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Presented in Partial Fulfillment of the Requirements for
the Degree Doctor of Philosophy in the Graduate
School of The Ohio State University

by

Dimite Joanne Buckley, B.Sc., M.Sc., M.Sc.

*** *** ***

The Ohio State University
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INTRODUCTION

The present study was undertaken to investigate the characteristics of calcium binding by alpha-s casein since knowledge in this area is essential for better understanding of micelle formation. Titration of the protein is done in the presence of calcium chloride using an ion exchange electrode to measure the calcium activity.

The 'milky' appearance of milk is due to formation of micelles composed primarily of alpha-s, beta and kappa caseins, colloidal calcium phosphate and bound calcium. The essential components for micelle formation are kappa casein, alpha-s casein and calcium ion. Kappa casein is considered to protect alpha-s casein from precipitation by calcium ion.

The phosphoester group has generally been accepted as being the group which binds calcium. Until the work of Ho and Waugh (1) and Yamauchi et al. (2) there has been no direct evidence for this. Ho and Waugh, using infrared spectroscopy, examined alpha-s casein in the presence and absence of calcium. They concluded the phosphate groups were the primary sites of calcium binding but that other groups might also be involved. Yamauchi compared the calcium binding capacities of native and dephosphorylated alpha-s casein and concluded that calcium bound to the phosphates, but that above pH 6 another type of calcium binding occurred.
The titration work of Österberg (3) on a phosphopeptide of alpha casein containing seven phosphate groups, indicated that all of these groups were monoesters. Alpha casein is a mixture of kappa and alpha-s casein. The phosphorus content of alpha-s casein is much higher than that of kappa casein. Thus the peptide is probably from alpha-s casein. Ho et al. (4) concluded from their phosphorus nuclear resonance study that all the phosphates of alpha-s casein, genetic variant B, are of the monoester type.
The total protein content of bovine skim milk is approximately 32 gm per liter of which about 25 gm is casein (5, 6). The whole casein of milk has usually been considered to be the group of proteins which precipitate from skim milk upon adjustment of the pH to 4.6 at various designated temperatures ranging usually from 0 to 37°C. The Committee on Milk Protein Nomenclature (7) of the American Dairy Science Association, A.D.S.A., has defined whole casein as "a heterogeneous group of phosphoproteins precipitated from skim milk at pH 4.6 and 20°C."

The whole casein of skim milk was first shown by moving boundary electrophoresis by Mellander (8) in 1939 to be composed of at least three components which he designated as alpha, beta and gamma casein in order of decreasing mobility.

Subsequently, von Hippel and Waugh (9) by addition of calcium chloride to skim milk, were able to prepare a fraction designated by them as first cycle casein which was a mixture of alpha, beta and possibly gamma caseins. By variation of temperature and of calcium chloride concentration they (10) were able to fractionate the first cycle casein and to demonstrate that the alpha casein was composed of a calcium sensitive alpha fraction, alpha-ς casein which is insoluble in calcium, and a calcium insensitive alpha fraction, kappa casein.
Kappa casein is soluble in calcium chloride and also acted upon by rennin.

McKenzie (11) has prepared an excellent review article on milk proteins. In one section he compares the casein fractionation procedures of various workers. Quite a few of the methods are based on the solubility of the caseins in urea or calcium chloride solutions at various temperatures.

The identification and characterization of the various caseins of bovine milk has been obscured to a large degree due to the interactions of the different caseins in the presence or absence of calcium ion.

The A.D.S.A. Committee has designated the alpha-s casein fraction as that fraction of the alpha casein complex insoluble in 0.40 M calcium chloride at pH 7.0 and 0 to 4°C and kappa casein as a fraction of the alpha casein complex soluble in 0.40 M CaCl₂ at pH 7.0 and 0 to 4°C and capable of stabilizing alpha-s casein against precipitation with calcium. The Committee has designated beta casein as the fraction of whole casein soluble in 4.6 M urea but insoluble in 3.3 M urea at pH 4.6 and states that beta caseins are precipitated with calcium at 35°C but not at 4°C.

The micelles of milk which are composed of the caseins and milk salts are spherical and range in diameter from about 30 to 300 μm. From electron microscopy studies of Knoop and Wortman (12) and Shimmin and Hill (13), the average diameter of the micelles is 93 and 80 μm, respectively. By light scattering D'yachenko and Vlodavets (14) deter-
mined an average size of 86 to 123 μm for the micelle diameter.

