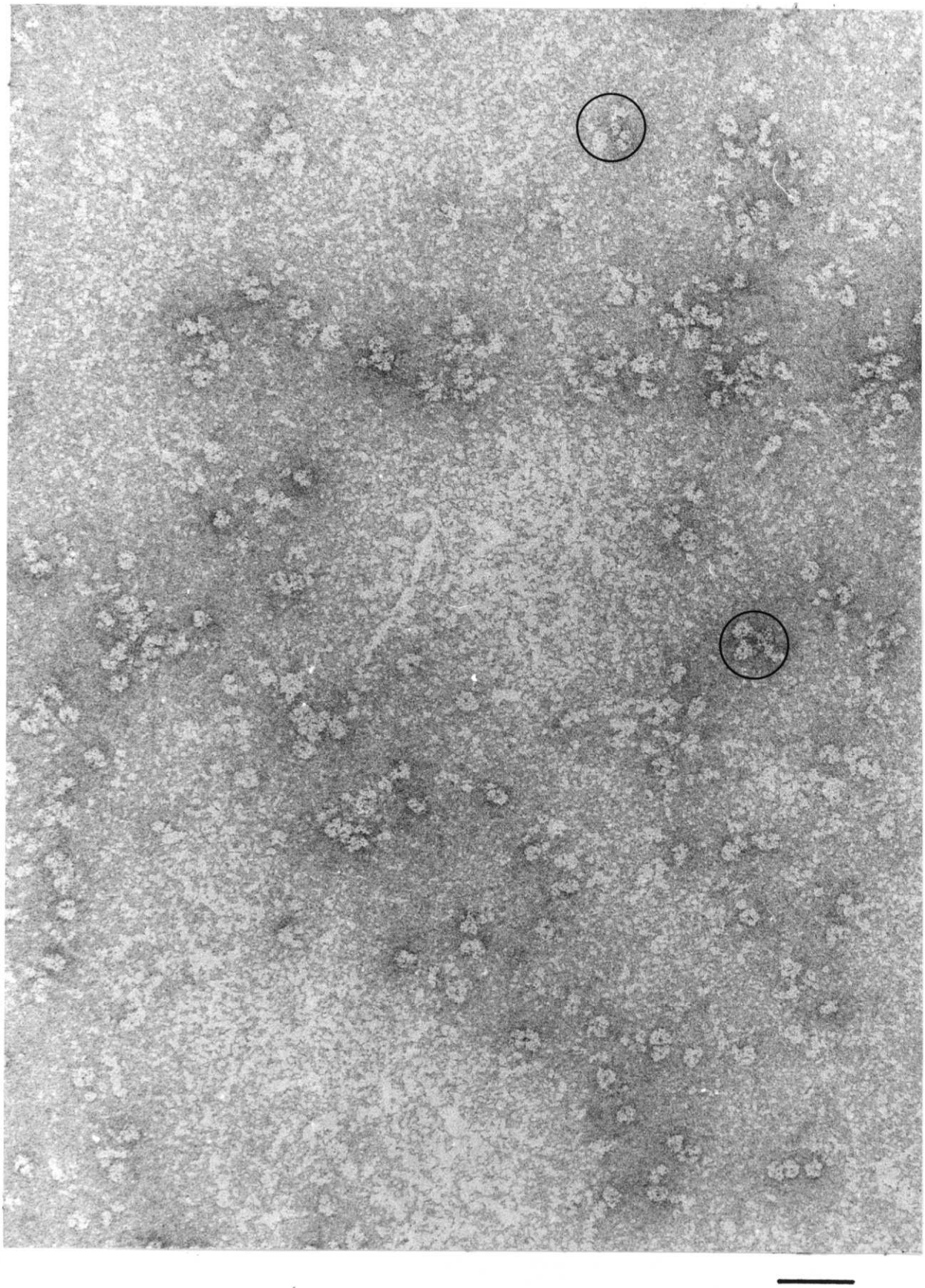


Fig. 12. Electron micrograph of 21S dynein. Negatively stained with 1% uranyl oxalate, pH 6.0. The particles with two heads and connecting thin rods are marked by circles. Bar indicates 50 nm.

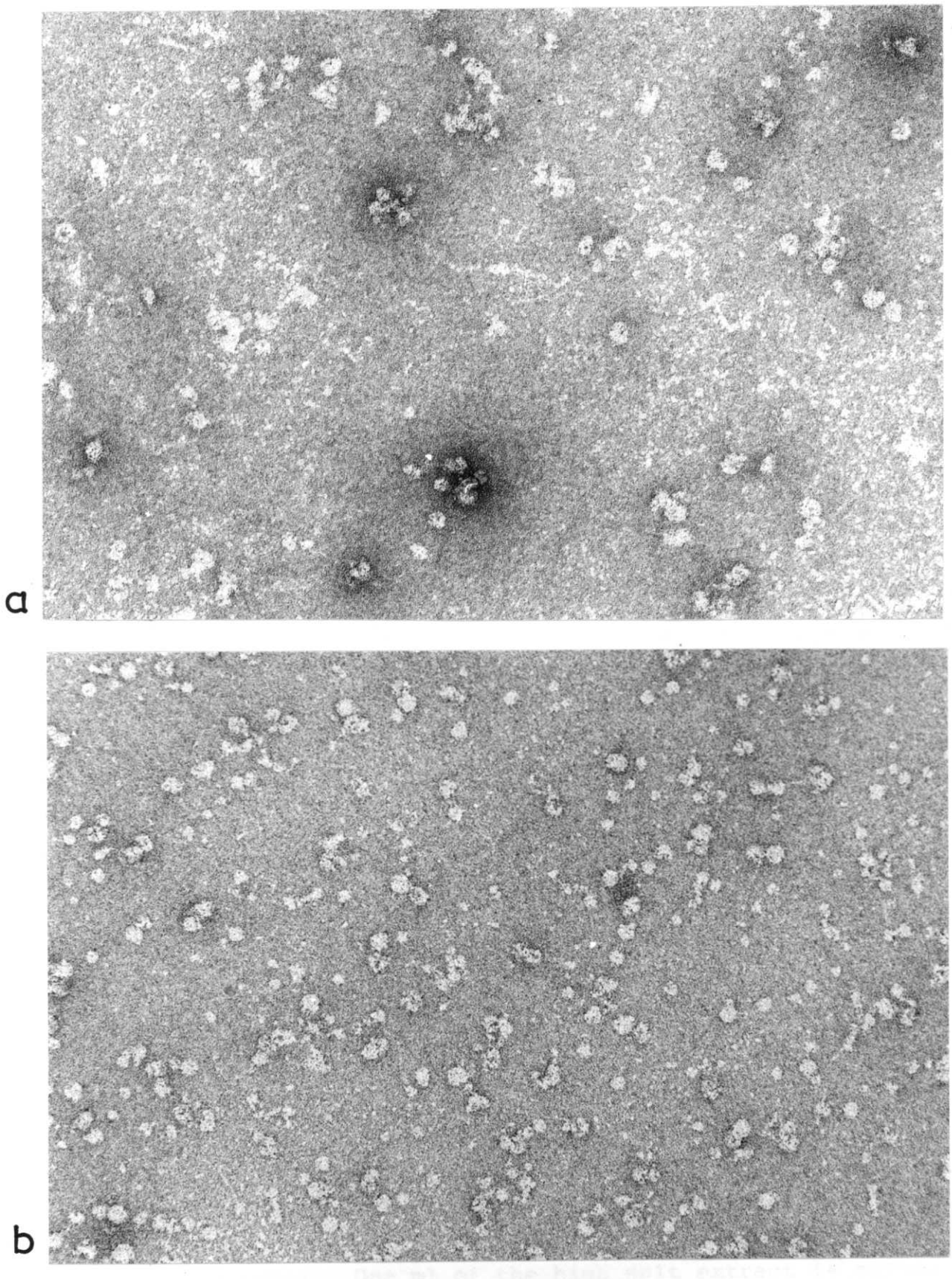


Fig. 13. Electron micrographs of dissociated products of 21S dynein. Aα fraction (a), and Aβ fraction (b). Negatively stained with 1% uranyl oxalate, pH 6.0. Bar indicates 50 nm.

