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Huxley and his coworkers (1) showed by electron-microscopy that muscle consists of two types of filaments, actin and myosin filaments with many cross-bridges between them. Furthermore, they demonstrated that neither type of filament changed in length, but that the distance between the actin filaments (H-zone) changed during muscle contraction. On the basis of these facts, they proposed the sliding theory of muscle contraction. According to this theory, contraction of cross striated muscle takes place by the sliding of myosin filaments and actin filaments past each other. Engelhardt and Ljubimova (2) found that a muscle protein, "myosin", has ATPase [ATP phosphohydrolase, EC 3.6.1.3] activity. Cain and Davies (3-5) established that hydrolysis of ATP occurs during a single twitch of living muscle, where creatine-kinase is inhibited by dinitrofluorobenzene. Hayashi and his coworkers (6-8) showed that ATP-induced contraction of actomyosin threads prepared by their method is dependent on the presence of actin. Therefore, it is generally accepted that muscle contraction is the result of an interaction between projections on myosin filaments, actin filaments and ATP. To elucidate the molecular mechanism of muscle contraction it is necessary to clarify the relationship between myofibrillar ATPase and tension development, and the structure and function of projections along ^{the} myosin filament.

Gordon et al. (9,10) showed that tension development in isometric tetanus at a sarcomere length of more than 2.0μ is proportional to the amount of overlap between myosin and actin filaments, as can be deduced from the sliding theory. On the other hand, Tonomura and his associates (11-17) concluded from kinetic studies on myosin ATPase that myosin is a double headed enzyme which hydrolyzes ATP through two different routes: (a) simple hydrolysis through a Michaelis complex, and (b) hydrolysis in which the Michaelis complex is converted to a "reactive myosin-phosphate complex" prior to liberation of P_i . They (17,18) showed that when F-actin binds to myosin the rate of decomposition of the "reactive myosin-phosphate complex" increases by more than 100-fold, and thus ATP is decomposed mainly by the so-called actomyosin type of ATPase. The activation of myosin ATPase by F-actin and the sliding of myosin filaments past actin filaments during contraction indicate that both the myofibrillar ATPase activity and tension development should be dependent on the sarcomere length when this is more than 2.0μ : myosin molecules in the region where myosin filaments do not overlap with actin filaments show only the myosin type of ATPase activity, but those in the region where myosin filaments overlap actin filaments show the actomyosin type of ATPase activity, besides the myosin type. Since the former ATPase is much more active than the latter, both the myofibrillar ATPase activity and the tension development must be proportional to the amount of overlap between myosin filaments and actin filaments. Furthermore, if the actomyosin type of ATPase is coupled with movement of actin filaments past myosin filaments, the myofibrillar ATPase activity should decrease at shorter sarcomere lengths, where

actin filaments no longer slide past myosin filaments.

The relation between sarcomere length and breakdown of phosphoryl creatine (19,20), and formation of inorganic phosphate has already been studied on living muscle. But analysis of results on living muscle is complicated owing to the ATPase activities in the protoplasmic membrane, sarcoplasmic reticulum etc. which are coupled with the contraction-relaxation cycle. Therefore, we investigated the relation between sarcomere length and ATPase activity with glycerol-treated muscle fiber bundles of which protoplasmic membranes were destroyed. Ward et al. (22) reported the dependence on sarcomere length of the ATPase activity of glycerol-treated muscle fiber bundles. They used 5mM ATP to avoid problems of diffusion of ATP into fiber bundles. But the concentration of ATP which they use seems unsuitable for quantitative studies on the relation between sarcomere length and ATPase activity, because it could cause destruction of the fine structure of myofibrils. Furthermore, they studied only the dependence of ATPase activity on the amount of overlap between the two types of filaments at sarcomere lengths of more than 2.0μ .

The dependence of ATPase activity on Ca^{++} concentration at low ionic strength in the presence of Mg^{++} has been studied by many investigators, mainly with myosin B and isolated myofibrils. Watanabe et al. (23) first showed that the ATPase activity of actomyosin was activated by a small amount of Ca^{++} . Furthermore, Weber (24) and Ebashi (25,26) showed that the activity of ATPase and the rate of superprecipitation of myosin B were regulated physiologically by the level of Ca^{++} . Thus, Ca^{++} is considered as the key substance regulating the actomyosin-ATP system.

Ward et al. (22) using glycerol-treated muscle fiber bundles found that the ATPase activity in the presence of 2mM ethylene-glycol bis(β -aminoethylether), N,N'-tetraacetate (EGTA) was much less than that in the absence of EGTA. Therefore, we investigated the dependence of ATPase activity on Ca^{++} concentration with glycerol-treated muscle fiber bundles at a fixed sarcomere length of 2.0 to 2.6 μ .

It is generally accepted (27-29) that the myosin molecule (molecular weight, 4.8×10^5) (60) is 1600 Å long and is composed of a helical portion, 1100 to 1400 Å long, and a less helical globular portion, 150 to 440 Å long (cf. Fig.28). Mihályi and Szent Györgyi (30,31) showed that the myosin molecule is converted into the two main fragments, heavy meromyosin (HMM) and light meromyosin (LMM), by tryptic digestion. HMM (molecular weight, about 3.4×10^5) (32-37) has the same globular portion as that of myosin at one end and a short rodlike tail at the other end. The whole length of HMM was reported to be 600 to 900 Å (27,39,40). LMM (molecular weight, about 1.4×10^5) (32,33,35-38) is a simple rod about 900 Å long (27,39,40). Mueller and Perry (41) isolated subfragment, S-1, from tryptic digests of HMM. Both the ATPase activity and the actin-combining ability, which are the characteristics of myosin, are found with HMM and also with S-1, but not with LMM. It is generally accepted that the two biological activities of myosin are confined to the globular head, and that S-1 is a primary component of the head.

Young et al. (42) reported that about 80 % of the mass of HMM was converted to S-1 during the early stages of digestion, and that the molecular weight of S-1 was 1.2×10^5 . Mueller (43)

showed that 55 to 60 % of the total mass of HMM was converted to S-1 on digestion with trypsin. He (43) obtained values of $1.1 - 1.3 \times 10^5$ as the molecular weight of S-1, depending on the digestion time. Similar values for the molecular weight were also reported by Jones and Perry (44), and Trotta et al. (45). Slayter and Lowey (39) showed by electron microscopy that the more globular portion at one end of myosin consists of two globular subunits. Therefore, they concluded that one molecule of myosin or HMM contains two molecules of S-1, derived from the globular head of the myosin molecule.

As described above, the projections on myosin filaments, which represent the HMM or S-1 ends of the myosin molecule, are indispensable both for development of tension and for the actomyosin type of ATPase activity. To elucidate the details of the molecular mechanism of muscle contraction, it is essential to see whether the two S-1 subfragments are identical, and it is also important to investigate their properties.

In the present report, some published results (46-48) by the author are summarized and discussed on the basis of (i) the dependence of the activity of myofibrillar ATPase on sarcomere length and calcium ion concentration, and (ii) the properties of the two subfragments, S-1, of the myosin molecule. The following conclusions were made: Maximum value of ATPase activity of glycerol-treated muscle fiber bundles during isometric contraction was observed in a range of sarcomere lengths of 2.0 to 2.5 μ . Myofibrillar ATPase activity during isometric contraction was proportional to the amount of overlap between myosin and actin filaments at sarcomere lengths

of more than 2.0μ . These results could be explained on the sliding-theory of muscle contraction. On the other hand, in the region where the sarcomere length was less than 2.0μ , the ATPase activity decreased as the sarcomere length decreased, and at a sarcomere length of about 1.0μ it reached 20 to 30 per cent of the maximum value. The ATPase activity of glycerol-treated muscle fiber bundles which had been stored in 50 per cent glycerol for a short period was very sensitive to Ca^{++} , but the activity of fiber bundles stored for a long period was quite insensitive to Ca^{++} . The capacities of myosin and HMM for ATP hydrolysis (per mole), both by simple hydrolysis and by phosphorylation, were preserved in the two moles of S-1 formed, though many intramolecular peptide bonds were broken on formation of S-1. It was also concluded that the active sites of ATPase were located in only one of the two S-1 molecules. Based on this non-identical subunit structure, we discussed the functions of these two S-1 portions of the myosin molecule in muscle contraction.

Experimental

Materials

Glycerol-treated muscle fiber bundles were prepared from rabbit psoas muscle by the method of Hanson and Huxley (49) with the slight modification of using 10mM Tris-maleate buffer instead of 6.7mM phosphate buffer.

Myosin B was extracted from glycerol-treated rabbit psoas muscle bundles which had been stored in 50 per cent glycerol at -10°C for 9 to 18 months and also from fresh rabbit psoas muscle. Myosin B was purified by precipitating it from 0.1 M KCl and dissolving it in 0.6 M KCl once or twice. All the procedures were done at below 4°C .

Pyruvate kinase [ATP: pyruvate phosphotransferase, EC2.7.1.40] was prepared from rabbit skeletal muscle by the method described by Tietz and Ochoa (50). The sedimentation pattern of pyruvate kinase showed a single and almost symmetrical peak, and the sedimentation coefficient, S_{20} , was 9.70S at the concentration of 3.45 mg per ml. The preparation gave a specific activity of 180 to 220 μmoles pyruvate per minute per mg of protein, when the enzyme was assayed at 25°C and pH 7.0, with 0.1 M histidine buffer, 0.1 M KCl, 4mM MgCl_2 , 1.0mM phosphoenol pyruvate (PEP) and 4mM ADP.

Myosin was prepared from rabbit skeletal muscle by the method of Perry (51) with slight modifications (52), and purified further by precipitating it with 0.04 M KCl and dissolving the precipitate in 0.5 M KCl at about pH 7. HMM was prepared essentially by the method of Tokuyama et al. (53), except that trypsin was purchased from Worthington Biochemical Corporation (2 x crystallized, salt-free) instead of from Sigma Chemical Co. HMM was precipitated with between 45 and 65 % saturation (at 4°C) of $(\text{NH}_4)_2\text{SO}_4$ containing 1 mM EDTA. Ammonium sulphate was passed through a column of Amberlite IR-120. The precipitated HMM was dissolved in, and dialyzed against 0.05 M KCl and 0.05 M Tris-HCl at pH 7.6 and 4°C , until the concen-

tration of sulphate ion titrated by barium ion became less than about 10^{-3} % saturation.

S-1 was prepared as follows (42): after dialysis, HMM was digested at 25°C *with* trypsin from Worthington Chemical Corporation (weight ratio of HMM to trypsin, 20 : 1) for 17 min, unless otherwise stated. The reaction was stopped with trypsin inhibitor (from soybean, type 1-S, Sigma Chemical Co.) and the digestion mixture (about 100 ml) was immediately chilled and applied to a column (6.0 x 69 cm) of Sephadex G-200 equilibrated with 0.05 M Tris-HCl and 0.5 - 1 mM EDTA at pH 7.6 and 4°C. The column was eluted with the same buffer at a rate of 24 to 36 ml per hr. The fractions of the central portion of the second peak (cf. Fig.16) were collected by precipitation with 70 % saturation of $(\text{NH}_4)_2\text{SO}_4$ at 4°C. The precipitate was dissolved in and dialyzed against the same buffer for several hours. The resulting clear solution was purified further by rechromatography on Sephadex G-200 (4.6 x 55 cm).

γ - ^{32}P -ATP, prepared from ATP and ^{32}Pi by the method of Glynn and Chappel (54), was kindly supplied by Dr. T. Kanazawa of this laboratory. Blue dextran was purchased from Pharmacia (Uppsala, Sweden). The crystalline sodium salt of ATP and PEP were purchased from Kyowa Hakko Kogyo Co. (Tokyo) and Sigma Chemical Co., respectively. EGTA was purchased from Dojindo & Co. IAA-1- ^{14}C was purchased from the Radio Chemical Center, Amersham, England. Other reagents were of analytical grade.

Methods

Measurement of myofibrillar ATPase activity at fixed

sarcomere lengths

As illustrated in Fig.1 the two ends (2 mm each) of a

Fig.1

fiber bundle were each placed between a pair of acrylic plastic plates, and the pairs of plates were fixed on a plastic frame by screws of stainless steel at a fixed distance apart to keep the fiber bundle from slackening. Photographs were taken under a phase contrast microscope (Olympus, model ECETP-2) at intervals of about 3 mm. The mean value of sarcomere length was plotted against the position where the photograph was taken. As shown in Fig.2, even the sarcomeres in fiber bundles

Fig.2

which had been forcibly stretched, were fairly uniform in length, except near the ends of the fiber bundle. Therefore, we usually measured the sarcomere length by the following method. One to six fiber bundles were taken out from 50 per cent glycerol, their diameters were reduced to an appropriate thickness, and then they were fixed in the acrylic plastic frame. The diameter of a fiber bundle was examined using a microscopic eyepiece micrometer,

and only bundles of uniform diameter were used. They were photographed in at least three positions, i.e., in the central part and at 5 to 10 mm from each end, and the sarcomere length of a muscle fiber bundle was calculated as the average of the mean values in each photograph (results obtained by this method are shown in Fig.9 as open circles). Fiber bundles of too wide or uneven diameter were cut off from the frame, leaving their ends fixed in the two pairs of plastic plates. The fiber bundles fixed to the plastic frame were incubated in a buffer solution of 50 mM KCl, 2 to 3.35 mM $MgCl_2$ and 20 mM Tris-maleate at pH 7.0 and 0 to 4°C for about 1 hour, changing the buffer three times, to wash out glycerol. Two or more fiber bundles were used, and bundles of a significantly different sarcomere lengths were not used at the same time for measurements. For measurement of ATPase activity of bundles of shorter sarcomere length, photographs were first taken at a fixed length of fiber bundle, and then the bundles were shortened to another length by the addition of ATP. The fiber bundles were shortened to the pre-determined length, and showed a constant activity of ATPase at the steady state within 15 seconds after the ATP-addition. The sarcomere length was assumed to be proportional to the length of the fiber bundle (the closed circles in Fig.9 were obtained by this method).

Pyruvate kinase was coupled with myofibrillar ATPase, and the ATPase activity was measured by determining the concentration of pyruvate liberated using the method described by Reynard et al. (55). Fig.3 shows a comparison of the time-course of pyruvate-liberation from HMM

Fig.3

ATPase coupled with pyruvate kinase with that of P_i -liberation from the ATPase uncoupled with the kinase. The two time-course of P_i -liberation from actomyosin ATPase was found to be unaffected by the presence of the kinase. Accordingly, we concluded that pyruvate kinase maintains the concentration of ATP in the system without affecting the myosin ATPase.

The ATPase reaction was usually initiated by adding ATP after the fiber bundles fixed to the plastic frame (cf. Fig.1) had been incubated in the reaction mixture for 10 to 15 minutes at 25°C, and aliquots (2 ml) were usually taken out at intervals of 1.5 minutes to determine the concentration of pyruvate liberated. We calculated the amount of pyruvate liberated per g of protein making a correction for the volume change of the reaction mixture due to sampling for the

determinations. First, the ATPase activity of the whole fiber bundles was measured, and second, the activity of the two end parts of the fiber bundles (2 mm, each) which were fixed between the plastic plates was measured after cutting off the other part. The difference between these values was adopted as representing the activity of a part of the fiber of constant sarcomere length. As stated above, fiber bundles of uneven diameter or of more than 300 μ in diameter at the moment of measurement of ATPase activity were cut off, leaving only their ends in the frame. Therefore, the number of the fiber bundles with ends contributing to the control activity was equal to, or more than the number of the fiber bundles which contributed to the activity at constant sarcomere length.

Measurement of ATPase activities of myosin, HMM, S-1 and myosin B.

The ATPase activities of myosin, HMM and S-1 were measured in 1.0 M KCl, 1 mM ATP and 0.05 M Tris-maleate at pH 7.0 and 25°C. As modifiers of ATPase, 7 mM CaCl_2 and 3 mM EDTA were used. The time-course of P_i -liberation was determined as described previously (15). The method for measurement of the initial burst of $^{32}\text{P}_i$ -liberation from the S-1- ^{32}P -ATP system was also reported previously (56). The ATPase activity of Myosin B was determined by measuring the time-course of pyruvate-liberation from myosin B ATPase coupled with pyruvate kinase. The reaction was initiated by adding ATP in the case of myosin B extracted from

glycerol-treated muscle fiber bundles, and by adding myosin B in the case of myosin B extracted from fresh psoas muscle, unless otherwise stated. In both cases the initial rate was adopted as the activity of myosin B.

Carboxamidomethylation of myosin and S-1

Myosin was subjected to carboxamidomethylation using IAA-1- ^{14}C , by the method mentioned in the preceding paper (57). Myosin was treated by the same procedures except that IAA was omitted, and was used as a control. A concentration of 1.17 mM IAA-1- ^{14}C (20 fold excess over S-1, on a molar basis) was added to a solution of 7.03 mg per ml S-1 in 0.5 M KCl and 0.1 M Tris-HCl at pH 8.5 and 0°C in the presence or absence of 0.1 M sucrose. At appropriate times after the start of the reaction, 2 ml aliquots were transferred to 4.7 ml of chilled saturated $(\text{NH}_4)_2\text{SO}_4$ solution containing 1 mM EDTA at pH 7.0. After carboxamidomethylation, S-1 was collected by centrifugation (13,000 x g, 30 min), and the precipitate was dissolved in, and dialyzed against 0.05 M KCl and 0.05 M Tris-HCl at pH 7.6 and 4°C. The radioactivity of samples was measured as described in the preceding paper (57). Correction for the quenching by proteins was made by the external standardization method in a Nuclear Chicago liquid Scintillation spectrometer, Mark II (Nuclear Chicago).

Protein concentration

The optical density at 280 m μ was measured after dissolving the myofibrils by treatment with 3 ml of 1 N NaOH either for 24 hours at room temperature, for 3 weeks at room temperature or for 2 hours at 100°C in a sealed test tube. Fig.4 shows the relation

Fig.4

between the optical density and the protein concentration calculated by multiplying the amount of nitrogen, determined in a Coleman, model 29, Nitrogen-Analyzer (Coleman, U.S.A.) by a factor of 6. The optical density at 280 m μ after treatment of myofibrils with NaOH at 100°C for 2 hours was the highest, indicating that this method was the best for dissolving muscle fiber bundles. Therefore, segments of muscle fiber bundles were cut off from the plastic frame after measuring the whole activity, and were treated twice with a buffer solution (40 mM KCl, 20 mM phosphate buffer pH 7.0) at 0°C for about 10 minutes to wash out nucleotide, pyruvate kinase and maleate. Then the optical density at 280 m μ was measured after dissolving them by treatment with 3 ml of 1 N NaOH at 100°C for 2 hours and then storage at room temperature for about 24 hours in a sealed test tube.

The concentrations of myosin B and myosin were estimated by the biuret reaction calibrated by nitrogen determination. The protein concentrations of HMM and S-1 were determined using values for $E_{1\text{cm}}^{1\%}$ at 280 m μ of 6.47 and 7.70, respectively (42).

Other methods

The concentration of total Ca^{++} was varied from 50 μM to 0.20 mM, and that of EGTA from 0.101 mM to 3.18 mM, and the concentration of free Ca^{++} was calculated by adopting 2.5×10^{-7} (cf.26) as the dissociation constant of the binding of Ca^{++} with

EGTA under our experimental conditions. All ultracentrifugal runs were performed in a Beckman Spinco Model E analytical ultracentrifuge operated with Schlieren optics using aluminium double sector cells or filled epon synthetic boundary cells.

Results

I. Contractile ATPase Activity on Sarcomere Length and Calcium Ion Concentration.

Diffusion of substrate into fiber bundle and ATPase activity

The ATPase activity of a fiber bundle of about 300 μ in diameter and 2.57 μ in sarcomere length was measured in the presence of 0.6 mM ATP, 8.4 μ g pyruvate kinase per ml and either 0.5 or 3.0 mM PEP in buffer solution (50 mM KCl, 2 mM $MgCl_2$ and 20 mM Tris-maleate buffer) at pH 7.0 and 25°C. As shown in Fig.5, the activity was scarcely affected by decrease in the

Fig.5

concentration of PEP from 3.0 to 0.5 mM. The activity was also independent of the concentration of pyruvate kinase between 5 and 32 μ g per ml. In the presence of 1.0 mM PEP and 65 μ g pyruvate kinase per ml, the ATPase activity of a fiber bundle of about 290 μ in diameter and 2.20 μ in sarcomere length was measured at first in 2.35 mM ATP and 3.35 mM $MgCl_2$, and then in 5.0 mM ATP and 6.0 mM $MgCl_2$ (Fig.6). The activities were

Fig.6

104 and 121 μ moles pyruvate per minutes per g of protein, repectively. As described later, the activity showed a tendency to increase on repeated measurement (cf. Fig.11). Furthermore, the mean value of myofibrillar ATPase activity at sarcomere lengths of between 2.0 and 2.5 μ was 92 ± 7 (S.E. of mean) μ moles pyruvate per minute per g of protein in the presence of 0.6 to 2.35 mM ATP, 1.0 mM PEP and 8.4 to 65 μ g pyruvate kinase per ml at pH 7.0 and 25°C (cf. Fig.9). Therefore, the ATPase activity of fiber bundles of less than 300 μ in diameter and 2.0 to 2.5 μ in sarcomere length was concluded to be independent of the ATP concentration between 0.6 and 5.0 mM in the presence of at least 1.0 mM PEP and 8.4 μ g pyruvate kinase per ml.

The ATPase activity of a fiber bundle of about 250 μ in diameter and 4.10 μ in sarcomere length was almost independent of the ATP concentration between 0.22 and 0.77 mM, when the reaction mixture contained 1.0 mM PEP and 8.4 μ g pyruvate kinase per ml. The ATPase activity of fiber bundles of about 310 μ in diameter and 5.33 μ in sarcomere length was independent of the PEP concentration between 0.5 and 1.0 mM in the presence of 0.6 mM ATP and 8.4 μ g pyruvate kinase per ml.

As shown in Fig.7, the ATPase activity of fiber bundles of

Fig.7

about 310 μ in diameter and 0.78 μ in sarcomere length was almost independent of the pyruvate kinase concentration between 25 and 250 μg per ml in the presence of 0.6 mM ATP and 1.0 mM PEP. Furthermore, the activity of fiber bundles of about 240 μ in diameter and 0.93 μ in sarcomere length was nearly independent of the ATP concentration between 2.35 and 5.0 mM in the presence of 1.0 mM PEP and 73 μg pyruvate kinase per ml. The ATPase activity of fiber bundles of less than 300 μ in diameter and less than 1 μ in sarcomere length was one-fifth to one-third of the maximum activity observed at sarcomere lengths of 2.0 to 2.5 μ in almost all cases, when the reaction mixture contained 0.6 to 5.0 mM ATP (see Fig.9). Thus, we can conclude that all fibers and fibrils are affected by ATP, when the diameter of fiber bundle is less than 300 μ and 0.6 mM ATP and 1.0 mM PEP are used as substrates in the presence of pyruvate kinase.

Dependence of ATPase activity on sarcomere length

The ATPase activity of muscle fiber bundles of less than 300 μ in diameter was measured in the presence of 0.6 to 2.35 mM ATP, 1.0 mM PEP and more than 8.4 μg pyruvate kinase per ml at different sarcomere lengths after storage in 50 per cent glycerol at -10°C during 1 to 4 months. The concentrations of MgCl_2 were

2.0 and 3.35 mM, respectively, with 0.6 and 2.35 mM ATP. No extra Ca^{++} was added to the reaction mixture.

Fig.8 (A) shows the time-course of pyruvate-liberation

Fig.8 A,B,C

from muscle fiber bundles of 3.40 μ in sarcomere length in the presence of $\overset{0.6}{\Delta}$ mM ATP, 1.0 mM PEP, and 10 μ g pyruvate kinase per ml. The activity was 64 μ moles pyruvate per minute per g of protein. A fiber bundle of 3.34 ± 0.10 μ in sarcomere length was shortened from 39 mm to 29 mm in muscle length, i.e., to 2.5 μ in sarcomere length, and then the ATPase activity was measured under the same conditions as above. As shown in Fig.8 (B), the activity was 93 μ moles pyruvate per minute per g of protein. Three fiber bundles with sarcomere lengths of 2.46 ± 0.03 μ , 2.24 ± 0.12 μ , and 2.39 ± 0.05 μ , respectively, were shortened from 35 to 15 mm in fiber length, i.e., to 1.0 μ in mean sarcomere length. They showed an activity of 31 μ moles pyruvate per minute per g of protein (Fig.8 (C)). In Fig.9, the ATPase activities of

Fig.9

30 preparations are plotted against sarcomere length. The

maximum activity was observed at sarcomere lengths of 2.0 to 2.5 μ , and was 92 ± 7 μ moles pyruvate per minute per g of protein (mean \pm S.E. of 6 values). The ATPase activity decreased linearly with increase in length beyond 2.5 μ , and showed a constant value of 30 μ moles pyruvate per minute per g of protein at sarcomere lengths above about 4 μ , as already reported by Ward et al. (22). On the other hand, with sarcomere lengths of less than about 2.0 μ , the ATPase activity decreased as the sarcomere length decreased, and reached one-fifth to one-third of the maximum activity at sarcomere lengths of less than about 1 μ .

The broken line above 2 μ of sarcomere length in Fig.9 was plotted on the basis of the correlation between sarcomere length and the number of bridges between myosin and actin filaments deduced from electron microscopic observations made by Huxley et al. (1). In this calculation, the activity at sarcomere lengths of above 3.5 μ or below 1.5 μ was assumed to be equal to the activity of the myosin type (see "DISCUSSION"). A value of 7.5 μ moles P_i per minute per g of protein was adopted as the activity of this type, since the ATPase activity of myosin under conditions similar to those used here was 15 μ moles P_i per minute per g of myosin, and since about 50 per cent of the protein in glycerol-treated muscle fibers is myosin (58). Our results on the relation between ATPase activity and sarcomere length deviate from those deduced from the above assumption at longer and shorter sarcomere lengths.

Fiber bundles of 2.91 ± 0.07 μ in sarcomere length, which

had been stored at -10°C in 50 per cent glycerol for about 4 months, were quickly put into reaction mixture containing 1.0 mM EGTA, 2.35 mM ATP, 1.0 mM PEP, 116 μg pyruvate kinase per ml and 3.35 mM MgCl_2 at pH 7.0 and 25°C . Then 3.5 minutes later, 2.0 mM Ca^{++} were added to the reaction mixture, and immediately the fiber bundles were cut in the middle with scissors. At the final stage, the pH of the reaction mixture was 6.7, and the sarcomere length and diameter of the fiber bundles were, respectively, about 0.7 μ and about 340 μ . The control activity was measured by the same procedures as above. Fig.10 shows

Fig.10

the time-course of pyruvate-liberation obtained by subtracting the pyruvate-liberation of the control from that of the whole. The ATPase activity was 134 μmoles pyruvate per minute per g of protein before cutting the fiber bundles and adding Ca^{++} , and was 54 μmoles pyruvate per minute per g of protein after these treatments. The high ATPase activity in the presence of 1 mM EGTA indicated a low Ca^{++} -sensitivity of the fiber bundles used (see "DISCUSSION"), and the low activity at about 0.7 μ in the presence of 1.0 mM free Ca^{++} was consistent with the results on the relation between sarcomere length and ATPase activity.

