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BIOCHEMICAL STUDIES ON THE FUNCTIONS OF CYTOPLASMIC ACTIN IN NON-MUSCLE CELLS

MASAHIRO ISHIURA

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GENERAL INTRODUCTION

Various contractile proteins, such as actin, myosin and tropomyosin have been isolated from various non-muscle cells and their localization in cells has been extensively studied by immunofluorescence microscopy and electron-microscopy (see Ref. 1 for review). However, no function has been established for these proteins (see Ref. 1 for review).

The cell fusion reaction induced by hemagglutinating virus of Japan(HVJ) involves several elementary steps: adsorption of the virus to cells, aggregation of the cells, fusion of the viral envelope with the cell membrane, and fusion of the cells. ATP-deficient conditions and the microfilament-disrupting mold metabolites, cytochalasin B and D, inhibit the cell-to-cell fusion of Ehrlich ascites tumor cells(ETC) without affecting other steps of the fusion reaction (2-5). These facts suggest that microfilaments(actin-containing filaments), a presumptive intracellular contractile system, are functionally involved in the HVJ-mediated cell-to-cell fusion.

As part of our endeavour to understand the mechanism of HVJ-mediated cell fusion reaction and the intracellular functions of actin in non-muscle cells, we prepared an actin-specific inhibitor, a chemically modified subfragment-1 of myosin(CMB-S-1) and studied the role of actin in temperature dependent gel-sol transformation of ETC extracts (assembly-disassembly of microfilaments) by use of the inhibitor.

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I. A CHEMICALLY MODIFIED SUBFRAGMENT-I OF MYOSIN AS AN ACTIN-SPECIFIC INHIBITOR

ABSTRACT

Two kinds of subfragment-1 of myosin, S-1(T) and S-1(CT), were prepared by two-step tryptic[EC 3.4.21.4] digestion of myosin that had been modified with about 1 mol of p-chloromercuribenzoate(CMB) per mol of myosin, and one-step chymotryptic[EC 3.4.21.1] digestion of the myosin, respectively. The amount of bound CMB was about 0.82 - 0.90 mol per 2 mol of S-1.

Both kinds of S-1 modified with CMB equally inhibited superprecipitation of myosin B from rabbit skeletal muscle. About 2 mol of CMB-S-1(1 mol of CMB-S-1A) inhibited the function of 1 mol of actin monomer on the superprecipitation of actomyosin reconstituted from myosin and fibrous actin (FA) with relaxing protein(RP). CMB-S-1 also effectively inhibited superprecipitation of myosin B from the plasmodia of the slime mold Physarum polycephalum.

The ATPase[EC 3.6.1.3] activity of CMB-S-1(T) was similar to that of CMB-S-1(CT) in the absence of FA, but was not enhanced as effectively by FA as the latter. In the presence of 0.3 mg/ml of FA with RP, the activity of CMB-S-1(T) was only one-fifth of that of CMB-S-1(CT).

CMB-S-1(T) did not affect the activities of ATPase from animal cells outside actomyosin systems, such as the Ca^{2+}-dependent ATPase[EC 3.6.1.3] of the SR prepared from rabbit skeletal muscle and the Na^{+}, K^{+}-dependent ATPase[EC 3.6.1.3] from porcine kidney. It also scarcely affected Ca^{2+}-uptake by the SR at concentrations lower than 0.2 mg/ml.

However, CMB-S-1(T) strongly inhibited the polymerization and depolymerization of tubulin prepared from bovine brain. At 0.15 mol per mol tubulin heterodimer, CMB-S-1(T) inhibited by 50 % the extent of polymerization of 0.80 mg/ml tubulin (7.3 μM tubulin heterodimer). S-1(T) also inhibited tubulin polymerization as effectively as CMB-S-1(T). CMB-S-1(CMB-S-1A) also weakly bound itself to polymerized tubulin.

It was concluded that CMB-S-1(T) can be used as a specific inhibitor of actin functions in non-muscle cells if the possible involvement of tubulin is excluded by other means.

INTRODUCTION

Actin, myosin, and tropomyosin have been isolated from many kinds of non-muscle cells and their localization in cells bas been studied (see review by Pollard and Weihing, Ref. 1). Actin has been visualized in cells by selective decoration of actin filaments with HMM (2) or by specifically staining them with fluorescent dye-labeled HMM (3). Recently, antibodies specifically reactive with each component of contractile proteins from non-muscle cells, such as actin (4), myosin (5), tropomyosin (6), and α-actinin (7), have been prepared and used successfully to show the intracellular distribution of each component. Much of the actin is localized on microfilaments (2,4), and in some cells, myosin (5), tropomyosin (6), and α-actinin (7) were also associated with actin filaments. While no function has been established for these proteins, they have been implicated in a variety of cell functions including motility, exocytosis, cytokinesis, membrane ruffling, maintenance of cell shape and cell adhesion to substratum (8-13, see also Ref. 1 for review).

Cytochalasin B and D, microfilament-disrupting mold metabolites, have been widely used to identify the functions of microfilaments in non-muscle cells (13-19). However, it remains difficult to identify actin-specific functions in non-muscle cells, since there is no direct evidence showing that cytochalasins specifically inhibit actin functions. Cytochalasin D was reported to bind with myosin and inhibit myosin ATPase[EC 3.6.1.3] (20). Cytochalasin B has the undesirable side effect of inhibiting some cellular transport systems (21-24).

To identify actin functions in non-muscle cells, first, we must find a substance which binds with actin specifically and stoichiometrically and inhibits actin functions. Second, we must elaborate a method of introducing the substance into cells without damaging cell functions. One such method enables us to introduce any substance into a target cell by HVJ (Sendai virus)-mediated cell fusion between the target cell and a human erythrocyte ghost loaded with the substance (25).

In the present paper, we describe experiments showing that subfragment-1 modified with CMB(CMB-S-1) meets the requirement for an actin-specific inhibitor. As described previously, CMB-S-1 is composed of equimolar S-1A modified with CMB(CMB-S-1A) and S-1B not modified with CMB, and that CMB-S-1 inhibits the superprecipitation of actomyosin induced by ATP, since CMB-S-1A binds very tightly with FA even in the presence of ATP, and thus occupies all the myosin binding sites of actin (26). In the present work, we prepared two kinds of CMB-S-1, CMB-S-1(T), and CMB-S-1(CT), and compared their inhibition of the superprecipitation of actomyosin and their ATPase activities in the presence of FA. We found that the two kinds of CMB-S-1 inhibited superprecipitation of actomyosin from rabbit skeletal muscle with the same effectiveness, while the acto-S-1-ATPase activity of CMB-S-1(T) was much lower than that of CMB-S-1(CT). Thus, using CMB-S-1(CT), we studied the stoichiometric relation between CMB-S-1 and actin in the inhibition of superprecipitation by CMB-S-1, and found that 2 mol of CMB-S-1 (1 mol of CMB-S-1A) inhibited the function of 1 mol of actin monomer. To see the effectiveness of CMB-S-1 on actomyosin of non-muscle origin, we prepared Physarum myosin B, and found that CMB-S-1 also effectively inhibited the superprecipitation of Physarum myosin B. To confirm the specificity of CMB-S-1, we prepared SR[EC 3.6.1.3] from rabbit skeletal muscle, Na^+, K^+-dependent ATPase[EC 3.6.1.3] from porcine kidney and tubulin from bovine brain, and studied their possible interactions with CMB-S-1. We found that they scarcely interacted with CMB-S-1 except for tubulin. Therfore, we concluded that CMB-S-1 can be used as a specific inhibitor of actin although it may also interact with tubulin.

MATERIALS AND METHODS

Materials - Myosin (MW=4.8 x 10^5) was prepared from rabbit skeletal white muscle by the method of Perry (27). HMM (MW=3.4 x 10^5) was prepared by tryptic digestion of myosin by the method of Szent-Györgyi (28) with slight modification (29). S-1(T) (MW=1.2 x 10^5) was prepared by tryptic digestion of HMM, followed by chromatography on Sephadex G-200, as described previously (26,30). S-1(CT) (MW=1.2 x 10^5) was prepared by chymotryptic digestion of myosin at low ionic strength, as described by Weeds and Taylor (31), and purified on Sephadex G-200.

G-actin with RP was extracted from an acetone powder of rabbit skeletal muscle at room temperature, and purified by a polymerization-depolymerization procedure (32). Purified G-actin was prepared from an acetone powder of rabbit skeletal muscle by the method of Spudich and Watt (33). After removal of free nucleotides from the G-actin solution by treatment with Dowex 1 x 4, actin was polymerized by addition of 1 mM $MgCl_2$ and 50 mM KCl.

Myosin B was prepared from rabbit skeletal muscle by the method described by Szent-Györgyi (34) with slight modification. Ca^{2+}-sensitive Physarum myosin B was prepared from the plasmodia of the slime mold, Physarum polycephalum, as described previously (35).

SR was prepared from rabbit skeletal muscle, as described previously (36). The activity of Ca^{2+}-dependent ATPase of the SR was responsible for more than 97 % of the total ATPase activity of the SR. The preparation used was kindly supplied by Dr. T. Yamamoto.

Na^+, K^+-dependent ATPase was prepared from the dark outer medulla of porcine kidney by the method of Jørgensen (37) with slight modification. The ATPase activity of the enzyme preparation was usually more than 2,000 μmol per mg protein per h at 37°C, and completely inhibited by ouabain (M. Yamaguchi & Y. Tonomura, unpublished observation.) The enzyme was a generous gift from Mr. M. Yamaguchi.

Bovine brain tubulin (αβ-heterodimer, MW=1.1 x 10^5) was purified from fresh brains essentially by the assembly-disassembly procedure described by

Shelanski et al. (38). The superficial blood vessels were removed, then the brain was washed with 0.34 M sucrose and minced with scissors in ATP-reassembly buffer, which contained 1 mM ATP, 1 mM EGTA, 0.5 mM $MgCl_2$, and 0.1 M Mes-NaOH (pH 6.5), at 4°C. Next, it was homogenized in 1 ml of buffer per g of tissue with a Sorvall Omnimixer for 1 min at half-maximum speed. The homogenate was centrifuged at 100,000 x g for 1 h at 4°C. The supernatant was collected, and mixed with an equal volume of the reassembly buffer containing 8 M glycerol, and incubated for 20 min at 37°C to polymerize tubulin. Polymerized tubulin was collected by 100,000 x g centrifugation for 1 h at 25°C, then dissolved in cold reassembly buffer, which contained 1 mM GTP instead of ATP (GTP-reassembly buffer), by gentle homogenization in a glass homogenizer. Next, the mixture was chilled on ice for 30 min to depolymerize tubulin. The tubulin solution was clarified by 100,000 x g centrifugation for 1 h at 4°C. The polymerization-depolymerization cycle was repeated twice in the presence of 1 mM GTP. After the second polymerization, the dissociated tubulin in GTP-reassembly buffer was brought to 8 M in glycerol and stored at -20°C, unless otherwise stated. The tubulin was more than 70 % pure according to electrophoresis on sodium dodecyl sulfate-polyacrylamide gels performed as described previously (30); it was contaminated mainly with tubulin-associated high molecular weight components.

Pyruvate kinase[EC 2.7.1.40] was prepared from rabbit skeletal muscle by the method of Tietz and Ochoa (39).

Trypsin was purchased from Worthington Chemical Co. Trypsin inhibitor and chymotrypsin were purchased from Sigma Chemical Co. Bovine serum albumin (fraction 5) was purchased from Nakarai Chemicals, Ltd. ^{203}Hg-CMB and CMB were purchased from the Radiochemical Centre. England, and Wako Pure Chemical Industries, Ltd., respectively, and purified by Boyer's method (40). $^{45}CaCl_2$ was purchased from the Radiochemical Centre. Phenylmethylsulfonylfluoride and phosphoenolpyruvate were purchased from Sigma Chemical Co., and β-mercaptoethanol from Nakarai Chemicals, Ltd. GTP and ATP were purchased

from P.L. Biochemicals Inc. and Kyowa Hakko Co., respectively.

Preparation of CMB-S-1(T) and CMB-S-1(CT) - Myosin was modified with CMB as described previously (26). CMB-HMM was prepared by tryptic digestion of CMB-myosin. CMB-S-1(T) and CMB-S-1(CT) were prepared by tryptic digestion of CMB-HMM and chymotryptic digestion of CMB-myosin, respectively. The radioactivity of ^{203}Hg-CMB bound to S-1 was measured in a Beckman liquid scintillation spectrometer, Model SL 150. CMB-S-1 contained 0.82 - 0.9 mol of CMB per 2 mol of S-1.

ATPase Activity and Ca^{2+}-Uptake - The ATPase activity of acto-S-1 in the steady state was determined at 25°C in a coupled system, with pyruvate kinase and phosphoenolpyruvate as an ATP-regenerating system, as described previously (29). The Ca^{2+}-dependent ATPase activity of SR was determined at 25°C as described previously (41). The amount of Ca^{2+}-uptake by SR was measured by the Millipore filtration method using $^{45}CaCl_2$ as substrate, as described previously (41,42). The activity of Na^+, K^+-dependent ATPase was measured as described by Jørgensen (37).

Superprecipitation of Actomyosin and Myosin B - The superprecipitation of actomyosin and myosin B was measured at 20°C by determining turbidity at 660 nm with a Cary Model 14 spectrophotometer, as described previously (26).

Polymerization of Tubulin - The polymerization of tubulin in GTP-reassembly buffer was measured at 25°C by the turbidimetric method described by Gaskin et al. (43). The time course of increase in absorbance at 350 nm was measured with a Zeiss Model PMQ3 spectrophotometer.

Protein Concentration - Protein concentration was estimated by the biuret reaction (44) calibrated by nitrogen determination, or by the Copper-Folin method (45).

RESULTS AND DISCUSSION

Stoichiometric Inhibition of the Superprecipitation of Actomyosin by CMB-S-1 - S-1(CT) holds the myosin-associated functions of interacting with actin, such as the activation of S-1-ATPase activity by FA and the acceleration of actin polymerization by S-1, more persistently than S-1(T) (46). We prepared two kinds of S-1 modified with CMB, CMB-S-1(T), and CMB-S-1(CT), and compared their inhibition of the superprecipitation of actomyosin. Since myosin B contains all the protein components of the muscle contractile system and is considered to be a more native contractile model than actomyosin reconstituted from myosin and FA, we used myosin B prepared from rabbit skeletal muscle.

When superprecipitation was initiated in the presence of CMB-S-1(T) or CMB-S-1(CT), both inhibited the rate and extent of superprecipitation of myosin B. Figure 1 shows that the two kinds of CMB-S-1 inhibited the extent of superprecipitation with same efficiency. This suggests that the tryptic and chymotryptic digestion for the preparation of CMB-S-1(T) and CMB-S-1(CT), respectively, did not damage the inhibitory function of myosin head modified with CMB. S-1 not modified with CMB, such as S-1(T) or S-1(CT), did not affect the superprecipitation of 0.28 mg/ml myosin B at the concentration of 0.6 mg/ml.

The ATPase activities of the two kinds of CMB-S-1 were measured at 25°C in the presence of 0 - 0.6 mg/ml FA with RP and 50 mM KCl at pH 7.6. Their ATPase activities in the absence of FA were about 50 - 100 μmol per min per g of S-1. The activity of CMB-S-1(CT) gradually increased with increasing concentration of FA with RP, and the activities in the presence of 0.3 and 0.6 mg/ml FA with RP were 780 and 1,060 μmol per min per g of S-1, respectively. However, FA did not enhance the activity of CMB-S-1(T) as effectively as that of CMB-S-1(CT). The activities of CMB-S-1(T) in the presence of 0.3 and 0.6 mg/ml FA with RP were only 160 and 240 μmol per min per g of S-1, respectively. This fact shows that the tryptic

digestion of CMB-myosin somehow damaged myosin molecules, decreasing the efficiency of FA activation of ATPase activity of CMB-S-1(T). When CMB-S-1 is used as a specific inhibitor of actomyosin motile systems, CMB-S-1-associated enzyme activities, such as ATPase, should be very low. We preferred CMB-S-1(T) as a candidate for a specific inhibitor of actomyosin motile systems.

CMB-S-1 inhibited the superprecipitation of actomyosin reconstituted from pure FA and myosin as well as that of actomyosin reconstituted from FA with RP and myosin. In addition, this inhibited superprecipitation was recovered by addition of pure FA, but not by addition of other protein components of myosin B. These facts clearly show that CMB-S-1 acted on actin to inhibit superprecipitation of myosin B. As described previously, CMB-S-1(T) contains equimolar S-1A modified with CMB(CMB-S-1A) and unmodified S-1B, and only CMB-S-1 binds with FA tightly in the presence of ATP (26). To show the stoichiometric inhibition of actin function by CMB-S-1 on the superprecipitation of actomyosin, we reconstituted actomyosin from 0.6 mg/ml of myosin and various amounts of FA with RP,and studied the quantitative relation between the actin content in the reconstituted actomyosin and the amount of CMB-S-1(T) required to inhibit superprecipitation.

Figure 2 shows the stoichiometric inhibition of superprecipitation of actomyosin by CMB-S-1(T). The amounts of CMB-S-1(T) required for half and complete inhibition of the extent of superprecipitation increased linearly with the amount of FA with RP in actomyosin. Assuming that the molecular weight of FA with RP per actin monomer is 6.3×10^4, the amounts of CMB-S-1(T) required for the half and complete inhibition were about 1.1 and 1.8 mol per mol of actin monomer, respectively. This fact clearly shows that 2 mol of CMB-S-1(T)(1 mol of CMB-S-1A) inhibited the function of 1 mol of actin monomer. Therefore, we can conclude that 1 mol of CMB-S-1A binds to 1 mol of actin monomer and inhibits the functional interaction between actin and myosin.

Inhibition of Superprecipitation of Myosin B Prepared from Nom-Muscle Cells by CMB-S-1 - CMB-S-1(T) inhibited superprecipitation of actomyosin

prepared from non-muscle cells as well as that of skeletal muscle actomyosin. Figure 3 shows that CMB-S-1(T) inhibited both the rate and extent of superprecipitation of Physarum myosin B. At the concentrations of 0.24 and 0.74 mg/ml, CMB-S-1(T) caused half and complete inhibition of the extent of superprecipitation of 1 mg/ml Physarum myosin B, respectively.

Effects of CMB-S-1 on the Activities of Ca^{2+}-Dependent ATPase of SR and Na^{+}, K^{+}-dependent ATPase Prepared from Porcine Kidney - To understand the specific inhibition of actin functions by CMB-S-1, we studied the possible interaction of CMB-S-1(T) with other ATPase prepared from animal cells. Figure 4 shows that the Ca^{2+}-ATPase activity of SR (50 µg/ml of SR protein) measured in the presence of 790 µg/ml CMB-S-1(T) was 93 % of that in the absence of CMB-S-1(T). This fact suggests that there was no interaction of CMB-S-1(T) with the Ca^{2+}-ATPase of SR. This view was supported by the following result of Ca^{2+}-uptake experiment.

When the Ca^{2+}-uptake by SR was measured in the presence of various amounts of CMB-S-1(T), it was scarcely affected at concentraions lower than 0.2 mg/ml, but partially inhibited with increasing concentration (Fig. 5). Ca^{2+}-uptake in the presence of 0.53 and 0.79 mg/ml CMB-S-1(T) was 74 and 47 % of that in the absence of CMB-S-1(T), respectively. The inhibition observed at high CMB-S-1(T) concentrations would be caused partially by the disturbing effect of high protein concentration on the Millipore filtration method, since protein concentrations higher than 0.5 mg/ml decrease the measured values of Ca^{2+}-uptake by SR.

CMB-S-1(T) also did not alter the activity of Na^{+}, K^{+}-dependent ATPase prepared from porcine kidney. Figure 6 shows that the Na^{+}, K^{+}-dependent ATPase activity measured in the presence of 100 µg/ml CMB-S-1(T) was 94 % of that in the absence of CMB-S-1(T).

Interaction of CMB-S-1(T) with Tubulin - The tubulin-dynein system commonly exists in animal cells, and is believed to be involved in various cell-associated phenomena, such as cell division, cell motility, transport,

pinocytosis and secretion. In addition, some authors recently reported that colchicine-binding protein from porcine brain (47) and blood platelets (48), and tubulin isolated from the outer fiber of the flagella of Tetrahymena (49) activate the Mg^{2+}-ATPase activity of skeletal muscle myosin as actin does, and suggested a homology between tubulin and actin. Thus, we examined the possible interaction of CMB-S-1(T) with tubulin.

Figure 7 shows the binding of CMB-S-1(T) to polymerized tubulin. When various amounts of ^{203}Hg-CMB-S-1(T) were centrifuged with 1.05 mg/ml of tubulin (9.5 μM tubulin heterodimer), a mixture of polymerized (74 % of total tubulin) and unpolymerized tubulin, the radioactivity due to ^{203}Hg-CMB bound to CMB-S-1A that coprecipitated with polymerized tubulin by ultracentrifugation became greater with increasing ^{203}Hg-CMB-S-1A concentration, although it was low even in the presence of excess CMB-S-1A. Only 0.22 mol of CMB-S-1A per mol of tubulin heterodimer coprecipitated with polymerized tubulin in the presence of 20 μM CMB-S-1A. The coprecipitation of CMB-S-1A with polymerized tubulin could not be attributed to actin contamination in the tubulin preparation since our tubulin was at least 70 % pure and showed no actin band on electrophoresis on sodium dodecyl sulfate-polyacrylamide gels.

CMB-S-1 not only bound to polymerized tubulin but also inhibited polymerization of tubulin (Fig. 8). Both CMB-S-1(T) and S-1(T) storongly inhibited polymerization of tubulin with the same efficiency. They affected the polymerization of 0.80 mg/ml tubulin (7.3 μM tubulin heterodimer) at concentrations greater than 45 μg/ml, and 0.12 mg/ml of CMB-S-1(T) or S-1(T) caused 50 % inhibition. The amount of CMB-S-1(T) or S-1(T) requierd for half inhibition was 0.15 mol per mol of tubulin heterodimer. The extent of the inhibition by CMB-S-1(T) or S-1(T) did not exceed about 74 % which was observed at the concentration of 1 mg/ml. Bovine serum albumin did not affect the polymerization at 1 mg/ml.

CMB-S-1(T) also inhibited depolymerization of polymerized tubulin

initiated by addition of 2 mM $CaCl_2$ in GTP-reassembly buffer at 25°C. These observations indicate that myosin head (at least head A) could interact with tubulin in vitro, and suggest that the modification of head A with CMB did not alter the interaction between myosin head and tubulin.

The mechanism for inhibition of tubulin polymerization and depolymerization by CMB-S-1(T) and S-1(T) is not clear. Tubulin requires equimolar GTP for polymerization (50). The GTPase activity associated with CMB-S-1(T) or S-1(T) could not account for the inhibition of tubulin polymerization, since the activity was not high enough to induce GTP depletion, which would inhibit the polymerization. When CMB-S-1(T) or S-1(T) was incubated in GTP-reassembly buffer at 25°C (conditions of the polymerization experiment), 0.12 mg/ml of CMB-S-1(T) and S-1(T), which inhibited by 50 % the polymerization of 0.80 mg/ml tubulin (Fig. 8), consumed only 25 and 39 % of the total GTP (1 mM) per 30 min, respectively, while the polymerization was almost complete within 30 min under the given conditions. Polymerization was inhibited 50 % by 0.15 mol of CMB-S-1(T) or S-1(T) per mol of tubulin heterodimer (Fig. 8). This amount of CMB-S-1(T) is equivalent to 0.07 mol of CMB-S-1A per mol of tubulin heterodimer, provided that 2 mol of CMB-S-1(T) contained 0.9 mol of CMB. Thus, in the presence of 9.5 μM tubulin, 0.7 μM of CMB-S-1A caused 50 % inhibition of tubulin polymerization. At the concentrations of tubulin and CMB-S-1A, only 0.02 mol of CMB-S-1A per mol of tubulin heterodimer bound to polymerized tubulin (Fig. 7). This calculation shows that if the binding of CMB-S-1A (or S-1A) to tubulin inhibited tubulin polymerization, the binding of only 0.02 mol of CMB-S-1A (or S-1A) per mol of tubulin heterodimer could inhibit the polymerization by 50 %. If so, we may consider two possible mechanisms for the inihibition. One is that CMB-S-1A (or S-1A) binds to polymerized tubulin and blocks the elongation and shortening of the tubulin polymer. The other is that CMB-S-1A (or S-1A) binds with microtubule-associated components that control the tubulin polymerization and depolymerization (51-54), and affects the control functions of the components. Further investigations are necessary to clarify the interaction between S-1 and tubulin.

