fatty acid content of the oil in crushed seed held at room temperature, suggests that enzymatic hydrolysis of the oils in crushed *Dimorphotheca* and *Lesquerella fendleri* seeds occurs slowly at low temperatures and rapidly at room temperaperature. To prevent hydrolysis, seeds should be processed promptly after crushing.

Extracted Meal

The amino acid compositions of *Lesquerella* and *Dimorphotheca* meal proteins have been reported (*1*, *5*). The proximate composition (Table II) permits a partial evaluation of the meals for livestock feed use; bioassay and nutritional studies will be necessary for a full evaluation of their potential.

Acknowledgment

The authors are indebted to Ann Gramps and Kenneth V. Smith for assistance in processing the large lots of seed.

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PHOSPHATE EFFECTS ON MEAT

Effect of Inorganic Polyphosphates on the Solubility and Extractability of Myosin B

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The solubility of myosin B, as well as the extractability of proteins from myofibrils in the presence of pyrophosphate, tripolyphosphate, and hexametaphosphate with or without sodium chloride, magnesium chloride, and calcium chloride, has been studied under various conditions. Data on the solubility of myosin B as a function of pH and salt concentration correspond well to those of the extractability of proteins from myofibrils. The influence of inorganic polyphosphates on the solubility of myosin B may be classified into two types of binding with the protein, viz., direct binding of highly polymerized polyphosphate ion such as hexametaphosphate and subsequent di- or triphosphate binding following well known preferential cation binding with myosin B. The importance of the formation of univalent metal-myosinate and soluble divalent metal-polyphosphate complexes is also discussed.

POLYPHOSPHATES are used in the manufacture of sausage to improve water-holding capacity and binding properties although there have been many arguments about their effects on the quality of meat and meat products.

Fukazawa *et al.* (*9, 10*) reported that the factor necessary for the binding properties of sausage was myosin A in muscle structural proteins and indicated that the effect of phosphate was due to the dissociation of actomyosin into myosin A and actin. Recently, Sherman (*26*) mentioned the importance of ion binding with muscle structural proteins for the improvement of the water-holding capacity of comminuted meat. The understanding of the interaction of myosin with ions is important in the study of muscle function. A great amount of evidence has accumulated to illustrate that the interaction of ions with muscle proteins plays an important role in the contractile process and therefore deserves special study.

Myosin, one of the principal contractile proteins, has been shown (*5, 11, 19, 21, 22, 27*) to have a great affinity for ions. The present paper reports a study of the solubility of myosin B and of the extractability of structural proteins from myofibrils as influenced by polyphosphate ions.

The results obtained in the present study enable us to classify those phosphates into two groups on the basis of their effect on the solubility of myosin B and on the extractability of structural proteins from myofibrils. One group contains polyphosphates of comparatively low molecular weight, such as pyrophosphate (PP) or tripolyphosphate (TP), which react as a salt with salt-free myosin B. Their affinity to myosin B is greatly improved in the presence of high salt concentration and divalent cations. The other group is made up of highly polymerized polyphosphates, such as hexametaphosphate (HP), in which the ratio of Na_2O to P_2O_5 is very close to 1:1. They bind directly with salt-free myosin B, but their binding is rather inhibited at high salt concentration and in the presence of divalent cation.

Materials and Methods

The reagents were commercial products of analytical grade and were used without further purification.

Myosin B was prepared from rabbit back and leg muscle by the method de-

scribed by Maruyama and Watanabe (*21*). However, the solvent used was 0.6*M* KCl in place of tetramethyl ammonium chloride. Thrice precipitated myosin B was dissolved in 0.6*M* KCl and clarified at 13,000 r.p.m. for 60 minutes by a high speed refrigerative centrifuge (Sakuma Model 150-B). The turbid and viscous upper solution was filtered through fine cloth to remove solid lipid and then stored in the ice box. All procedures were performed below 3° C. Myosin B preparations were usually used up within 10 days.

Myofibrils were prepared by the method previously reported (*6, 9*). To remove surplus (ethylenediamine)tetraacetate (EDTA) in the preparation, additional washings were made using deionized water and phosphate buffer solution. Freshly prepared myofibrils were used for the experiment within 48 hours after preparation.

Solubility Studies. The stock solution of myosin B in 0.6*M* KCl was dialyzed exhaustively with continuous stirring against 0.1*M* phosphate buffer at pH 7.0 (Na_2HPO_4/NaH_2PO_4) for 36 hours below 3° C. The outer solution was changed three times during dialysis. The myosin B suspension thus prepared was diluted to the desired protein concentration by addition of the outer solution with stirring so that a homogeneous suspension was obtained. To 4.5 ml. of solution containing known amounts of salts was added 0.5 ml. of myosin B suspension in 0.1*M* phosphate buffer, and the mixture (0.01*M* in phosphate buffer) was allowed to stand at 3° C. for 5 hours. The precipitate was then filtered off and the protein concentration in the filtrate was measured by the method of Lowry *et al.* (*20*) or by its absorption at 280 mμ. Since more than 5 m*M* $MgCl_2$ or $CaCl_2$ without phosphate caused a precipitation with Folin's phenol reagent, measurement of the ultraviolet absorption of the solution was used for determination of protein concentration under these circumstances.

In investigating the pH dependence of the solubility of myosin B, the stock solution of myosin B was dialyzed against deionized water instead of 0.1*M* phosphate buffer, and pH values were adjusted by adding adequate amounts of $^1/_{20}$*N* HCl or NaOH. Other procedures were similar to those described previously in this paper. The pH values were checked again after 5 hours of equilibration. All pH values of the polyphosphate solutions were adjusted beforehand with dilute HCl or NaOH.