Dependence of ATPase activity on Ca^{++} ion at fixed sarcomere lengths

After storage in 50 per cent glycerol at -10°C for about 3 months, the dependence on Ca^{++} -concentration of ATPase activity of a fiber bundle of $2.28\ \mu$ in the presence of $2.35\ \text{mM}$ ATP, $1.0\ \text{mM}$ PEP, either 58 or $39\ \mu\text{g}$ pyruvate kinase per ml and $3.35\ \text{mM}$ MgCl_2 . The activity of the fiber bundle was measured successively in the presence of $1\ \text{mM}$, $0.1\ \text{mM}$, $6\ \mu\text{M}$, $0.4\ \mu\text{M}$ and $1\ \text{mM}$ of free Ca^{++} . The ATPase activities were 80 , 98 , 124 , 99 and $124\ \mu\text{moles}$ pyruvate per minute per g of protein, respectively (Fig.11). The ATPase activity in the first measurement ($1\ \text{mM}\ \text{Ca}^{++}$) was lower than those in the third ($6\ \mu\text{M}\ \text{Ca}^{++}$) and fifth ($1\ \text{mM}\ \text{Ca}^{++}$). These results suggest that the fine structure of myofibrils is destroyed by repeating the measurement in the presence of $2.35\ \text{mM}$ ATP, and that the destruction leads to an increase in the activity.

The ATPase activity of fiber bundles of 2.07 to $2.65\ \mu$ in sarcomere length was $116 \pm 22\ \mu\text{moles}$ pyruvate per minute per g of protein (mean of 6 values) in the presence of $1\ \text{mM}\ \text{Ca}^{++}$. The ATPase activities of fiber bundles of $2.58\ \mu$ in sarcomere length in the presence of $1\ \text{mM}\ \text{Ca}^{++}$ and $58\ \mu\text{g}$ pyruvate kinase per ml and in the presence of $0.01\ \mu\text{M}$ free Ca^{++} and $19\ \mu\text{g}$ pyruvate kinase per ml were, respectively, 115 and $47\ \mu\text{moles}$ pyruvate per minute per g of protein (Fig.12). The ATPase activity at a sarcomere

Fig.12

length of $2.07\ \mu$, measured in the presence of $2\ \text{mM}$ EGTA under almost the same conditions as above, was $45\ \mu\text{moles}$ pyruvate per

minute per g of protein (Fig.13 (B)). In Fig.14, the ATPase

Fig.14

activity of fiber bundles of 2.00 to 2.65 μ in sarcomere length, which had been stored in 50 per cent glycerol at -10°C for about 3 months, is plotted against concentration of free Ca^{++} , taking the mean value of the activity in the presence of 1 mM Ca^{++} as 100. The half maximum activation was obtained at a concentration of about 0.04 μM of free Ca^{++} . In the presence of 1 to 2 mM EGTA and at 2.00 to 2.33 μ sarcomere length, the activity of muscle fiber bundles which had been stored in 50 per cent glycerol for about 3 months was 48 ± 14 μmoles pyruvate per minute per g of protein (mean of 6 values). On the other hand, the activity of fiber bundles which had been stored in 50 per cent glycerol for 13 days at a sarcomere length of 2.03 μ and in the presence of 1.0 mM EGTA was only 17 μmoles pyruvate per minute per g of protein (Fig.13 (A)). Table I summarizes the results on the

Fig.13

Table I

ATPase activities of fiber bundles stored for 3 to 13 days in the presence of 1.0 to 2.0 mM EGTA. The ATPase activity was 17 ± 5 μ moles pyruvate per minute per g of protein (mean of 8 values), which was about one third of that of bundles stored for about 3 months. Measurement of the ATPase activity of fiber bundles stored for a short period was attempted several times in the presence of 1 mM Ca^{++} , but was unsuccessful, because of breakdown of bundles within 1 minute after adding ATP. The ATPase activity of fiber bundles of 2.38 μ in sarcomere length, which had been stored for about 9 months, was measured only once in the presence of 1.0 mM EGTA under the same conditions as above. It was 126 μ moles pyruvate per minute per g of protein.

Dependence of ATPase activity of myosin B extracted from glycerol-treated muscle fiber bundles and fresh psoas muscle on Ca^{++} ion

The ATPase activity of myosin B, extracted from fresh psoas muscle and purified by dilution-precipitation treatment performed twice, was measured in the absence and presence of 2 mM EGTA.

Fig.15 shows the pyruvate-liberation from myosin B (0.0177 mg/ml)

Fig.15

ATPase in the presence of 0.094 mM ATP, 1.0 mM PEP, 58 μ g pyruvate kinase per ml, 54 mM KCl, 3.35 mM MgCl_2 and 20 mM Tris-maleate buffer at pH 7.0 and 25°C, as a function of time. The time-course was not linear when no extra Ca^{++} was added. The decrease in rate with time may be due to transformation of ATPase from the actomyosin type to the myosin type by ATP. The initial rate of ATPase activity was 180 μ moles pyruvate per minute per g of myosin B in the absence of EGTA, and 22 μ moles pyruvate per minute per g of myosin B in the presence of 1.0 mM EGTA. The

activity of myosin B was 250 μ moles pyruvate per minute per g of myosin B in the presence of 1.0 mM Ca^{++} . The activity in the absence of EGTA increased from 180 to 340 μ moles pyruvate per minute per g of myosin B, when the reaction of myosin-B (0.0177 mg/ml) ATPase was initiated by adding ATP after incubation for 5 minutes with F-actin (0.019 mg/ml). This increase in the activity on addition of F-actin shows that not all the myosin molecules in this preparation of myosin B represented the actomyosin type of ATPase, and that the amount of binding of myosin with F-actin was increased by adding F-actin. ATPase activities were measured with myosin B, extracted from glycerol-treated muscle fiber bundles stored in 50 per cent glycerol at -10°C for 9 to 18 months in the presence of various concentrations of free Ca^{++} , 0.094 mM ATP, 1.0 mMPEP, 58 μ g pyruvate kinase per ml, 52 mM KCl, 3.35 mM MgCl_2 and 20 mM Tris-maleate buffer at pH 7.0 and 25°C . ATPase activities in the presence of 1.0 mM, 0.05 mM, no added Ca^{++} , 0.05 μ M of free Ca^{++} and 1.0 mM EGTA were 250, 310, 255, 92 and 92 μ moles pyruvate per minute per g of myosin B, respectively. Table II also summarizes the results

Table II

obtained on the dependence of the ATPase activity of myosin B extracted from fresh psoas muscles on Ca^{++} concentration. In the presence of 1.0 mM Ca^{++} , the ATPase activity of myosin B extracted from glycerol-treated muscle fiber bundles was nearly

equal to the activity of myosin B extracted from fresh psoas muscle, but in the presence of 1.0 mM EGTA the ATAase activity of the former was about five times more than that of the latter.

II Subfragments, S-I, of Myosin Molecule

Yield of S-1 from HMM

HMM (14 mg/ml) was digested with trypsin (weight ratio of HMM to trypsin, 28 : 1) at pH 7.6 and 25°C for 13 min. The elution pattern of the reaction mixture (20 ml) from a column of Sephadex G-200 had two main peaks, as shown in Fig.16. The

Fig.16

specific activity of the Ca^{++} -activated ATPase in the central fractions of the first peak was equal to that of undigested HMM. When HMM was digested with trypsin of Sigma Chemical Co. (from bovine pancreas, 2 x cryst. EtOH ppt.) under the conditions used by Young et al.(42), only the first peak appeared, though Young et al. prepared S-1 using trypsin from Worthington Chemical Corp. Thus, the activity of trypsin from Sigma Chemical Co. seems to be less than that of trypsin from Worthington Chemical Corp. The second peak contained S-1, and on rechromatography was eluted as a single, symmetrical peak (cf. Fig.17). The amount of protein in the second peak and the amount in decrease in HMM (difference between the amount in HMM before digestion and that

in the first peak) were in the ratio of 0.65 : 1. Neglecting secondary degradation of S-1, this indicates that the yield of S-1 from HMM was 65 %.

S-1 obtained by the method described in the "EXPERIMENTAL" was applied to a column (3.6 x 92 cm) of Sephadex G-200, and eluted with the buffer solution (Fig.17). The specific activity

Fig.17

of Ca^{++} -ATPase in the central portion of the main peak was almost constant. The central portion of the main peak was concentrated by precipitation from 70 % saturated $(\text{NH}_4)_2\text{SO}_4$. After removing the $(\text{NH}_4)_2\text{SO}_4$, it was subjected to analytical ultracentrifugation in 0.20 M KCl, 0.05 M Tris-HCl and 1 mM EDTA at pH 7.6 and 4.2°C. The sedimentation diagram of the preparation 4 days after rechromatography revealed a fairly symmetrical main peak, and a slower moving component(s) as previously reported by Mueller (43).

The $S_{20,w}$ value of the main peak was 5.03 S, which was similar to the value of 5.04 S, calculated from the concentration dependency of the $S_{20,w}$ of S-1 obtained by Young et al. (42).

Therefore, we adopted the value of 1.2×10^5 , obtained by Young et al. (42), as the molecular weight of our preparation of S-1*. The molecular weights of HMM and myosin are about 3.4×10^5 (32-37) and 4.8×10^5 (60), respectively, so the myosin or HMM molecule seems to contain two regions which appear as S-1, as

* We (59) recently measured the molecular weight of S-1 with gel-filtration method, and it was found to be 1.22×10^5 .

already indicated by Lowey and her associates (61), Trotta et al. (45), Nauss et al. (62) and Bailin and Bárány (63).

Degradation of S-1 in 0.05 to 0.20 M KCl

Fig.18 shows that the amount of slower component(s) in the

Fig. 18

sedimentation pattern increases on storage of the preparation in 0.20 M KCl, 1 mM EDTA and 0.05 M Tris-HCl at pH 7.6 and 0°C. The amount of intact S-1, estimated from the ultracentrifugal pattern, decreased to 74 and 54 % of the initial value 4 and 10 days after rechromatography, respectively (values not corrected for the Johnston-Ogston effect). Degradation was markedly retarded by addition of sucrose. Thus, after 11 days storage in 0.05 M KCl, 0.05 M Tris-HCl and 0.5 mM EDTA containing 0.07 M sucrose at pH 7.6 and 0°C, only 18 % of the S-1 were degraded.

The decrease in the Ca^{++} -ATPase activity seemed to be proportional to the degree of degradation of S-1 to components of lower molecular weight (Fig.19). The rates of decrease in

Fig.19

Ca⁺⁺-ATPase activity were 9 and 0.5 % per day, respectively, in the absence and presence of 0.05 M sucrose. Lyophilized S-1 was very stable, and after 7 months' storage retained about 90 % of its specific Ca⁺⁺-ATPase activity before lyophilization.

Degradation of S-1 in guanidine-HCl or alkaline solution

The sedimenting boundary of S-1 (9.14 mg/ml) in 5 M guanidine-HCl at 4°C was broad, and the value of $S_{20,w}$ of the peak was 1.1 S (see inserted figure in Fig.20). S-1 was stored in 4.6 M guanidine-HCl solution (7.9 mg/ml S-1, 3 ml) at 4°C for 34 days, and then applied to a column (3.7 x 31 cm) of Sephadex G-200 equilibrated with 5 M guanidine-HCl and 2 mM EDTA. The column was eluted with the same solvent at a flow rate of 11 ml per hr at room temperature. Three peaks were eluted after that of blue dextran, as shown in Fig.20. About 30 % of the total protein were eluted after cytochrome C.*

Fig.20

A solution of S-1 was adjusted to pH 11.5 with triethylamine at 0°C, and then immediately subjected to ultracentrifugation at 5°C. The final composition of the solution was 9.1 mg of S-1 per ml, 0.05 M KCl, 5 mM Tris-HCl and 40 mM triethylamine. A broad peak appeared and the $S_{20,w}$ of its top was 1.5 S.

* Kindly supplied by Mr. T. Tanaka of Prof. Okunuki's laboratory, Department of Biology, Faculty of Science, Osaka University.

A solution of S-1 was adjusted to pH 11.5 with triethylamine, and 2 hours later it was applied to a column (2.7 x 87 cm) of Sephadex G-100, equilibrated with 0.05 M KCl and 0.025 M triethylamine-CO₂ buffer at pH 11.0 and room temperature. As shown in Fig.21, the

Fig.21

elution pattern was similar to that in guanidine-HCl, described above. According to Trotta et al. (45), S-1 prepared with trypsin dissociated into a heavy component (molecular weight 66,800): a light component (18,600) and peptide components (2,100, 16 % of the total material) on alkaline treatment. The present results also showed that the S-1 prepared with trypsin dissociated into small components on treatment with guanidine-HCl or alkali.

ATPase activities in the steady state

The ATPase activities of the control myosin used in the carboxamidomethylation experiment, measured in the presence of 7 mM CaCl₂, 20 mM MgCl₂ and 3 mM EDTA, were 189, 2.50 and 1,510 μ moles Pi/min/g, respectively. The specific Ca⁺⁺-, Mg⁺⁺- and EDTA-ATPase activities of HMM prepared from the control myosin were 236, 3.25 and 2,200 μ moles Pi/min/g, respectively. After correction for the presence of inactive small components (8 % in this case), the ATPase activities of S-1, prepared from the above preparation of HMM, were 363, 4.86 and 3,880 μ moles Pi/min/g in the presence of Ca⁺⁺, Mg⁺⁺ and EDTA, respectively.

As shown in Table III, the specific ATPase activities of myosin per mole were nearly equal to those of HMM, while the

Table III

activities of S-1 were about half those of myosin and HMM. The Ca^{++} -ATPase activities of preparations of myosin, HMM and S-1 were measured in 0.60 M KCl, 1 mM ATP, 0.05 M Tris-maleate and 10 mM CaCl_2 at pH 6.4 and 25°C, and were 230 ± 21 , 223 ± 32 and $110 \pm 11^*$ (mean \pm standard error of 4 values) moles Pi/min/mole of myosin, HMM and S-1, respectively. These results showed that on formation of S-1 there was no over-all change around the active site of steady state ATPase. Nauss et al. (62) obtained similar results on EDTA-ATPase activity, though the Ca^{++} -ATPase activity of their S-1 was higher than the value expected if two S-1 were produced from one HMM.

ATPase activity of HMM and S-1 prepared from myosin after carboxamidomethylation

Myosin was treated with IAA- $1\text{-}^{14}\text{C}$ in 0.5 M KCl and 0.1 M Tris-HCl at pH 8.5 and 0°C for 44 hr (molar ratio of myosin to IAA, 1 : 3) (57). The amount of IAA bound to myosin was 2.9 moles per mole of myosin. The Ca^{++} - and Mg^{++} -ATPase activities increased 11.7 and 9.7 fold, respectively, on this modification, but the EDTA-ATPase activity decreased to 24 % of the control value. The radioactivities of HMM isolated from tryptic digests of modified myosin showed that it contained

* No correction was made for degradation of S-1

2.3 moles of IAA per mole of HMM. The Ca^{++} - and Mg^{++} -ATPase activities of the IAA-HMM were 11.4 and 10 times those of control HMM, respectively, and the EDTA-ATPase activity was 19 % of the control value. S-1, produced by further tryptic digestion of IAA-HMM, was purified by chromatography on Sephadex G-200 (Fig.22). The elution pattern of S-1 was unchanged by

Fig.22

carboxamidomethylation. The fractions indicated by the symbol, \longleftrightarrow , contained 0.76 mole IAA per mole of S-1. The Ca^{++} - and Mg^{++} -ATPase activities of the IAA-S-1 were respectively 8.8 and 9.0 fold those of the control, while the EDTA-ATPase activity was 18 %. Ohe et al. (57) reported that the Ca^{++} - and Mg^{++} -ATPase activities were maximally activated on carboxamidomethylation of one specific cysteine residue in the myosin molecule by IAA and that this cysteine residue was blocked completely when about 3 moles of IAA were bound to myosin. The present results show that this cysteine residue was retained in S-1 (cf. Table III).

Carboxamidomethylation of S-1 with IAA-1- ^{14}C was carried out under the same conditions as those used with myosin, except that the molar ratio of S-1 to IAA was 1 : 20. The time-course of binding of IAA to S-1 was unaffected by the addition of sucrose. The extent of activation of the Ca^{++} -ATPase activity of S-1 by carboxamidomethylation (9 fold) was nearly

equal to that observed with myosin (Fig.23). In sharp contrast

Fig.23

with myosin where the extent of activation remained constant, even on binding of large amounts of IAA (see, Fig.4 of Ref. 57), the ATPase activity of S-1 decreased markedly with binding more than 2.3 moles of IAA per mole of S-1.

Initial stoichiometric burst of Pi-liberation from the HMM-and S-1-ATP system

Fig. 24 A shows the time-course of Pi-liberation from the HMM^{*}-ATP system in the presence of 2 mM MgCl₂. The amount of the initial stoichiometric burst with lyophilized HMM, 1.1 mole per mole, agreed with that with fresh HMM reported by Tokuyama et al. (53). Fig.24 B shows the time-course of Pi-liberation from the S-1-ATP system in the presence of 1 mM MgCl₂. The amount of the initial burst was 0.55 mole per mole

Fig.24 A,B

of S-1. The amount of the initial stoichiometric burst of Pi-liberation from the myosin-ATP system, measured at Mg⁺⁺-

* HMM lyophilized by the method of Yount and Koshland (64) was used.

concentrations above 1 mM, was 1.0 to 1.2 mole per mole of myosin (14, 15, 16, 56, 65).

Fig.25 shows the time-courses of P_i -liberation on mixing

Fig.25

1.0 mg per ml of S-1 with various concentrations of ATP (2-20 μ M) in 2.8 M KCl, 10 mM $MgCl_2$ and 20 mM Tris-HCl at pH 7.5 and 0°C. At ATP concentrations below 5 μ M, the time required for the initial P_i -liberation to reach half the maximum value, $\tau_{1/2}$, was independent of the ATP concentration and was about 24 sec, but the amount of P_i -liberation in the initial phase increased almost linearly with ATP concentration. At ATP concentrations above 5 μ M, the amount of the initial P_i -liberation remained constant at 1.2 mole per 2.4×10^5 g of S-1, as shown in Fig.26. The dependences of the amount and $\tau_{1/2}$ value of initial P_i -liberation per 2.4×10^5 g of S-1 on the ATP concen-

Fig.26

tration were similar to those per 4.8×10^5 g of myosin.

Discussion

The present results show that the diffusions of neither ATP nor PEP are limiting factors in the hydrolysis of ATP, if fiber bundles of less than 300 μ in diameter are used in the

presence of at least 0.6 mM ATP, 1.0 mM PEP and kinase. According to the Meyerhof-Schulz equation (66), the relation between the concentration of consumable substrate and the radius (γ) of a cylindrical shaped enzyme is given by

$$\gamma^2 = (C_e - C_i) \frac{4D}{A} ,$$

where C_e and C_i are the exterior and interior concentration of ATP, D is the diffusion coefficient, and A is the rate of ATP splitting. According to Bowen and Martin (67), D was 1.4 to 2.0×10^{-6} sq. cm per sec, which was one-half the rate of free diffusion of ATP through aqueous solution. Measurement of ATPase activity of myofibrillar fragments with no diffusion-problems indicated that it was sufficient for C_i to be 0.1 mM, and that A was 0.20 to 0.25 mmoles ATP split per sec per liter (68, 69). Substituting these values for D , A and C_i and using a value of 0.6 mM for C_e , which was the lowest concentration of ATP in our experiments, in the MEYERHOF-SCHULZ equation, γ was calculated at about 40 μ . This value (80 μ in diameter) is almost the order of diameter of a single fiber. This result indicates that when a fiber bundle of up to about 80 μ width, i.e., a single fiber, is used, the probability is that 0.6 mM ATP will affect all fibers and fibrils. But this width is much less than that of the bundles used in our experiments. Fiber bundle of less than 300 μ in diameter could be used in the presence of pyruvate kinase and PEP, since these substances could penetrate the endomysial zone between fibers, and maintain the concentration of ATP around each fiber at 0.6 mM.

Gordon et al. (9, 10) have studied the relation between sarcomere length and isometric tetanus tension, with special precautions to ensure uniformity of sarcomere length within the part of the fiber being studied. As shown in Fig.27,

Fig.27

in most respects the results were consistent with those found by Ramsey and Street (70), which were obtained without these special precautions. The peak of the curve observed by Gordon et al. was found to consist of a plateau between sarcomere lengths of 2.05 and 2.2 μ , and the decrease of tension above this plateau was steeper than that found by Ramsey and Street. Tension development became almost zero at sarcomere lengths of more than 3.65 μ . Many features of this length-tension relation at sarcomere lengths of more than 2.05 μ have been simply explained on the sliding-filament theory (see, Fig.27). Furthermore, the decline of tension below the plateau suddenly became steeper at a sarcomere length of about 1.67 μ and tension development became almost zero at a sarcomere length of about 1.3 μ .

Infante et al. (19) studied the dependencies on muscle length of ^{breakdown of} phosphoryl creatine and tension in isometric contractions on frog rectus abdominis muscle treated with 2,4-dinitrophenol. The results showed that these two relations were almost identical; both the amount of breakdown and the

tension development were almost zero both at about 130 per cent and 50 per cent of the resting length in situ, and their maximum values were obtained at the resting length. Similar results were also obtained by them (21) on the length dependence of P_i -liberation of frog sartorius muscle. The length dependence of splitting of phosphoryl creatine during isometric tetanus has since been determined in great detail with frog sartorius muscle treated with iodoacetate and nitrogen to block the resynthesis of ATP by Sandberg and Carlson (20). The results showed that the breakdown of phosphoryl creatine was maximum at a sarcomere length of about 2.3μ , and 50 per cent of the maximum value at sarcomere lengths of both 1.5μ and 3.2μ . They also suggested that there were two components in the utilization of chemical energy during an isometric tetanic contraction, that is, a tension (or length) dependent component and a tension independent component, and that the former was explainable on the sliding theory and the latter was a reflection of the energy utilization of the calcium pump found in the sarcoplasmic reticulum (71, 72).

The length dependence of ATPase activity during isometric contraction induced by ATP in glycerol-treated muscle fiber bundles was similar to those of tension development, breakdown of phosphoryl creatine and P_i -production during isometric tetanic contraction in living muscle, as described above. However, our results on the length-ATPase activity relation deviate from the theoretical relation at sarcomere lengths of above 2.0μ , as those on the length-tension relation by Ramsey and Street (70) do (cf. Fig.9 and Fig.27). This deviation may

be attributable to heterogeneity in sarcomere length in the end parts of the fiber bundles. The heterogeneity may also be caused by local shortening induced by ATP.

As the sarcomere length decreased below $2.0\ \mu$, the ATPase activity decreased, and, at sarcomere lengths of about $1\ \mu$, it reached one quarter to one third of the maximum activity. Gordon et al. (10) reported that muscle fiber does not develop tension when the sarcomere length decreases below about $1.3\ \mu$. One possible explanation for the decrease in ATPase activity and tension development is that the increase in fiber diameter which takes place as the muscle contracts allows the two types of filaments to move further apart and so alters the spatial situation at the cross-bridges (1). Furthermore, when muscle is shortened below a sarcomere length of $1.5\ \mu$ where myosin filaments hit the Z-discs, the filaments are expected to crumple or fold. This may reduce the number of bridges capable of generating both the actomyosin type of ATPase and tension. On the other hand, Gordon et al. (10) suggested that more than half the drop of tetanic tension represented an internal force opposing shortening rather than a decrease in intrinsic force of contraction. Hoyle et al. (73) and Osborne (74) reported in their studies of "supercontraction" that contraction down to below about 60 per cent of the resting length allowed the myosin filaments to pass through the Z-discs from one sarcomere to the adjacent sarcomere. This suggests the existence of an interaction between the projections of myosin filaments and actin filaments even at very short sarcomere lengths. Therefore, another

possibility is that the two types of filaments cannot move or oscillate past each other at sarcomere lengths below $1\ \mu$, because the ends of crumpled or folded myosin filaments ($1.6\ \mu$) are connected with the Z-discs and those of actin filaments ($1.0\ \mu$) come into collision with the opposite Z-discs. If the actomyosin type of ATPase is coupled with moving or oscillating of the two types of filaments past each other, and if the tension development is coupled with the actomyosin type of ATPase, both ATPase activity and tension development of muscle fibers must decrease at a short sarcomere length where the movement of the projections of myosin filaments cannot occur (75).

The ATPase activity of glycerol-treated muscle fiber bundles stored in 50 per cent glycerol at -10°C for about 3 months increased with increase in the Ca^{++} concentration. In the absence of Ca^{++} it was about 48 $\mu\text{moles pyruvate per minute per g of protein}$, and in the presence of sufficient Ca^{++} it was about 116 $\mu\text{moles pyruvate per minute per g of protein}$. The ATPase activity of myosin B extracted from fresh psoas muscles was almost the same as that of myosin B from glycerol-treated muscles stored for the long period in the presence of 1 mM Ca^{++} , but the latter was much higher than the former in the presence of 1 mM EGTA (cf. Table II). In the absence of Ca^{++} , the ATPase activity of fiber bundles stored for 3 to 13 days was about one ^h_A third of that of bundles stored for about 3 months. On the other hand, the ATPase activity of fiber bundles in the absence of Ca^{++} , which had been stored for about 9 months, was

about three times as large as that of bundles stored for about 3 months, and was almost the same as that of the latter bundle in the presence of Ca^{++} . These results showed that Ca^{++} -sensitivity of ATPase activity of fiber bundle conspicuously decreased as the period of storage became longer. These results on ATPase activity correspond to the results reported by Bozler (76, 77) that Ca^{++} -sensitivity of tension developed by ATP decreased as the period of storage became longer. It was shown by Ebashi (78, 79) that Ca^{++} -sensitivity of myosin-B in ATPase and superprecipitation was attributable to native tropomyosin which was composed of both tropomyosin and troponin. Therefore, there is a possibility that Ca^{++} -sensitivity of fiber bundle in ATPase activity might be disappeared by destruction of native tropomyosin on storing in glycerol for the long period.

To elucidate the number of ATPase site of myosin molecule, the bindings of PP_i , ADP and ATP to myosin have been investigated by many workers (Table IV). Tonomura and Morita (80)

Table IV

showed that two moles of PP_i were bound to one mole of myosin, and that the light-scattering intensity of myosin B decreased on binding of one mole of PP_i per mole of myosin in myosin B. Nauss et al. (62) and Kiely and Martonosi (81) recently confirmed that two moles of PP_i were bound per mole of myosin, though Gergely et al. (82) and Martonosi and Meyer (83) previously claimed

that the myosin molecule could bind only one PP_i molecule. Young (84) reported that two moles of ADP were bound to one mole of myosin with the same value of the association constant, and that one mole of ADP was bound to one mole of S-1. However, Morita (85, 86) recently showed that two moles of ADP combined with one mole of HMM, that the two had different association constants, and that the binding with the higher association constant induced the change in the ultraviolet absorbance of HMM.

Tomomura and his coworkers (11 - 14) have concluded that myosin has two kinds of active site of ATPase, i.e., site 1 and site 2 (referred to as the site for simple hydrolysis and the site for hydrolysis via phosphorylation, respectively). They demonstrated one mole of phosphorylation per mole of myosin at site 2, measured as the stoichiometric initial burst of P_i -liberation, and concluded that the binding of ATP or ADP to site 2 resulted in the change in ultraviolet absorbance of HMM (14). Nanninga and Mommaerts (88) using luciferase-luciferin system demonstrated that one mole of ATP was bound to 4.2×10^5 g of myosin. Imamura et al. (11) obtained the same result from kinetic analysis of the myosin-ATP system, using a Straus-Goldstein plot. Studies on the dependence of the difference spectrum of HMM on the ATP concentration by Morita (91) and Sekiya and Tomomura (87) also showed that one ATP molecule was bound to one HMM molecule.