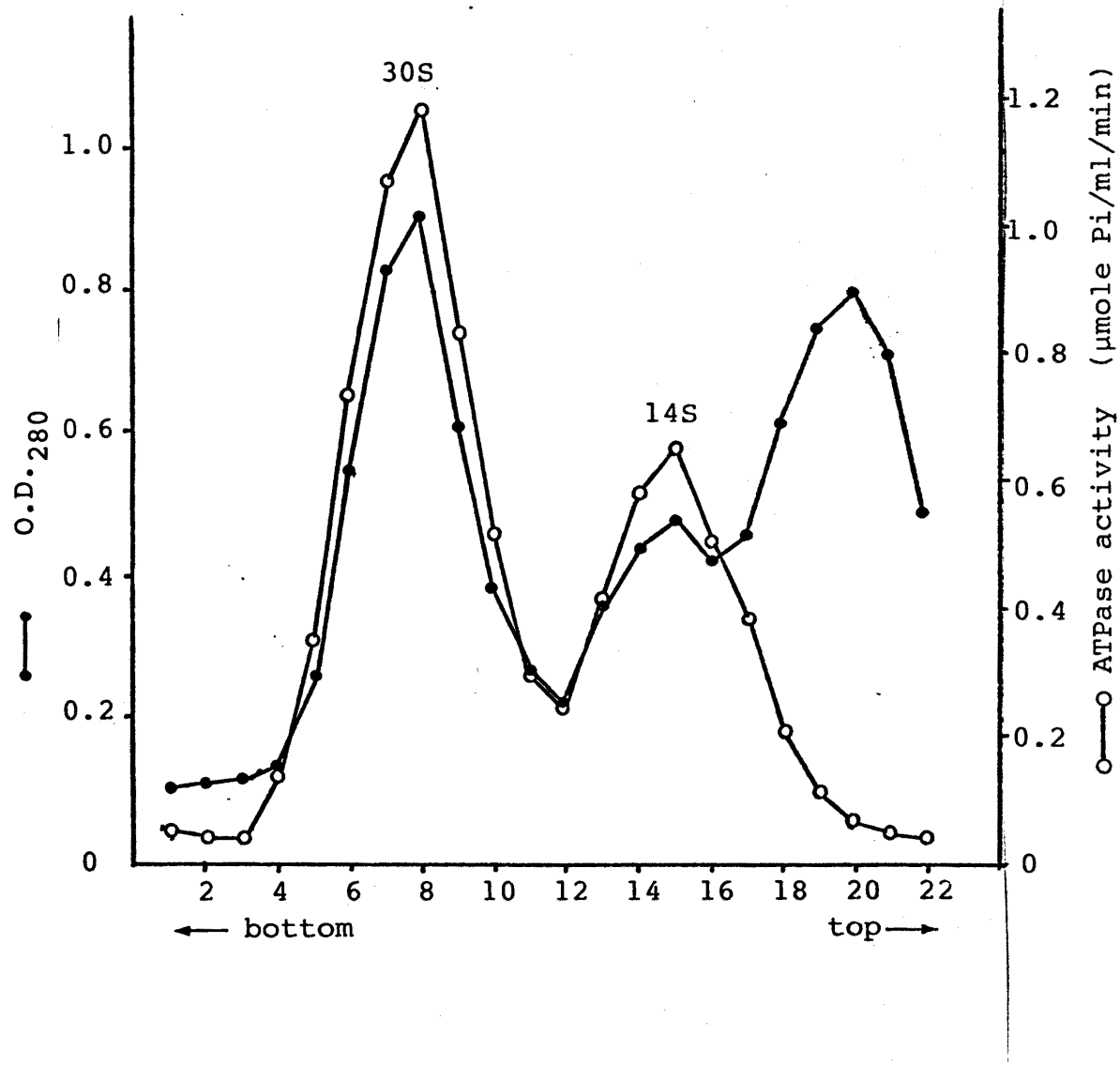


Fig. 14. Sedimentation profiles of protein concentration and ATPase activity of a high salt extract of Tetrahymena ciliary axonemes. One ml of the high salt extract (4 mg/ml) was applied on 15 ml of a 5-25% sucrose linear gradient in 10 mM HEPES, pH 7.4, 4 mM $MgCl_2$, 0.5 mM EGTA, 1 mM DTT, 0.1 mM PMSF, and centrifuged at 26,000 rpm for 20 hr at 4°C using Hitachi 55P ultracentrifuge with a RPS 27-2 rotor.

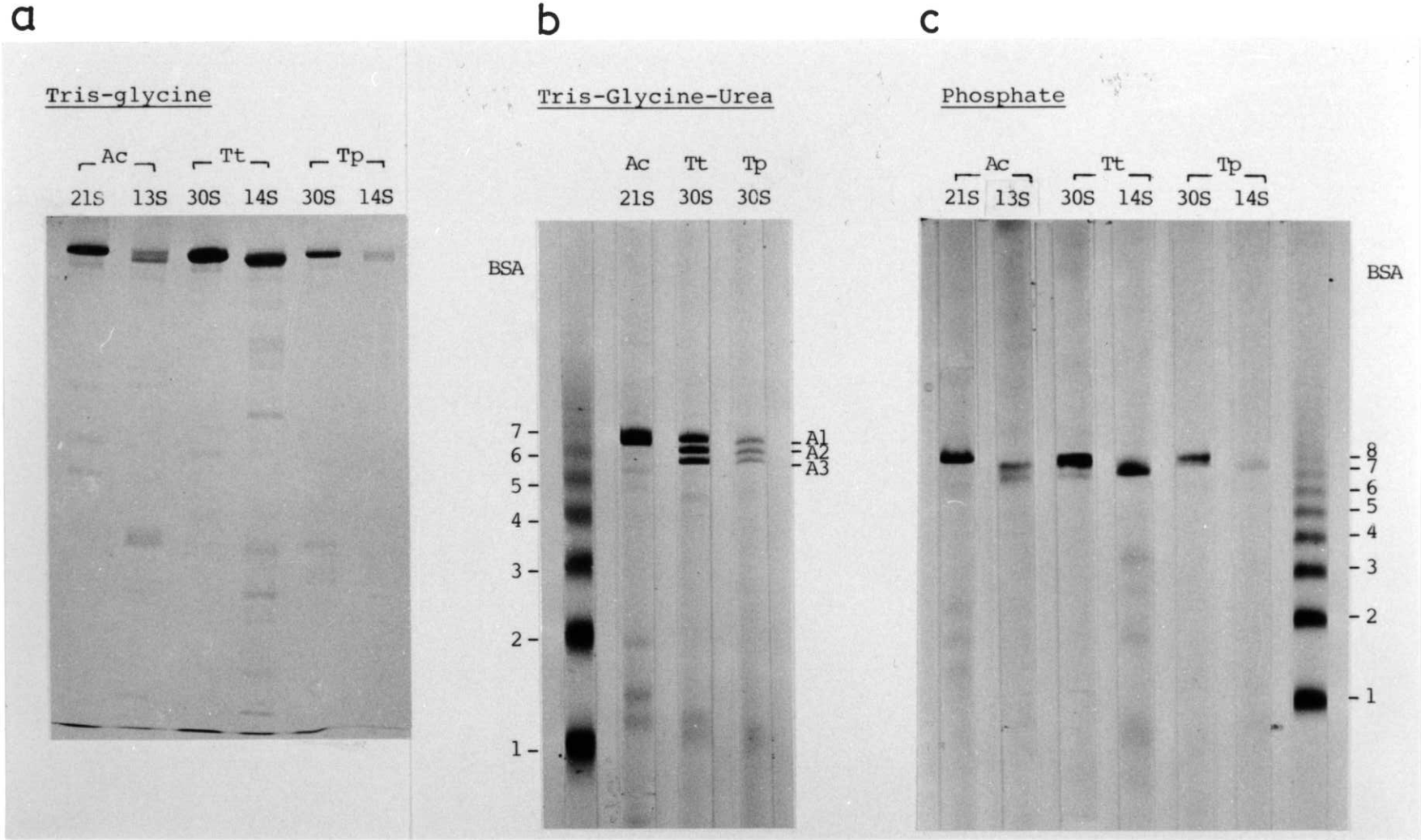
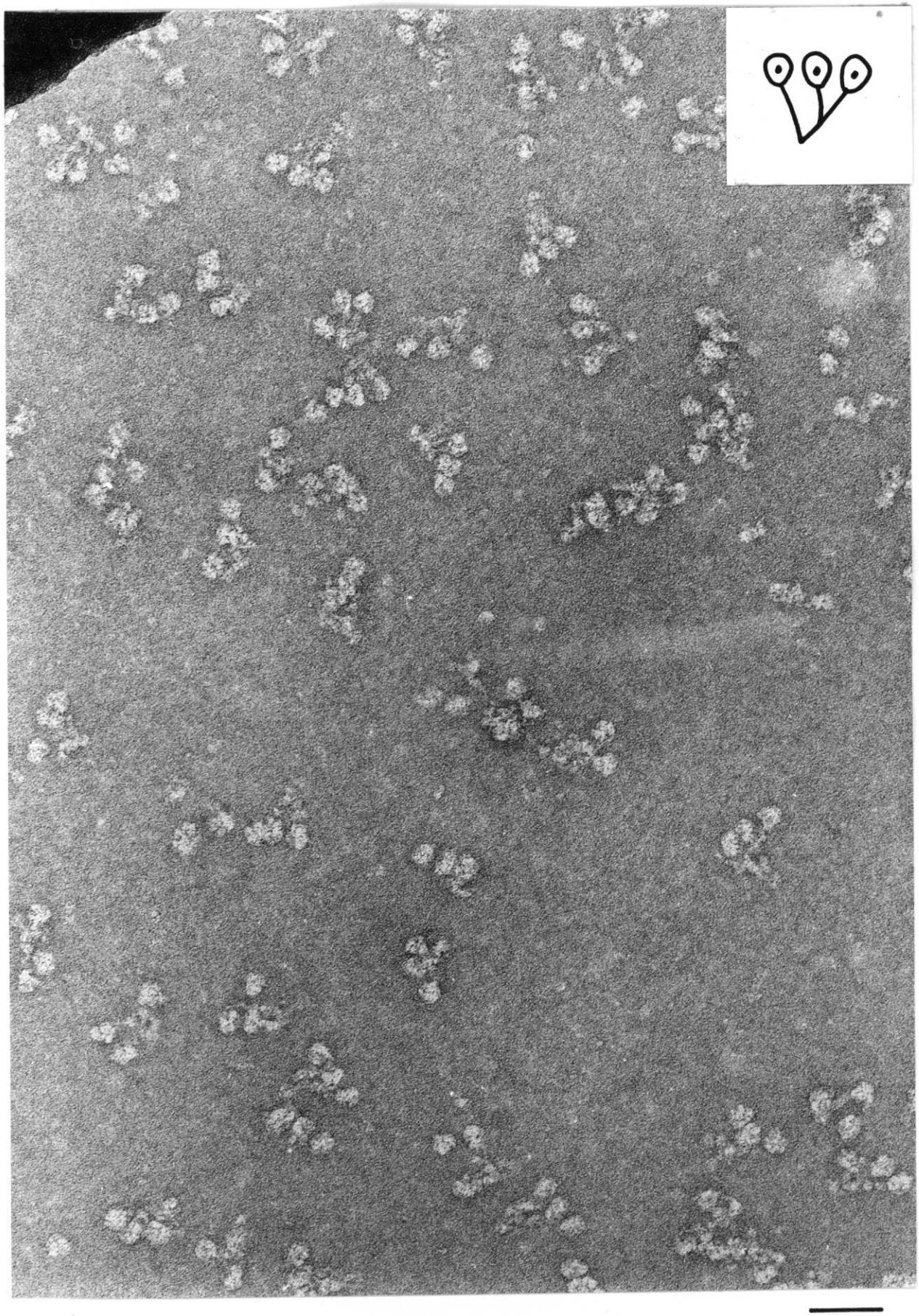
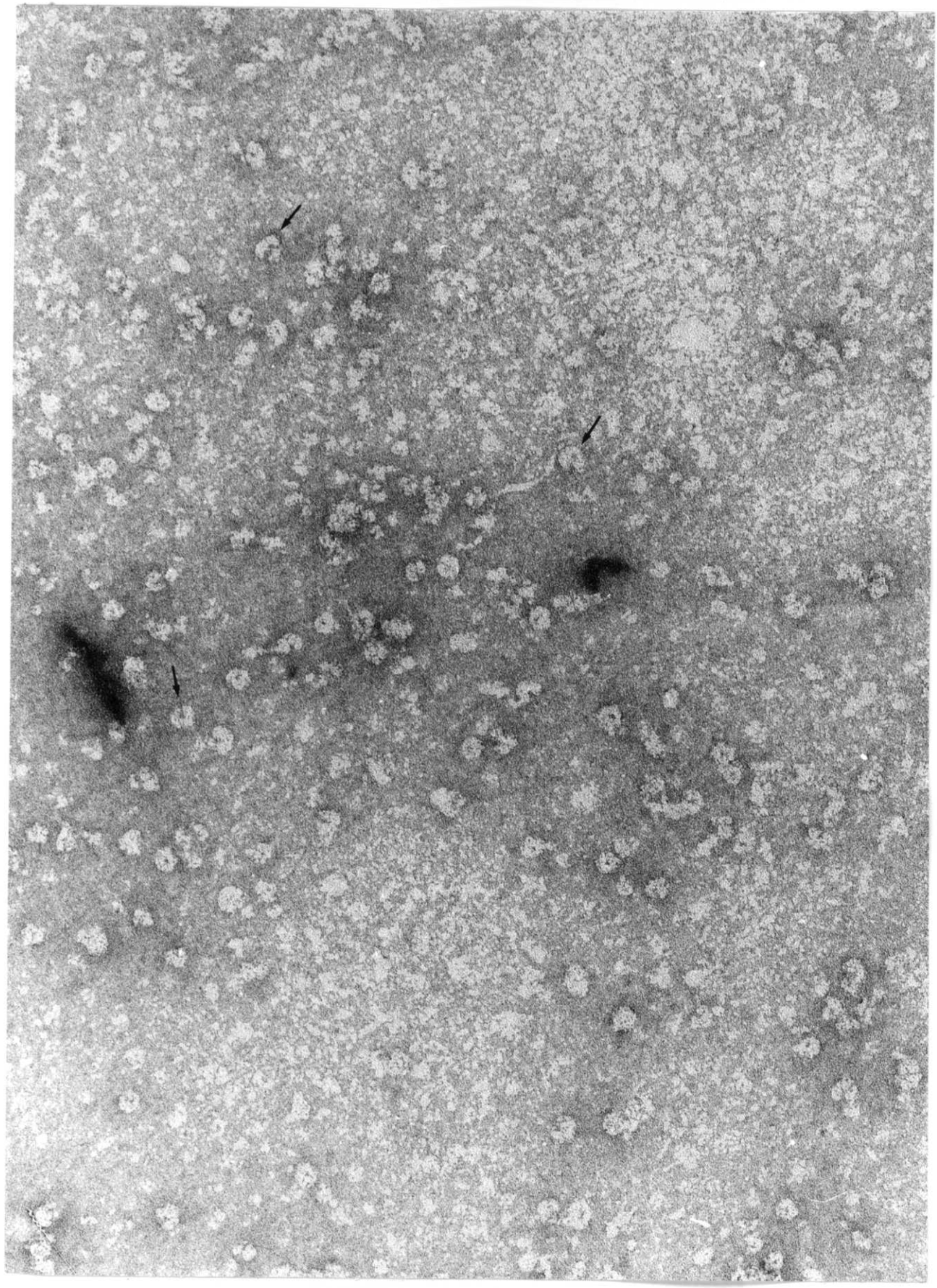