Extractability Studies. The slurry of myofibrils from which surplus EDTA had been removed was suspended in the basal medium (50 m*M* phosphate buffer, Na_2HPO_4/NaH_2PO_4 at desired pH values and known concentrations of various salts). The suspension was thoroughly mixed and incubated for 30 minutes below 3° C., and it was then centrifuged at 13,000 r.p.m. for 10 minutes at 0° C. The protein concentration in the upper clear layer was determined by a biuret method (*12*). Although the conditions of the experiments were standardized as strictly as possible, the absolute values of protein concentration in the supernatant fractions differed from one experiment to another, so that the curves from different figures may be compared only in general shape and not in absolute values.

Protein Concentration. The concentration of protein was calculated by multiplying the nitrogen content as determined by a micro-Kjeldahl procedure by a factor of 6.25, or it was estimated directly by the biuret reaction (*12*), or by the method of Lowry *et al.* (*20*).

Results and Discussion

The solubility studies were performed at a constant pH of 7.0 and a temperature below 3° C. Three inorganic polyphosphate-Na salt solutions and three metal chlorides were used to test the solubility of myosin B.

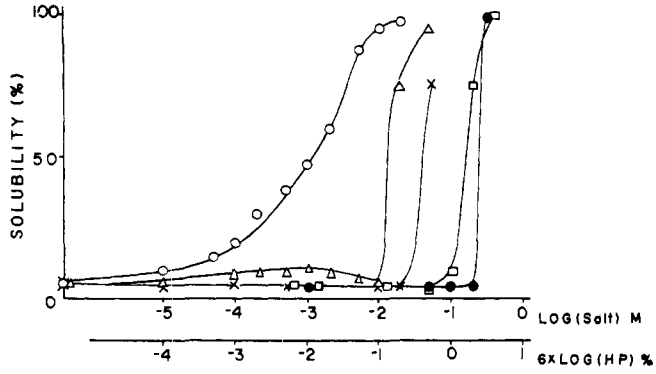

Figure 1. Effects of various ions on the solubility of myosin B

●, NaCl; □, $MgCl_2$ or $CaCl_2$; ×, PP; △, TP; ○, HP, 10mM phosphate buffer, pH 7.0; myosin B concn., 0.1 mg. per ml.

In Figure 1, the solubility of myosin B was determined in different salt solutions containing a constant cation (Na^{+1}) and different anions (Cl^{-1}, PP^{-3}, TP^{-4}, HP^{-x}), as well as a constant anion (Cl^{-1}) and different cations (Na^{+1}, Mg^{+2}, Ca^{+2}). The valency of the polyphosphate under the experimental conditions was determined by titration curves (*25*). The exact structure of HP has not yet been determined. Although it is considered to be $Na_{n+2}P_nO_{3n+1}$, in which $n = 50 \sim 150$, it is impossible to calculate its correct molecular weight. Therefore, the concentration of HP (Graham's salt) is expressed in terms of per cent by weight.

Under the experimental conditions used, the basal medium consisted of 0.01*M* phosphate buffer so that myosin B did not dissolve. On addition of NaCl to this myosin B solution, the protein was suddenly resoluble around $0.3M \sim 0.4M$ salt. As shown by the shift of the isoelectric point (I.P.) of myosin B quoted from the review of Szent-Györgyi [(*27*) Figure 4], this phenomenon can be explained by an initial cation absorption resulting in the formation of a metal-myosinate and resolubility of the protein due to subsequent anion binding. Although the range of concentration of salts in which resolubility occurs seems to depend on the particular cations and anions used, all salts tested redissolved myosin B at the range of ionic strength between 0.3 ~ 0.6 (the concentrations are converted to ionic strength by the equation $\Gamma/2 = {}^1/_2 \Sigma CZ^2$, in which C = mole concentration, Z = valency of ions at the pH value tested). This finding agrees well with the results obtained with KCl, the classic solvent. That is to say, resolubility of myosin B is a nonspecific, ionic-strength effect under the authors, experimental conditions.

Brahms (*5*) recognized that a cation which has a strong affinity for myosin A delays resolubility, and an anion with a strong affinity promotes it. However, in the present experiment, all salts (except HP) redissolve myosin B at almost the same range of ionic strength. This difference may possibly be ascribed to the presence of 10m*M* phosphate in the basal medium used here and to the modification of the physicochemical properties of the protein by the combination of actin to myosin A. Lewis and Saroff (*19*) reported that the number of sites in myosin A available for univalent cations is decreased through combination with actin, and that neither Na^{+1} nor K^{+1} combines with actin.

As demonstrated in Figure 1, HP shows a typical binding type curve. This tendency is also recognized in the case of TP, although it is very small (Figure 1 and 2), but it is not observable with PP. Inorganic polyphosphates can generally be shown as $Na_{n+2}P_nO_{3n+1}$, and the degree of polymerization, there-

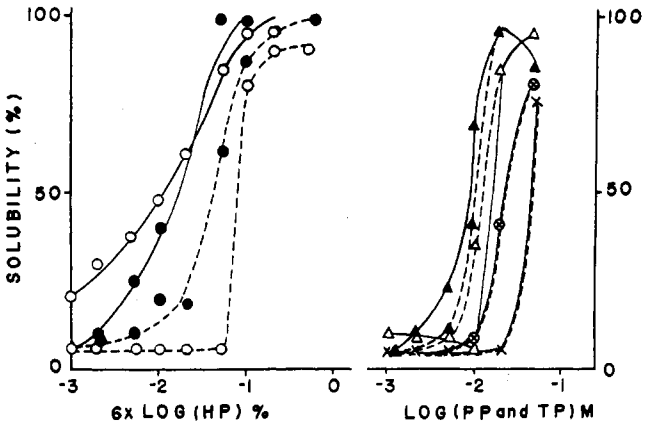

Figure 2. Effects of cations on the solubility of myosin B in the presence of various polyphosphates

○, HP; ●, HP + 0.5mM $MgCl_2$; △, TP; ▲, TP + 0.5mM $MgCl_2$; ×, PP; ⊗, PP + 0.5mM $MgCl_2$ in the absence (—) and presence (- - - -) of 0.05M NaCl. 10mM phosphate buffer, pH 7.0; myosin B concn., 0.1 mg. per ml.