The dependence of micelle size on numerous factors has been considered by various workers. McGann and Pyne (15) report that the micelle size is smaller in milk having a lower colloidal phosphate content. In addition, Sullivan et al. (16) have concluded from results on fractionation of skim milk by differential centrifugation that the micelle size decreases as the kappa casein to whole casein ratio increases. Decelles' (17) data supports this conclusion. However, it was found in this laboratory (18) that when three times concentrated milk was diluted and then fractionated by centrifugation, the ratio of kappa to total casein was the same regardless of the micelle size. In the concentrated milk the levels of soluble calcium, soluble phosphate and soluble citrate were approximately three times higher than for normal milk. Upon dilution of the milk the above levels were comparable to normal milk and the calcium ion concentration was 2.0 mM/l. The calcium ion concentration in normal skim milk as determined by the murexide method of Smeets (19) ranges from approximately 2.3 to 3.4 mM/l (20, 5, 21).

Rose and Colvin (22) prepared electron micrographs of a number of milks from individual cows in an attempt to relate micelle size to the analytical parameters and other characteristics of the individual milks. Studies on the milk from 15 individual cows gave no evidence of a relationship between beta casein content and micelle size. However, they related kappa casein content to average micelle diameter by the equation micelle diameter = 82.2 + 16.1 - (2.19 + 0.96)
kappa casein with a probability of 0.9 that the influence of kappa casein was statistically significant. They report that there is no apparent relationship between the average micelle diameter and the total or colloidal calcium, the total or colloidal phosphate, and the total magnesium but that the micelle size appears to be related to the amount of soluble calcium and citrate. They state that it is generally assumed that calcium ions can form salt bridges between adjacent casein molecules and thus influence agglomeration and micelle size. Their data indicates that calcium ion concentration is less closely related to micelle size than total soluble calcium, total soluble phosphate, or total soluble citrate and they therefore suggest that the more complex forms of soluble calcium such as (Ca citrate)$^-$, (Ca$_2$ citrate)$^+$, (CaH$_2$PO$_3$)$^+$, and (Ca$_2$HPO$_3$)$^{+2}$ are as effective as calcium ion in promoting cross linkages between casein molecules.

There has been considerable disagreement in the literature concerning the nature of the colloidal calcium phosphate in milk. There is the question of the composition of the colloidal calcium phosphate and also of the nature of its association with the micelle. The colloidal calcium phosphate may or may not be chemically bound to the casein.

Boulet and Marier (23) have studied the nature of calcium phosphate precipitated at the approximate ionic strength of milk and pH 6 to 8. Their results indicate that above 1.4 equivalents alkali added per P, freshly precipitated calcium phosphate can occur in two apatite forms. One is a gelatinous form that is probably octacalcium
phosphate with a Ca/P molar ratio of 1.33. This gelatinous precipitate can apparently equilibrate immediately exchanging H⁺ for the CaOH⁺ ions in solution to form a series of salts with compositions between that of octacalcium phosphate and hydroxylapatite. This gelatinous form can change into a granular form with time. This granular form probably nucleates from solution as dicalcium phosphate but with higher additions of alkali converts to a more alkaline salt with Ca/P molar ratio of 1.1 to 1.5.

At pH above 5, Visser (24) titrated phosphoric acid with sodium hydroxide in the presence of calcium chloride and casein. He concluded that between pH 5 and 12 calcium caseinate and tricalcium phosphate can occur together in a soluble chemical complex. By blocking the amino end groups or the carboxyl end groups, he determined that the soluble tricalcium phosphate formation took place only in the presence of free carboxyl groups associated with a colloidal matrix. He gives the general formula as follows:

\[
\text{colloid} - \text{Ca} - \text{O} - \text{P} - \text{O} - \text{Ca}
\]

When the carboxyl groups of casein were blocked off there was evidence up to pH 9 of formation of soluble dicalcium phosphate.

