STUDIES ON Ca^{2+}-BINDING PROTEINS IN CILIUM AND ITS ROLES IN Ca^{2+}-REGULATION OF CILIARY MOVEMENT OF TETRAHYMENA THERMOPHILA

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Contents

I. General Introduction

To elucidate the molecular mechanism of cell motility and its regulation is very important in respect of understanding many functions in living organisms and of understanding the cooperative functions of biological macromolecules. It has been known that two force generating systems, such as actomyosin system and tubulin-dynein system, are involved in cell motility, and that these systems include various subcomponents and regulatory proteins to sustain respective specialized functions, such as muscle contraction, amoeboid movement, axonal transport, chromosome movement, and ciliary (flagellar) movement.

Ciliary or flagellar motile system I have studied is an ubiquitous and conservative motile system (having so called "9+2" structure, Fig. 1, (1, 2)) in eukaryotic kingdom and is the most representative system of tubulin-dynein-mediated cell motility. This motile system has extensively been studied (3-5) and the following basic mechanism of ciliary movement has been accepted (Fig. 2). (i) Each neighbouring pair of outer-doublet microtubules slides longitudinaly by the force generated by arms (dynein ATPase) in a ATP-dependent manner. (ii) The active sliding is then converted to bending motion characteristic to ciliary beat by shear resistance due to certain sub-

ciliary structures, such as basal bodies, radial spokes, and interdoublet links which connecting outer-doublet microtubules. The same basic mechanism holds true for flagellar movement.

On the other hand, the swimming behavior of ciliated or flagellated cells (in other words, the pattern of ciliary or flagellar beat configuration) is affected by various stimuli, such as mechanic (6), electric (7), chemical (8), and light stimuli (9). For example, the direction of ciliary beat of Tetrahymena or Paramecium is reversed when the cell conflicts to an obstacle. This response is called "ciliary reversal" and the important role of Ca^{2+} in the response has been pointed out (6). In the course of this response, a mechanical stimulus induces depolarization of ciliary membrane and Ca^{2+}-influx through a Ca^{2+}-channel on the membrane into cilium. This Ca^{2+}-influx following membrane excitation is the triggering event common to the various responses of ciliary and flagellar movements to stimuli. However, the molecular mechanism by which the inpoured Ca^{2+} modulates the axonemal motile functions such as sliding of outer-doublet microtubules and/or its conversion to bending wave formation remains unsolved. I have thought that this problem is worth studying as a useful model for elucidating the general mechanism of Ca^{2+}-mediated regulation in various biological phenomena.

I have focused my studies on biochemical analysis of

Ca^{2+}-dependent ciliary reversal in Tetrahymena . As a clue for the biochemical analysis, I investigated a Ca^{2+}-binding protein in the cilium, since the most Ca^{2+}-dependent biological phenomena, such as muscle contraction (10), neurosecretion (11), cytoskeleton remodeling (12-15), and Ca^{2+}-stimulated activation of many enzymes (16), were known to be unexceptionally mediated by Ca^{2+}-binding proteins. As for the Ca^{2+}-binding protein of Tetrahymena, Suzuki et al. (17) of our laboratory succeeded in isolating a Ca^{2+}-binding protein (later on, it was identified as calmodulin (18)) for the first time from protozoa. The work made a contribution to the ubiquity, structural conservativeness and multifunctionality of calmodulin. The dissociation constant ($Kd= 4.6 \times 10^{-6}$) of Tetrahymena calmodulin nearly coincides with the free Ca^{2+} level ($10^{-6}M$) necessary to elicit a ciliary reversal in Tetrahymena cells (19). Moreover, Suzuki et al. (20) demonstrated that Tetrahymena cilia includes a protein which reacts specifically to anti-Tetrahymena calmodulin serum. From these evidences, calmodulin has been considered as a strong candidate for the target molecule of Ca^{2+} in the ciliary reversal response.

In this thesis, I present the proof showing the occurrence of calmodulin in Tetrahymena cilium, and demonstrate the subciliary localization of calmodulin and its counterpart molecule and structure. Based on these results, a

possible mechanism of ciliary Ca^{2+}-regulation is presented. Moreover, the occurrence of a new Ca^{2+}-binding protein (named TCBP-10) in cilium and the physicochemical properties of this protein purified to homogeneity are described.

II. Section 1

Occurrence of Calmodulin in *Tetrahymena* Cilium

II-1. Introduction

Changes in beat pattern of cilia and flagella have been known as one of Ca^{2+}-dependent biological phenomena; e.g., ciliary reversal in Paramecium (6) and Tetrahymena (21), ciliary arrest in molluscan gill (22, 23), flagellar waveform change in Chlamydomonas (24), reversal of flagellar wave propagation in Crithidia (25), asymmetrical (26) and intermittent (27, 28) flagellar beating in sea urchin sperm. In each phenomenon two Ca^{2+}-dependent steps appear to exist: (i) influx of Ca^{2+} into cilia or flagella occurs via the Ca^{2+} channels on ciliary or flagellar membrane; (ii) entered Ca^{2+} modulates ciliary or flagellar motile apparatus. However, in both steps the molecular targets of Ca^{2+} have not embodied.

Calmodulin has been considered as a strong candidate for the target molecule, since the protein is widely distributed in eukaryotes keeping its structural conservativeness and is involved in modulating a variety of cellular enzyme systems and cell motility systems in a Ca^{2+}-dependent manner (16, 29), and since a dissociation constant (Kd) of Ca^{2+}-binding to calmodulin (16, 18) coincides with the free Ca^{2+} level necessary for eliciting the change in beat pattern of cilia or flagella (21, 30-32).

In a ciliate, Tetrahymena, calmodulin from whole cells has been isolated by four different methods (18, 33-35).

The occurrence of calmodulin in the cilia has also been suggested from the finding that cilia include a protein which co-migrates with calmodulin in an alkali gel electrophoresis (33, 36), shows Ca^{2+}-dependent mobility shift in electrophoresis (36), reacts with an anti-Tetrahymena calmodulin antiserum (20), and binds to phenothiazine-Sepharose in a Ca^{2+}-dependent manner (33). In a flagellate Chlamydomonas, calmodulin from cell bodies has been purified (37, 38), and the occurrence of calmodulin in flagella has also been suggested: some flagellar fractions can activate brain cyclic nucleotide phosphodiesterase (PDE) and the flagellar fractions include a protein which co-migrates with the cell body calmodulin and shows a Ca^{2+}-dependent mobility change in SDS-polyacrylamide gel electrophoresis (37). Occurrence of calmodulin in Tetrahymena cilia or in Chlamydomonas flagella has been entertained, although the identification of the ciliary or flagellar protein as a calmodulin was done with a crude protein fraction for only two or three criteria.

However, Van Eldik et al.(38) reported that a protein isolated from Chlamydomonas flagella by phenothiazine-Sepharose affinity chromatography, by which calmodulins from various sources have been isolated (39, 40), did not activate brain PDE at all, in spite of the fact that the protein possesed several characteristic properties previously thought to be unique features of calmodulin;

e.g., the flagellar protein interacted with phenothiazine in a Ca^{2+}-dependent manner, was a small acidic protein, showed a Ca^{2+}-dependent mobility change in polyacrylamide gel electrophoresis, and reacted with an anti-calmodulin antiserum. The Chlamydomonas flagellar protein isolated by Van Eldik et al.(38) appears to be a calmodulin-like protein but not a calmodulin, since calmodulin is the name proposed for a brain PDE activator protein (41). Although it is not at present known whether calmodulin or calmodulin-like protein (or both) is included in Chlamydomonas flagella, the work of Van Eldik et al. (38) indicates that for identification of a protein such as calmodulin, a search for several criteria should be made directly with the protein purified to homogeneity. In such a strict sense, there has been no report showing that calmodulin is certainly included in cilia or flagella.

In this section, attempts were therefore made to purify a Tetrahymena ciliary protein previously thought to be a calmodulin and to ascertain whether the purified ciliary protein is identified as a calmodulin. I describe the evidence that Tetrahymena certainly includes calmodulin in the cilia.

II-2. Materials and Methods

Cell Culture ——— Tetrahymena pyriformis, strain W, and Tetrahymena thermophila, strain B 1868, were axenically cultivated at 26°C in a medium containing 1% proteose-peptone, 0.5% yeast extract and 0.87% dextrose, as described by Watanabe (42). In the present experiments, early stationary-phase cultures were used.

Preparation of Cilia and Deciliated Cell Bodies ——— This was performed by basically the same method described by Otokawa (43). Tetrahymena cells were harvested from 6 litters of the early stationary-phase culture by centrifugation at 1,600 g for 10 min. The cell pellet was quickly washed with distilled-deionized water (DDW) and gently resuspended in 4 volumes of cilium-detachment solution (12% ethanol, 30mM $CaCl_2$, 1mM EDTA, 2mM $MgSO_4$, 4mM KCl, 20mM sodium acetate, 10mM Tris-HCl, 1mM beta-mercaptoethanol, 0.1mM TLCK (a potent protease inhibitor), pH 7.0) which previously cooled to 0°C, and was kept standing for 10 min at 0°C. This suspension was centrifuged at 2,100 g for 10 min to separate cilia and deciliated cell bodies. The precipitate was used as the deciliated cell body fraction. The supernatant was centrifuged at 600 g for 10 min to remove contaminated cell bodies and then cilia were collected from the supernatant by centrifugation at 10,000 g for 20 min.

Electrophoresis ——— Alkali-glycerol-polyacrylamide gel electrophoresis and SDS-polyacrylamide gel electrophoresis were performed according to Perrie and Perry (44) and Laemmli (45), respectively.

Isolation of Calmodulin ——— Calmodulin from whole cells of Tetrahymena pyriformis W was isolated by the method of Suzuki et al. (18). Calmodulins from cilia and cilia-free cell bodies of Tetrahymena thermophila were isolated separately by the method of Suzuki et al. (18) except that the DEAE-cellulose step was omitted. About 1.1mg of ciliary calmodulin was obtained from 1.8g of ciliary acetone powder, and 6.0mg of cell body calmodulin from 10g of cell body acetone powder.

Antibodies ——— A rabbit antiserum against Tetrahymena whole cell calmodulin and its IgG fraction purified by calmodulin-affinity chromatography were the same as those prepared and characterized by Suzuki et al.(20).

Immunodiffusion test ——— This was performed by the method of Hirabayashi and Perry (46).

Assay for Phosphodiesterase Activity ——— Calmodulin-depleted 3′, 5′-cyclic nucleotide phosphodiesterase (PDE) was prepared from porcine brain after Teo and Wang (47). PDE activity was assayed by the method of Wickson et al. (48).

Protein Determination ——— Protein concentration was determined by the method of Lowry et al. (49) using bovine serum albumin as a standard.

II-3. Results

I purified a ciliary protein by applying an isolation method for Tetrahymena whole cell calmodulin (18) to the isolated cilia of Tetrahymena (see Materials and Methods). As shown in Fig. 3, the ciliary protein purified to homogeneity clearly co-migrated with Tetrahymena whole cell calmodulin in SDS-polyacrylamide gel electrophoresis. Hereafter, I shall refer to the ciliary protein as Tetrahymena ciliary calmodulin for the sake of my convenience. As for the ability of this protein to activate brain PDE, data will be shown later.

Both ciliary and whole cell calmodulins showed a Ca^{2+}-dependent mobility shift in alkali-glycerol gel electrophoresis (Fig. 4). This held true for calmodulin from cell bodies (data not shown). The protein band with the same mobility as Tetrahymena calmodulins (from cilia, cell bodies and whole cells) was detected in the total ciliary proteins (Fig. 4, lane b2).

Next, the antigenicity of ciliary calmodulin was tested with an antiserum specific for Tetrahymena whole-cell calmodulin. An Ouchterlony immunodiffusion test revealed that a precipitin line formed between Tetrahymena whole-cell calmodulin and anti-serum was completely confluent not only with the line formed with Tetrahymena ciliary calmodulin, but also with the respective lines formed

with the extract from whole cilia and with Tetrahymena cell body calmodulin (Fig. 5), suggesting that Tetrahymena ciliary calmodulin and cell body calmodulin hold just the same antigenic determinants in common.

Moreover, Tetrahymena ciliary calmodulin markedly activated brain PDE activity in the presence of Ca^{2+} (Fig. 6). The activation curve of PDE by varying amounts of the ciliary calmodulin was identical with that of the cell body calmodulin (also with that of whole cell calmodulin, data not shown).

Judging from these results, it is concluded that the ciliary protein I isolated is virtually a calmodulin.

II-4. Discussion

In the present experiments, I succeeded in isolating calmodulin from *Tetrahymena* cilia. This is, to my knowledge, the first instance of demonstrating in a strict sense the existence of calmodulin in cilia and flagella, since the flagellar protein isolated by Van Eldik *et al.* (38) was calmodulin-like protein but not calmodulin (see Introduction).

The *Tetrahymena* ciliary calmodulin I isolated was not distinguishable at all from *Tetrahymena* cell body calmodulin and *Tetrahymena* whole cell calmodulin in several properties, such as a molecular weight (Fig. 3), stabilities against heat and acetone, differential electrophoretic mobility in Ca^{2+} (Fig. 4), immunological reactivity against anti-calmodulin antiserum (Fig. 5), and ability to activate brain PDE activity (Fig. 6). I now consider that *Tetrahymena* ciliary calmodulin is identical with *Tetrahymena* cell body calmodulin, so that the former must possess various attributes which have so far investigated with *Tetrahymena* whole-cell calmodulin (17,18,50).

II-5. Summary

Tetrahymena calmodulins from cilia, cell bodies and whole cells were isolated separately and compared. These calmodulins showed just the same properties; they co-migrated in SDS-polyacrylamide gel electrophoresis, had a Ca^{2+}-dependent electrophoretic mobility shift in alkali-gel, held the same antigenic determinants in common, and activated brain cyclic nucleotide phosphodiesterase Ca^{2+}-dependently with identical activation curves.

III. Section 2

Localization of Calmodulin and Calmodulin-binding Protein in the Cilium

III-1. Introduction

I have investigated the intraciliary localization of Tetrahymena calmodulin as one way of explaining how this protein is involved in modulating ciliary movement. In this section, I present the evidence suggesting that ciliary calmodulin is localized on interdoublet links which laterally connect the outer-doublet microtubules.

III-2. Materials and Methods

Fractionation of Cilia ——— The fractionation of cilia was performed by the method of Gibbons (3) with slight modifications. The isolated cilia were resuspended in a Triton solution (1% Triton X-100, 30mM Tris-HCl, 2mM $MgSO_4$, pH 8.3) for 30 min at 0°C and centrifuged at 15,000 g. The resulting supernatant was dialysed against a Tris-Mg^{2+} solution (30mM Tris-HCl, 2mM $MgSO_4$, pH 8.3) for 4 h. This fraction was referred to as the membrane-matrix fraction. The pellet was washed with the Tris-Mg^{2+} solution and used as axoneme fraction. An aliquot of the fraction was further fractionated into two fractions; it was dialysed against Tris-EDTA solution (1mM Tris-HCl, 0.1mM EDTA, pH 8.0) for 4 h, centrifuged at 16,000 g, and the supernatant and pellet were used as crude dynein fraction and outer-doublet microtubule fraction, respectively. The purification of 30S dynein, 14S dynein, 6S tubulin from crude dynein fraction was performed by the method of Gibbons (3). ATPase activities were determined as described by Hayashi (51).

Immunoelectron microscopy ——— Samples in Tris-Mg^{2+}-NaCl solution (2mM $MgCl_2$, 145mM NaCl, 1mM beta-mercapto-ethanol, 30mM Tris-HCl, pH 8.3) were incubated with various concentrations of first antibody (anti-calmodulin IgG) at 18°C for 2 h, and various concentrations of

ferritin-conjugated second antibody (anti-rabbit IgG) were added to each preparation and incubated at 18°C for 2 h. These samples were negatively stained with 2% uranyl acetate and observed with a JEM-100C electron microscope at 80 kV. Appropriate dilutions for the first and second antibodies were about 1/80 and 1/1000, respectively. A ferritin-conjugated anti-rabbit IgG (goat) was purchased from Miles Yeda Ltd.

Other operations were performed by the same methods as described in the previous section.

III-3. Results

Distribution of Calmodulin and its Counterpart within Cilium ——— When the extract of whole cilia was subjected to an alkali-glycerol gel electrophoresis with Ca^{2+}, a fast-migrating calmodulin band disappeared (Fig. 4, b+), whereas with EGTA the band was clearly seen (Fig. 4). This suggests that calmodulin and its partner protein(s) form a large Ca^{2+}-dependent complex(es). To confirm the complex formation, two-dimensional gel electrophoresis was carried out: Cilia was first-dimensionally electrophoresed in the presence or absence of Ca^{2+} as in Fig. 4b, and each gel was subjected to the second-dimensional electrophoresis in the absence of Ca^{2+}. As shown in Fig. 7a, a calmodulin was released from the top of the gel which was firstly electrophoresed in the presence of Ca^{2+}, indicating that a Ca^{2+}-dependent complex was formed in the first-dimensional gel and this complex could not enter the first gel. On the other hand, no Ca^{2+}-dependent complex was shown to exist in the presence of EGTA (Fig. 7b).

These results urged me to pursue the localization of calmodulin and calmodulin-counterpart within a Tetrahymena cilium. For this purpose, cilia were fractionated into four fractions, such as membrane-matrix, axoneme, crude dynein and outer-doublet microtubule fractions.

Figure 8 shows the pattern of alkali-glycerol gel electrophoresis of these fractions. Calmodulin was found in membrane-matrix (lane b,c), axoneme (d) and outer-doublet microtubule (g) fractions, but not in crude dynein fraction (e). Though membrane-matrix fraction contains a relatively large amount of calmodulin, the amount of the fast-migrating calmodulin bands do not seem to make any difference when the samples are subjected to electrophoresis in the presence and absence of Ca^{2+} (b+, b-, c+, c-). On the contrary, calmodulin bands of axoneme (d+, d-) and outer-doublet microtubule (g_+, g_-) fractions showed clear differences in the apparent amount when electrophoresed in the presence and absence of Ca^{2+}: The fast-migrating calmodulin bands appeared in the presence of EGTA, whereas they disappeared completely in the presence of Ca^{2+}, having suggested that these fractions contained a certain counterpart molecule which formed Ca^{2+}-dependent complex with calmodulin. In this experimental conditions, crude dynein fraction contained no detectable calmodulin (e). Even when exogenous Tetrahymena calmodulin was added to this fraction, a Ca^{2+}-dependent complex was not detectable (f_+, f_-). These results held true for 14S and 30S dynein fractions separated on sucrose gradient centrifugation (Fig. 9).

In the preceding experiments, ciliary protein fractions were solubilized in 8M urea, so that microtubules would

be depolymerized into tubulin dimer. Therefore, there existed a possibility that a counterpart of calmodulin might be tubulin dimer. To varidate the possibility, I attempted co-sedimentation experiments in a condition in which ciliary outer-doublet microtubules remain intact. Outer-doublet microtubule fraction and Tetrahymena whole cell calmodulin were incubated at 18°C for 2 min in 1mM Tris-HCl buffer (pH 8.0) containing 2mM Ca^{2+} or 2mM EGTA. After centrifugation at 15,000 g for sedimenting microtubules, the supernatant and the pellet in the two conditions were electrophoresed in the presence of 5mM EGTA. Calmodulin was shown to bind to the outer-doublet microtubules when the mixture was incubated in the presence of Ca^{2+} (Fig. 10, b1), whereas almost all calmodulin was found to exist in the supernatant when the incubation of mixture was performed in the presence of EGTA (Fig. 10, a2). No tubulin dimer was detected in both supernatants (a1, a2), but Ca^{2+}-dependent calmodulin complex was demonstrated in the pellet from the mixture including Ca^{2+} (b1). Moreover, 6S tubulin dimer originated from central pair microtubules did not form Ca^{2+}-dependent complex with calmodulin (Fig. 9D). Under these conditions, tubulin dimer may not be a candidate for calmodulin-counterpart, but rather microtubules or microtubule-associated structures or both of them seem to be more possible candidates.

Calmodulin-binding Site on the Outer-doublet Microtubules

——— I then examined the calmodulin-binding site ultrastructurally. Before elucidating the binding site by immunoelectron microscopy, the outer-doublet microtubule fraction was observed. As seen in a thin-section electron micrograph (Fig. 11A), the fraction mainly consisted of outer-doublet microtubules free from dynein arms but fluffy or particulate materials were associated with them. In a negative-staining electron micrograph, periodic structures with regular intervals of about 90 nm were observed along the long axis of doublet microtubules (Fig. 11B, arrows).