CONCLUSION

Actomyosin exists commonly in various non-muscle cells (1) The intracellular distribution of actin is easily seen by specific decoration of the actin filaments with HMM (2) or immunofluorescence staining of the actin filaments using actin-specific antibody (4), and actin is tentatively located on microfilaments. Actomyosin in non-muscle cells is believed to be involved in various cell functions (1), mainly because the functions are inhibited by cytochalasin B or D, which disrupts the organization of microfilaments (13-19). However, actin functions in non-muscle cells are not yet clear mainly due to the lack of an actin-specific inhibitor.

Pure S-1, modified with CMB, is easily prepared in large amounts from rabbit skeletal muscle. CMB-S-1A binds with FA stoichiometrically at a ratio of 1 mol of CMB-S-1A per mol of actin monomer, and very tightly even in the presence of ATP. Thus it occupies all the myosin binding sites of actin, and inhibits the actin function to interact with myosin. Therefore, we can estimate the content of functional actin in cells using the specific binding of ^{203}Hg-CMB-S-1 with actin in the presence of ATP. The distribution of actin within a cell can also be seen under a fluorescence microscope using CMB-S-1 conjugated with a fluorescent dye, or using CMB-S-1 together with fluorescent dye-conjugated antibody reactive with S-1. Although HMM conjugated with fluorescein isothiocyanate has been successfully used to show the distribution of actin within a cell (3), CMB-S-1 may be preferable since the binding of S-1A to FA is greatly strengthened by the modification with CMB (26). We can also identify the actin functions in non-muscle cells using CMB-S-1 as a speficic inhibitor of actin. The ATPase activity associated with CMB-S-1(T) is low even in the presence of FA, and thus CMB-S-1(T) might affect the intracellular ATP level only slightly if introduced into a cell. If large amount of CMB-S-1A are isolated from CMB-S-1, it is preferable to CMB-S-1(T), since the ATPase activity of CMB-S-1A is not accelerated by FA (26). There is little possibility that CMB bound to S-1

is transferred to intracellular components and affects the functions of components other than actin, since CMB bound to S-1 is difficult to remove from CMB-S-1 even by treatment with ditiothreitol, and as reported previously (26), there is no indication of intramolecular and intermolecular transfer of CMB bound to S-1 during the preparation of CMB-S-1 from CMB-myosin and during incubation of CMB-S-1 with FA. CMB-S-1(T) does not interact with a cell-associated ATPase, such as the Ca^{2+}-dependent ATPase of SR from rabbit skeletal muscle or the Na^{+}, K^{+}-dependent ATPase from porcine kidney. Since both CMB-S-1(T) and S-1(T) show some interaction with tubulin from bovine brain, we can interpret the inhibition of cell functions by CMB-S-1 as a result of specific inhibition of actin functions, only when S-1 or a tubulin-specific inhibitor such as colchicine does not affect the same functions, excluding the possible involvement of tubulin in the functions. Furthermore, if the effect of S-1 to a cell function is dependent on the modification of S-1 with CMB, then the involvement of actomyosin, but not tubulin, in the function is highly probable, since the interaction of S-1 with tubulin is independent of the modification.

Kuroda has already used CMB-S-1 successfully to show the involvement of the actomyosin motile system in the protoplasmic streaming in Nitella cells (Kuroda, K., unpublished observation). She made Nitella drops permeable to proteins by treatment with EGTA, and showed that only 4 μM CMB-S-1 completely inhibits the rotation of chloroplasts within a Nitella drop as 1 mM N-ethylmaleimide, an SH reagent, does.

We can introduce CMB-S-1 into human erythrocytes (HRBC) by procedures described previously (25). A small amount of actin exists in HRBC, associated with spectrin (55), but actin functions in HRBC are not clear. Since there is no indication of the existence of tubulin in HRBC, the HRBC ghost loaded with CMB-S-1 is a very useful tool for studying actin functions in erythrocytes. CMB-S-1 also can be introduced into animal cells by HVJ(Sendai virus)-mediated cell fusion between a target cell and an HRBC

ghost loaded with CMB-S-1 (25). Successful inhibition of actin functions in non-muscle cells by introducing CMB-S-1 into the cells would depend on the efficiency of introduction into the target cell and the actin content in the cell, since the actin content in non-muscle cells of animal origin is rather high (0.2 - 10 % of the total cell proteins) (56-58). In addition, we must take into account the intracellular proteolysis of CMB-S-1 introduced into a target cell, since a foreign protein introduced into a target cell is degraded rather rapidly — the half life of the activity of bacteriophage T_4-endonuclease V introduced into the fibroblasts derived from Xeroderma pigmentosum patients is about 3 h (59), and that of bovine serum albumin in FL cells, an established line from human amnions, is about 4 h (Yamaizumi, M., Uchida, T., Furusawa, M., & Okada, Y., in preparation).

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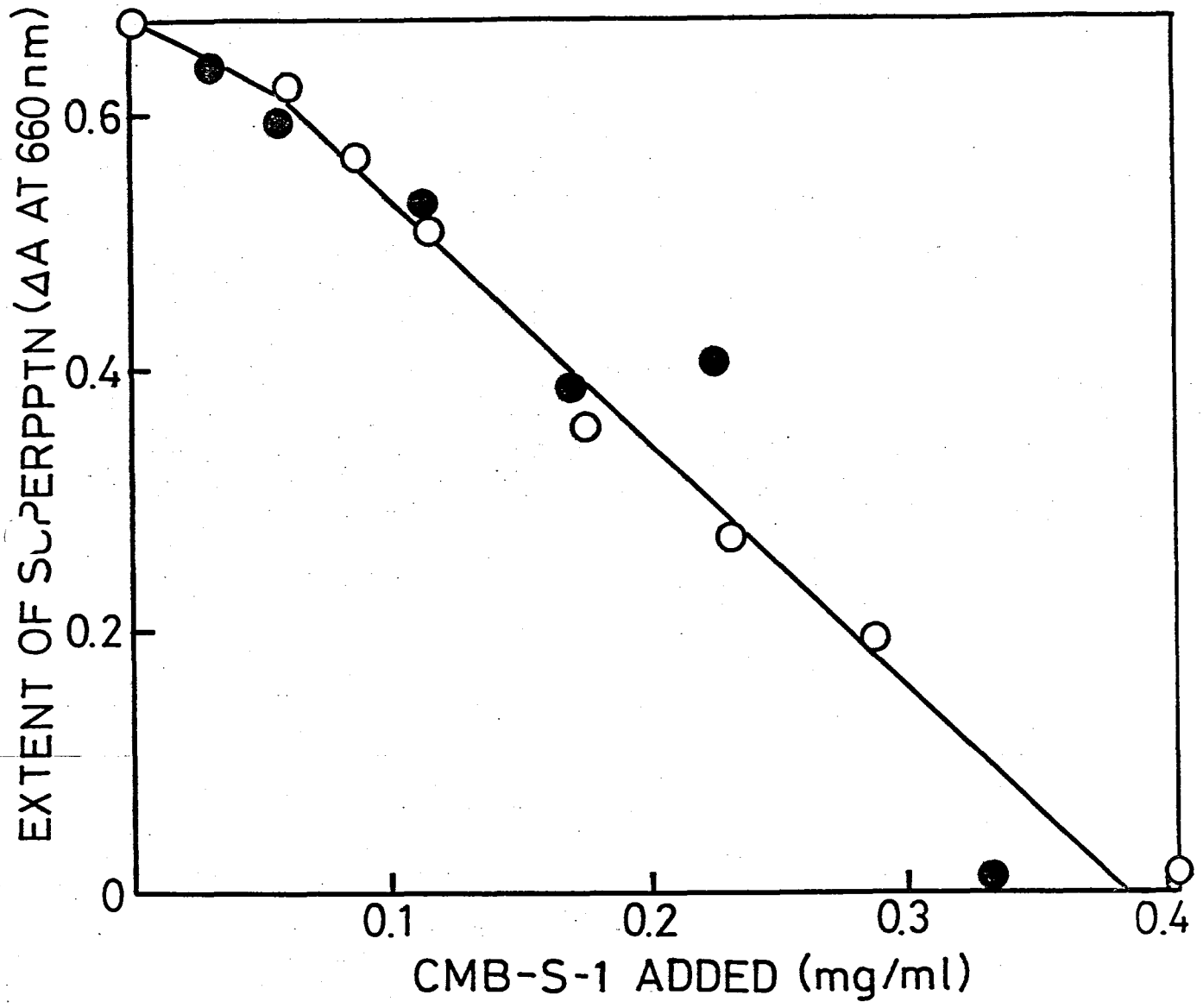


Fig. 1. Inhibition of the superprecipitation of rabbit skeletal muscle myosin B by CMB-S-1(T) and CMB-S-1(CT). Rabbit skeletal muscle myosin B (0.28 mg/ml) was mixed with various amounts of CMB-S-1(T) or CMB-S-1(CT), and preincubated at 20°C for 5 min. The clearing of myosin B was induced by adding 0.2 mM ATP in the presence of 62.5 mM KCl, 2 mM $MgCl_2$, 1 mM EGTA and 20 mM Tris-maleate (pH 7.0), and 1 min later, superprecipitation of myosin B was initiated by adding 3 mM $CaCl_2$. ΔA was defined as the final difference of absorbance at 660 nm in the presence and absence of $CaCl_2$. ○, CMB-S-1(T); ●, CMB-S-1(CT).

Fig. 2. Stoichiometric inhibition of the superprecipitation of rabbit skeletal muscle actomyosin by CMB-S-1(T). Actomyosin was reconstituted from 0.6 mg/ml rabbit skeletal muscle myosin and various amounts of rabbit skeletal muscle FA with RP, and then mixed with various amounts of CMB-S-1(T). Other conditions for the superprecipitation of actomyosin were the same as in Fig. 1. ○, amount of CMB-S-1(T) required for half inhibition of the extent of superprecipitation; ●, that required for complete inhibition.

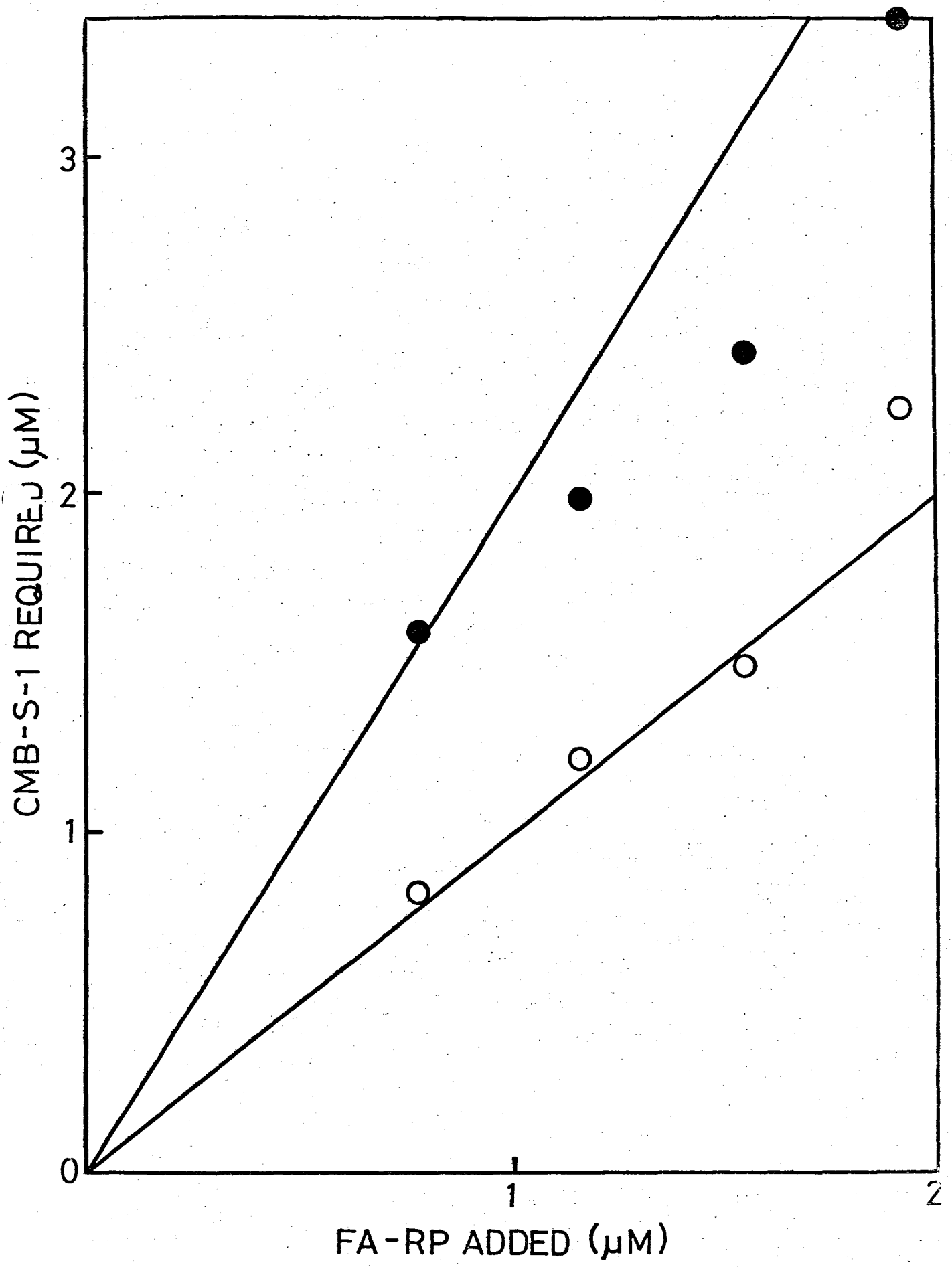
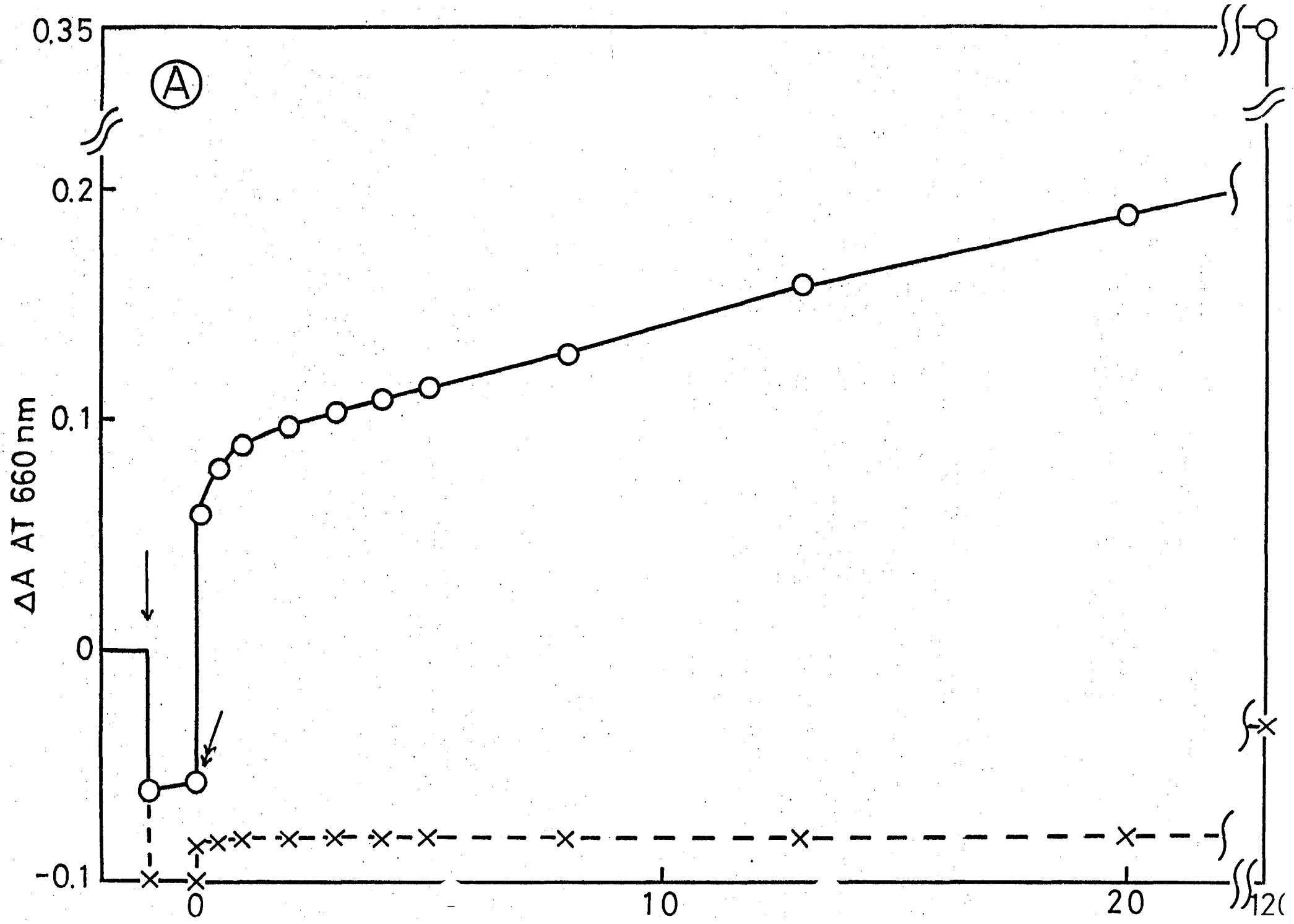

CMB-S-1 REQUIRED (μM)
0
1
2
3
1
2
FA-RP ADDED (μM)

Fig. 3. Inhibition of the superprecipitation of Physarum myosin B by CMB-S-1(T). Physarum myosin B (1 mg/ml) was mixed with various amounts of CMB-S-1(T) and preincubated for 5 min at 20°C. Next, the clearing and superprecipitation of Physarum myosin B were initiated by adding 0.2 mM ATP and 3 mM $CaCl_2$, respectively, in the presence of 75 mM KCl, 2 mM $MgCl_2$, 1 mM EGTA and 20 mM imidazole-HCl (pH 7.0), as described in Fig. 1. Other conditions were the same as in Fig. 1. A. Time course of superprecipitation. CMB-S-1(T) added: ○, 0 mg/ml; ×, 0.5 mg/ml. ↓, addition of 1 mM ATP; ↡, addition of 3 mM $CaCl_2$. B. Extent of superprecipitation.


0.35
Ⓐ
0.2
0.1
0
-0.1
ΔA AT 660 nm
0
10
20
120

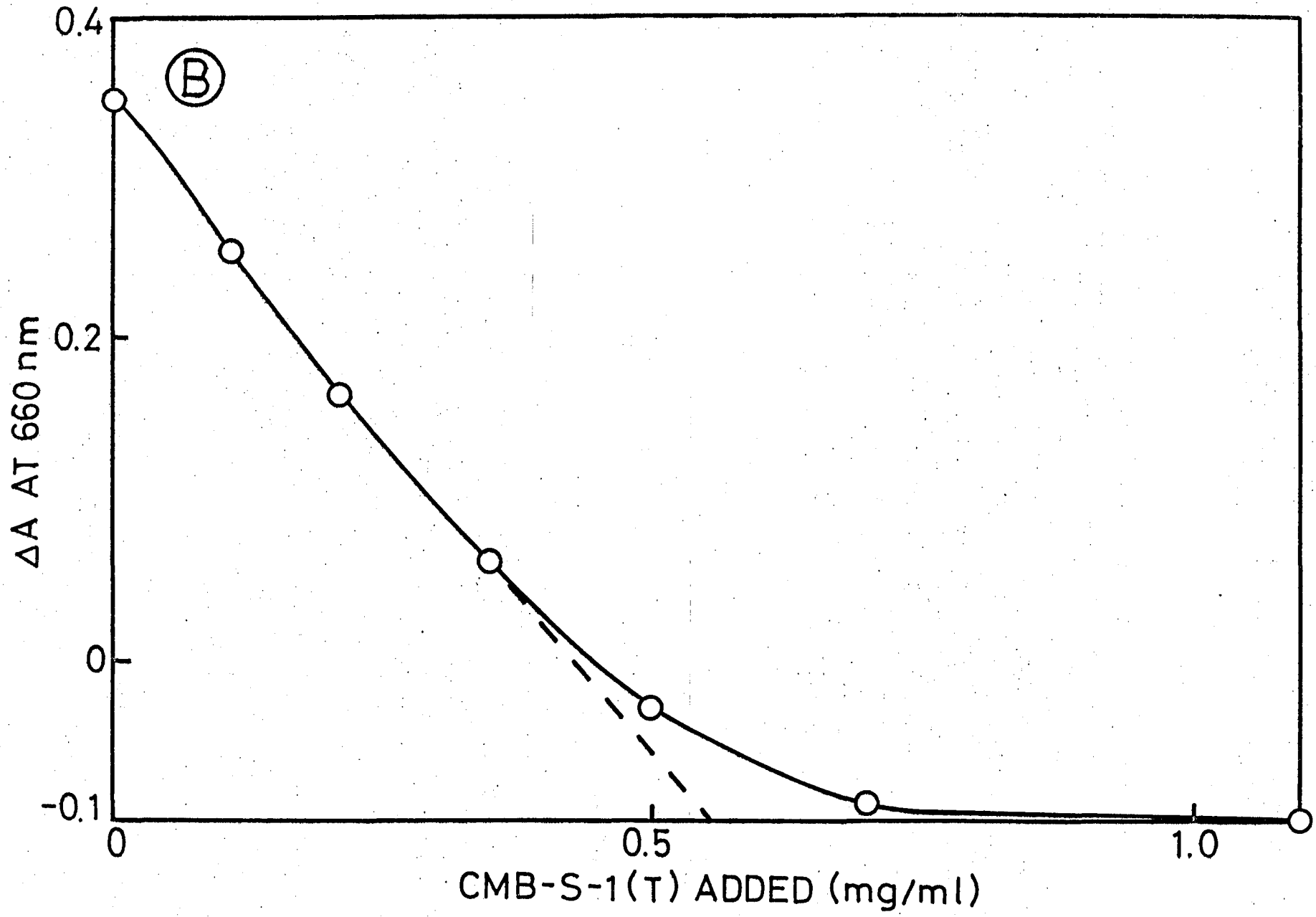

Ⓑ
0.4
0.2
0
-0.1
ΔA AT 660 nm
0
0.5
1.0
CMB-S-1(T) ADDED (mg/ml)

Fig. 4. Effect of CMB-S-1(T) on the Ca^{2+}-dependent ATPase activity of SR. ATPase activities of SR and/or CMB-S-1(T) were measured in the presence of 2 mM ATP, 70 mM KCl, 100 μM $CaCl_2$, 5 mM $MgCl_2$, and 60 mM Tris-maleate at pH 6.5 and 25°C. ○, SR (50 μg/ml SR protein); ×, CMB-S-1(T) (790 μg/ml); ●, SR (50 μg/ml SR protein) and CMB-S-1(T) (790 μg/ml); ---, ○ + ×.