Upon considering the results of Boulet and Marcus, Rose (25) has suggested formation of an ester phosphate-calcium-inorganic phosphate type bond for colloidal calcium phosphate of milk. He gives as the most probable structure the following:
Other theories on the nature of the colloidal phosphate of milk include that of Ter Horst (26) who postulates that the ion CaPO$_4^-$ is bound to the lysine of casein according to the reaction

$$\text{RNH}_3^+ \text{COO-R'} + \text{CaPO}_4^- \rightleftharpoons \text{RNH}_3\text{PO}_4\text{Ca} + \text{H}^+ \text{COO-R'}$$

and that any additional calcium is associated with the COO$^-$ groups.

In order to have greater insight into the nature of micelles in
skim milk it is important to study the characteristics and interactions of the caseins. Waugh and his group at M.I.T. have done extensive studies in this area. Alpha-s casein and kappa casein at neutral pH in the absence of calcium ion form a complex, the formation of which is temperature dependent. At 37°C but not at 2-6°C the complex is formed (27) as observed by ultracentrifugation studies. At 20-25°C the interaction has been reported to take place (28, 29) or not to take place (27, 30).

Noble and Waugh (27) have studied the solubility of alpha-s casein as a function of calcium concentration in 0.07 M KCl at pH 7 and 37°C. When calcium chloride is added above a concentration of 0.04 M to a 5 mg/ml alpha-s casein solution the protein precipitates out of solution. The alpha-s casein is only slightly more soluble at 0-4°C (31). On the other hand, beta casein is soluble in the presence of 0.07-0.4 M calcium in the 0-4°C range but insoluble from 0-37°C. In contrast, kappa is quite soluble in the presence of calcium ion from 0-37°C.

Noble and Waugh (27) studied the solubility relationships at pH 7 and 37°C of solutions containing alpha-s or mixtures of alpha-s and kappa caseins at different alpha-s:kappa weight ratios. Depending on protein concentration, precipitation of alpha-s casein takes place from both at the same calcium concentrations: 0.004 M for 0.5% and 0.005 M for 1% alpha-s casein. Precipitation probably of alpha-s casein from the mixture is progressively retarded by the presence of kappa casein, there being no precipitation when the weight
ratio is unity. The addition of calcium to the mixtures to give concentrations between 0.07 to 0.02 M leads to micelle formation and complete stabilization if the initial weight ratio is 10 or less. If the calcium is added incrementally, they found that there is some precipitation of protein at calcium levels where only micelle formation was observed in one aliquot addition, the apparent supernatant stabilization weight ratio being 2 to 3. The path dependencies are interpreted to mean that they are working with a nonequilibrium system under their experimental conditions. From the examination of centrifuged micelles Waugh and Noble (31) found the micelles to be highly solvated, the solvation decreasing as the initial weight ratio increased. At a weight ratio of unity and 20 the solvent volume to protein volume, assuming sphere packing, was 3.1 and 1.8 respectively. The authors proposed the following model (31):

Micelles consist, in simplest form, of cores of calcium $\alpha_s$-caseinate covered by a uniform coat of low weight ratio calcium $\alpha_s$-$\kappa$-caseinate. Calcium $\alpha_s$-caseinate in the core need not be in contact, therefore in exchange, with the environment.

Internally the coat subunits have a strong interaction with core calcium $\alpha_s$-caseinate and probably a lateral preference for each other. Surface components are in exchange with similar components in solution.

The model accepts $\beta$-casein to an extent. It places the carbohydrate moiety of $\kappa$-casein to the outside where it is accessible, even in extraordinarily stable micelles, to the action of rennin.

On the basis of observations regarding rapid action of rennin on micelles and the protective action of beta lactoglobulin on heating
of milk, McKenzie (11) concludes that the kappa casein must be near the surface of the micelle. Waugh and Noble (31) have noted that precipitates formed in the presence of kappa casein and at calcium concentrations sufficient for micelle stability are different from calcium alpha-s caseinate precipitates. These precipitates contain a small amount of kappa casein and, in contrast to the pure calcium alpha-s caseinate precipitates, are nonadherent.