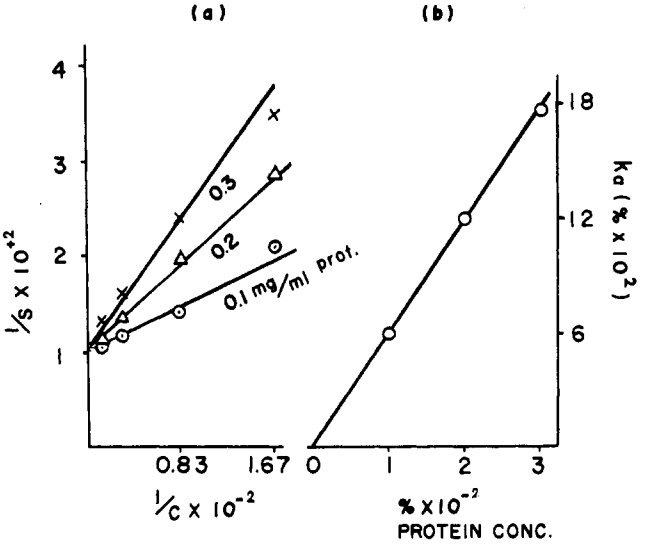

Figure 3. Dependence of the solubility of myosin B on the concentration of hexametaphosphate and on that of myosin B (*a*), and the apparent dissociation constant (K_a) versus myosin B concentration (*b*)

10mM phosphate buffer, pH 7.0

fore, can be expressed by the ratio of $Na_2O:P_2O_5$. Since this ratio is 2:1 for PP, 5:3 for TP, and 1:1 for HP, the above tendency seems to correspond to the polymerization grade of polyphosphate used. When 50m*M* NaCl, 0.5m*M* $MgCl_2$, or both are added in the presence of these phosphates, each solubility curve is greatly altered (Figure 2). Whereas the solubility curve of HP is changed from a binding type to an ionic-strength type by the addition of 50m*M* NaCl, those of TP and PP are scarcely changed. In the presence of 50m*M* $MgCl_2$, however, the solubility curves of PP and TP shift toward lower concentrations, while that of HP shows little alteration. Moreover, on addition of 50m*M* NaCl together with 0.5m*M* $MgCl_2$, the tendency observed with 50m*M* NaCl alone is clearly seen.

Therefore, it is conceivable that there are two kinds of effect of the polyphosphate on the solubility of myosin B. One is a general salt interaction; namely, after formation of the metal-myosinate the phosphate moiety combines and begins to show a solubility of the ionic strength type. The binding of the polyphosphate moiety with myosin B in this case is too strong to be easily released by Cl^{-1}. The other is the direct binding with myosin B shown in the case of polymetaphosphate. This type of binding could probably occur with positively charged groups of myosin B (*8*). Since the binding of K^{+1} or Na^{+1} to myosin appears (*12*) to be controlled by the imidazol and amino groups through the competition mechanism which involves hydrogen bonding between ammonium (or imidazolium) ions and the carboxylate ion, the binding of the polymetaphosphate with the protein should naturally compete with Na^{+1} for the sites. There is little doubt about the effect of the binding of the first type, because early investigations on ATP binding with myosin by Szent-Györgyi's school (*27*) and recent work (*1, 5, 11, 19, 28*) in the field of muscle biochemistry suggest the strong possibility of this kind of binding. Furthermore, more support is given to it from the fact (Figures 1 and 2) that the affinity of polyphosphates for myosin B is increased by the addition of divalent metals. As for the effect of the second type, metaphosphates bind with positively charged groups of the protein (*8*), and the shift of the solubility curve in the presence of 50m*M* NaCl seems to indicate the competition of univalent cation with phosphate for these sites.

If it is assumed that the increase in the solubility by HP reflects the number of sites occupied by HP and that it follows mass action law (*17*), the relationship between the extent of binding (ν_{HP}) and the free concentration of HP(C_{HP}) is given in the following formula, $\nu_{HP} = K_a C_{HP} n(1 + K_a C_{HP})$, where K_a and n represent the apparent intrinsic association constant and average maximum number of binding sites, respectively. The plot of the reciprocal of solubility ($1/s$) versus the reciprocal of concentration ($1/C$) gives a straight line (Figure 3). This indicates a direct binding of HP to myo-

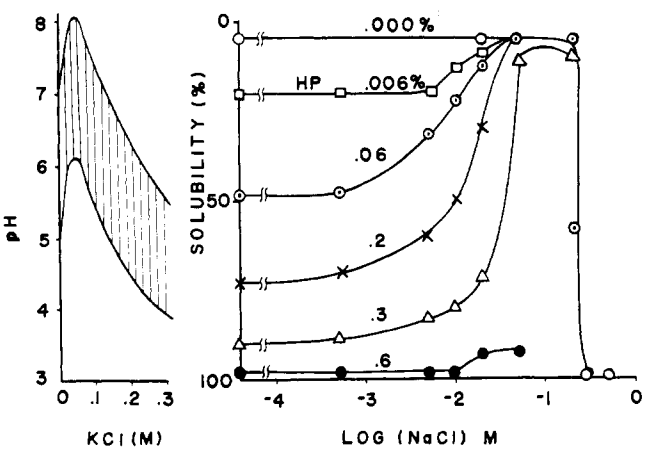

Figure 4. Changes of solubility of myosin B by NaCl with hexametaphosphate (*right*) and the "zone of isoelectric precipitation" copied from ref. 27 (*left*)

10mM phosphate buffer, pH 7.0

sin B in which the electrostatic interaction term is negligible (*17*). In the present experiments, the myosin concentration will influence the curves of solubility, for HP concentration is given as total HP instead of free HP [Figure 3*a*, see also (*22*)]. A proportionality of K_a to the increase in the protein concentration can be seen either in Figure 3*a*, or more directly in Figure 3*b*, where the value of K_a obtained at half maximal points of the solubility curves are plotted against protein concentrations. A molar concentration of HP cannot be shown; the value of n is also unknown. The only value which is derived from Figure 3 is a weight ratio of myosin B to HP—1:6. Although the value indicates that HP exists as a considerably large molecule in solution (mol. wt. $> \sim 5000$), at the same time, the results shown in Figure 3 prove the binding between myosin B and HP qualitatively.