Fig. 15. SDS polyacrylamide gel electrophoretic patterns of Tetrahymena dynein preparations. For comparison, those of sea urchin dynein preparations are also shown. (a) Electrophoresis on a 6% polyacrylamide gel using the discontinuous Tris-glycine buffer system (Laemmli, 1970). (b) Electrophoresis on 3[polyacrylamide gels using the continuous Tris-glycine buffer system containing 6 M urea. Note that three A-band heavy chains are clearly resolved. (c) Electrophoresis on 3% polyacrylamide gels in the phosphate buffer system (Weber & Osborn, 1969) for estimation of molecular weight. Ac, Anthocidaris crassispina; Tt, Tetrahymena thermophilla SB255; Tp, Tetrahymena pyriformis; BSA, BSA oligomers cross-linked with glutaraldehyde (Payne, 1973).



a

Fig. 16. Electron micorgraphs of 30S (a), and 14S (b) dynein fractions from Tetrahymena thermophilla SB255. Negatively stained with 1% uranyl oxalate, pH 6.0. Bars represent 50 nm. Inset in (a) shows a diagrammatic representation of 30S dyenin. Arrows in (b) indicate C-shaped particles (see text).



b

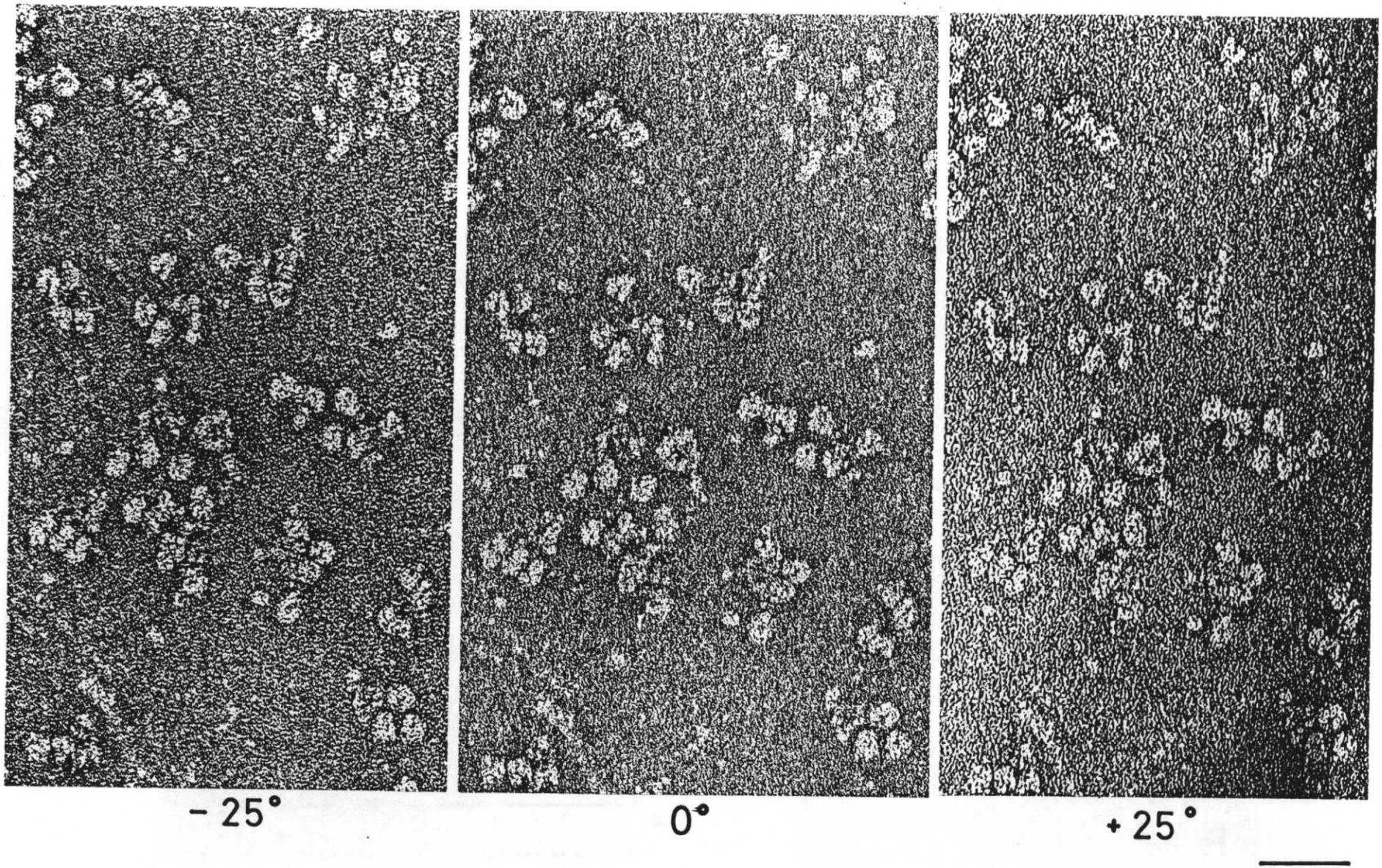


Fig. 17. Stereoscopic images of 30S dynein. Negatively stained with 1% uranyl oxalate, pH 6.0. Bar represents 50 nm. Numbers indicate tilting angles.

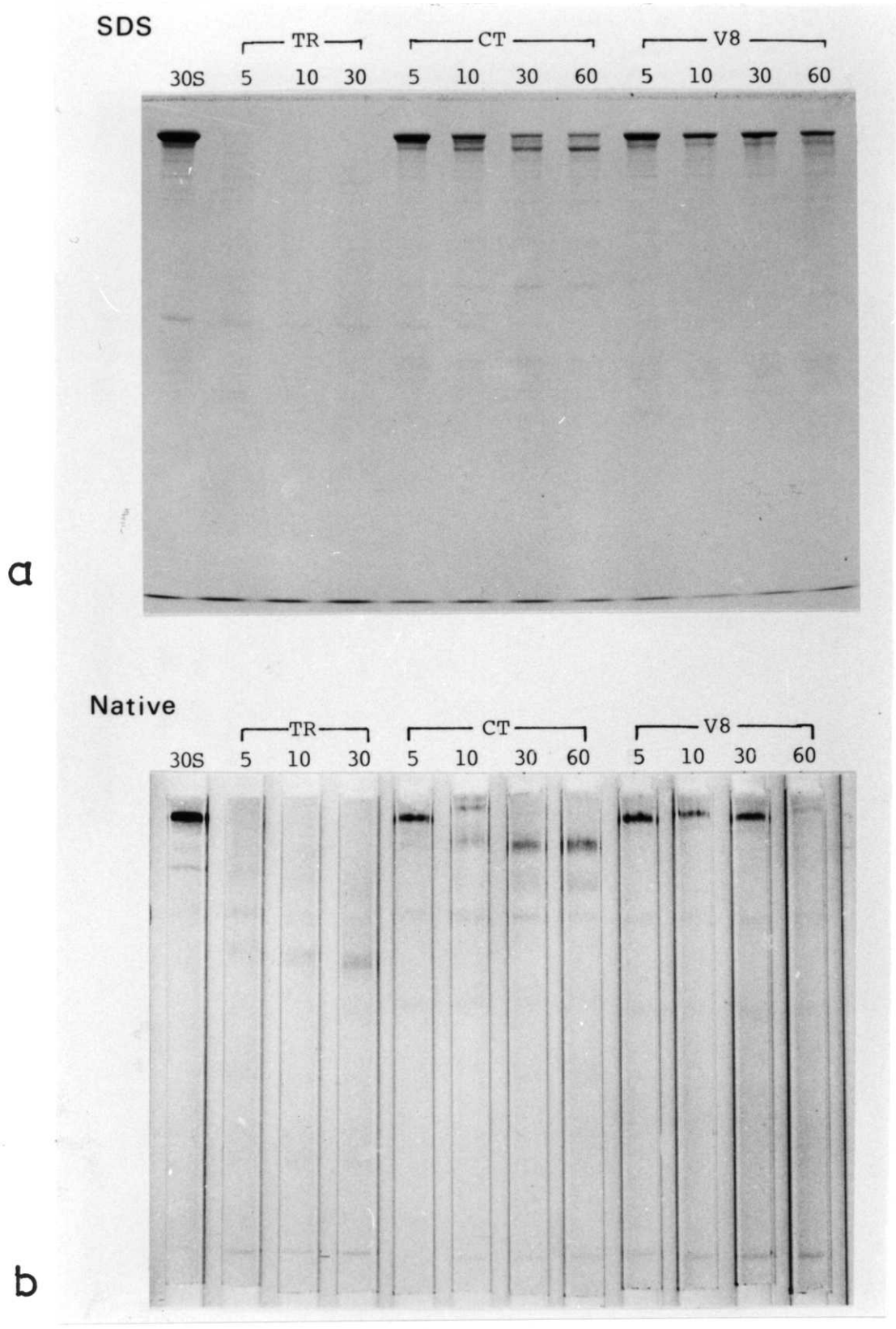


Fig. 18. Limited proteolytic digestion of 30S dynein. (a) SDS polyacrylamide gel electrophoresis on 6% gel using Tris-glycine buffer system (Laemmli, 1970). (b) Native polyacrylamide gel electrophoresis on 3% gels with Tris-glycine buffer system (Davis, 1964). T, trypsin; CT, chymotrypsin; V8, Staphylococcus aureus V8 protease.