Thompson and Kiddy (32) have identified three genetic variants of alpha-s casein which they named A, B, and C according to their mobility in starch gel electrophoresis. In order to determine physically the molecular weight of the alpha-s casein monomer which has a marked tendency to polymerize, a strongly dissociating media must be used such as urea (33, 34), guanidine hydrochloride (35), formic acid (36) or a high pH (37, 34). Waugh et al. (33) determined by osmotic pressure measurements in 6.5 M urea at pH 4.5 a molecular weight for the monomer of 26,900 ± 2000. By doing light scattering at pH 12 and ionic strengths between 0.3 and 1.2 Dreizen (37) determined a molecular weight of 27,300 ± 1500. Noelken (35) obtained by sedimentation equilibrium experiments approximations for the molecular weight of alpha-s casein B in 3 M guanidine hydrochloride at pH 7 and 25°C in the range of 24,100 to 32,600 depending on the value of v used. McKenzie and Wake (34) report a value of 27,600 ± 1000 obtained by sedimentation diffusion measurements in 6 M urea at pH 7.3. No correction was made for selective solvation. Their estimate of molecular weight at pH 12 in 0.1 ionic strength by the Archibald
sedimentation method is 25,500 ± 1000. Swaisgood and Timasheff (36) reported a molecular weight of the alpha-s casein C monomer of 26,900 ± 2700. They conducted light scattering experiments in anhydrous formic acid. In addition Waugh et al. (33) has reported an average molecular weight of 27,000 to 27,500 as measured by tryptophan release by carboxypeptidase A.

Just as the conditions leading to micelle formation are important, the degree of polymerization of alpha-s casein is influenced by temperature, ionic strength, protein concentration and type of salt present. Ho and Chen (38) employed osmotic pressure measurements to investigate the polymerization of alpha-s casein B under different conditions. They found that on increasing the KCl concentration from 0.01 to 0.1 M at 20°C and neutral pH the protein changed from monomer to the trimer and tetramer. The degree of polymerization appears to be much more dependent on ionic strength than on temperature. The intrinsic viscosities of the monomer vary from 11.8 ml per gm to 10.2 ml per gm as the temperature is increased from 4.9 to 37°C at the lower ionic strength. In 0.1 M KCl the intrinsic viscosities of the trimer or tetramer vary from 9.3 ml per gm to 7.7 ml per gm from 4 to 20°C. These values suggest that alpha-s casein B is not a spherical molecule and could be much more solvated than a globular protein. In the presence of 6 M guanidine hydrochloride the intrinsic viscosity increased at pH 7.1 and 20°C to 19.2 ml per gm. They therefore concluded that the protein is more flexible and possibly more solvated than a globular protein but much more compact than a denatured pro-
tein. Their optical rotation data suggest no or very little alpha helix formation in alpha-s casein B. In addition they state that the difference in $a_\text{o}$ values could reflect the fact that there is a difference in solvent-protein interactions between the monomer and the polymer of alpha-s casein. They also suggest that electrostatic interactions play an important role in the polymerization.

Swaisgood and Timasheff (36) studied the association of alpha-s casein C as a function of pH between 8.0 and 9.5 and at ionic strengths between 0.02 and 0.30 in NaCl. Their light scattering and sedimentation data are characteristic of a rapidly reequilibrating system at ionic strength of 0.1 and above. At pH values between 8.0 and 9.5 and ionic strengths above 0.1 the apparent molecular weight increases rapidly with increase in protein concentration. At pH values of 8 and 8.5 the data extrapolate to a molecular weight of 55,000 indicating that the dimer is the more stable form. Lowering the ionic strength to 0.02 causes considerable dissociation and the system is no longer in rapid equilibrium. Under these conditions there is a mixture of monomer and dimer. At pH 9.1 and 9.5 and at ionic strengths of 0.1 and 0.3 the data extrapolate to monomer indicating that while an increase in ionic strength favors association, the increased repulsion due to increased charge at the higher pH is sufficient to overcome the attractive forces between chains. Comparing their results with those of Payens and Schmidt (39) who at pH 6.4 and ionic strength 0.2 determined the fundamental aggregating unit to be a tetramer rather than a dimer, they conclude that a decrease in charge
results in formation of progressively large stable aggregates which undergo reversible polymerization and this would imply that the aggregation is accompanied by changes in conformation. Viscosity data of Swaisgood and Timasheff (36) further suggest that each step of the aggregation is accompanied by a conformational change from a compact solvent-impenetrable dimer to a rigid rod or stiff coil tetramer to a random coil hexamer. Optical rotation data indicates that aggregation is accompanied by some change in mutual orientations in space of the peptide bonds or a change in the environment and must reflect changes in the thermodynamic interactions of the various hydrophobic and hydrophilic residues with solvent and each other during the association process.