The properties of the active site of myosin ATPase have also been studied by chemical modifications. P-Nitrothiophenol bound specifically to about one mole of a specific glutamic acid residue located at site 2 per mole of myosin. As a result

the initial stoichiometric burst of P_i -liberation was completely lost, but the ATPase activity in the steady state remained unaffected (12, 18). Ohe et al. (57) recently showed that the Mg^{++} -, and Ca^{++} -ATPase activities of myosin were fully activated, while the EDTA-ATPase activity was inhibited, when one mole of a specific cysteine residue per mole of myosin in the sequence Ileu. CySH. Arg was modified with IAA. Tokuyama et al. (53) and Shimada (92) reported that the amounts of the sites for specific chemical modifications by 2,4,6-trinitrobenzenesulfonate and by 5-diazo-1H-tetrazole were about 1 mole per mole of myosin. All these results indicate that one mole of site 1 and one mole of site 2 are presented per mole of myosin.

Two S-1 molecules were produced from one HMM molecule, as described in the "RESULTS". The ATPase activity in the steady state and the amount of the initial stoichiometric burst per mole of myosin were substantially retained in the two moles of S-1, although many intramolecular peptide bonds were broken on formation of S-1 (cf. Table III, V). Moreover, the extra-burst of P_i -liberation per mole of myosin or HMM was twice that per mole of S-1 (Table V). These results show that both active

Table V

sites of myosin, site 1 and site 2, are located in these two molecular portions which appear as S-1 on tryptic digestion.

The dependence of $\tau_{1/2}$ and the amount of initial stoichiometric burst of P_i -liberation per $2 \times 1.2 \times 10^5$ g of S-1 on the ATP concentration were the same as for 4.8×10^5 g of myosin, as shown in Fig.26. According to the reaction mechanism of the myosin-ATP system proposed by Tonomura et al. (14), the formation of the Michaelis complex at site 1 (E_2^{1S}) occurs very rapidly and stoichiometrically. This rapidly formed E_2^{1S} is converted to E_{2S}^1 , and then to trichloroacetic acid-labile phosphoryl myosin ($E_{2P}^1 \cdot ADP$). When a sufficiently excess over the stoichiometric amount of substrate is present, ATP binds directly to site 2 ($E_2^1 + S \rightarrow E_{2S}^1$), besides being transferred from site 1 to 2 ($E_2^{1S} \rightarrow E_{2S}^1$). If site 2 is located in one S-1 and site 1 in the other S-1, there will be little transfer of ATP from site 1 to site 2 in the S-1-ATP system. Thus, the finding, that tryptic digestion of myosin to S-1 did not affect the dependences of $\tau_{1/2}$ and the amount of initial burst on the ATP concentration, indicates that both site 1 and site 2 are present in only one of the two S-1 of the myosin molecule.

However, Kielley and his associates (94-96) concluded that the myosin molecule is composed of identical subunits with a molecular weight of about 2.1×10^5 . Weeds and Hartley (97) also concluded that myosin was composed of identical subunits from analyses of the peptide structures around sulfhydryl groups. Ohe et al. (57) reported that one mole of cysteine residue in the Ileu. CySH. Arg sequence per mole of myosin was modified when native myosin was treated with IAA, but that two moles of residues in the same sequence were modified in the presence of urea. S-1 prepared from carboxamidomethyl

myosin showed almost the same extents of activation of Ca^{2+} -, and Mg^{2+} -ATPase (about 10 times the control value) and inhibition of EDTA-ATPase (to about 20 % of the control value) as myosin. Thus the above difference in the amount of IAA-binding could not be explained by supposing that the myosin molecule has two active sites of steady state ATPase but that IAA can bind to, or near, only one of them, probably due to steric hindrance between two S-1 parts. If we assume that there is one ATPase active site in each of the two S-1 portions, the EDTA-ATPase activity of IAA-S-1 prepared from IAA-myosin, in which one specific cysteine residue is modified, must be more than 1/2 that of control S-1. Furthermore, the Ca^{++} -ATPase activity of S-1 was activated by IAA to the almost same extent as that of myosin. These results seem to exclude the possibility that myosin has two identical active sites, and that in the myosin molecule one of them is buried.

Recently, Gershman et al. (98) showed that myosin is composed of 2 heavy subunits (molecular weight 212,000) and 2 light subunits (20,200). Furthermore, Murphy and Morales (99), and Stracher (100) demonstrated that the light subunit(s) was contained in the active site of myosin ATPase. The presence of only one active site in the myosin molecule can be explained in two ways: (i) Light chains are heterogeneous, and only one active light chain is present in the myosin molecule. The recent results of Gaetzen et al. (101) seems to support this possibility. Gazith et al. (102) recently showed that one of the two light subunits could be removed from myosin without any apparent changes in the ATPase activity, and that attempts to remove a large fraction of the subunits resulted

in a concomitant loss of enzymatic activity. Their results also support this possibility. (ii) The structures of the two heavy chains have minor differences as reported by Offer and Starr*, although they are identical in structure around the sulfhydryl groups. The proposed model of the myosin molecule was illustrated in Fig.28.

Fig.28

Jones and Perry (44) reported that all the S-1 subfragments can combine with actin. Onishi et al. (103) showed that the minimum amount of ATP required to produce the maximum decrease in the intensity of light-scattering of reconstituted actomyosin was 1 mole per mole of myosin. They (103) concluded that the decrease in light-scattering intensity was caused by the formation of a myosin-phosphate complex at site 2 ($E_2^1 \dots P$). Tonomura and Morita (80) showed that in myosin B only one mole of PP_i could bind to one mole of myosin and this binding induced a decrease in light-scattering, while in free myosin two moles PP_i could combine per mole of myosin. Nauss et al. (62) confirmed these results. Morales and his associates (104, 105) and Nihei and Tonomura (106)

* Presented at the Symposium on Molecular Biophysics of the International Union for Pure and Applied Biophysics (Cambridge, England, June 24-27, 1968).

showed that the weight average molecular weight of myosin B decreased slightly, but its gyration radius increased markedly on addition of ATP or PP_i . These facts could not be explained by supposing that myosin B dissociates completely into myosin and F-actin when ATP or PP_i is added. On the other hand, Gergely and Kohler (32, 107) demonstrated the dissociation of reconstituted actomyosin on addition of ATP. Weber (108) also reported the separation of myosin from actomyosin in the presence of ATP by ultracentrifugation. These apparently conflicting results may be interpreted as follows (Fig.29): The one S-1

Fig.29

part of the myosin molecule, which contains the active sites of ATPase, site 1 and 2, combines strongly with actin, while the other part combines less strongly. We will refer to these two kinds of S-1 as S-1a and S-1b, respectively (cf. Fig.28). When the myosin-phosphate or myosin- PP_i complex is formed in S-1a, S-1a dissociates from actin, but S-1b is still connected to actin through its weaker bond. However, this bond between S-1b and actin is partially decomposed at extremely low concentration of actomyosin as shown from light-scattering experiments, or by ultracentrifugal separation in the presence of ATP or PP_i . Complete dissociation was only observed with reconstituted actomyosin (32). This suggests that the bond between actin and S-1b in reconstituted actomyosin is weaker

than that in myosin B. However, at low ionic strength myosin molecules aggregate to form myosin filaments. Under these conditions, the bond between S-1b and F-actin might be very weak because of steric hindrance, and actomyosin dissociates into F-actin and myosin filaments on addition of ATP, as shown by electron-microscopy (109, 110).

Tonomura and his coworkers (14) proposed a molecular mechanism of muscle contraction on the basis of the reaction mechanism of the myosin-ATP system, especially transconformation of myosin from the α to the β state induced by decomposition of the phosphoryl intermediate and dissociation of actomyosin induced by formation of the myosin-phosphate complex. Assuming that the two head (S-1) portions of the myosin molecule have different functions, as discussed in this paper, this mechanism may be modified as follows (Fig.30). The binding of the S-1a portion with F-actin is initiated by release of

Fig.30

Ca^{2+} from the sarcoplasmic reticulum. The conformation of the S-1a portion is transformed from the α to the β state by direct decomposition of the phosphoryl intermediate, and the actin filament is shifted past the myosin filament. The bond between S-1a and F-actin is broken when a S-1a-phosphate complex is formed by ATP, and then the conformation of S-1a spontaneously

returns to the α -type. It is assumed that when S-1a takes the α -type shape, the S-1b portion of the myosin molecule can not bind to F-actin due to steric hindrance, but that it can bind when S-1a takes the β -type shape. The mechanism illustrated in Fig.30 will be readily understood by comparison with that given in Fig.24 of the previous paper (14).

Summary

I Dependence of contractile ATPase activity on sarcomere length and calcium ion concentration

1. Glycerol-treated muscle fiber bundles were prepared mainly by storage in 50 per cent glycerol at -10°C for about 3 months. Their ATPase activity was measured in the presence of 50 mM KCl, 2 to 3.35 mM MgCl_2 and 20 mM Tris-maleate buffer at pH 7.0 and 25°C during an isometric contraction induced by ATP. Pyruvate kinase was coupled with myofibrillar ATPase, and the activity was measured by determining the amount of pyruvate liberated. The process of liberation of pyruvate from fiber bundles was linear with time in all the measurements.

2. The relations between the ATPase activity of fiber bundles of less than 300μ in diameter and the concentration of ATP were investigated at sarcomere lengths from 0.8 to 5.3μ . It was concluded that the diffusion of ATP and phosphoenol pyruvate was not the limiting factor in the hydrolysis of ATP, if fiber bundles of less than 300μ in diameter were used in the presence of at least 0.6 mM ATP and 1.0 mM phosphoenol pyruvate.

3. The fiber bundles showed the maximum activity of 100μ moles pyruvate per minute per g of protein at sarcomere lengths of 2.0 to 2.5μ . As the sarcomere length increased beyond 2.5μ , the activity decreased and reached about 30μ moles pyruvate per minute per g of protein at sarcomere lengths of more than 4μ . Many features of this length-ATPase activity relation could be explained on the basis of the sliding filament theory of muscle contraction.

4. In the region where the sarcomere length was less than 2.0 μ , the ATPase activity decreased as the sarcomere length decreased, and, at a sarcomere length of 1 μ , it showed a quarter to a third of the maximum activity. These results suggested that both ATPase activity and tension development are coupled with the movement of myosin filaments past actin filaments.

5. The dependence on Ca^{++} concentration of the ATPase of glycerol-treated muscle fiber bundles, which had been stored for about 3 months, was investigated at fixed sarcomere lengths of 2.0 to 2.6 μ . When Ca^{++} was removed by EGTA, the activity was about 48 μ moles pyruvate per minute per g of protein, and in the presence of 1 mM Ca^{++} it was 116 μ moles pyruvate per g of protein. The concentration of Ca^{++} for the half maximum activation was about 0.04 μM . In the presence of 1 to 2 mM EGTA, the activity of fiber bundles stored for 3 to 13 days was only 17 μ moles pyruvate per minute per g of protein, and that of those stored for about 9 months was 126 μ moles pyruvate per minute per g of protein. Thus, Ca^{++} -sensitivity of myofibrillar ATPase activity was conspicuously decreased as the period of storage in glycerol became longer.

II. Subfragments, S-1, of myosin molecule

1. The yield of subfragment-1 (S-1) from tryptic digests of heavy meromyosin (HMM), which was prepared by tryptic digestion of myosin molecule, was 65 %. S-1 decomposed into smaller components at a rate of about 7 % of total material per day in neutral salt solution at 0°C. The Ca^{++} -ATPase activity of S-1 decreased in proportion to increase in the amount of small components. In 4.6-5 M guanidine-HCl and in alkaline (pH 11-11.5)

solution, S-1 showed a broad sedimentation pattern with $S_{20,w}$ values of the peak of 1.1 and 1.5 S, respectively. The pattern of elution from Sephadex G-200 and G-100 showed that S-1 was degraded into small components in 5 M guanidine-HCl or at pH 11.

2. The ratio of the ATPase activities of myosin (M.W. 4.8×10^5), HMM (3.4×10^5) and S-1 (1.2×10^5) on a molar basis was 1 : 1 : 0.5. This ratio was independent of the modifiers used. When myosin was subjected to carboxamidomethylation under conditions where one specific cysteine residue was completely modified with IAA, the Ca^{++} - and Mg^{++} -ATPase activities of myosin increased 11.7 and 9.7 fold, respectively, but the EDTA-ATPase activity decreased by 76 %. HMM and S-1 were prepared from this modified myosin. The extents of activation and inhibition of ATPase activities by IAA during their production of these fragments remained almost constant.

3. The amount of the initial stoichiometric burst of P_i -liberation per mole of S-1 was 0.55 to 0.6 mole, which was half of those of myosin and HMM. The rate of initial rapid P_i -liberation was independent of the ATP concentration when the latter was lower than 0.6 mole per mole of S-1, but at concentrations above this it increased with increase in ATP concentration. The amount of initial rapid P_i -liberation increased linearly with the ATP concentration until the amount of added ATP reached about 0.6 mole per mole of S-1, and at higher ATP concentrations remained constant at this value.

4. It was concluded that tryptic digestion of one mole of HMM produced two moles of S-1, one of which had the active sites of

ATP hydrolysis both via phosphorylation and by simple hydrolysis. The molecular mechanism of so-called dissociation of actomyosin by ATP was discussed, considering different functions of these two S-1 portions in the myosin molecule. The molecular mechanism of muscle contraction proposed previously by Tonomura and his coworkers was modified in consideration of the different functions of S-1.

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Table I

ATPase activity of glycerol-treated muscle fiber bundles stored in 50 % glycerol at -- 10°C for a short period in the presence of 1.0 mM EGTA.

2.35 mM ATP, 1.0 mM PEP, 19 µg pyruvate kinase/ml, 50 mM KCl, 3.35 mM MgCl₂, 20 mM Tris-maleate buffer, pH 7.0, 25°C.

Period of storage (days)	ATPase activity (µmoles pyruvate/ min/g of protein)	Sarcomere length (µ)
3	17.4	2.33 ± 0.07
4	9.1, 22	2.21
6	13	2.51
8	22.6, 14.3*, 19.7**	2.08
13	15.7	2.03

* 1.5 mM EGTA.

** 2.0 mM EGTA.

Table II

The dependence on Ca^{++} concentration of the ATPase activity of myosin B extracted from glycerol-treated muscle fiber bundles and from fresh psoas muscle.

Concentration of free Ca^{++} and EGTA	ATPase activity ($\mu\text{moles pyruvate/min/g of myosin B}$)	
	Extracted from glycerol-treated muscle fiber bundles	Extracted from fresh psoas muscle
1 mM Ca^{++}	250, 170*	250*
0.05 mM Ca^{++}	310	—
No added Ca^{++}	255	180*
0.05 μM Ca^{++}	92	—
1 mM EGTA	92, 107*	22*

* Myosin B was purified by two dilution-precipitation treatments and the others were purified once by this method. 0.094 mM ATP, 1.0 mM PEP, 50 μg pyruvate kinase/ml, 52 — 54 mM KCl, 3.35 mM MgCl_2 , 20 mM Tris-maleate buffer, pH 7.0, 25°C.

Table III

Effect of carboxamidomethylation on ATPase activities of myosin, HMM and S-1

ATPase activity was measured in 1 mM ATP, 1.0 M KCl and 0.05 M Tris-maleate at pH 7.0 and 25°C. A correction was made for the presence of inactive small components in S-1 (8 % in this case). Molecular weight : myosin, 4.8×10^5 ; HMM, 3.4×10^5 ; S-1, 1.2×10^5 .

Protein	Modifiers of ATPase *	v(mole P_i /min/mole)		CM/CON	IAA bound (mole/mole)
		CON **	CM **		
Myosin	Ca^{2+}	90.7	1,060	11.7	2.9
	Mg^{2+}	1.2	11.5	9.7	
	EDTA	725	173	0.24	
HMM	Ca^{2+}	80.2	915	11.4	2.3
	Mg^{2+}	1.1	11.5	10	
	EDTA	749	141	0.19	
S-1	Ca^{2+}	43.6	383	8.8	0.76
	Mg^{2+}	0.58	5.3	9.0	
	EDTA	465	84.2	0.18	

* 7 mM $CaCl_2$, 20 mM $MgCl_2$ or 3 mM EDTA

** CON : control protein, CM : protein after carboxamidomethylation

Table IV

The number of ^{the} substrate and ^{the} substrate-analogues bound to myosin and its active fragments

Author	Bound Amount* (mole/mole)	Method**	Conditions
Tonomura and Morita(80)(1959)	1.9-2.5 PPI to Myosin	Eq. Dial.	0.04-0.6M KCl, 0.3mM Mg ⁺⁺ , pH7.5, 25°C
Gergely et al.(82) (1959)	0.9-1.1 PPI to Myosin	Eq. Dial.	0.6M KCl, 1mM Mg ⁺⁺ , pH7.0, 2°C
Nanninga and Mommaerts (1960) (88)	1.1 ATP to Myosin****	Luciferase- Luciferin	0.36M Na ₂ SO ₄ , 0.05M NaHAsO ₄ , 26mM Mg ⁺⁺ , pH7.0-7.5, 25°C
Martonosi and Meyer(83)(1964)	1.0 PPI to Myosin	Eq. Dial.	0.6M KCl, 1mM Mg ⁺⁺ , pH7.6, 4°C
Sekiya and Tonomura(87)(1966)	0.98 ATP to HMM*** 0.98 ADP to HMM	Dif. Sp.	0.5-0.6M KCl, 1-2mM Mg ⁺⁺ , pH7.5, 24°C
Imamura et al.(11) (1966)	1.2 ATP to Myosin***	Kinetics	0.5M KCl, 5mM Mg ⁺⁺ , pH7.0, 25°C
Morita(85) (1967)	0.90 ATP to HMM	Dif. Sp.	0.06M KCl, 8.3mM Mg ⁺⁺ , pH8.0, 25°C
Young(84) (1967)	2.2 ADP to Myosin 2.6 ADP to HMM 1.1 ADP to S-1	Eq. Dially. Ultrac.	0.05 or 0.5M KCl, 1mM Mg ⁺⁺ , pH7.6, 4°C
Kiely and Martonosi(81)(1968)	1.4 PPI to Myosin	Eq. Dially.	0.6M KCl, 10mM Mg ⁺⁺ , pH8.0, 3 or 25°C
Schliselfeld and Bárdny (89)(1968)	1.4-1.7 ATP to Myosin 1.5 ADP to HMM	Gel Filt.	1.5M NaCl, 1mM Mg ⁺⁺ , pH7.4, 25°C
Morita(86) (1969)	2.0 ADP to HMM	Gel. Filt.	0.08-0.72M KCl, 10mM Mg ⁺⁺ , pH8, 25°C
Nauss et al.(62) (1969)	1.8 PPI to Myosin 1.7 PPI to HMM 0.95 PPI to S-1	Eq. Dially.	0.5M KCl, 1mM Mg ⁺⁺ , pH7.5, 3°C
Lowey and Luck(90) (1969)	1.3-1.6 ADP to Myosin	Eq. Dially.	0.5M KCl, 1mM Mg ⁺⁺ , pH7.7,

* Molecular weight: Myosin, 4.8×10^5 ; HMM, 3.4×10^5 ; S-1, 1.2×10^5 .

** Eq. Dially., equilibrium dialysis; Dif. Sp., difference spectrum; Gel Filt., gel filtration; Ultrac., ultracentrifugal transport.

*** coupled with pyruvate kinase

**** coupled with creatine kinase

Table V

Amount of initial burst of P_i -liberation from Myosin-, HMM-, and S-1-ATP systems

	Concentration of Mg^{++}	Amount of "initial-burst" (mole P_i /mole of protein)*		
		Myosin	HMM	S - 1
Stoichiometric-burst	above 1 mM	(1.1 - 1.2)**	1.1	0.55 - 0.60
Extra-burst	10 μ M	13***	11***	6 ***

0.50 M KCl, 20 mM Tris-maleate, pH 7.0, 25°C

* Molecular Weights : Myosin, 4.8×10^5 ; HMM , 3.4×10^5 ; S-1, 1.2×10^5 .

** Average of many values measured in our laboratory (12, 14, 15, 93)

*** values measured by Kinoshita et al. (12)

Legends for Figures

Fig. 1. Apparatus for measurement of ATPase activity of fiber bundles at a fixed sarcomere length.

The two ends of a fiber bundle were each placed between a pair of acrylic plastic frame with stainless steel screws. The frame can be easily taken out from, or put into reaction mixture.

Initially, the volume of the reaction mixture was either 34 or 40 ml. A magnetic stirrer was put into the reaction bath. The temperature was maintained at 25°C by running water, adjusted at 25°C, around the bath.

Fig. 2. Distribution of sarcomere lengths in isolated glycerol-treated muscle fiber bundle, which had been forcibly extended.

The abscissa shows the distance from one end of the muscle fiber bundle which was fixed to an acrylic plastic frame. Circles are mean values of sarcomere length, measured by the method described in the text. 50 mM KCl, 2.0 mM MgCl₂, 20 mM Tris-maleate buffer, pH 7.0, room temperature.

Fig. 3. Effect of pyruvate kinase on ATPase activity of H-meromyosin.

0.5 M KCl, 2 mM MgCl₂, 20 mM Tris-HCl (pH 7.5), 25°C.

-X- 0.2 mg HMM/ml, 0.1 mM ATP, 0.4 mM PEP, 40 µg pyruvate kinase/ml.

The amount of pyruvate liberated was measured as described in the text.

-O- 1 mg HMM/ml, 1 mM ATP. P_i liberation was determined by the Martin-Doty method.

Fig. 4. Determination of amount of protein in fiber bundles from the optical density at 280 mµ.

The optical density at 280 mμ was measured after dissolving homogenized fiber bundles with 3 ml of 1 N NaOH for 24 hours (—○—), for 3 weeks (---X---) at room temperature and for 2 hours at 100°C (—●—) in a sealed test tube. Protein concentration was calculated by multiplying the amount of nitrogen by a factor of 6.

Fig. 5. Dependence of liberation of pyruvate from muscle fiber-pyruvate kinase system on concentration of PEP.

Two fiber bundles (0.405 mg protein, 2.57 μ in sarcomere length, about 310 μ in diameter), 0.6 mM ATP, 8.4 μg pyruvate kinase/ml, 50 mM KCl, 2 mM MgCl₂ and 20 mM Tris-maleate buffer at pH 7.0 and 25°C.

—○— , —●— Liberation from the whole system. ---X--- Liberation from ends of fiber bundles which were held between plastic plates, i.e., the control system (see text for details).

Concentration of PEP;

—●— 3.0, —○— 0.5, ---X--- 1 mM.

Fig. 6. Dependence of myofibrillar ATPase at a sarcomere length of 2.20 μ on concentration of ATP.

One fiber bundle (0.170 mg protein, 2.20 μ in sarcomere length, about 290 μ in diameter), 1.0 mM PEP, 65 μg pyruvate kinase/ml, 50 mM KCl and 20 mM Tris-maleate buffer at pH 7.0 and 25°C.

—○— , —●— Liberation from the whole system.

---△--- , ---▲--- Liberation from ends of 5 fiber bundles which were held between two plastic plates (see text for details).

Concentrations of ATP and MgCl₂;

—○— , ---△--- 5.0 mM ATP, 6.0 mM MgCl₂.

—●— , ---▲--- 2.35 mM ATP, 3.35 mM MgCl₂.

Fig. 7. Dependence of myofibrillar ATPase at a sarcomere length of 0.78 μ on concentration of pyruvate kinase.

Two fiber bundles (0.249 mg protein, 0.78 μ in sarcomere length, about 310 μ in diameter), 0.6 mM ATP, 1.0 mM PEP, 50 mM KCl, 2 mM MgCl₂ and 20 mM Tris-maleate buffer at pH 7.0 and 25°C. The pyruvate liberated was measured after shortening fiber bundles from 31.5 mm in muscle length (2.33 μ in sarcomere length) to 11 mm by adding ATP.

—○— , —●— Liberation from the whole system.

---X--- Liberation from the control system(see text for details).

Concentration of pyruvate kinase;

—○— 25, —●— 250, ---X--- 25 μ g/ml.

Fig. 8. ATPase activities at three different sarcomere lengths.

The ATPase activity of fiber bundles of less than 300 μ in diameter was measured in a solution containing 0.6 mM ATP, 1.0 mM PEP, 50 mM KCl, 20 mM MgCl₂ and 20 mM Tris-maleate buffer at pH 7.0 and 25°C. ○ , Liberation from the whole system. X , Liberation from the control system. (A) Two fiber bundles (0.732 mg protein, 3.40 μ in sarcomere length), 10 μ g pyruvate kinase/ml. (B) One fiber bundle (0.195 mg protein, 2.5 μ in sarcomere length), 21 μ g pyruvate kinase/ml. Pyruvate liberated was measured after shortening the fiber bundle from 39 to 29 mm in muscle length by adding ATP. (C) Three fiber bundles (0.312 mg protein, 1.0 μ in sarcomere length), 33 μ g pyruvate kinase/ml. Pyruvate liberated was measured after shortening the fiber bundles from 35 to 15 mm in muscle length.

Fig. 9. Dependence of myofibrillar ATPase activity on sarcomere length.

0.6 — 2.35 mM ATP, 1.0 mM PEP, 8.4 — 73 μ g pyruvate kinase/ml, 50 mM KCl, 2.0 — 3.35 mM MgCl₂ and 20 mM Tris-maleate buffer at pH 7.0 and 25°C. See text for explanation of open circles, closed circles and broken line.

Fig. 10. Decrease in ATPase activity with decreasing length of fiber bundle.

The time-course of pyruvate-liberation was obtained by subtracting the pyruvate liberation by the control system from that by the whole system. Five fiber bundles (0.825 mg protein), $2.91 \pm 0.07 \mu$ in sarcomere length, $171 \pm 13 \mu$ in diameter. 1.0 mM EGTA, 2.35 mM ATP, 1.0 mM PEP, 116 μ g pyruvate kinase/ml, 50 mM KCl, 3.35 mM $MgCl_2$ and 20 mM Tris-maleate buffer at pH 7.0 and 25°C. The first arrow indicates the addition of 2.0 mM Ca^{++} , and the second arrow, the cutting of fiber bundles. At the final stage the sarcomere length was about 0.7 μ and pH 6.7.

Fig. 11. Increase in myofibrillar ATPase at a sarcomere length of 2.28 μ on repeated ATPase assay.

One fiber bundle (stored in 50 % glycerol at - 10°C for about 3 months, 0.209 mg protein, 2.28 μ in sarcomere length, about 260 μ in diameter), 2.35 mM ATP, 1.0 mM PEP, 50 mM KCl, 3.35 mM $MgCl_2$ and 20 mM Tris-maleate buffer at pH 7.0 and 25°C.

Symbol	Sequence of measurement	Concentration of Ca^{++} (μ M)	Concentration of pyruvate kinase (μ g/ml)	System
○	1	1,000	58	} whole system
△	2	100	58	
□	3	6	58	
■	4	0.4	39	
●	5	1,000	58	} control system
●	6	1,000	58	
X	7	0.4	39	

Fig. 12. Increase in ATPase of fiber bundles at 2.58 μ in sarcomere length with increasing Ca^{++} concentration.