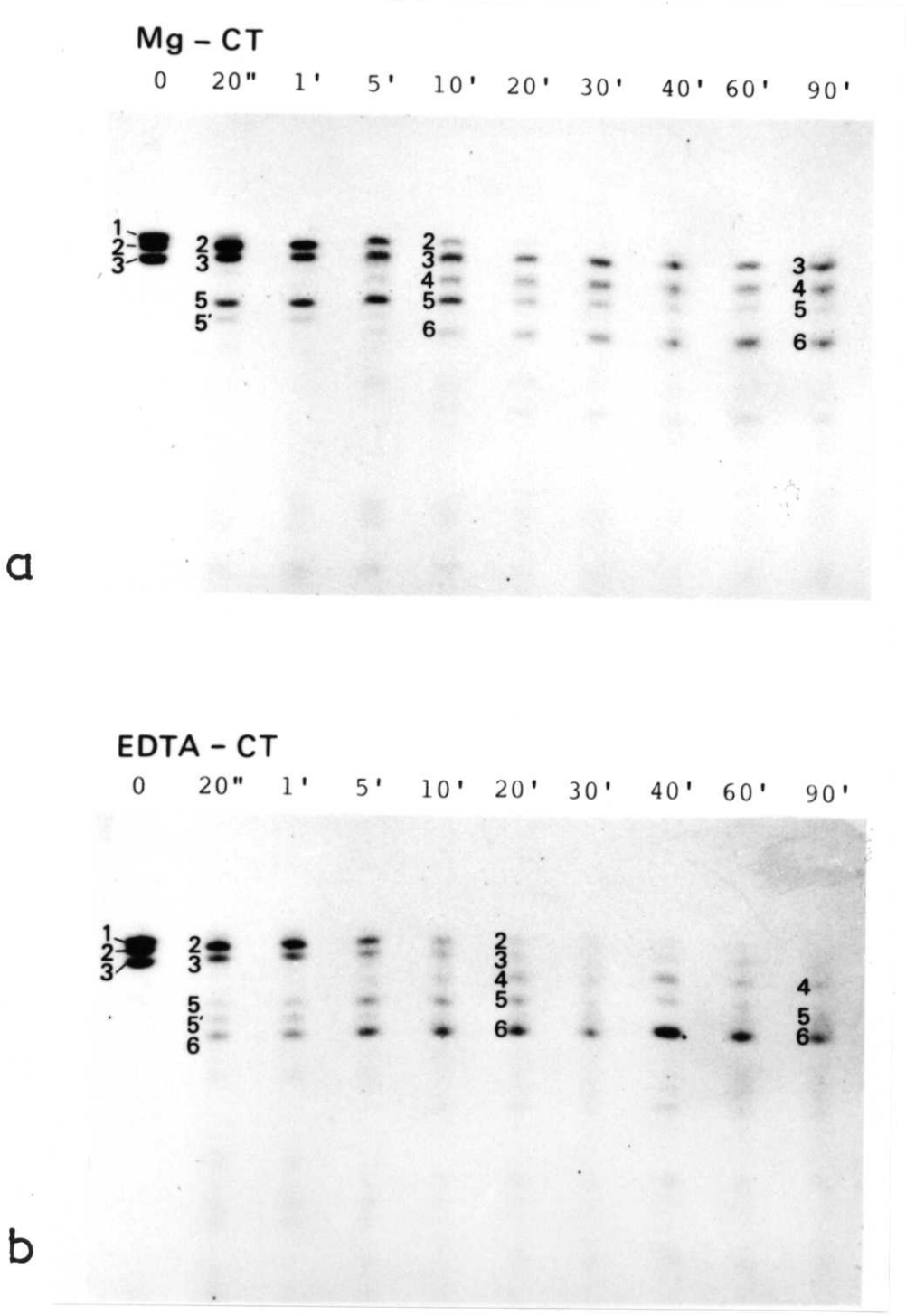


Fig. 19. SDS-urea gels showing the time course of chymotryptic digestion of 30S dynein. Thirty S dynein was digested with chymotrypsin at the weight ratio of 1/25 for 20 sec to 90 min at 25°C in the presence of either Mg^{2+} (a), or EDTA (b). Alliquots were loaded on 3% SDS-urea gels. Gels were stained with Coomassie brilliant blue. Heavy chains and their major fragments are numbered. Co-migration experiments showed that the heavy chain fragments to which the same numbers are given in (a) and (b) were apparently identical.

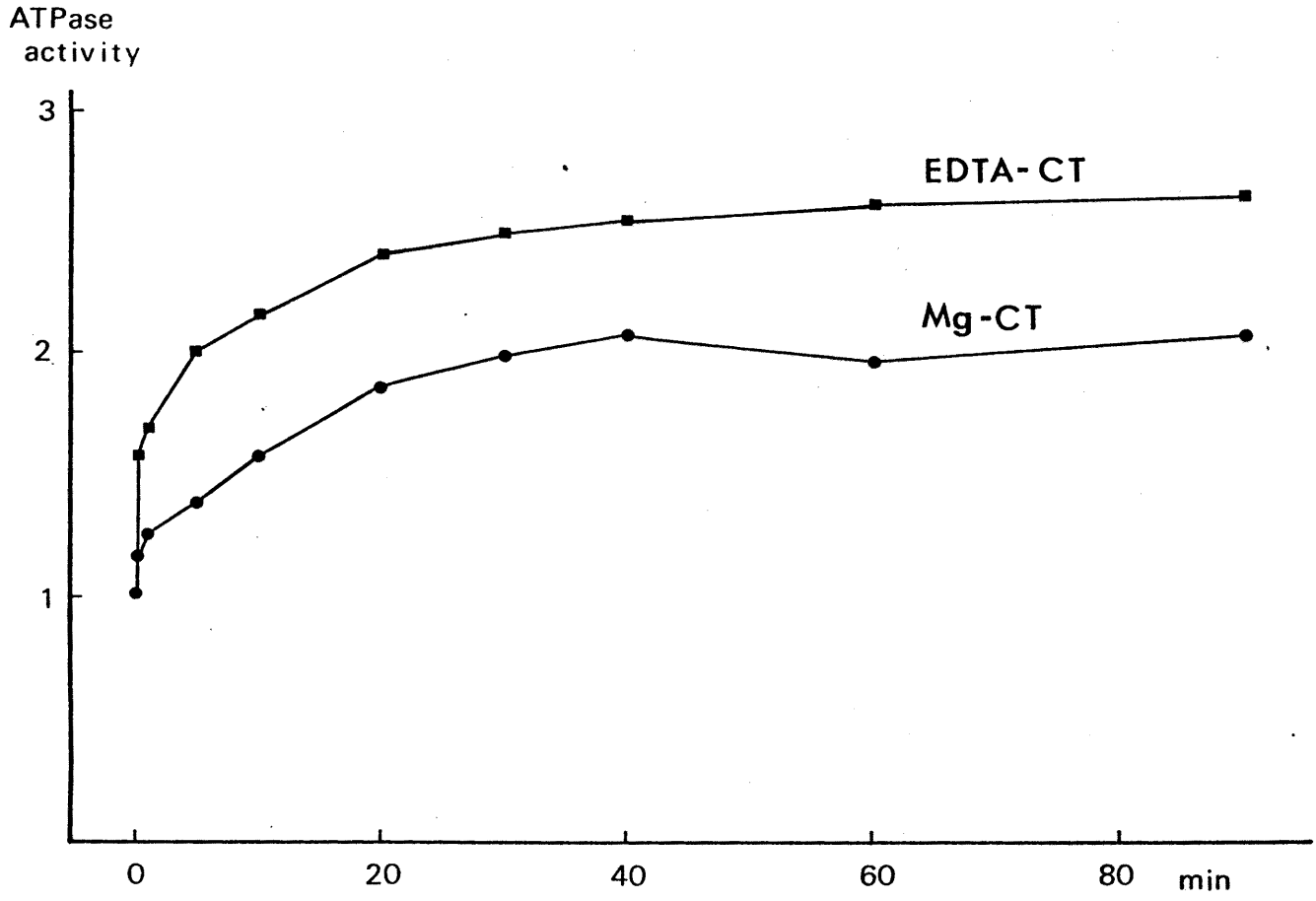


Fig. 20. Effect of chymotryptic digestion on ATPase activity of 30S dynein. Thirty S dynein was digested with chymotrypsin at the weight ratio of 1/25 for 1 to 90 min at 25°C in the presence of either 4 mM $MgCl_2$ (•), or 0.5 mM EDTA (▪). Ordinate, ratio of ATPase activity relative to that of non-digested 30S dynein; abscissa, digestion time.