Österberg (40) has prepared a phosphopeptide from a tryptic hydrolysate of bovine alpha casein which contains in addition to seven phosphorylserine and threonine residues a high concentration of carboxyl residues. Swaisgood and Timasheff (36) suggest that this peptide may be partially buried with only the charged groups protruding or completely extended and hydrated, depending on the conditions. On the basis of the sensitivity of the protein to pH and ionic strength they postulate that this high local charge concentration may lead to strong electrostatic repulsion.

Österberg (40) further hydrolyzed the tryptic phosphopeptide into four peptides by peptic hydrolysis. The partial structure of two of the peptides in the amino half of the original peptide are as follows:
These two peptides contain seven phosphoamino acid residues and because of the high concentration of phosphorus, they would have to originate from alpha-\(\text{S}\) casein which has approximately 1% P rather than from kappa casein which reportedly contains only 0.22-0.33% P (11).

Österberg (3) studied the phosphorus linkages of the tryptic peptide by acid-base titration at 25°C in 0.15 M KCl. His results indicate that all of the peptide phosphorus is bound in 0-monophosphate ester form. The rates of heat-induced dephosphorylation (41) of caseins fall between those for phosvitin and phosphoserine, both of which are phosphomonoesters, thus supporting the view that the caseins contain phosphate exclusively in the monoester form. In addition, Ho et al. (4), on the basis of their phosphorus nuclear magnetic resonance study of alpha-\(\text{S}\) casein B, conclude that the protein contains phosphate all in the monoester form. On changing the pH from 4 to 9 they found a change in chemical shift of approximately 4 ppm as was found in other monoester compounds.

From his data on the fully reversible titration of the phosphopeptide Österberg calculated according to the procedure generally used for proteins, the intrinsic ionization constants and the electrostatic parameter, \(W\). The pK\(_{i}\) values of 4.1 for the \(\beta,\gamma\) carboxyl groups and
5.3 for the second phosphate ionizations are lower than expected. However, Österberg points out that the individual groups may be exposed to different kinds of electrostatic interaction or they may differ in intrinsic identity. The electrostatic parameter for the phosphate group was higher than that for the carboxyl groups but much lower than for a rigid impenetrable sphere. Österberg suggests that the results may be partly explained by different intrinsic identity among the individual phosphate groups and partly by some existing electrostatic interaction similar to that of a rigid spherical polyelectrolyte containing a cluster of similar charges as analyzed theoretically by Tanford (42).

There have been several studies done on the binding of calcium to whole casein (43, 44, 45, 46), usually employing sedimentation or equilibrium dialysis methods. The calcium binding was found to be pH dependent with little or no binding below pH 5. Zittle et al. (45) found that the sedimentation and equilibrium dialysis results agreed suggesting that binding to soluble and insoluble casein phases is the same. In addition to calcium binding they report that inorganic phosphate is not bound to calcium caseinate except above pH 6 where there is also a simultaneously large increase in bound calcium, the molar ratio of additional bound calcium to bound phosphate being 1.5.

It has been generally assumed that calcium binds to the organic phosphate esters. Ho and Waugh (1) were the first to give direct evidence of this. Their infrared data on phosphoserine, alpha-s casein, kappa casein, and alpha-s - kappa casein mixtures with and
without calcium at pH 7 indicate that the primary site for calcium binding is the organic phosphate group. The shift in absorption peaks due to interaction of ionic phosphate with calcium are the only changes observed in a calcium-casein system when the Ca/P ratio is 0.2. These shifts are complete when Ca/P equals 2.0. It is at ratios of 2.5 to 4 that micelle formation takes place in systems which have a 2% or above concentration of kappa- alpha-s casein mixtures. They also found that when Ca/P is 1.4 there is a decrease in the relative size of the peak at 1395 cm\(^{-1}\). They make no specific assignment for this peak but state that changes in absorption in the region 1440-1590 cm\(^{-1}\) could be due to effects on carboxylate or ammonium ions or on phenolic or unsubstituted amide groups. On the basis of solubility curves of alpha-s casein Noble and Waugh (27) estimate that seven calcium ions are bound per 27,000 gm of alpha-s casein just prior to its precipitation by calcium followed by an increase of 4 more upon formation of precipitate.