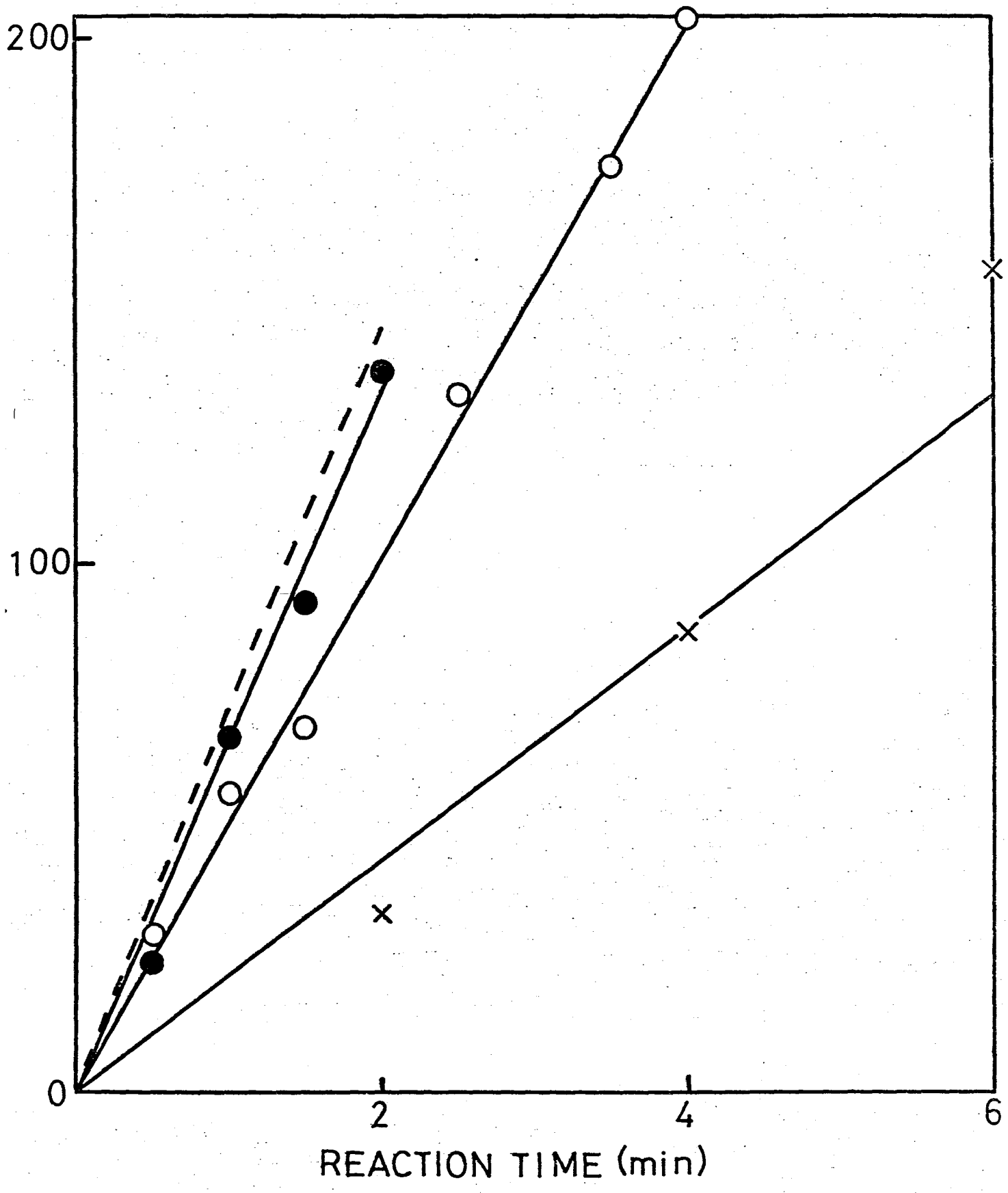
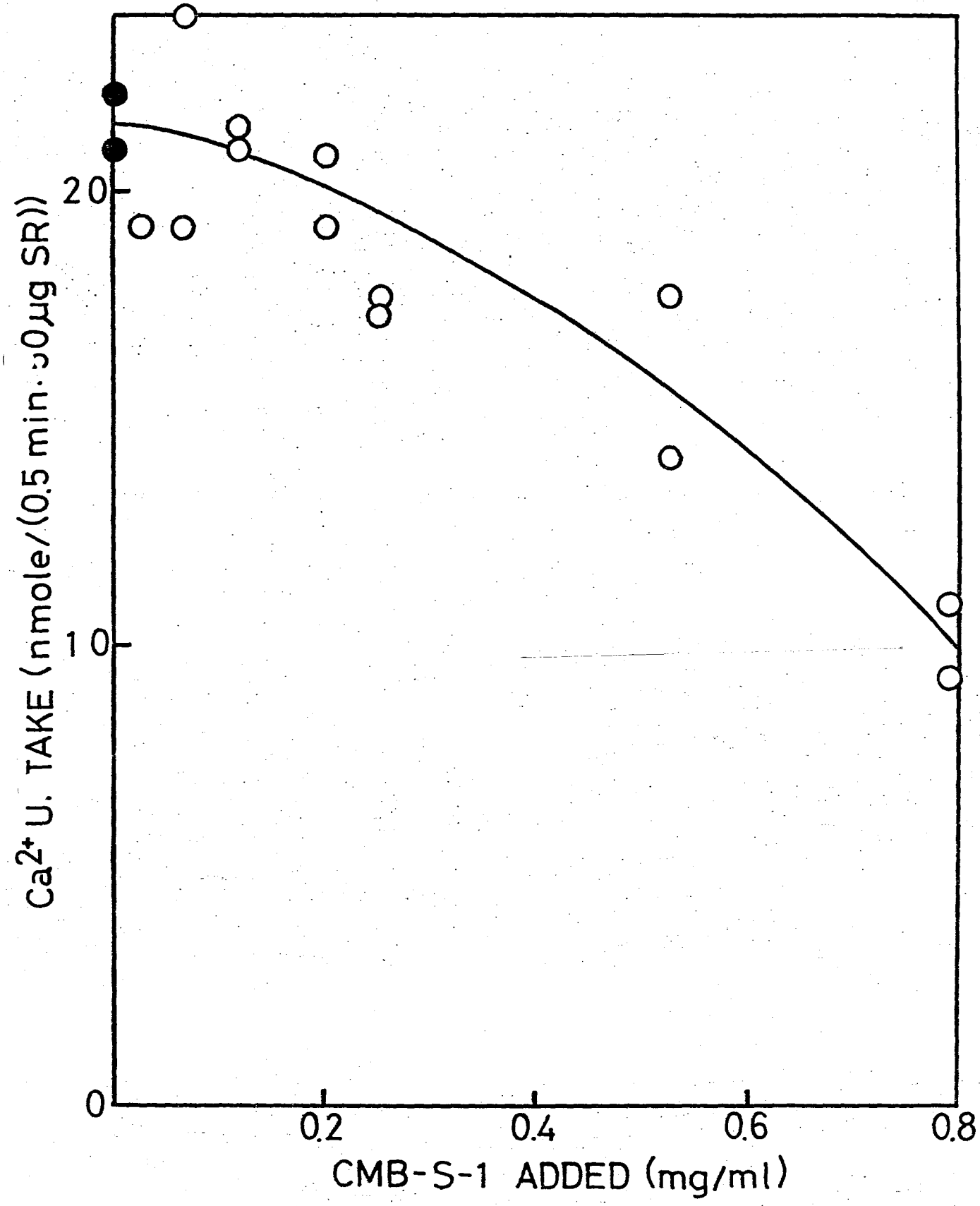

200
100
0
2
4
6
REACTION TIME (min)

Fig. 5. Effect of CMB-S-1(T) on the Ca^{2+}-uptake activity of SR. Ca^{2+}-uptake by SR (50 μg/ml protein) was started by adding 2 mM ATP, and measured by the Millipore filtration method in the presence of 2 mM ATP, 70 mM KCl, 100 μM $^{45}CaCl_2$, 5 mM $MgCl_2$, and 60 mM Tris-maleate at pH 6.5 and 25°C.


Ca2+ U. TAKE (nmole/(0.5 min·50μg SR))
20
10
0
0.2
0.4
0.6
0.8
CMB-S-1 ADDED (mg/ml)

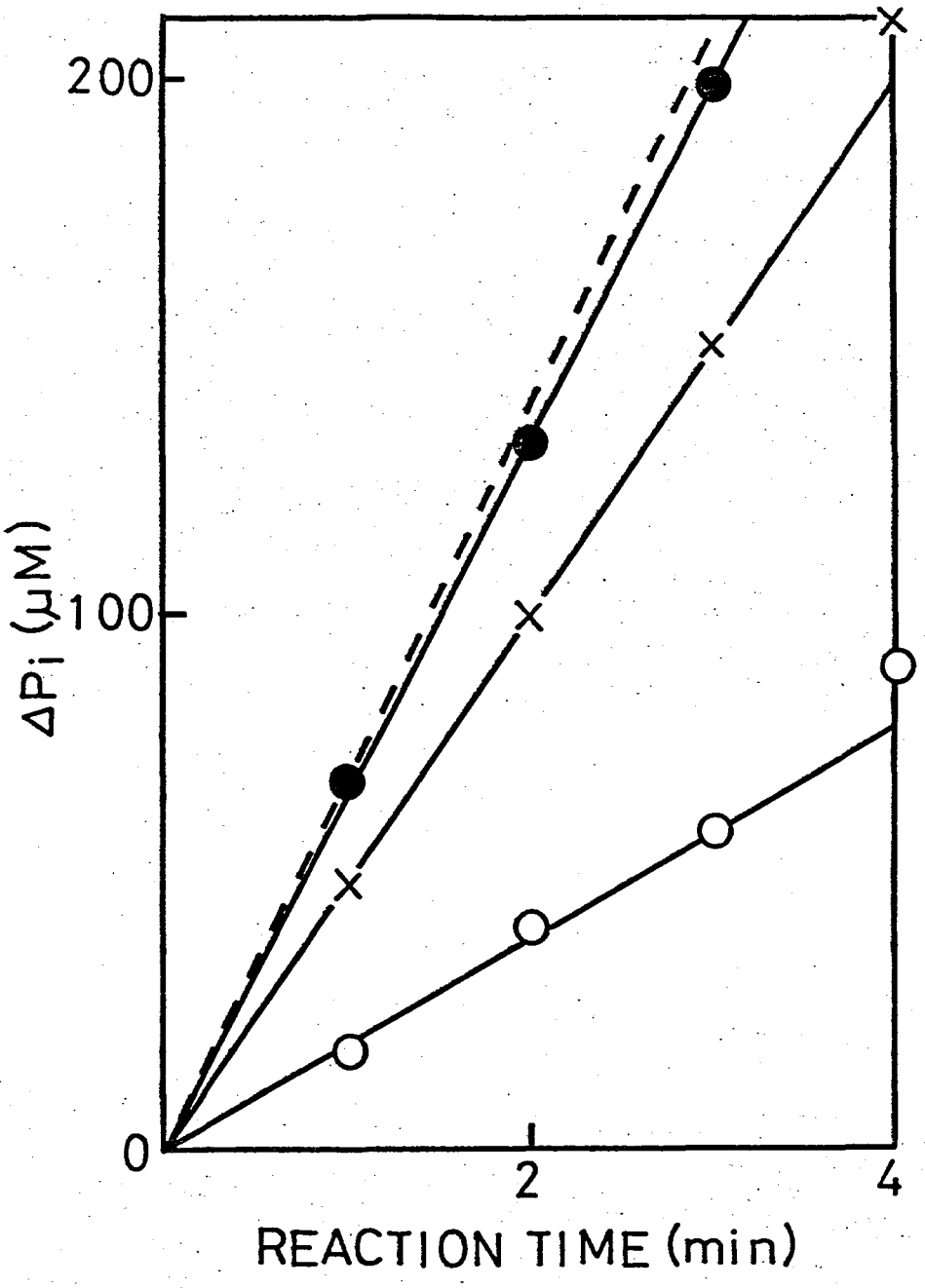


Fig. 6. Effect of CMB-S-1(T) on the activity of Na^+, K^+-dependent ATPase from porcine kidney. ATPase activities of Na^+, K^+-dependent ATPase and/or CMB-S-1(T) were measured in the presence of 2 mM ATP, 140 mM NaCl, 15 mM KCl, 5 mM $MgCl_2$, and 206 mM Tris-HCl at pH 7.6 and 37°C.
○, Na^+, K^+-dependent ATPase (680 ng/ml); ×, CMB-S-1(T) (100 μg/ml);
●, Na^+, K^+-dependent ATPase (680 ng/ml) and CMB-S-1(T) (100 μg/ml):
---, ○ + × .

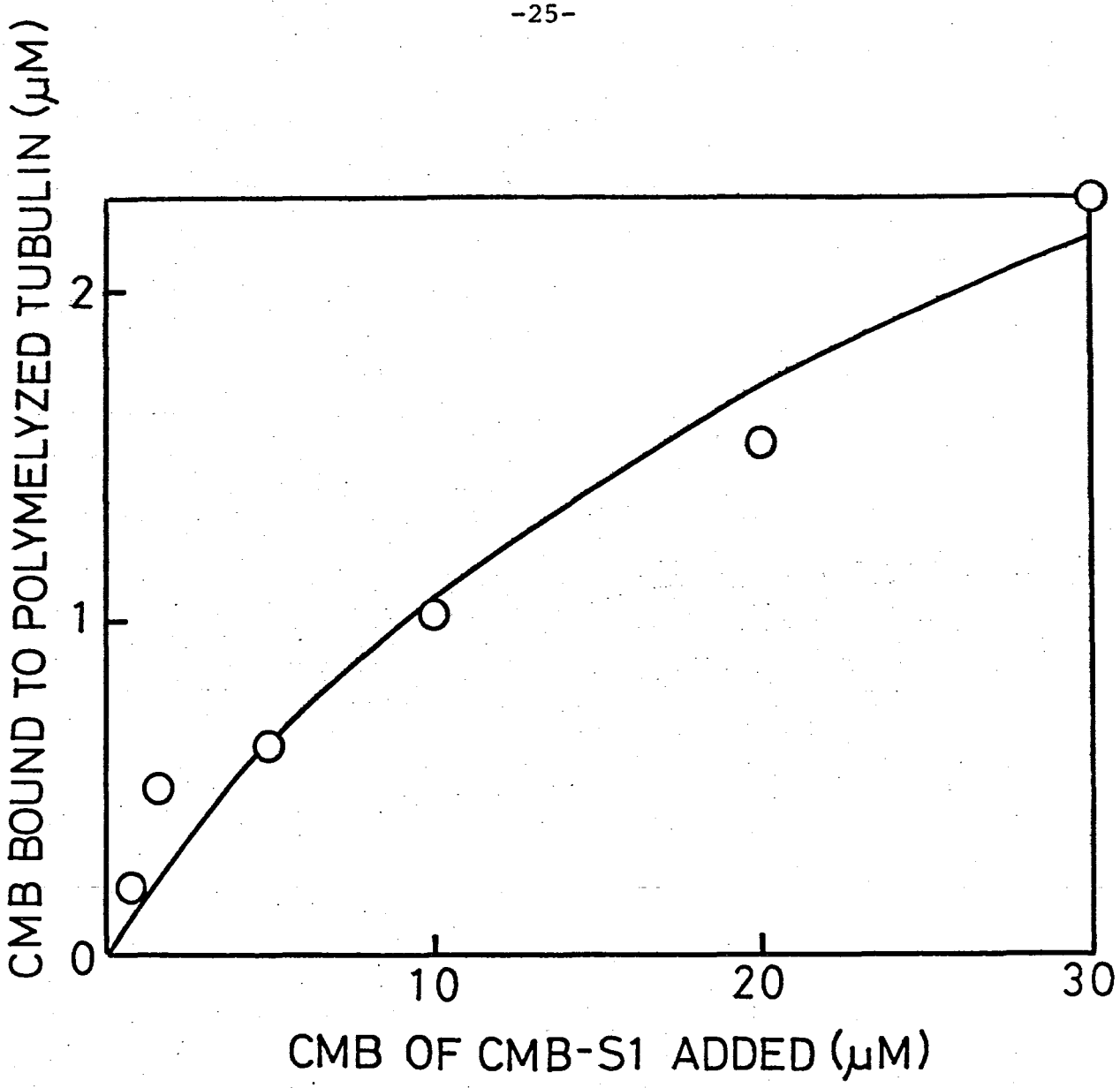
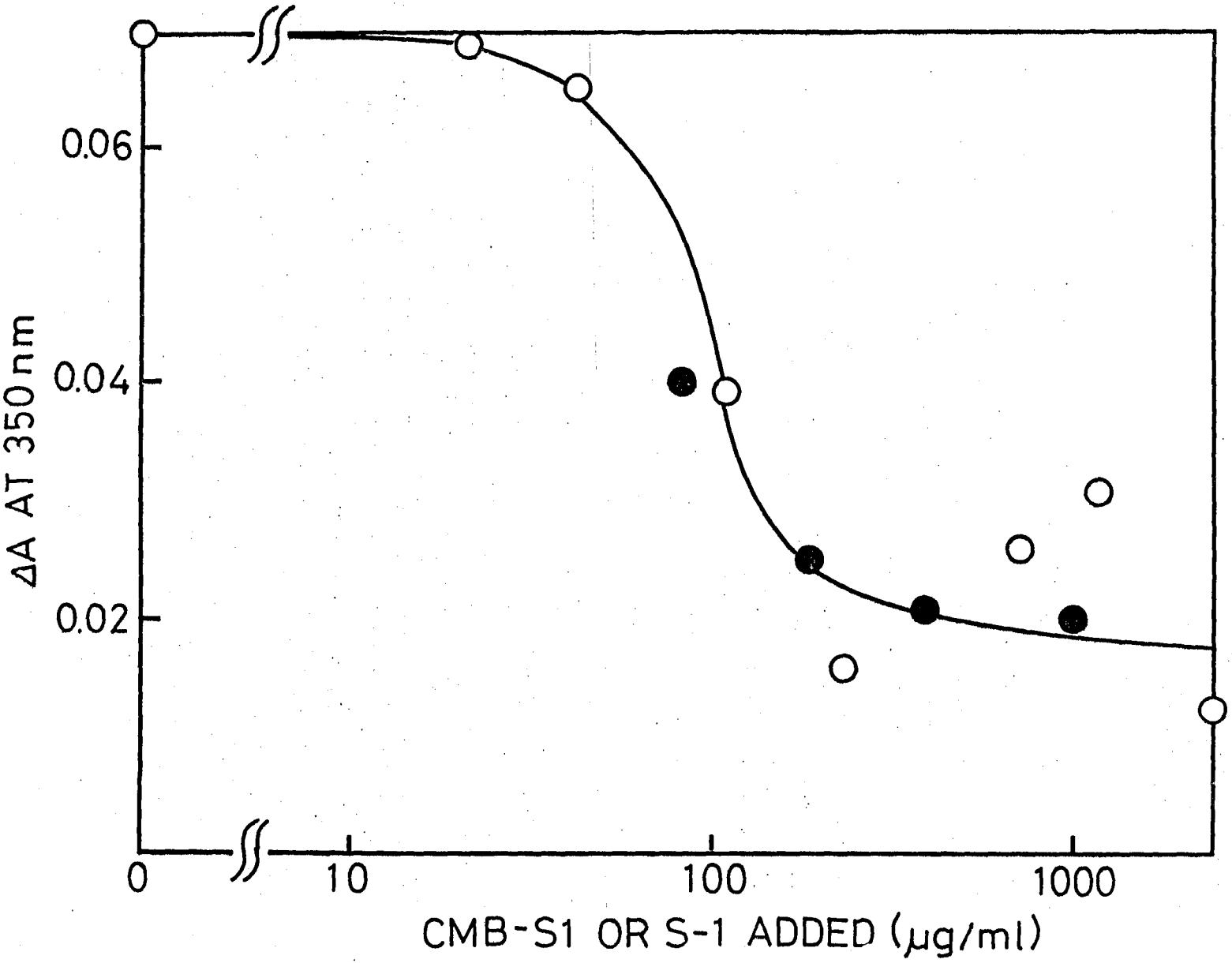


Fig. 7. Coprecipitation of CMB-S-1(T) with polymerized tubulin by ultracentrifugation. Tubulin (1.05 mg/ml= 9.5 μM tubulin heterodimer) was incubated for 20 min at 37°C in GTP-reassembly buffer (1 mM GTP-0.5 mM $MgCl_2$-0.1 M Mes-NaOH at pH 6.5) for polymerization of tubulin. Under the conditions, 74 % (7.0 μM) of total tubulin polymerized. The tubulin was mixed with various amounts of ^{203}Hg-CMB-S-1(T) in GTP-reassembly buffer and centrifuged at 100,000 x g for 1 h at 25°C. ^{203}Hg-CMB-S-1(T) and the tubulin were also centrifuged separately. The amounts of ^{203}Hg-CMB in the supernatant and precipitate were measured by the radioactivity. The amount of ^{203}Hg-CMB coprecipitated with polymerized tubulin was calculated by subtracting the amount of ^{203}Hg-CMB precipitated in the absence of tubulin from that in its presence.

Fig. 8. Effect of CMB-S-1(T) on tubulin polymerization. Tubulin (0.8 mg/ml= 7.3 μM tubulin heterodimer) was mixed with various amounts of CMB-S-1(T) or S-1(T), and preincubated for 5 min at 4°C in GTP-reassembly buffer. Polymerization was initiated by incubation at 25°C, and measured at 25°C in GTP-reassembly buffer by the turbidimetric method. Δ A was defined as the increase of absorbance at 350 nm during the incubation for 60 min at 25°C. ○, CMB-S-1(T); ●, S-1(T).


0.06
0.04
0.02
ΔA AT 350nm
0
10
100
1000
CMB-S1 OR S-1 ADDED (μg/ml)

II. THE ROLE OF ACTIN IN TEMPERATURE-DEPENDENT GEL-SOL TRANSFORMATION OF EXTRACTS OF EHRLICH ASCITES TUMOR CELLS

ABSTRACT

Ehrlich ascites tumor cell extracts gel when warmed to 25°C at pH 7.0 in sucrose solution, and the gel rapidly becomes a sol when cooled to 0°C. This gel-sol transformation was studied quantitatively by determining the volume or the total protein of pellets of gel obtained by low-speed centrifugation. The gelation depended on nucleotide triphosphates, Mg^{2+}, KCl, and a reducing agent. Gelation was inhibited reversibly by 0.5 μM free Ca^{2+} and 25 to 50 ng/ml of cytochalasin B or D but it was not affected by 10 mM colchicine. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis demonstrated that the gel was composed of six major proteins with molecular weights of >300,000, 270,000, 89,000, 51,000, 48,000, and 42,000 daltons. The last component was identified as cell actin by the findings that it had the same molecular weight as muscle actin and bound with muscle myosin and tropomyosin.

The role of actin in gelation was studied by use of actin-inhibitors. Gelation was inhibited by a chemically modified subfragment-1 of myosin, which binds with F-actin even in the presence of ATP, and by bovine pancreatic DNase I, which tightly binds with G-actin. Muscle G-actin neutralized the inhibitory effect of DNase I when added at an equimolar ratio to the latter, and it also restored gelation after its inhibition by DNase I. These findings suggest that gelation depends on actin. However, the extracts showed temperature-dependent, cytochalasin-sensitive, and Ca^{2+}-regulated gelation just like the original extracts when the cell actin in the extracts was replaced by muscle actin, suggesting that components other than cell actin might be responsible for these characters of the gelation.

INTRODUCTION

Recently, it has become possible to study the assembly and disassembly of actin-containing filaments(microfilaments) in vitro. Kane first found that actin filaments form a gel when extracts of sea urchin eggs are warmed in the presence of ATP, ethyleneglycol-bis(β-aminoethylether)-N,N'-tetraacetate(EGTA), and KCl(1,2). Similar temperature-, and ATP-dependent gelation of cell extracts and subsequent contraction of the gel have also been reported for various kinds of non-muscle cells including rabbit pulmonary macrophages (3,4), HeLa cells (5,6), human leukocytes from patients with chronic myelogenous leukemia (7), platelets (8), Acanthamoeba (9,10), Amoeba proteus (11), Dictyosterium discoideum (12,13), and Ehrlich ascites tumor cells(ETC) (14). It is interesting in connection with cytochalasin effects on cells, that cytochalasin B reversibly inhibits the gelation of cell extracts (3,5,10,12). Hartwig and Stossel demonstrated that cytochalasin B reversibly inhibits the gelation of actin caused by the interaction of actin and actin-binding protein (3). In spite of these previous studies, however, much remains to be determined about the gelation, such as its temperature-dependence, Ca^{2+}-regulation, and ATP-requirement, and the protein components involved. The difficulty in studying the gelation is partly due to lack of a reliable quantitative method for measuring the gelation.