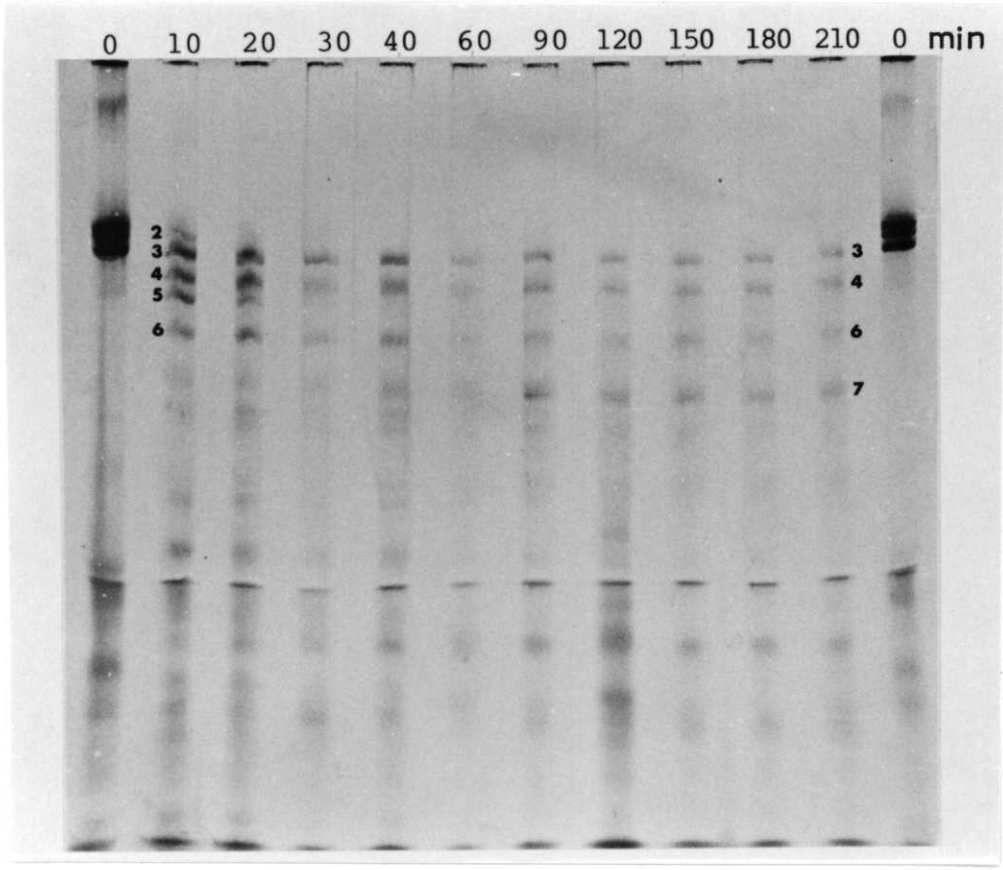
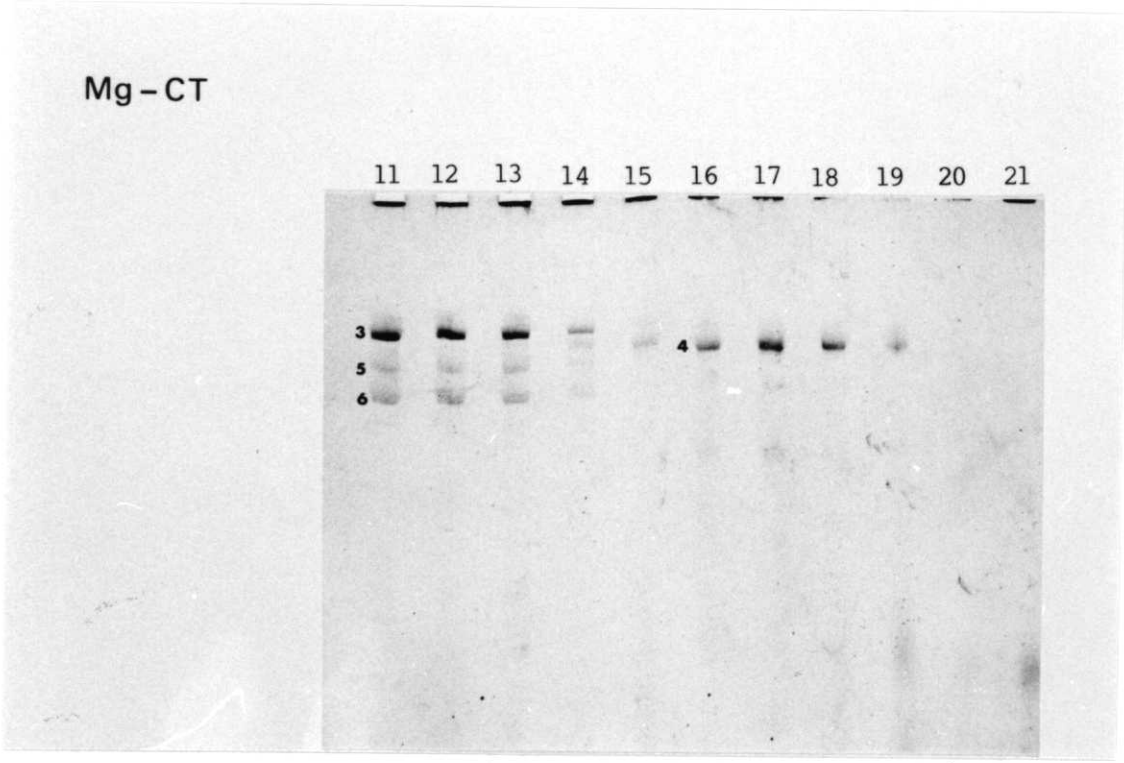


Fig. 21. Time course of chymotryptic digesion of 30S dynein. Same expereiment as shown in Fig. 19 but digested for longer period in the presence of Mg^{2+}. A 3% SDS-urea gel stained with silver. Note that band-7 heavy chain appears much denser in silver-stained gels than dye-stained gels (Compare this figure with Fig. 19a).



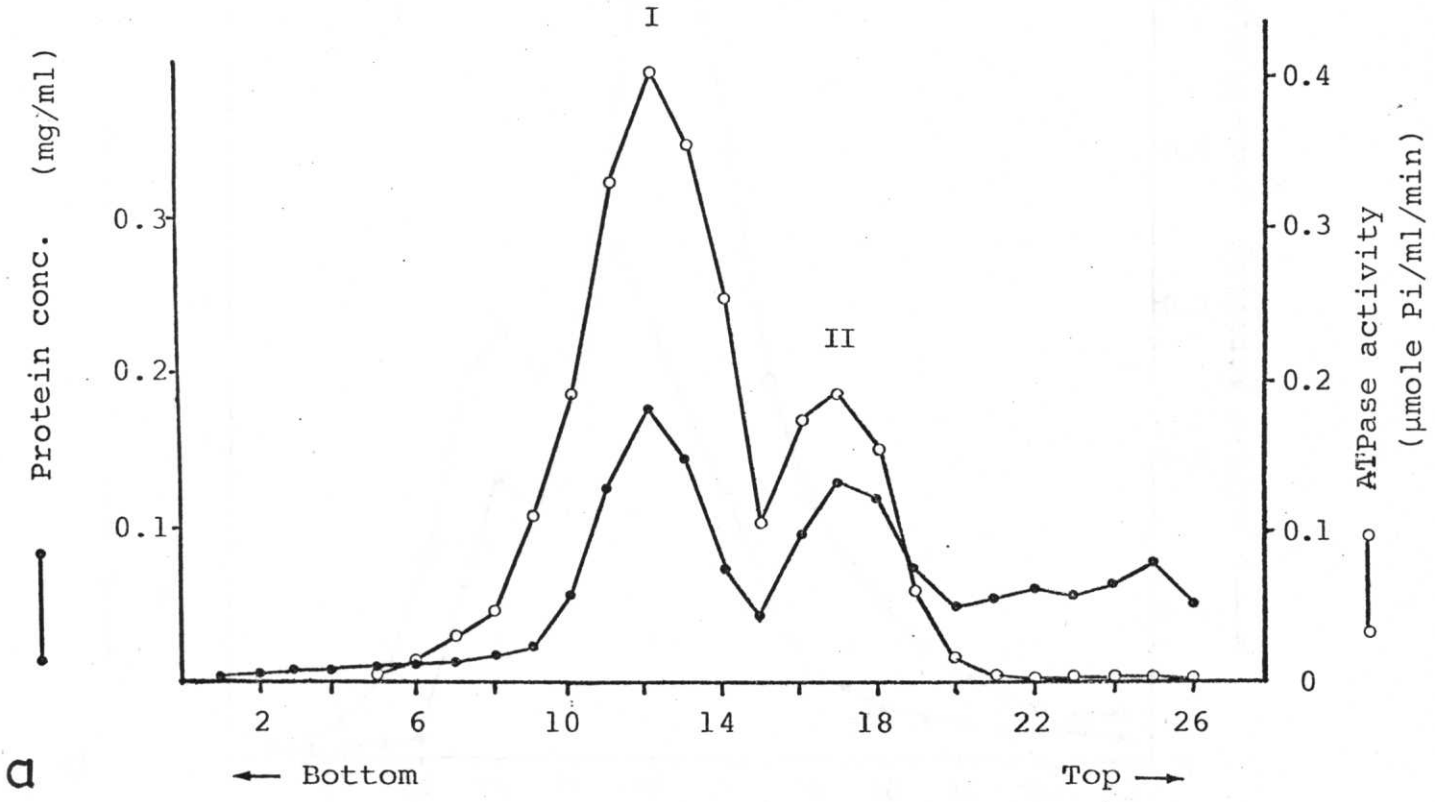


Fig. 22. Sucrose denstiy gradient centrifugation of digestion products of 30S dyenin in the presence of Mg^{2+}. Chymotrypsin digestion was carried out for 60 min in the presence of 4 mM Mg^{2+}. Centrifugation was done on 5-20% sucrose density gradient. (a) Sedimentation profiles of protein concentration and ATPase activity. Protein concentration was measured by Bradford method. (b) SDS-urea gel electrophoretic pattern on a 3% gel. The gel was stained with silver. Numbers indicate fraction numbers in (a).

