and the optical purity of the product were unsatisfactory. In the present method these disadvantages could be largely avoided. Comparisons of resolution results for DL-Trp-BS and DL-Trp-*p*-PhS are summarized in Table III. The undesired isomer of Trp could be racemized easily by dissolving it in water and heating at 180°. Scheme I shows the flow sheet of L-Trp production.

The optical resolution methods now presented are very advantageous because they require neither an optically active resolving agent nor conversion of amino acids into complicated derivatives, the yield per unit volume is very high, and the operation is very simple. Industrial productions of optically active 6-Cl-Trp and Trp by this method are considered to be very promising if combined with a proper synthetic method for 6-Cl-DL-Trp and DL-Trp.

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Binding of Mg(II) by the 11S Fraction of Soybean Proteins

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Binding of Mg(II) by the 11S fraction of soybean proteins has been measured in 0.1 *M* borate buffer of pH 7.8 by equilibrium dialysis. The binding sites are possibly the imidazole groups of the histidine residues of the protein molecule. NaCl at 0.5 *M* concentration suppresses binding. Addition of urea or prior treatment of the protein with EDTA reduces binding. EDTA treatment causes dissociation of the protein which is partly reversed by the addition of Mg(II). No conformational change occurs in the protein due to the binding of Mg(II). The protein, both before and after EDTA treatment, is quantitatively precipitated by 1×10^{-2} *M* Mg(II). NaCl at 0.5 *M* concentration suppresses precipitation. Addition of Mg(II) to the water extract of defatted soybean yields a precipitate which consists almost entirely of the 11S fraction.

Calcium salts have been used to precipitate the 11S fraction of soybean proteins (Koshiyama, 1965). This protein binds Ca(II) ions (Appu Rao and Narasinga Rao, 1975). Mg(II) does not appear to have been used for such precipitation. In this investigation the binding of Mg(II) by the 11S fraction has been measured and compared with Ca(II) binding. The effect of Mg(II) binding on the physicochemical properties of the protein has also been determined. Addition of Mg(II) to unfractionated soybean proteins yields a precipitate which consists almost entirely of the 11S fraction.

EXPERIMENTAL SECTION

Preparation of 11S Fraction. Improved Pelican variety soybeans were used. The 11S fraction was obtained by the method of Wolf et al. (1962). Homogeneity of the preparation was determined by analytical ultracentrifugation. The sedimentation velocity pattern in 0.05 *M* phosphate buffer (pH 7.9) containing 0.5 *M* NaCl indicated the presence of a small amount of 7S fraction which did not amount to more than 2–3% of the total (Figure 1). The protein was free from phosphorus and carbohydrate impurities.

Protein Concentration. This was determined by measuring the absorbance at 280 nm and using a value of 9.2 for $E_{1cm}^{1\%}$ (Wolf and Briggs, 1959).

Equilibrium Dialysis. Aliquots (2.5 ml) of 1% protein solution in 0.1 *M* borate buffer (pH 7.8) were dialyzed against 5 ml of buffer solution containing varying amounts of $MgCl_2$, for 48 hr at 30°. Corresponding "blanks" containing only the buffer solution were also run. An interval of 48 hr was found sufficient for equilibrium to be attained. At the end of the period, the concentration of Mg(II) of the outside solutions was estimated. From the difference in Mg(II) concentration, the number of Mg(II) ions bound by 100,000 g of protein was calculated. When the experiments were done in the presence of urea, all the solutions were prepared in 0.1 *M* borate buffer containing 8 *M* urea.

Estimation of Mg(II). The colorimetric method of Smith (1955) was used. The color was developed with 0.1% Eriochrome Black T. The dye solution was prepared freshly for each set of measurements. A calibration curve of absorbance at 520 nm vs. Mg(II) concentration was constructed. Beer's law was obeyed over a concentration range of 0 to 5×10^{-4} *M* Mg(II). A separate calibration

