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PHOSPHATE EFFECTS ON MEAT

Specific Interaction of Inorganic Polyphosphates with Myosin B

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The specific interaction of inorganic polyphosphates with myosin B has been investigated by several different methods. The protein extracted from myofibrils is found to be almost identical to myosin B by means of diethylaminoethylcellulose column chromatography and ultracentrifugal analysis. Viscosity measurements and the determination of orthophosphate liberation indicate that, among the polyphosphates examined, only pyrophosphate can cause changes in the size and shape of myosin B and that tripolyphosphate is effective only after it is first hydrolyzed by myosin B–tripolyphosphatase. Hexametaphosphate has almost no effect on the viscosity of myosin B solutions. The tripolyphosphatase activity of myosin B is recognizable only in the presence of divalent cation and at a high ionic strength. The inorganic tripolyphosphatase activity, unlike the myosin B adenosine triphosphatase activity, is favored in an acid medium. The appearance of a fraction more soluble than the original myosin B is found upon the addition of pyrophosphate to a myosin B solution containing 0.6M potassium chloride and 0.5mM magnesium chloride, thus suggesting the dissociation of actomyosin into myosin A and actin. The possible role of a specific interaction of inorganic polyphosphate with myosin B in improving the binding properties and water-holding capacity of meat is discussed.

Yasui *et al.* (*27*) studied the effects of three inorganic polyphosphates on the solubility of myosin B (natural actomyosin) and on the extractability of structural protein from myofibrils in various conditions, and have classified the effect of inorganic polyphosphates into two types. The first is polyphosphates of comparatively low molecular weight, such as pyrophosphate (PP) or tripolyphosphate (TP), which react with salt-free myosin B as a salt. Their affinity to myosin B is greatly improved in the presence of high salt concentrations and divalent cations. The other type is highly polymerized polyphosphates such as hexametaphosphate (HP), in which the ratio of H_2O to P_2O_5 is very close to 1:1. These bind directly with salt-free myosin but their binding is somewhat inhibited by the presence of high salt concentration and divalent cations.

Muscle is known to contain sufficient salt and divalent cations for the muscle structural protein to react with organic polyphosphates (*1, 5*). Moreover, the sausage manufacturing process is always undertaken in the presence of at least 2% NaCl. Therefore, polyphosphates which belong to the former group may play a substantial role in meat processing.

Helendoorn (*11*) investigated the water-holding capacity of meat in the presence of various polyphosphates and NaCl, and found that only PP and TP in combination with NaCl show an improvement over NaCl alone. Sherman (*20*) emphasized the positive correlation between ion absorption and the water-holding capacity of lean pork. He also found that commercial polyphosphates containing PP had such a strong effect that it seemed likely a mechanism different from that in effect with a common neutral salt must be operative. As early as 1954, Bendall (*3*) investigated the effect of polyphosphates on the swelling of comminuted whale meat, and predicted that the effective polyphosphates should have chemical structures similar to that of ATP—that is, PP and TP.

Bendall's prediction was confirmed by the studies of Fukazawa *et al.* (*7, 8*) on experimental sausage made from myofibrils and on the effects of PP, TP, and HP on the phyosicchemical properties of structural proteins extracted from myofibrils. Kotter (*13*) stressed independently the importance of low molecular weight polyphosphates which interact specifically with muscle structural proteins. The work by Yasui *et al.* (*27*) on the solubility of muscle structural proteins appears to be useful in providing for a comprehensive interpretation of the effect of inorganic polyphosphates on the properties of meat.