In Figure 4, the inhibitory action of NaCl to such binding of HP is shown. In the range from 50mM to 0.2M NaCl, HP does not induce the resolubility of myosin B up to 0.3%, and then it suddenly resolubilizes the protein between 0.3 and 0.6%. This is the explanation for the change of solubility curves in Figure 2, and under such a condition HP functions only as a polyphosphate of type I. Also the range of ionic strength over $0.05 \sim 0.2$ has been found to be important for physiological functions of muscle and determines the colloidal state of myosin (*1*, *27–29*, *33*). Myosin A is saturated by the presence of 0.05M KCl (*22*), and an association constant of Na^{+1} with the protein is greater than that of K^{+1} (*19*).

In Figure 4, the inhibitory effect of Na^{+1} (probably K^{+1} may also show the same result) on HP binding with myosin B has been clarified, but, in the case of divalent cations such as Ca^{+2} and Mg^{+2}, the situation becomes quite different from the case with univalent cation (Figure 5). The important point in this case is metal-phosphate complex formation. Divalent cations and HP bind stoichiometrically and form a complex. If more metal than phosphates exists, myosin B is precipitated. The solubility change on that occasion can be interpreted as a binding of the metal-HP complex with myosin B. Since an inverse relationship exists (*16*) between the dissociation of the metal-polyphosphate and complex-formation ability, and Ca^{+2} is generally bound more tightly than Mg^{+2} by phosphate, the results in Figure 5 may be regarded as the effect of Ca-HP and Mg-HP on the solubility of myosin B. The two types of effects of phosphates reasoned from the results in Figure 2 are in good agreement with the experimental facts obtained so far, and moreover, the results in Figure 5 show the importance of metal-polyphosphate complexes. Divalent metals such as Mg^{+2} and Ca^{+2} remarkably increase the affinity of PP and ATP for myosin (*27–30*).

If it is assumed that HP exhibits only an ionic strength effect (Figures 2 and 4), then the ionic strength of HP is calculated to be about 50. If so, HP becomes 10 $(NaPO_3)H_2O$, and it contradicts the result, suggesting a large molecular weight calculated from the ion binding studies. Although HP is a large molecule, as an electrolyte it might behave like $\sim 6(NaPO_3)H_2O$, as its name "hexametaphosphate" implies.

Hamm and Grau (*14*) claimed that the effect of polyphosphates on the water-holding capacity of meat is accounted for by their ability to remove the metal ions from saltbridges in muscle structural protein molecules. Currently, this

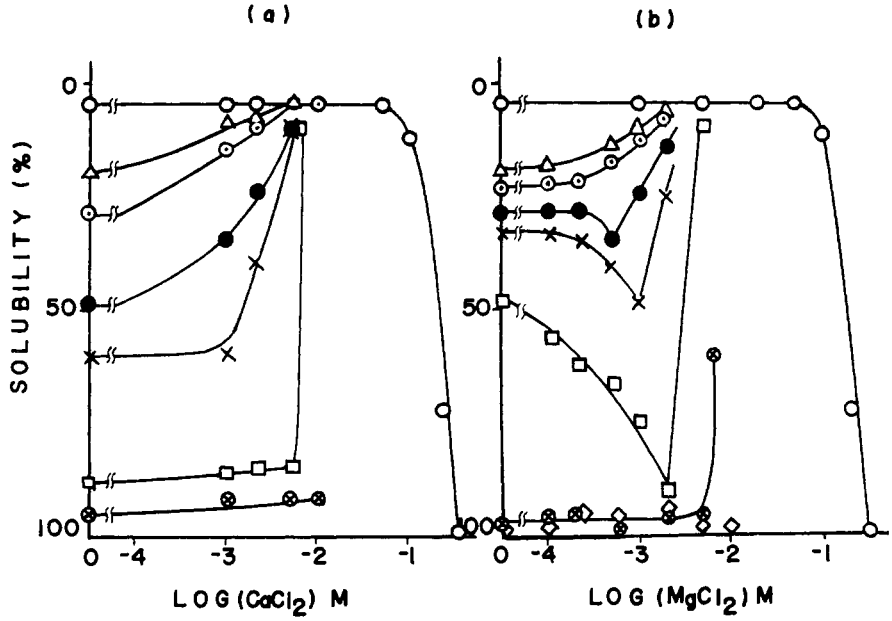

Figure 5. Changes of solubility of myosin B by $CaCl_2$ (*a*) and $MgCl_2$ (*b*) with hexametaphosphate

10mM phosphate buffer, pH 7.0
(*a*) $CaCl_2$: ○, 0% HP; △, 0.012% HP; ⊙, 0.03% HP; ●, 0.06% HP; ×, 0.12% HP; □, 0.3% HP; ⊘, 0.6% HP; myosin B concn., 0.1 mg. per ml
(*b*) $MgCl_2$: ○, 0% HP; △, 0.006% HP; ⊙, 0.012% HP; ●, 0.03% HP; ×, 0.06% HP; □, 0.12% HP; ⊘, 0.3% HP; ◇, 0.6% HP; myosin B concn., 0.14 mg. per ml.