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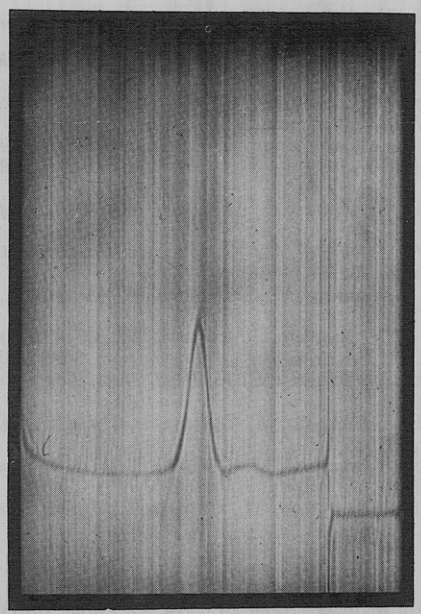

Figure 1. Sedimentation velocity pattern of 11S protein in 0.05 *M* phosphate buffer (pH 7.9) containing 0.5 *M* NaCl. Sedimentation proceeds from right to left.

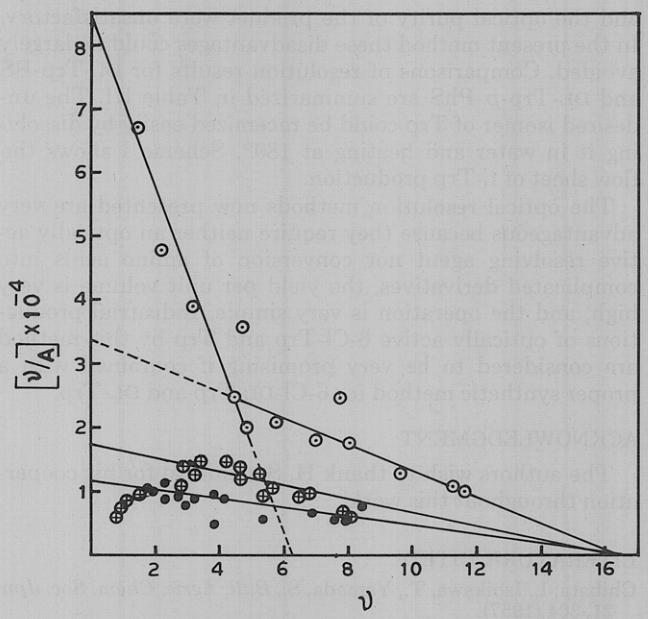

Figure 3. Scatchard plot of binding data in 0.1 *M* borate buffer (pH 7.8): (⊙) 11S fraction; (⊕) EDTA-treated 11S fraction; (●) 11S fraction in buffer containing 8 *M* urea.

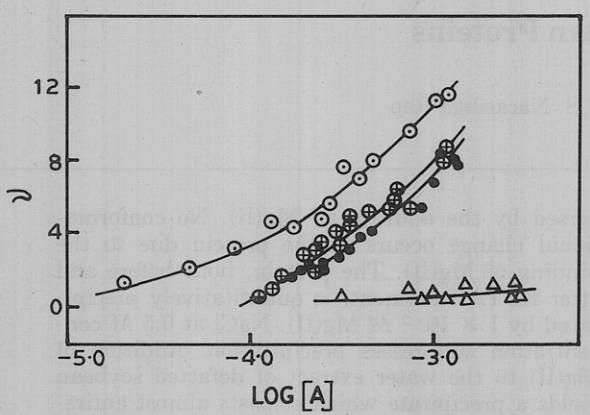

Figure 2. Binding of Mg(II) by the 11S fraction (0.1 *M* borate buffer, pH 7.8): (⊙) 11S fraction; (●) 11S fraction in buffer containing 8 *M* urea; (△) 11S fraction in buffer containing 0.5 *M* NaCl; (⊕) EDTA-treated 11S fraction.

curve was prepared for estimation in the presence of urea.

Sedimentation Velocity. The measurements were made at room temperature (~25°) with a Spinco Model E ultracentrifuge fitted with phase-plate schlieren optics and RTIC unit. Protein (1%) in 0.1 *M* borate buffer solution was used. The $s_{20,w}$ value was calculated by the standard procedure (Schachman, 1959).