I performed indirect immunoelectron microscopy with ferritin conjugates to know the calmodulin-binding site in outer-doublet microtubules and their associated structures. As shown in Fig. 12A, ferritin particles were clustered on the structures laterally linking the doublet microtubules at regular intervals of about 90 nm when antibody directed to Tetrahymena calmodulin was charged. In a disconnected doublet microtubule sample, ferritin particles were spaced about 90 nm intervals along the microtubules (Fig. 12B). When a sample was pre-washed thoroughly with an EGTA buffer in place of Ca^{2+} buffer or incubated with non-immune rabbit IgG as a first antibody, the periodic localization of ferritin was not observed. Ferritin particles were scattered at random and

barely localized on the link structures (Fig. 12C, D). From the localization of ferritin-antibodies, it is most likely that the interdoublet link is a calmodulin-counterpart in the ciliary axoneme.

III-4. Discussion

Concerning the distribution of calmodulin in *Tetrahymena* cilium, a considerable amount of calmodulin was shown to be present in the membrane plus matrix fraction (Fig. 8). Calmodulin might be involved in membrane fractions such as excitation (signal transduction) with accompanying membrane protein phosphorylation (52). Suzuki *et al.* (20) observed that 40μM trifluoperazine brought about ciliary reversal in *Tetrahymena*. Therefore, it is tempting to speculate that calmodulin might be a component of the Ca^{2+}-channel on the ciliary membrane. However, non-reversal mutants of *Tetrahymena thermophila*, *tnr A* and *tnr B* (19), which possess some defect in membrane Ca^{2+}-channel, have been shown to include normal calmodulin like that of wild-type cells (36). In the present experiments, I failed to detect any calmodulin-counterpart in this fraction (Fig. 8). This may be due to a very small amount of the counterpart molecule or extraordinarly conformational change of the counterpart protein induced by 8M urea treatment or injury of membrane geometry with Triton X-100. Calmodulin-binding protein in this membrane-matrix fraction would be very important for the ciliary membrane function, but little is known about the protein at the present time.

As for dynein fraction, Jamieson *et al.*(33) detected

calmodulin in this fraction. Furthermore, Blum et al. (53) reported that Tetrahymena calmodulin markedly stimulated 14S dynein ATPase in the presence of Ca^{2+} and that dynein ATPase could be purified by Ca^{2+}-dependent calmodulin-Sepharose affinity chromatography. The activation of dynein ATPase by calmodulin, as well as that of membrane-bound guanylate cyclase (50), seems to be noteworthy as function in situ of Tetrahymena calmodulin, in contrast to the fact that the function in situ of calmodulins from some lower eukaryotes remains to be established (54-56).

Gitelman and Witman tried to detect calmodulin from Chlamydomonas 12S and 18S dynein fractions, but they failed to detect any calmodulin in these dynein fractions (37). The results of the present experiment also showed that neither calmodulin nor calmodulin-counterpart was detected in Tetrahymena ciliary dynein fraction (Fig. 6e,f). Therefore, the calmodulin-dynein interaction found by Blum et al. (53) might be much weaker than other Ca^{2+}-dependent calmodulin complexes such as calmodulin-troponin I complex.

In crude dynein fraction, tubulin dimer from central pair microtubules is certainly included. Kumagai et al. (34) reported that Tetrahymena calmodulin bound Ca^{2+} dependently to an affinity column of tubulin-Sepharose 4B. However, as stated before, I failed to detect any calmodulin or calmodulin-counterpart in the crude dynein fraction (Fig. 8e, f) and 6S tubulin fraction (Fig. 9D).

They used porcine brain tubulin with microtubule-associated proteins (MAPs) to prepare their affinity column, so that it is not known whether calmodulin interacts with tubulin or MAPs.

Concerning the existence of calodulin in outer-doublet microtubule fraction, Gitelman and Witman reported that calmodulin appeared to be present in Chlamydomonas flagellar axonemes isolated in the presence of EGTA and EDTA (37). In the preparation used in the present experiment, the outer-doublet microtubule fraction corresponds to the axoneme after extracting dynein with Tris-EDTA, and the fraction included both calmodulin and its counterpart (Fig. 8g). However, unlike Chlamydomonas axoneme, calmodulin was removed from Tetrahymena outer-doublet microtubule fraction when the fraction was thoroughly washed with EGTA (see Fig. 10). Blum et al. (53) also demonstrated that considerable amounts of (^{35}S)calmodulin could bind to twice-extracted axonemes (with Tris-EDTA). They inferred that calmodulin might intract with MAPs or residual ATPases. In this regard, I demonstrated for the first time the localization of calmodulin in the ciliary axonemes by indirect immunoelectron microscopy (Fig. 12).

From my observation, calmodulin was found to be localized along the longitudinal axis of outer-doublet microtubules at a regular interval of about 90 nm (Fig. 12A, B). The interval corresponds with that of both radial spokes and

interdoublet links. However, it has been known that radial spokes are extractable from ciliary axonemes with Tris-EDTA, whereas interdoublet links are not (3). In outer-doublet microtubule fraction, no spoke head was seen (Fig. 11), although I do not know whether the remnants of spokes still remain. In addition, immunoferritin particles were shown to be clustered on the structures laterally linking a microtubule to the adjacent one (Fig. 12). From these, it is most likely that calmodulin is localized on the interdoublet links. In other words, the calmodulin counterpart in the ciliary axoneme seems to be the interdoublet links.

This interaction would occur in a Ca^{2+}-dependent manner even in 8M urea like the interaction between calmodulin and troponin I (Fig. 7a, 8g). The biological significance of the specific localization of calmodulin on the interdoublet links can be considered as follows. As stated before, it has been accepted that the basic mechanism of ciliary movement is the sliding of outer-doublet microtubules by dynein arms and its conversion to bending by shear resistance against sliding (see General Introduction, Fig. 2). It has also been suggested that the interdoublet links are the very elastic ultrastructures which bring about the shear resistance against the sliding of outer-doublet microtubules (57). Strength of the shear resistance bears a key role in the bending wave formation of the cilia,

and modulation of the resistance may result in the change of ciliary beat configuration. Taking account of these circumstances, occurrence of the following serial molecular events during Ca^{2+}-dependent regulation of ciliary reversal is conceivable (58). i) An influx of Ca^{2+} into cilium is brought about by ciliary membrane depolarization which is triggered by various stimuli, such as mechanical, chemical, electrical, and light stimuli. ii) Inpoured Ca^{2+} binds to calmodulin and turns calmodulin into activated form. iii) Activated calmodulin binds to interdoublet links and modifies link's elastic nature (strength of shear resistance). As a consequence, the mode of sliding-bending conversion is altered and the change in pattern of ciliary beat is induced (Fig. 13A).

Recently, Gibbons and Gibbons (59,60) reported that the Ca^{2+}-induced change in flagellar beat pattern (symmetrical swimming to asymmetrical swimming) of sea urchin sperm flagella is mimicked by some hydrophobic organic solvents in the absence of Ca^{2+}, whereas another group of hydrophobic organic solvents is able to abolish the Ca^{2+}-induced asymmetrical flagellar beat (Fig. 13B). They also showed that Ca^{2+}-induced asymmetrical swimming is irreversibly converted to symmetrical swimming when the sperm flagella is mildly digested with trypsin. From these findings, they suggested two important informations concerning the molecular mechanism of Ca^{2+}-reguration of

flagellar movement; i) Ca^{2+} works to modulate flagellar movement through hydrophobic interaction of molecules. ii) The axonemal site directly responsible for the modulation of flagellar wave form is sensitive to mild digestion with trypsin. These deductions seem to consolidate my hypothesis on molecular mechanism of ciliary reversal mentioned before, since it is well-known that Ca^{2+}-activated calmodulin binds to various calmodulin-binding proteins (for example, brain phosphodiesterase) with hydrophobic bonds (61, 62) and the interdoublet link is the most trypsin-sensitive structure in a cilium or flagellum (63). I further examined this hypothesis by using calmodulin inhibitors and Triton-extracted cell model (see succeeding section).

III-5. Summary

Distribution of calmodulin and calmodulin-counterpart in Tetrahymena cilium were investigated by using alkali gel electrophoresis in the presence of Ca^{2+} or EGTA, and by immunoelectron microscopy. Calmodulin was detected in the membrane plus matrix fraction and outer-doublet microtubule fraction, and its Ca^{2+}-dependent counterpart existed exclusively in the latter fraction. However, neither calmodulin nor its counterpart was detected in the crude dynein fraction. Immunoelectron microscopy revealed that calmodulin was localized along the longitudinal axis of outer-doublet microtubules at regular intervals of about 90 nm. The calmodulin-binding site in the ciliary axoneme was suggested to be interdoublet links. From these results, a possible mechanism by which Ca^{2+} regulates ciliary movement is presented.

IV. Section 3

Isolation and Characterization of *Paramecium* Calmodulin and Effects of Calmodulin Inhibitors on Ciliary Movement

IV-1. Introduction

Previously, Suzuki et al. of our laboratory observed that a potent inhibitor of calmodulin function, trifluoperazine or chlorpromazine, caused living Tetrahymena cells to shift from forward swimming to backward swimming at a final concentration of 40μM (20). This urged me to investigate the effect of trifluoperazine or chlorpromazine on Ca^{2+}-dependent ciliary reversal in the Triton-model of Paramecium, since reactivation of the Triton-model of Tetrahymena is not as easily achieved.

In this section, I describe evidences that calmodulins isolated from Tetrahymena and Paramecium are indistinguishable from each other in terms of several characteristics such as their antigenic properties, potency to activate brain phosphodiesterase and Tetrahymena guanylate cyclase (50) and in addition confirmed that trifluoperazine is able to potently inhibit the activation of enzymes by Tetrahymena calmodulin (64). I also describe evidence that Ca^{2+}-dependent ciliary reversal in the Triton-model of Paramecium is not affected at all by the addition of trifluoperazine or chlorpromazine (65).

IV-2. Materials and Methods

Cell Culture ——— Paramecium caudatum, strain 27AG3 (mating type VI), was grown in 2.5% fresh lettuce juice in Dryl's solution inoculated with Klebsiella pneumoniae (66), or in hey infusion at 26°C.

Purification of Paramecium Calmodulin ——— Calmodulin was isolated from Paramecium acetone powder by the same method as that used for isolation of Tetrahymena calmodulin (see Section 1).

Preparation of Triton-extracted Model of Paramecium ——— This was performed by the method described by Naitoh and Kaneko (30). Paramecium cells were washed three times with 1mM Tris-HCl (pH 7.0) buffer containing 1mM KCl, and 1mM $CaCl_2$ and the cell suspension was cooled to 0°C in an ice bath for 30 min. Hereafter, the buffers used were previously cooled to 0°C. The cells were washed with "extraction buffer" which contains 0.01% Triton X-100 (V/V), 20mM KCl, 10mM EDTA and 10mM Tris-maleate (pH 7.0). The suspension was kept standing in an ice bath for 40 min. Then, the cells were washed with the buffer containing 50mM KCl, 2mM EDTA and 10mM Tris-maleate (pH 7.0), and then resuspended in the buffer containing 50mM KCl and 10mM Tris-maleate (pH 7.0). One drop of the suspension containing 100-200 cells was transferred to 1ml of "reactivation buffer" which is containing 36mM KCl, 4mM $MgCl_2$,

4mM ATP, 10mM Tris-maleate (pH 7.0) and 3mM EGTA. The swimming behavior of the model was observed by dark-field photomicroscopy. In order to induce backward swimming (ciliary reversal), $CaCl_2$ was added to the re-activation medium to a final concentration of 2.9mM. This $CaCl_2$ addition yields free Ca^{2+} concentration of 10^{-5}M in the reactivation medium.

Chemicals ——— Trifluoperazine (TFP) was kindly gifted by Yoshitomi Pharmaceutical Co. Chlorpromazine (CPZ) was purchased from Sigma Chemical Co.

IV-3. Results

Isolation and Characterization of Paramecium Calmodulin ——— Paramecium calmodulin was isolated by the same procedure used for isolation of Tetrahymena calmodulin. This procedure includes heat extraction of Paramecium acetone powder, ammonium sulfate fractionation and preparative polyacrylamide gel electrophoresis. By this procedure, 530μg of calmodulin was isolated from 1.24g of Paramecium acetone powder. The purified protein showed the same electrophoretic mobility and of Ca^{2+}-dependent mobility shift on alkali-glycerol polyacrylamide gel electrophoresis (Fig. 14). The activation profile of brain phosphodiesterase by the protein is indistinguishable from that by Tetrahymena calmodulin (Fig. 15). It was also shown that Paramecium calmodulin activates Tetrahymena guanylate cyclase in a Ca^{2+}-dependent manner (Ca^{2+} concentration required for half maximal activation is 3.0×10^{-6}M), and this activation was completely inhibitted by 50μM trifluoperazine (*1, 64).

Effect of Calmodulin Inhibitors on Ciliary Movement of Paramecium Triton-model ——— From the results de-

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*1, This work was performed as a joint research with Drs. S. Kudo, Y. Muto, and Y. Nozawa, Gifu University School of Medicine.

scribed in the previous subsection, it is shown that Paramecium Triton-model is able to be used in place of Tetrahymena model. As a probe to know the role of calmodulin in ciliary reversal, I tested the effect of trifluoperazine and chlorpromazine on the swimming patterns of Paramecium Triton-model. As summarized in Table 1, addition of each inhibitor to a concentration ranging from 10μM to 100μM did not exert any significant effect on Ca^{2+}-dependent ciliary reversal reaction. Moreover, also the preincubation of models with 40μM of these drugs for 30 min did not affect ciliary reversal reaction (conversion from forward swimming to backward swimming). However, in these experiments, the swimming velocity (beat frequency) of the model was reduced significantly when these drugs were added to a concentration more than 100μM. The addition of excess EGTA to a reactivation medium containing 10^{-5}M Ca^{2+} introduced forward swimming of the model. Trifluoperazine and chlorpromazine did not affect on this conversion (backward swimming to forward swimming), either.

It was also shown that Ba^{2+} in concentration ranging from 7.5μM to 7.5mM could induce backward swimming of Triton-model. Trifluoperazine (5μM) did not exert any effect on the Ba^{2+}-induced ciliary reversal of the model.

IV-4. Discussion

In this section, I described the properties of Paramecium calmodulin purified to homogeneity and the effect of calmodulin inhibitors on ciliary reversal reaction of Paramecium Triton-model. It was shown that Paramecium calmodulin bore nearly the same properties as that of Tetrahymena calmodulin in terms of heat resistibility, acetone resistibility, electrophoretic mobility on alkali-glycerol-polyacrylamide gel electrophoresis, Ca^{2+}-dependent electrophoretic mobility shift, potency for the activation of brain phosphodiesterase and Tetrahymena guanylate cyclase. These evidences, especially its potency for guanylate cyclase activation, suggest that the Paramecium calmodulin is geneologically close to Tetrahymena calmodulin, since any other calmodulins from various animals are not able to activate guanylate cyclase (50). This also suggests the homological functions of these calmodulins in Tetrahymena and Paramecium cells. Moreover, the abolishment of calmodulin-dependent activation of guanylate cyclase by trifluoperazine guarantees the use of trifluoperazine as a calmodulin inhibitor in these organisms.

On the basis of these observations, effects of trifluoperazine and chlorpromazine on ciliary reversal of Paramecium Triton-model was examined. Unexpectedly, these drugs did not exert any significant effect on the Ca^{2+}-

regulation of ciliary beat direction, though the involvement of calmodulin in Ca^{2+}-regulation was considered from its specific localization in the cilium (see Section 2). Possible explanations for this discord is that i) trifluoperazine might not inhibit all the diverse functions of calmodulin; ii) trifluoperazine-binding sites of calmodulin might have been occupied by the association between calmodulin and a certain ciliary protein before the addition of trifluoperazine; or iii) a ciliary Ca^{2+}-binding protein other than calmodulin might play a crusial role in the Ca^{2+}-dependent ciliary reversal.

IV-5. Summary

I succeeded in isolating Paramecium calmodulin. It was shown that Paramecium calmodulin bore nearly the same properties as those of Tetrahymena calmodulin in terms of heat and acetone resistibilities, molecular weight and Ca^{2+}-dependent mobility shift on alkali-glycerol polyacrylamide gel electrophoresis, potency for the activation of brain phosphodiesterase and Tetrahymena guanylate cyclase, and sensitivity to trifluoperazine. Next, the effect of trifluoperazine and chlorpromazine on ciliary reversal reaction of Paramecium Triton-model were tested. These drugs did not exert any significant effect on Ca^{2+}-induced and Ba^{2+}-induced ciliary reversal reactions. The ineffectiveness of calmodulin inhibitors is discussed.

V. Section 4

Purification and Some Properties of a Second Ca^{2+}-binding Protein (TCBP-10) Newly Found in a Cilium and Cell Body of *Tetrahymena*

V-1. Introduction

In section 1, I presented the direct evidence for the occurrence of calmodulin in the cilia of Tetrahymena by purifying calmodulin from the isolated cilia (67). Furthermore, I demonstrated that the bulk of calmodulin in the cilium was localized on the interdoublet links (nexin links) (Section 2, 67). Since the interdoublet link is very elastic and is responsible for the shear resistance against active sliding of outer-doublet microtubules, the conditions which exert an influence on the elasticity of the link may affect the bending wave form of the cilium. A Ca^{2+}-dependent modulator which control the elasticity of the interdoublet link is likely to be calmodulin.

However, a few observations difficult to be explained by the simple mechanism in which only calmodulin plays a role in Ca^{2+}-dependent regulation of ciliary or flagellar movement have recently been presented. For example, (i) Ca^{2+}-induced ciliary reversal of Triton-extracted model of Paramecium was not affected at all by the addition of calmodulin inhibitors, such as trifluoperazine and chlorpromazine (Section 3, 65). (ii) Ca^{2+}-sensitivity of Triton-extracted model of sea urchin sperm flagella was shown to be significantly influenced by the Ca^{2+} concentrations in both of the demembranation medium and

reactivation medium. According to the data of Gibbons (27, 28), sperms demembranated with a solution including EGTA showed Ca^{2+}-dependent changes of flagellar wave form at a micromolar level of Ca^{2+}, whereas those demembranated with Ca^{2+}(5mM) showed the wave form changes at a millimolar level of Ca^{2+}, in spite of a likelihood that the former case make calmodulin bound with its binding protein so that leakage of calmodulin from the model does not take place. Thus, it became necessary to consider a more complicated mechanism for the Ca^{2+}-dependent ciliary reversal or flagellar wave form change. As a possible clue to solve this problem, I have tried to ascertain whether a second Ca^{2+}-binding protein different from calmodulin is present in Tterahymena cilia.

In this section, I describe evidence for the occurrence of a second Ca^{2+}-binding protein in a cilium and cell body of Tetrahymena. I also describe some properties of this protein purified to homogeneity.

V-2. Materials and Methods

Heat-resistant Ciliary Protein Fraction ——— Cilia fraction was resuspended with the Cilium-detachment solution (see Section 1) and homogenized by sonication with the Ultrasonic Disruptor Model UR-200 (Tomy Seiko Co., Tokyo) at an out put intensity of 9 for 2 min. The homogenate was heated at 95°C for 10 min in a boiling water and cooled quickly in an ice bath. The cooled homogenate was centrifuged at 20,000 g for 20 min, and the heat-resistant proteins of the supernatant were recovered by adding 100% TCA to a final concentration of 10%. The precipitate which recovered by centrifugation at 20,000 g for 20 min was resolved with 1M Tris and dialysed against DDW. This fraction was used as heat-resistant ciliary protein fraction.

Ammonium Sulfate Fractionation of Ciliary Proteins ——— Cilia fraction was resuspended with the Buffer A (250mM sucrose, 0.5mM EGTA, 1mM beta-mercaptoethanol, 10mM Tris-HCl, pH 7.5) and homogenized as described above. The homogenate was centrifuged at 20,000 g for 20 min and the supernatant was fractionated by adding fine powder of ammonium sulfate to the appropriate concentrations. Salted out proteins were precipitated by the centrifugation at 20,000 g for 20 min and dialysed against DDW. The proteins soluble in 80%-saturated ammonium sulfate

were precipitated by adding 100% TCA to a final concentration of 10%. The precipitate was dissolved with 1M Tris and dialysed against 8M urea, and then analysed on alkali-glycerol-polyacrylamide gel electrophoresis.

Partial Purification of Ca^{2+}-binding Proteins ——— Ca^{2+}-binding proteins were partially purified from deciliated cell body fraction as follows. All operations were performed at 4°C.

Step 1; heat treatment ——— About 200g of freshly prepared deciliated cell bodies were suspended in the equal volume of Cilium-detachment solution and heated at once at 95°C for 10 min in a boiling water bath. The suspension was homogenized with the Omni-mixer (Ivan Sorvall Inc., U.S.A.) for 1 min at an out put intensity of 8 and cooled quickly in an ice bath. To the homogenate, beta-mercaptoethanol was added to a final concentration of 1mM and the homogenate was incubated at 0°C for 10 min. The heat-denatured proteins were removed by centrifugation at 22,000 g for 20 min.