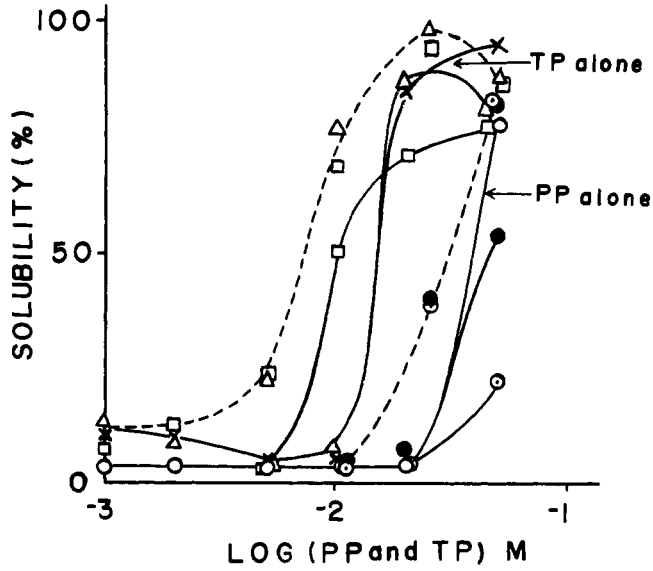

Figure 6. Changes of solubility of myosin B by pyrophosphate and tripolyphosphate with $CaCl_2$ and $MgCl_2$

10mM phosphate buffer, pH 7.0; ○, PP alone; ●, PP + $MgCl_2$; ⊙, PP + $CaCl_2$; ×, TP alone; □, TP + $MgCl_2$; △, TP + $CaCl_2$; ——, 5mM divalent cations; - - -, 0.5mM divalent cations; myosin B concn., 0.14 mg. per ml.

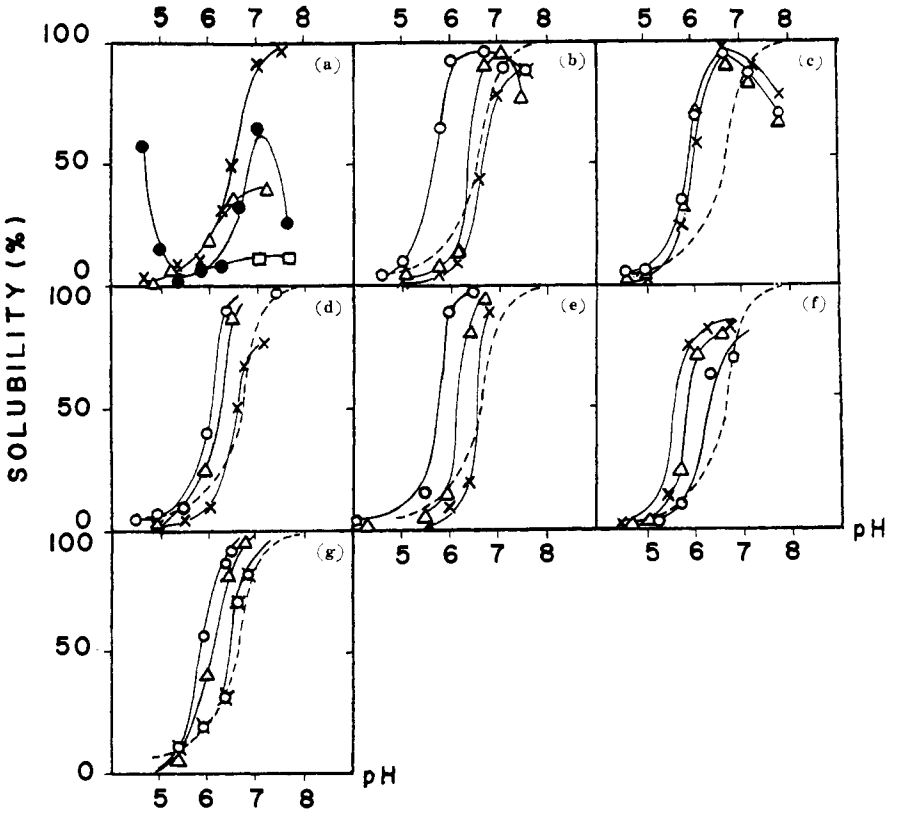

Figure 7. Effect of pH on the solubility of salt-free myosin B under various conditions

(a) ●, salt-free myosin B; ⊏, 0.01M NaCl; △, 0.3M NaCl; ×, 0.4M NaCl
(b) ○, 0.6% HP; △, 0.02M TP; ×, 0.05M PP; dotted line stands for a control (0.4M NaCl)
(c) ○, 0.4M NaCl + 0.6% HP; △, 0.4M NaCl + 0.02M TP; ×, 0.4M NaCl + 0.05M PP; dotted line stands for control (0.4M NaCl)
(d) ○, 0 5mM $MgCl_2$ + 0.6% HP; △, 0.5mM $MgCl_2$ + 0.02M TP; ×, 0.5mM $MgCl_2$ + 0.05M PP; dotted line is control (0.4M NaCl)
(e) ○, 0.5mM $CaCl_2$ + 0.6% HP; △, 0.5mM $CaCl_2$ + 0.02M TP; ×, 0.5mM $CaCl_2$ + 0.05M PP; dotted line is control (0.4M NaCl)
(f) ○, 0.4M NaCl + 0.5mM $MgCl_2$ + 0.6% HP; △, 0.4M NaCl + 0.5mM $MgCl_2$ + 0.02M TP; ×, 0.4M NaCl + 0.5mM $MgCl_2$ + 0.05M PP; dotted line, 0.4M NaCl alone
(g) ○, 0.4M NaCl + 0.5mM $CaCl_2$ + 0.6% HP; △, 0.4M NaCl + 0.5mM $CaCl_2$ + 0.02M TP; ×, 0.4M NaCl + 0.5mM $CaCl_2$ + 0.05M PP; dotted line, 0.4M NaCl alone