Precipitation of Protein with Mg(II). Aliquots (2.5 ml) of 1% protein solution in 0.1 *M* borate buffer of pH 7.8 were mixed with Mg(II) solution (also in the buffer) of varying concentration and the volume made to 5 ml with the buffer solution. The mixture was left for 6 hr in a water thermostat maintained at 30°. The resulting precipitate was removed by centrifugation and the absorbance of the supernatant, after dilution where necessary, was read at 280 nm. From the absorbance of the original solution and the supernatants, the percent of protein precipitated was calculated. Mg(II) concentration varied from 1×10^{-3} to 5×10^{-2} *M*.

Fluorescence Spectrum. A Perkin-Elmer fluorescence spectrophotometer Model 203 was used for fluorescence measurements. Protein solution having an absorbance of 0.13 at 280 nm was used. Fluorescence emission was measured at various wavelengths from 300 to 400 nm after excitation at 280 nm; excitation spectrum was measured between 250 and 310 nm, after fixing the emission at 330 nm. The measurements were made at room temperature (~25°).

Other Measurements. Absorption measurements were made with a Carl-Zeiss UV spectrophotometer. A Radiometer pH meter Model TTT2 was used for pH measurements.

RESULTS AND DISCUSSION

The binding data are given in Figure 2. The number of Mg(II) ions bound by 100,000 g of protein, ν, is plotted against the logarithm of free Mg(II) concentration, [A]. Mg(II) was bound by the protein under the experimental conditions. The binding was, however, almost completely suppressed by the addition of 0.5 *M* NaCl.

The binding data were analyzed with the Scatchard equation (Scatchard, 1949) to obtain the association constant, *K*, and the maximum number of ions bound, *n*. The equation is $\nu/[A] = K(n - \nu)$. For a set of equivalent binding sites the plot of $\nu/[A]$ vs. ν would be a straight line with slope equal to *K* and the intercept on the abscissa equal to *n*. The Scatchard plot is given in Figure 3. The plot could be fitted to a set of two straight lines. From the initial linear portion, values of $K = 1.4 \times 10^4$ and $n = 6$ were obtained. From the second linear portion, $K = 2 \times 10^3$ and $n = 16$ were obtained. Thus, the binding sites appeared to be nonequivalent; the first few ions were bound more avidly than the remaining. The value of $n = 16$ agrees remarkably well with the histidine content of the 11S fraction (Shvarts and Vaintraub, 1967). This agreement would suggest that the binding sites could be the imidazole groups of the histidine residues.

The nonequivalence of the binding sites might suggest that some of the imidazole groups were not easily accessible for interaction with Mg(II) because of conformational restraints. To determine if this could be the cause for the nonlinear Scatchard plot, binding measurements were made in buffer solution containing 8 *M* urea. The binding in 8 *M* urea was considerably less (Figure 2). The Scatchard plot consisted of a single straight line (Figure 3). Although $n \simeq 16$, the value of *K* had decreased to 1×10^3. In view of the scatter in the experimental points, a

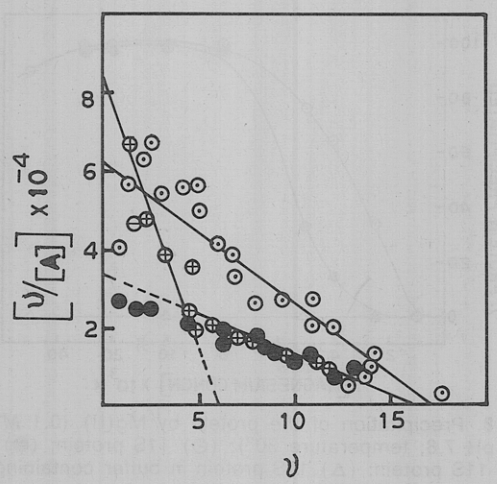

Figure 4. Scatchard plot of competitive binding data in 0.1 *M* borate buffer (pH 7.8): (⊙) Ca(II) binding; (⊕) Mg(II) binding; (●) Ca(II) binding in presence of 8×10^{-4} *M* Mg(II).

very precise estimate of slope (and hence K) was, however, difficult. Thus, 8 *M* urea rendered all the binding sites equivalent.

