Phosphate Complexes of Soluble Fish Proteins

Their Formation and Possible Uses

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Sarcoplasmic fish proteins in dilute aqueous concentrations were quantitatively precipitated from solution as condensed phosphate complexes. The ratio of phosphorus to nitrogen in the complexes was dependent on the concentration of the condensed phosphate present in the solution, the type of phosphate, and the pH of the solution. Available lysine and feeding data indicate that the nutritional characteristics of the complexed proteins are not impaired. The work suggests that the reaction product between condensed phosphates and soluble proteins could be used to recover waste proteins from industrial effluents.

Under acidic conditions, certain condensed phosphates react with soluble proteins to form insoluble protein phosphate complexes (PPC) that possess unusual properties. Perlmann and Herrmann (1938) have shown, for example, that complexes formed, even at a pH below that of the isoelectric point of the protein, will yield regenerated proteins having unaltered chemical properties. More recent investigations have shown that by careful control of pH and phosphate concentrations, discrete protein complexes can be formed from a protein mixture. Nitschmann *et al.* (1959) have advantageously used this property to fractionate protein mixtures.

In the laboratory, investigations of PPC have been concerned mainly with the chemistry of their formation and the utility of this reaction as an aid in fractionating complex protein systems. In the food industry, these reactions have quite naturally been oriented toward the modification of food. Proteinaceous foods treated with condensed phosphates, such as pyrophosphates, polyphosphates, and hexametaphosphates, display increased water-holding capacity and other interesting properties. In 1940, for example, Grettie showed that condensed phosphates improved the whipping quality of gelatin.

Although it is known that the food-modifying properties of condensed phosphates are due to their interreaction with the protein fraction of the food, *e.g.*, the dissociation of actomysin by tripolyphosphate and pyrophosphate or the formation of protein complexes with sodium hexametaphosphate, the utility of using PPC directly as food adducts has received little attention. One disclosure in this area was made by McKee and Tucker (1966), who found that the metaphosphate complex of lactalbumin was useful as a substitute for dried milk solids in cake and cookie formulations.

The low solubility characteristics of PPC also suggest another area of investigation that has received little attention, *i.e.*, the utility of using condensed phosphates to remove and recover soluble proteins from industrial effluents.

The objectives of the work here were to:

1. Determine the efficacy of different condensed phosphates in precipitating protein from dilute aqueous solutions.
2. Evaluate some of the nutritional characteristics of the complex of sodium hexametaphosphate and the sarcoplasmic proteins from fish tissue.
3. Obtain data on the utility of using condensed phosphates for the recovery of proteins from industrial effluents.

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EXPERIMENTAL AND RESULTS

Materials and Methods. SARCOPLASMIC FISH PROTEINS. Sarcoplasmic proteins were obtained by mincing the flesh of rockfish (*Sebastodes melanops*) and extracting the proteins with cold 0.1*M* NaCl solution. The sarcoplasmic fraction was separated from the myofibrillar proteins and connective tissue by centrifugation at 2000 r.p.m. (16-inch diameter head) for 10 minutes at 5° C.

AVAILABLE LYSINE. K. J. Carpenter (1960). Calculation assumes epsilon DNP lysine equals 40% lysine.

PROTEIN EFFICIENCY RATIO. Official Methods of Analysis, AOAC (1960a).

NITROGEN. Official Methods of Analysis, AOAC (1960b); Lowery *et al.*, 1951.

PHOSPHORUS. About 100 mg. of sample were digested in 5 ml. of concentrated HNO_3 and 0.5 ml. of concentrated HCl for one-half hour. The digests were brought to 25 ml. with distilled water. Phosphorus was then determined by the method of Fiske and SubbaRow (Hawk *et al.*, 1949).

CONDENSED PHOSPHATES. Metaphosphoric acid, reagent grade. Sodium trimetaphosphate obtained from Monsanto Chemical Co. Sodium hexametaphosphate (HP) obtained from the Calgon Co. A molecular weight of 610 was assumed as a basis for calculating the molar concentrations of this compound. Sodium tetrametaphosphate prepared by the method of Bell *et al.* (1952).