However, since solubility studies tell

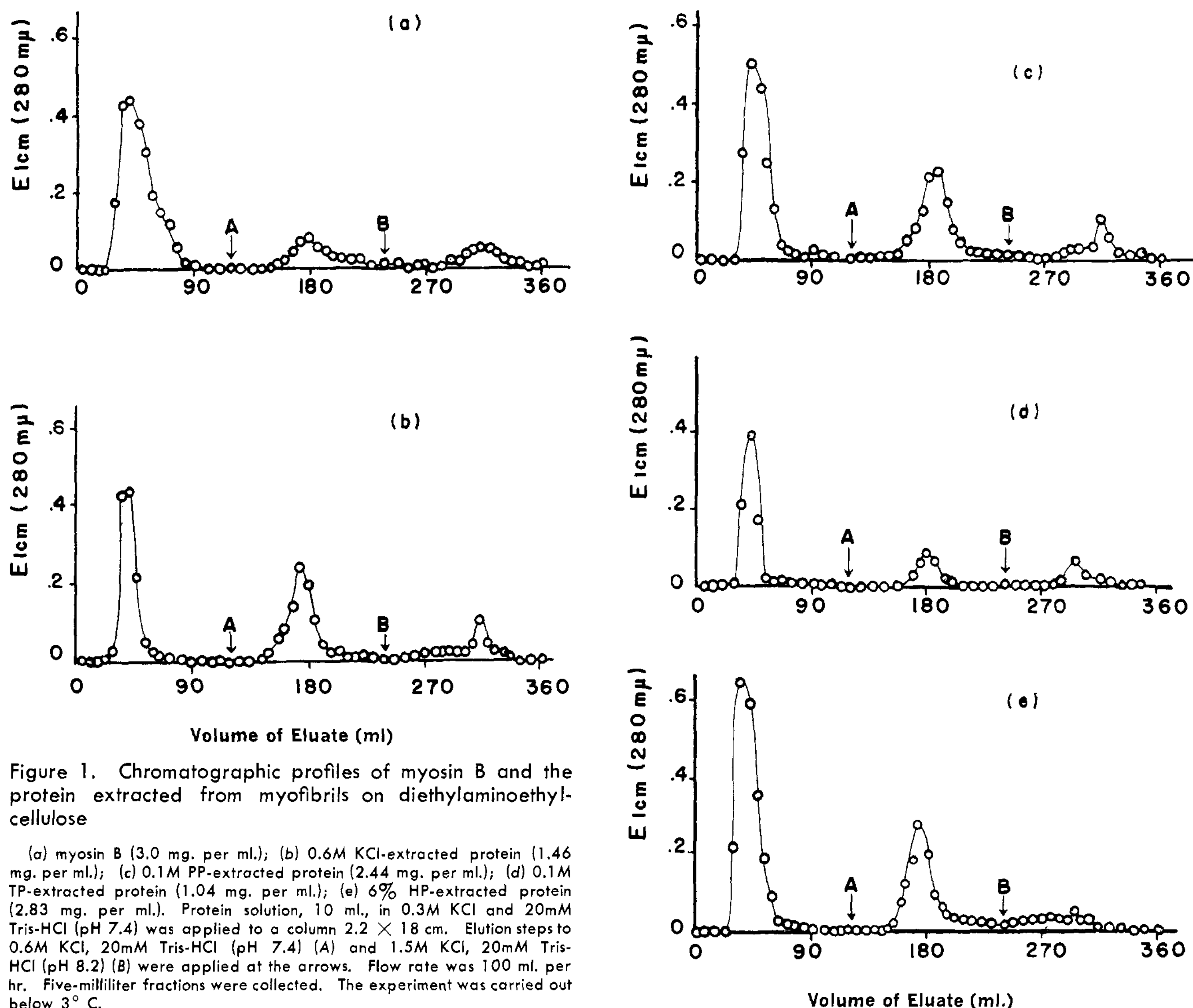


Figure 1. Chromatographic profiles of myosin B and the protein extracted from myofibrils on diethylaminoethylcellulose

(*a*) myosin B (3.0 mg. per ml.); (*b*) 0.6*M* KCl-extracted protein (1.46 mg. per ml.); (*c*) 0.1*M* PP-extracted protein (2.44 mg. per ml.); (*d*) 0.1*M* TP-extracted protein (1.04 mg. per ml.); (*e*) 6% HP-extracted protein (2.83 mg. per ml.). Protein solution, 10 ml., in 0.3*M* KCl and 20m*M* Tris-HCl (pH 7.4) was applied to a column 2.2 × 18 cm. Elution steps to 0.6*M* KCl, 20m*M* Tris-HCl (pH 7.4) (*A*) and 1.5*M* KCl, 20m*M* Tris-HCl (pH 8.2) (*B*) were applied at the arrows. Flow rate was 100 ml. per hr. Five-milliliter fractions were collected. The experiment was carried out below 3° C.

only a little about what is actually occuring at the molecular level, more detailed studies are needed to provide further evidence of the specific interactions between inorganic polyphosphates which belong to group I and myosin B. In this report, therefore, the physicochemical changes of myosin B in a solution 0.6*M* in KCl in the presence of inorganic polyphosphates and divalent cations are investigated further by measuring the viscosity and enzymic activity of myosin B. The results indicate that the basic function of the inorganic polyphosphates affecting the binding properties of meat is to cause a shift to the right of the following equilibrium: actomyosin ⇌ actin + myosin.