Two fiber bundles (stored in 50 % glycerol at -10°C for about 3 months, 0.388 mg protein, 2.58 μ in sarcomere length, 270 μ in diameter), 2.35 mM ATP, 1.0 mM PEP, 50 mM KCl, 3.35 mM MgCl_2 and 20 mM Tris-maleate buffer at pH 7.0 and 25°C .

—○— , —●— Liberation from the whole system.

---△--- , ---▲--- Liberation from the control system (see text for details).

Concentrations of Ca^{++} and pyruvate kinase;

—○— , ---△--- 1.0 mM Ca^{++} , 58 μg pyruvate kinase/ml.

—●— , ---▲--- 0.01 μM free Ca^{++} , 19 μg pyruvate kinase/ml.

Fig. 13. Effect of storage of muscle fibers in 50% glycerol on ATPase activity in the presence of a high concentration of EGTA.

2.35 mM ATP, 1.0 mM PEP, 19 μg pyruvate kinase/ml, 50 mM KCl, 3.35 mM MgCl_2 and 20 mM Tris-maleate buffer at pH 7.0 and 25°C .

—○— Liberation from the whole system.

--X-- Liberation from the control system (see text for details).

(A) Two fiber bundles which had been stored in 50% glycerol at -10°C for 13 days (0.277 mg protein, 2.03 μ in sarcomere length, 290 μ in diameter), 1.0 mM EGTA.

(B) Two fiber bundles which had been stored for about 3 months (0.255 mg protein, 2.07 μ in sarcomere length, 260 μ in diameter), 2.0 mM EGTA.

Fig. 14. Dependence of ATPase of fiber bundle at resting length on Ca^{++} concentration.

Glycerol-treated muscle fiber bundles of less than 300 μ in diameter were used after storage in 50% glycerol at -10°C for about 3 months.

In all the measurements except two, the sarcomere length was fixed between 2.00 and 2.65 μ .

The ATPase activity was plotted against concentration of free Ca^{++} , taking the mean value of the activity in the presence of 1 mM Ca^{++} as 100. 2.35 mM ATP, 1.0 mM PEP, 19--58 μg pyruvate kinase/ml, 50 mM KCl, 3.35 mM MgCl_2 and 20 mM Tris-maleate buffer at pH 7.0 and 25°C.

Fig. 15. Inhibition of myosin B-ATPase by the addition of EGTA.

17.7 $\mu\text{g}/\text{ml}$ of myosin B extracted from fresh psoas muscle, 0.094 mM ATP, 1.0 mM PEP, 58 μg pyruvate kinase/ml, 54 mM KCl, 3.35 mM MgCl_2 and 20 mM Tris-maleate buffer at pH 7.0 and 25°C.

- X— 1.0 mM EGTA.
- , —●— No EGTA added.
- 19 μg F-actin/ml, no EGTA.

Fig. 16. Chromatography of tryptic digest of HMM on Sephadex G-200.

HMM (14 mg/ml) in 0.05 M KCl and 0.1 M Tris-HCl was digested with trypsin (weight ratio of HMM to trypsin, 28 : 1) for 13 min at pH 7.6 and 25.6°C. The digestion mixture (20 ml) was applied to a column (5.8 x 43 cm) of Sephadex G-200 equilibrated with 0.05 M KCl and 0.1 M Tris-HCl at pH 7.6 and 4°C. The column was eluted with the same solvent at a flow rate of 12 ml per hr, and 4.0 ml fractions were collected. ATPase activities were measured in 1 mM ATP, 9 mM CaCl_2 , 0.45 M KCl and 45 mM Tris-maleate at pH 6.4 and 25°C. —○— , E279 mp ; --●-- , Ca^{++} -ATPase activity (mM P_i /min/ml of fraction).

Fig. 17. Rechromatography of S-1 on Sephadex G-200.

S-1 (14 mg/ml, 15 ml) was applied to a column (3.6 x 92 cm) of Sephadex G-200 equilibrated with 0.05 M KCl, 0.05 M Tris-HCl and 0.5 mM EDTA at pH 7.6 and 4°C. The column was eluted with the same solvent at

a flow rate of 15 ml per hr, and 4.0 ml fractions were collected. ATPase activities were measured in 1 mM ATP, 10 mM CaCl_2 , 0.50 M KCl and 50 mM Tris-maleate at pH 6.4 and 25°C. \bigcirc , $E_{280} \text{ m}\mu$; $\text{---}\bigcirc\text{---}$, Ca^{++} -ATPase activity (1 in $E_{270} \text{ m}\mu = 2.9$ in mM P_i /min/ml of fraction); $\text{---}\times\text{---}$, Specific activity of Ca^{++} -ATPase (1 in $E_{720} \text{ m}\mu/E_{280} \text{ m}\mu = 2,100$ in μ moles P_i /min/g).

Fig. 18. Ultracentrifugal patterns showing increase in amount of slower components of S-1 preparation on storage in 0.20 M KCl solution.

S-1 (14.2 mg/ml) was stored in 0.20 M KCl, 0.05 M Tris-HCl and 1 mM EDTA at pH 7.6 and 0°C.

(A): 4 days after rechromatography. 56,100 rpm, bar angle 70°, 4.2°C, 77 min after reaching full speed, aluminium double sector cell.

(B): 10 days after rechromatography. 42,040 rpm, 65°, 4.4°C, 90 min, filled upon synthetic boundary cell.

Fig. 19. Degradation and decrease in ATPase activity of S-1 on storage in 0.05 - 0.20 M KCl solution in the presence and absence of sucrose.

ATPase activities (\bigcirc , \times) were measured in 1 mM ATP, 7 mM CaCl_2 , 1.0 M KCl and 0.05 M Tris-maleate at pH 7.0 and 25°C.

Degradations of S-1 were estimated from the ultracentrifugal pattern shown in Fig. 3 (\bigcirc) or elution pattern on column chromatography

(2.8 x 18 cm) on Sephadex G-200 (\bigcirc). Conditions of storage: \bigcirc ,

14.2 mg/ml S-1, 0.20 M KCl, 0.05 M Tris-HCl, 1 mM EDTA, pH 7.6, 0°C;

$\text{---}\bigcirc\text{---}$, 12 mg/ml S-1, 0.07 M sucrose, 0.05 M KCl, 0.05 M Tris-HCl, 0.5 mM

EDTA, pH 7.6, 0°C; $\text{---}\bigcirc\text{---}$, 9.9 mg/ml S-1, 0.20 M KCl, 0.05 M Tris-HCl, 1 mM

EDTA, pH 7.6, 0°C. The arrow indicates the time of addition of 0.05 M

sucrose to the storage-solution 3 days after rechromatography; $\text{---}\times\text{---}$,

lyophilized in 0.06 M sucrose a few days after rechromatography.

Fig. 20. Degradation of S-1 in guanidine-HCl.

S-1 was incubated in 4.6 M guanidine-HCl, 1.9 mM EDTA, 0.03 M KCl, 0.043 M sucrose and 0.03 M Tris-HCl (pH 7.6) at 4°C for one month. The solution ($E_{280} \text{ mp} = 5.24$, 3.0 ml) was applied to a column (3.7 × 31 cm) of Sephadex G-200 equilibrated with 5 M guanidine-HCl and 2 mM EDTA at room temperature. The column was eluted with the same solvent at a flow rate of 11 ^{ml} per hr. Blue dextran and cytochrome c (about 2 ml, each) in 5 M guanidine-HCl and 2 mM EDTA were applied separately to the same column, and were followed by measuring $E_{280} \text{ mp}$ and $E_{530} \text{ mp}$, respectively. Fractions of 3.5 ml were collected. —○—, $E_{280} \text{ mp}$ of S-1; ---●---, $E_{530} \text{ mp}$ of cytochrome c; ---X---, $E_{280} \text{ mp}$ of blue dextran. The inserted figure is the sedimentation pattern of S-1 (9.34 ml/ml) in 5 M guanidine-HCl, 7.2 mM EDTA, 0.13 M KCl and 0.033 M Tris-HCl (pH 7.6), taken 203 min after reaching the full speed of 42,040 rpm, at 20°C and a bar angle of 55°. A synthetic boundary cell was used.

Fig. 21. Chromatography of S-1 on Sephadex G-100 in alkali.

Two hours after adjusting the pH to 11.5 with triethylamine, an alkaline solution of S-1 ($E_{280} \text{ mp} = 7.24$, 0.05 M KCl, 5 mM Tris-HCl, 0.035 M Triethylamine, 5.7 ml) was applied to a column (2.7 × 87 cm) of Sephadex G-100 equilibrated with 0.05 M KCl and 0.025 M triethylamine -CO₂ buffer at pH 11 and room temperature. The column was eluted with the same solvent at a flow rate of 17 ml per hr. Cytochrome c, in the same solvent, was applied to the same column, and was followed by measuring $E_{530} \text{ mp}$. Fractions of 3.7 ml were collected. —○—, $E_{280} \text{ mp}$ of S-1; ---●---, $E_{530} \text{ mp}$ of cytochrome c.

Fig. 22. Elution profile of radioactivity and E₂₈₀ mp on gel filtration of a tryptic digest of ¹⁴C-carboxamidomethyl HMM.

¹⁴C-IAA-HMM (19.1 mg/ml, 2.3 moles IAA bound/mole), prepared from ¹⁴C-IAA-myosin (2.9 moles IAA bound/mole), was digested with trypsin (weight ratio of HMM to trypsin, 25 : 1) in 0.05 M KCl and 0.05 M Tris-HCl at pH 7.6 and 25°C for 18 min.

The reaction was stopped by addition of trypsin inhibitor (in 2 fold excess over trypsin on a weight ratio), and the digestion mixture (34 ml) was applied to a column (6.0 × 69 cm) of Sephadex G-200, equilibrated with 0.05 M KCl, 0.05 M Tris-HCl and 0.5 mM EDTA at pH 7.6 and 4°C. The column was eluted with the same solvent at a flow rate of about 20 ml per hr. Fractions of 3.95 ml were collected. Those indicated by the symbol, \longleftrightarrow , were collected as fractions of S-1. The radioactivity of 50 μ l of each fraction was measured. \bigcirc , E₂₈₀ mp; $--\bullet--$, Radioactivity (cpm).

Fig. 23. Time-courses of IAA-binding to S-1 and changes in Ca⁺⁺- and EDTA-ATPase activities of S-1.

S-1 (7.03 mg/ml) in 0.50 M KCl and 0.1 M Tris-HCl at pH 8.5 and 0°C was subjected to carboxamidomethylation with IAA-1-¹⁴C (20 fold excess over S-1 on a molar basis) in the presence (\bullet , \blacktriangle) or absence (\bigcirc , \triangle , \square) of 0.1 M sucrose. At appropriate times after the start of the reaction, S-1 was isolated from the reaction mixture as described in the "EXPERIMENTAL". Its Ca⁺⁺- and EDTA-ATPase activities were measured in 1 mM ATP, 1.0 M KCl and 0.05 M Tris-maleate at pH 7.0 and 25°C in the presence of 7 mM CaCl₂ and 3 mM EDTA, respectively. The radioactivity of S-1 was also measured. The ratios of Ca⁺⁺-, and EDTA-ATPase activities at various times to Ca⁺⁺-ATPase activity of S-1 before addition of IAA are plotted as relative values.

\bigcirc , \bullet , Ca⁺⁺-ATPase activity; \square , EDTA-ATPase activity; \triangle , \blacktriangle , IAA bound to S-1 (mole/mole of S-1).

Fig. 24. Initial stoichiometric burst of P_i -liberation from the HMM-, and S-1-ATP systems.

ATPase activity was measured in 0.25 mM ATP, 0.60 M KCl and 20 mM Tris-maleate at pH 7.0 and 25°C.

(A) 5.0 mg/ml HMM, 2.0 mM $MgCl_2$. (B) 1.35 mg/ml S-1, 1.0 mM $MgCl_2$.

Fig. 25. Time-courses of initial rapid P_i -liberation after mixing S-1 with various concentrations of ATP.

1 mg/ml S-1, 2.8 M KCl, 10 mM $MgCl_2$, 20 mM Tris-HCl, pH 7.5, 0°C.
Concentrations of ATP: \square , 2; \bullet , 5; \triangle , 10; \circ , 20 μM .

Fig. 26. Dependence of the amount and $\tau_{1/2}$ value of the initial P_i -liberation on ATP concentration.

Experimental conditions were as described for Fig. 25.
The values of S-1 (\triangle , \blacktriangle) were plotted adopting twice of molecular weight as one unit. \circ , \triangle , amount of P_i -liberation; \circ , \triangle , time, $\tau_{1/2}$, required for initial P_i -liberation to reach half the maximum value of myosin and S-1, respectively. The values for myosin (\circ , \bullet) were taken from results reported by Onishi et al. (65).

Fig. 27. Tension development and ATPase activity of muscle fiber in isometric contraction as functions of sarcomere length.

—, ATPase activity of rabbit muscle fiber bundle by us.
----, tension development of single frog muscle fiber by Ramsey and Street (70).
- - - - , tension development of single frog muscle fiber by Gordon et al. (9, 10).

The amount of overlap between actin filaments and "bridges" along the myosin filaments was illustrated at various sarcomere lengths on the basis of the structure of double array of filaments and the sliding filament theory of muscle contraction. The values of 1.6μ , 1.0μ and 0.2μ were adopted as the lengths of myosin filament, actin filament and bare region along myosin filament, respectively (29,110).

Fig. 2^e
A Schematic representation of the myosin molecule with two nonidentical S-1 portions

One (S-1a) of the two S-1 portions which were released by tryptic digestion of one molecule of HMM have the active sites of ATP hydrolysis both via phosphorylation and by simple hydrolysis. The other portion (S-1b) have not the sites of ATP hydrolysis. However, all the S-1 subfragments can combine with actin. References to these molecular weights and dimensions can be found in ref. 27, 32-40, 42, 60 and 98.

Fig. 29. Schematic representation of the dissociation of actomyosin induced by ATP and PP_i at high ionic strength.

S-1a and S-1b indicate the S-1 portions at the head of the myosin molecule with and without active sites of ATPase, respectively. $\cdots P(PP_i)$ represents the myosin-phosphate (or pyrophosphate) complex. This figure shows only the essential part of actomyosin, i.e. its F-actin (F-A) and HMM portion. For further explanation see text.

Fig. 30. Molecular mechanism of muscle contraction based on different functions of the two S-1.

This figure shows the part of the muscle where the essential reactions of muscle contraction occur, i.e. the projection of the myosin filament (HMM) and the actin filament (F-A). $\sim P$ and $\cdots P$ indicate phosphoryl myosin and myosin-phosphate complex, respectively. For further explanation see text and compare this figure with Fig. 24A of the previous paper (14).