Step 2; ammonium sulfate fractionation ——— The resulting supernatant was subjected to ammonium sulfate fractionation(60-90% saturation). In this procedure, fine powder of ammonium sulfate was added to the supernatant with stirring and the stirring was continued for 30 min. Salted out proteins were precipitated by centrifugation at 22,000 g for 20 min. The pellet was resuspended in

a small volume of DDW and dialysed against DDW for 8 h.

Step 3; preparative polyacrylamide gel electrophoresis ——— The sample from step 2 was dialysed for 6 h against an 8M urea solution containing 1mM beta-mercaptoethanol, 1mM EGTA and 0.02% bromophenol blue and subjected to alkali-glycerol-polyacrylamide gel electrophoresis. The system is the same as that described by Perrie and Perry (44) except that the size of the slab gel was 1 x 12 x 21cm (cross-sectional area is 21cm^2) and the run was performed at 600 V for 6 h. The pattern of the bands on the gel was transferred onto a filter paper (Whitman Biochemicals, number 1) by being overlaid on the slab gel. After blotting for 10 min, the filter paper was stained with 0.2% Coomasie Brilliant Blue in 50% TCA for 10 min and destained with 10% acetic acid containing 20% ethanol. After drying the filter paper, the slab gel was superimposed on it and the each portions of the gel corresponding to the Ca^{2+}-binding protein bands was cut out. Each protein was recovered electrophoretically from the gel pieces.

Anti-serum and Immunodiffusion Test ——— A rabbit anti-serum against Tetrahymena calmodulin used was the same as that prepared and characterized by Suzuki et al.(20). Ouchterlony immunodiffusion test was performed by the method of Hirabayashi and Perry (46).

Cleavage of Proteins with Cyanogen Bromide ——— Cleav-

age was performed under the conditions similar to those described by Koide and Ikenaka (68). A protein sample was dissolved in 70% formic acid to a concentration of 2mg/ml, and cyanogen bromide was added to the sample at a 100-fold molar excess. The reaction mixture was incubated at 25°C for 24 h, then diluted with 9 volumes of water and lyophilized. The dried material was dissolved in 8M urea and analysed by alkali-urea-polyacrylamide gel electrophoresis.

Isolation of TCBP-10 ——— TCBP-10 (abbreviation of a new Ca^{2+}-binding protein, see Results) was purified to homogeneity using the procedure described below. As a starting material 800g of deciliated cell bodies were used.

Steps 1 and 2; ——— Heat-treatment (step 1) and ammonium sulfate fractionation (step 2) were performed by the same procedure as those used for partial purification described above.

Step 3; DEAE-cellulose column chromatography ——— A DEAE-cellulose column (2.7 x 45cm) was equilibrated with Buffer B (6M urea, 20mM potassium phosphate buffer, beta-mercaptoethanol, pH 7.0) and the solution from step 2 was applied to the column after dialysis against Buffer B. The column was washed with 1 litter of Buffer B and eluted with a linear gradient of 0.02-0.5M potassium phosphate in Buffer B (800ml). The fractions were ana-

lysed by alkali-glycerol- and SDS-polyacrylamide gel electrophoreses and the fractions containing TCBP-10 were pooled and dialysed against 1mM sodium bicarbonate.

Step 4; Hydroxylapatite column chromatography ——— A hydroxylapatite column (0.9 x 10cm) was equilibrated with Buffer C (6M urea, 20mM potassium phosphate buffer, 1mM beta-mercaptoethanol, pH 6.0) and the fraction from step 3 was applied to the column after dialysis against buffer C. The elution was conducted with a linear gradient of 0.02-0.3M potassium phosphate in Buffer C. The fractions containing only TCBP-10 were thoroughly dialysed against 0.1M KCl and then against 1mM sodium bicarbonate, and stored at -20°C.

Electrophoreses and Isoelectric Focusing ——— Alkali-glycerol- and alkali-urea-polyacrylamide gel electrophoreses were performed after Perrie and Perry (44) and Head and Perry (69), respectively. Isoelectric focusing on an agarose gel was performed according to the method of Hirabayashi (70). For these electrophoreses, samples solubilized with 8M urea were used. SDS-polyacrylamide gel electrophoresis was performed as described by Laemmli (45).

Amino Acid Analysis ——— A sample containing 0.4mg protein was hydrolysed with HCl at 110.6°C for 20 and 70 h. The hydrolysate was dried with an evaporator at 55°C, and analysed on a Durrum Model D-500 Amino Acid Analyser.

Ca^{2+}-Binding Assay ——— For measuring Ca^{2+}-binding site(s) and dissociation constant of a test protein for Ca^{2+}, conventional equilibrium dialysis was performed at various concentration of Ca^{2+}, using ^{45}Ca. Sample solutions (0.5ml) containing 61.4μg of TCBP-10 were placed in dialysis tubes (8/32 cellulose tubing, Visking Company) and dialysed against 100ml of solutions containing 0.1M KCl, 0.05M Tris-HCl (pH 7.5) and various concentrations of $CaCl_2$. Definite amount of ^{45}Ca was initially added to the outside solution. Equilibrium dialysis was performed under the conditions in which outside solution was continuously stirred at 4°C for 48 h. Quantitative sampling (0.2ml x 2) from inside and outside the dialysis bag was made and radioactivities of the aliquotes were determined with a Beckman 3155T scintillation spectrometer. Data from these determinations were treated by the method of Scatchard (71). To minimize background Ca^{2+} in this experimental system, we took possible cares as follows. All glasswares used were of Pyrex and thoroughly washed with 1N HCl and subsequently with DDW. Buffers were passed through the Chelex-100 ion-chelating resin. As a result, the background Ca^{2+} concentration was shown to be less than 0.01ppm using a Jarrel-Ash Model 975 plasma luminescence spectrophotometer. Dialysis membranes were boiled in 1mM EGTA solution for 10 min and thoroughly washed with DDW.

Analytical Techniques ——— Protein concentrations

were determined by UV absorption or by the method of Lowry *et al.* (49). UV absorption spectra were obtained using a Beckman 34 spectrophotometer.

Other operations were performed by the same methods as described in the foregoing sections.

V-3. Results

Occurrence of Ca^{2+}-binding Proteins Different from Calmodulin in the Cilium and Cell Body ——— In order to know whether a Ca^{2+}-binding protein(s) other than calmodulin was present in Tetrahymena cilium, I used an alkali-glycerol-polyacrylamide gel electrophoretic system deviced by Perrie and Perry (44). In this system, Ca^{2+}-binding proteins, such as troponin C and calmodulin, can easily be detected by its Ca^{2+}-dependent mobility shift. When ammonium sulfate fractions of ciliary proteins (see Materials and Methods) were analysed by the electrophoretic system, one protein band, besides calmodulin, showing Ca^{2+}-dependent mobility shift was detected in 60-80% and 80%-TCA fractions (Fig. 16). This protein was proved to be resistant to heat (95°C, 10 min) and TCA (10%), since the heat-resistant ciliary protein fraction (see Materials and Methods) certainly contained this protein (Fig. 17). The mobility of the protein is 51% of that of calmodulin when electrophoresed in the absence of Ca^{2+}. The findings strongly suggest the occurrence of a second Ca^{2+}-binding protein, in addition to calmodulin, in Tetrahymena cilia. From the densitometric analysis of the electrophoresed gel, the content of the protein in the cilium was estimated to be about 0.5% of the total ciliary proteins (data not shown).

Next, attempts were made to know whether the second Ca^{2+}-binding protein also exist in a cell body. A heat-resistant protein fraction of deciliated cell bodies was prepared by the same procedure as that used for ciliary fraction shown in Fig. 17, and it was analysed on the same electrophoretic system. As shown in Fig. 18B, three protein bands showing a Ca^{2+}-dependent mobility shift were found besides calmodulin band. The mobilities of the three protein bands were 51%, 63% and 76% of that of calmodulin when these proteins were electrophoresed in the absence of Ca^{2+}, so that I tentatively designated these proteins as P51, P63 and P76, respectively. One of these proteins, P51, showed exactly the same mobility and the same extent of Ca^{2+}-dependent mobility shift as those of the ciliary Ca^{2+}-binding protein mentioned above. The result clearly indicates that P51 is identical with the ciliary Ca^{2+}-binding protein. This urged me to purify the newly found Ca^{2+}-binding proteins from Tetrahymena cell bodies with the aims of knowing the properties of these proteins and compareing the properties with those of calmodulin, because I had considered that purification of the second Ca^{2+}-binding protein from cilia would be very difficult in respect of its amount.

Partial Purification and Some Properties of Newly Found Ca^{2+}-binding Proteins from Cell Bodies ——— Three Ca^{2+}-

binding proteins (P51, P63 and P76) were partially purified from Tetrahymena cell bodies by using the preparative gel electrophoretic technique (see Materials and Methods). The partially purified samples retained the original electrophoretic mobilities and the original extent of Ca^{2+}-dependent mobility shifts on alkali-glycerol electrophoresis. However, P63 and P76 preparations included several minor Ca^{2+}-sensitive bands which seemed to be of the products degraded during the purification (Fig. 18C). The P76 fraction was contaminated with a trace amount of calmodulin.

SDS-polyacrylamide gel electrophoresis of these three fractions revealed that major components of these fractions were the polypeptides whose molecular weights were approximately 10,000, although the P63 and P76 fractions contained the major polypeptides somewhat smaller than that of P51 fraction (Fig. 18D). The fact that the molecular weight of these proteins were much smaller than that of calmodulin urged me to examine a possibility that the proteins were degraded fragments of calmodulin. This possibility was examined as follows. First, the immunological cross-reactivity of these proteins to an anti-Tetrahymena calmodulin serum was tested. As shown in Fig. 19, partially purified fractions of P51 and P63 were not reacted at all with the anti-calmodulin serum, whereas P76 fraction gave rise to a very weak precipitin line,

presumably due to the contamination of calmodulin in the fraction (Fig. 18C, lane d). Secondly, cyanogen bromide-fragments of P51 and P63 were compared with that of calmodulin. By the treatment of cyanogen bromide, calmodulin was cleaved into more than four small subfragments, whereas P51 and P63 were not cleaved at all, suggesting that these proteins did not contain methionine (Fig. 20). Moreover, any fragment derived from calmodulin did not co-migrate with P51 or P63. Thirdly, proteolytic fragments of calmodulin and P51 were compared. In order to reproduce the proteolytic conditions in the cell, the supernatant fraction of Tetrahymena cell homogenate was used for the source of proteolytic enzyme. As shown in Fig. 21A, digested fragments of calmodulin retained the nature of Ca^{2+}-dependent mobility shift but were electrophoresed faster than intact calmodulin. On the other hand, two fragments derived from P51 showed the same mobilities with those of P63 and P76 (Fig. 21B).

These results mentioned above indicates that the protein, P51, is not a degraded product of calmodulin, and that P63 and P76 are proteolytic fragments of P51. Thus, it is concluded that P51, in other sense, a small ciliary Ca^{2+}-binding protein, is a new Ca^{2+}-binding protein species different from calmodulin. Hereafter, I will designate this protein as TCBP-10 (Tetrahymena Ca^{2+}-Binding Protein whose molecular wight is about 10K), although the exact Ca^{2+}-binding

ability and the molecular weight of this protein will be described later.

Isolation of TCBP-10 ——— Isolation of TCBP-10 was performed by the four-step procedure including heat-treatment (95°C, 10 min), ammonium sulfate fractionation (60-90% saturation), DEAE-cellulose column chromatography, and hydroxylapatite column chromatography (for the details, see Materials and Methods). At the begining, I attempted the chromatography with relatively anti-caotropic buffer conditions (for example, 10mM potassium phosphate buffer, 1mM beta-mercaptoethanol, pH 7.5). However, TCBP-10 and its degraded proteins (P63 and P76) were eluted loosely by a linear gradient of potassium phosphate buffer and did not form any prominent peak. Therefore, DEAE-cellulose and hydroxylapatite chromatographies were performed under the presence of 6M urea. Elution profiles of DEAE-cellulose column chromatography and hydroxylapatite column chromatography were shown in Figs. 22 and 23, respectively. As shown in Fig. 22, TCBP-10 was eluted from the DEAE-cellulose column with 0.14M potassium phosphate and the fractions indicated with a braket were pooled. This fraction mainly contained TCBP-10, but still contained its degraded fragments.

Chromatography of this fraction on the hydroxylapatite column resulted in the separation of TCBP-10 from contami-

nation without potassium phosphate gradient (Fig. 23), presumably reflecting a difference between TCBP-10 and degraded fragments in affinity to calcium immobilized in the resin. TCBP-10 fractions indicated with a bracket in the Fig. 23 were proved to be homogeneous by SDS- and alkali-glycerol-polyacrylamide gel electrophoreses (Fig. 24), so that I used them for further analysis described later. Figure 24 also shows the SDS-polyacrylamide gel electrophoresis of proteins in each purification step (see Materials and Methods). By this purification procedure, TCBP-10 was purified to homogeneity and 3.6mg of TCBP-10 was recovered from about 100g of Tetrahymena cell body proteins. A representative purification is summarized in Table 2.

Physicochemical Properties of TCBP-10 ——— Molecular weight of TCBP-10 was determined by SDS-polyacrylamide gel electrophoresis and by Sephadex G-50 gel filtration. In the SDS-polyacrylamide gel electrophoresis, the molecular weight of TCBP-10 was estimated to be 10,000 (Fig. 25A). On the other hand, in gel filtration on Sephadex G-50, the peak of TCBP-10 was situated at a K_{av} corresponding to the molecular weight of 22,000, the value close to the molecular weight of 20,000 predicted if the native form of TCBP-10 was a dimer of the 10,000-Mr polypeptide (Fig. 25B). The presence of 1mM EGTA during gel filtration did not exert any significant influence on the elution

profile of TCBP-10 (data not shown).

Isoelectric point of TCBP-10 was estimated to be 4.5 in the agarose gel isoelectric focusing (Fig. 26b). The protein was considerably acidic, but had slightly basic isoelectric point as compared with Tetrahymena calmodulin (pI=4.2, Fig. 26a).

From the analysis of amino acid composition of TCBP-10, it was acceptable that the protein contained a large amount of acidic amino acid like other proteins belong to calmodulin family (Table 3). The protein was lacking in methionine like vitamine D-dependent Ca^{2+}-binding proteins found in mammalian tissues (73, 76). However, there had some clear differences between TCBP-10 and other proteins of calmodulin family.

Figure 27 shows the UV absorption spectrum of TCBP-10. The spectrum had a prominent peak at 282nm and two shoulders at 285nm and 273nm. The molar extinction coefficient of TCBP-10 based on the protein concentration determined by the method of Lowry et al. was calculated to be about 9,000. The value suggest that the protein contains a single tryptophane residue.

Finally, the affinity and binding capacity of TCBP-10 for Ca^{2+} were determined by equilibrium dialysis. Figure 28 is a Scatchard plot of the results. From the slope and the intercept of abscissa, the dissociation constant (Kd) and the number of Ca^{2+}-binding site were calculated

to be 2.7×10^{-5}M and 1.07, respectively.

V-4. Discussion

While I was studying a role of calmodulin in Ca^{2+}-dependent ciliary reversal, the occurrence of another Ca^{2+}-binding protein(s) in a cilium was suggested (65). In this section, I could demonstrate that a new Ca^{2+}-binding protein, TCBP-10, existed in a cilium and cell body of Tetrahymena. In addition, some fundamental properties of the protein purified to homogeneity were presented. The new protein showed a Ca^{2+}-dependent mobility shift on alkali-glycerol-polyacrylamide gel electrophoresis and was heat (95°C, 30 min) and TCA (10%) resistant, and was recovered in 60-90% ammonium sulfate fraction (Fig. 16 and 17). Although these properties were similar to those of calmodulin (16-18), the molecular weight of TCBP-10 (Mr=10,000) was much smaller than that of calmodulin (Mr=16,500, Fig. 18). Consequently, as a possibility that TCBP-10 is a degraded fragment of calmodulin has been considered formerly. However, the possibility was excluded by the following evidences: (i) TCBP-10 did not cross-react with anti-Tetrahymena calmodulin serum (Fig. 19), (ii) TCBP-10 was not cleaved by cyanogen bromide and did not co-migrate with any of cyanogen bromide fragments derived from calmodulin on alkali- urea-polyacrylamide gel electrophoresis (Fig. 20), (iii) none of proteolytic fragments of calmodulin showed the same

electrophoretic mobility as that of TCBP-10 (Fig. 21), (iv) UV absorption spectrum of TCBP-10 was quite different from that of calmodulin (Fig. 27), (v) TCBP-10 was lacking in methionine (Table 3), whereas Tetrahymena calmodulin is known to contain eight methionines in the molecule in a fashion that the molecular weight of maximum intra-molecular sequence devoid of methionine is about 3,900 (72).

In higher organisms, several species of Ca^{2+}-binding protein called calmodulin family, including troponin C, S-100 protein (PAP I-b), vitamin D-dependent Ca^{2+}-binding protein, parvalbumin and oncomodulin (77), have been reported (78). I believe it is reasonable to classify TCBP-10 into the calmodulin family from the standpoints described below. The molecular weight of TCBP-10 and its affinity to Ca^{2+} (Fig. 28) are comparable to those of some calmodulin family proteins. Moreover, the properties that TCBP-10 was considerably acidic (pI=4.5) and composed of a high content of aspartic and glutamic acids are discriminating characteristics common to calmodulin family.

Two minor forms of TCBP-10 (P63 and P76) were found in the preparation from cell bodies and were proved to be proteolytic fragments of TCBP-10 (Fig. 21). Fullmer and Wasserman reported that the two of three Ca^{2+}-binding proteins, which possesed nearly the same molecular weights, from bovine intestinal mucosa were proteolytic fragments

of another one which was identified later to be a vitamin D-dependent Ca^{2+}-binding protein, and they also noted similar states of such proteins from chick and guinea pig (79-81). There seems to be the similar protease sensitivity between TCBP-10 and vitamin D-dependent Ca^{2+}-binding protein, presumably reflecting the similar primery and ternaly structures of the proteins. Besides the similar protease-sensitivity, it is noteworthy that both proteins lack methionine commonly (Table 3).

The molecular weight of TCBP-10 was estimated to be 10,000 by the SDS-polyacrylamide gel electrophoresis (Fig. 25A) and the molecular weight calculated from amino acid composition was 10,838 (Table 3). However, the molecular weight estimated by the gel filtration in non-denaturing condition was 22,000 (Fig. 25B). It is most likely that native molecule exists in a dimer form. These figures of the molecular weight and the tendency to form dimer molecules were similar to those reported for S-100 protein (PAP I-b) by Isobe *et al.* (74). They determined the monomer molecular weight as 10,507 by amino acid sequencing and dimer molecular weight as 20,000 by gel filtration for PAP I-b protein.

The number of Ca^{2+}-binding site and the dissociation constant (Kd) of TCBP-10 for Ca^{2+} were determined by equilibrium dialysis to be 1 and 27μM, respectively (Fig. 28). Although these values are unique and somewhat under-

estimated as compared with those of calmodulin family molecules (16, 82, 83), it seems unlikely that the protein is damaged to reduce the Ca^{2+}-affinity during the purification processes, because the purified pritein showed the same extent of Ca^{2+}-dependent mobility shift as that observed in the starting material for purification. Therefore, I believe that the values reflect the intrinsic nature.

After all, TCBP-10 seems to bear some properties similar to those of different Ca^{2+}-binding proteins such as vitamin D-dependent Ca^{2+}-binding protein and PAP I-b rather than that of calmodulin. In genealogic study of Ca^{2+}-binding proteins, it is reported that calmodulin and troponin C situated on a branch of genealogical tree different from that of vitamin D-dependent Ca^{2+}-binding protein and PAP I-b (78). Therefore, as well as in higher animals, Tetrahymena cell contains both of two different groups of Ca^{2+}-binding proteins, that is calmodulin and TCBP-10 (vitamin D-dependent Ca^{2+}-binding protein- or PAP I-b-like protein). Considering that both vitamin D-dependent Ca^{2+}-binding protein and PAP I-b protein have so far been found only in mammalian cells and tissues, an analysis of amino acid sequence of TCBP-10 will be very much informative for the studies on the evolution of calmodulin family molecules.