The polyphosphate which plays a direct role in this is PP, and TP becomes effective only after its terminal phosphate is split by myosin B–tripolyphosphatase (TPase). On the other hand, HP may not be effective unless it is degraded to PP through spontaneous reversion in solution or by decomposition by some mechanism such as enzymatic reaction in meat.

Materials and Methods

Myosin B and myofibrils were prepared by the methods reported previously (*27*). Extractions of proteins from myofibrils by various solvents (0.6*M* KCl, 0.1*M* PP, 0.1*M* TP, and 6.3% HP) were performed at pH 6.4 (50 m*M* phosphate buffer) and 3° C., with gentle stirring. The myofibrillar proteins extracted were precipitated at ionic strength 0.1 and redissolved in 0.6*M* KCl. For HP-extracted myofibrillar proteins, dilution was made until precipitation took place, since an exact molecular weight and valency for this salt has not yet been ascertained. The protein solutions were then dialyzed exhaustively against 0.3*M* KCl solution (for chromatographic analysis) or 0.6*M* KCl solution (for ultracentrifugal analysis) at 3° C. and pH 7.4 (20m*M* tris-HCl buffer).

Partially purified pyrophosphatase (PPase) was obtained from rabbit skeletal muscle by the method developed in the authors' laboratory. A water extract of rabbit muscle was dialyzed for 24 hours at 3° C. against neutralized water in which 5m*M* $MgCl_2$ was present, and the insoluble precipitates were removed by centrifugation for 20 minutes at 9000 r.p.m. From the supernatant fluid, the fraction which precipitated over the range of 43 ~ 65% saturation of ammonium sulfate was collected. The PPase thus prepared was activated only by magnesium and was quite heat stable; its optimum pH value was 7.3.

No Kielly-Meyerhof adenosinetriphc phatase (ATPase) (*12*) was present in tl fraction and stability of this enzyme (pended on the presence of magnesi PP and TP were hydrolyzed by enzyme, but HP was little affected.

Column Chromatography. Diethylaminoethylcellulose(DEAE-cellulose) was prepared by the method of Peterson and Sober (*19*). The chromatography of myofibrillar proteins on DEAE-cellulose was carried out, in general, as reported by Takahashi *et al.* (*22*). A myofibrillar protein solution was equilibrated by dialysis against 0.3*M* KCl and 20m*M* Tris-HCl buffer (pH 7.4), and then run into a column (2.3 × 18 cm.) of DEAE-cellulose equilibrated against the same buffer. After the unabsorbed fraction had passed through the column, a stepwise ascending gradient from 0.6*M* KCl and 20m*M* Tris-HCl (pH 7.4) to 1.5*M* KCl and 20m*M* Tris-HCl (pH 8.2) was applied. The ultraviolet absorption at 280 mμ and the ATPase activity of the effluents were measured.

Ultracentrifugal Analysis. The ultracentrifugal sedimentation pattern was determined with a Spinco Model E ultracentrifuge at 16.5° C. and 56,100 r.p.m.

Viscosity Change. After removal of gross aggregates by centrifugation for 20 minutes at 13,000 r.p.m. and 0° C., the viscosity of a 0.6*M* KCl myosin B solution (protein concentration, ~ 5.0 mg. per ml.) was estimated by a viscometer of the Ostwald type at 21° C. and pH 7.0 (20m*M* Tris-maleate buffer) in the presence of 1m*M* $MgCl_2$. From the measured values, the reduced viscosity could be obtained by the following formula:

$$\text{reduced viscosity} = \frac{\eta\ \text{rel.} - 1}{C} \times 100 \quad (1)$$

where C = protein concentration.

Enzymatic Activity. Hydrolysis of various polyphosphates by myosin B was measured in a reaction mixture where 0.6 or 0.06*M* KCl, 20m*M* buffer (Tris-maleate-NaOH was used as a buffer in the pH ranges of 5.5 to 7.5) and modifiers (1 or 2m*M* $MgCl_2$, or 2m*M* $CaCl_2$) were present. Final concentrations of polyphosphates in the mixture were 1 or 2m*M* for ATP, 1 or 2m*M* for PP and TP, and 0.065 or 0.13% for HP. Protein concentrations in the reaction mixture were approximately 0.5 mg. per ml. The temperature of incubation was 21° C. At measured intervals of time, 2-ml. samples were withdrawn and pipetted into 2 ml. of 16% trichloroacetic acid. The polyphosphatase activity in the reaction mixture was determined by measuring the liberated orthophosphate according to the method of Martin and Doty (*14*). Since the polyphosphate sequestering agents tend to hydrolyze to orthophosphate in aqueous solution (reversion), data of polyphosphatase activity shown in this paper were corrected by subtracting values of spontaneous orthophosphate liberation in the reaction mixture without myosin B.