Fig. 1

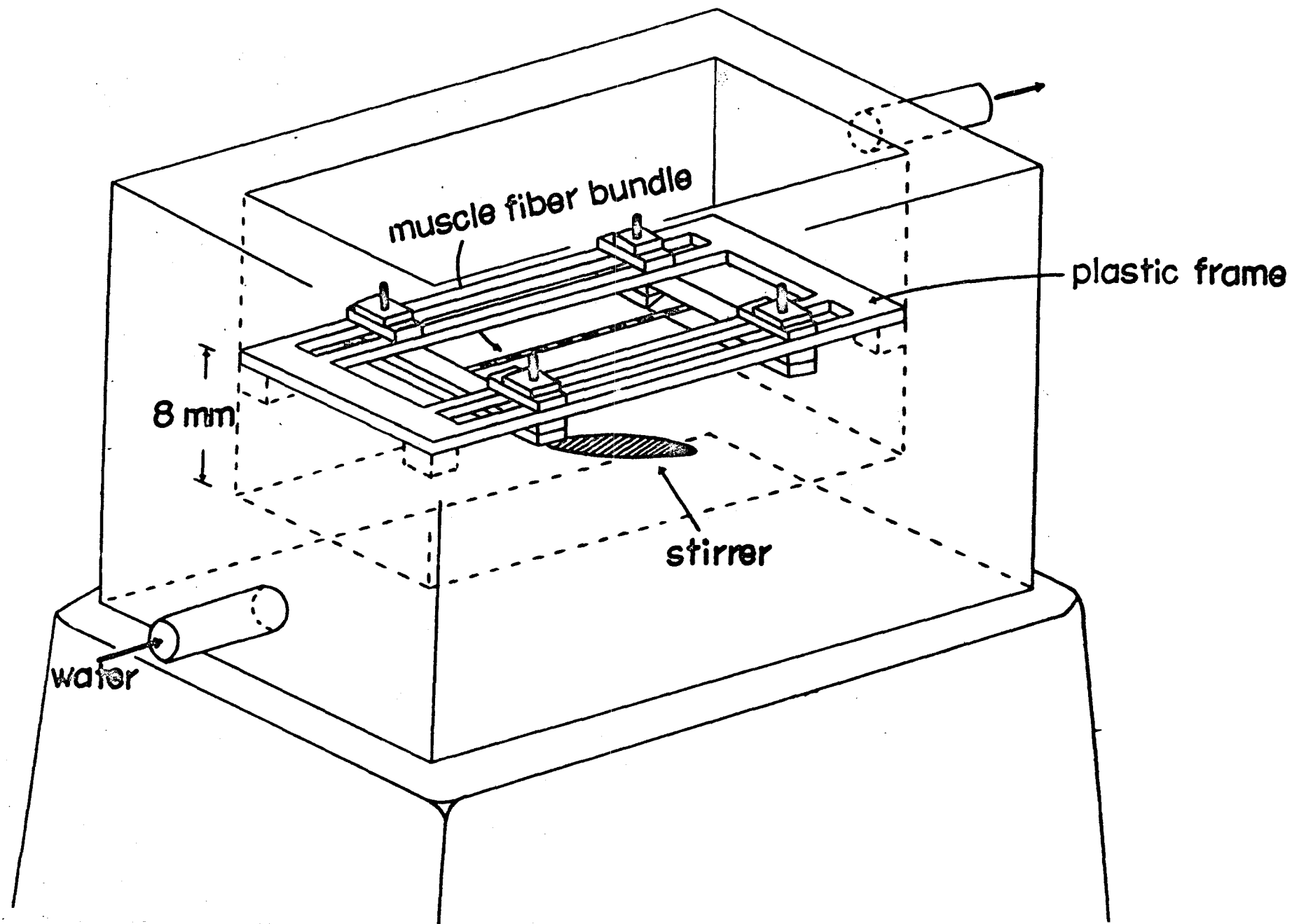


Fig. 2

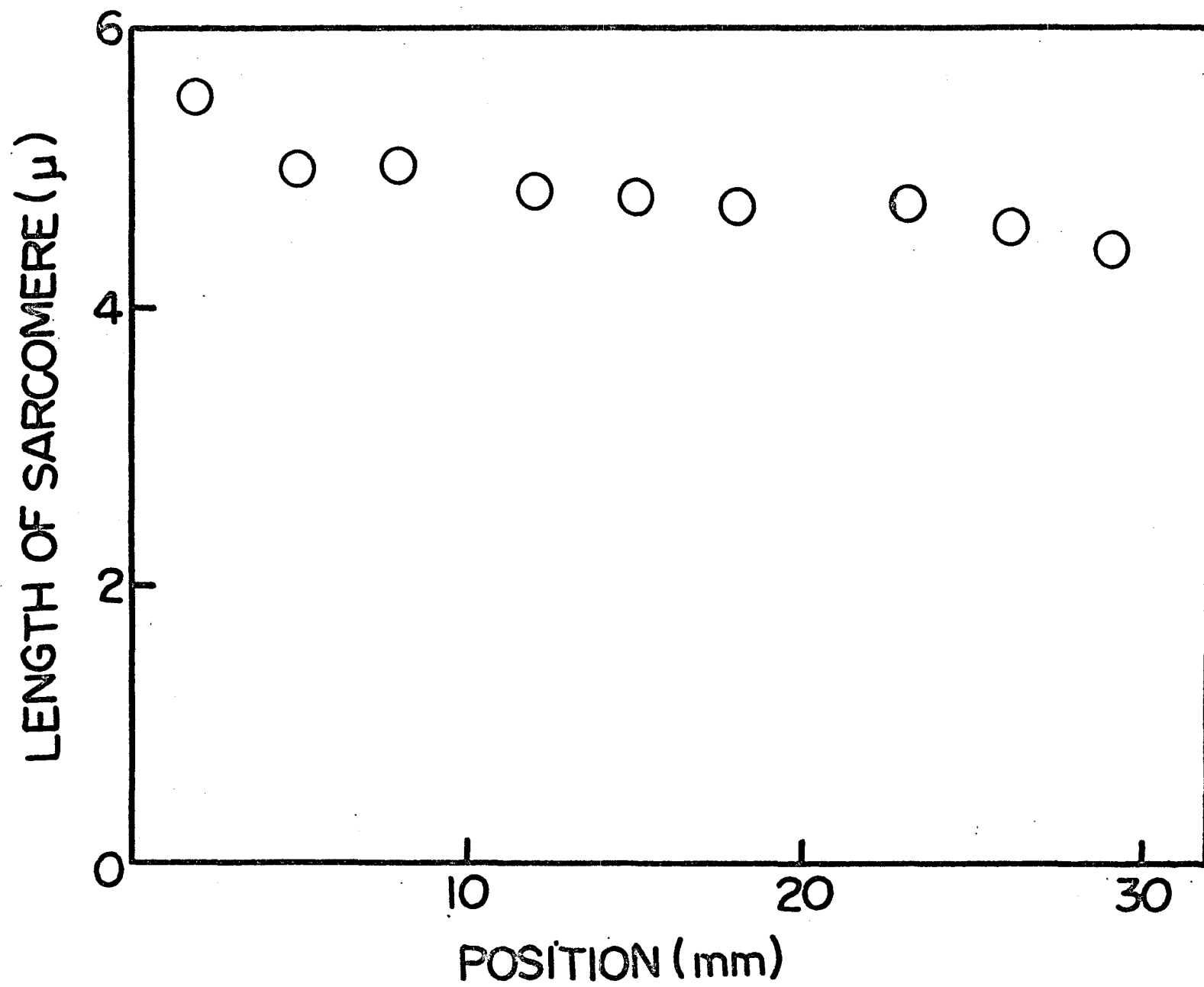


Fig. 3

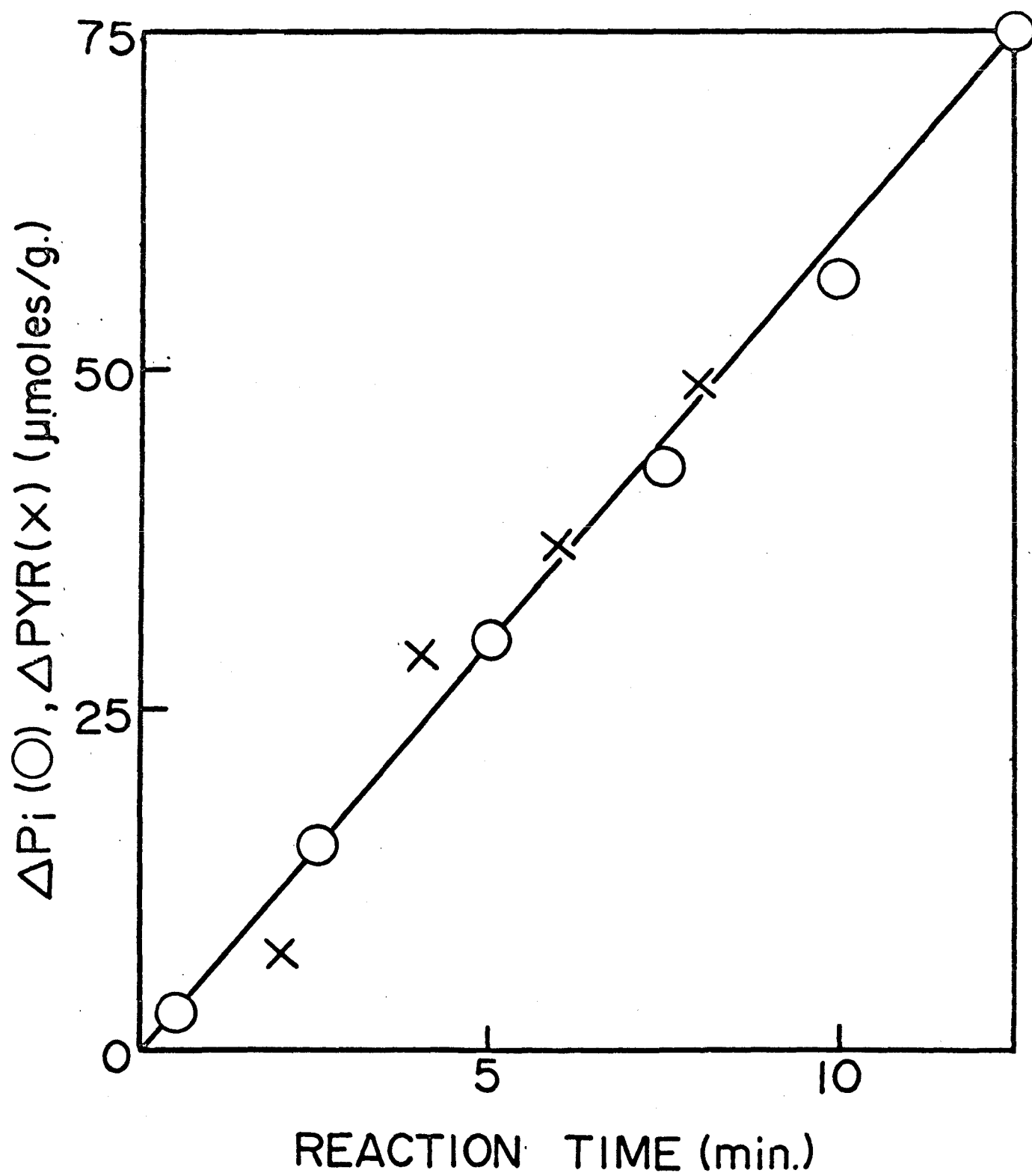


Fig. 4

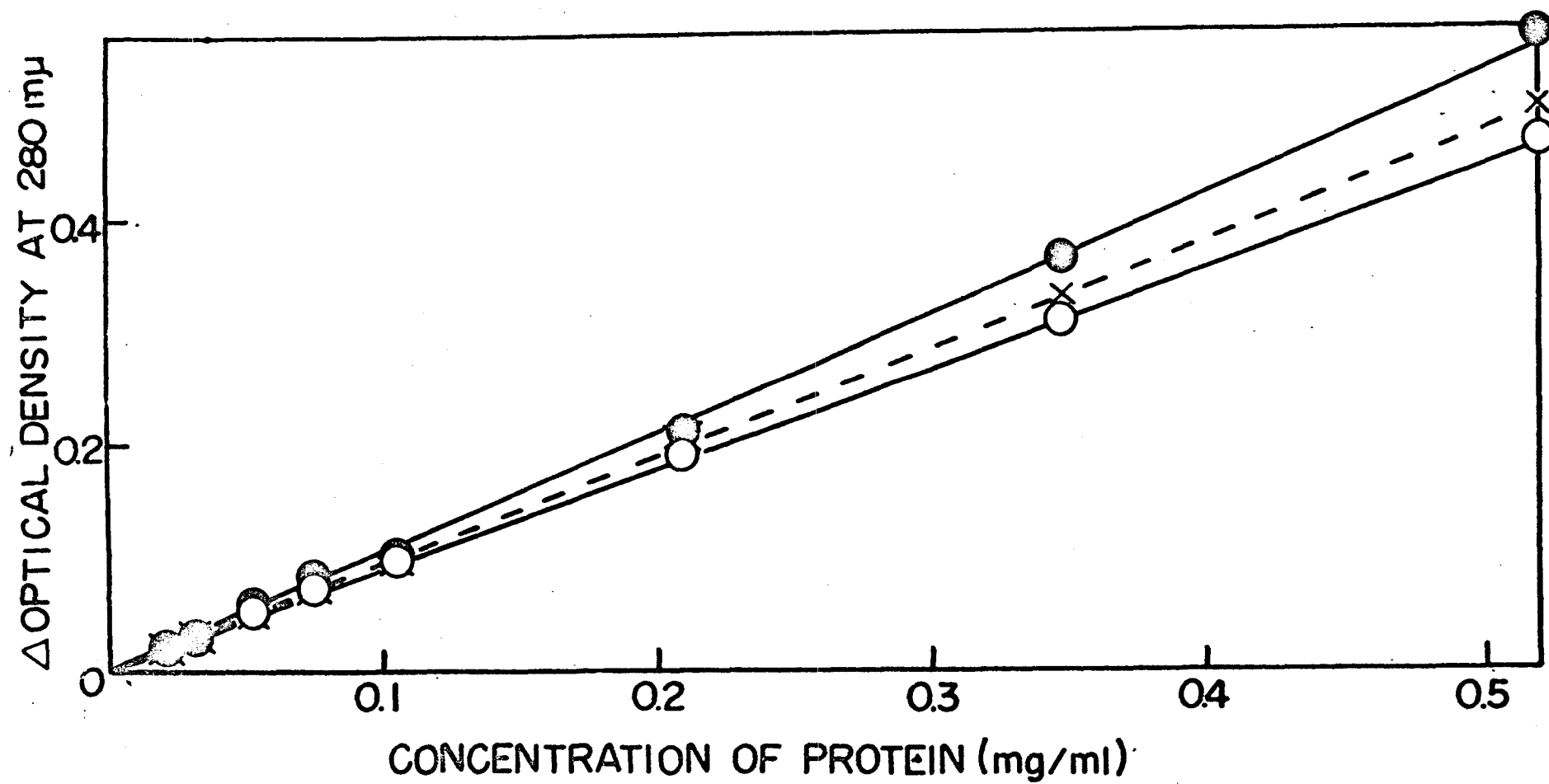


Fig. 5

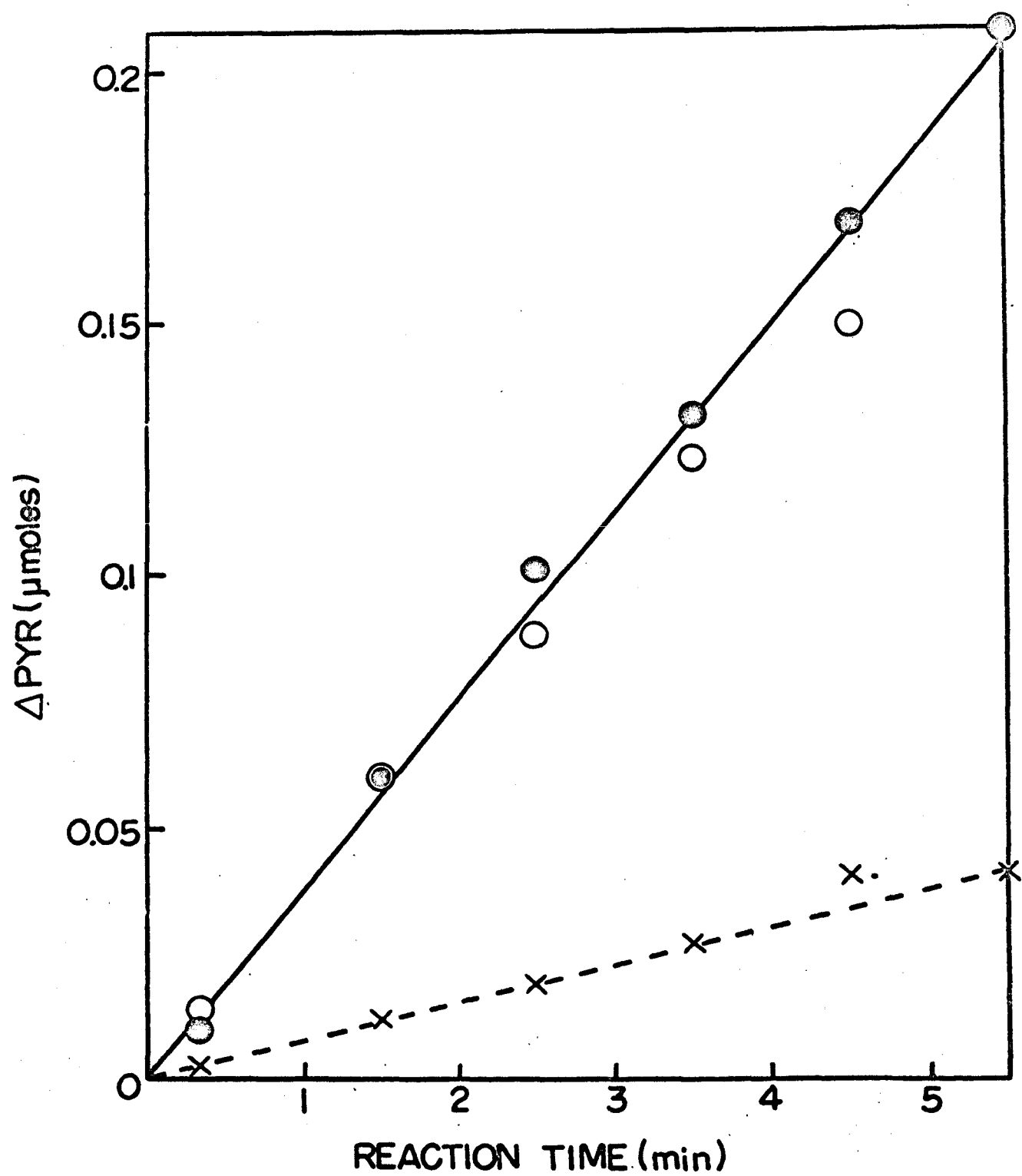


Fig. 6

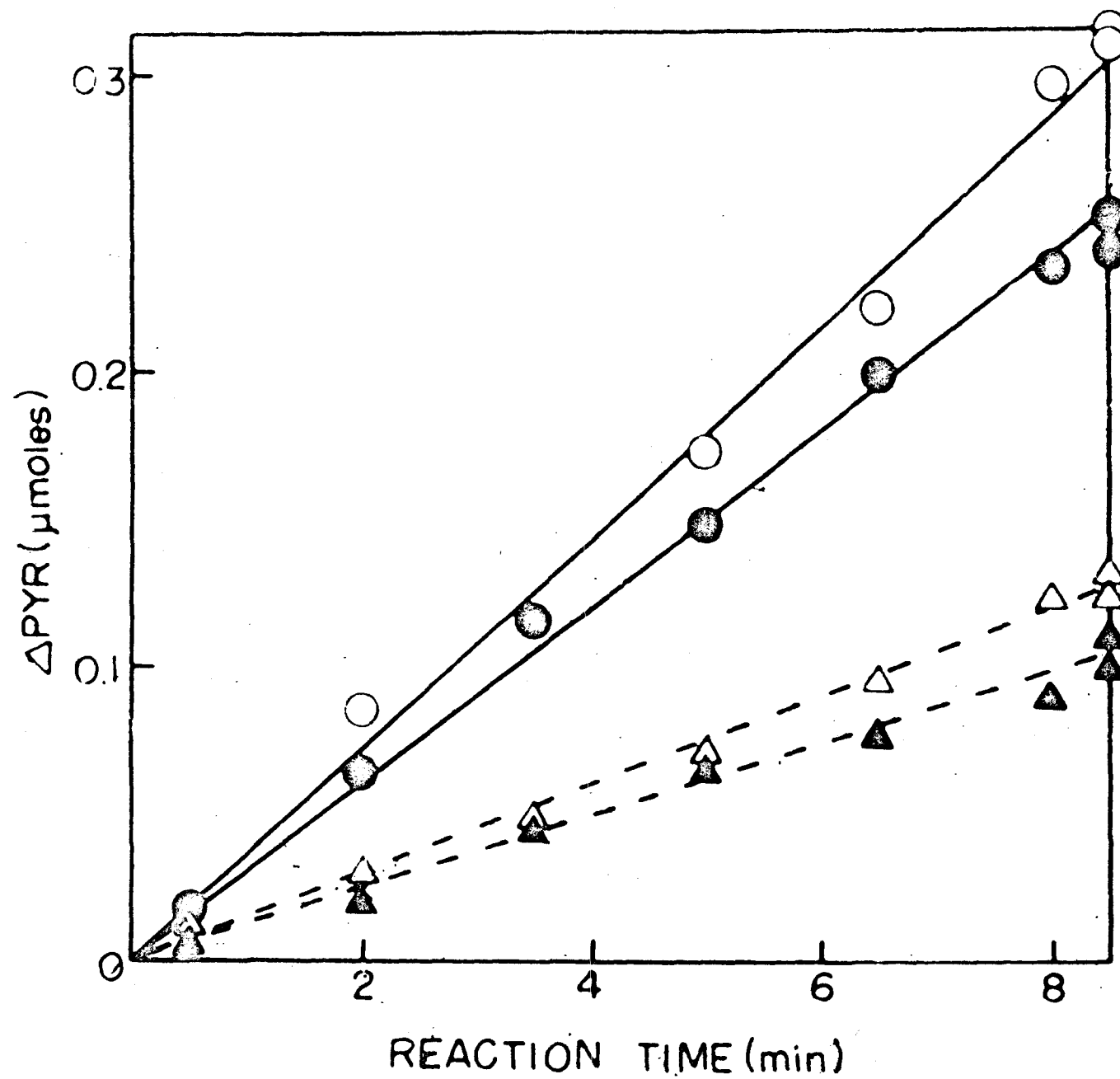


Fig. 7

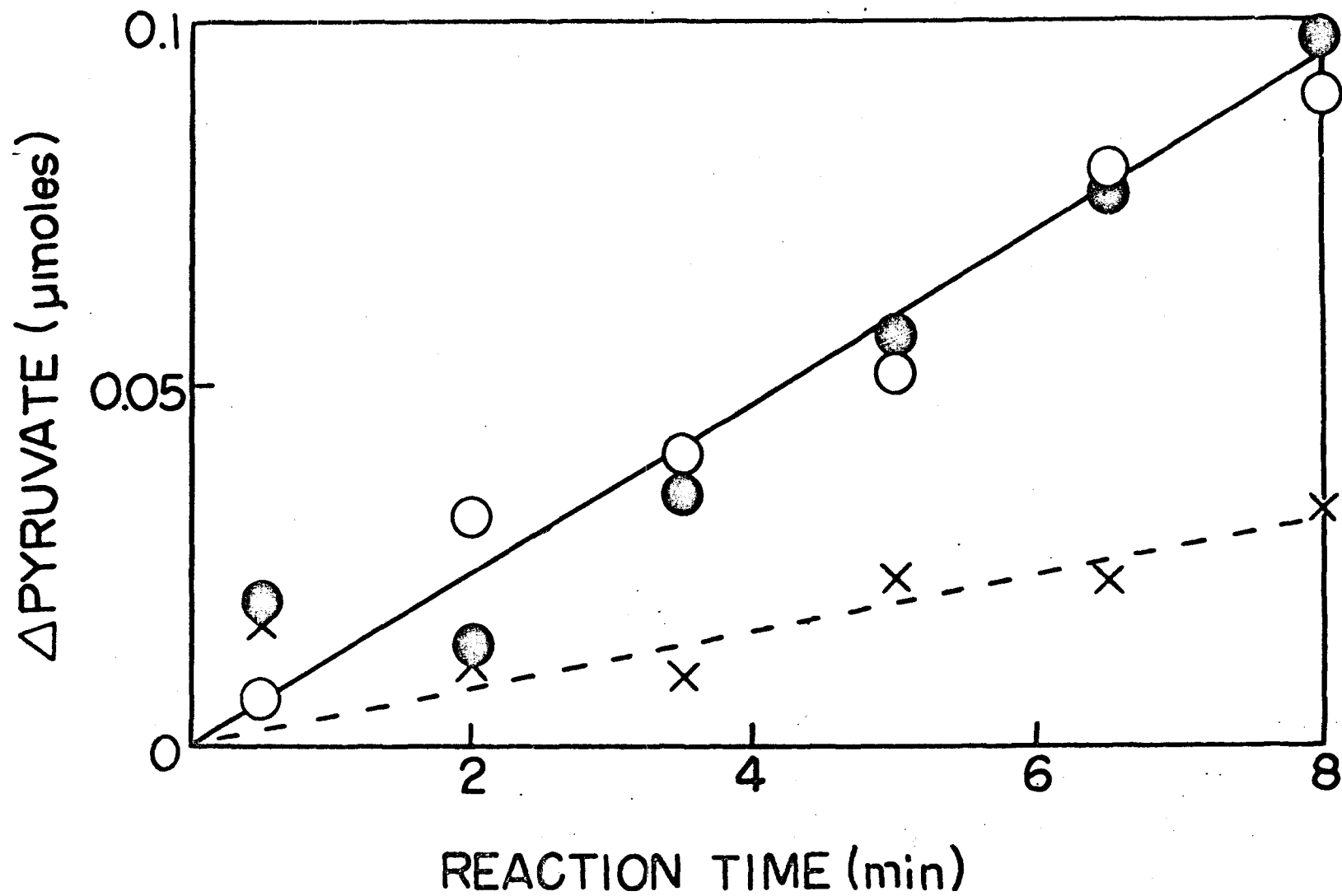


Fig. 8(A)

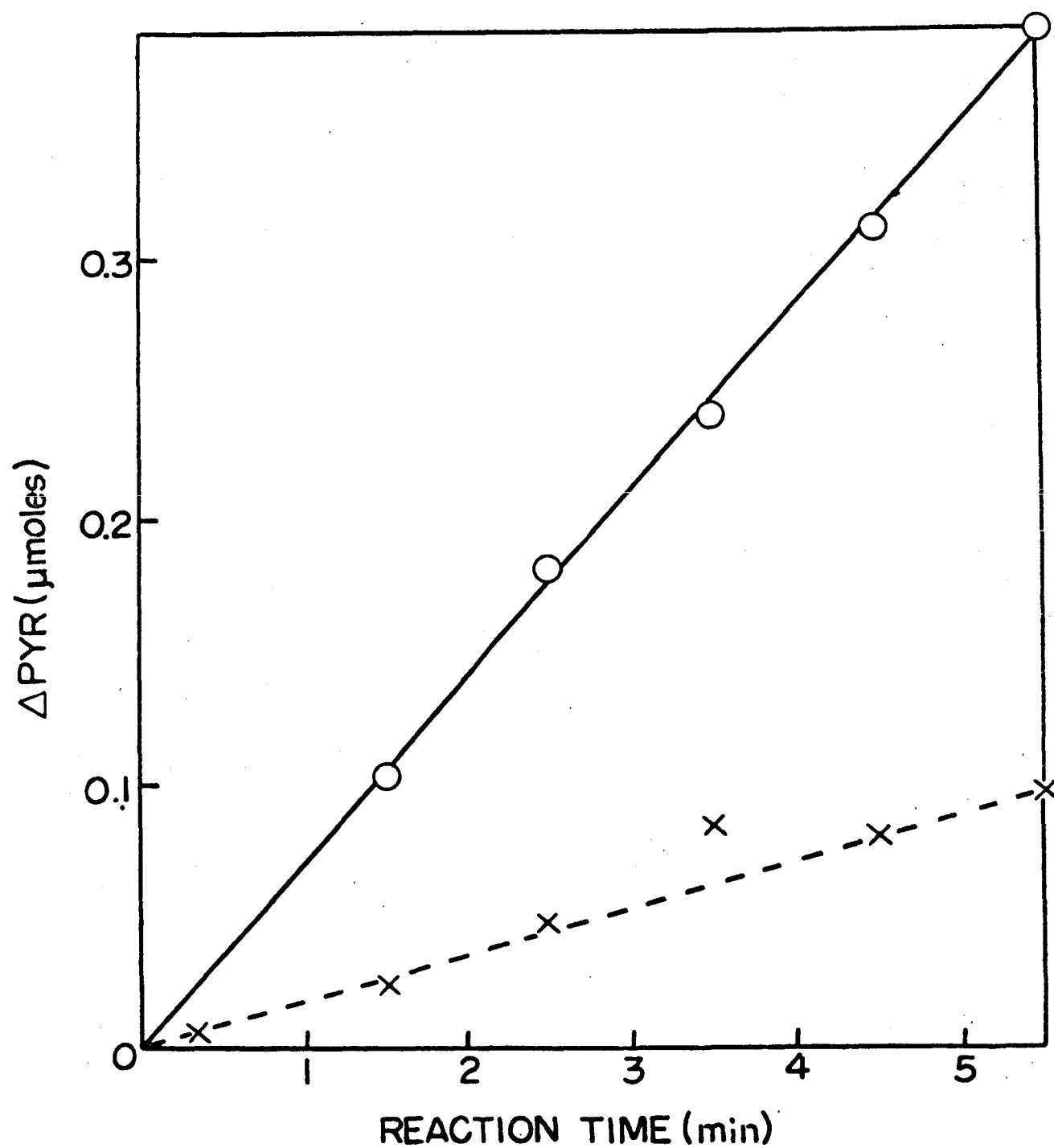


Fig. 8(B)

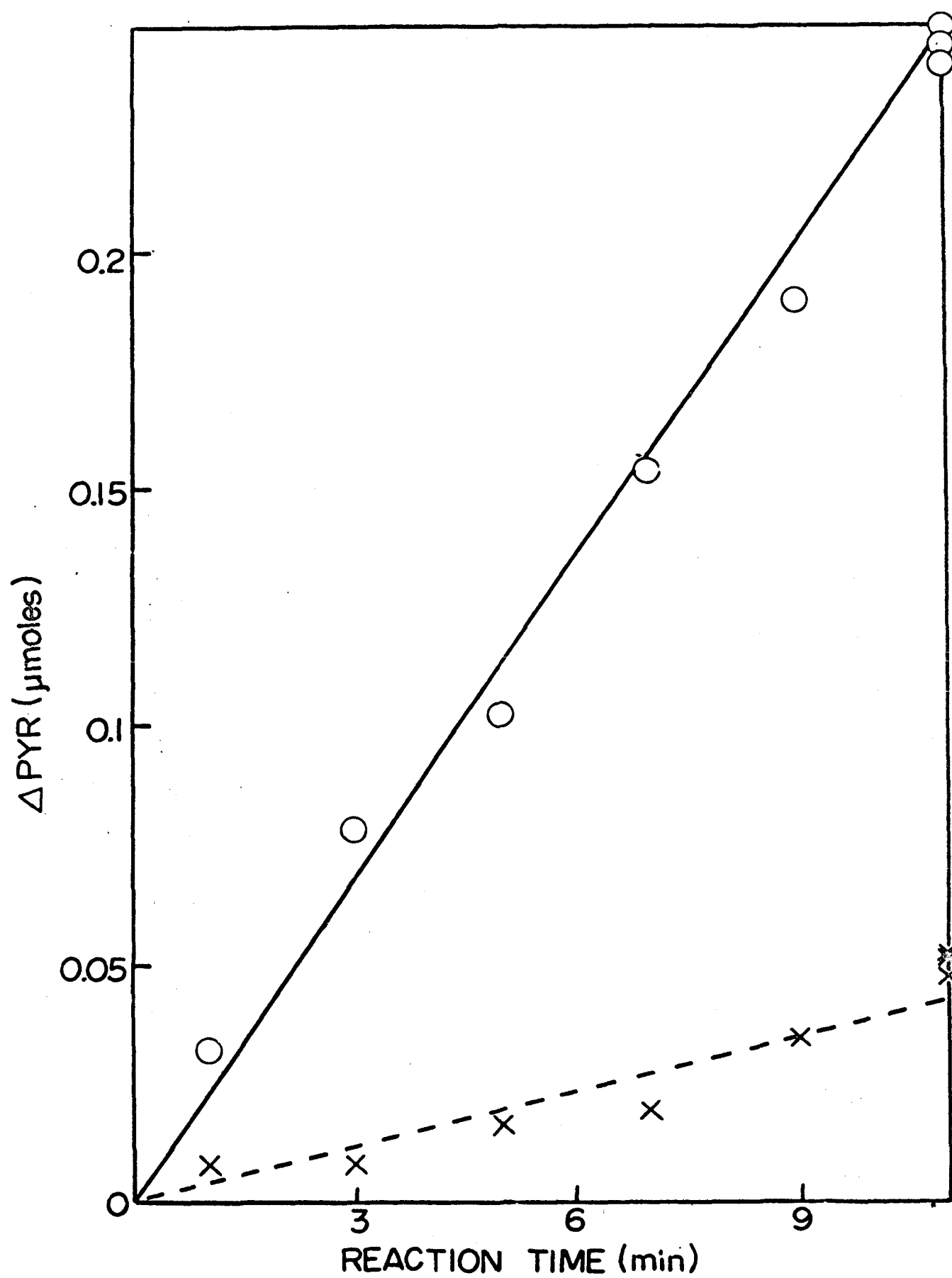


Fig. 8(c)