Precipitation of Sarcoplasmic Fish Proteins with Condensed Phosphates. METHOD. Sarcoplasmic protein solutions were prepared as described and adjusted to a protein concentration of 1% with distilled water. Sufficient phosphate in aqueous solution was added to the protein solutions to yield specific molar concentrations of the phosphate. With constant stirring, pH adjustments were made by slowly adding 0.1*N* H_2SO_4 to the protein-phosphate solution. At specific pH values, the complex that was formed was collected after about 10 minutes by centrifuging (2000 r.p.m.) from solution. Proteins not precipitated by phosphate were measured by the method of Lowery *et al.* (1951). To remove occluded protein, residual lipids and unreacted phosphates, the precipitates were resuspended and centrifuged twice in distilled water and once in anhydrous isopropanol. They were then dried in a vacuum at 80° C.

EFFICACY OF PROTEIN REMOVAL. Four different phosphates—metaphosphoric acid, cyclic sodium trimetaphosphate, cyclic sodium tetrametaphosphate, and sodium hexametaphosphate—were reacted with the sarcoplasmic fish proteins to determine the amount of proteins that could be precipitated from solution as the concentration of phosphate

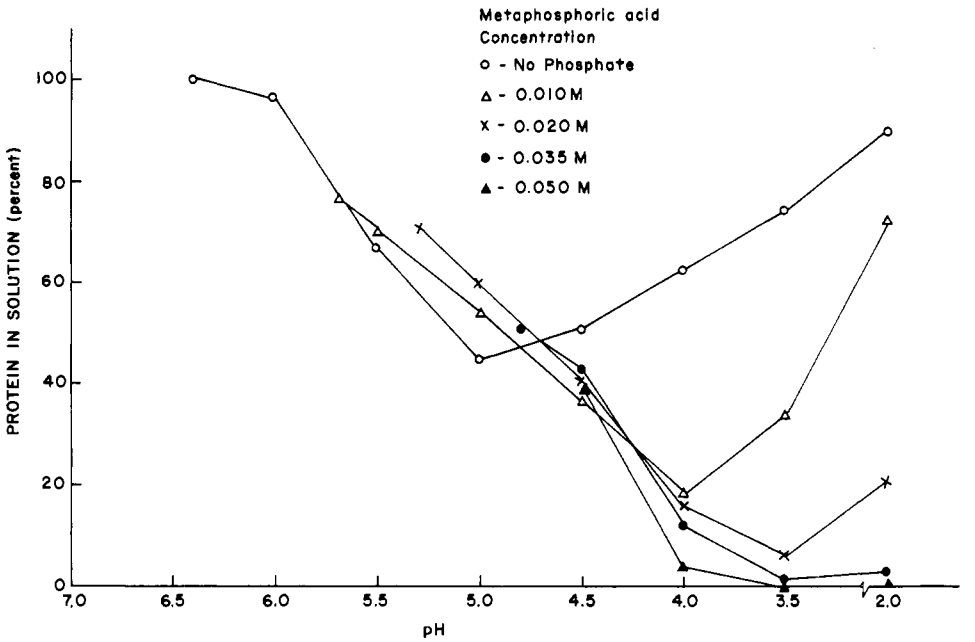

Figure 1. Amount of sarcoplasmic fish protein complexed from a 1% solution at various pH's and concentrations of metaphosphoric acid