Protein concentration was determined by the same method as described previously (*27*). The reagents were also the same (*27*). Adenosine triphosphate (ATP) was purchased from Sigma Chemical Co.

Results and Discussion

Fukazawa *et al.* (*8*) reported that the main protein component extracted from myofibrils by NaCl-Weber-Edsall solution is myosin B. In this work, chromatographic profiles as well as ultracentrifugal patterns of proteins extracted from myofibrils by solutions of KCl, PP, TP, and HP were compared with those of myosin B. Figure 1 illustrates the elution pattern from a column of myosin B and various extracts from myofibrils (0.6*M* KCl extract, 0.1*M* PP-extract, 0.1*M* TP-extract, and 6.3% HP-extract). Under these experimental conditions, all the chromatographic profiles are nearly identical, thus indicating that the proteins are qualitatively the same. The enzymatic characteristics of all but the last peak in each chromatographic pattern were examined by determining the rate of inactivation of ATPase activity in the presence of $CaCl_2$. ATPase activity in all peaks examined belonged more or less to a myosin B, but not to a myosin A (*25, 26*).

In the ultracentrifugal patterns of the same protein preparations, a peak of myosin A and a preceding heavy component were clearly observed. The results agreed well with those of Fukazawa *et al.* (*8*) and of the DEAE-column chromatography described above.

Viscosity. Figure 2 illustrates the time course of viscosity change which occurs when 1m*M* PP or TP, or 0.063% HP were present in a 0.6*M* KCl solution of myosin B containing 1m*M* $MgCl_2$ at pH 7.0 (Figure 2*a*), as well as that of orthophosphate liberation accompanying the above change (Figure 2*b*). As controls, distilled water and ATP (1m*M*), whose interaction mechanism has been established (*5, 16, 17, 21, 24*), were used in

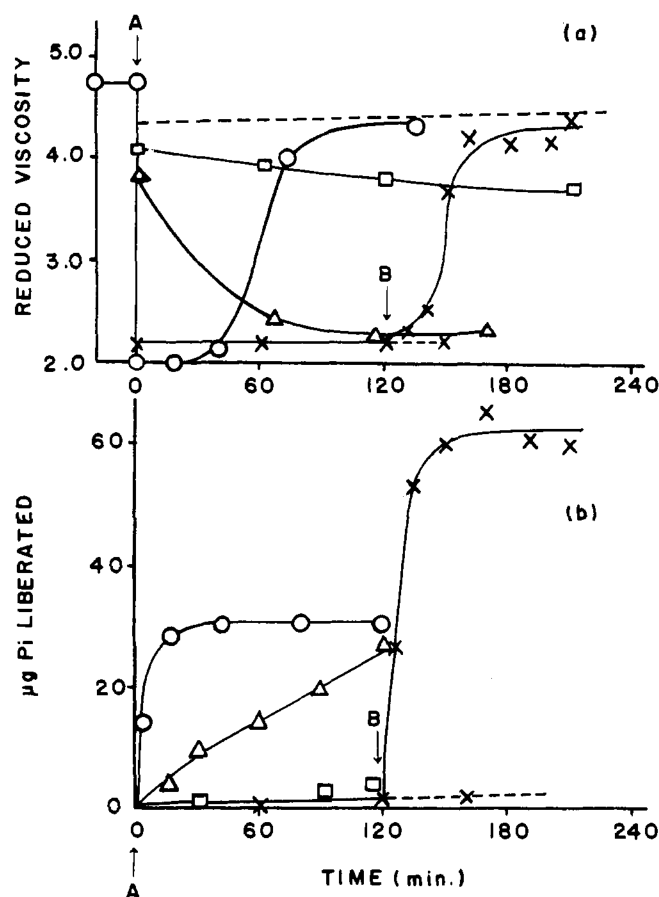


Figure 2. Time course of changes in viscosity (*a*) of myosin B, and in orthophosphate accumulation in the medium (*b*)

Myosin B, 5 mg. per ml., was incubated in 0.6M KCl, 1mM $MgCl_2$, and 20mM buffer at 21° C. (total volume, 4 ml.). At the first arrow (*A*) from the left, 0.4 ml. of distilled water [dotted line in (*a*)], 10mM ATP (O), 10mM PP (×), 10mM TP (△), and 0.6% HP (□) were added to the reaction mixture. At the second arrow (*B*), 1.2 ml. of PPase (10 mg. per ml.) in 0.6M KCl–12mM $MgCl_2$ solution were added to the PP–myosin B system

place of the various phosphates. As is generally known (*16*, *21*), the addition of ATP to a 0.6*M* KCl solution of myosin B remarkably decreased the viscosity of the solution due to ATP-induced conformational changes of myosin B. However, at the same time, the added ATP is hydrolyzed to ADP by the myosin B–ATPase. Since the resulting product, ADP, does not exhibit the specific functions of ATP (*17*), the viscosity of the myosin B will revert to a level close to the original after a certain period of reaction time (Figure 2).