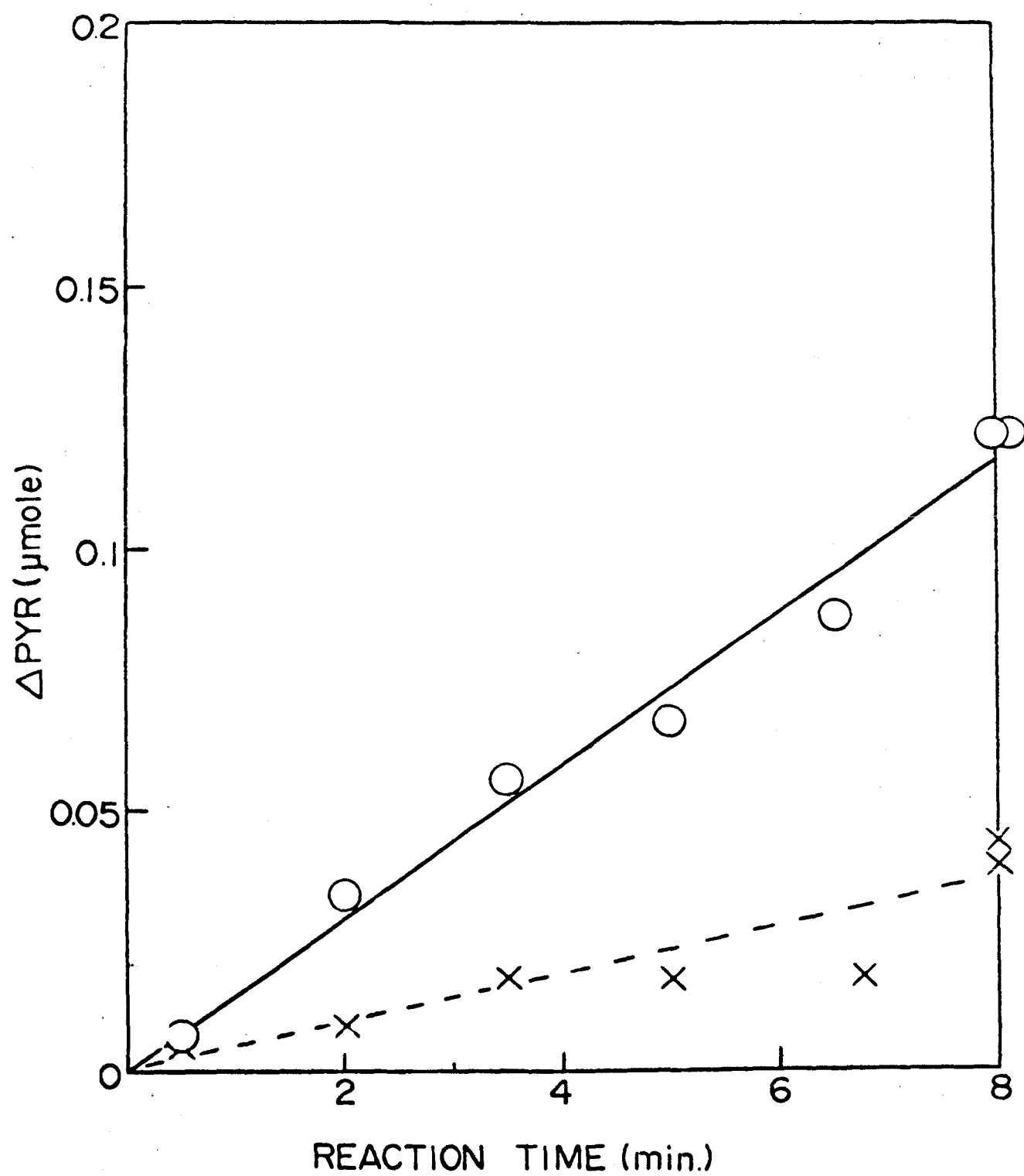


Fig. 9

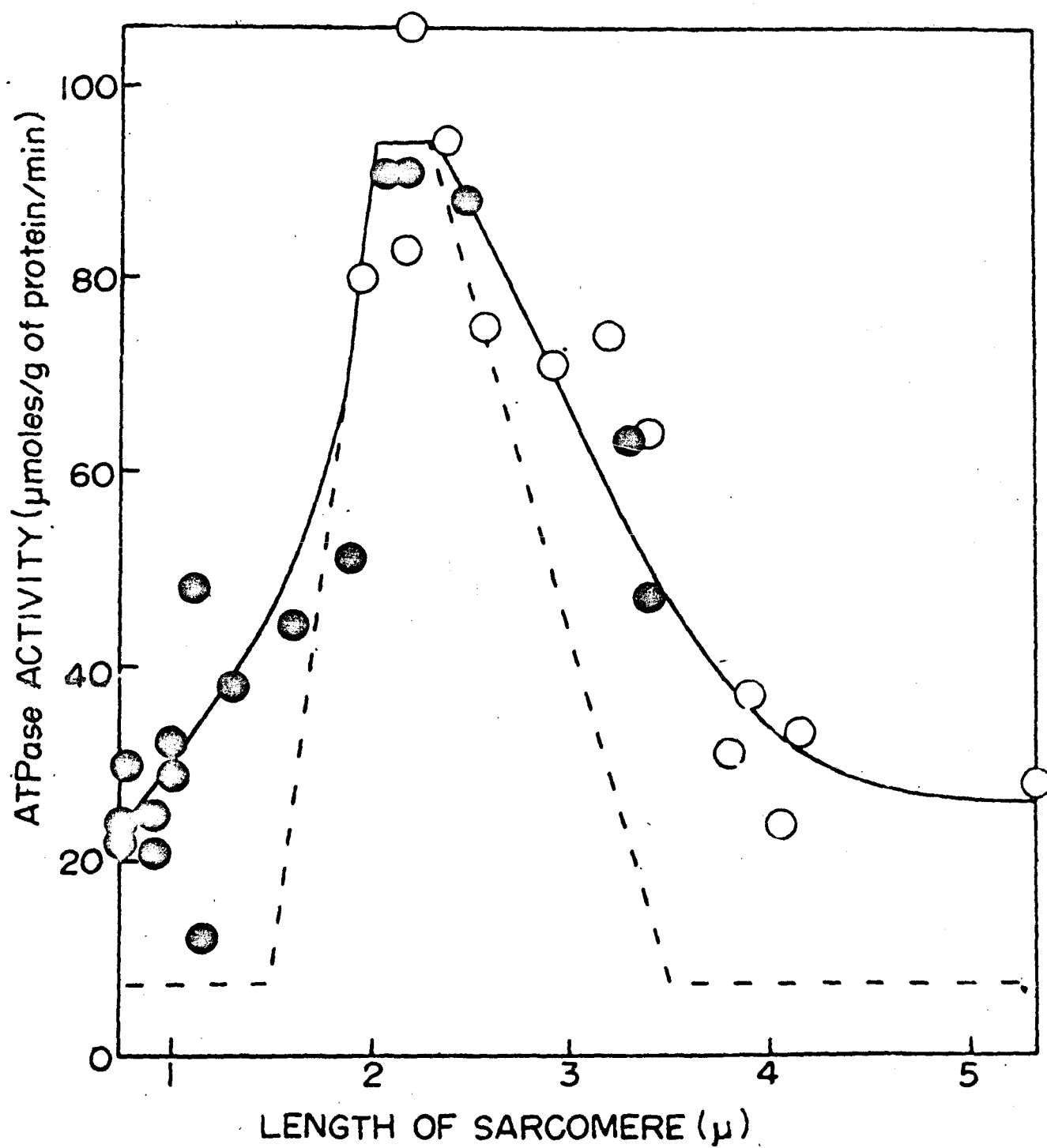


Fig. 10

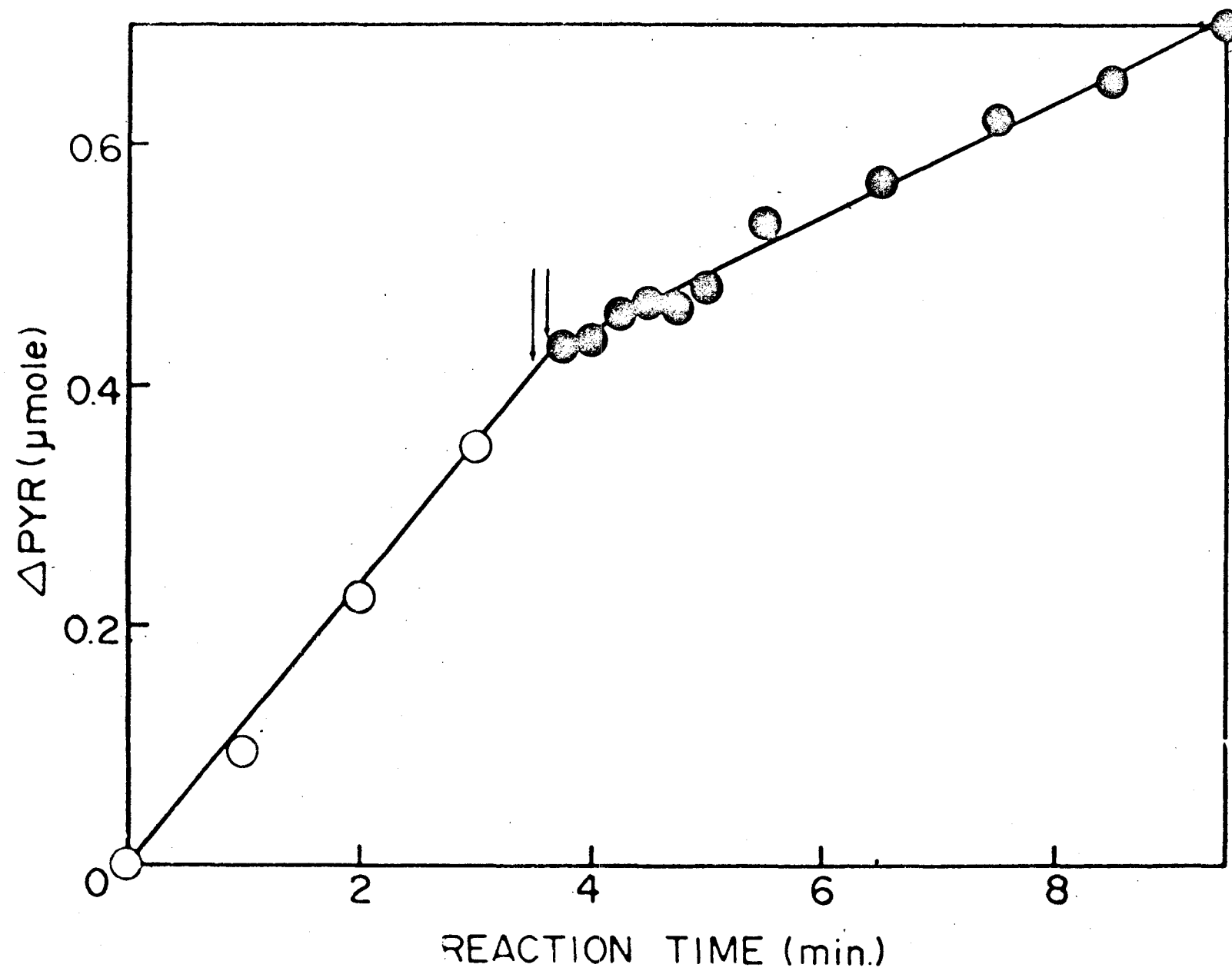


Fig. 11

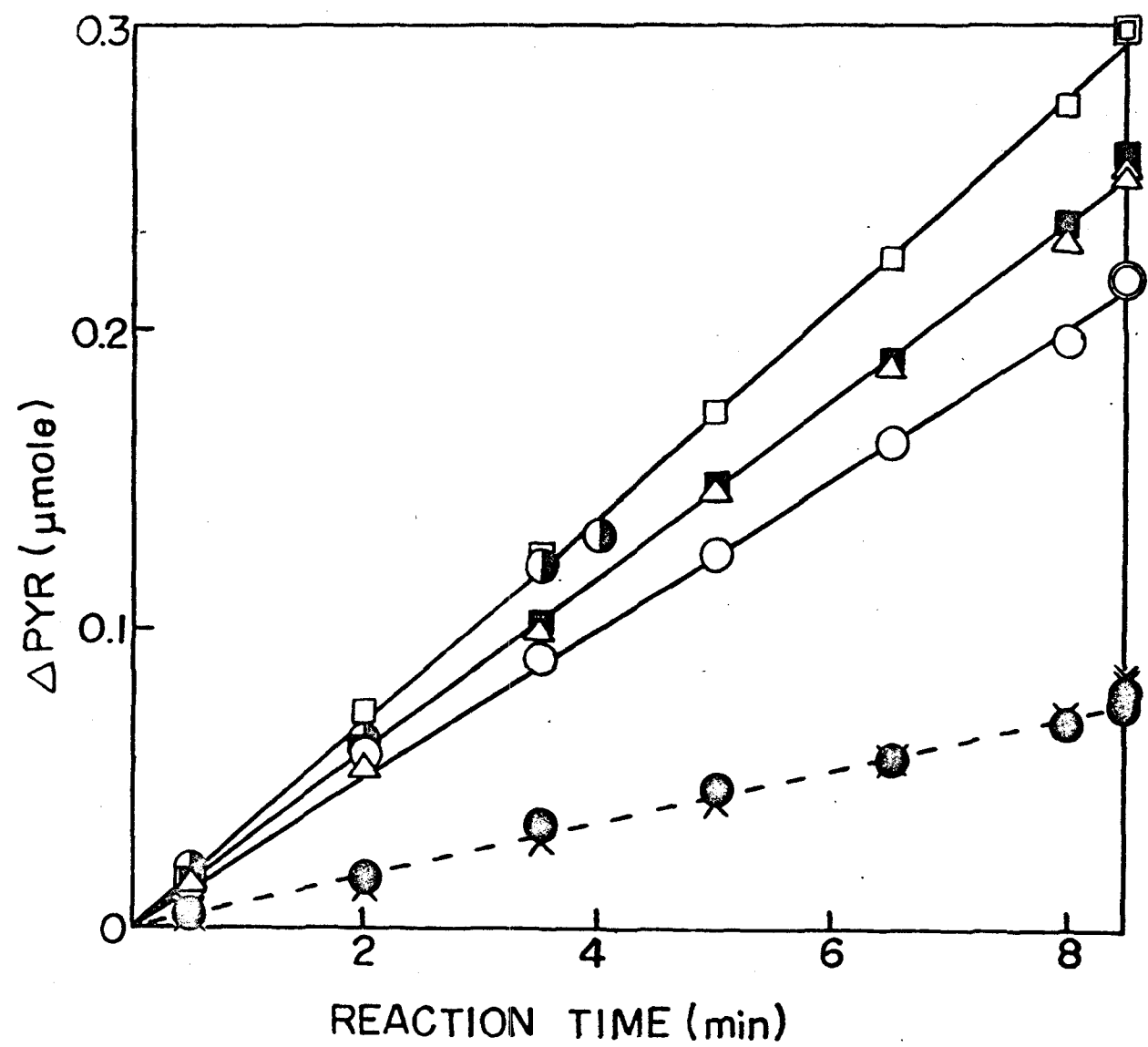


Fig. 12

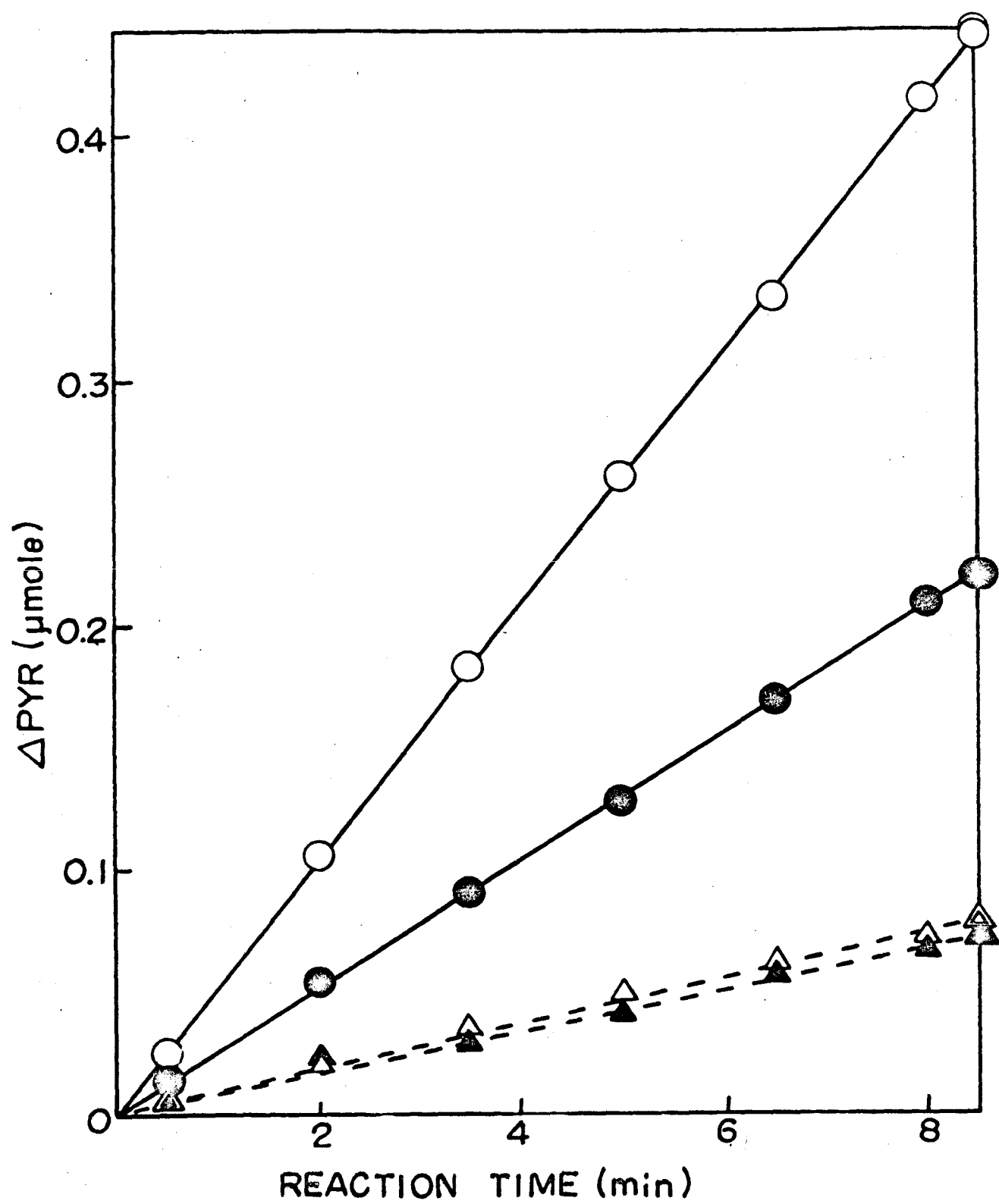


Fig. 13

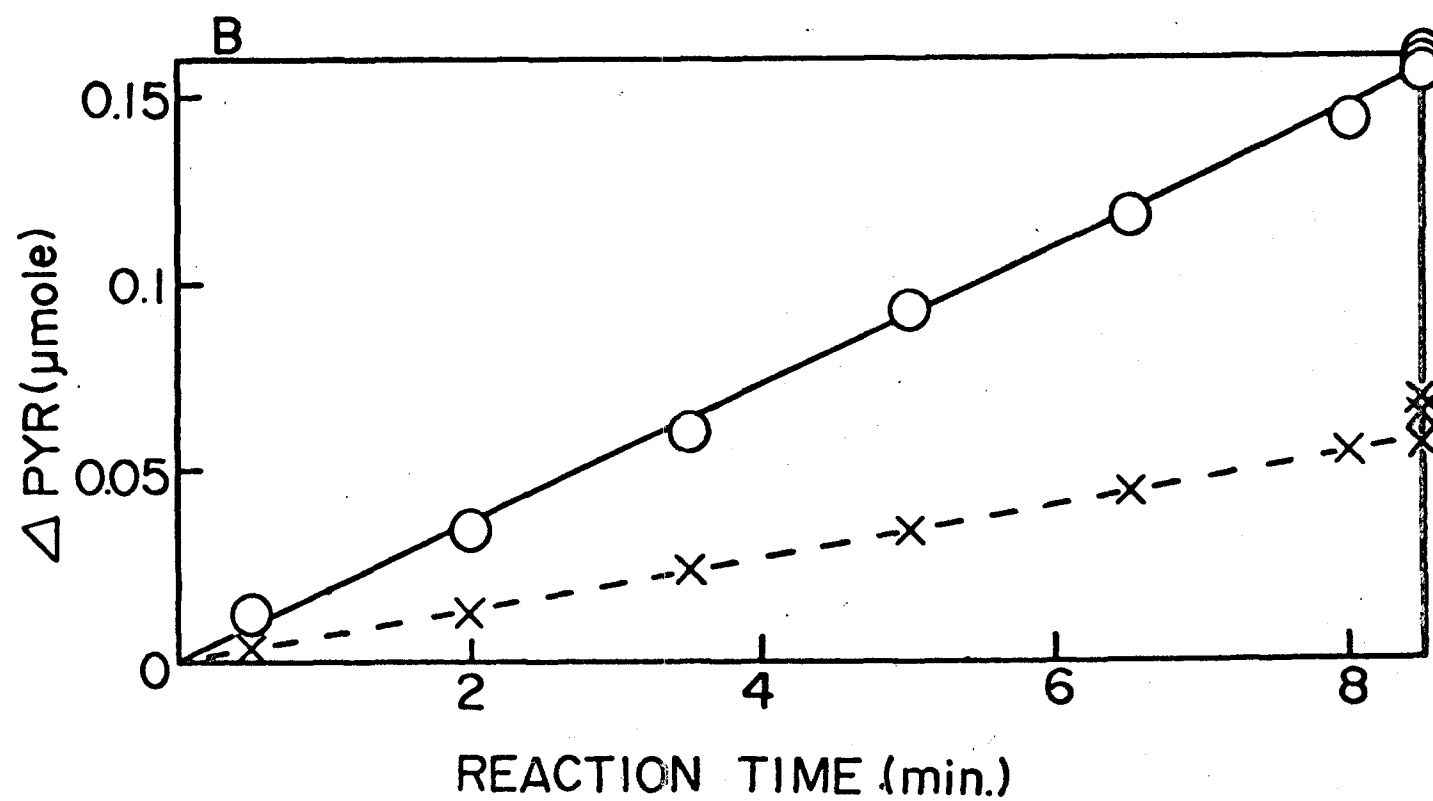
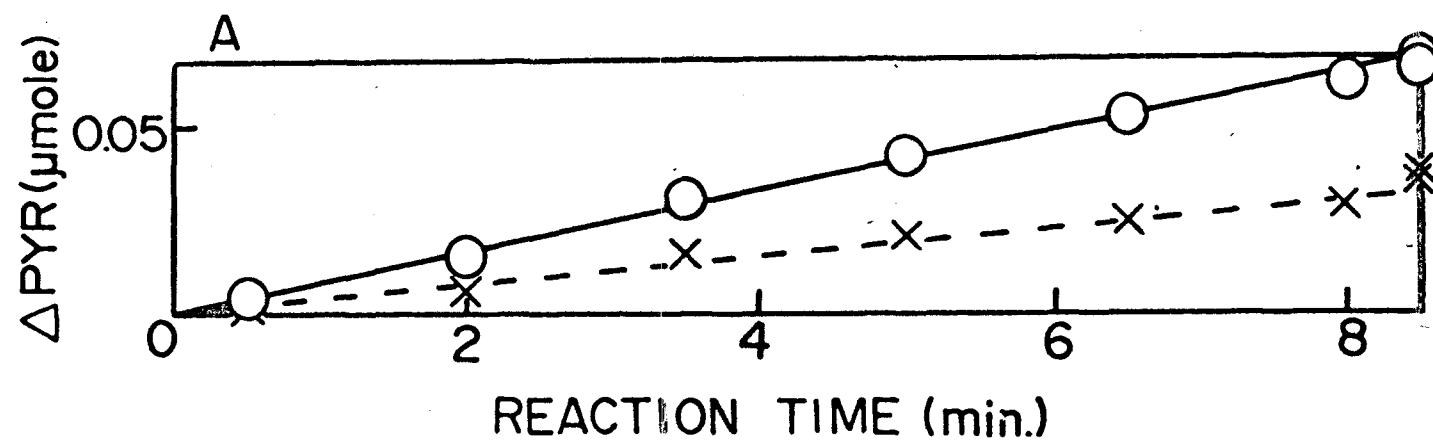