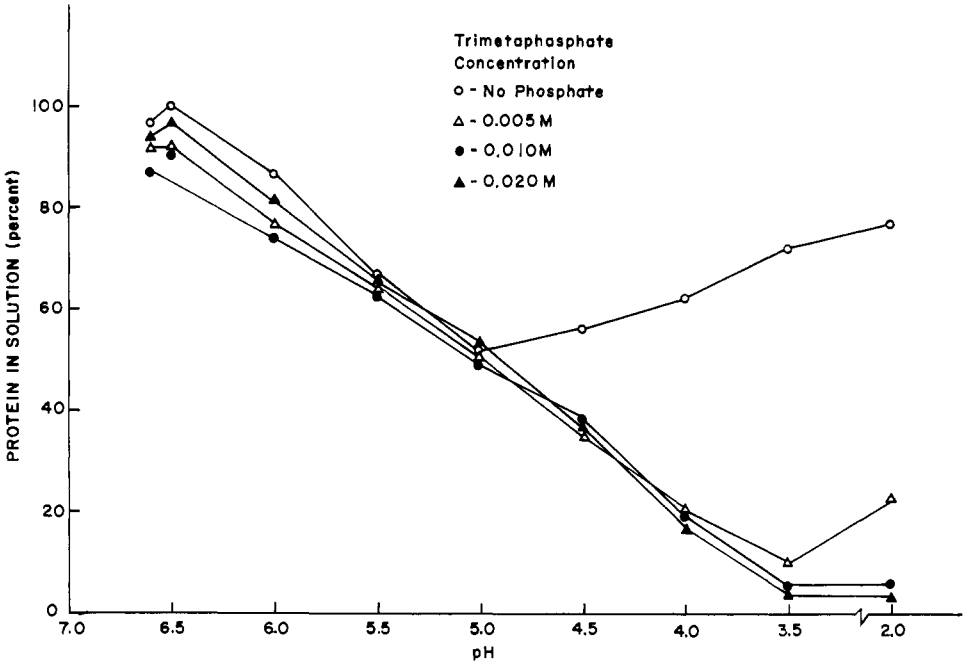

Figure 2. Amount of sarcoplasmic fish protein complexed from a 1% solution at various pH's and concentrations of sodium trimetaphosphate

and the pH of the solution were varied. The results of these experiments are shown in Figures 1, 2, 3, and 4.

Data from these experiments (Figures 1–4) show that flocculation of the protein complex is related to the molecular size of the phosphate moiety. In the presence of sodium hexametaphosphate (HP), quantitative precipitation of the proteins occurred at an HP concentration of 0.001*M* at pH 4.0 (Figure 4). When comparable recoveries of protein were obtained with other phosphates, the phosphates had to be used in higher concentrations and a pH of 3.5 or lower was necessary. Examination of Figure 1 shows that with metaphosphoric acid at pH 4.0 and at a molar concentration of 0.01, about 80% of the proteins were precipitated from solution. As the pH was lowered, however, proteins were resolubilized at a rate similar to that of the control containing no phosphate. The proteins passing into solution were not believed to be complexed. This was determined by washing the protein precipitates (collected and washed at pH 4.0). A portion of these proteins passed into solution as the pH was lowered to 2.0, but there was no corresponding liberation of phosphate. Similar but less obvious resolubilization reactions were observed with trimetaphosphate but not with tetrametaphosphate.

Composition of the HP Complexes. The composition of protein-HP complexes was determined at all phosphate concentrations ranging from 0.005 to 0.05*M* and at pH values

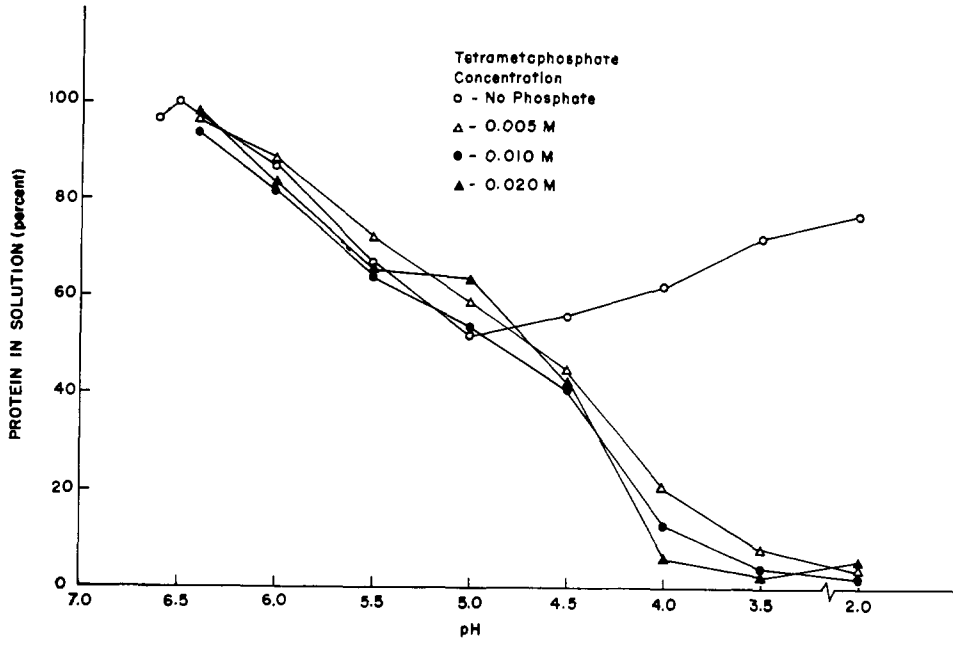

Figure 3. Amount of sarcoplasmic fish protein complexed from a 1% solution at various pH's and concentrations of sodium tetrametaphosphate