This paper reports a quantitative method to measure the extent of gelation or solation of cell extracts. By this method, we studied the temperature-dependent reversible gel-sol transformation of ETC extracts, and also examined the role of actin in the transformation quantitatively by use of two actin inhibitors, a chemically modified subfragment-1 of myosin(CMB-S-1) and bovine pancreatic DNase I.

MATERIALS AND METHODS

ETC - A mutant of ETC, resistant to 8-azaguanine (15), was propagated in the abdomen of ddo mice. After 10 to 13 days of in vivo culture, the cells were harvested from the abdomen. All subsequent treatments of the cells were carried out in ice-cold conditions. The cells were washed three to four times with 0.15 M NaCl containing 15 mM sodium citrate by centrifugation at 120 x g for 3 min to remove ascites fluid and blood cells. Then the cells were suspended in 15 volumes of distilled deionized water to lyse residual erythrocytes. The preparation was mixed by pipetting, and then rapidly centrifuged at 1,150 x g for 10 min. When there was no apparent contamination with blood, the water treatment was omitted. The packed cells were immediately used for preparation of cell extracts.

ETC extracts - Cell extracts were prepared by a procedure based on the methods of Stossel and Hartwig (4), and Weihing (5). Freshly prepared packed ETC were suspended in an equal volume of an extraction solution containing 0.45 M sucrose, 5 mM ATP, 1 mM EGTA, 10 mM DTT, 1 mM phenylmethylsulfonylfluoride(PMSF), 0.6 % Triton X-100, and 20 mM Tris-maleate(pH 7.0 at 25°C), and gently stirred for 10 min under ice-cold conditions. This procedure effectively ruptures all the cells, as monitored by phase microscopy. In some experiments the extraction solution was slightly modified. The ruptured cells were centrifuged at 160,000 x g for 90 min at 3°C, and the resulting clear supernatant fraction(ETC extract) was collected. Unless otherwise mentioned, the supernatant fraction was dialysed against 25 volumes of buffered sucrose solution [0.34 M sucrose and 20 mM Tris-maleate(pH 7.0 at 25°C)] containing 0.25 mM ATP, 0.5 mM EGTA, 0.5 mM DTT, and 0.2 mM PMSF, for 12 hr in ice-cold conditions, with one change of the buffer. After dialysis, the fraction was clarified by centrifugation of 1,150 x g for 30 min at 4°C, and used for experiments. In some experiments, ETC extracts were desalted by chromatography on

Sephadex G-25.

Rabbit Skeletal Muscle Proteins - Myosin was prepared from rabbit skeletal white muscle by the method of Perry (16). Heavy meromyosin(HMM) was prepared by tryptic digestion of myosin by the method of Szent-Györgyi (17) with slight modification (18). Subfragment-1 of myosin(S-1) [MW=1.2 x 10^5] was prepared by tryptic digestion of HMM, followed by chromatography on Sephadex G-200 (19,20).

Myosin was chemically modified with p-chloromercuribenzoate(CMB) as described previously (20). S-1 modified with CMB(CMB-S-1) was prepared by two-step tryptic digestion of the modified myosin by the procedure used for preparation of S-1.

Purified G-actin[MW=4.2 x 10^4] was prepared from an acetone powder of rabbit skeletal white muscle by the method of Spudich and Watt (21). F-actin was prepared by polymerizing G-actin by adding 1 mM $MgCl_2$ and 50 mM KCl.

Tropomyosin[heterodimer, MW=6.8 x 10^4] was extracted with 1 M KCl from the residue of rabbit skeletal white muscle remaining after extraction of actin, and purified in the presence of 10 mM β-mercaptoethanol by a procedure based on the method of Bailey (22). Briefly, the 1 M KCl extracts were acidified to pH 4.5 with 1 N HCl. The resulting precipitate was collected by centrifugation and dispersed in 10 volumes of water, and the pH was readjusted to 7.0 with 1 N NaOH. The solution was clarified by centrifugation and protein was salted out with 41 % to 70 % saturation of $(NH_4)_2SO_4$. Then, isoelectric fractionation at pH 4.5 in the presence of 1 M KCl followed by fractionation at pH 7.0 with 53 % to 60 % saturation of $(NH_4)_2SO_4$ was repeated three times.

Reagents - Bovine pancreatic DNase I (DL-CL) [MW=3.1 x 10^4] was purchased from Sigma Chemical Co. It was more than 90 % pure as judged by electrophoresis

on sodium dodecyl sulfate(SDS)-polyacrylamide gel. Cytochalasin B was purchased from Aldrich Chemical Co., Inc., and cytochalasin D was a gift from Shionogi Co. CMB, 5,5'-dithio-bis(2-nitrobenzoate)(DNTB), and iodoacetamide(IAM) were obtained from Nakarai Chemicals, Ltd., and p-chloromercuriphenylsulfonate(CMBS) from Sigma Chemical Co. N-Ethylmaleimide (NEM) was obtained from Wako Pure Chemical Industries, Ltd. PMSF was purchased from Sigma Chemical Co. All other chemicals were reagent grade.

Temperature-Dependent Gel-Sol Transformation of ETC Extracts - The temperature-dependent gel-sol transformation of ETC extracts was measured by determining the gel volume or total protein in the gel. Gelation was defined as the increase of the gel volume or total protein in the gel, and solation as their decrease. Gelation was induced by warming the preparation to 25°C in a small test tube graduated in 25 µl(6 x 80 mm), and solation by cooling it to 0°C. After incubation for gelation or solation, extracts were separated into gel and liquid layers reproducibly by centrifugation at 1,150 x g for 30 min or at 2,060 x g for 10 min. After removing the liquid layer carefully with a Pasteur pipette, the gel volume and/or total protein in the gel was determined. To obtain reproducible results, gelled extracts were not stirred: this is very important because the gels are fragile and easily broken, giving white precipitates when stirred.

SDS-polyacrylamide Gel Electrophoresis - Polyacrylamide gel electrophoresis in the presence of SDS was performed on gels containing 5 % or 7.5 % acrylamide, 0.1 % SDS, and 50 mM sodium phosphate buffer at pH 7.2, by the method of Weber and Osborn (23), with slight modification (19). After electrophoresis, the gels were stained at 45°C for 1 hr with 0.1 % Coomassie Brilliant Blue R 250. Electrophoreograms were scanned with a Gilford Model 2000 spectrophotometer at a wavelength of 550 nm.

The molecular weights of polypeptides were calculated by the method of Weber and Osborn (23) using the following proteins as standards: human erythrocyte spectrin[MW=2.4 x 10^5 and 2.2 x 10^5], rabbit skeletal muscle myosin[heavy chain, MW=2.0 x 10^5], E. coli β-galactosidase[MW=1.3 x 10^5], rabbit skeletal muscle α-actinin[MW=9.0 x 10^4], bovine serum albumin [MW=6.8 x 10^4], rabbit muscle pyruvate kinase[MW=5.7 x 10^4], bovine brain tubulin[MW=5.5 x 10^5], pig cardiac muscle fumarase[MW=4.9 x 10^4], rabbit skeletal muscle actin[MW=4.2 x 10^4], and rabbit muscle glyceraldehyde-3-phosphate dehydrogenase[MW=3.6 x 10^4].

Protein composition was estimated roughly by quantitative densitometry of SDS-polyacrylamide gels stained with Coomassie Blue after electrophoresis. Using the procedure of Gorovsky et al. (24), the peak areas of polypeptides in the densitometric scans were cut out and weighed on an analytical balance.

Protein Determination - Protein concentration was estimated by the biuret reaction (25) or by the Copper-Folin method (26) with bovine serum albumin as a standard. Proteins were precipitated from crude extracts with 5 % trichloroacetic acid(TCA), and contaminating nucleic acids were hydrolysed by incubating the precipitates at 90°C for 10 min in 5 % TCA. The precipitates were washed three times with 5 % TCA, dissolved in 0.1 N NaOH, and used for protein determination. The concentration of tropomyosin was estimated from the ultraviolet absorbance using a value of $A^{1\ \%}_{277} = 3.3$ (27).

RESULTS

GENERAL FEATURES

Quantitative Measurement of the Gelation of ETC Extracts - To study the gel-sol transformation of ETC extracts in detail, we first establish a quantitative method to measure the extent of gelation of the extracts. Recently, the gelation of cell extracts of rabbit pulmonary macrophages (3), Acanthamoeba (9), human leukocytes (7), and Amoeba proteus (11) was measured turbidimetrically. This method, however, was not applicable to ETC extracts because the turbidity of the extracts easily changed independently of the extent of gelation. Therfore, in this study we measured the gelation of ETC extracts quantitatively by the following procedure. After incubation for gelation or solation, the extracts were separated into gel and liquid layers by low-speed centrifugation without stirring. After removing the liquid layer, the gel volume or the total protein in the gel was measured.

Fig. 1 shows the linear relations between the protein concentrations of ETC extracts and the extent of gelation, measured as the volume or total protein of the gel. Above a critical protein concentration of the extracts, the gel volume and the percentage of total protein in the gel increased linearly until saturation, with increase in the protein concentration of the extracts. This shows that we can measure the extent of gelation of ETC extracts quantitatively, as the volume or total protein of the gel.

Temperature-Dependent Gel-Sol Transformation of ETC extracts - we studied the temperature-dependent gel-sol transformation of ETC extracts quantitatively by the centrifugation method described above. Fig. 2A shows the time course of gelation induced by warming, and subsequent solation induced by cooling. ETC extracts formed a gel when warmed to 25°C, and the gel liquified when cooled to 0°C. The rate and extent of gelation increased with increase

in the protein concentration of the extracts. At a protein concentration of about 5 to 6 mg/ml, the extracts gelled within 30 min when warmed to 25°C, and the gelled extracts liquified rapidly within 15 min when cooled to 0°C. When warmed to 25°C again, the liquified extracts gelled again within 5 min; that is, more rapidly than the original extracts. Even if F-actin prepared from rabbit skeletal muscle was present in the reaction mixtures for gelation, the extracts never gelled without warming. This indicates that the presence of F-actin only is not sufficient for inducing gelation.

Fig. 2B shows the reversibility of the temperature-dependent gel-sol transformation. When ETC extracts in reaction mixtures were alternately warmed to 25°C for gelation and cooled to 0°C for solation, the extracts showed reversible gel-sol transformation. This transformation cycle could be repeated at least 6 times. On repeating the cycle the extent of gelation decreased and unliquified material increased. Addition of 1 mM Mg-ATP partially restored the extent of gelation of extracts after several transformation cycles (Fig. 2B), suggesting the ATP-requirement for gelation described below in detail.

Ion- and Nucleotide-Requirements for the Gelation - To show ion- and nucleotide-requirements for the gelation, we rapidly desalted the extracts by chromatography on Sephadex G-25, and then added various amounts of KCl, $MgCl_2$, or various nucleotides and phosphoric compounds to the reaction mixtures for gelation. Fig. 3 shows the dependence of gelation on the concentrations of KCl and $MgCl_2$. When warmed in the absence of either KCl or $MgCl_2$ the extracts formed only a slight precipitate, showing no gelation. The extent of gelation increased linearly with increase in the KCl concentration and became maximal at 75 mM KCl; high concentrations of KCl were rather inhibitory. The extent of gelation also increased with increase in the $MgCl_2$ concentration, and in the presence of 1 mM ATP, it reached a saturation

level at 2 mM $MgCl_2$.

Table 1 shows the nucleotide-requirements for the gelation. When added to reaction mixtures at concentrations of 1 mM, nucleotide triphosphates, such as ATP, CTP, GTP, ITP, and UTP, were all effective for sustaining the gelation, whereas inorganic phosphate, pyrophosphate, AMP, and ADP had no effect. Fig. 4 shows the dependence of gelation on the concentration of ATP. The gelation was detectable on addition of 10 μM ATP. Addition of 250 μM to 1 mM ATP resulted in maximal gelation, but high concentrations of ATP were rather inhibitory. In addition to sustaining gelation, ATP apparently stabilized the gelation activity of ETC extracts, because the extracts irreversibly lost activity in the absence of ATP but not in its presence.

Effects of Reducing or SH-Blocking Reagents on the Gelation - When ETC extracts were dialysed for 12 hr against buffered sucrose solution without a reducing agent, they formed insoluble material containing 42,000-dalton polypeptide, presumably cell actin, as a main protein component, and lost gelation activity. Addition of DTT did not restored the lost activity. Inclusion of DTT in the dialysis solution stabilized the activity, presumably by reducing the SH-groups of protein components.

Fig. 5 shows the inhibitory effects of SH-blocking reagents on the gelation. When added to reaction mixtures containing 0.5 mM DTT just before warming to 25°C, 1 mM DTNB, 2 mM NEM, 2 mM CMB, or 2 mM CMBS caused complete inhibition, and 7 mM IAM caused 50 % inhibition. The gelation inhibited by 1 mM DTNB or 2 mM CMBS was restored to 72 % and 61 %, respectively, of the control value without the reagents by adding 39 mM DTT. These findings indicate that SH-groups of protein components involved in the gelation should be kept reduced for their functional interactions resulting in gelation.

Regulation of the gelation by Free Ca^{2+} - When EGTA was not included in the solutions for extraction, dialysis, and incubation of ETC extracts for gelation, the gelation was partially or almost completely inhibited. This inhibition seemed to be due to contaminating calcium or heavy metals, because the inhibited gelation was restored when EGTA was added to the reaction mixture for gelation. This fact also indicates the reversibility of the inhibition. To examine the possible role of Ca^{2+} in regulating the gelation of ETC extracts, we first measured the extent of gelation at various concentrations of free Ca^{2+} obtained with Ca-EGTA buffer. Fig. 6 shows that low concentrations of free Ca^{2+} inhibited the gelation: 50 % inhibition was observed with 0.06 μM Ca^{2+} and complete inhibition with 0.5 μM Ca^{2+}. In addition, Table 2 shows that the gelation inhibited by low concentrations of free Ca^{2+} was partially restored by adding excess EGTA.

We also examined the solation of gelled extracts by Ca^{2+}. Table 3 shows the results. When 1 ml of $CaCl_2$ in 100 mM Tris-HCl buffer (pH 7.6) was overlaid on 1 ml of gelled extracts containing 1 mM EGTA, for 1.5 hr at 25°C, 1 mM $CaCl_2$ solution (final concentration, about 10^{-6} M free Ca^{2+}) caused 50 % reduction of the gel volume. Because ETC extracts do not show contraction phenomena such as those reported for ther cell extracts, this suggests that low concentrations of free Ca^{2+} caused weak solation of gelled extracts.

Effects of Cytochalasins and Colchicine on the Gelation - Fig. 7 shows the inhibition of gelation by cytochalasins, which are metabolites of mould that affect microfilaments. Cytochalasin B and D were equally effective for inhibiting the gelation, and both inhibited the gelation of ETC extracts (5.8 mg/ml extract protein) by 50 % at 25 ng/ml and completely at 50 ng/ml (about 0.1 μM). The amounts of cytochalasins required for inhibition increased with increase in the protein concentration of the extracts.

Table 4 shows the reversibility of the inhibition of gelation by cytochalasin B. When extracts treated with 0.1 μg/ml cytochalasin B were extensively dialysed against buffered sucrose solution, the gelation activity was partially recovered. In addition Table 3 shows that when cytochalasin B was overlaid on gelled extracts it caused weak solation of the extracts.

In contrast to cytochalasins, colchichine, which prevents the assembly of tubulins into microtubules, did not affect the gelation even at a concentration of 10 mM, suggesting that assembly of tubulins was not involved in the gelation.

INVOLVEMENT OF ACTIN

Protein Composition of the Gel - After completion of gelation, the gelled extracts were broken by vigorous agitation in a voltex mixer, and then separated into a small volume of broken gels and supernatants by low-speed centrifugation. Then, the protein compositions of the broken gels, the supernatants, and the original extracts were analysed by SDS-polyacrylamide gel electrophoresis. The relative amounts of the major gel components in the gels and the original extracts were also estimated roughly by quantitative densitometry of SDS-polyacrylamide gels stained with Coomassie Blue after electrophoresis.

When ETC extracts immediately after preparation were gelled by warming to 25°C for 1 hr, their pellets contained polypeptides of 42,000, 48,000, 51,000, 89,000, and 270,000 daltons, and a very large polypeptide (VLP, the largest component easily detected on SDS-polyacrylamide gels of ETC extracts stained with Coomassie Blue after electrophoresis) with a molecular weight of more than 300,000 daltons, with numerous minor unidentified polypeptides. Washing the gels several times with gelation solution at 25°C removed minor polypeptide bands, but did not significantly

alter the relative amounts of the major polypeptide bands (Fig. 8d)[1]. The amount of the polypeptides of 42,000, 51,000, 89,000, and 270,000 daltons in the supernatants[2] were less than in the original extracts, and most of the VLP in the original extracts was concentrated in the gels (Fig. 8a and 8b). Fig. 9 and Table 5 show the relative amounts of the major polypeptides of the washed gels.

When the gels were prepared from extracts dialysed against buffered sucrose solution or stored under ice-cold conditions in the absence of PMSF for 12 hr, the gels contained only two major polypeptides of 42,000 and 230,000 daltons with a trace of the VLP (Fig. 9 and Table 5). The extracts before gelation contained only a trace of the VLP but there was much more of the 230,000-dalton polypeptide than in the ETC extracts just after preparation. This suggests that the VLP was degraded into 230,000-dalton polypeptide by endogenous protease. If ETC extracts were further dialysed or stored, they lost their gelation activity and these extracts were found to have scarcely any VLP or 230,000-dalton polypeptides.

The polypeptide of 42,000 daltons co-migrated with rabbit muscle actin on SDS-polyacrylamide gels. To determine whether it was in fact cell actin, first we examined its selective binding with muscle myosin. ETC extracts and rabbit muscle myosin were extensively dialysed together against a solution of low ionic strength without ATP, and then centrifuged at low speed in the absence or presence of ATP. Then the supernatants

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1. Washing the gels also removed some of the 270,000-dalton polypeptide, but a significant amount of the polypeptide remained in the gels even after extensive washing.

2. The supernatants prepared without the agitation also showed the same SDS-polyacrylamide gel pattern.

and washed pellets were analysed by SDS-polyacrylamide gel electrophoresis. In the absence of ATP, about 40 % of the 42,000-dalton polypeptide selectively co-precipitated with myosin. On the other hand in the presence of ATP, neither this polypeptide nor muscle myosin precipiteted. This indicates that at least 40 % of the 42,000-dalton polypeptide was functional cell actin.

Second, we studied the binding of muscle tropomyosin with presumptive cell actin. Rabbit muscle tropomyosin, up to 0.6 mg/ml, did not affect the gelation. So, the gelled extracts were made in the presence of various amounts of muscle tropomyosin. Then, the protein composition of the washed gels was analysed by SDS-polyacrylamide gel electrophoresis. Fig. 10 shows that muscle tropomyosin was co-precipitated with the gels by low-speed centrifugation. Assuming that all the 42,000-dalton polypeptide in the gel was cell actin and that muscle tropomyosin was co-precipitated with the gels by binding with cell F-actin, quantitative densitometry of SDS-polyacrylamide gels stained with Coomassie Blue after electrophoresis showed that the maximal amount of tropomyosin co-precipitated with cell F-actin in the gels was about 0.18 mole per mole of actin monomer. This value is consistent with the value of 0.15 mole tropomyosin per mole of actin monomer reported for the binding of rabbit muscle tropomyosin with rabbit muscle F-actin (28,29). This indicates that most of the 42,000-dalton polypeptide in the gels was cell F-actin.

Functional Involvement of Actin - Cell F-actin is the main protein component in the gels. Thus, to determine whether actin is functionally involved in the gelation, we used bovine pancreatic DNase I and CMB-S-1 as actin inhibitors.

DNase I binds very tightly with an equimolar amount of G-actin (30,31) and inhibits the polymerization of G-actin to F-actin (32). In addition,

it depolymerizes F-actin to G-actin (32,33). Thus, DNase I can block F-actin-dependent functions by forming an inactive equimolar G-actin-DNase I complex.

Fig. 11 shows the inhibition of gelation by DNase I. ETC extracts and DNase I were incubated together at 0°C for 30 min to allow the formation of a G-actin-DNase I complex, and then warmed to 25°C for gelation. The extent of gelation decreased linearly with increase in the DNase I concentration, and addition of 75 μg/ml (2.4 μM) DNase I resulted in complete inhibition of gelation of ETC extracts (4.0 mg/ml extract protein).

To determine whether the inhibition of gelation by DNase I resulted from the formation of an equimolar cell G-actin-DNase I complex, we examined the neutralization of the inhibitory function of DNase I by muscle actin and the reversal of the inhibition by muscle actin. Fig. 12A shows that 196 μg/ml (4.6 μM) muscle G-actin neutralised the inhibitory function of 148 μg/ml (4.9 μM) DNase I when DNase I was preincubated with G-actin at 0°C for 10 min before its addition to the reaction mixtures for gelation. Fig. 12B shows that muscle G-actin also restored gelation after its inhibition by DNase I. The amount of actin required for complete recovery of the gelation inhibited by 296 μg/ml (9.4 μM) DNase I was calculated as 585 μg/ml (14 μM) by extrapolation of the results. The molar ratio of actin to DNase I of 1.5 required for recovery of the gelation is consistent with the value of 1.0 for neutralization described above. These facts clearly show that DNase I inhibited the gelation by forming an equimolar cell G-actin-DNase I complex, and that muscle actin sustained the gelation as effectively as cell actin.

S-1 binds to F-actin in the absence of ATP, but dissociates from it on addition of ATP. CMB-S-1 is composed of equimolar amount of S-1A[1]

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1. S-1A, S-1 without the initial burst of P_i-liberation; S-1B, S-1 with the P_i-burst. For further explanation, see Inoue, A., and Y. Tonomura. 1976. J. Biochem. (Tokyo). 79, 419-434.

modified with CMB(CMB-S-1A) and S-1B not modified with CMB (20). In contrast to S-1, CMB-S-1A binds with F-actin very tightly and stoichiometrically in a ratio of 1 mole of CMB-S-1A per mole of actin monomer, even in the presence of ATP. Thus, finally it occupies all the myosin binding sites of F-actin, and inhibits the ability of F-actin to interact with myosin. Because CMB-S-1 specifically binds with F-actin, it can be used as an actin-specific inhibitor (20, 34).

Fig. 13 shows the effects of CMB-S-1 and S-1 on the gelation. When reaction mixtures for gelation contained high concentrations of ATP[1], gelation of ETC extracts (8.5 mg/ml extract protein) was inhibited 50 % and completely by 0.38 mg/ml and 0.66 mg/ml of CMB-S-1, respectively, but only slightly by 1.9 mg/ml of S-1. This difference in the effects of S-1 and CMB-S-1 indicates that the inhibition by CMB-S-1 was due to the tight binding of CMB-S-1A with cell F-actin, because the gelation was measured in the presence of high concentrations of ATP.

In consistent with the results of the actin-inbihitor experiments, the gelation activity of ETC extracts was lost partially or almost completely when cell F-actin was removed from the extracts by ultracentrifugation after polymerization of cell actin, and the actin-deficient extracts, however, regained the full activity on addition of muscle actin. The extent of the gelation increased linearly with increase in the amount of muscle actin added. (Fig. 14).