The 11S protein is a high molecular weight protein which dissociates into lower molecular weight fractions in the presence of substances such as urea (Catsimpoolas et al., 1969). Because of this effect, the linear Scatchard plot obtained in 8 *M* urea solution could not be interpreted in terms of unmasking of the groups. The decreased binding of Mg(II) in the presence of urea could be due to the binding of urea by the protein (Klotz and Shikama, 1968).

Wolf and Briggs (1958) obtained evidence that the 11S protein may contain cationic impurities such as Ca(II). Such bound ions may affect the binding of Mg(II). Therefore, binding measurements were made after treating the 11S fraction with EDTA. The protein in borate buffer was mixed with 5×10^{-3} *M* EDTA and then dialyzed extensively against the buffer to remove EDTA. Binding data obtained with the EDTA-treated protein are given in Figure 2. The binding was considerably less than in the case of the untreated protein. The Scatchard plot consisted of a single straight line (Figure 3). The values of n and K were 16 and 1×10^3, respectively.

Thus, binding of Mg(II) was reduced in 8 *M* urea and by EDTA treatment of the protein; furthermore, the binding sites were rendered equivalent. Catsimpoolas et al. (1969) have reported that urea dissociates the 11S protein. Wolf and Briggs (1958) have shown that addition of EDTA also causes dissociation. This dissociation can be reversed to some extent by the addition of metal ions (Wolf and Briggs, 1958). It was probable that the reduced binding observed could be due to the dissociative effect of these reagents; in the case of urea there could be the additional effect discussed above.

The electrometric titration curve of the protein, before and after EDTA treatment, was determined in the range of pH 2–8 to see if there was any difference in the number of ionizable groups or their pK values. In this pH range the dissociation of carboxyl and imidazolium groups would occur (Tanford, 1962). The titration curves were superimposable. The reduced binding by the EDTA-treated protein thus could not be due to any difference in either the number of ionizable groups or the pK values.

Ca(II) also appears to bind to the imidazole groups of the 11S protein (Appu Rao and Narasinga Rao, 1975). Therefore, binding of Ca(II) in the presence of 8×10^{-4} *M* Mg(II) was measured at various Ca(II) concentrations. The measurements were made with ^{45}Ca(II). The binding data, in the form of a Scatchard plot, are given in Figure 4. The binding of Ca(II) was considerably less in the presence of Mg(II). Although $n \simeq 16$, K had decreased from 3×10^3 to 2×10^3. Thus, Ca(II) and Mg(II) appeared to bind to the same set of binding sites. However, this conclusion cannot be unequivocal since Mg(II) bound at other sites could conceivably affect the binding of Ca(II) by electrostatic effects.

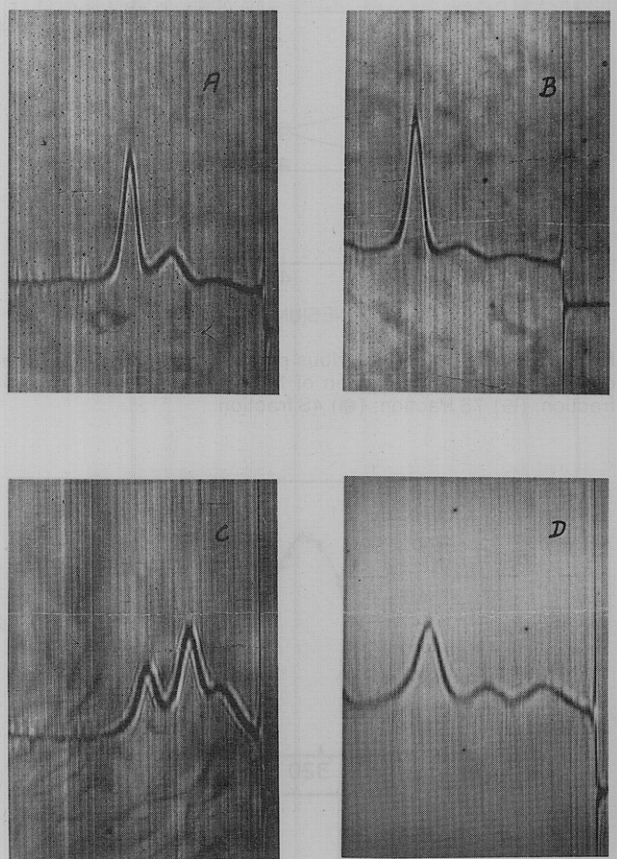

Figure 5. Sedimentation velocity patterns in 0.1 *M* borate buffer (pH 7.8): (A) 11S fraction; (B) 11S fraction containing 8×10^{-4} *M* Mg(II); (C) EDTA-treated 11S fraction; (D) EDTA-treated 11S fraction containing 8×10^{-4} *M* Mg(II). Sedimentation proceeds from right to left.