Using the sedimentation method, Yamauchi et al. (2) have studied the calcium binding of both the native and the dephosphorylated states of whole, alpha-s and kappa caseins. The increase in calcium binding capacity of native whole casein going from pH 5.5 to 7.0 was very much larger than that of dephosphorylated whole casein which had little binding taking place below pH 6. They studied the calcium binding of both types of alpha-s casein at pH 7 and 9. Their results indicate equality of removal of phosphorus to decrease in calcium binding for both alpha-s and whole caseins and they suggest that the phosphate
groups are almost completely saturated with calcium ions at neutral pH when the calcium concentration is above 10 mM. At 15 mM CaCl₂ they calculated that 8.7 moles of calcium ion was bound per 27,000 gm protein by the phosphate groups. Their phosphate analysis was 1.04% which corresponds to a total of 9.2 phosphate groups per 27,300 grams protein. In the pH region of 7 to 9 the increase in calcium binding for both native and dephosphorylated alpha-s casein was almost identical. They ascribe this additional binding to sites other than phosphate groups providing the phosphate groups have been saturated at pH 7. Studies on the native and dephosphorylated whole casein give similar results at pH levels above 7.

Caseins (46, 47) are some of the few proteins which bind sodium and potassium ions. Carr and Engelstad (46) attribute this ability to bind alkali metals to concentration of high negative charges in the protein molecule. They isolated an octaphosphopeptide from casein containing two glutamic acids, two serine phosphates and two other carboxyl groups. They did sodium binding studies of the peptide, whole casein and phosvitin, all of which bound the sodium. They suggest that sites for the binding of small cations by casein arise from localization of negative charges in the form of phosphate and carboxyl grouping since they found the binding to be stronger than expected for an isolated phosphate group. They expect the binding to be of a relatively nonspecific type.

Ho and Waugh (47) studied by means of e.m.f. measurements the binding of sodium and potassium ions by alpha-s casein by addition of
salt to deionized protein, pH 4.8, and at a few higher pH values between 5.7 and 7.2. They did titration curves on dialyzed protein at ionic strengths 0.05 and 0.4 at 20°C. They suggest two classes of binding sites with \( n_1 = 2 \) and \( n_2 = 3 \) per 27,300 gm protein with 323 and 7.1 as binding constants, respectively. They make no specific site assignments. However, at pH 4.8 and \( 8.00 \times 10^{-2} \) M KCl there are 4.7 ions bound per 27,300 grams protein. They had precipitate formation over part of the titration curves. They found no decrease in binding below pH 7.5 as Carr and Engelstadt (46) found with whole casein.

Most proteins that contain only carboxylate ions as their acidic groups do not bind sodium ions. Exceptions are zein (48), myosin (49) and \( \beta \)-lactoglobulin (50). Baker and Saroff (50) found no binding of sodium ion at the isionic point of \( \beta \)-lactoglobulin despite a decrease in pH upon addition of NaCl to the deionized protein. With increasing pH the sodium binding increased to 4.28 per mole of protein at pH 9.48 and at 0.06 M sodium ion concentration. Employing the general electrostatic theory for protein binding (51) they determined a binding constant of 150-200 for a chelation site of sodium ion between a carboxyl and an imidizole side chain. They make an interesting comparison between calcium and sodium binding constants for the above type chelation in serum albumin and myosin and binding constants for various other types of ligands.

Sodium ions are considered to have a coordination number of four and calcium ions one of six (52). By comparison of the order of as-
sociation constants of calcium and sodium ions for various types of ligands, assuming the same site binds calcium and sodium, they propose the following structure for serum albumin which binds calcium ions (53) but not sodium ions (49).

They note that the ligands capable of forming the tetradentate and higher order complexes with calcium ion also complex sodium ion with constants ranging from 50 to 2000. Since the sodium binding constant for β-lactoglobulin is 150-200 and that for myosin, 1000-1600, they propose the following model for the binding of calcium and sodium ions to β-lactoglobulin and myosin.

The calcium binding constant of myosin is $10^{-4.7\pm0.3}$. The calcium binding constant of myosin is $10^{-5.3\pm0.3}$ a chelation such as Baker and Saroff propose might take place in alpha-s casein for calcium binding sites other than organic phosphate groups. He
also suggests that some phosphate groups may also participate in such a reaction.
EXPERIMENTAL

Preparation of Whole Casein.