Gelation of ETC Extracts Reconstituted from Actin-Blocked or Actin-Deficient ETC extracts by Addition of Muscle Actin - To determine whether cell actin is responsible for the temperature-dependence,

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1. At low concentrations of ATP, S-1 also inhibited the gelation. This inhibition, however, probably resulted from mere ATP deficiency.

cytochalasin-sensitivity, or Ca^{2+}-regulation of the gelation, we inactivated cell actin in ETC extracts by addition of excess DNase I, or removed it from the extracts by ultracentrifugation after its polymerization, and then reconstituted extracts with gelation activity by addition of muscle G-actin, and examined these actin reconstituted extracts for reversibility of temperature-dependent gel-sol transformation, cytochalasin-sensitivity, and Ca^{2+}-regulation of gelation.

Irrespective of whether the extracts were reconstituted from actin-blocked extracts or actin-dificient extracts, the actin reconstituted system behaved like the original extracts. (Fig. 15 and Table 6).

DISCUSSION

Quantitative Measurement - In this paper, we established a very simple quantitative method to measure the gelation or solation of cell extracts. With this method it became possible to study the gel-sol transformation of ETC extracts quantitatively and even to examine the stoichiometry of actin in the gel-sol transformation. Therefore, this method is useful in studies on the interactions between protein components in the gel-sol transformation of cell extracts.

Protein Components - SDS-polyacrylamide gel electrophoresis showed that the main components of ETC extract gels had molecular weights of 42,000, 48,000, 51,000, 89,000, 270,000, and more than 300,000 daltons (VLP). Washing the gels several times with gelation solution did not significantly alter the relative amounts of the major polypeptides. This indicates that these polypeptides are either structural components or associated with the true structural components. The polypeptide of 42,000 daltons was identified as cell actin by the facts that it had the same mobility on SDS-polyacrylamide gel as muscle actin, and that it bound with muscle myosin and muscle tropomyosin. However, the protein components and their functions remain to be established.

Functional Involvement of Actin -Bovine pancreatic DNase I inhibited gelation. Muscle G-actin neutralized the inhibitory function of DNase I when added in equimolar amount, and addition of muscle actin also restored the gelation inhibited by DNase I. These facts indicate that DNase I inhibited the gelation by forming an equimolar G-actin-DNase I complex, because DNase I binds tightly with an equimolar amount of G-actin (30,31) and inhibits the polymerization of actin (32). Because the association between G-actin and DNase I is very tight and almost irreversible (30,31), and DNase I also depolymerizes F-actin to form a G-actin-DNase I complex (32,33), in the presence of excess DNase I essentially all the cell actin

is probably fixed as an equimolar complex. Thus, the concentrations of free G-actin and F-actin, if any, are probably very low. Therefore, inhibition of gelation by DNase I was caused by lack of available free G-actin or F-actin.

CMB-S-1(CMB-S-1A), which binds with F-actin even in the presence of ATP (20), inhibited the gelation, whereas S-1, which dissociates from F-actin in the presence of ATP, did not. Because the gelation was measured in the presence of ATP, this differential inhibition of gelation indicates that CMB-S-1 inhibited the gelation as a result of the tight binding of CMB-S-1A with cell F-actin, suggesting the functional involvement of F-actin in the gelation. This view is consistent with the following facts: (1) The gelation depended on the conditions for polymerization of actin, namely, the presence of ATP, Mg^{2+} and KCl, and warming. (2) Removal of cell F-actin from ETC extracts by ultracentrifugation resulted in loss of the gelation activity. (3) Muscle tropomyosin bound stoichiometrically with cell actin in the gels, indicating that most of actin in the gels was F-actin. (4) The cyclic peptide phalloidin, a toxic component of the toad stool Amanitia phalloides, which induces polymerization of actin and which prevents depolymerization of F-actin (35,36), scarcely affected the gelation (Ishiura and Okada, in preparation). However, it is possible that another type of actin, distinct from G-actin of F-actin, might be involved in the gelation (cf. 12).

The content of functional actin in ETC extracts can be calculated from data on the inhibition of the gelation by CMB-S-1 or DNase I. Assuming that 2 moles of CMB-S-1 (1 mole of CMB-S-1A) inhibits the function of 1 mole of actin monomer in the gelation, the results shown in Fig. 13 indicate that functional actin comprised about 1.4 % of the total protein of ETC extracts. Similarly, if DNase I inhibits the functin of actin

in the gelation when added in equimolar amount, the results shown in Fig. 11 indicate that functional actin comprised about 2.5 % of the total protein of the extracts. These values are consistent with each other, although quantitative densitometry of SDS-polyacrylamide gels stained with Coomassie Blue after electrophoresis demonstrated that the component of 42,000 daltons presumed to be cell actin comprised about 14 %[1] of the total stainable protein of ETC extracts. The band of 42,000-dalton polypeptide may contain denatured actin or a protein other than actin, because some of the polypeptide could not be co-precipitated with muscle myosin.

Interactions between Actin, Myosin, and Gelation Protein - The inhibition of gelation by CMB-S-1 also indicates that when all the myosin-binding sites of F-actin are occupied by CMB-S-1A (34), it is difficult for a protein component inducing gelation of F-actin (gelation protein) to interact functionally with the actin. Thus, it is possible that binding of myosin with F-actin might prevent the functional interaction of the gelation protein with F-actin, and vice versa. In accordance with this possibility, Maruta and Korn reported that Acanthamoeba proteins that induce the gelation of F-actin inhibit actin-activated HMM-ATPase activity (37). Weihing reported that HMM inhibits the gelation of HeLa cell extracts (6). In addition, Condeelis and Taylor reported that treatments that inhibit the gelation and induce the solation of Dictyosterium extracts enhance the

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1. This value might be an overestimation, because the content of functional actin (the 42,000-dalton polypeptide co-precipitated with muscle myosin) estimated by quantitative densitometry of SDS-polyaclylamide gels stained with Coomassie Blue after electrophoresis was two to three times greater than that calculated from data on the inhibition of the gelation by CMB-S-1 or DNase I.

functional interaction of myosin and actin (contraction) in the extracts (12). However, in contrast to these observations, Stoossel and Hartwig reported that actin-binding rpotein isolated from rabbit pulmonary macrophages, which causes the gelation of F-actin, does not affect the macrophage myosin-ATPase activity activated by actin and a cofactor protein (4). Therefore, further studies are necessary on the interactions between actin, myosin, and a gelation protein such as actin-binding rpotein.

Ca^{2+}-regulation - We showed that concentrations of 10^{-8} to 10^{-6} M free Ca^{2+} control the gelation. This concentration range is physiologically significant, since in animal cells the concentration of Ca^{2+} in the cytosol or free cytoplasm is in the range of 10^{-8} to 10^{-5} M (38-43). Thus, it is possible that intracellular free Ca^{2+} controls the interaction between actin and gelation protein, namely, the assemble and disassembly of actin-containing filaments (microfilaments) in cells, and therby the cell shape (cf. 12,13). In fact, recently, Goshima et al. found that micromolar free Ca^{2+} controls the cell shape and rhythmical beating of cultured mouse cardiac cells (44).

In some non-muscle cells, Ca^{2+}-regulation of actin-myosin interactions seems to be carried out by the troponin-tropomyosin system (45-47, see also 48 for review). But the system has not yet been found in many non-muscle cells. Ca^{2+}-regulation of actin-myosin interaction via gelation protein might explain the Ca^{2+}-regulation of non-muscle cell contractility demonstrated in intact cells (49-51), cell models (52,53), and isolated cytoplasm (11, 54-57 see also 48,58 for review). Condieelis and Taylor have also proposed the same mechanism for Ca^{2+}-regulation of cell contractility (12).

In reconstitution experiments we demonstrated that even if cell actin of ETC extracts was replaced by muscle actin, the reconstituted

extracts showed Ca^{2+}-regulated gelation. This suggests that cell actin is not directly involved in Ca^{2+}-regulation. Similar Ca^{2+}-regulated gelation of cell extracts has also been reported in sea urchin eggs (1) and Dictyosterium (12). However, it is unknown what component is the Ca^{2+}-binding protein responsible for Ca^{2+}-regulation.

Temperature-Dependence and Nucleotide Triphosphate-Requirement - Reconstitution experiments demonstrated that reconstituted extracts in which cell actin was replaced by muscel actin showed temperature-dependent reversible gel-sol transformation as the original extracts did. This suggests that the temperature-dependent reversible G-F transformation of cell actin (59,69), if any, is not the main cause of the temperature-dependent gel-sol transformation of ETC extracts, because rabbit skeletal muscle actin does not show significant reversible G-F transformation in response to temperature changes. Instead, a temperature-sensitive component other than actin may be responsible for the temperature-dependence of the gelation. This possibility is also supported by the fact that addition of muscle F-actin did not cause gelation at 0°C and warming was always required for the functional interaction between F-actin and gelation protein. In this connection it is important to note that the purified macrophage actin-binding protein solution shows a reversible turbidity change on alternate warming and cooling (4).

The general features of assembly-disassembly of microtubules are very similar to those of temperature-dependent gel-sol transformation of ETC extracts: the assembly is induced by warming and depends on GTP, and the disassembly is induced by cooling; this reversible process occurs very rapidly; moreover, low concentrations of Ca^{2+} not only inhibit the assembly but also induce the disassembly (61). But we can eliminate the possibility of involvement of temperature-dependent reversible assembly -disassembly of microtubules in the gel-sol transformation of

ETC extracts, because the gelation did not depend on a polypeptide corresponding to tubulins, and because 10 mM colchicine, an alkaloid that inhibits the assembly of microtubules, did not affect the gelation of ETC extracts at all.

The gelation of ETC extracts depended on nucleotide triphophates. The temperature-dependence and the nucleotide triphosphate-requirement suggest that assembly of actin-containing filaments (microfilaments) might depend on the hydrolysis of nucleotide triphosphates or the the phosphorylation of a protein component involved in the gelation. However, the true substrate involved in the assembly is unknown, because ATP, GTP, ITP, and UTP were equally effective for sustaining the gelation, and the role of the substrate in the assembly is also unknown.

Cytochalasin-Sensitivity - The reconstitution experiments also demonstrated that extracts reconstituted by addition of muscle actin showed cytochalasin-sensitive gelation. Low concentrations (5×10^{-8} to 10^{-7} M) of cytochalasin B and D inhibited the gelation, but earlier studies showed that these low concentrations of cytochalasin B do not markedly influence the viscosity, morphology, function, or polymerization of muscle actin (36, 62-64). The lack of influence of low concentrations of cytochalasin B has also been reported with macrophage actin (3). Furthermore, assuming that functional actin comprised 1.4 to 2.5 % of the total protein of EXT extracts, as discussed above, from the results shown in Fig. 7 the amount of cytochalasins required for the complete inhibition of gelation was calculated as 0.03 to 0.05 mole per mole of actin monomer, indicating a lack of stoichiometry between actin and cytochalasins. These facts suggest that a component other than actin might be responsible for the cytochalasin-sensitivity of the gelation. The nature of the cytochalasin-sensitive component has not been established, but it should

be noted that Hartwig and Stossel demonstrated that low concentrations of cytochalasin B reversibly prevent the gelation of muscle actin by purified macrophage actin-binding protein (3). In addition, similar cytochalasin-sensitive gelation has also been reported with cell extracts of HeLa cells (5), Acanthamoeba (10), and Dictyostelium discoideum (12), and interestingly, these gels all contain a VLP apparently corresponding to the actin-binding protein.

During the preparation of this maunuscript, we learned that Mimura and Asano also observed Ca^{2+}-regulated and cytochalasin-sensitive gelation of ETC extracts (14).

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Table 1. Nucleotide-Requirements for Gelation

Nucleotide and phosphoric compound	Total protein* in gel (μg)	Gel volume* (μl)
None	114 ± 49	< 10
Inorganic phosphate	174 ± 2	< 10
Pyrophosphate	185 ± 9	< 10
AMP	164 ± 5	< 10
ADP	142 ± 12	< 10
ATP	1591 ± 563	210 ± 90
CTP	730 ± 112	80 ± 10
GTP	1418 ± 225	200 ± 30
ITP	1851 ± 132	270 ± 10
UTP	1854 ± 239	260 ± 50

* Means ± standard deviations for triplicate experiments. Reaction mixtures contained the nucleotide or phosphoric compound indicated at 1 mM concentration, 6.8 mg/ml extract protein, 50 mM KCl, 2.5 mM $MgCl_2$, and 1 mM DTT in 1 ml of buffered sucrose solution. Other conditions were the same as for Fig. 3.

Table 2. Reversibility of the Inhibition of Gelation by Free Ca^{2+}

Condition of free Ca^{2+}	Total protein in gel (μg)
Control with 1 mM EGTA	3431 ± 199 (100)*
Ca^{2+} ⟶ + 0 mM EGTA	321 ± 111 (9)
Ca^{2+} ⟶ + 5 mM EGTA	1388 ± 128 (40)
Ca^{2+} ⟶ + 10 mM EGTA	1713 ± 155 (50)

* Percentage of the value for the 1 mM EGTA control.

ETC extracts were incubated at 25°C for 1 hr in the presence of 0.35 mM $CaCl_2$ and 0.5 mM EGTA(about 5 x 10^{-7}M free Ca^{2+}) in 1 ml of reaction mixture for gelation. Then 0.1 ml of EGTA(pH 7) was added to the extracts at the final concentrations indicated and the extracts(6.1 mg/ml extract protein) were further incubated at 25°C for 1 hr. Other conditions were the same as for Fig. 1. The initial free Ca^{2+} concentration of about 5 x 10^{-7}M was calculated as described for Fig. 6.

Table 3. Solation of Gelled Extracts by Addition of $CaCl_2$ or Cytochalasin B

Addition	Total protein in gel (μg)	Gel volume (μl)
A. $CaCl_2$ (1 ml)		
0 mM	8280 ± 732 (100)*	840 ± 70 (100)*
1 mM	4475 ± 510 (54)	410 ± 90 (49)
2 mM	4921 ± 160 (59)	430 ± 40 (51)
B. Cytochalasin B (0. 1 ml)		
0 μg/ml	NM‡	310 ± 10 (100)
10 μg/ml	NM	200 ± 40 (65)

* Percentage of the value for the buffer control.

‡ NM, not measured.

(A). The gelled extracts(10.3 mg/ml extract protein) in 1 ml of reaction mixture were prepared by warming ETC extracts at 25°C for 1.5 hr in the presence of 1 mM EGTA, as described for Fig. 1. Then 1 ml of various concentrations of $CaCl_2$ in 100 mM Tris-HCl (pH 7.6) was overlaid on 1 ml of gelled extracts and the extracts were further incubated at 25°C for 1.5 hr. Other conditions were the same as for Fig. 1.

(B). The gelled extracts(4.2 mg/ml extract protein) in 0.9 ml of reaction mixture for gelation were prepared, and then 0.1 ml of 0 or 10 μg/ml cytochalasin B in 10 mM Tris-maleate(pH 7.0) was overlaid on 0.9 ml of gelled extracts. Other conditions were the same as for (A).

Table 4. Reversibility of the Inhibition of Gelation by Cytochalasin B

Condition of cytochalasin B	Total protein in gel (μg)	Gel volume (μl)
(-) ⟶ (-)	6190 ± 287 (100)*	1000 (100)*
(-) ⟶ (+)	94 ± 12 (2)	< 10 (< 1)
(+) ⟶ (-)	3925 ± 198 (63)	640 (64)
(+) ⟶ (+)	232 ± 23 (4)	10 (1)

* Percentage of the value for the control without cytochalasin B.

ETC extracts treated with 0.1 μg/ml cytochalasin B for 30 min at 25°C in reaction mixture for gelation were dialysed against 42 volumes of buffered sucrose solution containing 1 mM EGTA, 0.25 mM ATP and 0.5 mM DTT, under ice-cold conditions. The buffer was changed twice at 6 hr intervals. ETC extracts without cytochalasin treatment were also dialysed similarly. Then extracts(6.4 mg/ml extract protein) in 1 ml of reaction mixture were incubated at 25°C for 30 min in the presence or absence of 0.1 μg/ml cytochalasin B, for gelation. Other conditions were the same as for Fig. 7.

Table 5. Protein Composition of the Gel

Polypeptide (dalton)	% of total stainable polypeptide		
	Extract*	Gel I††	Gel II§
>300,000	0.33 ± 0.09	6.4 ± 0.7	< 0.1
270,000	NM‖	1.6 ± 0.5	< 0.1
230,000	NM	< 0.1	14.5 ± 0.2
89,000	NM	5.2 ± 0.8	ND¶
51,000	NM	19.5 ± 0.1	9.4 ± 0.8**
48,000	NM	6.3 ± 0.7	
42,000	13.9 ± 1.7	37.2 ± 6.4	73.0 ± 0.8
Others	NM	23.8 ± 5.8	3.1 ± 0.3

* Fresh extracts just after preparation.

†† Gels prepared immediately after preparation of ETC extracts.

§ Gels prepared after dialysis of the extracts against buffered sucrose solution without PMSF for 12 hr.

‖ NM, not measured.

¶ ND, not detected.

** 51,000-dalton polypeptide plus 48,000-dalton polypeptide.

The extracts immediately after preparation or dialysis against buffered sucrose solution without PMSF for 12 hr were warmed at 25°C for 1 hr for gelation. The gels were washed three times with reaction mixture for gelation, as described for Fig. 8. The relative amounts of the major components in the gels and in the extracts just after preparation were roughly estimated by quantitative densitometry of SDS-polyacrylamide gels stained with Coomassie Blue after electrophoresis.

Table 6. Inhibition of Gelation of the Reconstituted Extracts by Cytochalasin B or Ca^{2+}

Inhibitor	Gel volume (ul)
A. Extracts reconstituted from actin-deficient extracts	
water	1000
cytochalasin B (1.7 µg/ml)	100
$CaCl_2$ (1.7 mM)	50
B. Extracts reconstituted from actin-blocked extracts	
water	310 ± 20
cytochalasin B (0.5 µg/ml)	10 ± 10

Extracts with gelation activity were reconstituted from actin-deficient or actin-blocked extracts by addition of muscle actin, as described for Fig. 15.

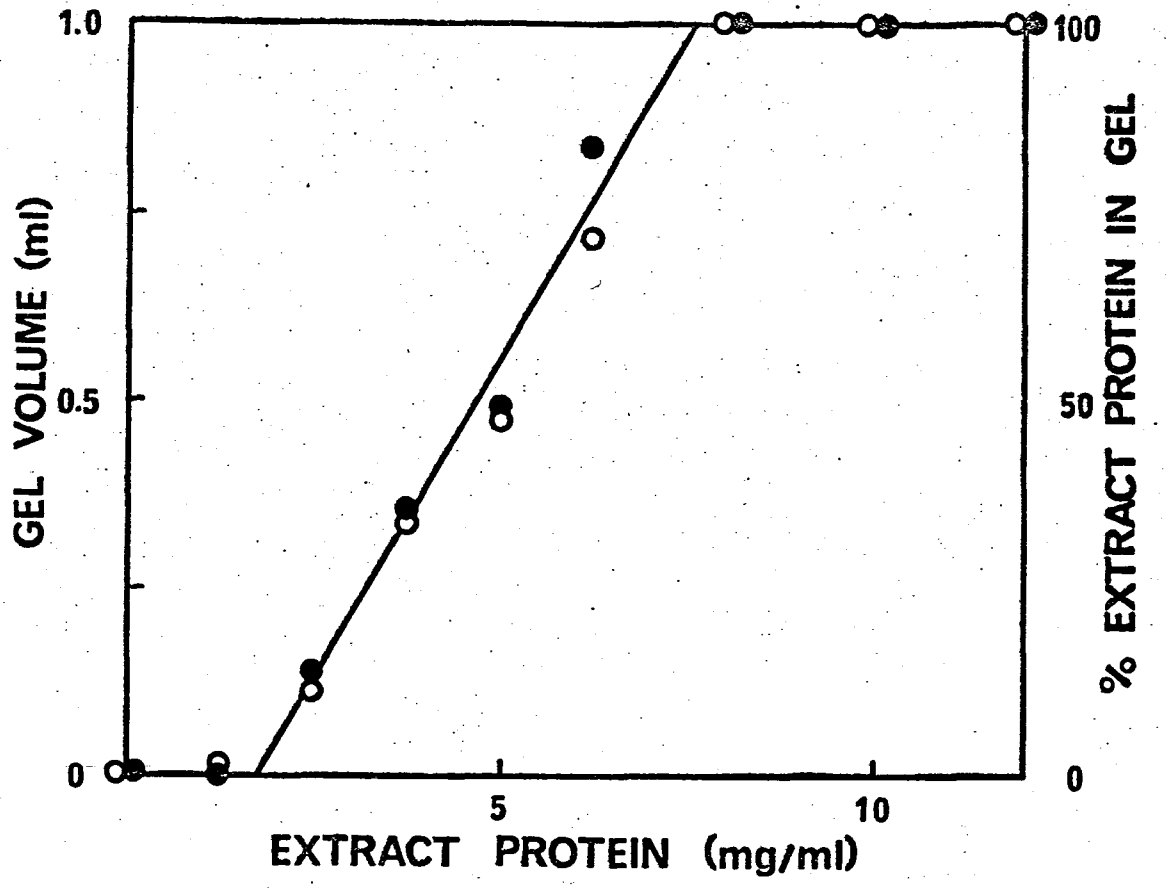
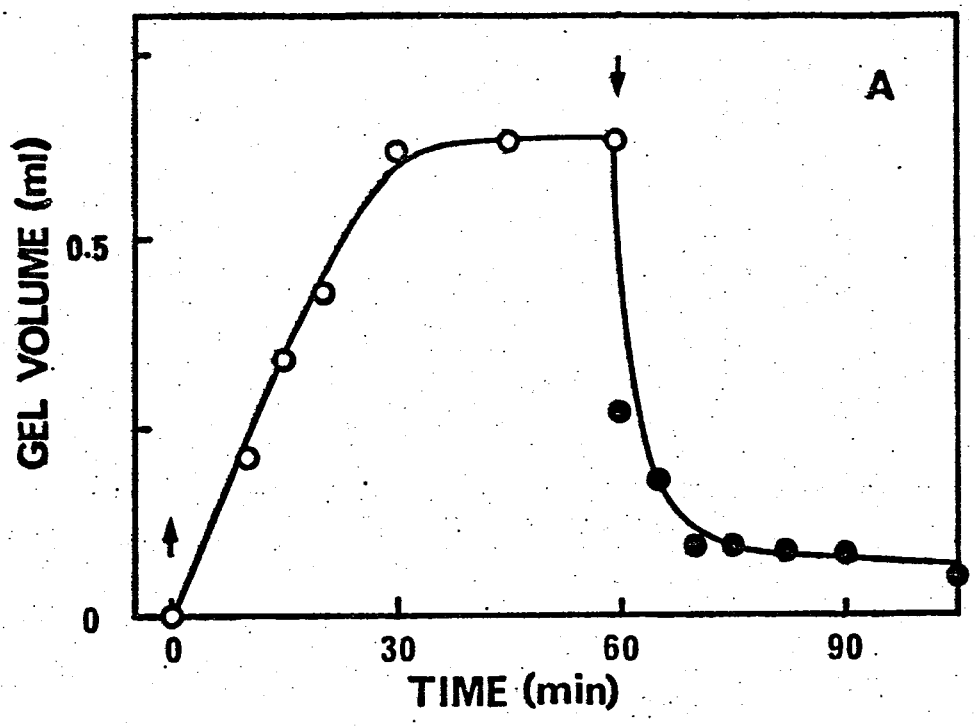


Fig. 1. Linear relations between the protein concentration of ETC extracts and the gel volume and the percentage of the total protein in the gel. ETC extracts were dialysed against 25 volumes of buffered sucrose solution (0.34 M sucrose, 20 mM Tris-maleate, pH 7.0 at 25°C) containing 0.25 mM ATP, 0.5 mM EGTA, 0.5 mM DTT, and 0.2 mM PMSF for 12 hr under ice-cold conditions, with one change of buffer. Reaction mixtures containing various amounts of the extracts, 57 mM KCl, 1.4 mM ATP, 3.9 mM $MgCl_2$, 2.5 mM EGTA, 2.5 mM DTT, and 0.2 mM PMSF were incubated at 25°C for gelation, and the resulting gels were separated by centrifugation at 1,150 x g for 30 min at 25°C. ○, percentage of total protein in the gel; ●, gel volume.