The Scatchard plot of Mg(II) binding data consisted of two straight lines whereas that of Ca(II) binding data consisted of a single straight line. Thus, in the case of Mg(II), the first few ions were bound more avidly than the other ions. It would have been interesting to study how the binding of the first few ions would be affected by Ca(II) ions. However, these measurements could not be made since Ca(II) interfered in the estimation of Mg(II).

Sedimentation velocity measurements of the 11S fraction in 0.1 *M* borate of pH 7.8 gave a pattern with a major peak of 11 S and two minor peaks of 7 and 4 S (Figure 5A). The sedimentation velocity pattern of the protein in 0.05 *M* phosphate buffer (pH 7.9) containing 0.5 *M* NaCl did not contain either the 7S or 4S fraction (Figure 1). Thus, a decrease in ionic strength had caused dissociation. The addition of Mg(II) at 8×10^{-4} *M* Mg in 0.1 *M* borate buffer did not cause any marked changes except that the proportion of 7S peak had decreased, suggesting that Mg(II) caused the aggregation of this protein to 11S protein (Figure 5B).

EDTA treatment of the 11S protein caused considerable dissociation of the protein as could be judged from the increase in the proportion of the lower molecular weight components (Figure 5C). The sedimentation velocity pattern consisted of three peaks, 11S, 7S, and 4S fractions; their percentage proportions were 29, 45, and 26, respec-

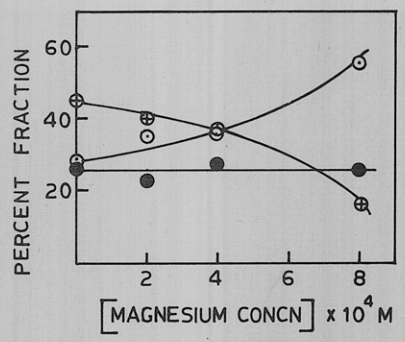

Figure 6. Percent of the various protein fractions of EDTA-treated 11S protein as a function of Mg(II) concentration: (⊙) 11S fraction; (⊕) 7S fraction; (●) 4S fraction.

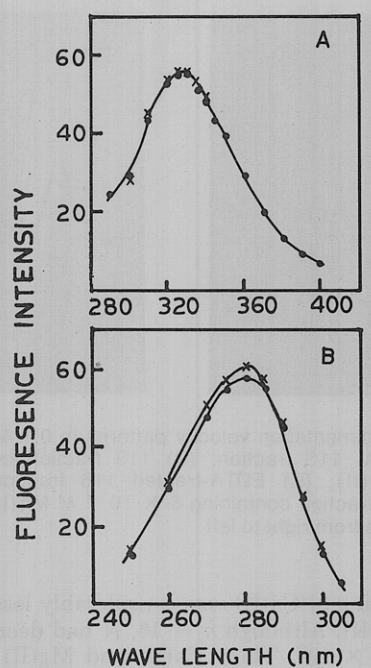

Figure 7. Fluorescence spectrum (measured in 0.1 *M* borate buffer, pH 7.8): (A) emission spectrum; (●) 11S protein; (x) 11S protein + 8 × 10^{-4} *M* Mg(II); (B) excitation spectrum; (●) 11S protein; (x) 11S protein + 8 × 10^{-4} *M* Mg(II).

tively. The addition of Mg(II) brought about marked changes in the pattern (Figure 5D). The following changes were observed: (1) the proportion of the 7S fraction decreased with an increase in Mg(II) concentration while that of the 11S fraction increased and (2) the proportion of the 4S fraction remained constant. These data are given in Figure 6. In the estimation of the proportion of the fractions from area measurements no correction was applied for radial dilution. Thus, Mg(II) binding appeared to reverse the dissociation of the 11S fraction to the 7S fraction caused by EDTA treatment but not the dissociation to the 4S fraction. Metal ions such as Mg(II) and Ca(II) perhaps play a key role in maintaining the structure of the 11S fraction. This suggestion has been made by Wolf and Briggs (1958).