The function of TCBP-10 in the organism, especially in Ca^{2+}-dependent ciliary reversal, has not yet been

elucidated. However, considering that the S-100 protein is known to be specific for nervous system (84) and the vitamin D-dependent Ca^{2+}-binding protein is recently shown to exist in excitatory cells in brain (85), I can assume that the TCBP-10 plays a role similar to that of S-100 or vitamin D-dependent Ca^{2+}-binding protein in Tetrahymena cell, a unicellular excitatory organism. In preliminary experiments, partially purified TCBP-10 failed to activate several enzymes, such as adenylate cyclase, guanylate cyclase and phosphodiesterase of Tetrahymena and porcine brain phosphodiesterase (86). However, the fact that a considerable amount of TCBP-10 is present in cilium strongly suggests the involvement of this protein in Ca^{2+}-dependent phenomena of cilium, such as membrane excitation and ciliary reversal. It is well-known that Ba^{2+} is able to introduce the intermittent ciliary reversal, called Ba^{2+}-dance, on intact Paramecium or Tetrahymena cells when added in an extra-cellular medium (87), and to introduce the continuous ciliary reversal on Triton-extracted model of these cells when added in a reactivation medium (Section 3). These effects of Ba^{2+} may be interpreted as its attack on Ca^{2+}-channels on ciliary membrane and on Ca^{2+}-sensitive axonemal machinery which is controlling and modifying a ciliary beat direction. My preliminary results suggested a high affinity of TCBP-10 for Ba^{2+}, in that Ba^{2+}as well as Ca^{2+} could induced obvious mobility

shift of TCBP-10 on alkali-glycerol-polyacrylamide gel electrophoresis (88). On the other hand, a relatively slight mobility shift of calmodulin by Ba^{2+} has been reported by Suzuki *et al.* (17). Thus, the function of TCBP-10 on ciliary movement is now under the extensive investigation.

V-5. Summary

A new Ca^{2+}-binding protein occurring in the cilium and cell body of Tetrahymena was found out. This protein, designated as TCBP-10, was purified to homogeneity and its basic properties were investigated. TCBP-10 is a polypeptide which bears the following characteristics: 1) Its molecular weight is 10,000; 2) The protein is resistant to heat and trichloroacetic acid; 3) The protein is recovered with 60-90% saturated ammonium sulfate; 4) The protein shows Ca^{2+}-dependent mobility shift on alkali-glycerol-polyacrylamide gel electrophoresis; 5) The protein tends to form dimer molecule in native condition; 6) Its isoelectric point is 4.5; 7) Its dissociation constant and the number of binding site for Ca^{2+} is 27μM and 1, respectively; 8) Its amino acid composition is similar to that of calmodulin family protein, especially to vitamin D-dependent Ca^{2+}-binding protein and S-100 protein (PAP I-b). Since the considerable amount of TCBP-10 existed in a cilium, its involvement in Ca^{2+}-regulation of ciliary movement besides calmodulin is expected.

VI. General Conclusion

For the purpose of elucidating the mechanism by which Ca^{2+} regulates cell motility, I focused my study on the phenomenon of "ciliary reversal" in Tetrahymena as a representative Ca^{2+}-dependent phenomenon in tubulin-dynein-mediated cell motility. The new findings and notions revealed in the course of this study are enumerated below.

(1) In order to obtain a direct evidence showing the presence of calmodulin in Tetrahymena cilia, I isolated calmodulin from the cilia. Calmodulin from the cilia had the same characteristics as that of calmodulin from the cell bodies in terms of molecular weight, Ca^{2+}-dependent mobility shift on alkali-glycerol-polyacrylamide gel electrophoresis, antigenicity, and Ca^{2+}-dependent activation of brain phosphodiesterase.

(2) Alkali-glycerol-polyacrylamide gel electrophoresis revealed that calmodulin in the cilium was localized on the membrane-matrix fraction and outer-doublet microtubule fraction. Moreover, the presence of calmodulin-binding protein was shown in the outer-doublet microtubule fraction.

(3) Immunoelectron microscopic observations using anti-calmodulin antibody revealed that Ca^{2+}-dependent calmodulin-binding subciliary structure is the interdoublet links, which connect each neighbouring pair of outer-doublet microtubules. From the evidence, I presented a new possible

mechanism that Ca^{2+}-dependent ciliary reversal is brought about by the change in elasticity of the interdoublet links through its interaction with Ca^{2+}-calmodulin complex.

(4) In order to further investigate the role of calmodulin with a Triton-extracted model, I used Triton-extracted Paramecium model which was able to be prepared much easier than Tetrahymena model. As a prerequisite for using a Paramecium model, I isolated calmodulin from Paramecium for the first time and confirmed that the properties of calmodulins from both ciliates were the same with each other.

(5) Ca^{2+}-induced ciliary reversal of Triton-extracted Paramecium model was not affected at all by the addition of calmodulin inhibitors, such as trifluoperazine and chlorpromazine. From the result, I predicted that a second Ca^{2+}-binding protein might be exist in a cilium.

(6) As I predicted, I could find out such a second Ca^{2+}-binding protein in Tetrahymena cilium, using alkali-glycerol-polyacrylamide gel electrophoresis. The protein was designated as TCBP-10.

(7) I succeeded in isolating TCBP-10. TCBP-10 was apperently different from calmodulin in terms of antigenicity and the pattern of cyanogen bromide fragments. Its molecular weight and isoelectric point were determined to be 10,000 and 4.5, respectively. Gel filtration analysis

suggested that under native conditions TCBP-10 was present in a dimer form.

(8) The dissociation constant and the number of binding site of TCBP-10 for Ca^{2+} was determined to be 27μM and 1, respectively.

(9) Amino acid analysis of TCBP-10 revealed that the protein was rich in aspartic acid and glutamic acid like other proteins of calmodulin family but lacking in methionine unlike calmodulin and troponin C.

(10) TCBP-10 resembled to mammalian brain S-100 protein (PAP I-b) and vitamin D-dependent Ca^{2+}-binding proteins which have hitherto been found only in avian and mammalian tissues in terms of the molecular weight and Ca^{2+}-binding capacity, and amino acid composition.

(11) In the research field of ciliary or flagellar movement, I showed for the first time the fact that a second Ca^{2+}-binding protein like TCBP-10 coexists with calmodulin in cilium.

(12) A Ca^{2+}-binding protein like TCBP-10 has not so far been reported in lower eukaryotes. Hence, the detailed information on the structure and function of TCBP-10 will provide an important clue for elucidating the evolutional and functional relations among the proteins of calmodulin family.

(13) Concerning the mechanism of Ca^{2+}-dependent ciliary reversal, I expect that , in addition to the possible effect

of calmodulin on the function of interdoublet links (see (3)), TCBP-10 may play an important cooperative role with calmodulin. Scrutiny of cooperative role of TCBP-10 will uncover the essential mechanism of Ca^{2+}-reguration of ciliary movement.

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Table 1

Effects of Calmodulin Inhibitors on the Direction of Ciliary Movement of Triton Model of *Paramecium*

Preincubation		Reactivation		Swimming[b]
Ca^{2+}	TFP(CPZ)[a]	Ca^{2+}	TFP(CPZ)	
		-	-	F
		+	-	B
		+	+	B
-	+	-	+	F
-	+	+	+	B
+	+	-	+	F[c]
+	+	+	+	B[c]
		- → +	-	F → B
		- → +	+	F → B
		+ → -	-	B → F
		+ → -	+	B → F

a; Trifluoperazine (chlorpromazine)
b; F, forward swimming; B, Backward swimming
c: Swimming velocity was markedly reduced.

Table 2

Purification and Recovery of TCBP-10 from Tetrahymena Cell Body

	total vol.	Protein concentration	Total protein	Total TCBP-10	Yield	Purification
	(ml)	(mg/ml)*	(mg)	(mg)**	(%)	(fold)
Deciliated cell body	1800	54.6	98280	(1191.0)	(100)	(1)
Step 1; Heat treatment	2124	4.6	9770	1191.0	100	10.06
Step 2; $(NH_4)_2SO_4$ fractionation	360	5.01	1728	278.2	23.4	13.28
Step 3; DEAE-cellulose	15.5	0.61	9.5	6.9	0.6	59.90
Step 4; Hydroxylapatite	36.0	0.10	3.6	3.6	0.3	82.52

* The amount of protein was determined by the method of Lowry et al. (49).

** The amount of TCBP-10 in deciliated cell body is assumed to be equal to the amount recovered in heat-treatment extract. Values are calculated from densitometric analysis.

Table 3

Amino Acid Composition of TCBP-10 and Other Ca^{2+}-binding Proteins

	*1 TCBP-10	*2 Tetrahymena calmodulin	Vitamin D *3 -dependent CaBP	S 100 *4 protein (PAP I-b)	Parvalbumin *5
	(residures/mol)				
Asx	18	23	6	9	12
Thr	6	11	2	3	5
Ser	7	4	6	5	5
Glx	23	25	17	19	13
Pro	6	2	4	0	1
Gly	6	11	5	4	9
Ala	7	11	2	5	11
Cys	N.D.	0	0	2	0
Val	4	6	3	6	5
Met	0	8	0	3	3
Ile	4	9	2	4	6
Leu	4	12	12	8	9
Tyr	1	1	1	1	0
Phe	1	8	5	7	9
His	1	2	0	5	2
Lys	8	7	10	8	16
Arg	1	6	0	1	1
Trp	(1) *6	0	0	0	0

*1: Values for TCBP-10 correspond to a molecular weight of 10,000

*2: From Yazawa. M, et al.,(72)

*3: From Fullmer, C. S. and Wasserman, R. H., (73)

*4: From Isobe, T., et al., (74)

*5: From Lehky, P., et al., (75)

*6: Value was determined spectrophotometrically.

X. Figure Legends

Fig. 1; An illustration of representative ultrastructures of cilium. (A) Cross-sectional view. (B) Longitudinal view (a part contains two outer-doublet microtubules and central pair microtubules are illustrated).

Fig. 2; Schematic display of a accepted basic mechanism of ciliary beating. A sliding of each neighbouring pair of outer-doublet microtubules by dynein arms is converted to bending by a shear-resistance which works to fix corresponding points on outer-doublets (A and B) against sliding. The complicated undulation of a ciliary movement is achieved by a delicate cooperativity of these two basic forces. It is likely that the Ca^{2+} affects either or both of these forces to modify the pattern of ciliary beat. As a candidates for the ciliary ultrastructure responsible for the shear-resistance, basal body, spoke, arm, and interdoublet link have been considered.

Fig. 3; Co-migration of calmodulin in SDS-polyacrylamide gel electrophoresis. (a) Tetrahymena ciliary calmodulin, (b) a mixture of calmodulins from whole cells and cilia, (c) Tetrahymena whole-cell calmodulin, (d) a mixture of molecular weight markers, ovalbumin (43,000), chymotrypsinogen A (25,000) and ribonuclease A (13,700). Electro-

phoresis was performed in 12.5% polyacrylamide gel including 1mM EGTA in running gel, electrode buffer and sample buffer.

Fig. 4; Ca^{2+}-dependent electrophoretic mobility shift of calmodulin in 10% alkali-glycerol-polyacrylamide gel. (a) Tetrahymena calmodulin from whole cells, (b) extract from whole cilia, (c) Tetrahymena calmodulin from cilia. Samples were solubilized in 8M urea and electrophoresed in the presence of 2mM Ca^{2+}(+) or 2mM EGTA (-), respectively.

Fig. 5; Ouchterlony immunodiffusion tests with anti-Tetrahymena calmodulin serum. (a) Anti-Tetrahymena calmodulin serum, (b) 10μg of Tetrahymena cell body calmodulin, (c) 10μg of Tetrahymena ciliary calmodulin, (d) 1.6mg of ciliary lysate (whole cilia were sonicated in 10mM Tris-HCl (pH 7.5), containing 250mM sucrose, 1mM dithiothreitol and 0.5mM EGTA), (e) 10μg of Tetrahymena whole cell calmodulin. Note that no spur formation was seen.

Fig. 6; Activation of porcine brain cAMP-phosphodiesterase by Tetrahymena calmodulins. □, Tetrahymena cell body calmodulin; ○, Tetrahymena ciliary calmodulin. Reactions were performed in the presence of 1mM Ca^{2+} (filled symbols) or 1mM EGTA (open symbols). Each value represents the mean of triplicate experiments.

Fig. 7; Two-dimensional gel electrophoresis of extract of whole cilia. 8M urea extract of cilia was electrophoresed in the presence of 2mM Ca^{2+} and 2mM EGTA, as described in Fig. 4b. The two lanes were loaded separately on the second-dimensional gel and subjected to electrophoresis in the presence of 5mM EGTA. Two-dimensional gel electrophoretic patterns (a, b) represent those of cilia extract first electrophoresed in the presence of 2mM Ca^{2+} and 2mM EGTA, respectively.

Fig. 8; Alkali-glycerol gel electrophoresis of subciliary fractions. (a) Whole cilia fraction, (b) membrane-matrix fraction, (c) membrane-matrix fraction (double the quantity of (b)), (d) axoneme fraction, (e) crude dynein fraction, (f) crude dynein fraction plus exogenous Tetrahymena calmodulin, (g) outer-doublet microtubule fraction. For fractionation of cilia, see Materials and Methods. Volumes of these fractions were adjusted equally except (c). Each fraction was solubilized in 8M urea and electrophoresed in the presence of 2mM Ca^{2+} (lane +) and 2mM EGTA (-).

Fig. 9; Alkali-glycerol gel electrophoresis of the mixture of calmodulin and purified dyneins. Crude dynein fraction was subjected to 5-20% continuous sucrose density gradient centrifugation (A). The peaks indicated as (a), (b) and (c) were used as purified 6S tubulin, 14S dynein and

30S dynein fractions. (B) and (C), Alkali-glycerol gel electrophoresis of 30S dynein (B) and 14S dynein (C) fractions with various amount of exogenous calmodulin. The protein amount of dyneins are 170μg/lane and the amount of calmodulin is 20μg (a), 10μg (b), 5μg (c) and 1μg (d). (D) The mixture of 6S tubulin (100μg/lane) and calmodulin (10μg/lane).

Fig. 10; Ca^{2+}-dependent binding of calmodulin to outer-doublet microtubule fraction. 1.43mg of outer-doublet microtubule fraction and 25μg of exogenous Tetrahymena calmodulin were mixed and incubated at 18°C for 2 min in 1mM Ca^{2+} or 2mM EGTA. The suspensions were centrifuged at 15,000 g and the resulting pellets were washed twice with respective incubation buffers. The supernatants and pellets were solubilized in 8M urea and subjected to alkali-glycerol gel electrophoresis in the presence of 5mM EGTA. Electrophoretic patterns shown are of supernatants (a) and of pellets (b). (1) and (2) represent the test samples incubated with Ca^{2+} and EGTA, respectively.

Fig. 11; Thin-section (A) and negative-stain (B) electron micrographs of outer-doublet microtubule fraction. Arrows indicate periodic structures on doublet microtubules. Bar, 0.2μm.

Fig. 12; Indirect immunoelectron micrographs. Outer-doublet microtubule fraction (1.6mg protein) was incubated with exogenous Tetrahymena calmodulin (10μg) in a Tris-Mg-NaCl solution including either 1mM Ca^{2+} (A) or 1mM EGTA (C). Each sample was washed with respective buffers and incubated with anti-calmodulin antibody and ferritin-conjugated anti-rabbit IgG antibody as described in Materials and Methods. Arrows indicate periodic structures which are connecting neighbouring doublet microtubules to each other. In (A) ferritin particles were concentrated on the structures. A similar tendency was shown in the sample with no exogenous Tetrahymena calmodulin (data not shown). (B) and (D) represent another series of experiments. In (B) the sample was treated as in (A) but a disconnected microtubule region is shown. In (D) the sample was treated as in (A), except that normal rabbit IgG was used in place of anti-calmodulin antibody. Bar, 0.2μm.

Fig. 13; (A) Schematic display of possible mechanism by which Ca^{2+} inpoured into cilium modulates the pattern of ciliary beat. (B) Schematic display of the effects of two groups of organic solvents and trypsin on Ca^{2+}-induced conversion of flagellar beat pattern. For detailed explanation, see text.

Fig. 14; Alkali-glycerol-polyacrylamide gel electro-

phoresis of Paramecium calmodulin and Tetrahymena calmodulin. Paramecium calmodulin (a, 10μg) and Tetrahymena calmodulin (b, 20μg) were electrophoresed in the presence of 1mM Ca^{2+} or 1mM EGTA.

Fig. 15; Activation of porcine brain cAMP-phosphodiesterase by Tetrahymena calmodulin and Paramecium calmodulin. ○ , Tetrahymena calmodulin; □ , Paramecium calmodulin. Reactions were performed in the presence of 1mM Ca^{2+} (filled symbols) or 1mM EGTA (open symbols).

Fig. 16; Alkali-glycerol-polyacrylamide gel electrophoresis of the ammonium sulfate fractions of ciliary proteins. Supernatant fraction of cilia homogenate was fractionated by ammonium sulfate as described in Materials and Methods. (a) 0-40%, (b) 40-60%, (c) 60-80% and (d) 80%-TCA fractions.

Fig. 17; Alkali-glycerol-polyacrylamide gel electrophoresis of heat-resistant ciliary protein fraction. Heat-resistant ciliary protein fraction was obtained from cilia homogenate by the method described in Materials and Methods.

Fig. 18; Electrophoreses of heat-resistant cell body proteins and partially purified Ca^{2+}-binding proteins.

(A) and (B), Alkali-glycerol gel electrophoresis of heat-resistant ciliary proteins (A) and heat-resistant cell body proteins (B). (C) and (D), Alkali-glycerol gel electrophoresis (C) and SDS gel electrophoresis (D) of Ca^{2+}-binding proteins partially purified by preparative electrophoresis; (a) calmodulin, (b) P51, (c) P63 and (d) P76. Arrows indicate the main polypeptide bands of P51, P63 and P76 fractions. For the preparation of these fractions, see Materials and Methods.

Fig. 19; Ouchterlony immunodiffusion test with an anti-Tetrahymena calmodulin serum. (a) Anti-Tetrahymena calmodulin serum, (b), (d) and (f) Tetrahymena calmodulin (10μg), (c) P-51 fraction (10μg), (e) P-63 fraction (10μg), (g) P76 fraction (10μg).

Fig. 20; Alkali-urea-polyacrylamide gel electrophoresis of cyanogen bromide-fragments of calmodulin, P51 and P63. Proteins were digested with cyanogen bromide as described in Materials and Methods. (a), (b) and (c) are original samples of calmodulin, P51 fraction and P63 fraction, respectively. (d), (e) and (f) are cyanogen bromide-digested samples of calmodulin, P51 fraction and P63 fraction, respectively. For each lane, 200μg of proteins are loaded.

Fig. 21; Digestion of calmodulin and P51 with crude protease fraction. 200μg of calmodulin and P51 were re-suspended with 200μl of 10mM Tris-HCl buffer (pH 7.0) containing 1mM Ca^{2+}, and crude protease fraction of 30μl and 10μl were added to the calmodulin containing fraction and P51 containing suspension, respectively. Incubation was performed at 20°C and the aliquotes of 60μl were withdrawn at appropriate time. The reaction was stopped by heating the aliquotes at 95°C for 5 min. Heat-resisant proteins of each aliquot were dialysed against 8M urea and subjected to alkali-glycerol gel electrophoresis. Electrophoretic patterns shown are time course of calmodulin (A) and P51 (B) digestion; (a) 0 min, (b) 30 min, (c) 120 min. (D), heat-resistant cell body proteins. The crude protease fraction used was supernatant of Tetrahymena cell lysate which was obtained by the homogenization of cells with a equal volume of 10mM Tris-HCl buffer (pH 7.0).

Fig. 22; DEAE-cellulose chromatography of the sample from purification step 2. Sample from purification step 2 (ammonium sulfate fraction of Tetrahymena heat-resistant proteins, see Materials and Methods) was dialysed against 0.02M potassium phosphate buffer (pH 7.0) containing 6M urea and 1mM beta-mercaptoethanol, and loaded on DEAE-cellulose column (2.7 x 45cm) pre-equilibrated with the same buffer. The column was eluted with a linear gradient

of 0.02-0.5M potassium phosphate buffer (pH 7.0) containing 6M urea and 1mM beta-mercaptoethanol. (a), (b) and (c) represent the alkali-glycerol gel electrophoresis of the fraction 95, 100 and 131, respectively. The fractions indicated with bracket a were pooled and were rendered next purification step. The fractions indicated with bracket c were pooled and used as pure calmodulin fraction (see Fig. 24A, lane 7).

Fig. 23; Hydroxylapatite chromatography of the sample from purification step 3. The sample from purification step 3 (DEAE-cellulose chromatography fraction, see Fig. 22) was dialysed against 0.02M potassium phosphate buffer (pH 6.0) containing 6M urea and 1mM beta-mercaptoethanol, and loaded on hydroxylapatite column (0.9 x 10cm) pre-equilibrated with the same buffer. Two peakes were eluted from the column without increasing the potassium phosphate concentration. (a) and (b) represent the alkali-glycerol gel electrophoresis of the fraction 5 and 10, respectively. The fractions indicated with bracket b were pooled and used as TCBP-10 fraction.