Fig. 2. Temperature-dependent reversible gel-sol transformation of ETC extracts. Gelation was induced by warming the mixture to 25°C, and solation by cooling it to 0°C. After various incubation times, the gelled extracts were separated into gel and liquid layers by centrifugation at 2,060 x g for 10 min at 25°C, whereas solled extracts were centrifuged at 4°C to prevent their regelation. Other conditions were the same as for Fig. 1. ↑, warming to 25°C; ↓, cooling to 0°C; ↓↓, addition of 1 mM Mg-ATP; △, extracts to which 1 mM Mg-ATP was added at 5 hr; ○△, extracts centrifuged at 25°C; ●, extracts centrifuged at 4°C. (A). Time course of the gelation on warming and the subsequent solation on cooling. ETC extracts(5.4 mg/ml extract protein), in 1 ml of reaction mixture, were incubated at 25°C, and then at 0°C. (B). Reversibility of the gel-sol transformation. ETC extracts(8.0 mg/ml extract protein), in 1 ml of reaction mixture, were incubated at 25°C for 30 min for gelation, and then at 0°C for 30 min for solation. This warming-cooling cycle was repeated.


A
GEL VOLUME (ml)
0.5
0
0
30
60
90
TIME (min)

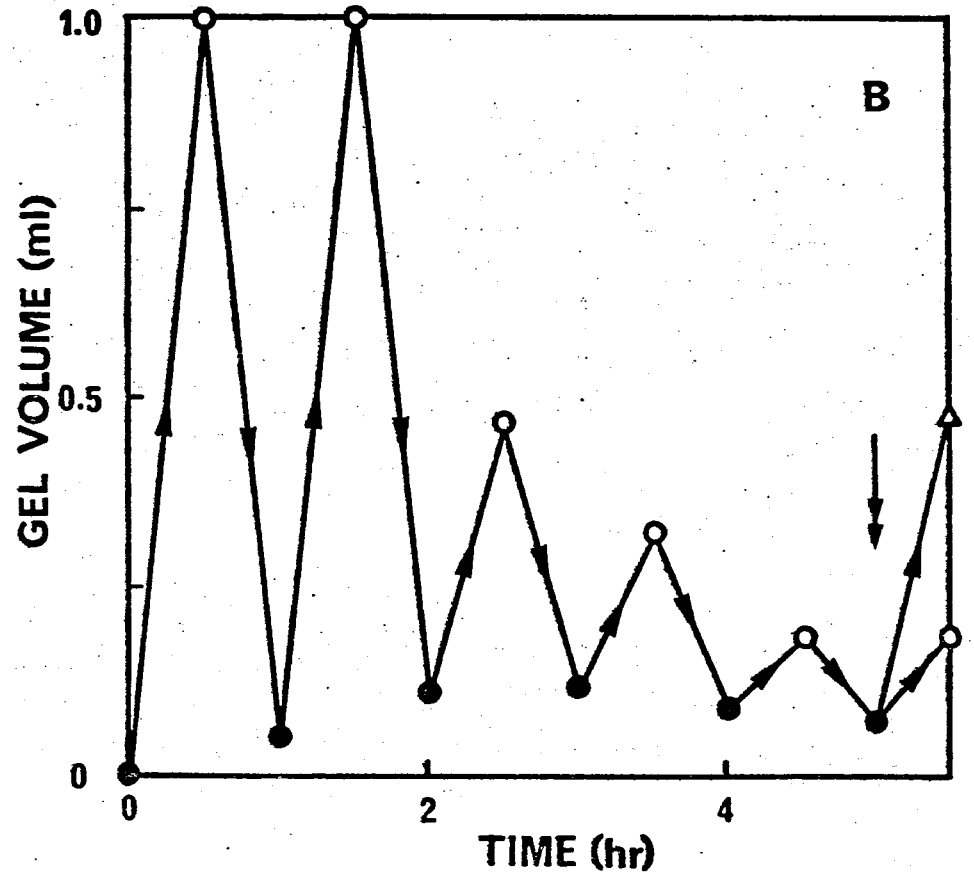
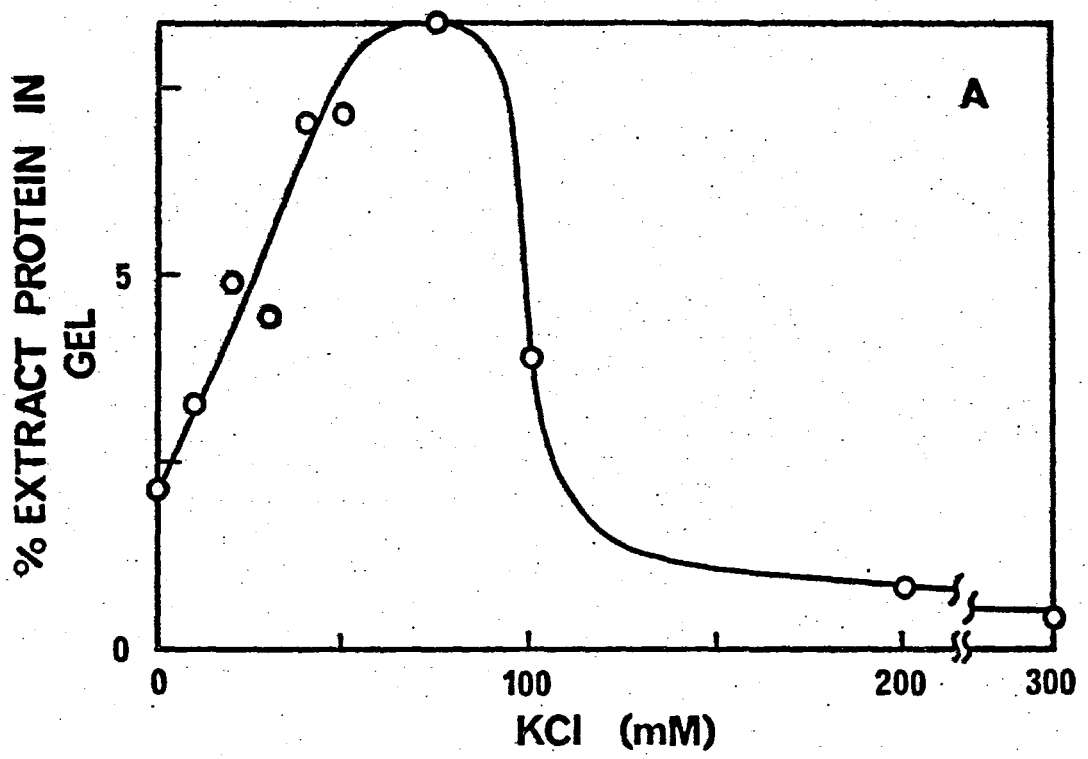
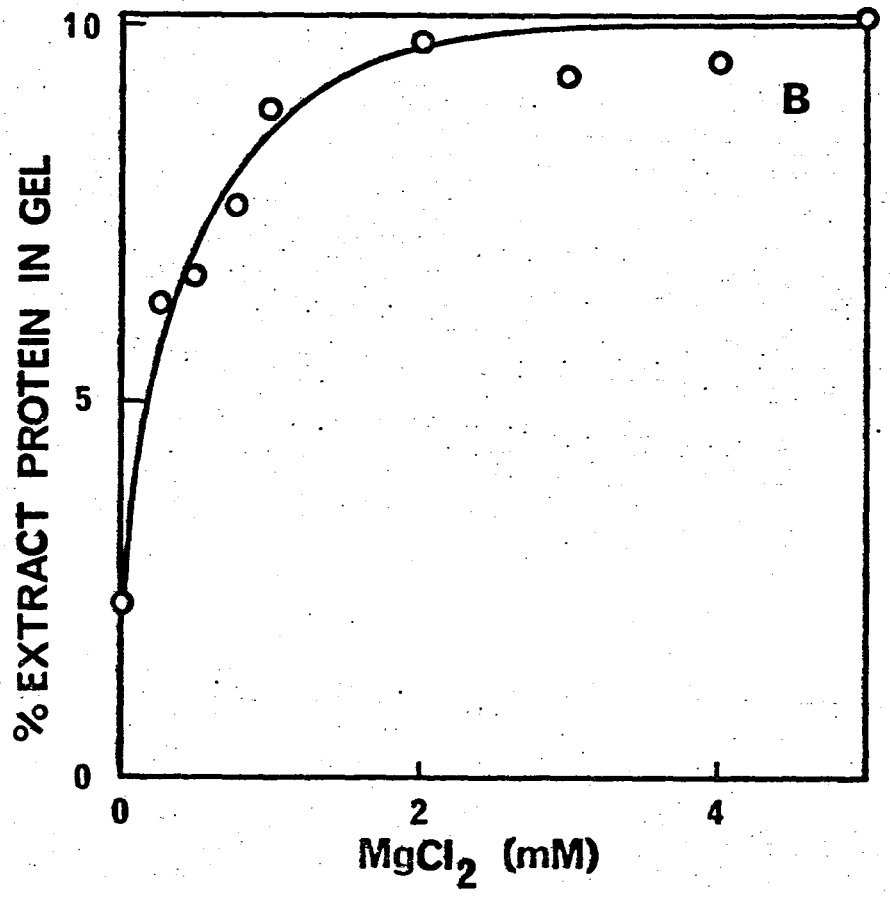

B
GEL VOLUME (ml)
1.0
0.5
0
0
2
4
TIME (hr)

Fig. 3. Effects of KCl and $MgCl_2$ on gelation. Eight ml of ETC extract was desalted by chromatography on Sephadex G-25(3 x 25 cm) equilibrated with buffered sucrose solution containing 0.5 mM EGTA and 1 mM DTT, and the void fractions were collected. The void fractions were incubated in 1 ml of reaction mixture at 25°C for 1 hr for gelation. The gels were separated by centrifugation of 1,150 x g for 30 min at 25°C, and the total protein in the gels was determined. (A). Effect of KCl. Reaction mixtures contained various amounts of KCl, 5.0 mg/ml extract protein, 0.5 mM ATP, 1 mM $MgCl_2$, 0.5 mM EGTA, and 1 mM DTT in buffered sucrose solution. (B). Effect of $MgCl_2$. Reaction mixtures contained various amounts of $MgCl_2$, 5.0 mg/ml extract protein, 50 mM KCl, 1 mM ATP, 0.5 mM EGTA, and 1 mM DTT in buffered sucrose solution.


% EXTRACT PROTEIN IN GEL
A
5
0
0
100
200
300
KCl (mM)


% EXTRACT PROTEIN IN GEL
B
10
5
0
0
2
4
MgCl2 (mM)

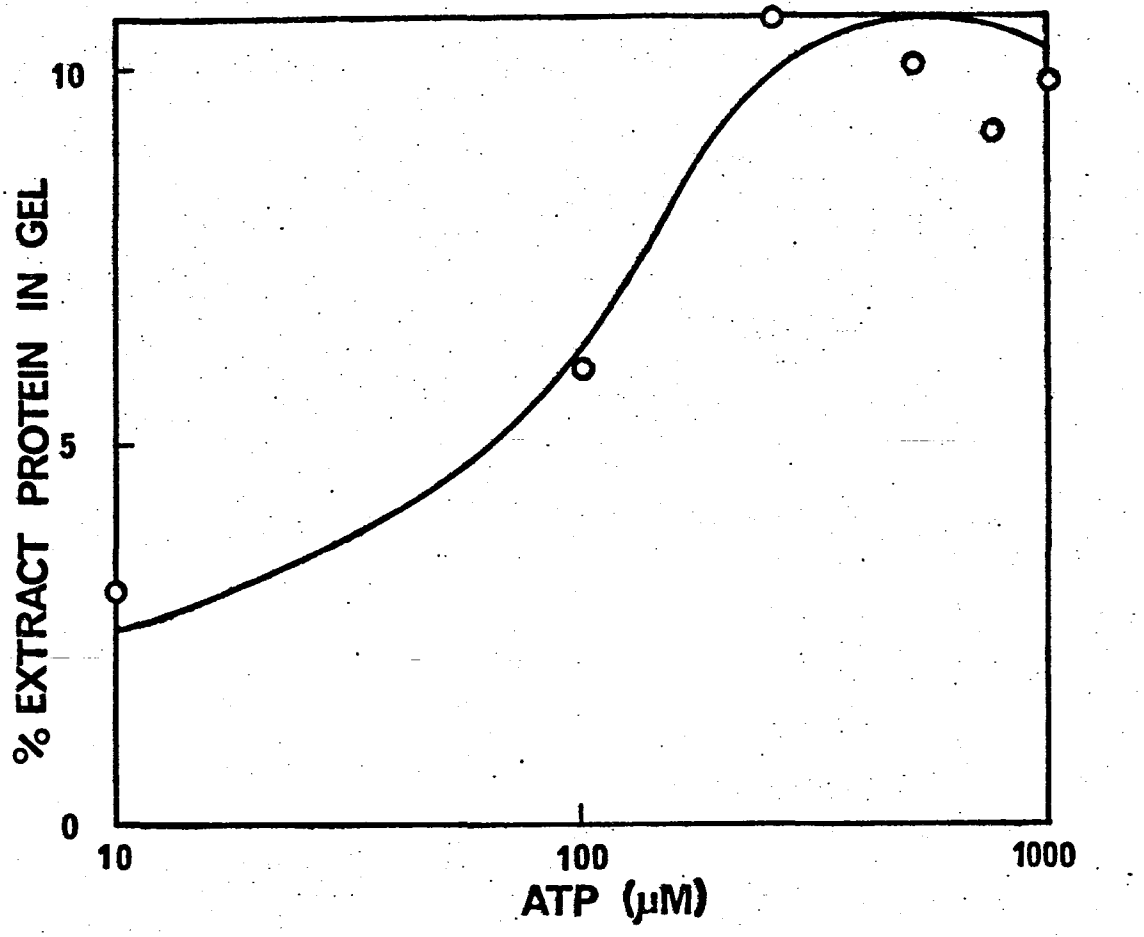


Fig. 4. Dependence of gelation on ATP concentration. Reaction mixtures contained various amounts of Mg-ATP, 5.0 mg/ml extract protein, 50 mM KCl, 1 mM $MgCl_2$, 0.5 mM EGTA, and 1 mM DTT in 1 ml of buffered sucrose solution. Other conditions were the same as for Fig. 3.

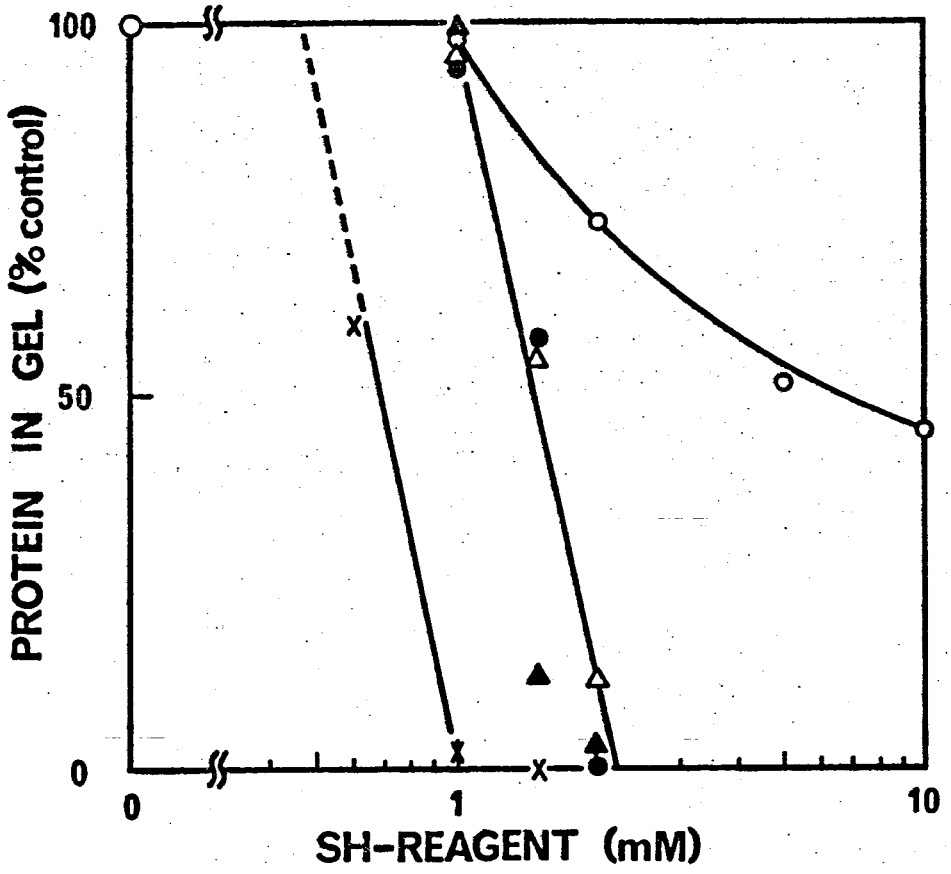


Fig. 5. Inhibition of gelation by SH-reagents. ETC extracts were dialysed against 25 volumes of buffered sucrose solution containing 0.25 mM ATP, 0.5 mM EGTA, and 0.5 mM DTT, with one change of buffer. The extracts (10.5 mg/ml extract protein), in 1 ml of reaction mixture, were incubated at 25°C for 30 min for gelation in the presence of 0.5 mM DTT and various amounts of SH-reagents. Other conditions were the same as for Fig. 1. ○, IAM; ●, NEM; △, CMB; ▲, CMBS; x, DTNB.

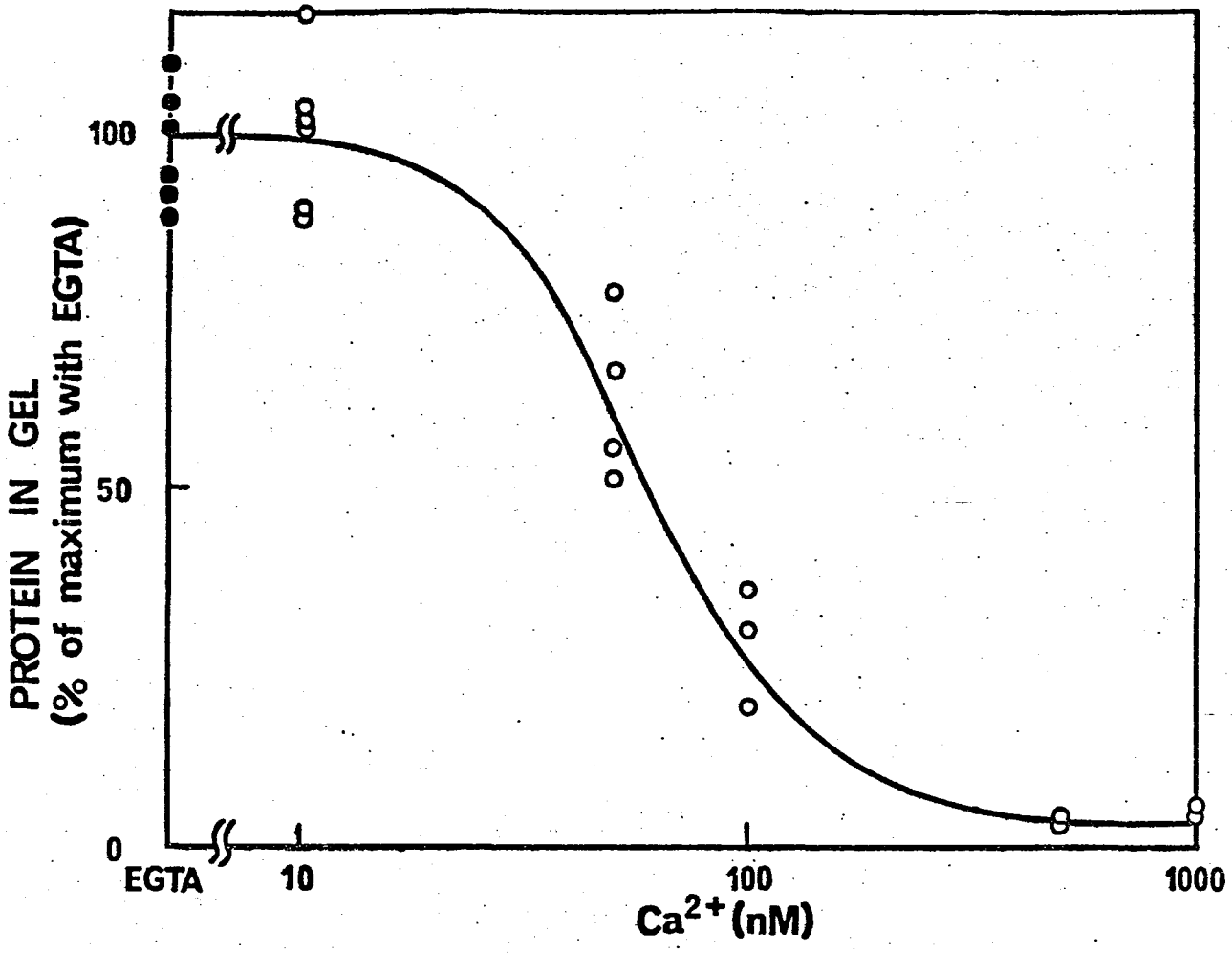


Fig. 6. Inhibition of gelation by free Ca^{2+}. ETC extracts were dialysed against 25 volumes of buffered sucrose solution containing 0.25 mM ATP, 0.5 mM EGTA, and 1 mM DTT, with one change of buffer, and then against 25 volumes of buffered sucrose solution containing 0.25 mM ATP and 1 mM DTT, with one change of buffer. The extracts(6.0 mg/ml extract protein), in 1 ml of buffered sucrose solution containing 50 mM KCl, 1 mM ATP, 2 mM $MgCl_2$, 1 mM EGTA, and 1 mM DTT, were incubated at 25°C for 1 hr for gelation, at various free Ca^{2+} concentrations obtained with Ca-EGTA buffer. Free Ca^{2+} concentrations of reaction mixtures were calculated adopting the value of 5.13×10^6 M^{-1} as the apparent stability constant of the Ca-EGTA complex at pH 7.0(65). Other conditions were the same as for Fig. 1.