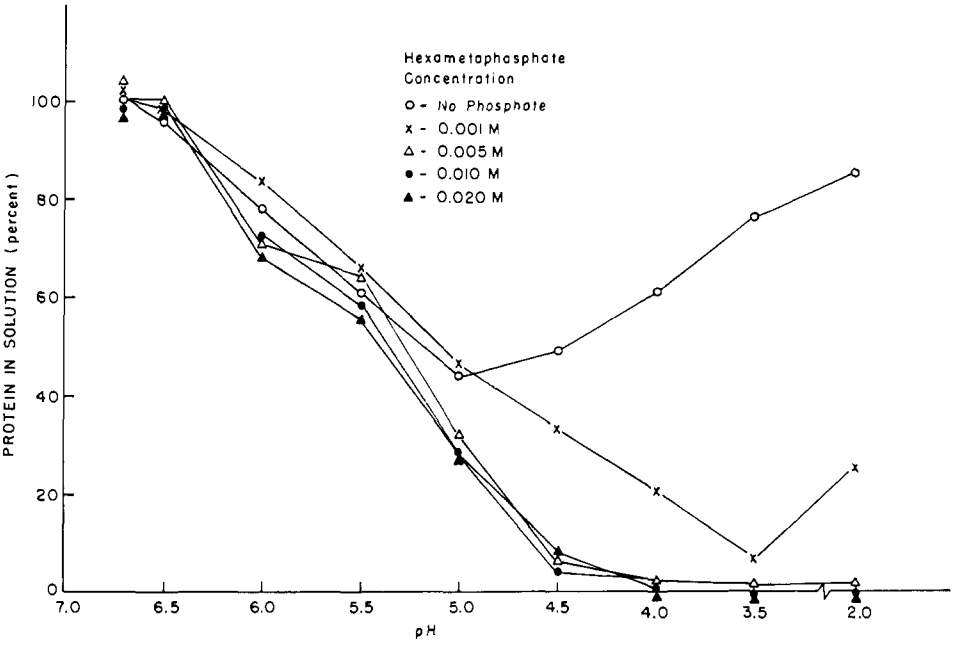

Figure 4. Amount of sarcoplasmic fish protein complexed from a 1% solution at various pH's and concentrations of sodium hexametaphosphate

from 5.5 to 2.0. The amount of nitrogen and phosphorus in the complexes and the phosphorus-to-nitrogen (P/N) ratios are shown in Table I. At HP molar concentrations of 0.005, 0.01, and 0.02, P/N ratios were reasonably constant at any pH.

The increase in the phosphorus content of the complexes as the pH was lowered to 4.0 is probably due to protein fractionation (high molecular weight proteins precipitating at the higher pH ranges) and an increase in phosphate binding as the pH is lowered. Figure 4 indicates that at pH 4.0 complete precipitation of the proteins had occurred; yet the phosphate content in the precipitated complexes continued to increase as the pH was lowered (Table I).

When the concentration of the phosphate was increased from 0.02*M* to 0.05*M*, a significant increase in the P/N ratio was evident at all of the pH levels examined. These findings suggest that, under these conditions, the P/N ratios of the complexes had reached a maximum value at HP concentrations of 0.005*M*. The increase in the phosphorus content of the complexes at HP concentrations exceeding 0.02*M* was probably due to a mass action effect (Briggs, 1940) that results in further binding of the phosphate ion within the complex.

Composition of Tri- and Tetrametaphosphate Protein Precipitates. A complete study of the composition of the protein complexes obtained by precipitation from sodium

Table I. Composition of Complexes of Sarcoplasmic Fish Proteins Prepared from a 1% Solution of Proteins at Various pH's and in the Presence of Sodium Hexametaphosphate Concentrations of 0.005, 0.01, 0.02, and 0.05*M*

Phosphate Molarity	pH	Nitrogen, %	Phosphorus, %	Ratio P/N
0.005	5.5	15.1	2.2	0.146
	5.0	14.9	2.6	0.174
	4.5	14.9	3.0	0.201
	4.0	14.5	3.6	0.225
	3.5	14.1	4.0	0.283
	3.0	13.8	4.4	0.318
	2.0	13.4	4.7	0.350
0.01	5.5	14.8	2.7	0.182
	5.0	14.8	2.6	0.176
	4.5	14.4	3.1	0.225
	4.0	14.0	3.8	0.270
	3.5	13.7	4.3	0.315
	3.0	13.7	4.5	0.337
	2.0	13.7	4.6	0.336
0.02	5.5	14.8	2.5	0.169
	5.0	14.6	2.6	0.178
	4.5	14.3	3.2	0.223
	4.0	14.0	3.9	0.278
	3.5	13.8	4.4	0.320
	3.0	13.6	4.7	0.346
	2.0	13.7	4.8	0.350
0.05	5.5	14.1	2.8	0.198
	5.0	14.1	3.3	0.233
	4.5	13.9	4.2	0.310
	4.0	12.7	5.0	0.393
	3.5	12.7	5.4	0.425
	3.0	12.4	5.2	0.420
	2.0	12.2	5.6	0.460