Fig. 24; SDS-polyacrylamide gel electrophoresis of the fractions of each purification steps (A) and alkali-glycerol polyacrylamide gel electrophoresis of purified proteins(B). (A); (a) and (h), molecular weight markers

(chymotrypsinogen A (25K), ribonuclease A (13.7K) and cytochrome C (11.3K)), (b) starting materials (total cell body proteins), (c) fraction from purification step 1 (heat-treatment), (d) step 2 fraction (ammonium sulfate fractionation), (e) step 3 fraction (DEAE-cellulose chromatography), (f) step 4 fraction (hydroxylapatite chromatography), (g) calmodulin fraction of DEAE-cellulose chromatography. (B); (a) purified calmodulin (the same sample as A(e)), (b) purified TCBP-10 (the same sample as A(g)).

Fig. 25; Molecular weight determination of TCBP-10 with SDS-polyacrylamide gel electrophoresis (A) and Sephadex G-50 gel filtration (B). (A) SDS gel electrophoresis were performed by the method of Laemmli. (B) Gel filtration on a Sephadex G-50 column (2.6 x 95cm) equilibrated with 50mM sodium bicarbonate in the presence or absence of 1mM EGTA. Molecular weight markers used were chymotrypsinogen A (25K), ribonuclease A (13.5K), cytochrome C (11.3K) and Tetrahymena calmodulin (16.5K).

Fig. 26; Isoelectric point of TCBP-10 and Tetrahymena calmodulin. Tetrahymena calmodulin (a, arrow) and TCBP-10 (b, arrow) were subjected to isoelectric focusing in the presence of 7M urea. As a markers for pH, acetylated cytochrome C were used. For the detail, see Materials and Methods.

Fig. 27; UV absorption spectra of TCBP-10 (A) and Tetrahymena calmodulin (B). The spectra were recorded in 0.1M imidazole-HCl buffer (pH 7.0) containing 1mM EGTA, using protein concentrations of 0.1mg/ml.

Fig. 28; Ca^{2+}-binding by TCBP-10. The data shown are from equilibrium dialysis in 50mM Tris-HCl (pH 7.5) containing 0.1M KCl as described in Materials and Methods.

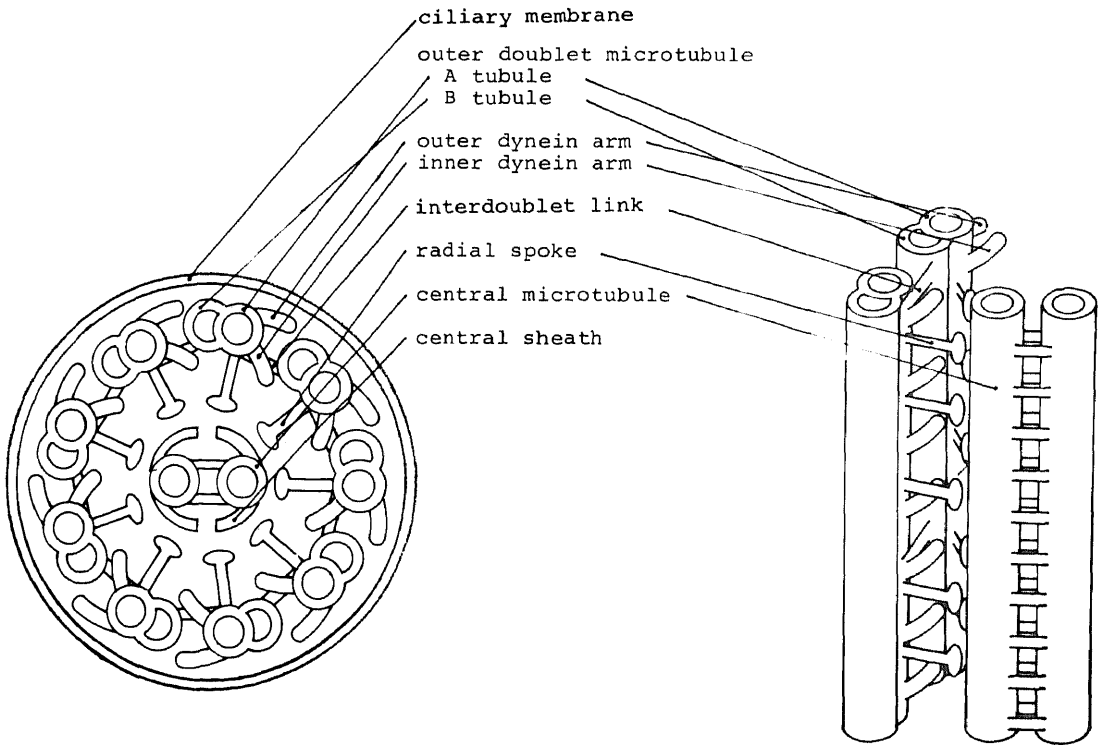

ciliary membrane
outer doublet microtubule
A tubule
B tubule
outer dynein arm
inner dynein arm
interdoublet link
radial spoke
central microtubule
central sheath

Fig. 1

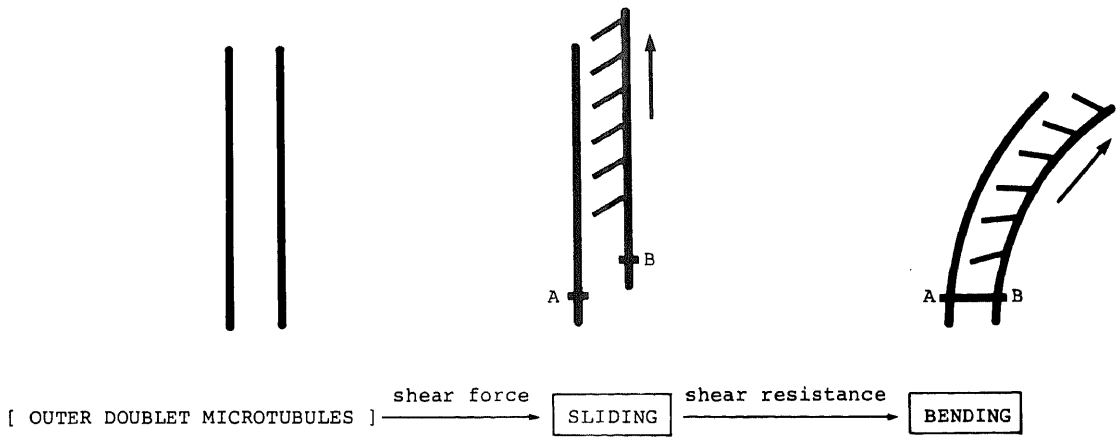

B
A
A
B
[OUTER DOUBLET MICROTUBULES]
shear force
SLIDING
shear resistance
BENDING

Fig. 2

Fig. 3

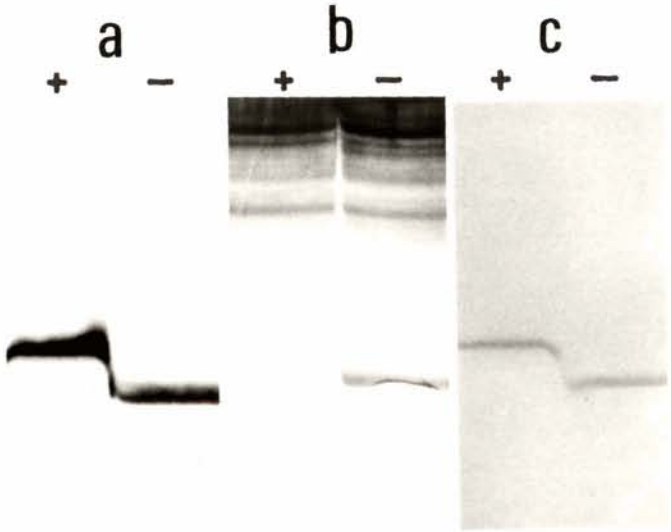


Fig. 4

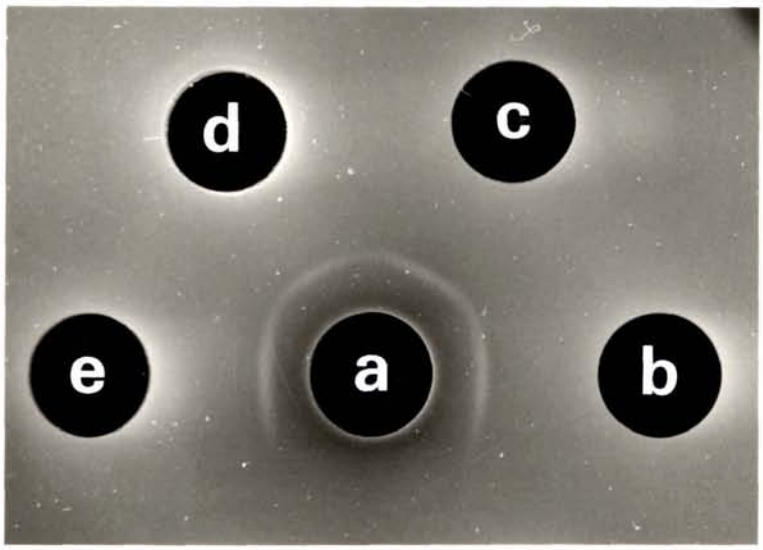


Fig. 5

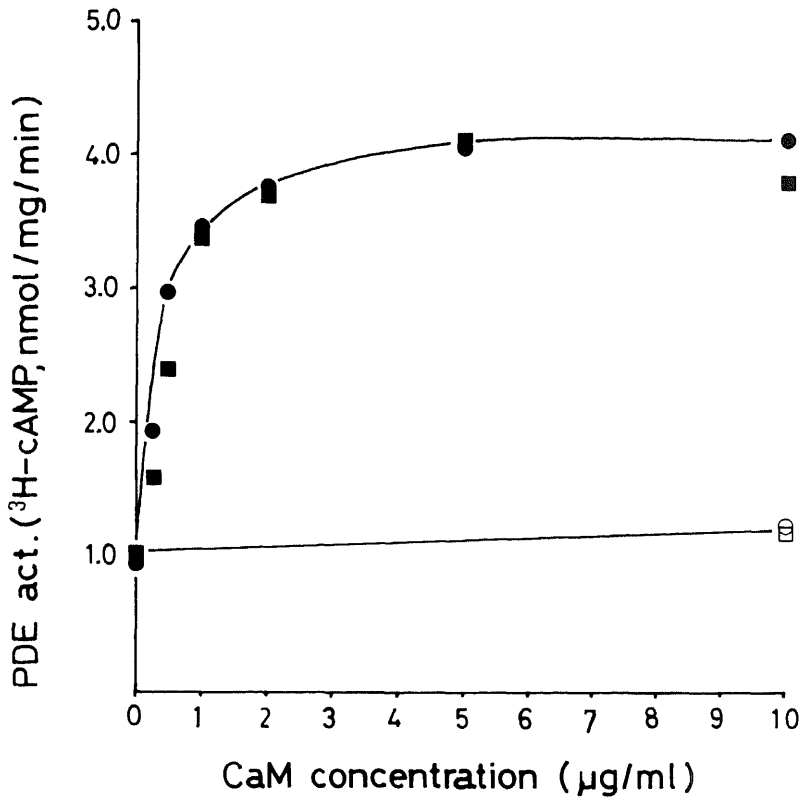

5.0
4.0
3.0
2.0
1.0
PDE act. (^{3}H-cAMP, nmol/mg/min)
0
1
2
3
4
5
6
7
8
9
10
CaM concentration (µg/ml)

Fig. 6

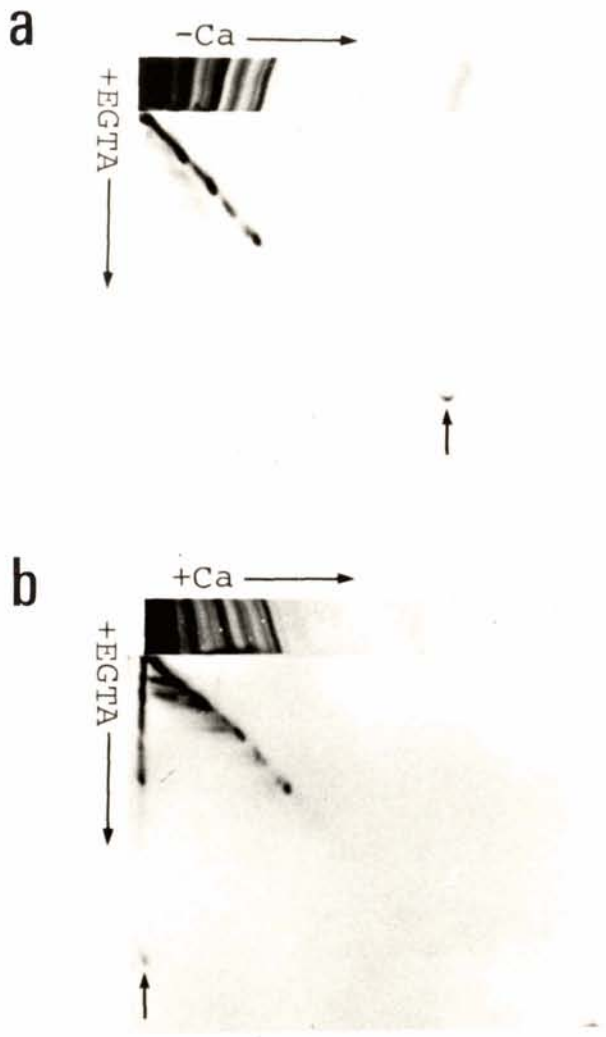


Fig. 7

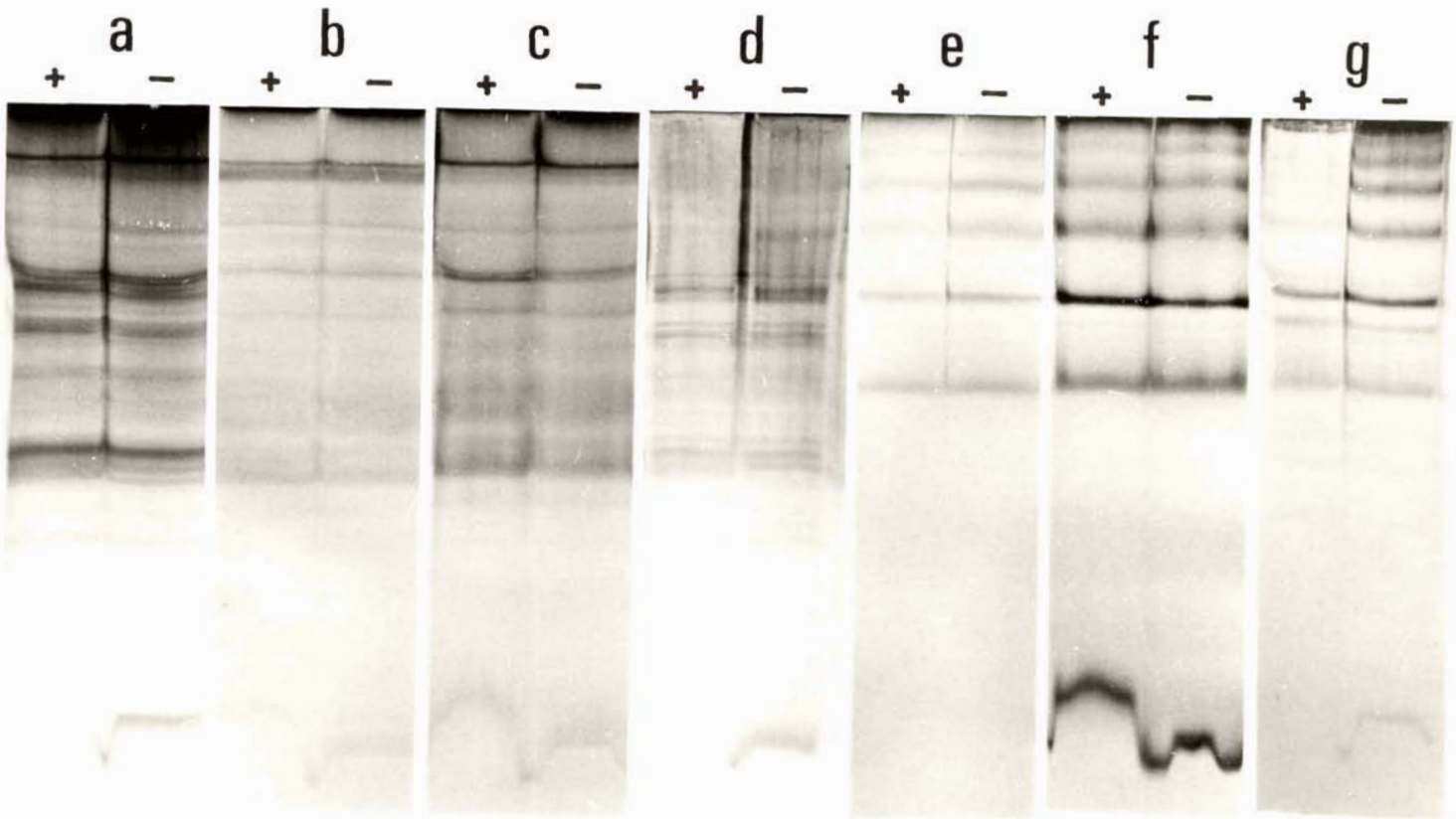
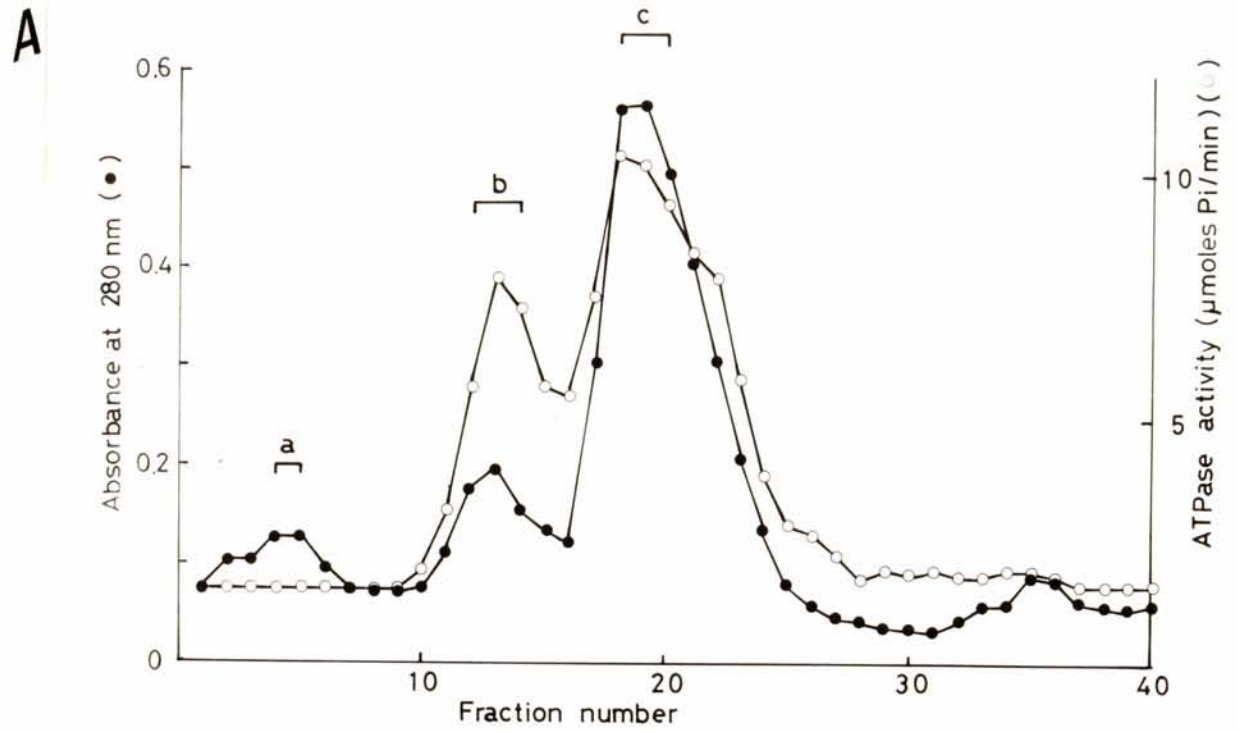
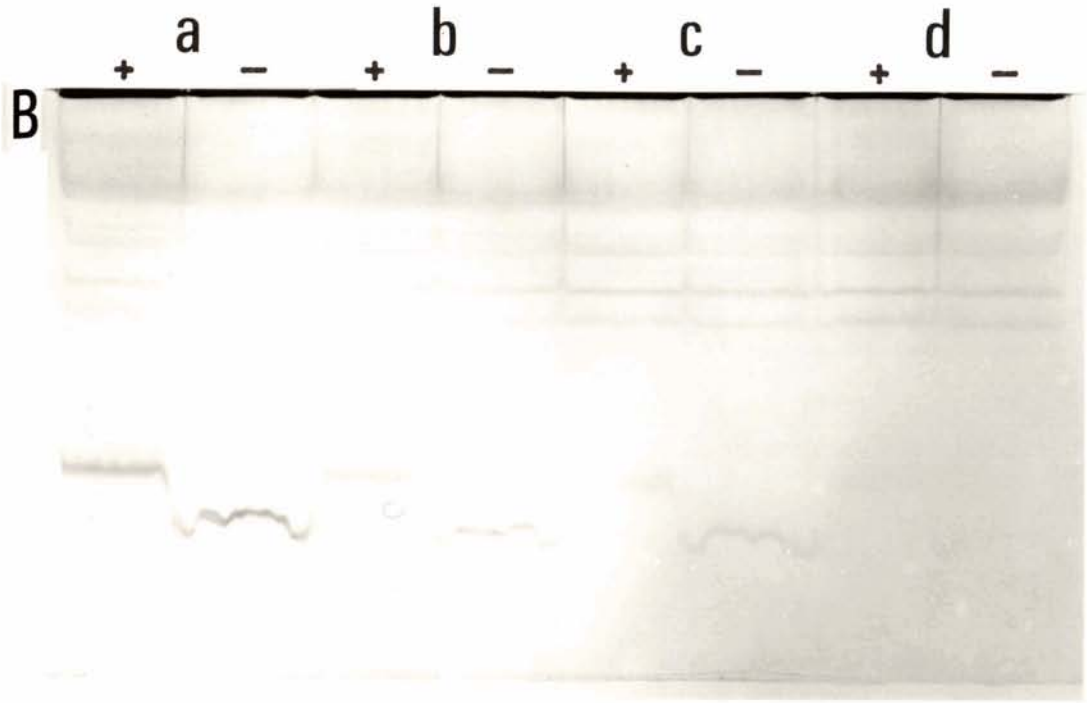
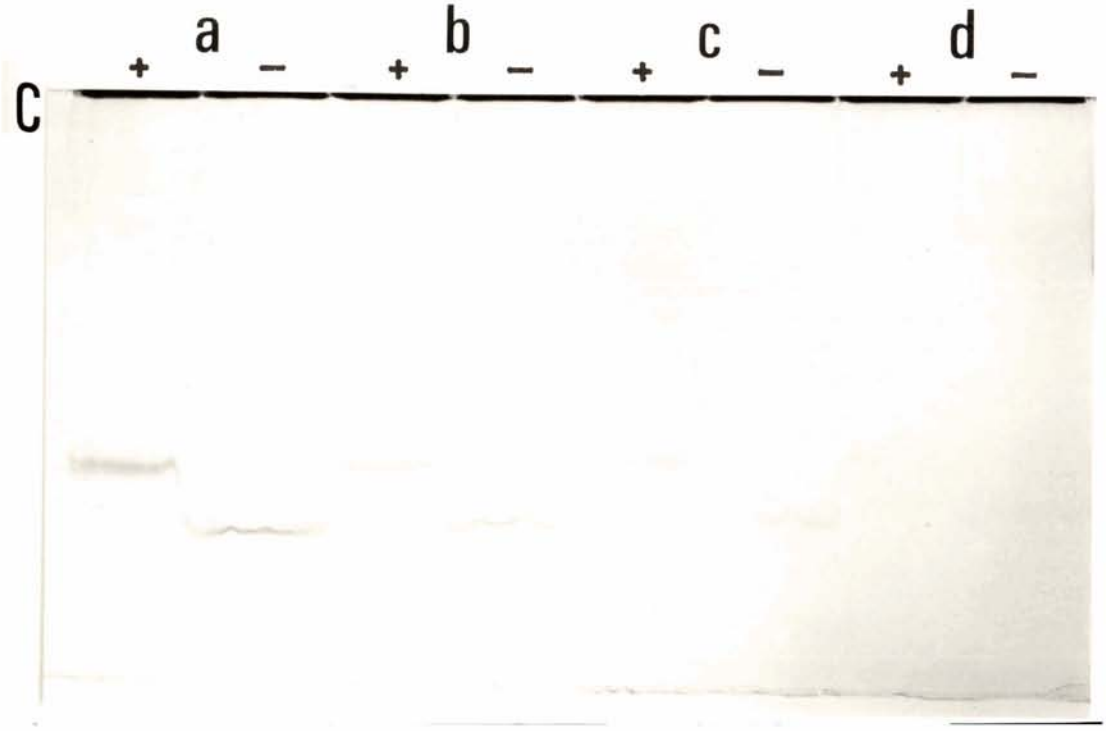