PP causes (*9*, *15*) a conformational change of myosin B in 0.6*M* KCl solution without being hydrolyzed by myosin B. Therefore, a reversal of the viscosity change of myosin B once lowered by PP cannot be observed unless the PP is hydrolyzed by the addition of PPase (Figure 2). Fukazawa *et al.* (*8*) reported that myosin B extracted from myofibrils showed a response to PP, but not to TP or HP.

In this report, as shown in Figure 2*a*, the viscosity of myosin B is decreased slightly by both TP and HP in the presence of a sufficient concentration of myosin B ($\sim$5 mg. per ml.); the decrease in viscosity is greater in the case of the TP. These results, however, may be due to contamination of the TP or HP with PP rather than from a direct interaction between myosin B and these polyphosphates, for the amounts of orthophosphate in these polyphosphates are in the order of TP > HP > PP.

As has been already reported (*2*, *6*), myosin A in 0.5 or 0.6*M* KCl is an inorganic tripolyphosphatase(TPase) in the presence of $CaCl_2$ as well as an ATPase, though the TPase activity is far weaker than the ATPase activity, and the pH dependence of the TPase activity is entirely different from that of the ATPase activity. In Figures 3 and 4, TPase activity of myosin B is compared with its ATPase activity of myosin B. The TPase activity of myosin B occurs only in 0.6*M* KCl solution with $MgCl_2$ and $CaCl_2$, but not in the absence of divalent metal and at low salt concentration (0.06*M* KCl). Moreover, the pH-dependence curve of TPase activity differs completely from that of ATPase activity, showing activation on the acid side. [For details, see Azuma *et al.* (*2*) and Friess and Morales (*6*).] The results are in good agreement with those on the TPase activity of myosin A, and particularly the latter part is of importance when the actual pH value in meat is considered. The fact that myosin B–TPase is revealed only in 0.6*M* KCl solution with divalent cations appears to be related to the fact reported by Yasui *et al.* (*27*) that the affinity of PP or TP for myosin B is increased drastically in the presence of a sufficient amount of univalent and divalent cations. It suggests that the increase in the binding ability of inorganic polyphosphates of low molecular weight for myosin B observed in solubility studies may involve the specific binding of those compounds which trigger the biological functions of the protein.

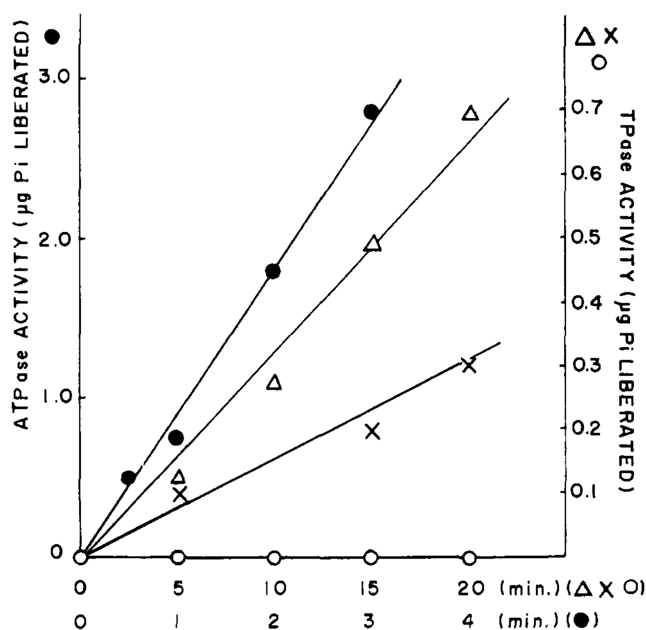


Figure 3. ATPase and TPase activity of myosin B

●, ATPase activity, 0.6M KCl, 20mM Tris-maleate buffer (pH 7.0), 1mM ATP, 2mM $CaCl_2$, and at 21° C.
Δ, TPase activity, conditions were the same as ATPase activity except 1mM TP and 2mM $MgCl_2$ were used in place of ATP and $CaCl_2$
X, TPase activity, conditions were the same as ATPase activity except 1mM TP was used in place of ATP
O, TPase activity of myosin B in 0.6M KCl without divalent metal ions and of myosin B in 0.06M KCl either with or without 2mM $MgCl_2$ or $CaCl_2$
The concentration of myosin B was 0.7 mg. per ml.