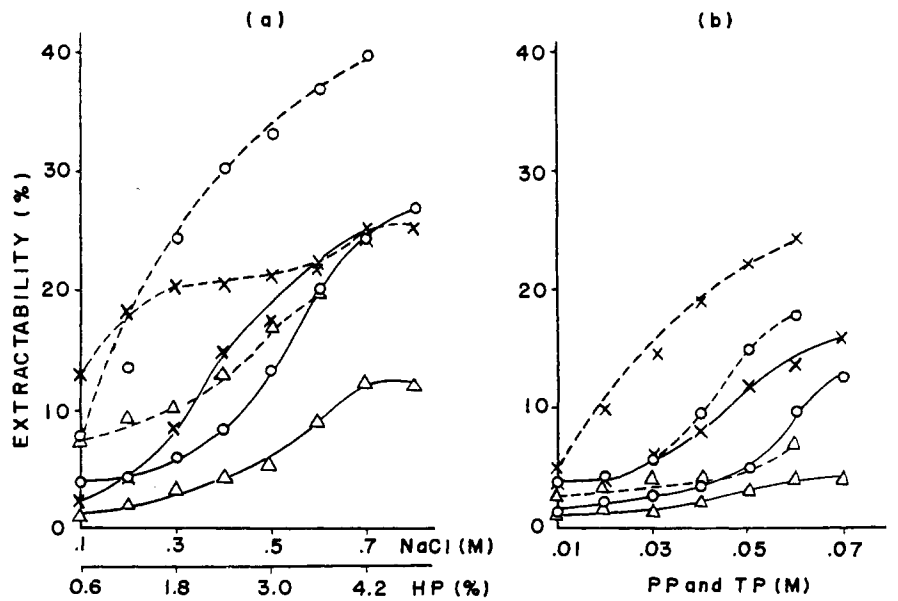

Figure 8. Extractability of protein from myofibrils by NaCl and inorganic polyphosphates in 50mM phosphate buffer

△, pH 5.5; ○, pH 6.4; ×, pH 7.5; solid lines stand for NaCl in (a) and PP in (b), and dotted lines for HP in (a) and TP in (b)

theory seems to be unfavorably viewed by many meat scientists, because strong chelators such as EDTA and oxalate, which show higher values of stability constants than those of polyphosphate (*1*, *16*), do not exhibit any favorable effect on the water-holding property of meat (*15*, *26*). As shown in Figure 7, this protein is soluble on both sides of its I.P. Thus the graphs indicate that HP evidently reverses the solubility of myosin B to the original level or far beyond it through its own binding or the binding of metal-HP complex. Although in such conditions the claim of Hamm and Grau (*13*, *14*) does not apply to polyphosphates which have only the function of type I, it may be true of a highly polymerized polyphosphate such as HP, provided that the function of the metal-polymetaphosphate complex is taken into account.

Figure 6 illustrates the influence of Mg^{+2} and Ca^{+2} on the solubility curves of myosin B in the presence of PP and TP. The importance of the metal-polyphosphate is emphasized again here. The binding between polyphosphates and metals is generally called sequestering (*16*), and the following two reactions are considered to occur.

$$\text{Me} + \text{polyphosphate} \rightleftharpoons \text{Me-polyphosphate (ppt.)} \quad (1)$$

$$\text{Me-polyphosphate (ppt.)} + \text{polyphosphate} \rightleftharpoons \text{Me-polyphosphate complex (soluble)} \quad (2)$$

Since the order of relative sequestering ability is HP > TP > PP for Ca^{+2}, and PP > TP > HP for Mg^{+2} (*16*), the effect of polyphosphates on the solubility of myosin B is likely to depend greatly on the formation of metal-polyphosphate complexes. The decrease in solubility of myosin B at a high concentration of divalent metals may be attributable to a shift of the second equilibrium to the left. The differences noted with different anions can also be correlated to the products of the above equilibria.

Effect of pH. Figure 7 summarizes data on the solubility of metal-free myosin B. In the absence of other salts, the pH dependency of the effects of polyphosphates on the solubility of myosin B (Figure 7*a*) exactly follows the titration curves (*25*) of these phosphates. Clearly what is necessary for the effects of these phosphates is HP^{-2}, TP^{-4}, and PP^{-3}. In comparison with the effect of NaCl, which is used as the control (Figure 7), the effects of PP and TP are ionic strength effects, and only HP shows an exceptionally strong affinity. If the influence of each phosphate on the solubility in the presence of Na^{+1}, Mg^{+2}, and Ca^{+2} is plotted against varying pH values, changes in the solubility become apparent, as illustrated in Figure 7. *c*, *d*, and *e*. In the presence of enough Na^{+1} (0.4*M*), the affinity of TP

and PP is increased (Figure 7*c*), and that of HP is conversely decreased. As a result, differences in affinity of the three phosphates disappear. A similar tendency is observed in the presence of 0.5m*M* Mg^{+2} or Ca^{+2}, although it is smaller in comparison with 0.4*M* Na^{+1}.

With Mg^{+2}, the affinity of PP and TP for myosin B increases, while that of HP shows almost no change. With Ca^{+2}, whereas the affinity of TP increases, the affinity of PP is improved only slightly, and HP is not influenced at all. Under such conditions, Ca^{+2} or Mg^{+2} might form only a slightly dissociable complex with HP and not react. On the other hand, it is supposed that the complex of Mg-TP and Ca-TP similarly increase the affinity to myosin B, and PP produces complexes having different affinities for myosin B (Mg-PP > Ca-PP) (*16*, *28*). Examination of the influence of polyphosphates in the presence of 0.4*M* NaCl in addition to Mg^{+2} or Ca^{+2} (0.5m*M*) (Figure 7, *f* and *g*) shows that the most drastic effects on the solubility of myosin B are observed when the affinity for PP is remarkably increased, while that of HP is decreased. In a Ca^{+2}–NaCl–polyphosphate system, the effect of the phosphates, except TP, tends to be less promoted than in a NaCl–polyphosphate system.