Fig. 8


A
Absorbance at 280 nm (•)
ATPase activity (μmoles Pi/min) (○)
Fraction number
a
b
c
0
0.2
0.4
0.6
5
10
10
20
30
40


B
a
b
c
d
+
−


C
a
b
c
d
+
−


D
+
−

Fig. 9

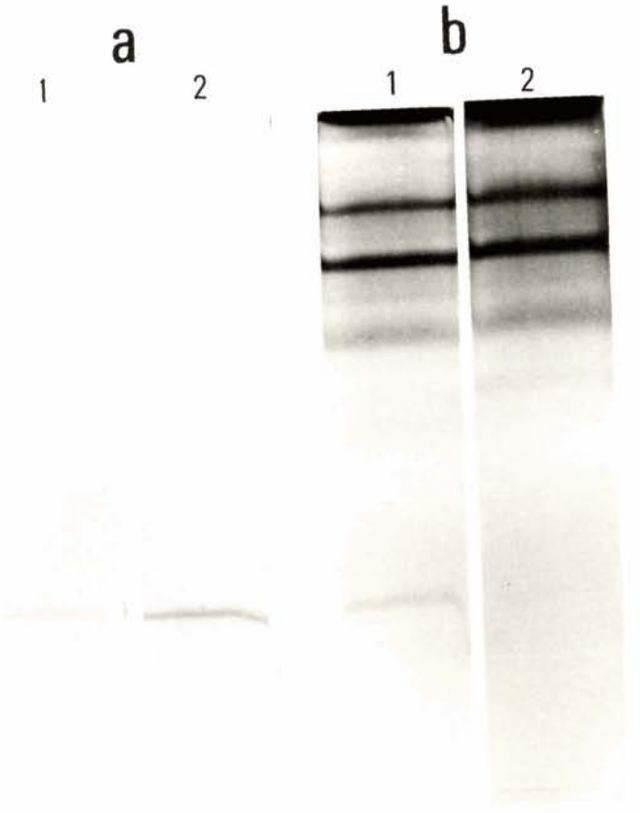


Fig. 10

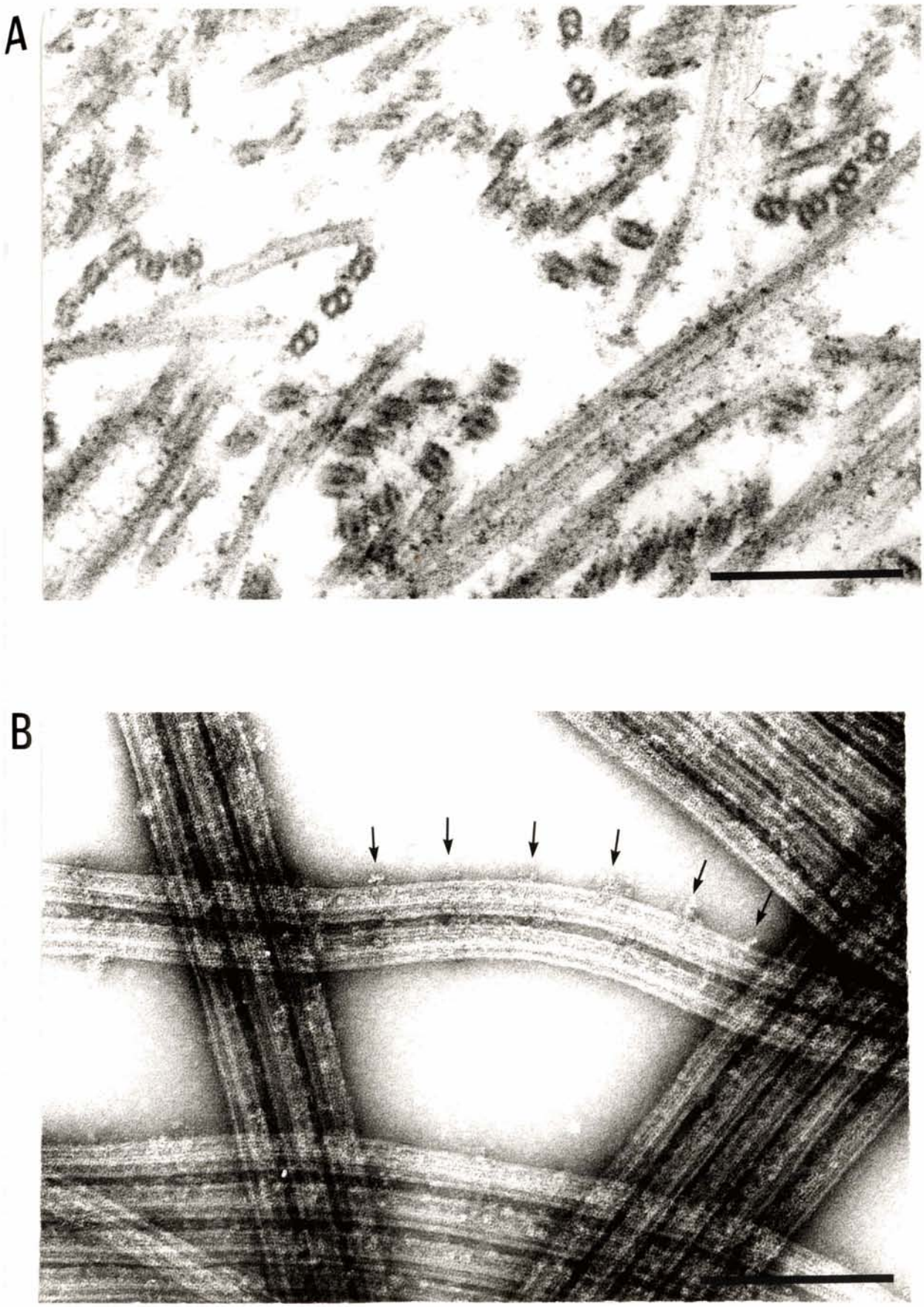


Fig. 11

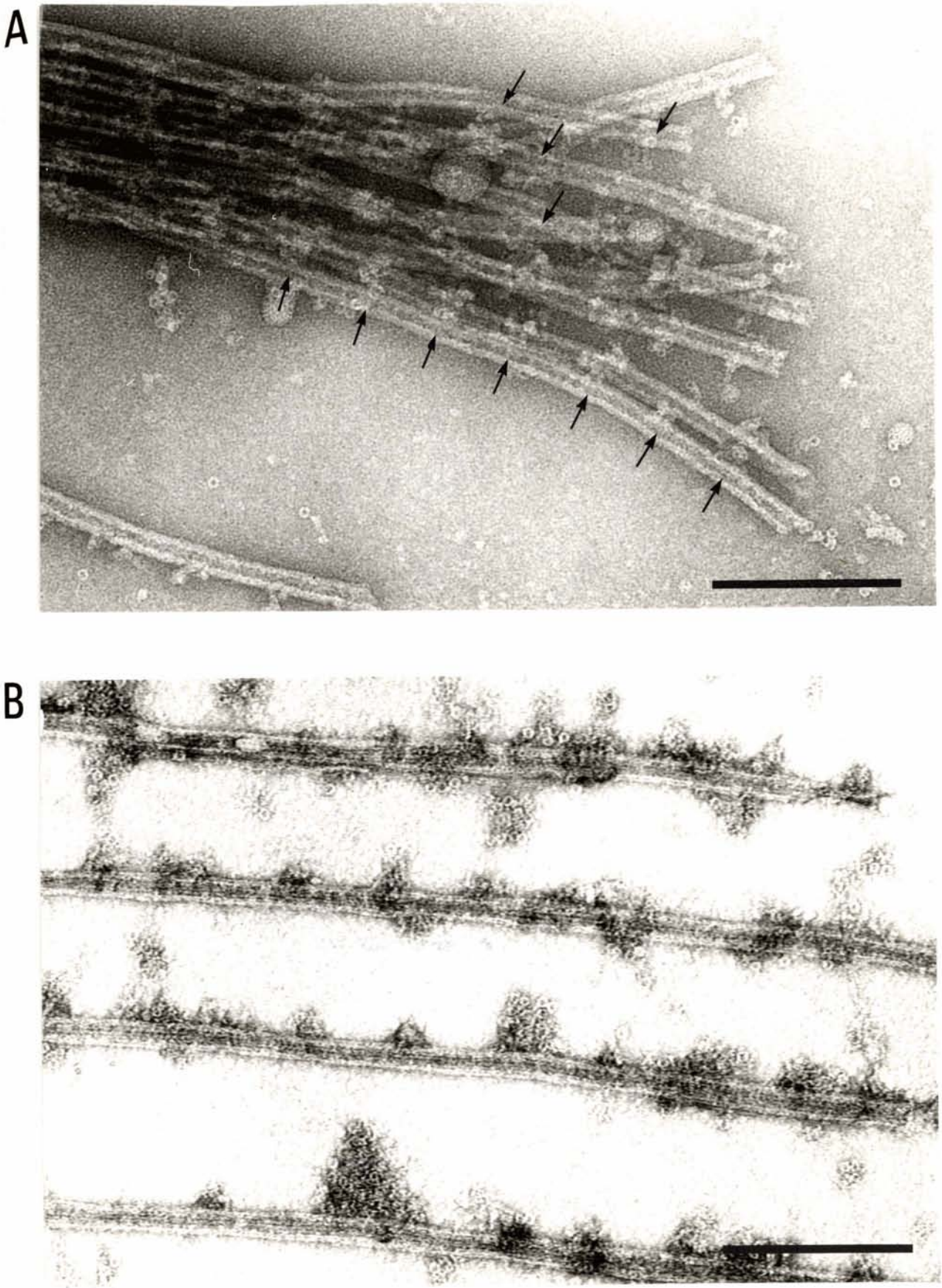
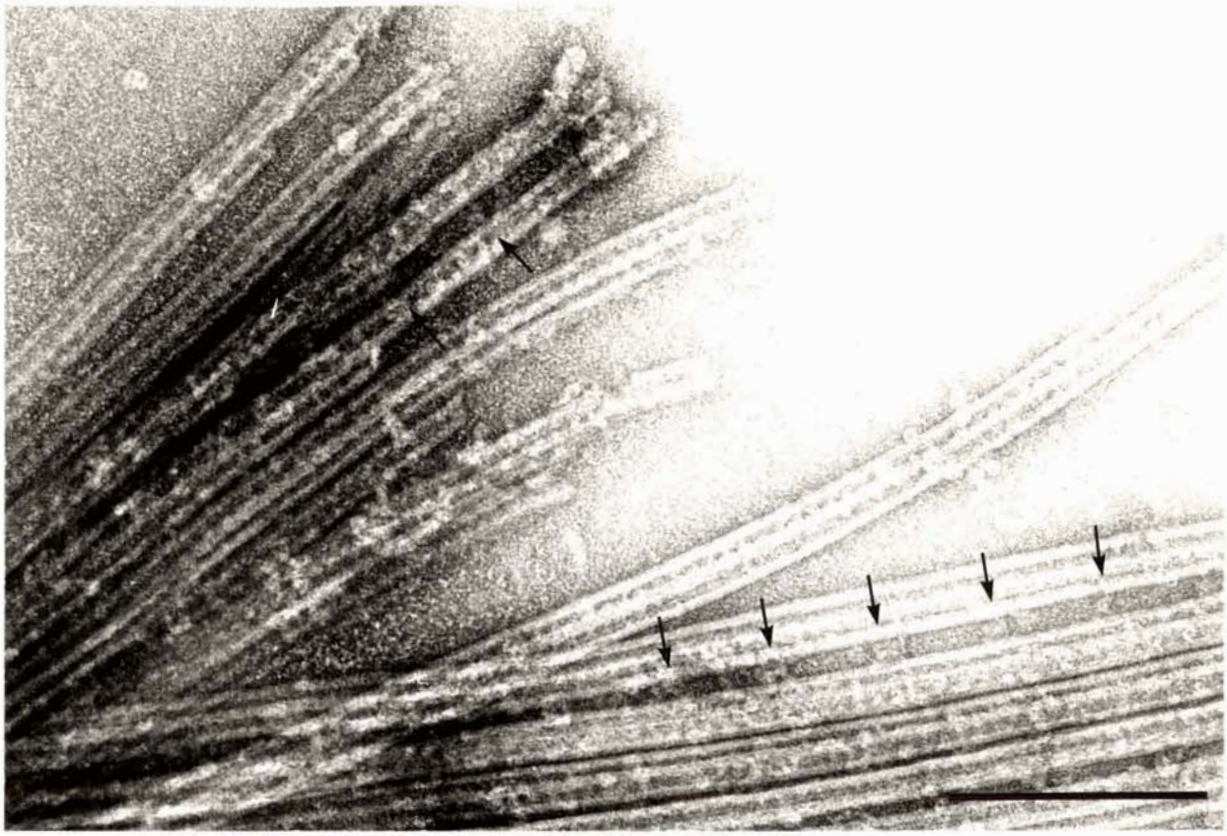


Fig. 12

C



D

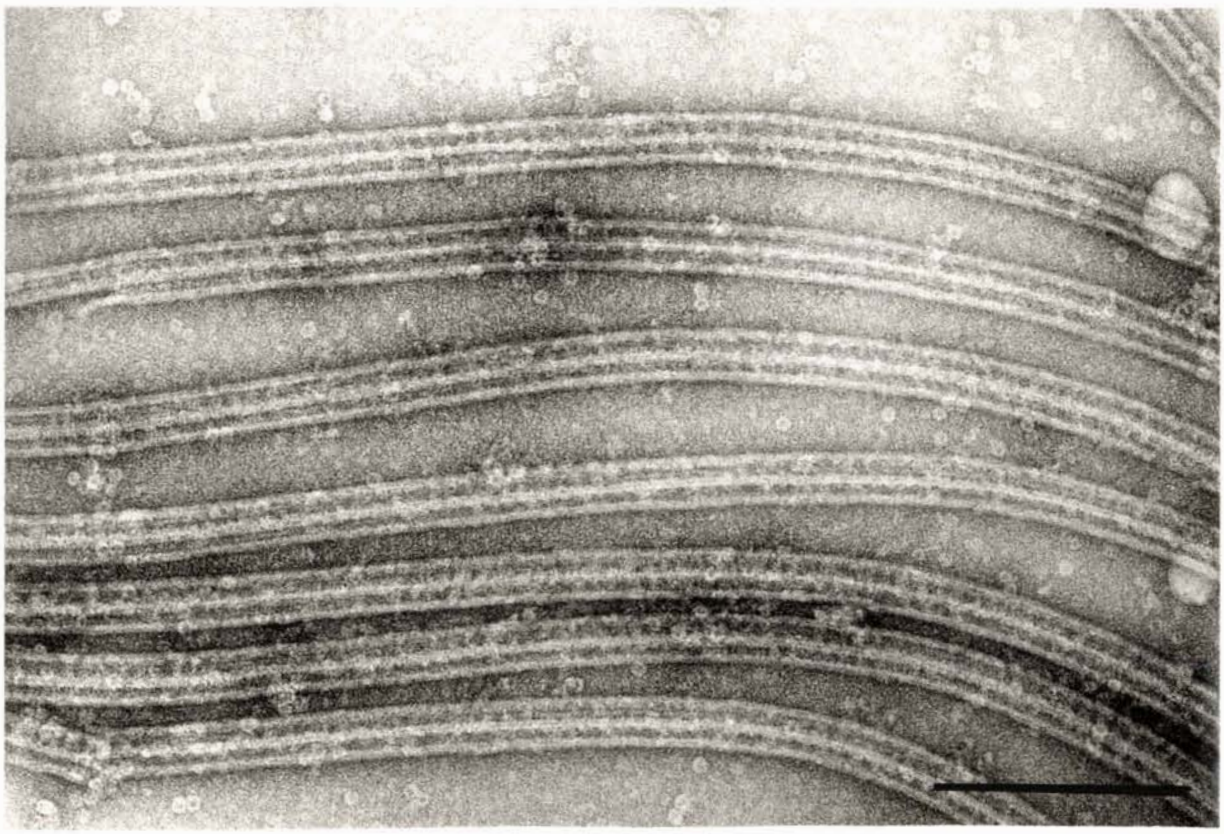
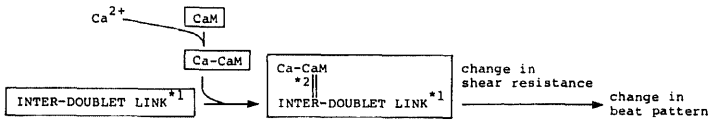