Fluorescence spectral measurements have been used to investigate conformational changes in proteins (Chen et al., 1969). Hence, the fluorescence spectrum of the protein, with and without Mg(II), was determined. The emission spectrum (Figure 7A) had a maximum at 330 nm. The 11S protein contains both tyrosine and tryptophan: 24 groups of tyrosine and 7 groups of tryptophan per 100,000 g of protein (Shvarts and Vaintraub, 1967). The addition of Mg(II) did not bring about any change either

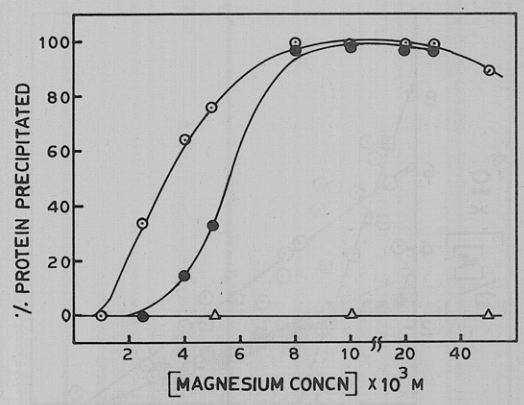

Figure 8. Precipitation of the protein by Mg(II) (0.1 *M* borate buffer, pH 7.8; temperature 30°): (⊙) 11S protein; (●) EDTA-treated 11S protein; (△) 11S protein in buffer containing 0.5 *M* NaCl.

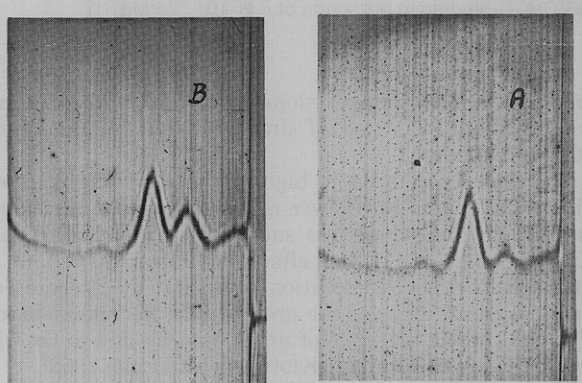

Figure 9. Sedimentation velocity patterns in 0.05 *M* phosphate buffer (pH 7.8) containing 0.5 *M* NaCl: (A) Mg(II) precipitate from the water extract of soybean proteins; (B) Ca(II) precipitate from the water extract of soybean proteins.

in fluorescence intensity or the wavelength of maximum fluorescence. The same result was obtained with excitation spectral measurements also (Figure 7B). Thus, addition of Mg(II) did not appear to bring about any change in the conformation of the 11S protein. Sedimentation velocity measurements indicated that a slight dissociation of the protein occurred in 0.1 *M* borate buffer and this could be reversed by the addition of Mg(II) (Figures 5A and B). Apparently this effect was not reflected in the fluorescence measurements. Cowgill (1968) and McCubbin and Kay (1973) have reported that the addition of Ca(II) to troponin A increased the fluorescence intensity of the protein and this could be attributed to an increase in helical content of the protein molecule upon adding Ca(II).