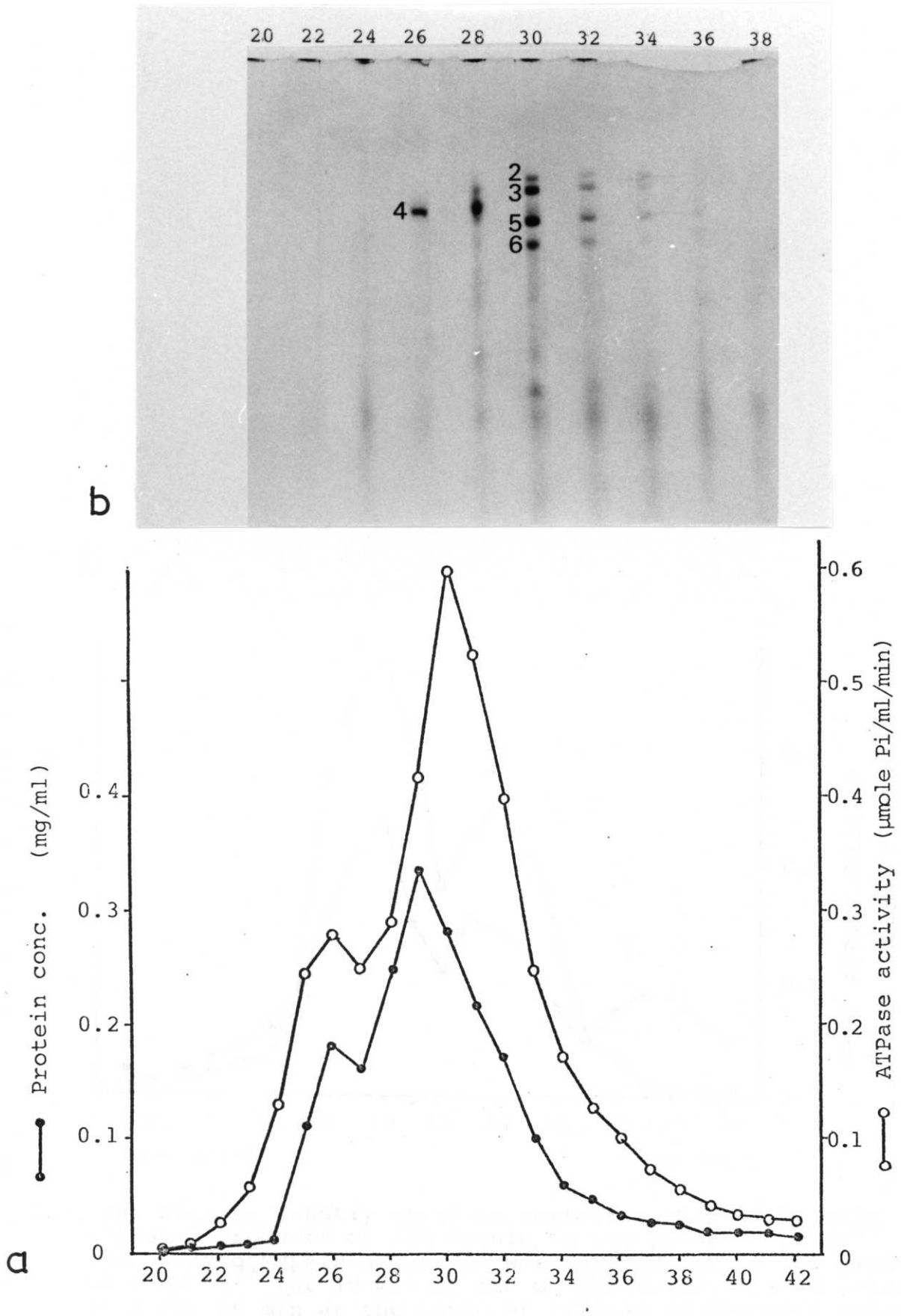


Fig. 23. DEAE-Sephacel column chromatography of digestion products. Digestion conditions were the same as in Fig. 22. About 1 mg of specimen was applied on a DEAE-Sephacel column, and eluted with a linear gradient of 0-0.5 M NaCl at pH 7.4. (a) Elution profiles of protein concentration and ATPase activity. Protein concentration was measured by Bradford method. b) SDS-urea gel electrophoretic pattern on a 3% gel. The gel was stained with silver. Numbers indicate fraction numbers in (a).

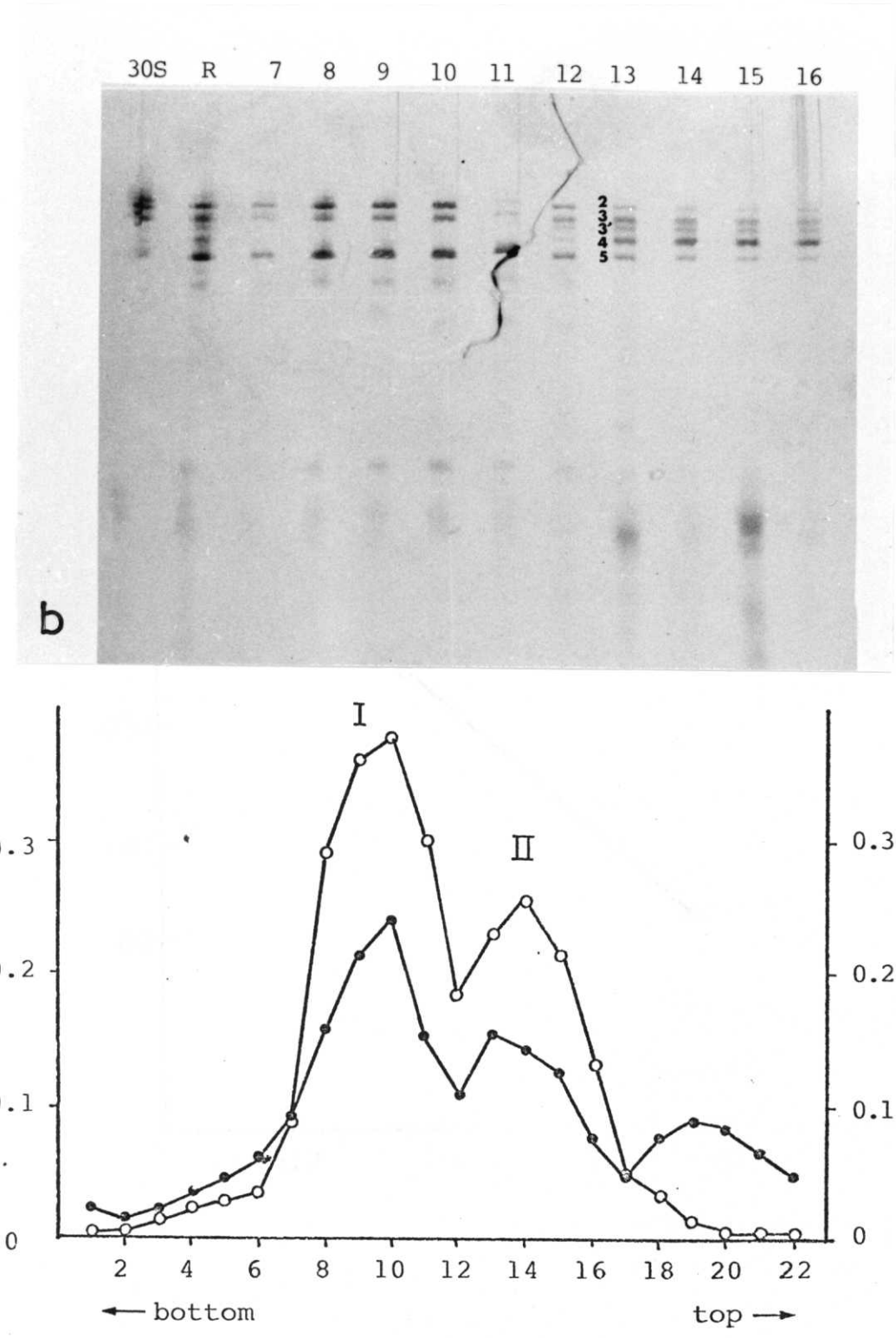


Fig. 24. Sucrose denstiy gradient centrifugation of briefly digested products of 30S dyenin in the presence of Mg^{2+} ion. Chymotrypsin digestion was carried out in the presence of 4 mM Mg^{2+} for 10 min at the weight ratio of 1/10 rather than for 60 min at the ratio of 1/25 as in the most other experiments. (a) Sedimentation profiles of protein concentration and ATPase activity. Protein concentration was measured by Bradford method. (b) SDS-urea gel electrophoretic pattern on a 3% gel. The gel was stained with silver. Numbers indicate fraction numbers in (a). R, Digestion products before fractionation.

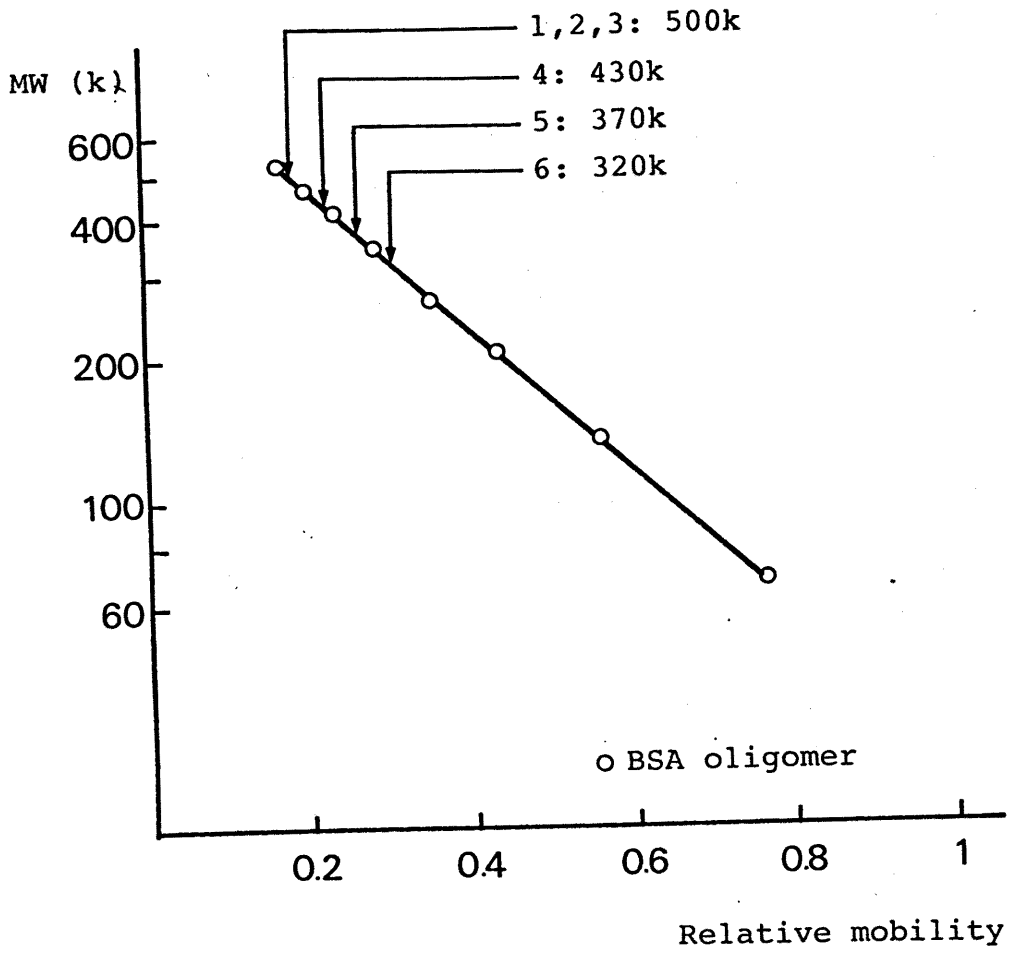


Fig. 25. Molecular weight determination of 30S dynein heavy chains and their fragments produced by chymotryptic digestion. Plot of the logarithm of molecular weight versus relative mobility on 3% polyacrylamide gels run in the phosphate buffer system (Weber & Osborn, 1969). Arrows specify relative mobilities of 30S dynein heavy chains and their chymotryptic fragments. o , BSA oligomers cross-linked with glutaraldehyde.