Five liters of raw skim milk, which for our studies was obtained from The Ohio State University Dairy, is warmed to between 24 and 30°C and diluted with an equal volume of double distilled water. While being stirred, the diluted milk is then adjusted to pH 4.6 by adding dropwise 1 N hydrochloric acid. The precipitate is allowed to settle and most of the clear supernatant is syphoned off. The precipitate is then collected in two portions by filtering with suction through a large Buchner funnel using Whatman #40 paper. To facilitate the filtration, a plastic screen somewhat smaller than the filter paper was placed under the paper. The casein is then washed a total of five times with double distilled water. For each wash the precipitate is first suspended in a small amount of water and then diluted with a total volume of five liters of water. The resulting suspension is adjusted to pH 4.6, if necessary, stirred for at least 20 minutes, then allowed to settle and finally collected by filtration. If the above precipitation had been carried out at 20°C, centrifugation rather than filtration would have had to have been used because of the resulting smaller particulate size of the precipitate.

In order to remove lipids and some of the water, the washed whole casein is then treated five times with ethanol-ether (50-50)
by stirring the suspension in first 1400 ml. and finally 700 ml. of solvent, allowing the precipitate to settle and collecting the casein by filtration. This whole defatted casein is air dried for approximately 24 hours and then stored until use at \(-20^\circ\text{C}\).

**Preparation of Alpha-s Casein.**

Two hundred grams of whole defatted casein are dissolved in 3.3 liters of 6.6 M urea containing 21 grams sodium chloride per liter. Holding the temperature between 0 and \(4^\circ\text{C}\) while stirring the preparation, crude alpha-s casein is precipitated by diluting to 2.2 M urea and adjusting the pH to between 4.3 and 4.4. All urea solutions from this point on contain at 6.6 M urea concentration and 21 gm sodium chloride per liter. The precipitate, Fraction A-1 of Figure 1, is collected by centrifugation at 4100 xg for 30 minutes at \(2^\circ\text{C}\) solution temperature after at least one half hour additional stirring.

Fraction A-1 in Step 1 is dissolved in 1.5 liters of 6.6 M urea, diluted while stirring by adding dropwise 1.5 liters water, adjusted to pH 4.3, and then centrifuged at 4100 xg for 30 minutes at \(2^\circ\text{C}\), the supernatant being discarded. Steps 2, 3 and 4 are carried out under the same conditions of pH and temperature as Step 1 but adding only one liter each of urea and distilled water. Steps one through four must be done at \(4^\circ\text{C}\) or lower or there is much greater likelihood of beta casein contamination.

Step 5 is usually necessary in order to prepare a clear preparation. If urea is not present, the neutralization with potassium
200 grams crude defatted casein
dissolve in 3.3 l 6.6 M urea (NaCl)

0-4°C
2.2 M urea
pH 4.3-4.4
centrifuge 4100 xg min., 2°C

Fraction A-I (crude $\alpha_s$ casein) Fraction A-I
Supernatant
dissolve in 1.5 l 6.6 M urea (NaCl)

STEP 1
0-4°C
add slowly 1.5 l H2O
pH 4.3-4.4
centrifuge 4100 xg 30 min.

DISCARD

STEP 2, 3, 4
dissolve in 1.0 l 6.6 M urea (NaCl)
0-4°C
add slowly 1.0 l H2O
pH 4.3-4.4
centrifuge 4100 xg 30 min.


DISCARD

STEP 5
dissolve in 300 ml 6.6 M urea (NaCl)
neutralize with 1 N KOH
dilute to 2-2.5 M urea
centrifuge 35,000 xg 10-20 hrs.

SUBNATANT
alpha-s casein

dialyze
freeze-dry

DISCARD top layer and sediment

Fig. 1.—Outline for preparation of alpha-s casein from crude whole defatted casein.
hydroxide keeping the pH below 8, is very difficult and time consuming. After the centrifugation of Step 5, the clear subnatant is syphoned off and is dialyzed for approximately one week against many changes of cold double distilled water. The final product is lyophilized.

During dialysis the volume of the preparation becomes fairly large due to intake of water. If the lyophilization is done in two or three steps, a step consisting of dissolving and thawing the partially lyophilized protein and then refreezing and relyingophilizing it, the final product is more compact and much easier to handle.

Amino Acid Analysis.