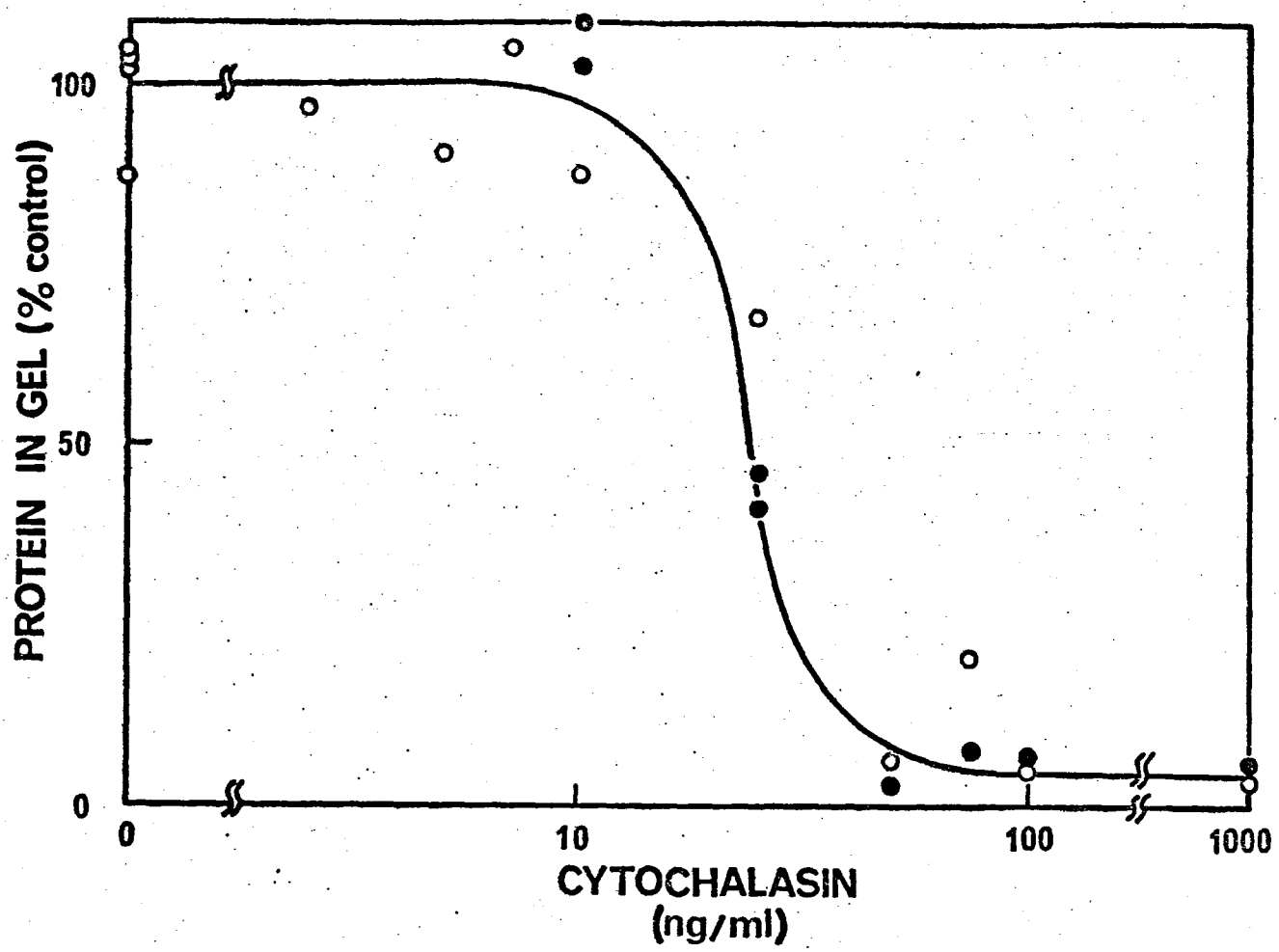
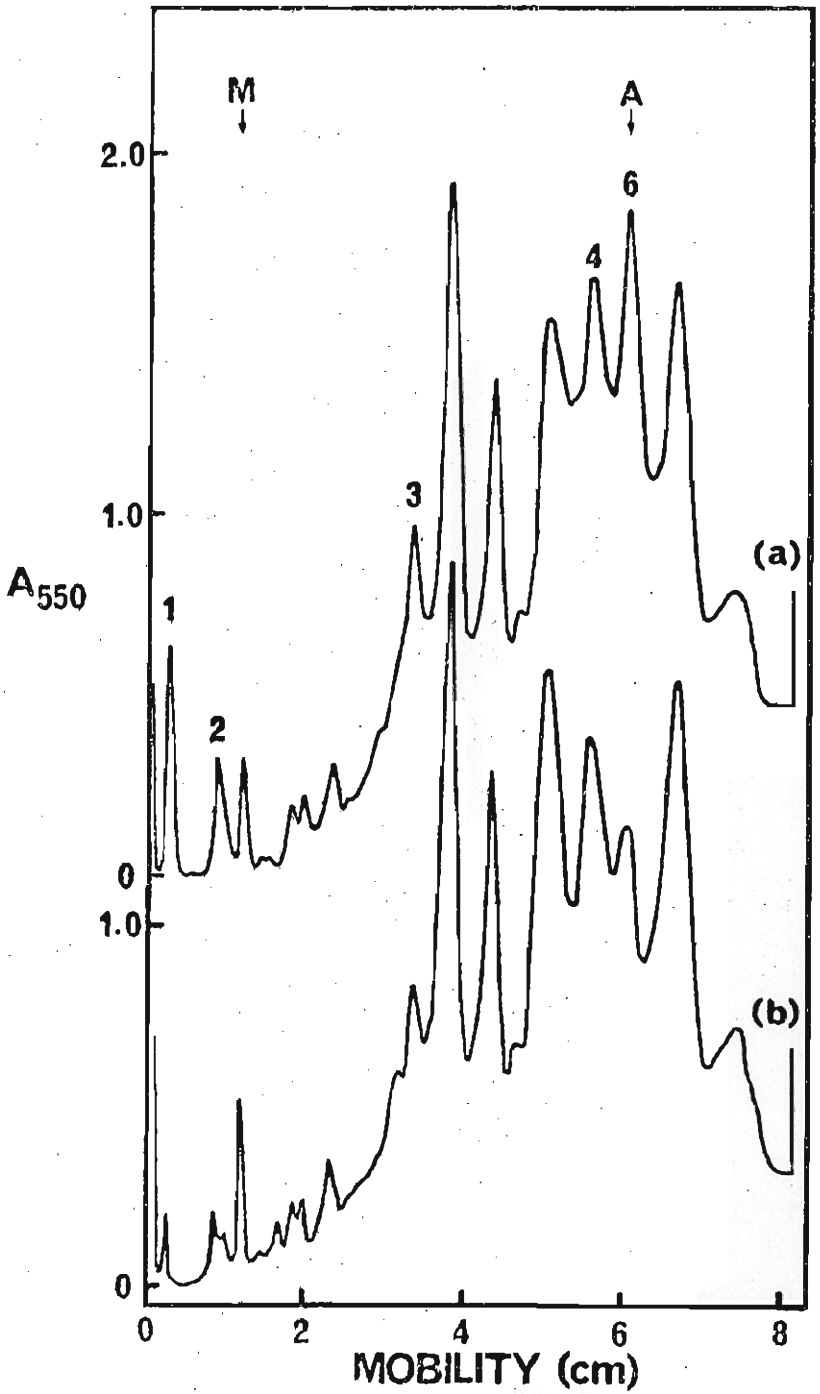


Fig. 7. Inhibition of gelation by cytochalasin B and D. Cytochalasin B and D dissolved in dimethylsulfoxide at a concentration of 1 mg/ml were diluted with water, and added to ETC extracts. Dimethylsulfoxide did not affect the gelation at concentrations of 0.1 % or higher. ETC extracts (5.8 mg/ml extract protein), in 1 ml of buffered sucrose solution containing 50 mM KCl, 1.3 mM ATP, 2.3 mM $MgCl_2$, 0.5 mM EGTA, and 1 mM DTT, were incubated at 25°C for 1 hr for gelation, with various concentrations of cytochalasin B and D. Other conditions were the same as for Fig. 1. ○, cytochalasin B; ●, cytochalasin D.

Fig. 8. Protein composition of the gels analysed by SDS-polyacrylamide gel electrophoresis. ETC extracts immediately after preparation, in 1 ml of reaction mixture for gelation, were gelled by warming them to 25°C for 1 hr. The gelled extracts were broken by vigorous agitation in a voltex mixer, and the broken gels were collected by low-speed centrifugation(1,150 x g for 30 min at 25°C). The precipitated gels were resuspended in 2 ml of reaction mixture for gelation at 25°C, and collected again by low-speed centrifugation. The gels were washed again. The protein compositions of the broken gels, the supernatants after gelation, and the original extracts were analysed by SDS-polyacrylamide gel electrophoresis on 5 % polyacrylamide gels. Other conditions were the same as for Fig. 1. The numbers indicate the polypeptide peaks(or bands) corresponding to molecular weights of more than 300,000(1), 270,000(2), 89,000(3), 51,000(4), 48,000(5), and 42,000 daltons(6), respectively. The arrows indicate the position of heavy chain of rabbit muscle myosin(M) and actin(A), respectively. Densitometric scans of electrophoretic gels stained with Coomassie Blue: (a), original extracts; (b), supernatants after gelation. (c), SDS-polyacrylamide gels, from left to right, of the unwashed, once washed, and twice washed gels.


M
A
2.0
1.0
0
1.0
0
1
2
3
4
6
(a)
(b)
A_{550}
0
2
4
6
8
MOBILITY (cm)

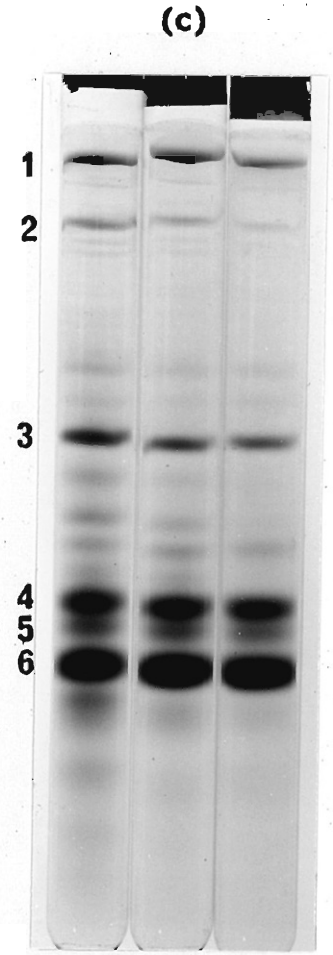

(c)
1
2
3
4
5
6

Fig. 9. Densitometric scans of SDS-polyacrylamide gels of ETC extract gels stained with Coomassie Blue. The extracts immediately after preparation or dialysis against buffered sucrose solution without PMSF for 12 hr were warmed at 25°C for 1 hr for gelation. The gelled extracts were broken by vigorous agitation in a voltex mixer, and the broken gels were collected by low-speed centrifugation and washed three times with reaction mixture for gelation. Other conditions were the same as for Fig. 8. The numbers indicate the polypeptide peaks corresponding to molecular weights of more than 300,000(1), 270,000(2), 230,000(2'), 89,000(3), 51,000(4), 48,000(5), and 42,000 daltons(6), respectively. (a), gels prepared immediately after preparation of ETC extracts; (b), gels prepared after dialysis of the extracts against buffered sucrose solution without PMSF.

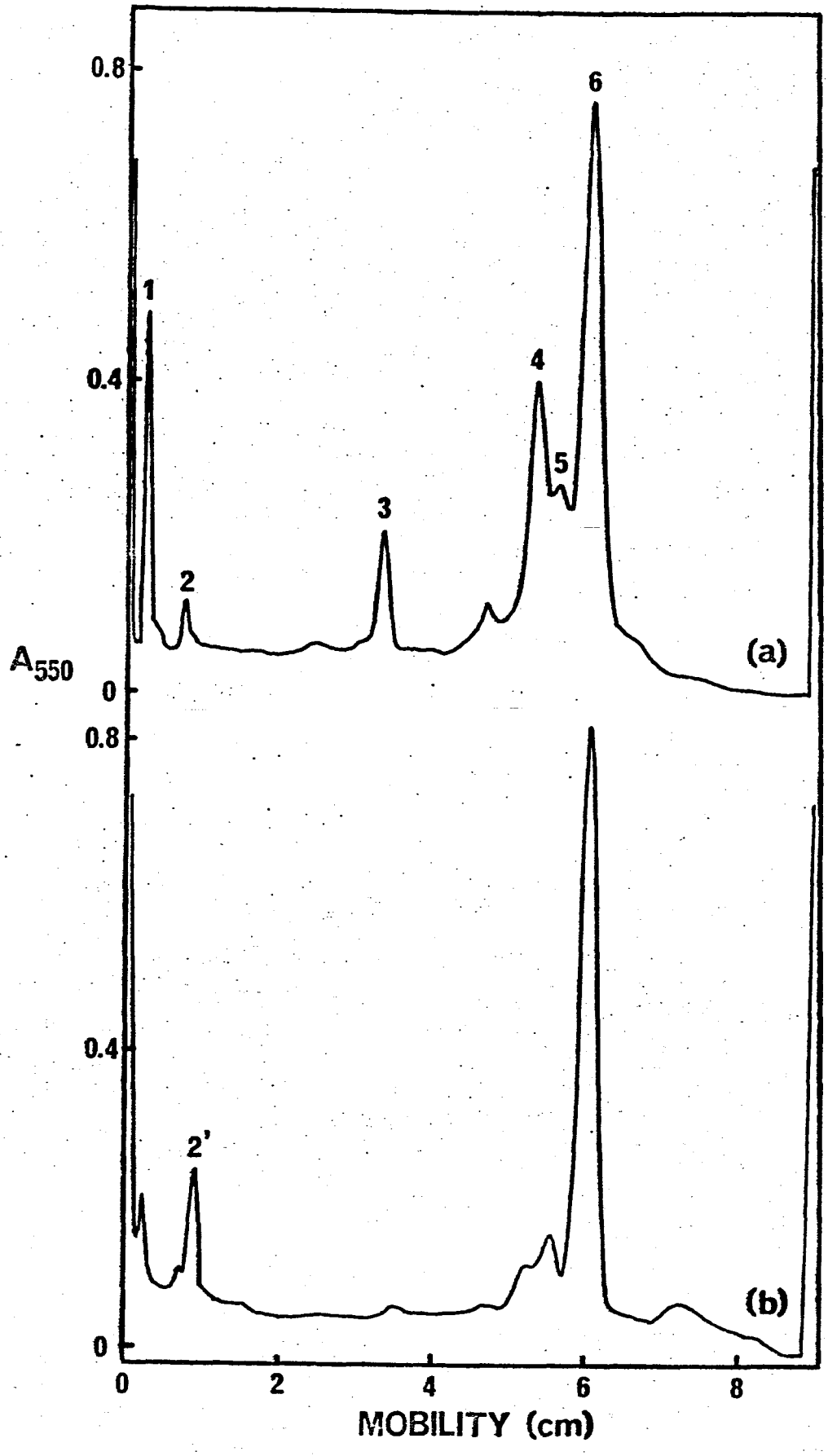
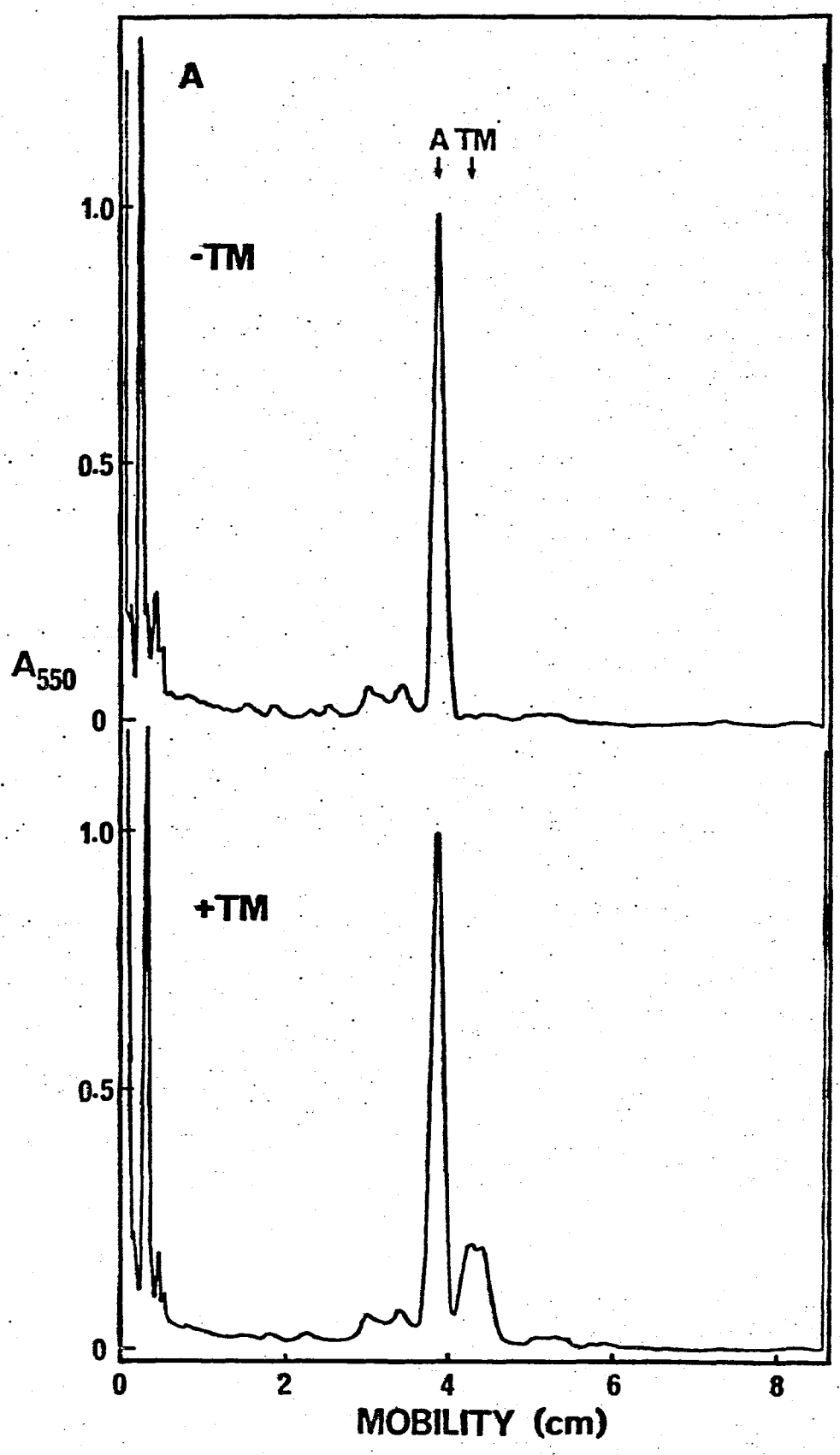
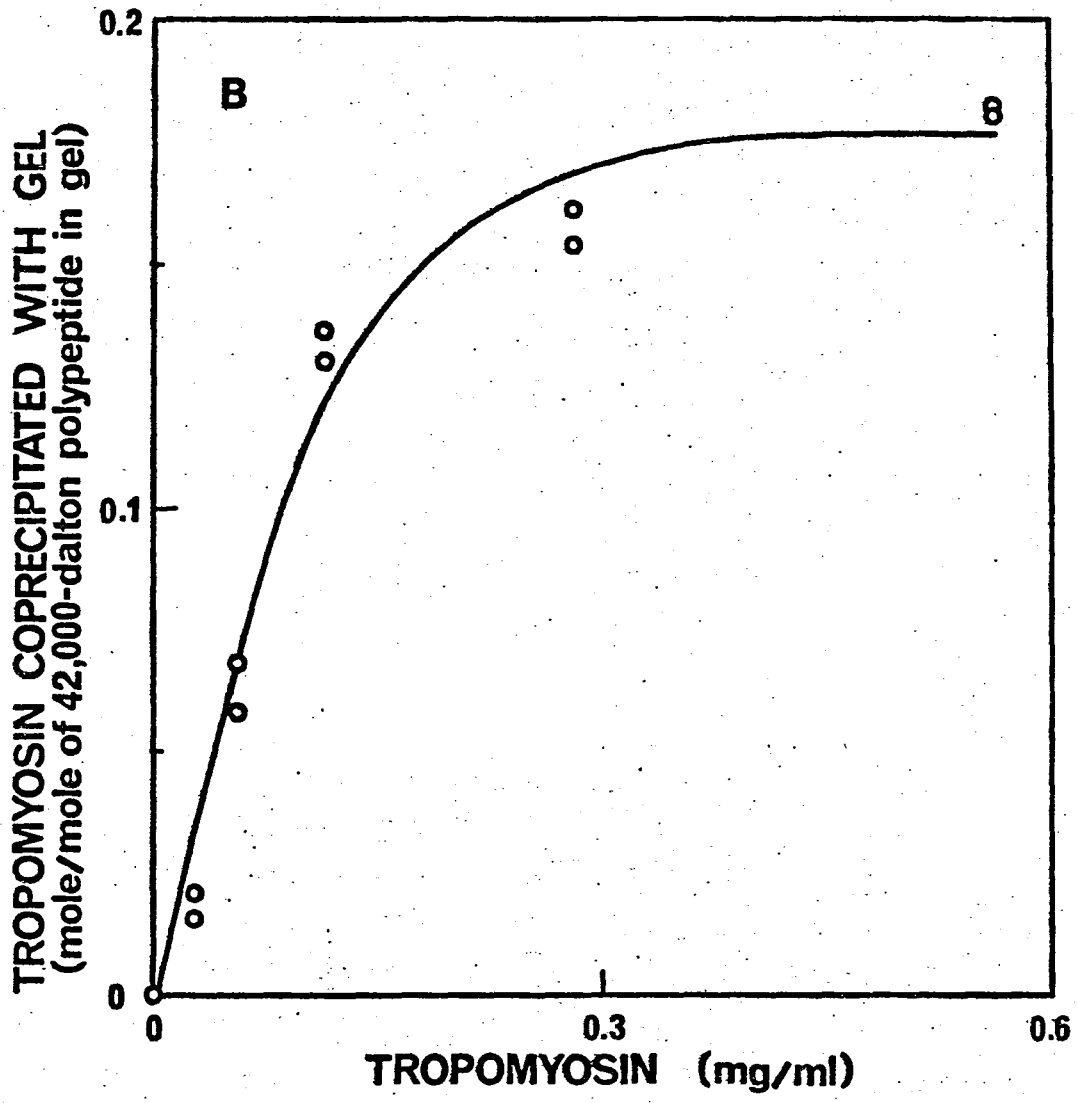

0.8
0.4
0
A_{550}
1
2
3
4
5
6
(a)
0.8
0.4
0
2'
(b)
0
2
4
6
8
MOBILITY (cm)

Fig. 10. Co-precipitation of tropomyosin with the gels by low-speed centrifugation. For preparation of gels containing only two major protein components, ETC extracts were used after dialysis against buffered sucrose solution for 12 hr in the absence of PMSF. Samples of extracts (4.2 mg/ml extract protein), in 1 ml of reaction mixture for gelation, were incubated at 25°C for 30 min for gelation in the presence or absence of rabbit muscle tropomyosin. The gelled extracts were broken by vigorous agitation in a voltex mixer, and the broken gels were collected by low-speed centrifugation and washed twice with 2 ml of reaction mixture for gelation, as described for Fig. 9. The protein composition of the washed gels was analysed by SDS-polyacrylamide gel electrophoresis on 7.5 % polyacrylamide gels. Other conditions were the same as for Fig. 1. (A). Densitometric scans of electrophoretic gels stained with Coomassie Blue. (B). Amount of muscle tropomyosin co-precipitated with the gels, estimated by quantitative densitometry of electrophoretic gels stained with Coomassie Blue after electrophoresis.