Fig. 12(continued)

A

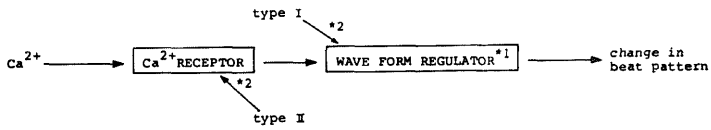
Ohnishi et al. 1982
(Tetrahymena cilia)



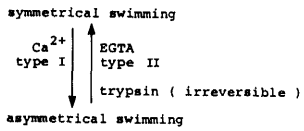
*1; trypsin sensitive structure
*2; hydrophobic interaction

B

Gibbons 1982
(Sea urchin sperm flagella)



*1; trypsin sensitive factor
*2; hydrophobic interaction



★ type I organic solvents
(mimic the action of Ca^{2+})
ethylene glycol
methanol
2-propanol

★ type II organic solvents
(block the action of Ca^{2+})
p-dioxane
N,N'-dimethylformamide
formamide

Fig. 13

a b

\+ − + −

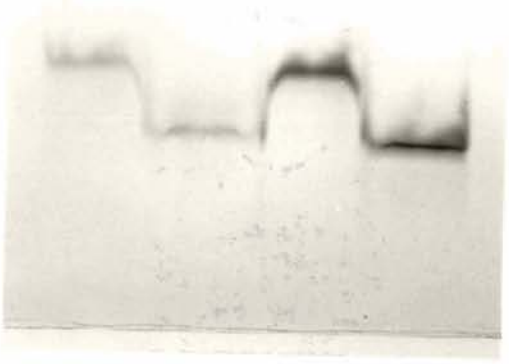


Fig. 14

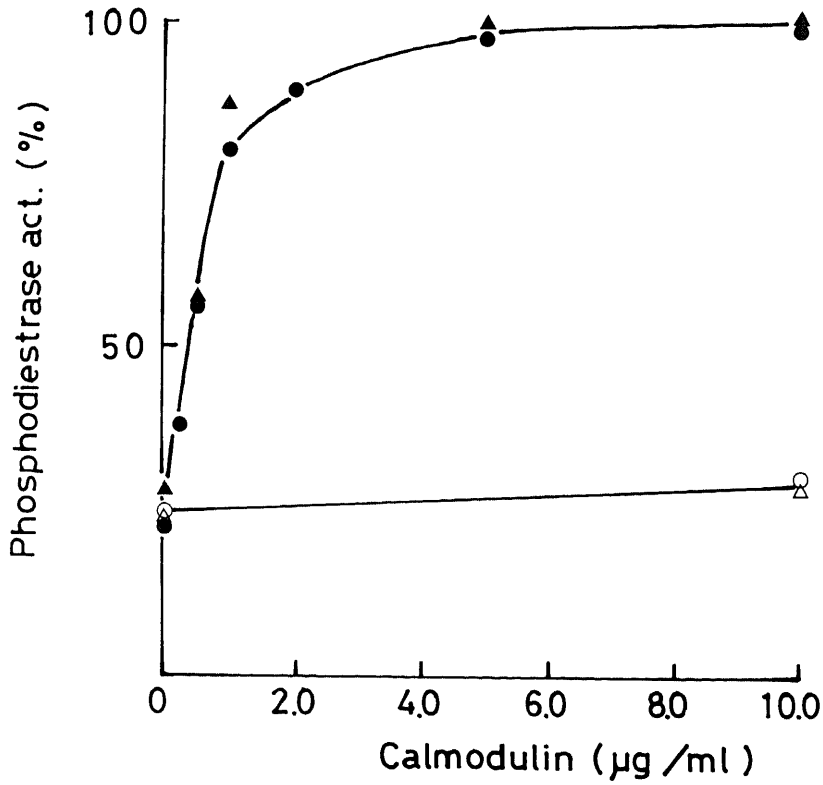

100
50
Phosphodiestrase act. (%)
0
2.0
4.0
6.0
8.0
10.0
Calmodulin (μg/ml)

Fig. 15

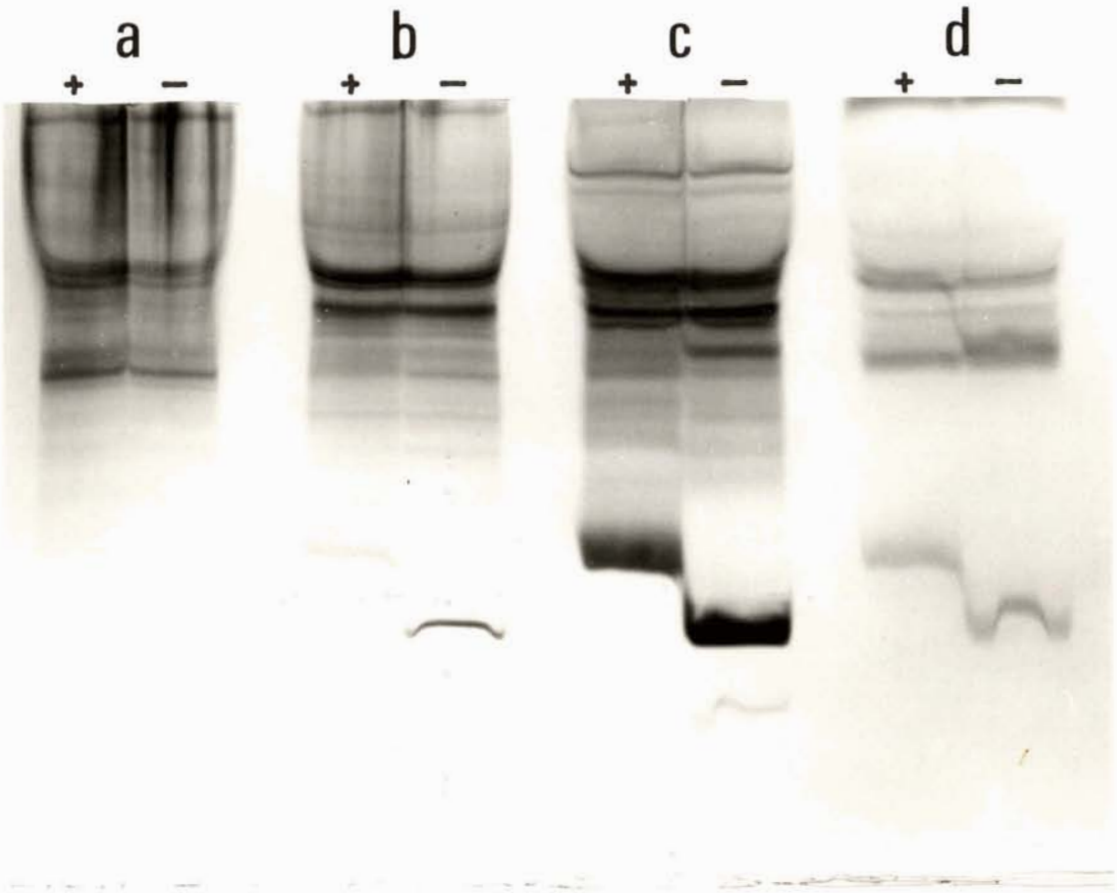


Fig. 16

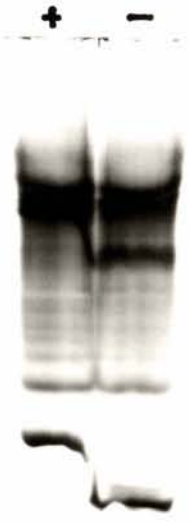


Fig. 17

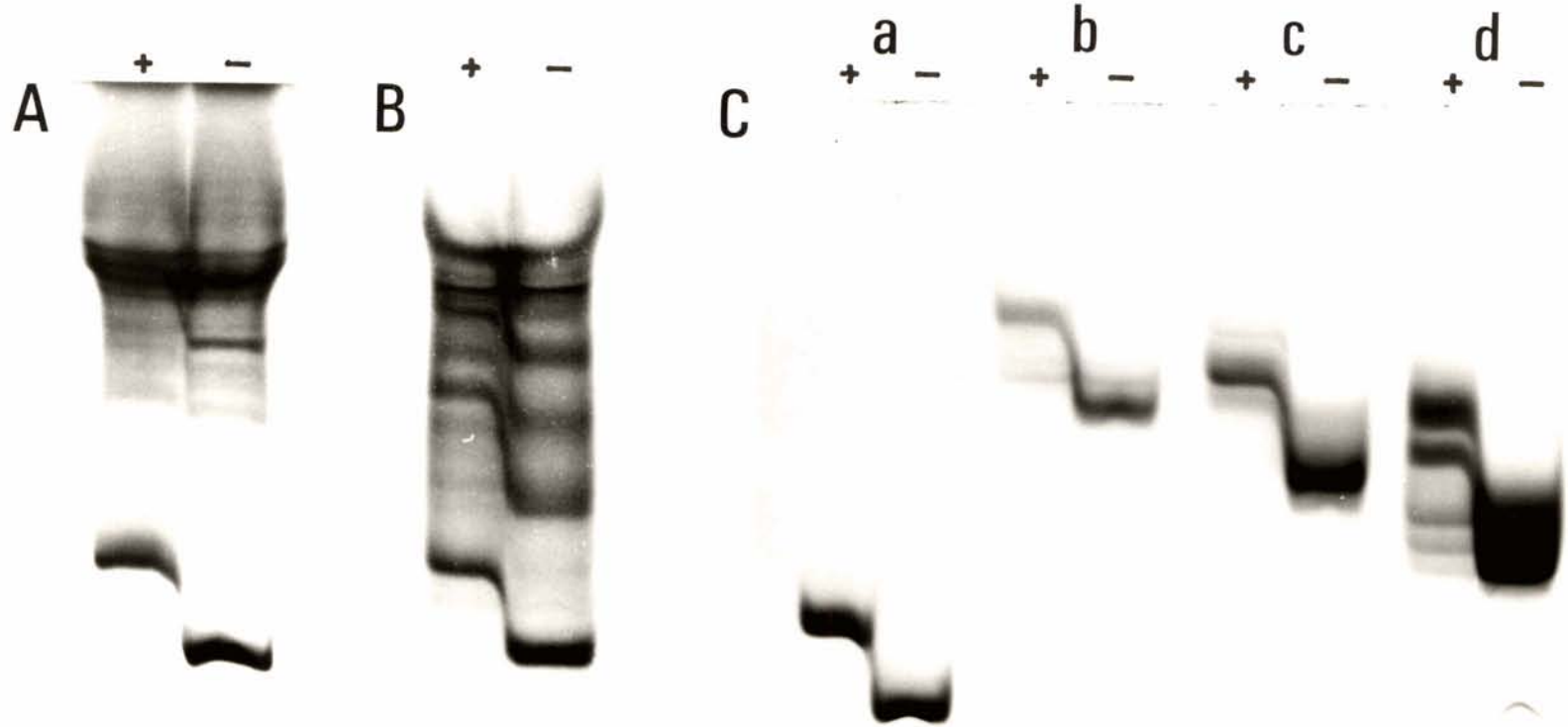


Fig. 18

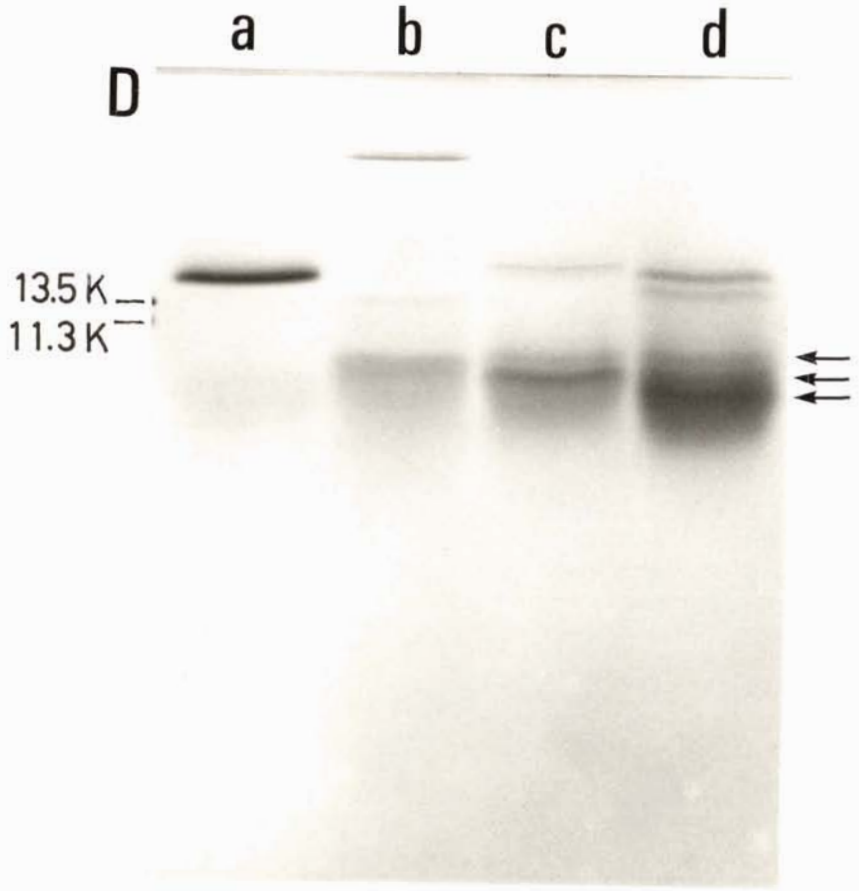


Fig. 18(continued)