Fig. 14

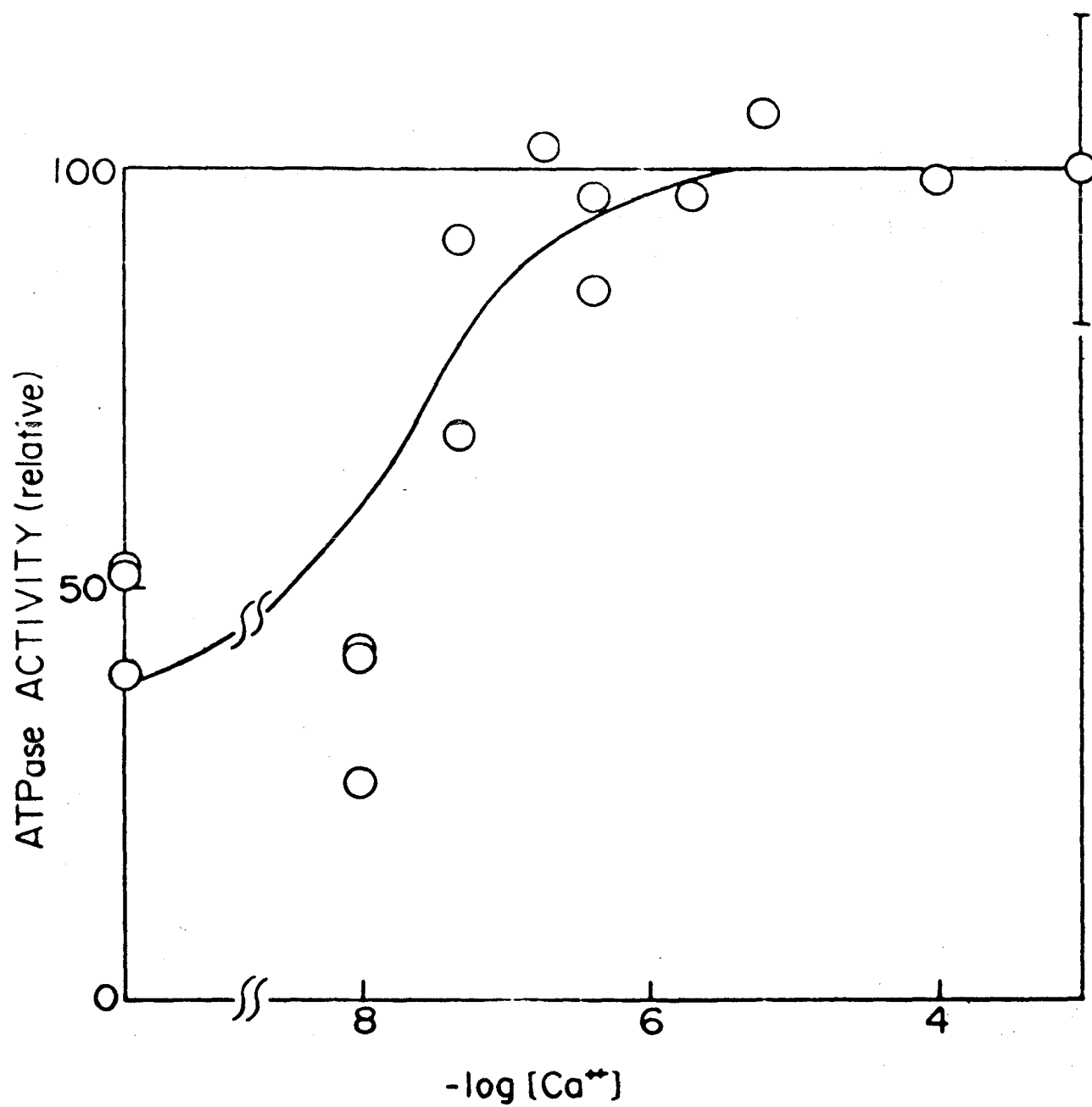


Fig. 15

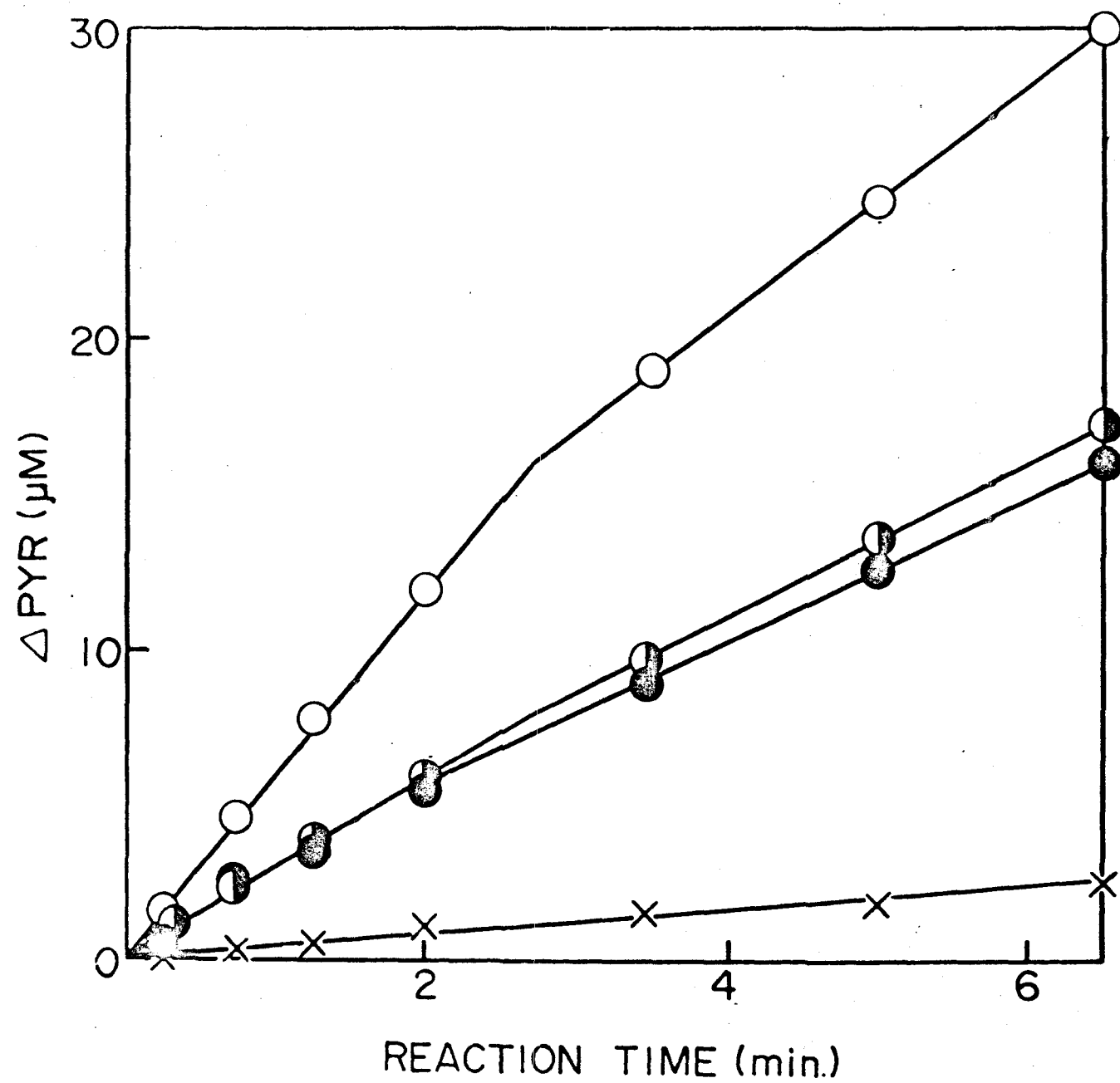


Fig. 16

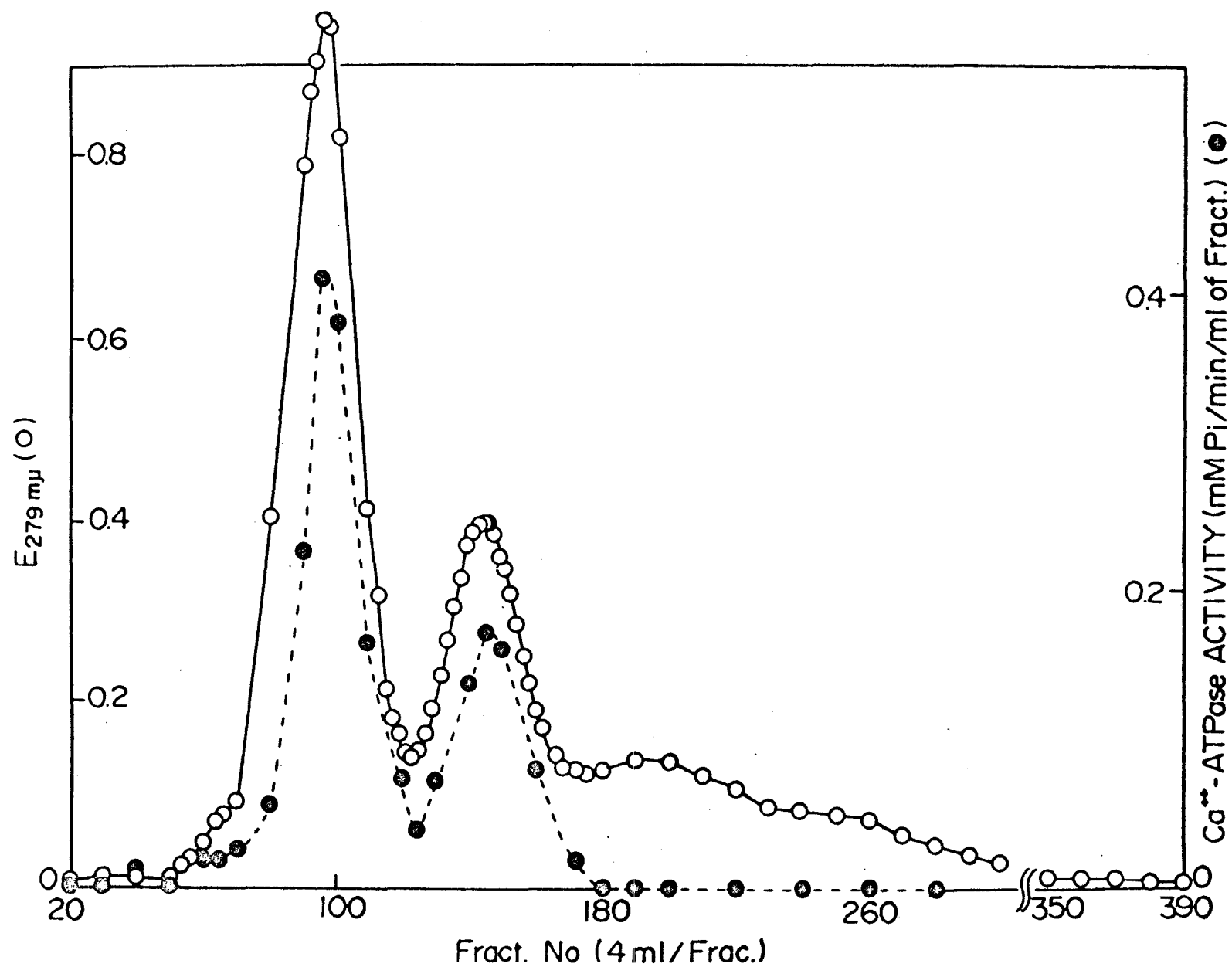


Fig. 17

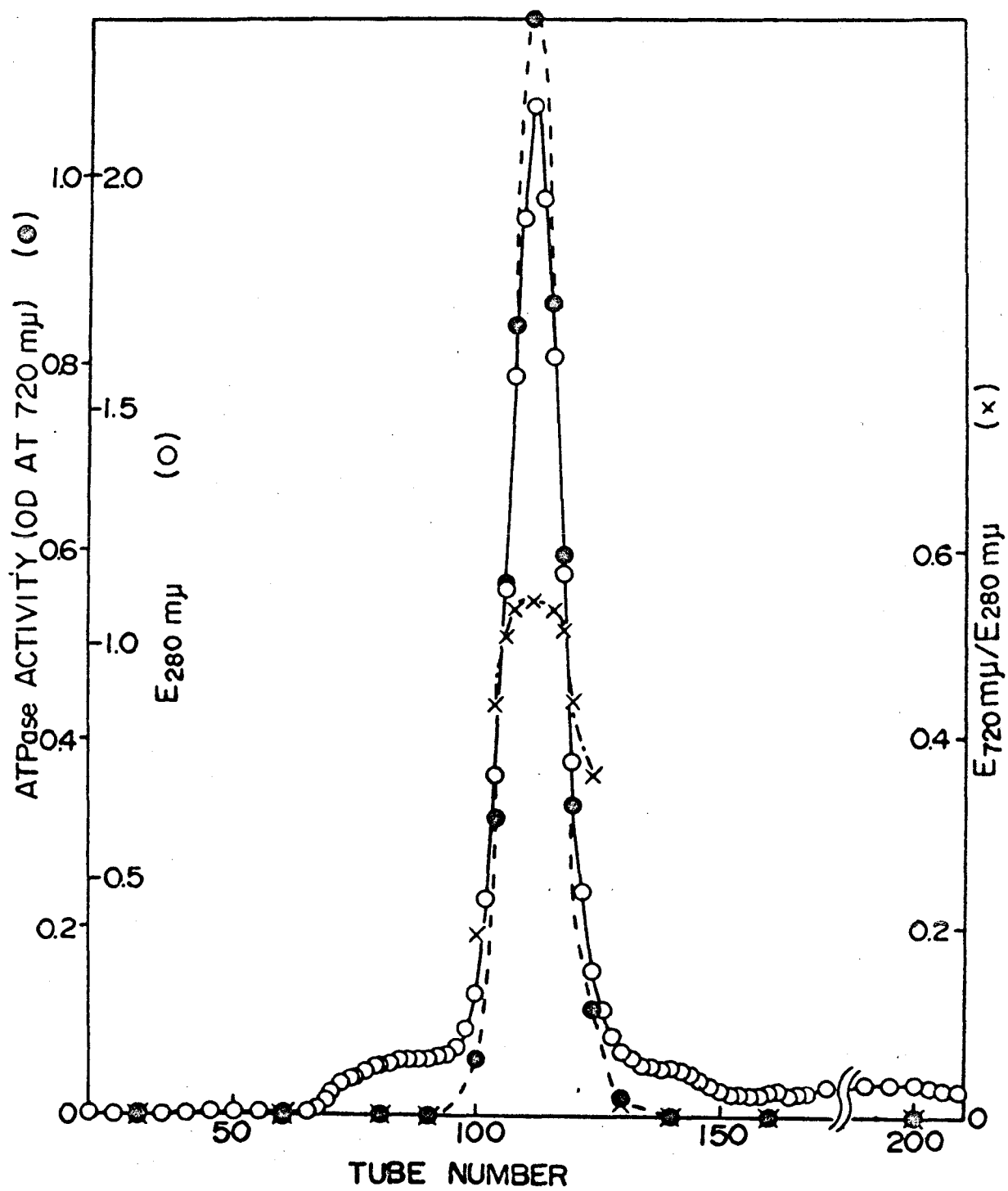


Fig. 18

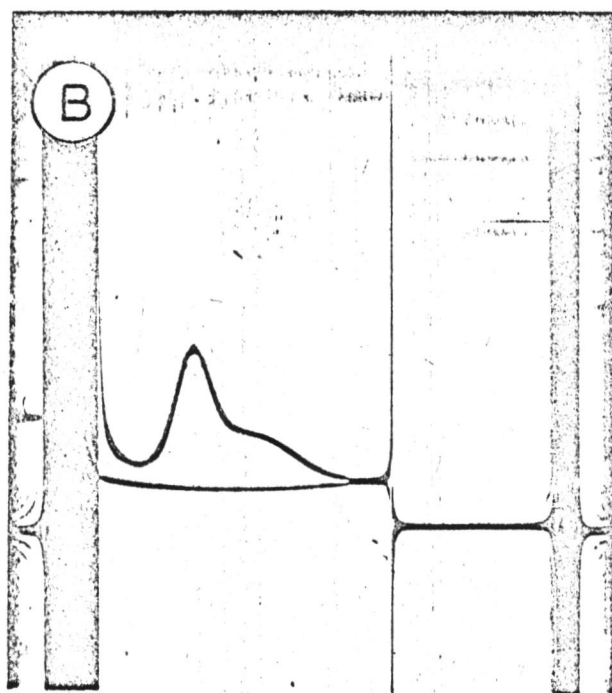
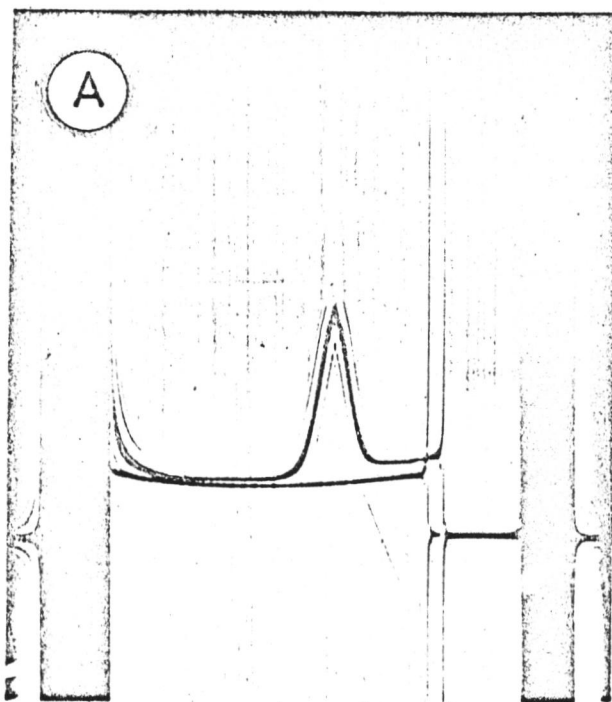


Fig. 19

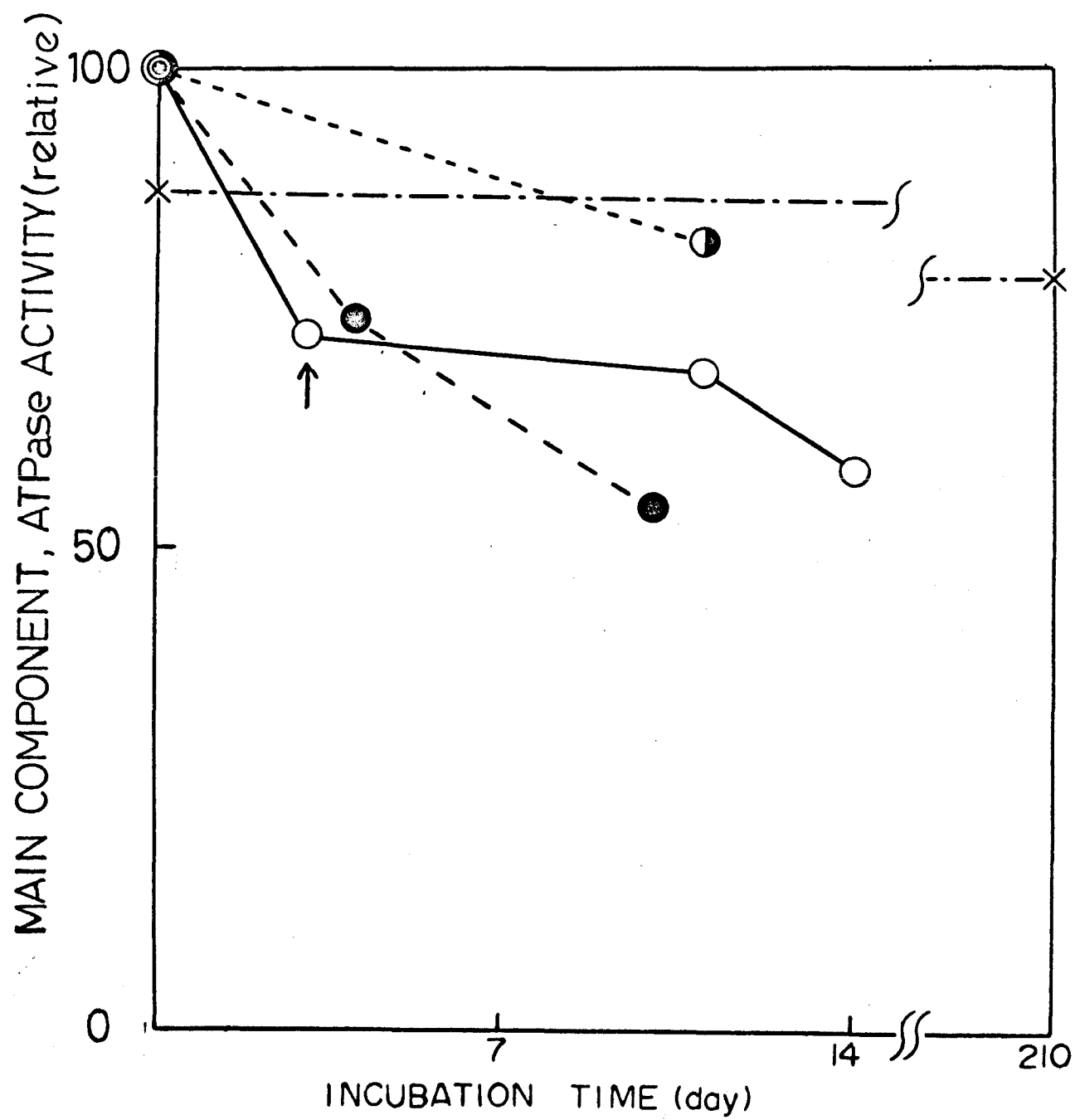


Fig. 20

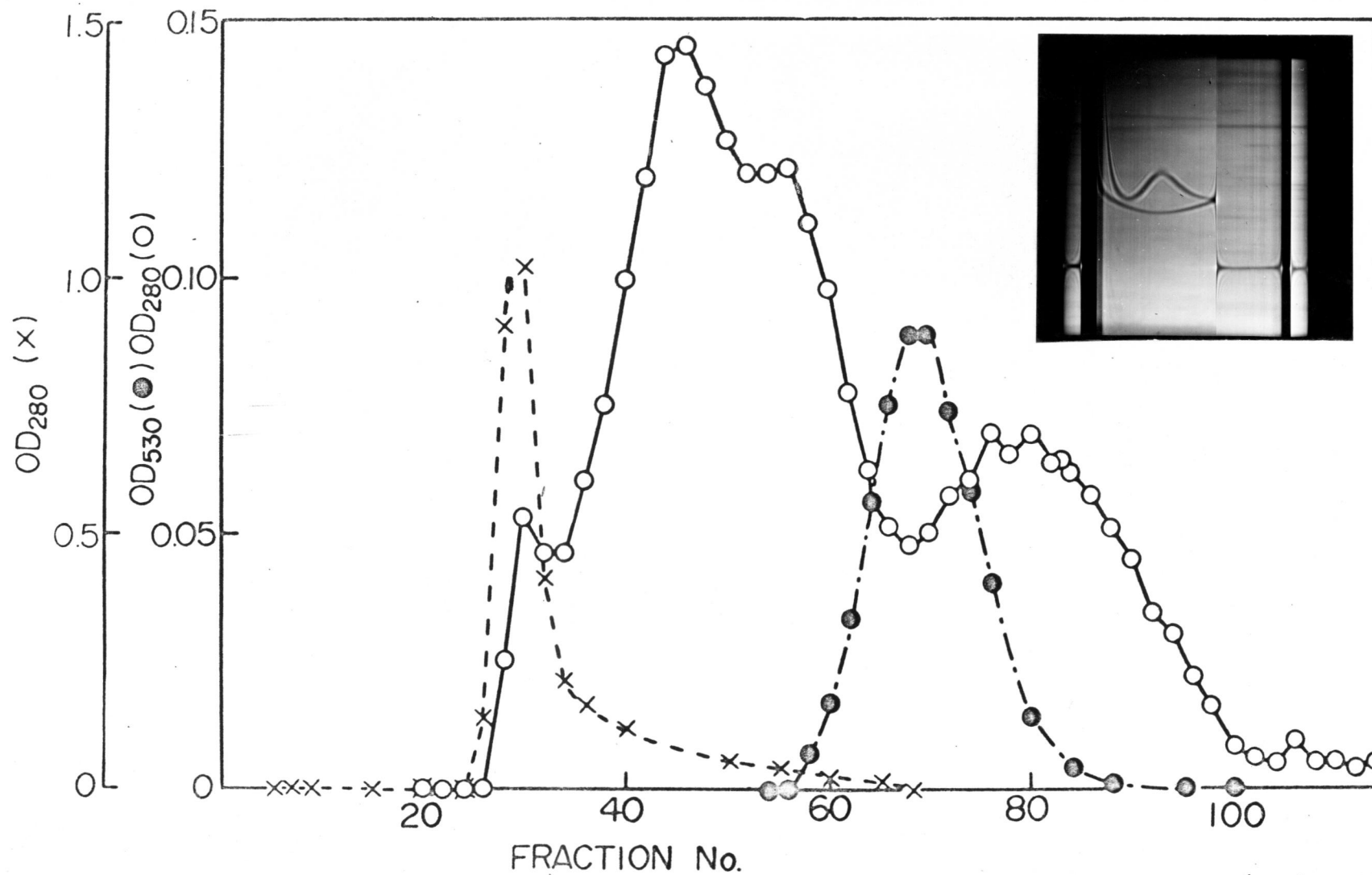


Fig. 21

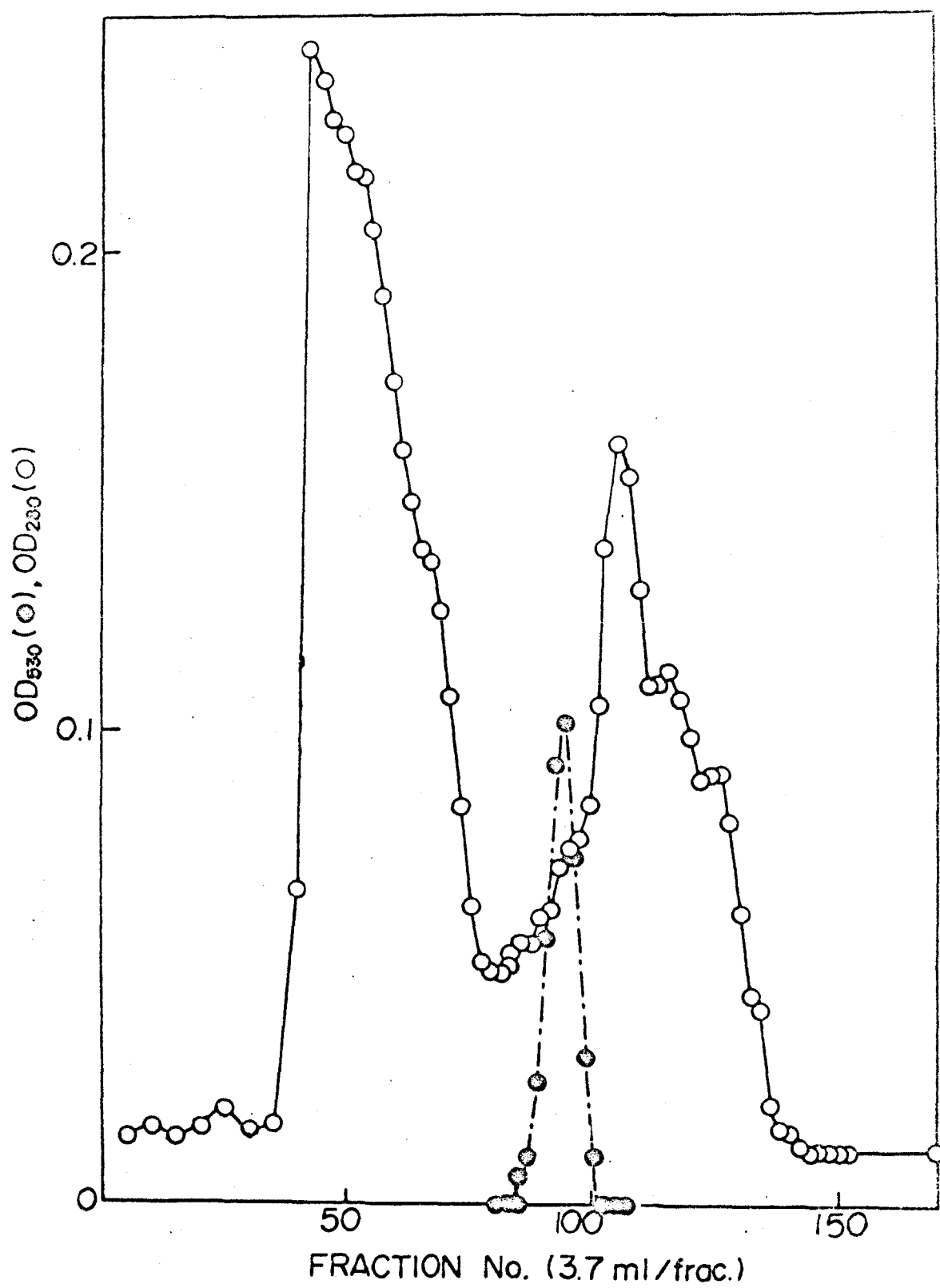


Fig. 22

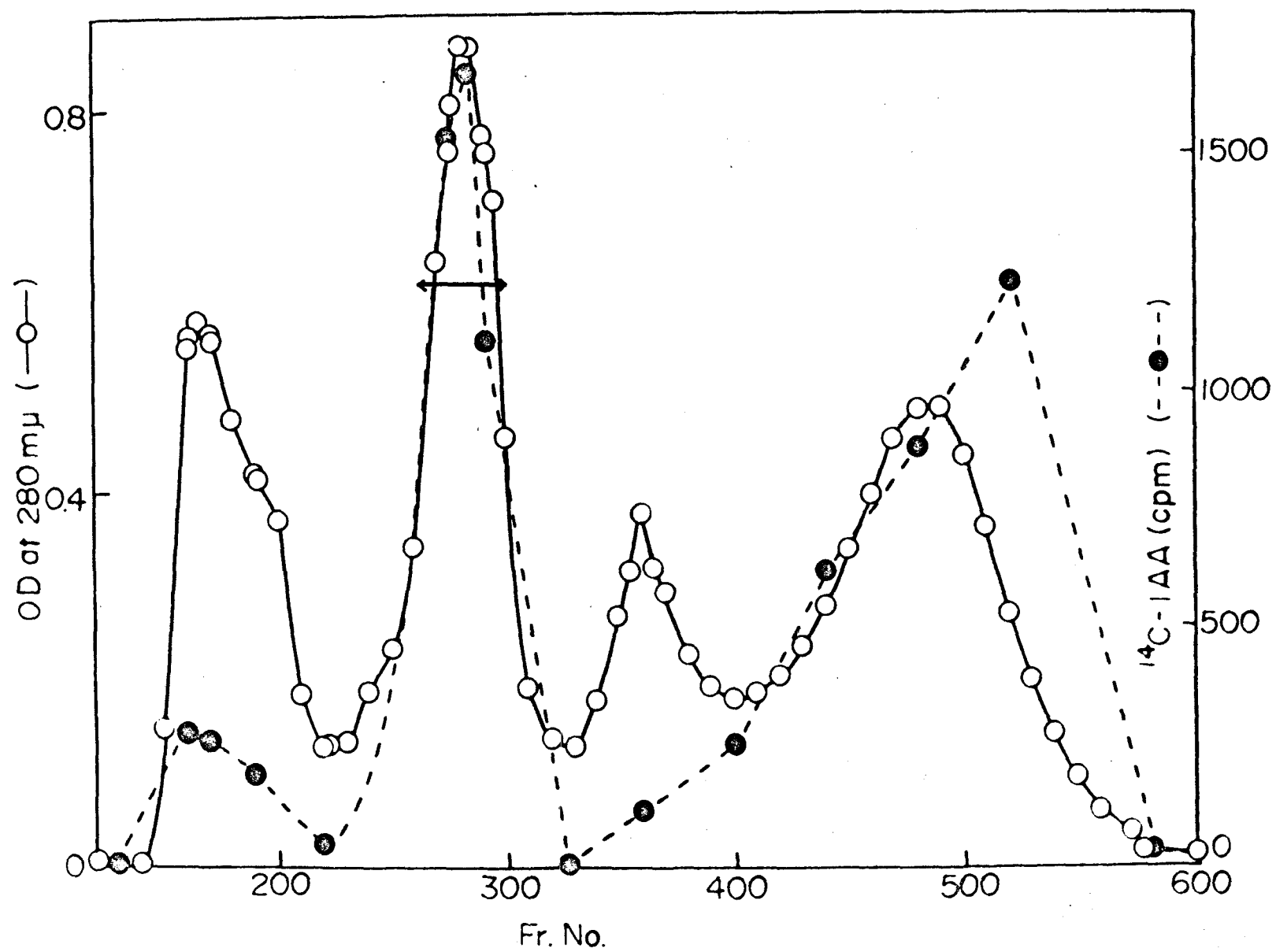


Fig. 23

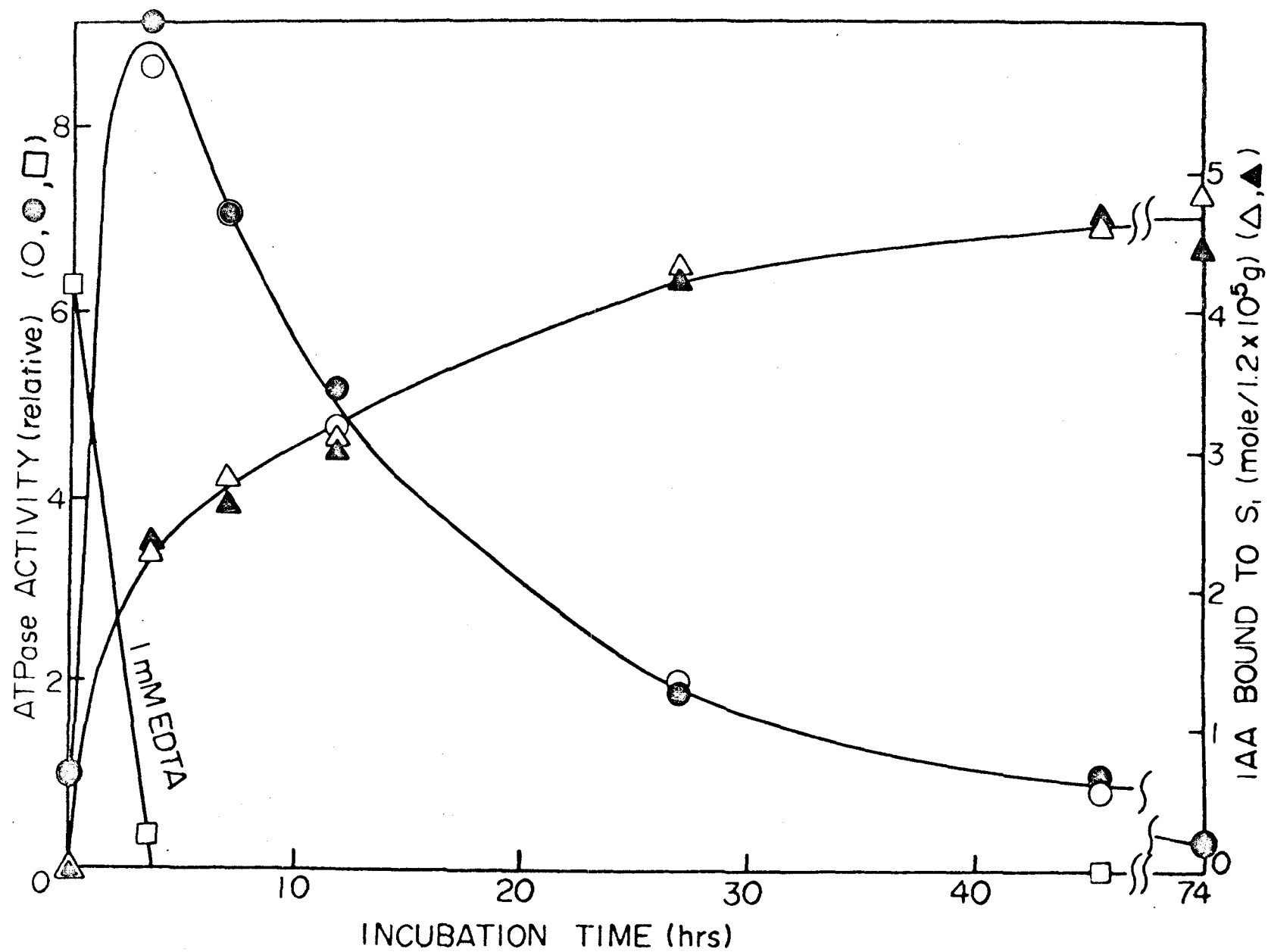


Fig. 24 (A)