Table II. Effect of Temperatures on Available Lysine Content of Complexed Sarcoplasmic Fish Proteins and Sarcoplasmic Fish Proteins Precipitated from Solutions with 70% Isopropanol

Sample	Drying Conditions	Temperature, ° C.	Available Lysine G. Lysine/ 16 G. N
Complex	Vacuum	70	6.7
IPA precipitated	Vacuum	70	6.5
Complex	Air	100	6.8
Complex	Air	120	6.2
Complex	Air	150	3.8
IPA precipitated	Air	150	3.4

tri- and tetrametaphosphate solutions was not made. We did find, however, that the phosphate content of some of the protein precipitates (isolated at pH 4.0 from 0.02*M* phosphate) that were analyzed was about one-third that of the precipitates that were obtained in the HP experiments. This observation verifies those of Rane and Newhouser (1965) who found that when blood plasma was precipitated in the presence of sodium tetrametaphosphate the isolated fractions did not contain bound phosphate. This phenomenon is probably characteristic of the cyclic compounds because the trimetaphosphate compound behaves similarly. Further work in this area is currently being done at our laboratory.

Nutritional Characteristics. PROTEIN EFFICIENCY RATIO AND AVAILABLE LYSINE. About 2 pounds of complexed sarcoplasmic proteins from rockfish were prepared by complexing from a 0.01*M* solution of HP at pH 4.0. The complexed proteins were washed twice with water and then resuspended in isopropanol prior to drying (80° C. for 16 hours in vacuum) and milling. Control proteins were prepared by precipitation from approximately 70% isopropanol.

Tables II and III show the protein efficiency ratio (PER) and available lysine values of these preparations. No significant differences in either the PER or available lysine values were found between the two preparations.

The effect of drying at different temperatures on the available lysine content of the protein-phosphate complexes (above preparations) was also determined. The complexes were dried for 16 hours at 70°, 100°, 120°, and 150° C. prior to analysis (Table II). The available lysine content showed no significant decrease until drying temperatures reached 120° C. At 150° C., available lysine values decreased to one-half their original values.

Rate of Reaction. The rate at which protein is precipitated from solution was determined by precipitating sarcoplasmic fish proteins in the presence of 0.01*M* concentrations of HP. Two experiments were made as follows: a 1% solution of sarcoplasmic fish protein was brought to a specific pH with 0.1*N* H_2SO_4, and a portion of the reaction mixture was immediately filtered; the experiment was repeated, but the reaction mixture was allowed to stand one hour before filtering. The amount of proteins remaining in solution was determined in the filtrates.

Figure 5 reports the results from these experiments. The data show that at the pH values of 6.0 to 5.0, flocculation of the complexes are incomplete, and that about 5 to 15% of additional proteins precipitate from solution after the initial floc is centrifuged from the solution. As the pH is lowered from 5.0, the rate of flocculation becomes very rapid and at pH 4.0 no differences were found in the amounts of proteins left in solution between the two time intervals investigated.

Industrial Application of Protein Complexing. In many industries where proteinaceous products are produced, aqueous plant effluents often contain soluble proteins in dilute concentrations. If discharged untreated into streams or other bodies of water, these effluents create serious pollution problems. Conventional treatment to reduce the organic load usually consists of discharging these effluents into stabilizing ponds of various designs where organic components are biologically degraded into CO_2, H_2O, and other simple

Table III. Comparative Protein Efficiency Ratios of Complexed Sarcoplasmic Fish Proteins and Sarcoplasmic Fish Proteins Precipitated from Solution in 70% Isopropyl Alcohol

Assay Group	Average Weight Start, grams	Average Weight End, grams	Average Weight Gain, grams	Average Protein Intake, grams	Average Food Intake, grams	PER	Nitrogen, %
ANRC reference casein	67.7	159.1	91.4	293.4	333.7	3.11	14.1
Protein-phosphate complex	71.8	190.5	118.7	296.3	367.6	4.0	12.9
IPA precipitated sarcoplasmic proteins	69.2	209.5	140.3	366.9	410.6	3.82	14.3