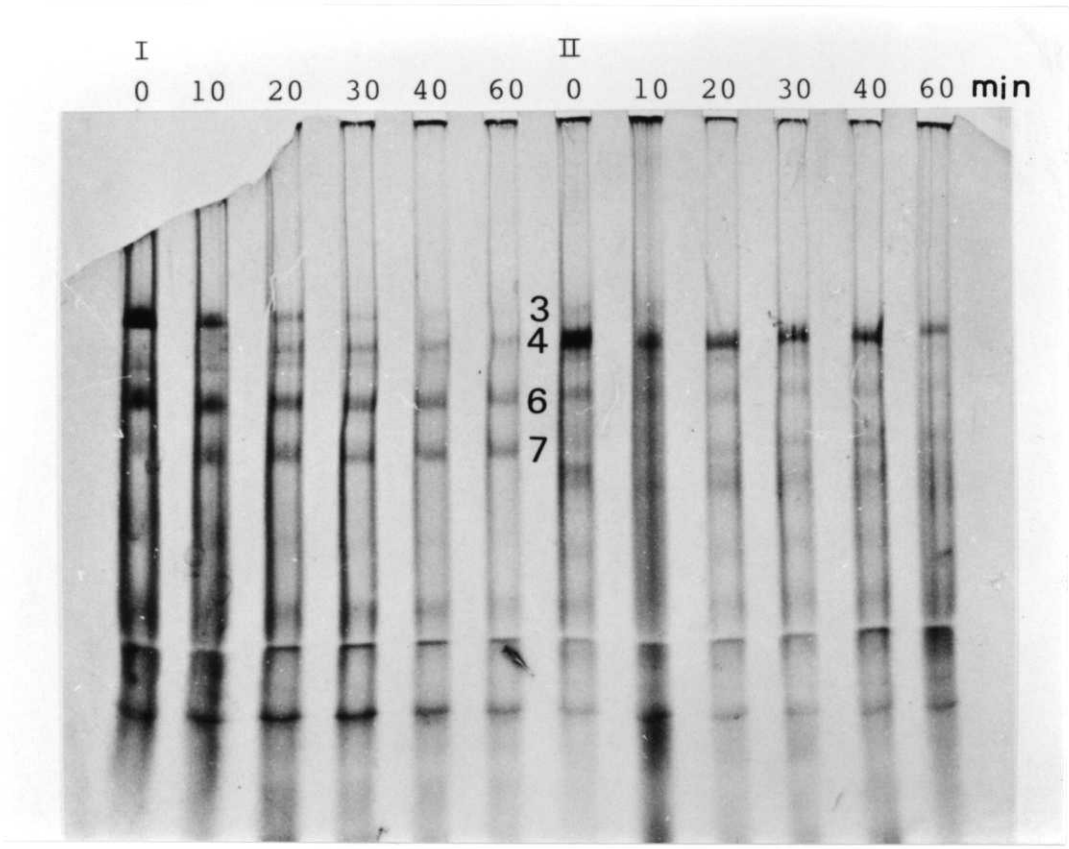


Fig. 26. SDS-urea gel electrophoresis of the digestion products of peak I and II fractions on sucrose density gradient. Peak I and peak II fractions (about 0.08 mg/ml) were digested with chymotrypsin at 0.02 mg/ml for 10 to 60 min in the presence of Mg^{2+}. Silver stained. Note the appearnace of band-4 heavy chain in digestion products of peak I.

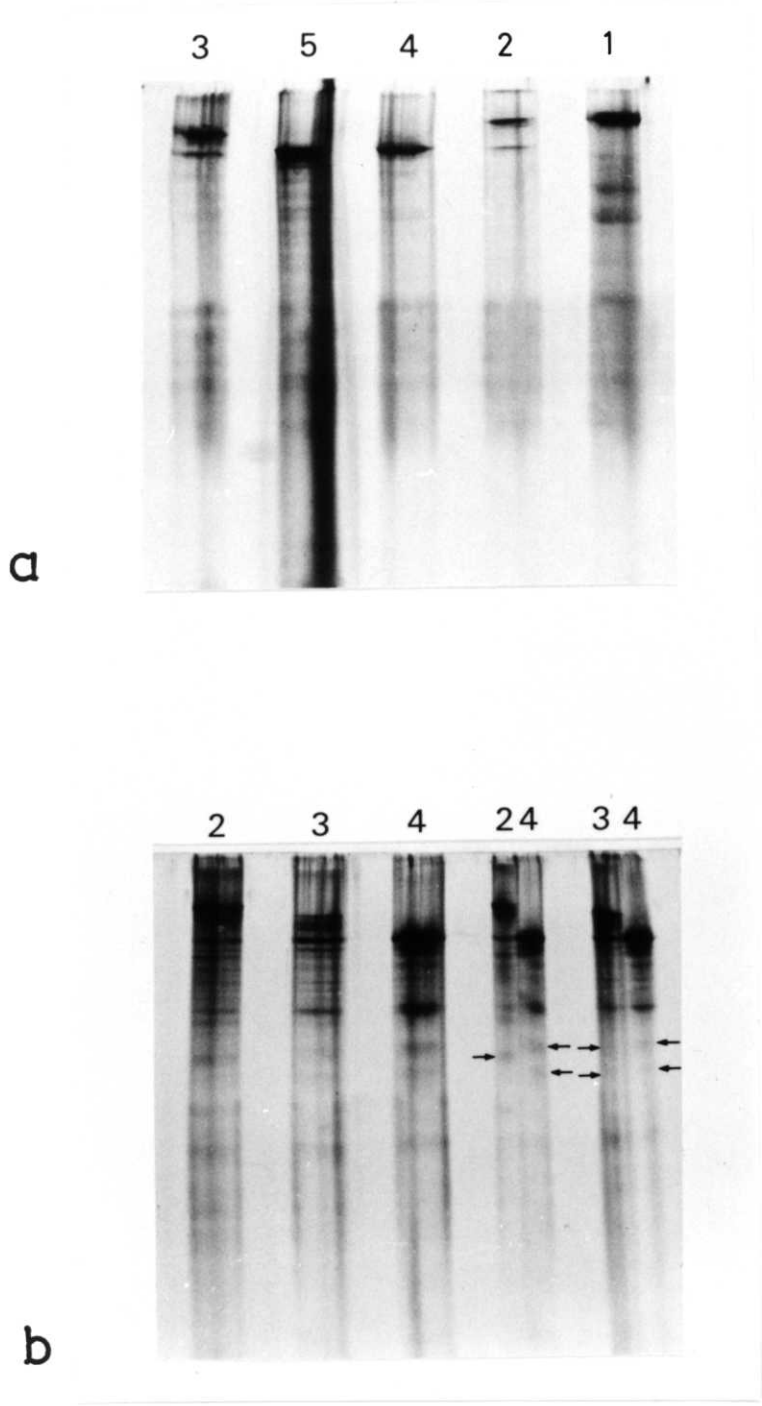


Fig. 27. One-dimensional peptide mapping of 30S dynein heavy chains and their chymotryptic fragments. DACM labeled heavy chain bands were cut out from SDS-urea gels, and put into the slots of stacking gels of the second electrophoresis. When detailed comparison was intended, two bands to be compared were put into the same slot. After applying specified amounts of γ-chymotrypsin (0.1 μg (a) or 2 μg (b)), second electrophoresis was done for 14 hr using 6% separating gels according to Cleveland et al. (1977). The gels were stained with silver. The numbers represent heavy chain bands speicified in Fig. 19a.