Since Mg(II) precipitates the unfractionated soybean proteins (Wolf, 1972) and the 11S fraction forms a major component of soybean proteins, measurements of the precipitation of the 11S protein by Mg(II) were made. The protein, before and after EDTA treatment, was used. There was almost quantitative precipitation of the protein at an Mg(II) concentration of 8 × 10^{-3} *M* (Figure 8). Below this concentration the extent of precipitation increased with Mg(II) concentration. The EDTA-treated protein was precipitated considerably less at Mg(II) concentrations below 8 × 10^{-3} *M*. However, above this concentration both the proteins were quantitatively precipitated. The addition of NaCl at 0.5 *M* concentration completely suppressed precipitation. Thus, there was a parallel between precipitation and Mg(II) binding. Either EDTA treatment or addition of NaCl reduced binding of Mg(II) and they also reduced precipitation. This contrasts with the precipitation behavior observed with Ca(II)

(Appu Rao and Narasinga Rao, 1975). Although EDTA treatment reduced the binding of Ca(II), it had no effect on precipitation of the protein. If anything, the precipitation was higher. The mechanism of precipitation of the 11S protein by the two cations thus appears to be different.

Difference in the precipitation of the unfractionated soybean proteins by Ca(II) and Mg(II) was revealed by the following experiment. Soybean proteins were extracted with water at a meal to solvent ratio of 1 to 10. The carbohydrate residue was removed by centrifugation. To the supernatant Mg(II) was added in the cold to a concentration of 5×10^{-2} *M*. After standing in the cold for 6 hr the precipitate was separated and dissolved in 0.05 *M* phosphate buffer of pH 7.8 containing 5×10^{-3} *M* EDTA. It was then dialyzed extensively against 0.05 *M* phosphate buffer of pH 7.8 containing 0.5 *M* NaCl to remove EDTA and Mg(II). The sedimentation velocity pattern of this preparation consisted of a major fraction, 11 S, and two minor fractions, 15 S and 7 S (Figure 9A). The proportion of the minor fractions was 5–10% of the total. A similar experiment was conducted using Ca(II) for precipitation. The sedimentation velocity pattern consisted of 3 peaks, 11, 7, and 2 S (Figure 9B). The proportion of the 11S fraction was only 60% of the total. Thus precipitation with Mg(II) appeared to yield a more homogeneous 11S fraction and thus offers a single step procedure for the preparation of this protein in a fairly homogeneous form.

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Removal of Mercury from Fish Protein Concentrate by Sodium Borohydride Reduction

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Mercury removal from fish protein concentrate (FPC) can be accomplished by reduction with sodium borohydride in aqueous FPC slurries at a pH between 9 and 10. Investigation of the effects of contact time and temperature revealed that both inorganic and methylmercury were most effectively removed in 20 to 30 min at a temperature of 23 ± 2°. Studies relating sodium borohydride concentration to effectiveness of mercury removal indicated that for each part per million of mercury in 100 g of FPC, 1.2 g of sodium borohydride was required to obtain a final level of 0.5 ppm of mercury in the FPC. A 100% removal of mercury may be achieved with excess sodium borohydride. Metaborate ion, formed during $NaBH_4$ treatment, may be washed out of the FPC with water. A test of possible changes in the nutritional value of the treated FPC was made using chicks as the subject of a growth–response study which lasted 3 weeks. The growth of chicks fed a treated FPC diet was equal to that for chicks fed a diet employing untreated FPC.

There has been considerable recent interest in the removal of mercury from FPC. The methods described heretofore involve competitive complexation (Regier, 1972; Yannai and Saltzman, 1972; Spinelli et al., 1973; Lee and Richardson, 1973) or a chemical modification of the FPC (Archer et al., 1973). In the former cases, the extent of mercury removal is limited by the relatively small equilibrium constants possible for competitive complexation processes. On the other hand, removal of mercury coupled with extensive modification of the FPC does not always produce a product with the most desirable qualities.

Our research was initiated for two reasons. One was to find a method of removing mercury which could be tailored to any production process for FPC and could produce any desired level of mercury removal up to 100%. The second reason was to examine the possibility of using Lake Erie fish as the input for an FPC processing plant. Although the commercial catch of fish from Lake Erie over the past 150 years has exceeded that of the other four Great Lakes combined, a variety of factors has reduced the value of the fish caught in recent years even though there has been little, if any, fall-off in their abundance. Fish of low commercial value such as carp and sheepshead are now in such abundance that they make fishing for the more valuable species difficult. Use might be made of these fish to produce FPC (Finch, 1970). Because of heavy previous contamination of some of the Great Lakes with mercury (Pillay et al., 1971), consideration of the mercury removal problem comes into any plan for utilization.

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