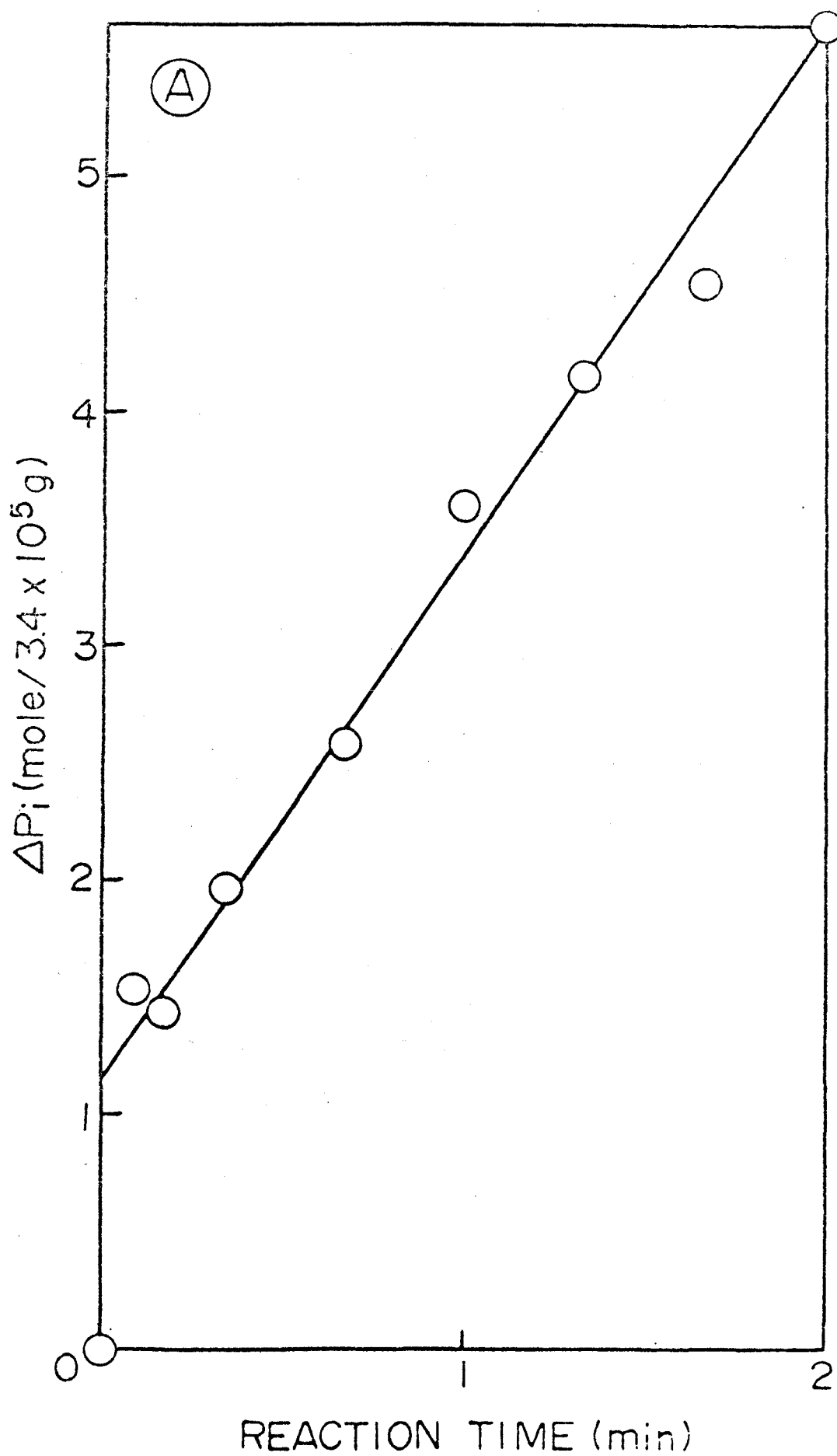


Fig. 24 (B)

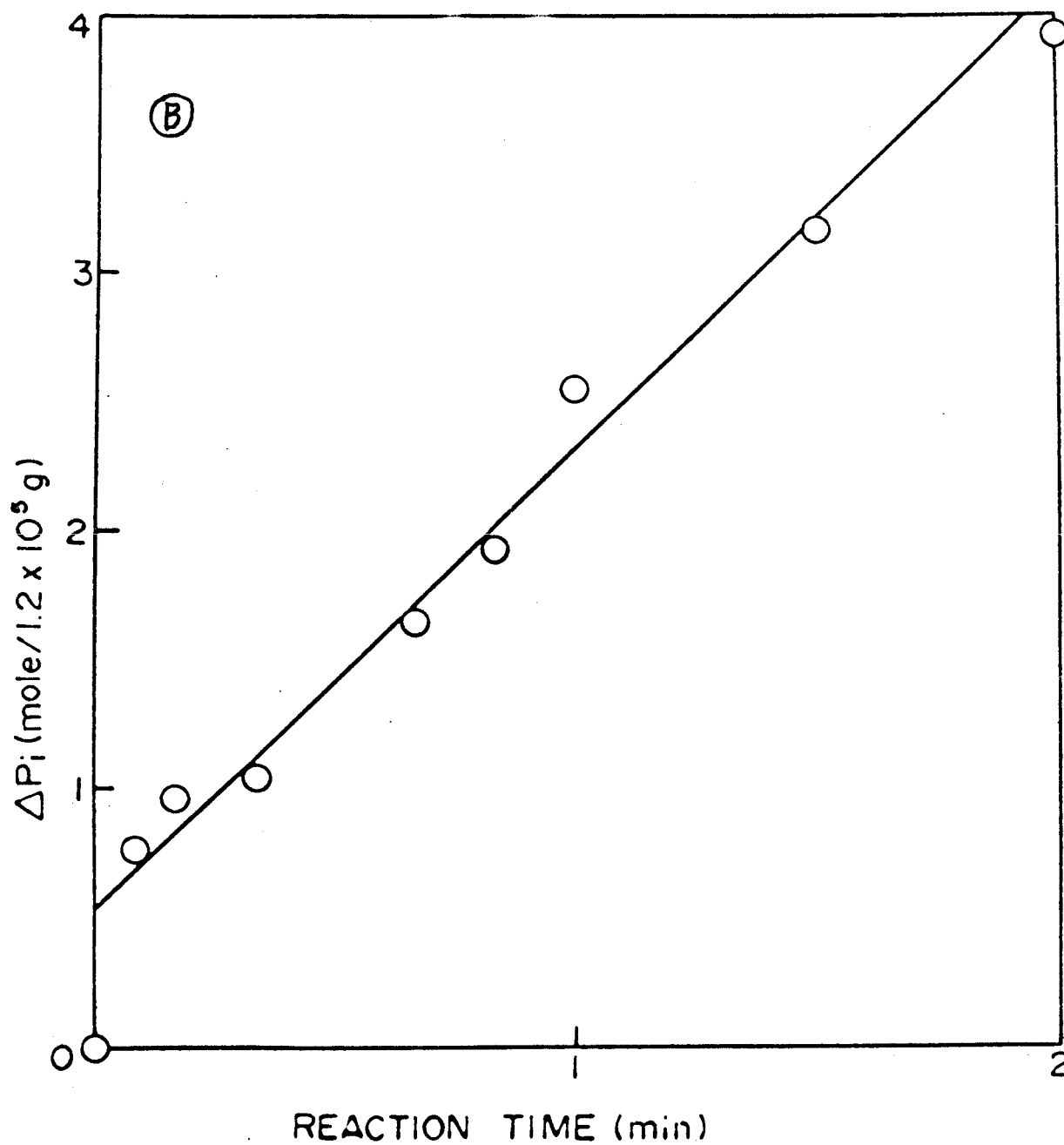


Fig. 25

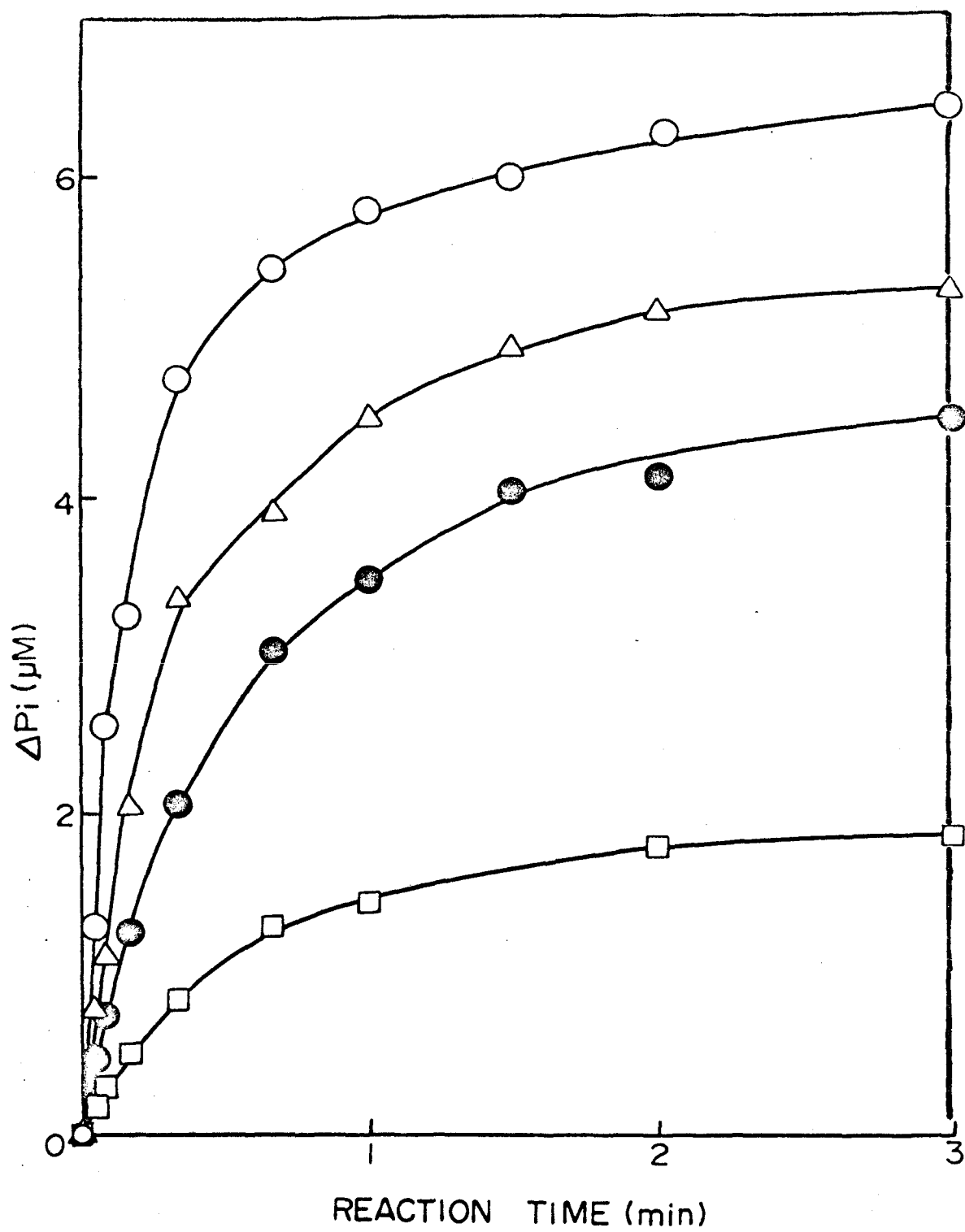


Fig. 26

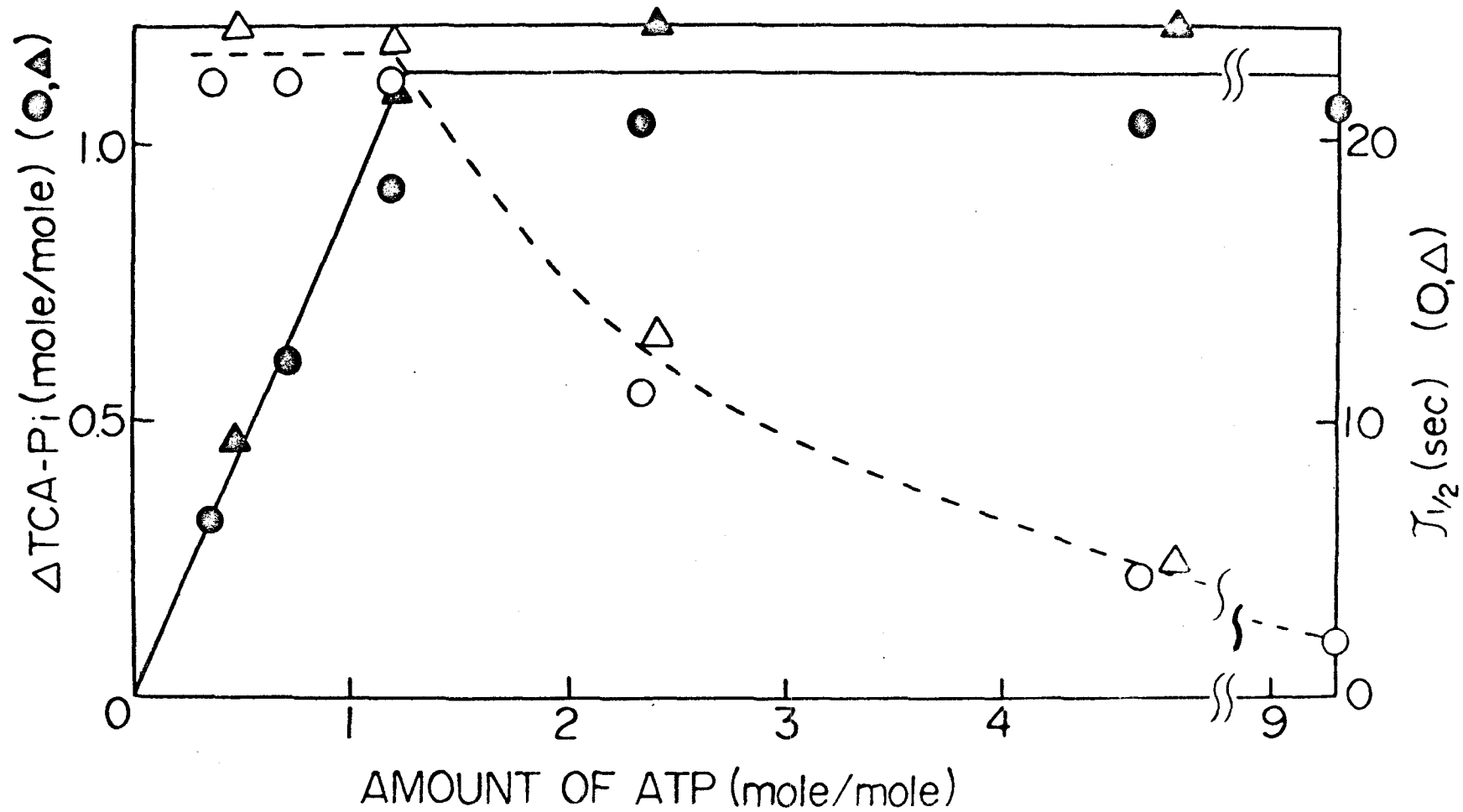


Fig. 27

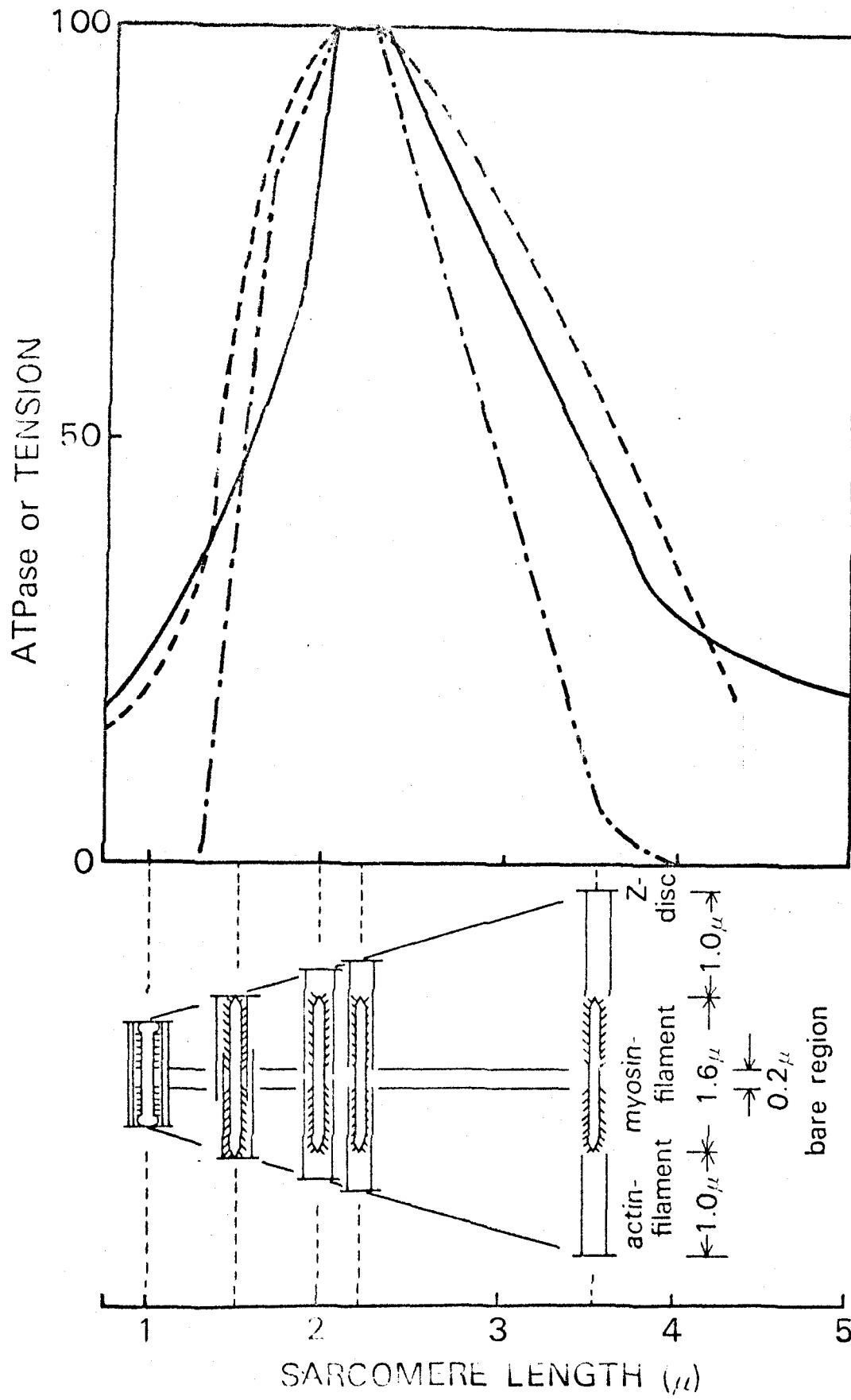


Fig. 28

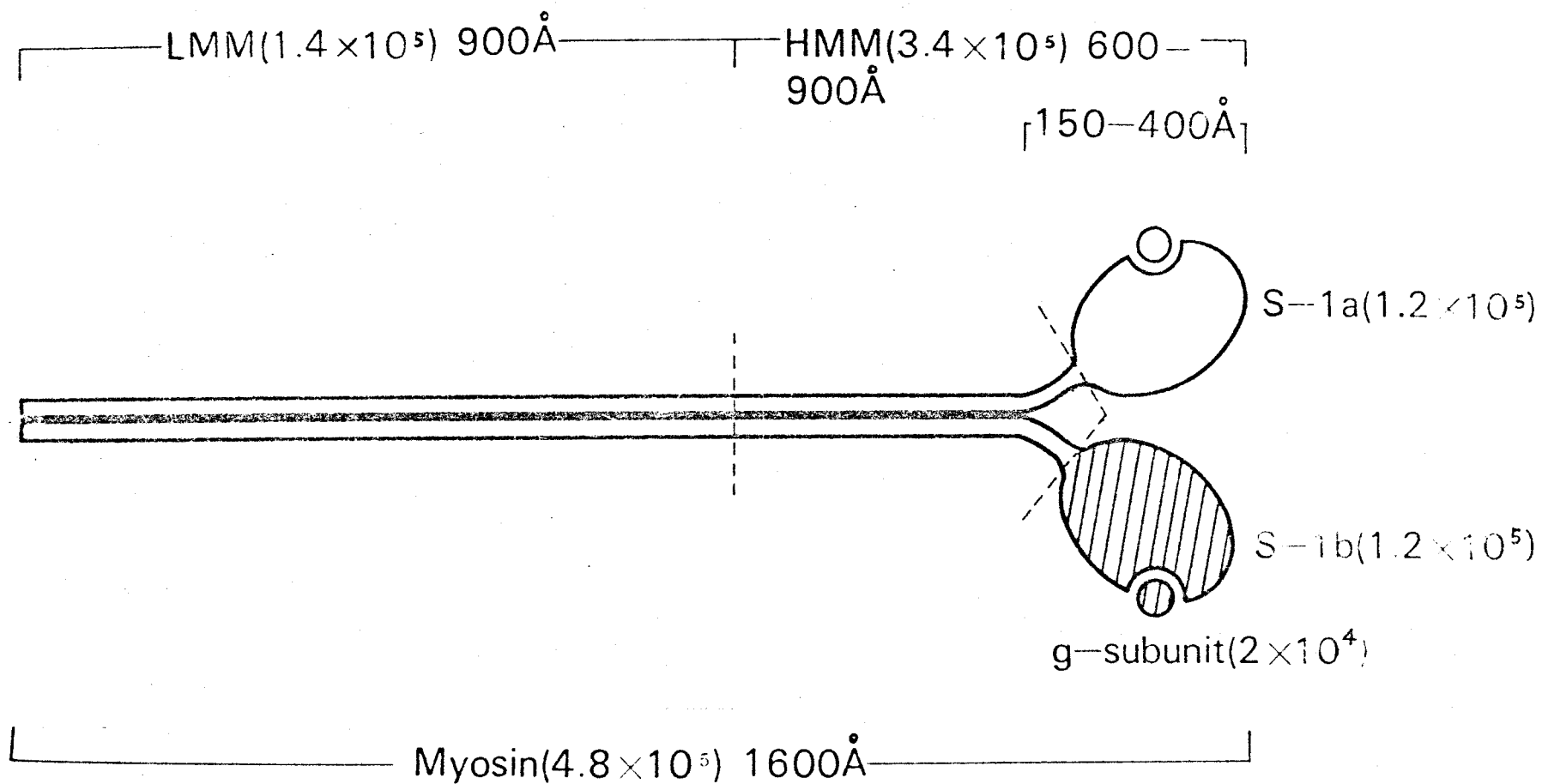


Fig. 29

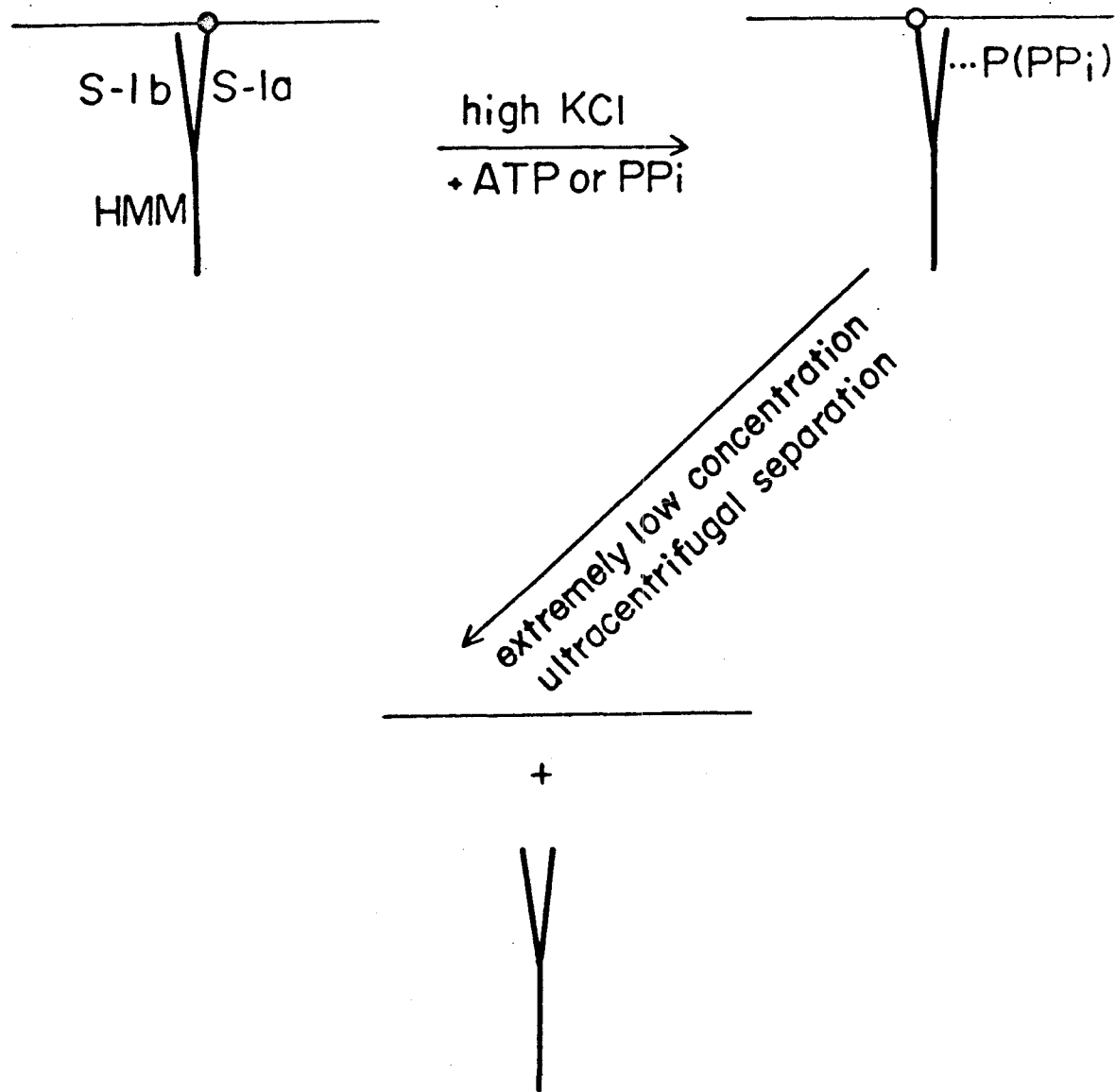


Fig. 30