The viscosity of myosin B, which decreased slightly on addition of TP, decreases further until it finally reaches the level induced by PP (Figure 2*a*).

Besides this viscosity change, the amount of orthophosphate liberated in the system increases to 25 µg. per ml. when the lowest viscosity of myosin B is obtained (Figure 2*b*). The effect of TP on the physicochemical properties of myosin B shown in Figure 2 may be explained from the fact that TP is converted to PP through hydrolysis of its terminal phosphate bond by myosin B–TPase. HP is also slightly hydrolyzed (Figure 2*b*). This may be due to the decomposition by myosin B–TPase of TP contaminating the HP, because the decrease in viscosity of the myosin B is accompanied by an increase in accumulated orthophosphate. A slight increase in the amount of orthophosphate in the system containing PP has already been shown to be caused by the presence of contaminating PPase in the myosin B preparation (*23*, *26*).

Solubility. Physical changes, such as changes in viscosity or light-scattering, caused by the interaction between myosin B in a solution of 0.6*M* KCl (or reconstituted actomyosin) and PP or ATP in the presence of divalent cation, have been attributed to the fact that these polyphosphates weaken the binding

Table I. The Effect of Various Phosphate-Na Salts on the Protein Content of the Upper Clear Layer after Centrifugation at 13,000 R.P.M. and 0° C.

	% of Protein Concn. of Upper Clear Layer of Myosin B[a]					
	Experiment I		Experiment II		Experiment III	
Salts[b]	With $MgCl_2$[c]	Without $MgCl_2$	With $MgCl_2$	Without $MgCl_2$	With $MgCl_2$	Without $MgCl_2$
PP	10.01	2.68	20.46	6.38	13.22	8.75
TP	6.72	15.20	4.84	10.81	9.87	12.37
HP	4.03	4.70	7.39	6.27	6.85	7.42
Control (KCl)	2.68	1.34	6.71	14.43	...	6.94

[a] Myosin B in 0.2*M* KCl and 50m*M* Tris-maleate buffer (pH 6.8).
[b] Final concentrations: 1m*M* PP, TP, KCl, and 0.06% HP.
[c] Final concentration: 0.5m*M* $MgCl_2$.

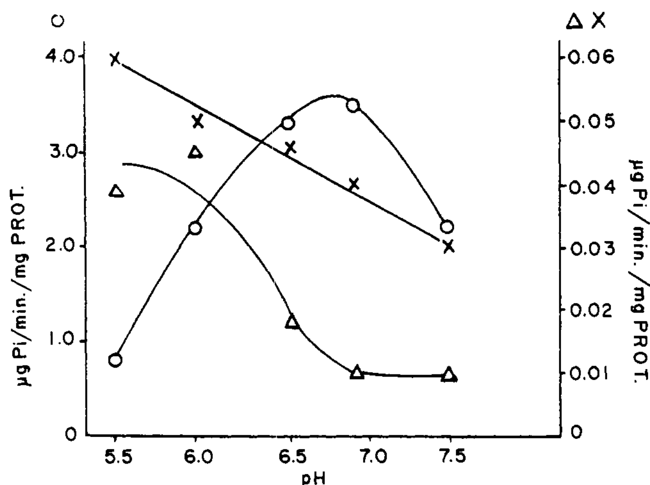


Figure 4. Effect of pH on TPase activity of myosin B

1 mg. per ml. of myosin B was incubated in 0.6*M* KCl, 20m*M* Tris-maleate buffer (at various pH values), and at 21° C.
○, ATPase activity 1m*M* ATP, 2m*M* $CaCl_2$
Δ, TPase activity, 2m*M* TP, 2m*M* $CaCl_2$
×, TPase activity, 2m*M* TP, 2m*M* $MgCl_2$