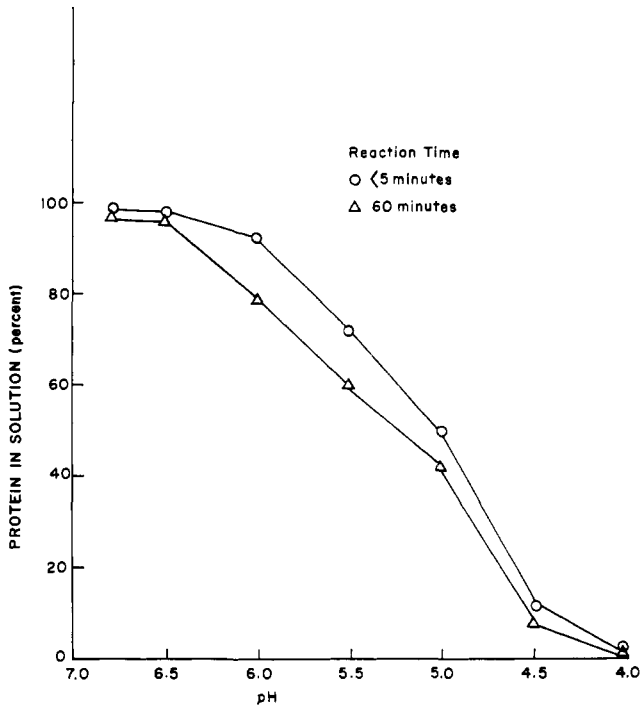

Figure 5. Amount of sarcoplasmic fish protein complexed from a 1% solution with 0.1*M* sodium hexametaphosphate at two different time intervals

organic and inorganic constituents. Other treatments can consist of treating the effluents with metallic salt such as $FeCl_3$, $Al_2(SO_4)_3$, and combinations of these salts and $Ca(OH)_2$ (lime) or NaOH (Quinn, 1968). Recently Claggett and Wong (1969) used some of the above combinations and also a polyelectrolyte derived from lignosulfonic acid (F Flok) to recover proteins from salmon cannery wastes.

Recovery of Proteins from Plant Processing Liquors. Based on our observations, on the reaction between condensed phosphates and proteins, some preliminary observations were made to determine whether the complexing reaction might afford a means of recovering and utilizing proteins that might be lost as waste or else be recovered in such a way that their full economic potential might not otherwise be realized. For these experiments, we used stickwater from a herring fish meal plant and also the aqueous discharge from a shrimp processing plant.

Experiments with the stickwater showed that approximately 70% of the proteins can be recovered as an HP-protein complex. The complex was formed by adding 12 grams of HP per gallon of stickwater, followed by a lowering of the pH to 3.8 to 4.0 with 1*N* sulfuric acid. The complex can be centrifuged to approximately 40 to 45% solids in a high-speed centrifuge and dried to a nonhygroscopic meal. Similar results for protein recovery were obtained when liquors from shrimp processing were treated with HP and acid. One set of feeding tests was made from herring stickwater proteins recovered as described. The PER values were 2.3, compared with 3.0 for casein. No feeding tests have been made on the complexed shrimp protein.

Removal of Unreacted Phosphates. Unreacted metaphosphates and other normally occurring orthophosphates present in the treated effluents were removed by treatment with $Ca(OH)_2$ slurries or by treatment with $Al_2(SO_4)_3$ and lime $Ca(OH)_2$ (Dryden and Stern, 1968). Lime treatment reduced the residual phosphate content by 90%; the combination $Al_2(SO_4)_3$-lime treatment reduced the residual phosphate content by 98%.

Discussion. Based on the above experiments, it appears that phosphate complexing is more effective in removing proteins from processing effluents than treatment with metallic salts and equivalent to the polyelectrolyte F Flok. Claggett and Wong reported protein recoveries of approximately 50% with $Al_2(SO_4)_3$ and lime and 70% with F Flok.

The economics of recovering protein by any of the proposed methods would in large measure be dependent on the utilization of the recovered products. Claggett and Wong reported on some preliminary feeding tests run with chicks on meals recovered by aluminum precipitation. They found that the meals could be used in chick-starting diets as a source of supplemental protein when included in the diet at a level of 5%. Poor growth was recorded at levels higher than 5%.

Tests on the nutritional characteristics of proteins complexed with condensed phosphates are continuing at our laboratory.

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