The results of the studies of pH dependence on the solubility of myosin B not only do not contradict the results of solubility tests performed at pH 7.0, but also reaffirm the ideas discussed here—that is, the affinity of PP and TP to myosin B is greatly improved by the formation of Na-myosinate, and that of HP is conversely depressed. In the presence of Mg^{+2} or Ca^{+2}, the influence of each phosphate on the solubility of myosin B depends upon the affinity of the divalent metal-polyphosphate complex formed with myosin B.

Extractability. All of the above experiments have been undertaken using myosin B, one of the main constituents in muscle structural proteins. To approach more closely the actual situation with meat, protein extractability with various polyphosphates was studied with metal-free myofibrils as samples. The results are illustrated in Figures 8 and 9. Since 50m*M* phosphate buffer has been used as the basal medium in these experiments, possibly the formation of Na-myosinate is sufficient to make the effects of all polyphosphates used a non-specific ionic strength type.

The results of the extractability tests (Figure 9), conducted with various salts at three different pH values as well as at pH 6.4 in the presence of NaCl (0.4*M*), $MgCl_2$ (5m*M*), and $CaCl_2$ (5m*M*), were similar to those results obtained with the solubility studies previously described in this report. The non-specific ionic strength effect on solubility is considered to be due to secondary

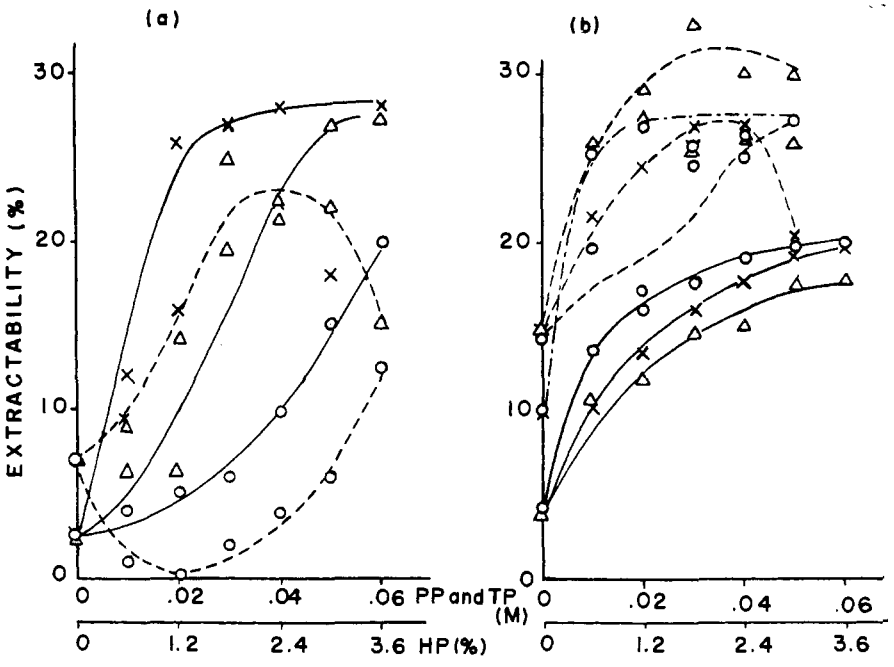

Figure 9. Effect of divalent metals on the extractability of proteins from myofibrils by inorganic polyphosphates in 50mM phosphate buffer, pH 6.4

(a) Without 0.4M NaCl; O, PP; △, TP; ×, HP; ——, 5mM $MgCl_2$; - - -, 5mM CaC'_2
(b) With 0.4M NaCl; O, PP; △, TP; ×, HP; ——, no divalent metal; —·—, 5mM $MgCl_2$; - - -, 5mM $CaCl_2$

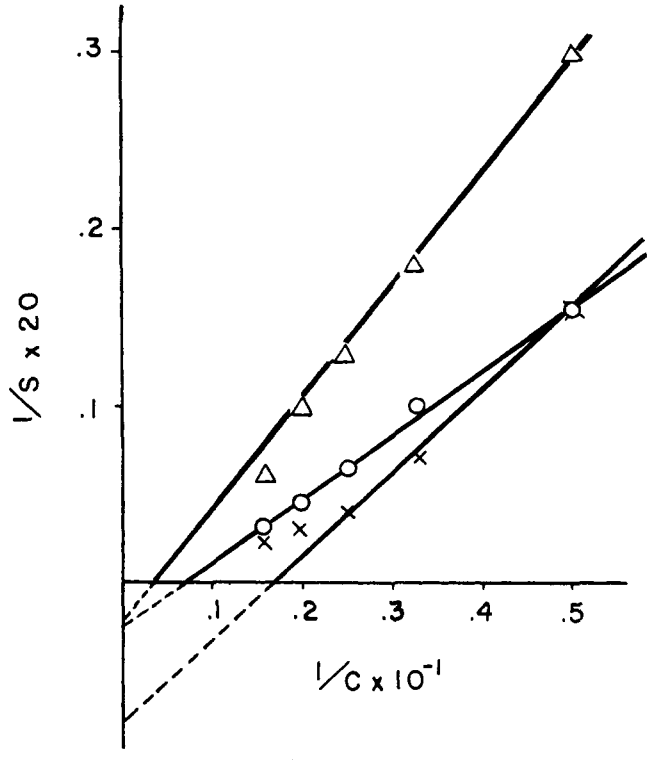

Figure 10. Figure 8 (a) curves of NaCl in reciprocal ordinates

Points are arbitrary points of the curves of NaCl in Figure 8 (a); △, pH 5.5; O, pH 6.4; ×, pH 7.5
C = NaCl concentration (M),

$$S = \frac{\text{extractability (\%)}}{\text{maximum extractability (\%)}} \times 100$$

anion binding following the primary preferential cation binding. Na-polymetaphosphate and NaCl, acting as a normal electrolyte, should show this type of effect under these experimental conditions, but the similarity of extractability curves by these two salts falls short in this expectation (Figure 8*a*).