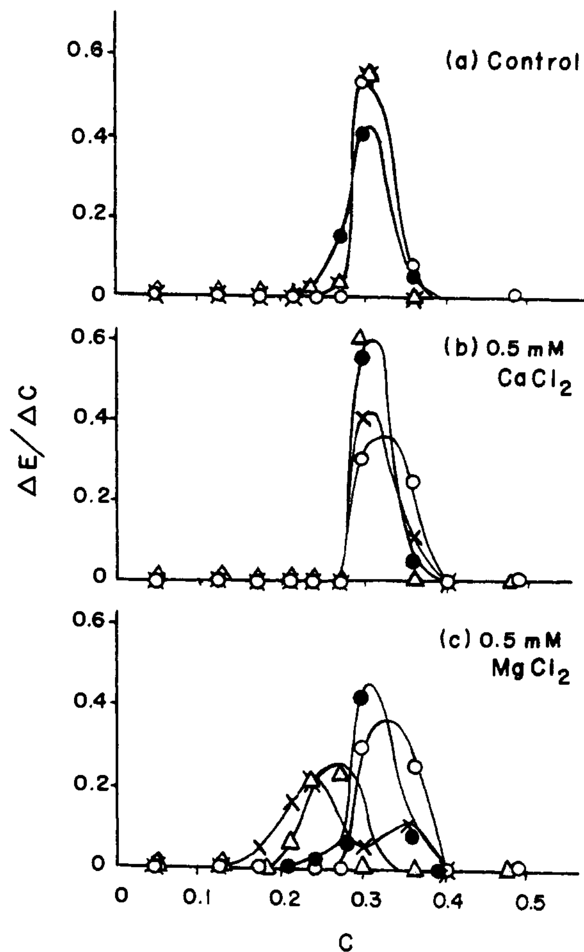


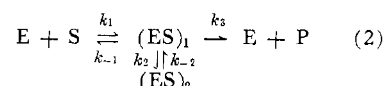
Figure 5. Differential salting-in diagrams of myosin B in the absence (a) and the presence of 0.5m*M* $CaCl_2$ (b) and $MgCl_2$ (c)

○, no phosphate; ×, 1m*M* PP; Δ, 1m*M* TP; ●, 0.06% HP; protein concentration, 0.2 mg. per ml.; 10m*M* phosphate buffer (pH 7.0); S, extinction at 750 mμ; C, molarity of NaCl solution

of myosin A with F-actin in actomyosin, and result in the formation of free F-actin and myosin A (*9*, *18*, *21*). Fukazawa *et al.* (*8*) studied the changes in nitrogen distribution of NaCl-Weber-Edsall solution extracts from myofibrils after ultracentrifugation in 0.6*M* KCl solution containing 1m*M* of various polyphosphates and 1m*M* $MgCl_2$; the increase in nitrogen content in the upper clear layer occurred only when PP was used. Since myosin A is soluble at a lower ionic strength than myosin B (*4*, *16*, *21*), the solubility of the protein should be changed if the dissociation of myosin B into myosin A and actin takes place upon addition of polyphosphates to the system.

To a myosin B suspension, dialyzed against 0.2*M* KCl and 20m*M* Tris-maleate buffer at pH 7.0, were added amounts of PP, TP, and $MgCl_2$, so that the final concentration of the phosphate mixtures would be 1m*M* for PP and TP, 0.065% for HP, and 0.5m*M* for $MgCl_2$. The suspensions were then centrifuged at 13,000 r.p.m. for 2 hours at 0° C. In Table I, the protein content in the supernatant fractions after centrifugation is shown. The results demonstrate clearly that the protein content increases only when $MgCl_2$ and PP are present. If ordinary solubility curves of myosin B under various conditions are converted to salting-in diagram in which the increase of solubility (ΔS) per unit concentration of NaCl (ΔC) is plotted as a function of NaCl concentration, differences in the effects of the various inorganic polyphosphates on the solubility of myosin B become more obvious as shown in Figure 5. Only slight effects on the solubility of the protein are observed in the presence of polyphosphates or divalent cations alone, while a more soluble fraction than the control appears in the presence of polyphosphate together with divalent cations. This effect of polyphosphates becomes stronger in the order of PP > TP > HP; HP shows almost no effect. As pointed out earlier, if the PP contamination of TP is taken into consideration, the net difference of effectiveness on solubility between PP and TP is presumably greater than the apparent one. The facts found in this experiment have added further support to the report on the mechanism of the effect of inorganic polyphosphate on the solubility of myosin B by Yasui *et al.* (*27*).