A
ATM
1.0
-TM
0.5
A_{550}
0
1.0
+TM
0.5
0
0
2
4
6
8
MOBILITY (cm)


B
0.2
0.1
0
0
0.3
0.6
TROPOMYOSIN COPRECIPITATED WITH GEL
(mole/mole of 42,000-dalton polypeptide in gel)
TROPOMYOSIN (mg/ml)

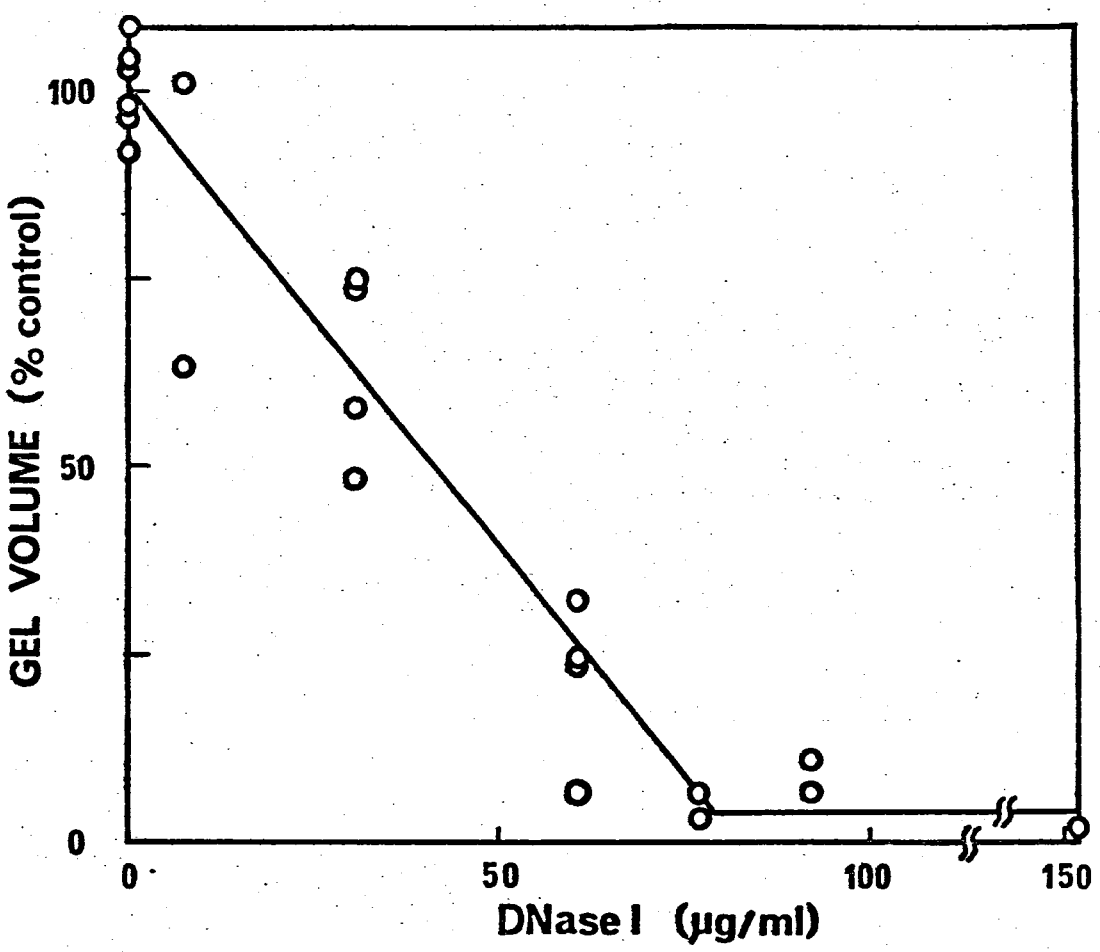
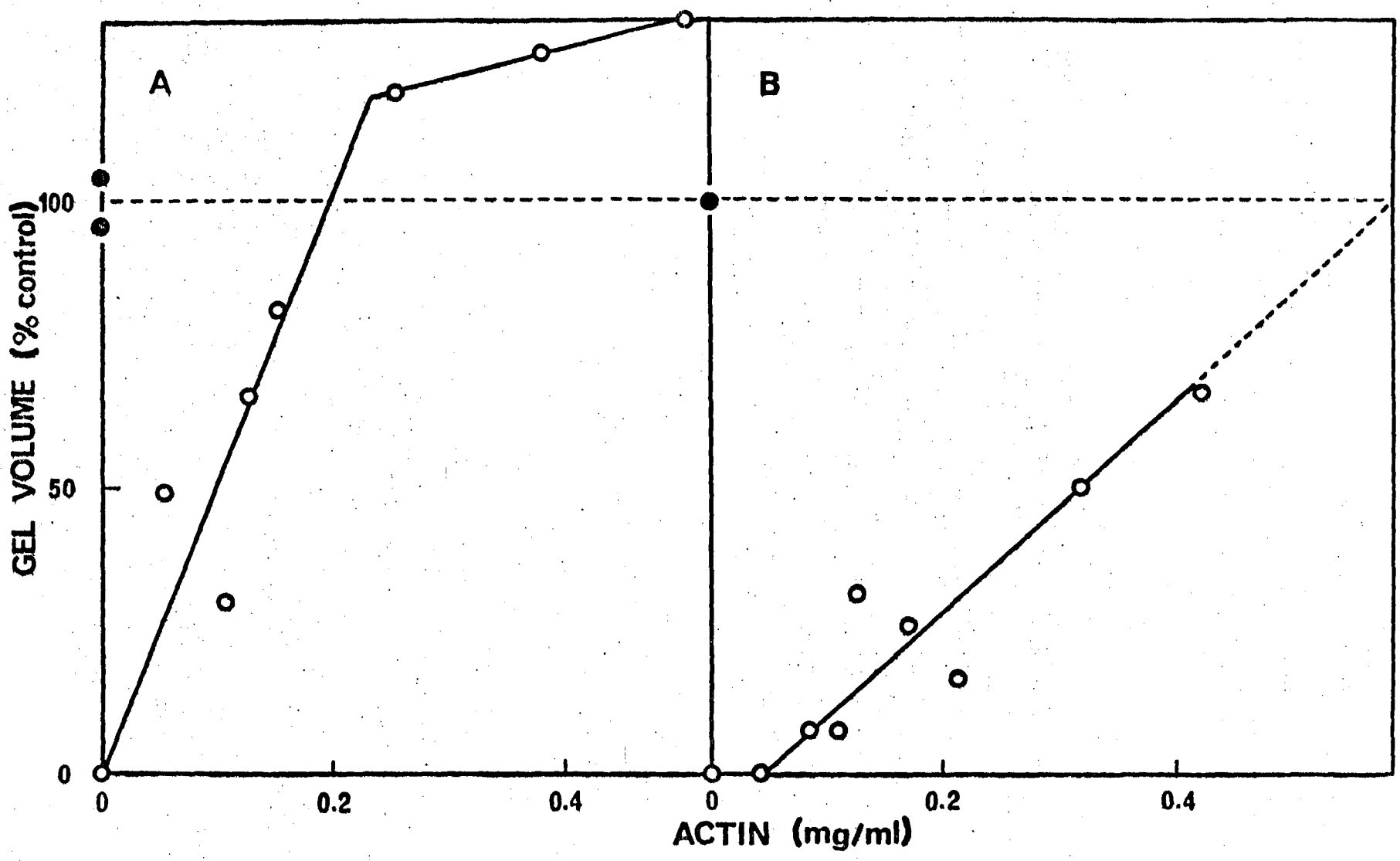


Fig. 11. Inhibition of gelation by DNase I. ETC extracts(4.0 mg/ml extract protein), in 1 ml of reaction mixture, were incubated with various amounts of DNase I for 30 min at 0°C, and then at 25°C for 30 min for gelation. Other conditions were the same as for Fig. 1.

Fig. 12. Neutralization by muscle action of the inhibitory function of DNase I and reversal by muscle actin of inhibition of gelation by DNase I. (A). Neutralization of the inhibitory function of DNase I. DNase I and various amounts of rabbit muscle G-actin were incubated at 0°C for 10 min in the presence of 114 mM KCl, 2.8 mM ATP, 7.8 mM $MgCl_2$, 5 mM EGTA, and 0.2 mM PMSF to allow the formation of an equimolar G-actin-DNase I complex. After addition of the mixtures, ETC extracts(3.7 mg/ml extract protein), in 1 ml of reaction mixture, were incubated at 0°C for 30 min and then at 25°C for 30 min in the presence of 148 μg/ml DNase I and various amounts of muscle actin. Other conditions were the same as for Fig. 1. ○, gelation in the presence of DNase I and various amounts of muscle actin; ●, gelation in the absence of both DNase I and muscle actin. (B). Reversal of the inhibition of gelation by DNase I. When ETC extracts (5.0 mg/ml extract protein), in 0.9 ml of reaction mixture, were incubated at 25°C for 30 min in the presence of 329 μg/ml DNase I and 0.5 mM PMSF, no gelation occurred. After addition of 0.1 ml of various amounts of rabbit muscle G-actin, the extracts in 1 ml of reaction mixture were further incubated at 25°C for 30 min in the presence of 296 μg/ml DNase I and various amounts of muscle actin. Other conditions were the same as for Fig. 1. ○, gelation in the presence of DNase I and various amounts of muscle actin; ●, gelation in the absence of both DNase I and muscle actin.


A
B
GEL VOLUME (% control)
100
50
0
0
0.2
0.4
0
0.2
0.4
ACTIN (mg/ml)

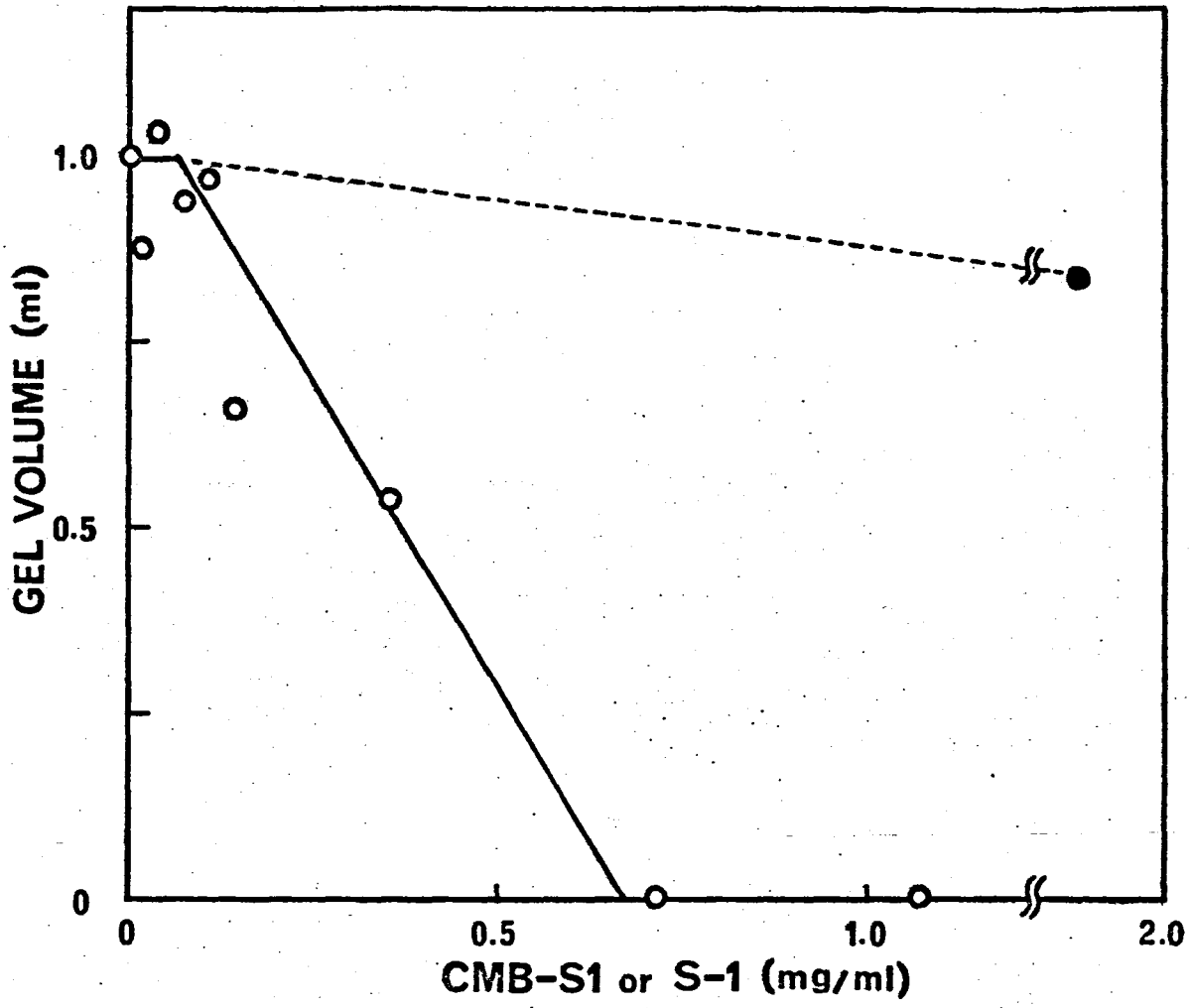


Fig. 13. Effects of CMB-S-1 and S-1 on gelation. ETC extracts were dialysed against 50 volumes of buffered sucrose solution containing 0.25 mM ATP and 0.5 mM EGTA for 6 hr to decrease the DTT concentration. The extracts (8.5 mg/ml extract protein), in 1 ml of buffered sucrose solution containing 55 mM KCl, 6 mM ATP, 7 mM $MgCl_2$, 0.5 mM EGTA, and 0.1 mM DTT, were incubated at 25°C for 30 min for gelation in the presence of various amounts of CMB-S-1 or S-1. Other conditions were the same as for Fig. 1. ○, CMB-S-1; ●, S-1.

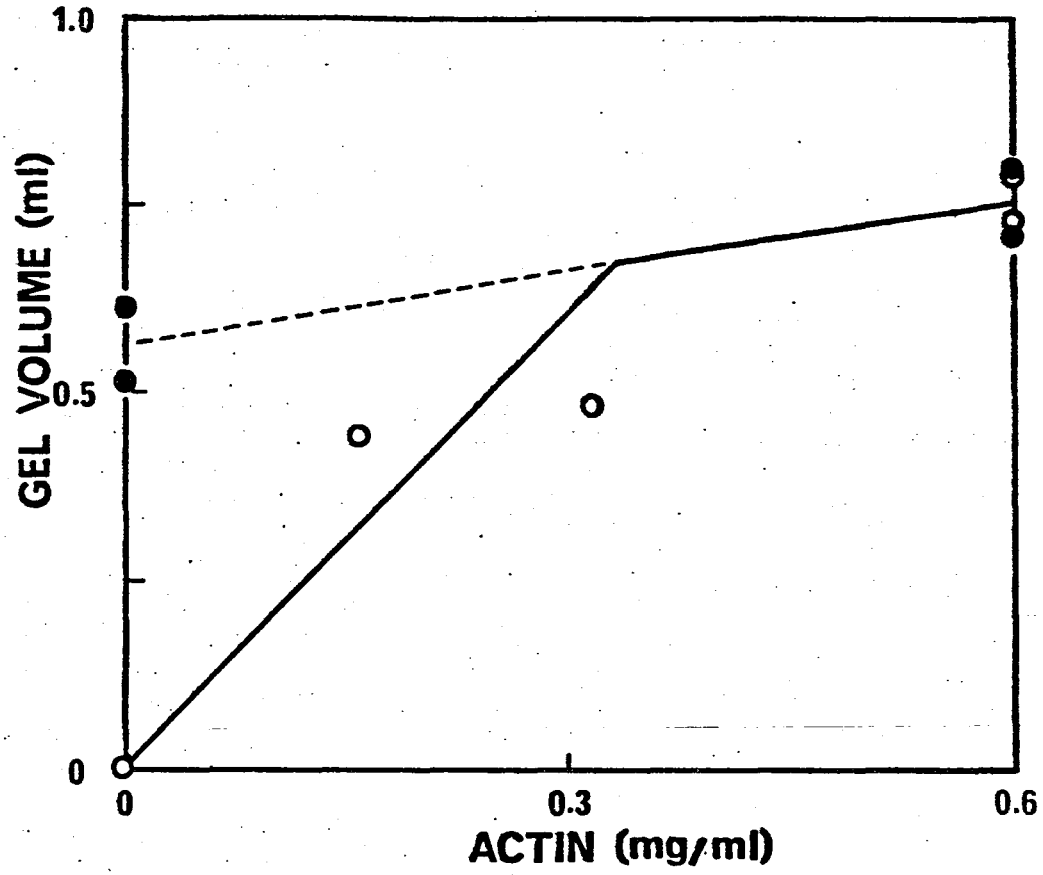
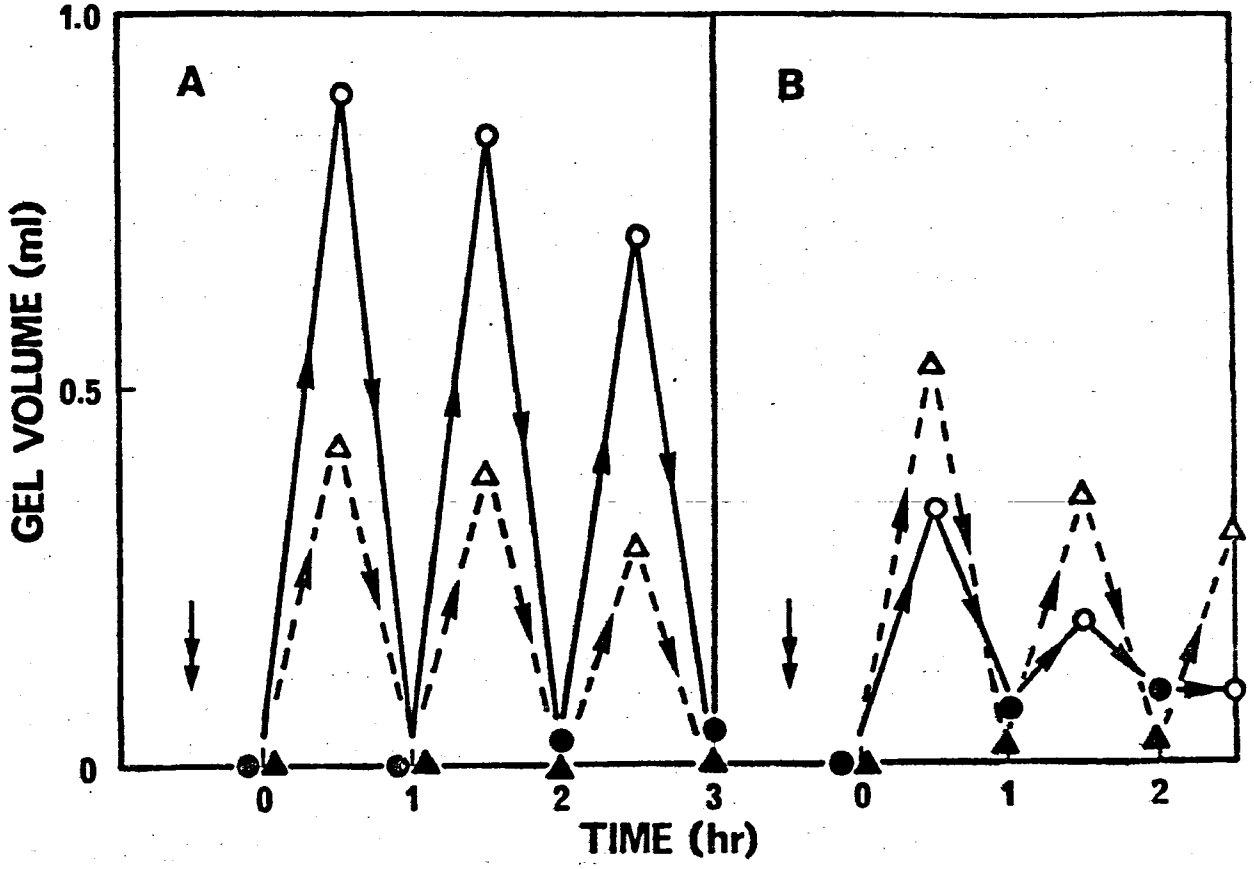


Fig. 14. Promotion of gelation by muscle actin. ETC extracts were incubated at 0°C for 12 hr in buffered sucrose solution containing 57 mM KCl, 1.4 mM ATP, 3.9 mM $MgCl_2$, 2.5 mM EGTA, 2.5 mM DTT, and 0.2 mM PMSF, and then centrifuged at 160,000 x g for 2 hr at 3°C to remove cell F-actin. The high-speed supernatants and the original extracts(5.0 mg/ml extract protein), in 1 ml of reaction mixture, were incubated at 25°C for 30 min for gelation in the presence of various amounts of rabbit muscle G-actin. Other conditions were the same as for Fig. 1. ○, high-speed supernatants; ●, original extracts.

Fig. 15. Temperature-dependent reversible gel-sol transformation of the reconstituted extracts from actin-blocked or actin-deficient extracts by addition of muscle actin. ETC extracts in which actin was blocked with DNase I were prepared by incubating ETC extracts in reaction mixtures for gelation in the presence of 329 μg/ml (10 μM) DNase I and 0.5 mM PMSF, at 0°C for 30 min and then at 25°C for 30 min. Actin-deficient high-speed supernatants of ETC extracts were prepared as for Fig. 14. Extracts with gelation activity were reconstituted from the actin-blocked extracts or actin-deficient high-speed supernatants by addition of 1 mg/ml of rabbit muscle G-actin. Temperature-dependent gel-sol transformation was measured in 1 ml of reaction mixture, as for Fig. 2. Neither actin-blocked extracts nor actin-deficient high-speed supernatants showed gelation on incubation at 25°C for 2 hr. Other conditions were the same as for Fig. 2B. (A), extracts reconstituted from actin-deficient high-speed supernatants; (B), extracts reconstituted from actin-blocked extracts. Protein concentration of original ETC extracts in reaction mixtures for gelation: (A), 4.2 mg/ml; (B), 4.7 mg/ml. ↡, addition of muscle actin; ↑, warming to 25°C; ↓, cooling to 0°C; ○●, reconstituted extracts; △▲, original extacts; ○△, gelled extracts centrifuged at 25°C; ●▲, solled extracts centrifuged at 4°C.


1.0
0.5
0
GEL VOLUME (ml)
A
B
0
1
2
3
0
1
2
TIME (hr)

GENERAL DISCUSSION

In PART I, we demonstrated that CMB-S-1 meets the requirement for an actin-specific inhibitor. After completion of this study, several authors showed that bovine pancreatic DNase I also meets this requirement (1-4).

In PART II, first we described the basic features of the temperature-dependent gel-sol transformation of ETC extracts. Second, using CMB-S-1 and DNase I we showed that cytoplasmic actin is functionally involved in the transformation.

Since cells always grow and multiply at about 37°C, we do not know whether there is any physiological significance in the gelation of ETC extracts on warming and their solation on cooling. But the temperature-dependent reversible gel-sol transformation of ETC extracts (assembly-disassembly of microfilaments) has interesting features in relation to the mechanism of HVJ-mediated cell fusion reaction. The early stage of cell fusion reaction induced by HVJ, which proceeds at 0°C, involves adsorption of the virus to cells and aggregation of the cells, and the late one, which is initiated by warming the cells to 37°C, involves fusion of the viral envelope with the cell membrane and fusion of the cells. For effective cell fusion, the cells should be preincubated with HVJ at 0°C for 5 to 15 min in the presence of a millimolar Ca^{2+} concentration(5). Without cooling and Ca^{2+}, poor cell fusion occurs. When the cells are incubated at 0°C in the presence of Ca^{2+}, Ca^{2+} passively penetrates into the cells, resulting in increase of the intracellular Ca^{2+} concentration(6), and interaction of HVJ with the cells also enhances this Ca^{2+}-penetration(7, cf. 8). Both cooling and increase of Ca^{2+} concentration inhibit the assembly of microfilaments and initiate the disassembly. When the cells are warmed again to 37°C to initiate the envelope fusion and cell-to-cell fusion, Ca^{2+} is rapidly pumped out by energy-dependent Ca^{2+}-pumps, resulting in

decrease of intracellular Ca^{2+} concentration(6). Warming and decrease of Ca^{2+} concentration initiate the reassembly of microfilaments. Cytochalasin B and D(9,10), which inhibit the assembly of microfilaments, or ATP-deficient conditions(5,11), which inhibit the assembly of microfilaments as well as Ca^{2+}-pumps(6), inhibit the cell fusion in the late stage. These facts tempt us to hypothesize that disassembly and reassembly of microfilaments play an important role(s) on the cell fusion reaction induced by HVJ.

In connection with the possible involvement of cytoskeleton in cell fusion reaction, there are some interesting observations. Several authors demonstrated that redistribution of intramembrane particles of the plasma membrane occurs during virus-induced(12) and chemically induced cell fusion (13-16). Recently, Sekiguchi and Asano showed that antispectrin antibody which is introduced into human erythrocyte ghosts inhibits the cell fusion of the ghosts and the redistribution of intramembrane particles associated with the cell fusion(17). However, the role of cytoskeleton in cell fusion reaction is unknown.

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