In the case of the weak electrolytes, TP and PP, the direction of the shift of the following equilibria—$TP^{-4} \rightleftharpoons TP^{-3}$, $PP^{-3} \rightleftharpoons PP^{-2}$—may play an additional role, because the ionic strength of salts depends greatly on the valency of the ions dissociated in solution. One might question the crossing of the extractability curves performed at pH 6.4 and 7.5 using NaCl and HP. Now, if it is assumed the secondary anion binding with the protein electrostatically overcomes the primary preferential Na^{+1} binding, the theory of mass action may give a clue to what is going on with the protein molecules.

In Figure 10, the normal extractability curves of NaCl (in Figure 8*a*) are transformed in a plot of 1/relative extractability versus 1/NaCl concentration.

The slope and intercept show that at pH 7.5, in spite of the increase in the maximum number of ions bound (the intercept is the lowest), the apparent association constant becomes smaller (the slope becomes steeper) than the one at pH 6.4. According to Lewis and Saroff (*19*), who studied the binding of Na^{-1} and K^{+1} with myosin A and B, the apparent discrepancy observed between the association constant and the maximum number of bound ions around pH 7.4 is due to the competition of H^{+1} with Na^{+1} for the sites. The results of Figure 10 may be interpreted in this way, thus indicating a dominant role of the primary Na^{+1} binding in the first stage of the reaction in the case of the nonspecific ionic strength type of effect.

The effects of univalent cations, divalent cations, and a mixture of both on the extractability of proteins in the presence of inorganic polyphosphates (Figure 9) may be reasonably interpreted from the results on the solubility studies. That is, the formation of Na-myosinate strengthens the affinity of the protein with PP and TP, but weakens that of HP, and the effect of divalent cations seems to depend on the nature of each metal-phosphate complex formed The decrease in extractability at higher concentrations of phosphate in the presence of divalent cations might be accounted for by the competition for the binding sites of free phosphate with the metal-phosphate complexes as has been frequently observed with pyrophosphatase (*4*).

On the basis of the results obtained in this study, two underlying processes for the effect of polyphosphates on the solubility changes of myosin B, which is believed to play a substantial role in the water-holding capacity or binding properties of sausage, should be mentioned: the formation of univalent metal myosinate which improves the affinity of myosin B for polyphosphates; and the formation of divalent metal-polyphosphate complexes which modify the reactivity of polyphosphates to myosin B.

For a description of the first point, the early works of Szent-Györgyi's school (*27*), as well as many more recent works in the field of muscle biochemistry (*24, 28, 31*), may be consulted. Despite important experimental results by Sherman (*26*) and Helendoorn (*15*), the first point has been overlooked in the field of meat science. Ammonium ion (*24*) and tetramethylammonium ion (*21*) have been found to substitute for potassium and sodium ions to induce the reaction in an actomyosin-ATP system at low ionic strength.

As to the second point, it is not difficult to find references in the literature (*23, 28–30*) in which the authors put much emphasis on the importance of metal-phosphate complex in the (acto)myosin-ATP system. For example, the essential substrate for muscle contraction has been regarded as a metal-ATP complex, and the important roles played by Mg-ATP or Mg-PP have been pointed out in studies on the changes in size and shape of myosin A and B at high ionic strength (0.6*M* KCl) (*2, 28–30*). What is more, the TPase activity of myosin A and B can be demonstrated if Mg^{+2} or Ca^{+2} are present together with a sufficient amount of univalent metal ion (*1, 7, 32, 33*). Along with the concepts described so far, it may now be possible to classify the effects of inorganic polyphosphates on the solubility of myosin B into two types. Type I consists of inorganic polyphosphates of relatively low molecular weight, such as PP and TP, with which Bendall (*3*), Fukazawa *et al.* (*10*), and Kotter (*18*) observed similar effects. The feature of these phosphates is a marked increase in their affinity for myosin B in the presence of univalent cations, and a remarkable improvement of their reactivity with the protein through the formation of divalent metal-phosphate complexes, though they act as a normal salt, such as NaCl, with salt-free myosin B. In addition, these phosphates behave similarly to organic polyphosphates such as ATP or inosine triphosphate (ITP) in a reaction system where both univalent and divalent cations are present. A more detailed discussion of this appears elsewhere (*32*).

Type II are highly polymerized inorganic phosphates represented by HP in this paper, and their effect becomes stronger as the ratio $Na_2O:P_2O_5$ gets closer to 1:1. Where little or no salt exists, this type of phosphate solubilizes myosin B by a direct binding, presumably with positively charged groups on the myosin B molecule. This effect, however, is diminished in the presence of a sufficient amount of univalent cation, and the phosphate acts like a normal salt by merely increasing ionic strength in the reaction mixture. Although the phosphate also acts as a metal-complex in the presence of divalent cation, the affinity of the complex for myosin B decreases according to its dissociability. The poorest affinity is observed in the presence of a sufficient amount of NaCl together with a divalent cation such as Mg^{+2}. As opposed to the Type I phosphates, no evidence is available as yet showing ATP-like function of Type I phosphates. The ion exchange theory developed by Hamm (*13, 14*) may be true only of this type of inorganic polyphosphate under the particular conditions described previously in this report.

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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 Na_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