Inorganic polyphosphates of low molecular weight, such as PP and TP, not only increase their affinity for myosin B in the presence of sufficient amounts of univalent and divalent cations, but also show the specific reaction with myosin B similar to that of ATP. Tonomura and Morita (*23*) expressed the reaction schematically in the actomyosin-ATP system as:



$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} (ES)_1 \overset{k_3}{\longrightarrow} E + P \quad (2)$$
$$k_2 \downarrow\uparrow k_{-2} \quad (ES)_2$$

where E is actomyosin, $(ES)_1$ is an actomyosin-substrate complex, $(ES)_2$ is a stable actomyosin-substrate complex [actomyosin is deformed by the formation of $(ES)_2$], P is the product of the enzymatic activity, and *k*'s are velocity constants of each stage. The inorganic polyphosphates, PP and TP, possess only one of the two functions—*i.e.*, the configurational changes of the myosin molecule and the hydrolysis of the terminal P—O—P linkage by myosin B of ATP. PP has only the former, and TP only the latter. Then, a question arises from a practical viewpoint: that is, which function is necessary for the effect on the binding properties of salted comminuted meat?

Fukazawa *et al.* (*7*) have indicated that myofibrils deficient in myosin A appreciably decreased the binding properties initially present in the control. Therefore, the factor which influences most directly the improvement of the binding properties of meat in sausage

manufacturing may be dissociation of actomyosin to myosin A and actin by PP together with Mg^{+2}. On the other hand, TP becomes effective after its decomposition to PP by the TPase in meat. Actually, however, TP could be of use when a long curing period is used, for, unlike PP which becomes ineffective through its hydrolysis by PPase in meat, TP exhibits its influence for longer time period through its hydrolysis by the TPase in meat. The effect of polyphosphates such as HP may be confined to an enhancement of solubility and extractability of myosin B by increasing the ionic strength under the conditions of sausage manufacture, unless TP or PP are produced from HP spontaneous reversion or other type of decomposition. As pointed out by Hashimoto *et al.* (*10*), the important factor for the binding properties of sausage is the quality of the extracted proteins, and not the amount of protein extracted from meat.

These experimental results show that the effects of various inorganic polyphosphates on the physicochemical properties of myosin B will reflect the behavior of the muscle structural protein in meat in the presence of these phosphates.

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FOOD FLAVORS AND ODORS

Volatile Sulfur Compounds in Potatoes

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A LARGE VARIETY of simple sulfur compounds have been isolated from higher plants (*36*), and many such compounds have been found in vegetables (*1*, *12*, *14*, *17*, *24*), fruit juices (*22*, *30*), meat extract (*4*), beer (*7–9*), wine (*38*), tea (*23*), and coffee (*21*, *28*). The importance of sulfur-containing compounds to flavor lies in their extremely low odor thresholds, ranging in the order of 5 to 20 p.p.b. in water solution for compounds such as hydrogen sulfide, methyl mercaptan, and dimethyl mono- and disulfides (*7–9*, *29*, *34*).

Although gas chromatography has long since proved itself to be indispensable in flavor research, it has had limited application in the analysis of volatile sulfur compounds. The chromatographic behavior of a number of mercaptans and sulfides, including their response using a thermistor detector, has been studied by Baumann and Olund (*3*).

The first part of the work presented here is concerned with gas chromatography of a series of simple organic sulfur compounds, and preliminary studies of the response of the hydrogen flame ionization detector to these compounds. Reports on the behavior of the highly sensitive hydrogen flame ionization detector toward classes of compounds other than hydrocarbons are relatively few and have been reviewed by Ettre (*19*). In the second part, the volatile sulfur compounds from cooking potatoes were investigated by these gas chromatographic techniques. With the exception of hydrogen sulfide, which was identified chemically, identification of potato volatiles is based on two criteria: functional reaction—i.e., precipitation of mercaptans, sulfides, and disulfides with mercuric chloride—and retention time.

Part I. Gas Chromatography of Sulfur Compounds

Experimental Procedure. GAS CHROMATOGRAPHY APPARATUS. The dual-column gas chromatography apparatus, including the detectors, was constructed in the laboratory (*11*). Three different columns were used. Two were of 5-foot × 0.21-inch i.d. stainless steel—one packed with 80 to 100-mesh firebrick coated with 20% diethylene glycol succinate polyester (DEGS) and the other packed with 60 to 80-mesh firebrick coated with 30% Apiezon M. The third column was a 1000-foot × 0.034-inch i.d. nylon capillary coated with silicone SF 96(100). The DEGS column was operated at 38° C. and 28 ml. per minute nitrogen flow; the Apiezon column was operated at 115° C. and 30 ml. per minute nitrogen flow; and the capillary column was operated at 40° C. at 10 and 20 pounds per sq. inch nitrogen pressure. The nitrogen carrier gas was moistened by passing it through a glass-fiber wick which dipped into distilled water. Temperature fluctuations were minimized with "Thermistemp" temperature controllers utilizing thermistor probes. The columns were operated with hydrogen flame ionization