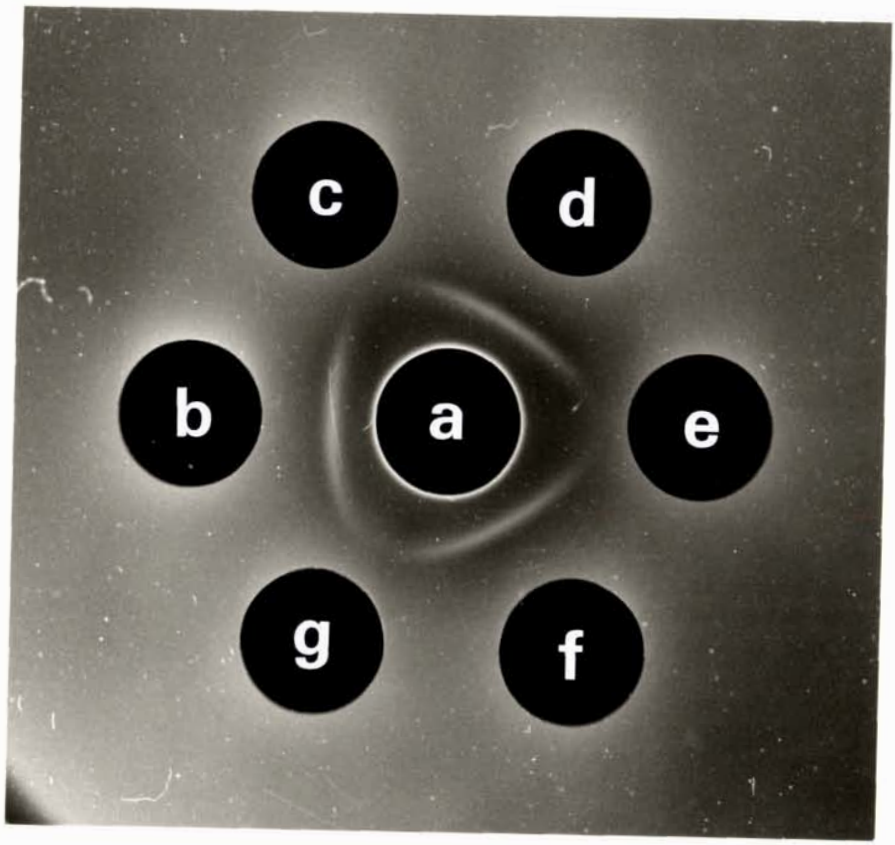


Fig. 19

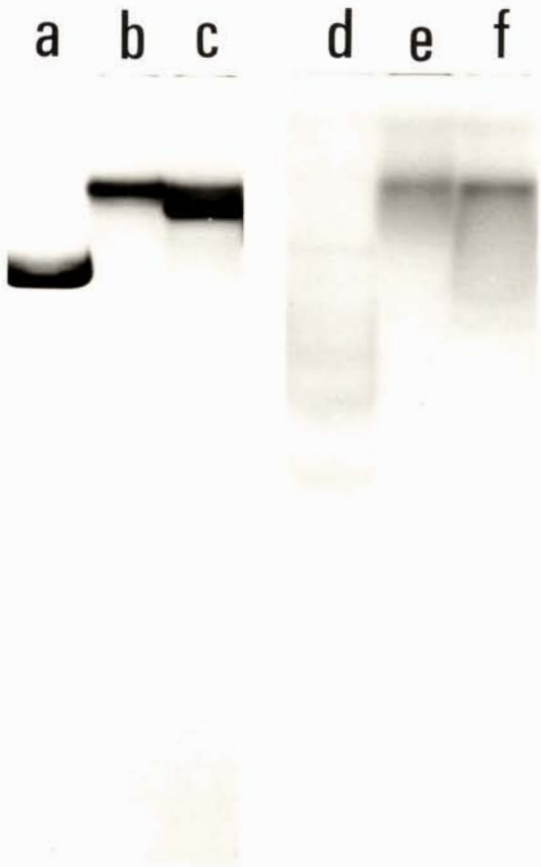


Fig. 20

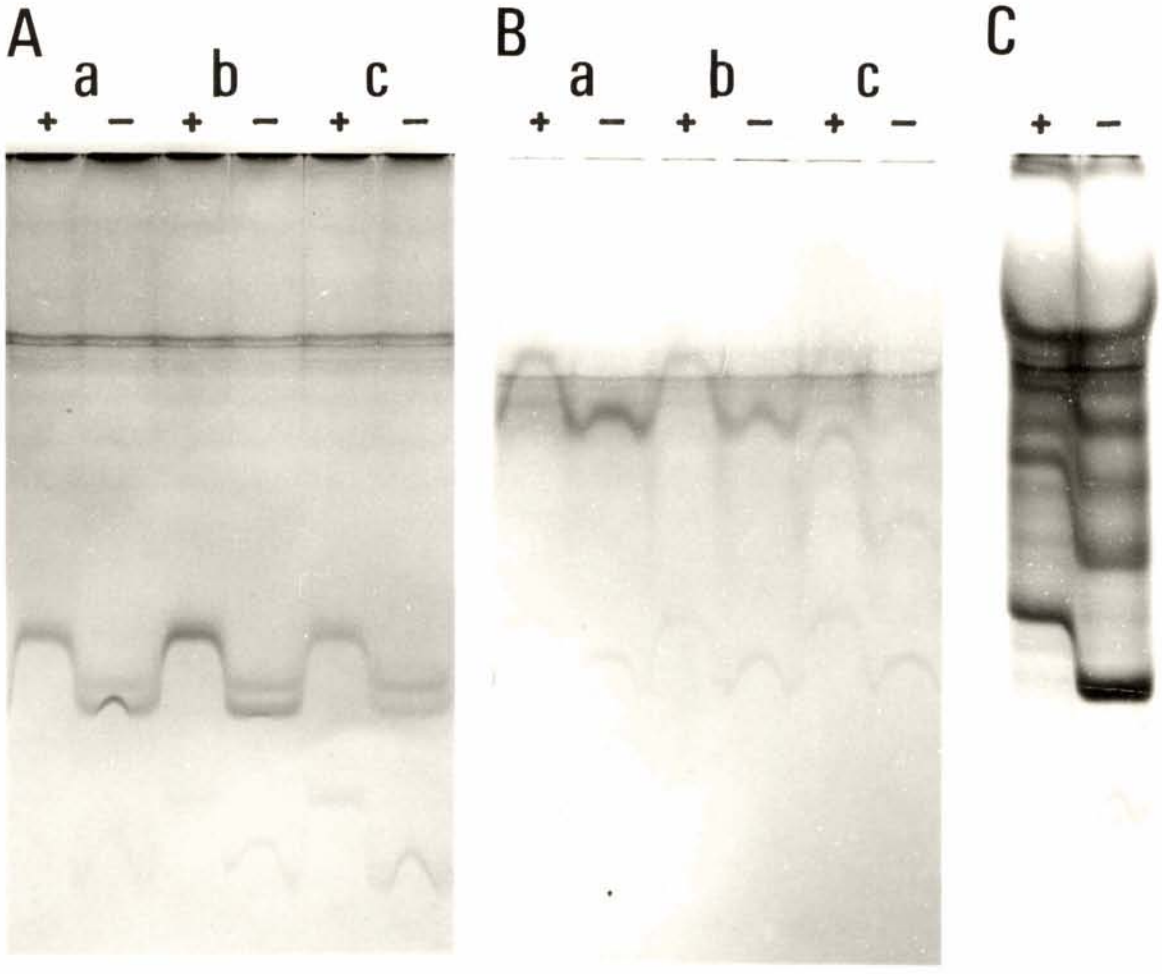


Fig. 21

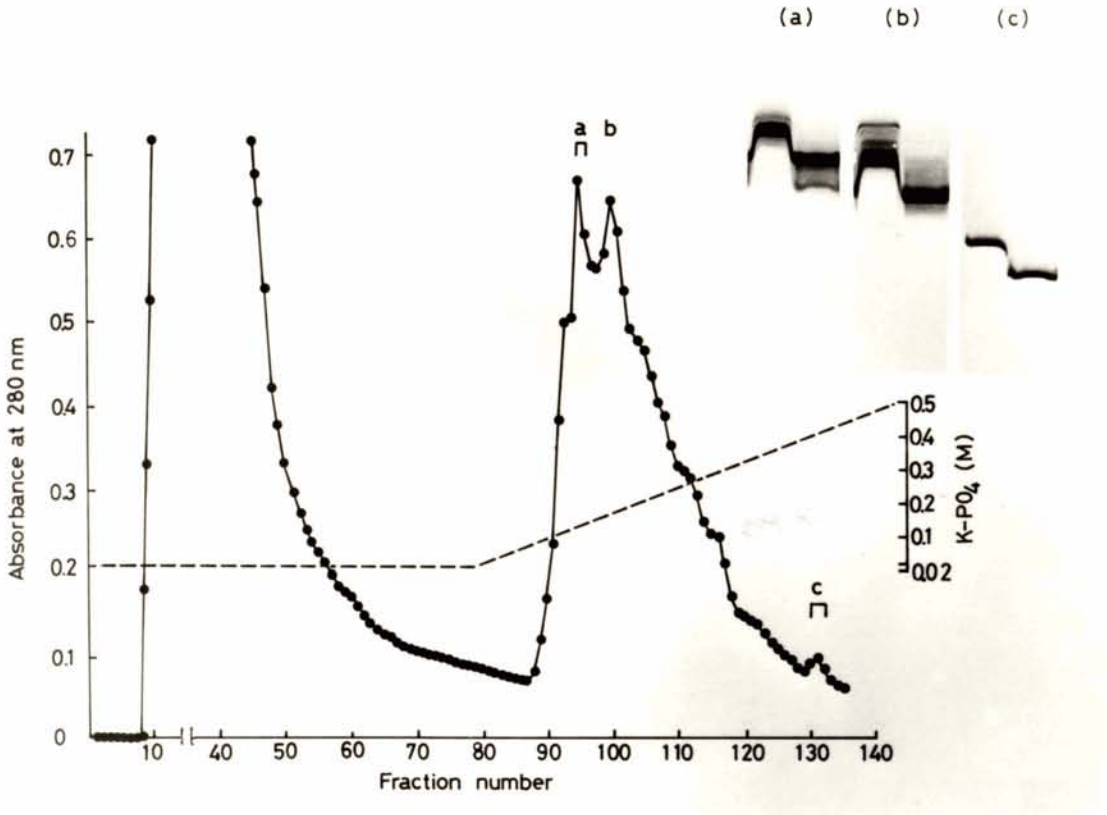

(a)
(b)
(c)
a
b
c
Absorbance at 280 nm
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
10
40
50
60
70
80
90
100
110
120
130
140
Fraction number
0.5
0.4
0.3
0.2
0.1
0.02
K-PO4 (M)

Fig. 22

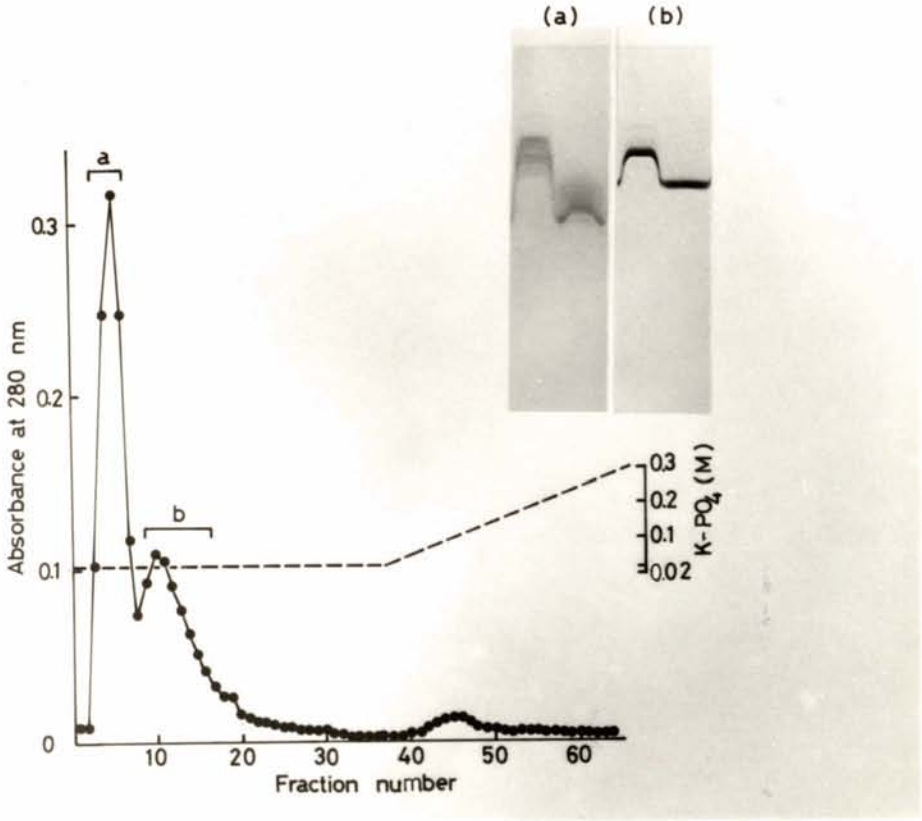

(a)
(b)
a
b
0.3
0.2
0.1
0
Absorbance at 280 nm
10
20
30
40
50
60
Fraction number
0.3
0.2
0.1
0.02
K-PO4 (M)

Fig. 23

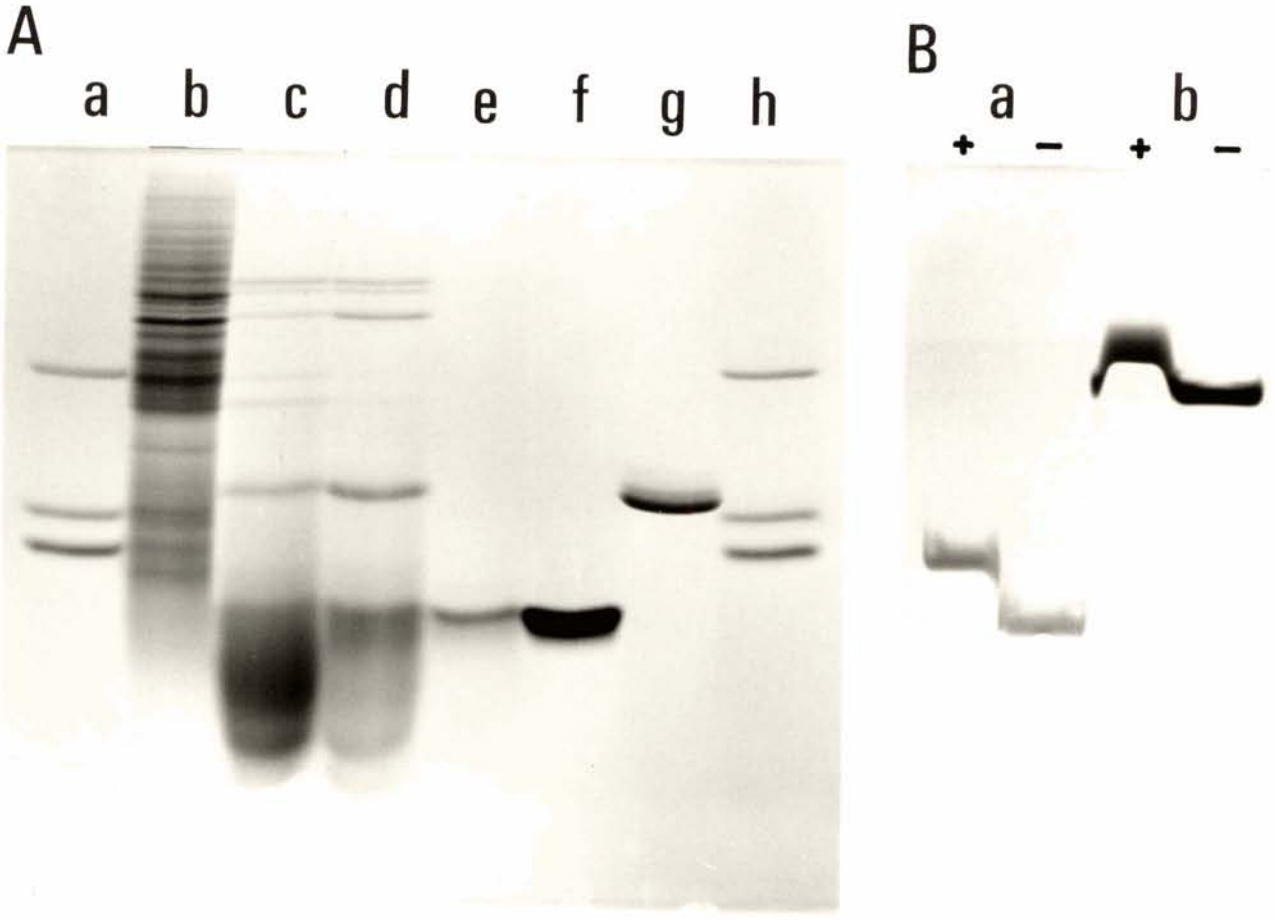


Fig. 24

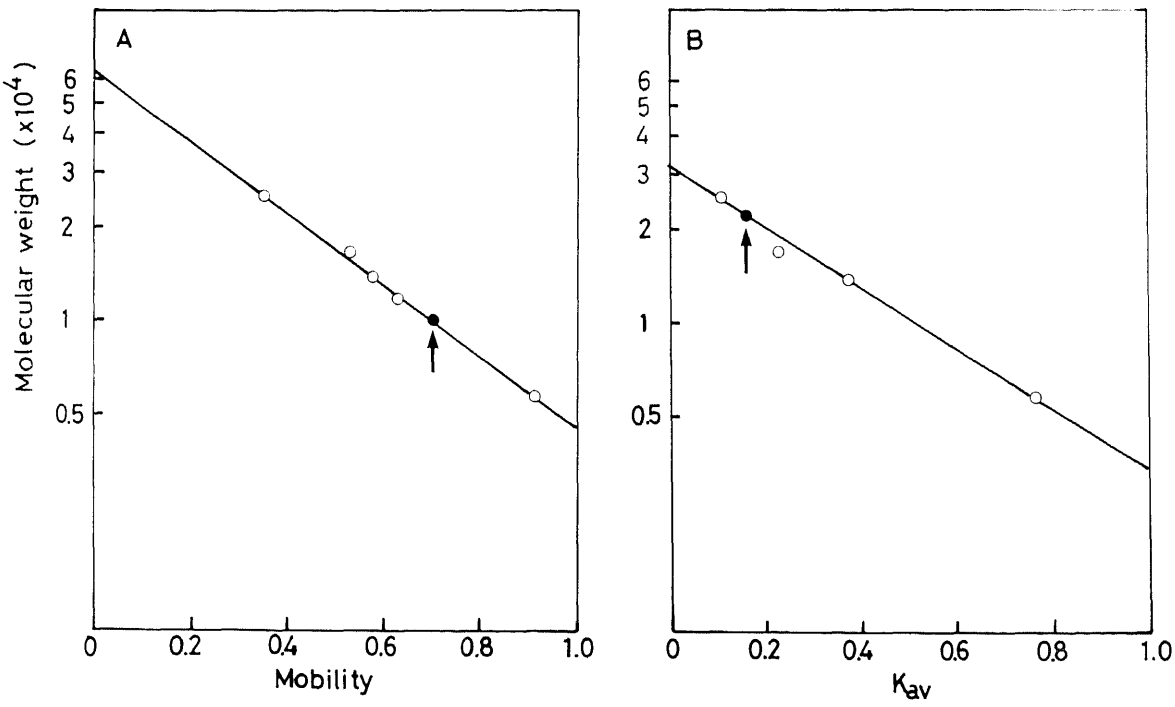

A
B
Molecular weight (x10⁴)
6
5
4
3
2
1
0.5
0
0.2
0.4
0.6
0.8
1.0
Mobility
Kav

Fig. 25

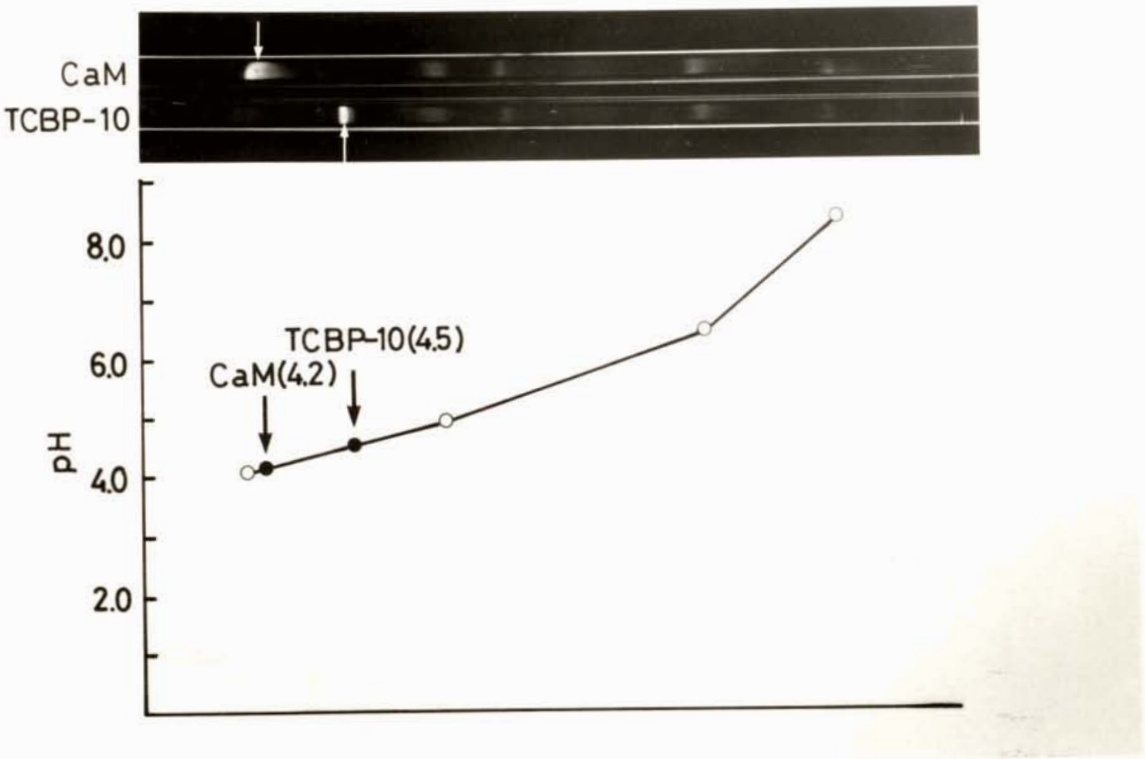


Fig. 26

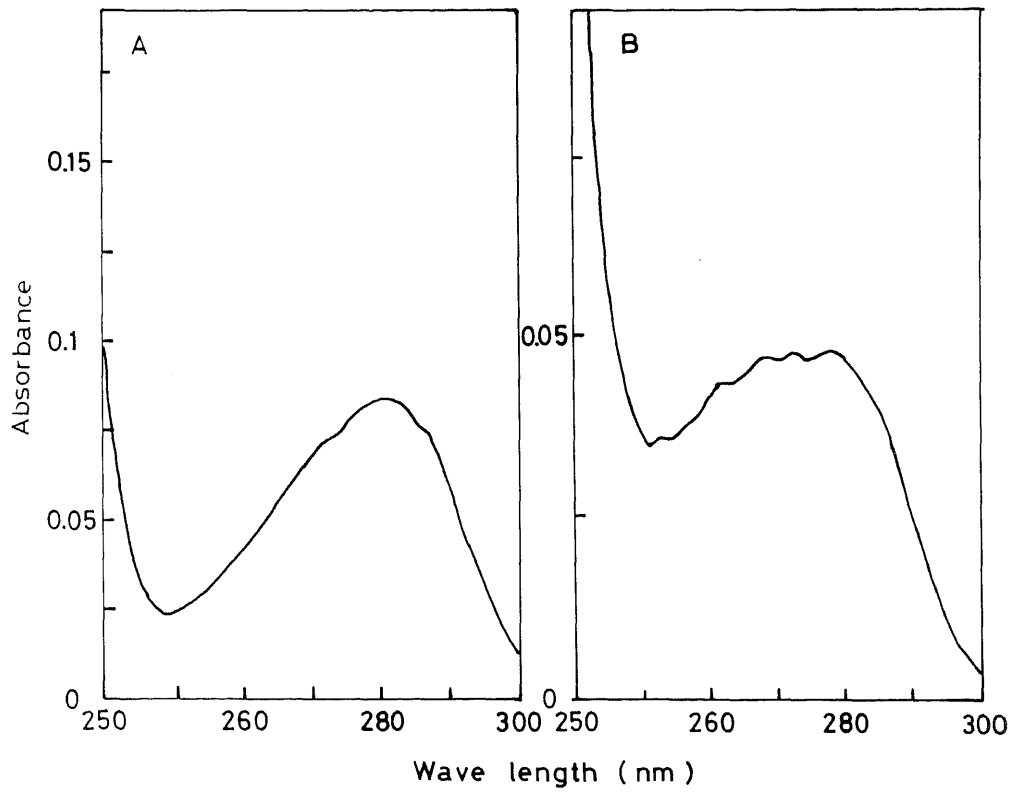

A
B
0.15
0.1
0.05
0
0.05
0
Absorbance
250
260
280
300
250
260
280
300
Wave length (nm)

Fig. 27

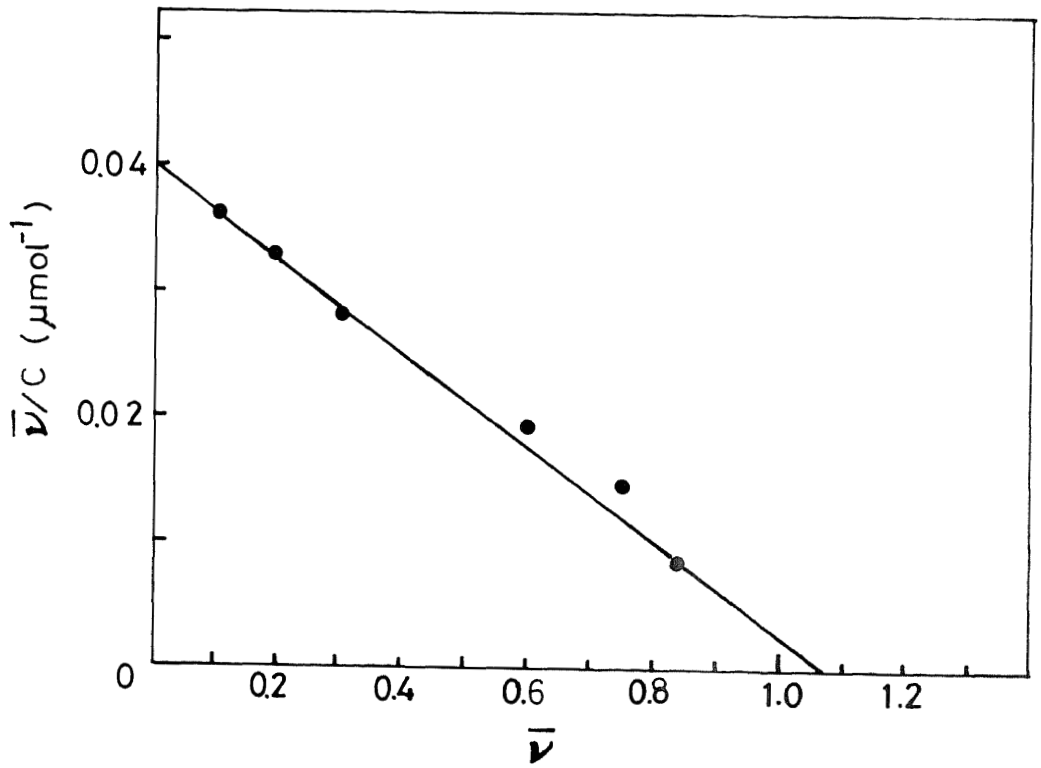


Fig. 28