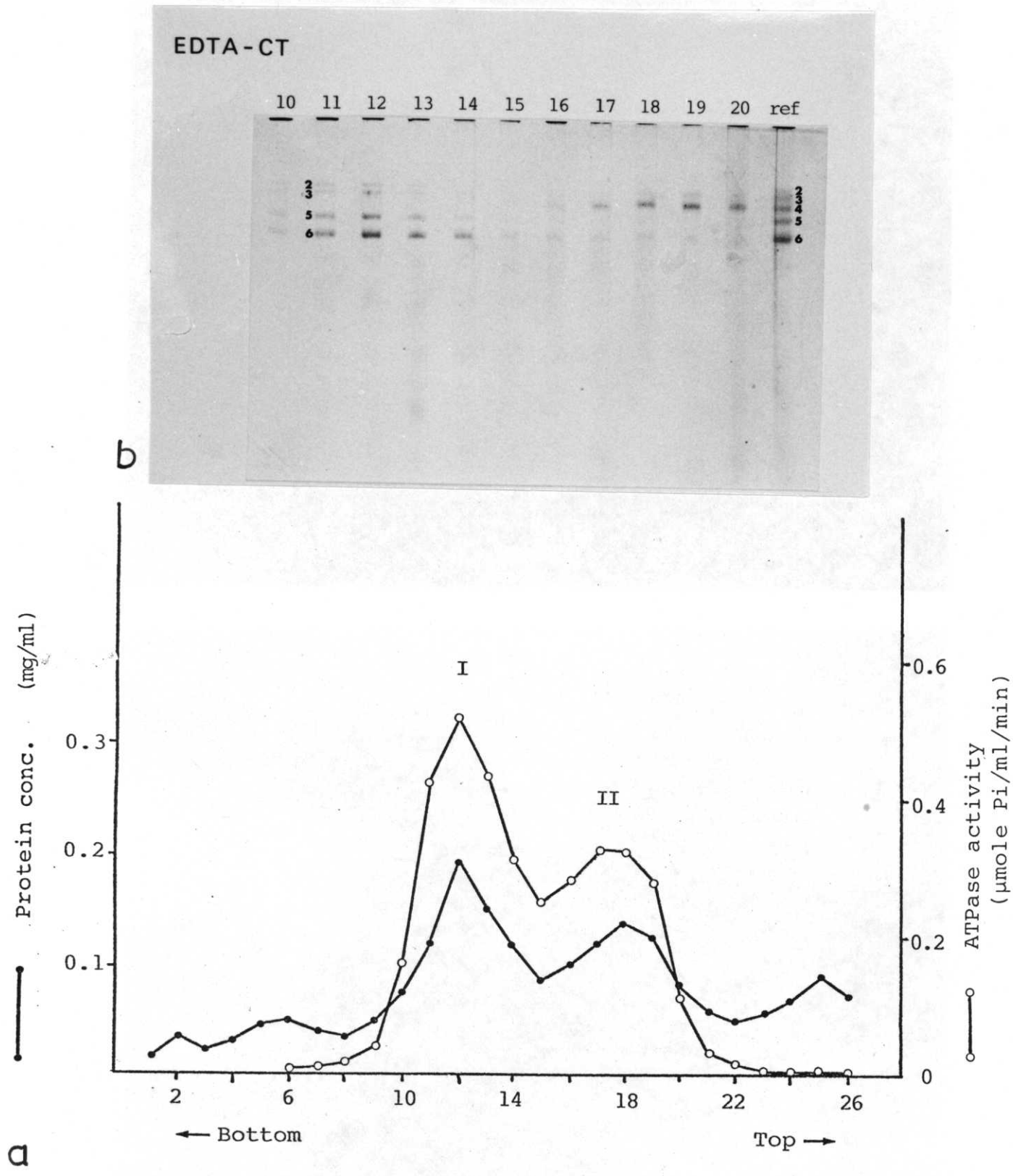


Fig. 28. Sucrose denstiy gradient centrifugation of digestion products of 30S dynein in the presence of EDTA. Chymotrypsin digestion was carried out for 60 min in the presence of 0.5 mM EDTA. Centrifugation was done on 5-20% sucrose density gradient. a) Sedimentation profiles of protein concentration and ATPase activity. Protein concentration was measured by Bradford method. b) SDS-urea gel electrophoretic pattern on a 3% gel. The gel was stained with silver. Numbers indicate fraction numbers in (a). ref, digestion products before fractionation.

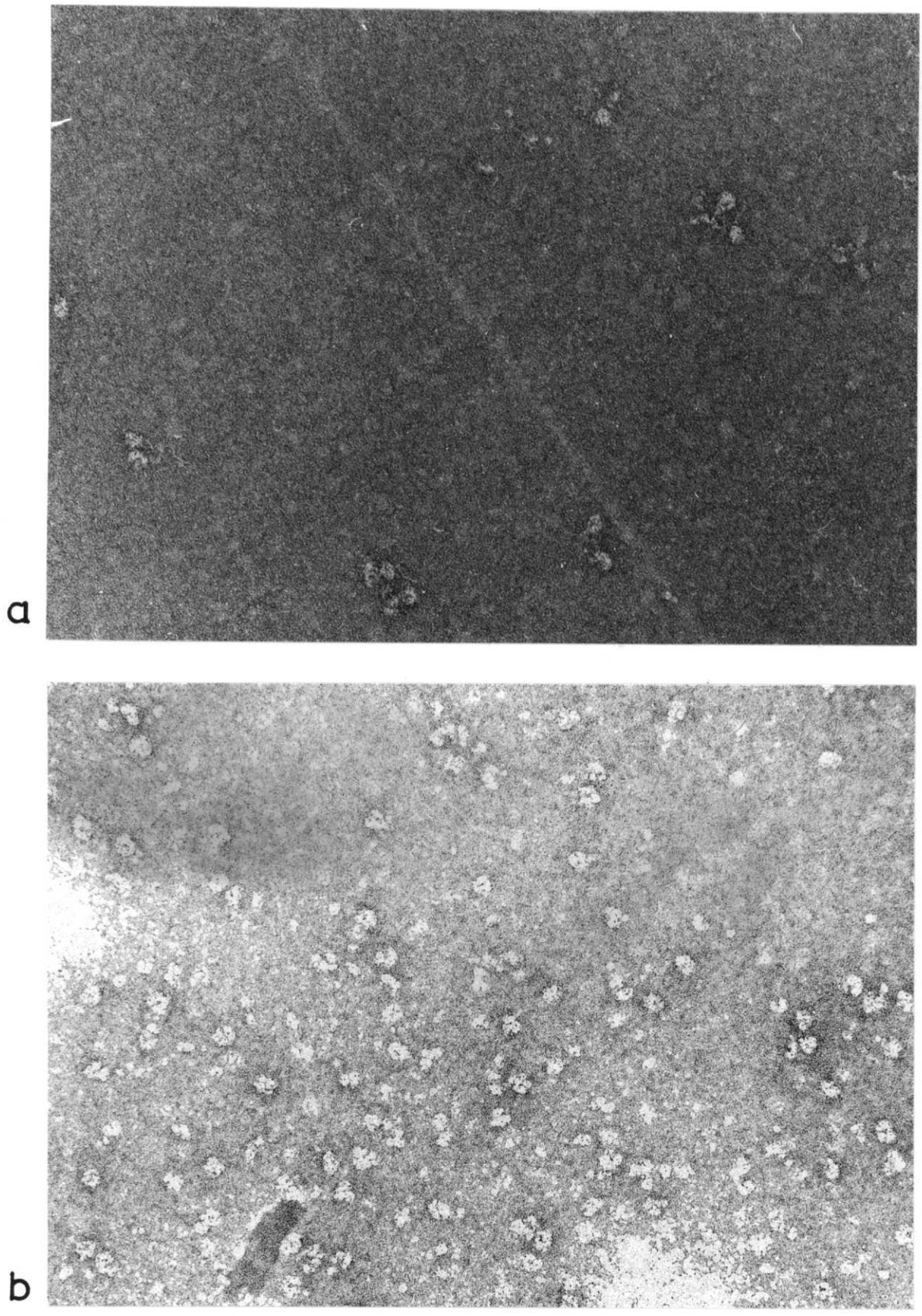


Fig. 29. Electron micrographs of digestion products of 30S dynein. Digestion was done in the presence of Mg^{2+} and fractionation on sucrose density gradient was performed. Peak I (a), and peak II (b) fractions of the digestion products. Negatively stained with 1% uranyl oxalate, pH 6.0. Bar represents 50 nm.

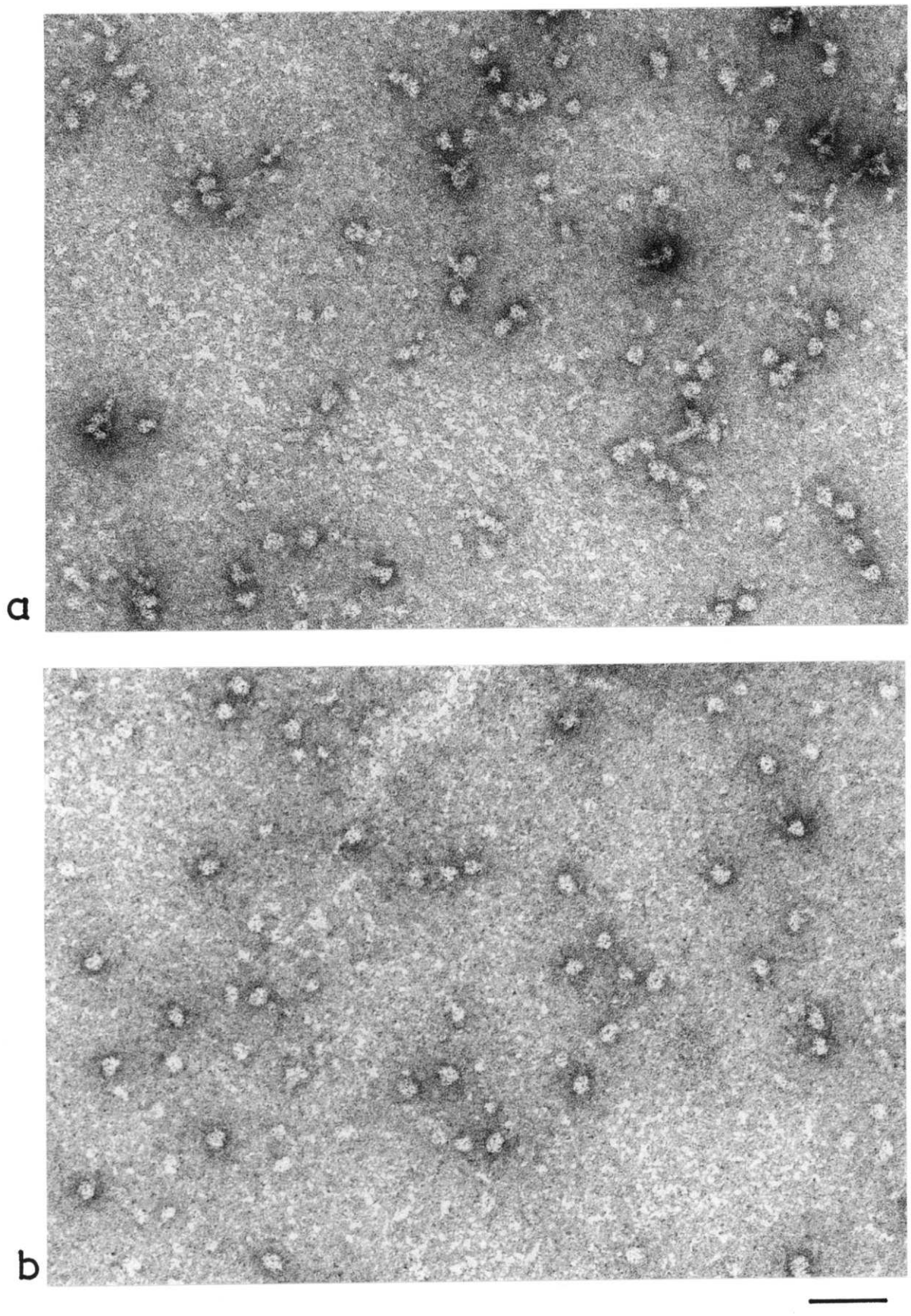


Fig. 30. Electron micrographs of digestion products of 30S dynein. Digestion was done in the presence of EDTA and fractionation on sucrose density gradient was performed. Peak I (a), and peak II (b) fractions of the digestion products. Negatively stained with 1% uranyl acetate (unbuffered). Bar represents 50 nm.

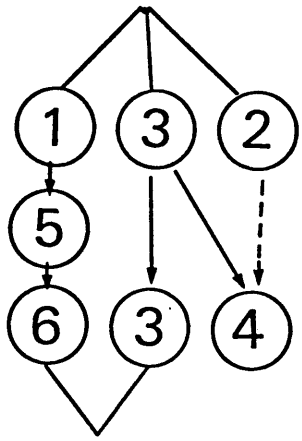


Fig. 31. Possible pathway of the chymotryptic degradation of 30S dynein heavy chains in the presence of Mg^{2+}. Numbers correspond to those of heavy chain bands revealed in SDS-urea gels (Fig. 19). Solid lines represent the paths confirmed as actual in this study.