Acid hydrolysis of the alpha-s casein was carried out primarily according to the procedure described by Moore and Stein (54). Protein aliquots of 0.5 ml containing 2.79 mg alpha-s casein and norleucine as an internal standard, were hydrolyzed in heavy walled Pyrex digestion tubes after addition of 0.5 ml concentrated hydrochloric acid. The tubes had been washed with chromic acid, rinsed well first with double distilled water and finally with 1 N HCl. The residual hydrochloride acid was removed in an air oven and the tubes stored inverted in a covered container to prevent deposition of ammonium chloride from the laboratory air.

Upon addition of the acid and using a small glass rod sealed inside the rim of the tube as a handle, the digestion tube is constricted at one point to about a one millimeter bore. The handle is removed and the lower half of the tube inserted in an alcohol-dry ice bath. The tube is then connected to a high vacuum system. When
the pressure is below 50 micron, the tube is withdrawn from the dry ice bath and the sample is slowly allowed to thaw allowing bubbles to form and repeating the process of freezing and thawing until gas removal is complete. The tube is then sealed off. The removal of traces of dissolved air is necessary for highest recovery of tyrosine. Hydrolysis is conducted at 110°C for 20, 40, 70 and 140 hours.

After the tube has cooled to room temperature, it is opened and the hydrolysate is transferred by means of several washings to a 50 ml round-bottom flask. The hydrochloric acid is removed under reduced pressure by attaching the flask to a rotary evaporator. The rinsing of the tube with distilled water followed by evaporation of the flask contents is repeated two more times.

Cysteine plus cystine is determined as cysteic acid after performic acid oxidation of the protein at -10°C according to the procedure of Hirs (55) followed by acid hydrolysis for 20 hours.

Hydrolysates were analyzed with a Tecnicon Amino Acid Analyzer in the laboratory of Dr. Hanns Gruemer of the Department of Pathology, The Ohio State University.

Tryptophane was determined by the spectrophotometric method of Goodwin and Morton (56).

**Determination of Amide Nitrogen.**

The amide nitrogen of the protein is determined at 35°C by a Conway (57) microdiffusion technique as applied by Stegemann (58) using alkaline hydrolysis with simultaneous diffusion of the ammonia into dilute sulfuric acid and nesslerization of the solution in the
same container. The diffusion was performed in a 50 ml pyrex beaker sealed by a Obrink (59) type glass cover. A blank determination is done on the sample after removal of the alpha-s casein by precipitation at pH 4.6.

**Chemical Analysis.**

A microdetermination of kjeldahl nitrogen was done using the nesslerization procedure of Lang (60). Phosphorus (61) and calcium (62) determinations were also done.

**Electrophoresis.**

Polyacrylamide-gel electrophoresis was done in Tris buffer at pH 9 and 4.5 M urea according to Peterson (63). Mercaptoethanol was added to the protein sample as suggested by Wozchik (64) in order to at least partially dissociate kappa casein so that it can enter the gel during electrophoresis. The vertical cell is similar to the E-C Apparatus Company commercially available cell. Instead of using water cooling, the electrophoresis runs were done in a refrigerated cold room.

**Deionization of Alpha-s Casein.**

A 2.9% alpha-s casein solution is deionized at room temperature in a two step procedure by adding Amberlite MB-1 ion exchanger resin (20-50 mesh, Mallenchrordt) while stirring the solution with a magnetic stirrer in a 100 ml nalgene beaker. When the pH has dropped to about 5.4 the solution becomes milky in appearance. The resin is then removed by centrifugation and the supernatant is refrigerated
overnight. The next morning the deionization is completed under a nitrogen atmosphere. The resin is removed by centrifuging it down in an International Clinical Centrifuge two times for five minutes each. The final protein concentration ranged usually from 2.1 to 1.6%. Attempt to use the Bio-Rad Laboratories mixed bed ion exchange resin that Ho and Waugh (47) used to deionize alpha-s casein, resulted in much greater loss of protein.

Titration of Alpha-s Casein.

All pH and millivolt measurements were made with a Corning Model 12 Expanded Scale pH meter. The pH meter has an accuracy of 0.002 pH units. Boiled glass double distilled water was used for the reagents and the titration studies. The studies were done in a temperature controlled (25 ± 0.2°C) nitrogen atmosphere box. Dry high purity nitrogen was first passed over ascarite and then through distilled water before entering the box.

Plexiglass dishes (inside diameter, 3 cm) with covers were used as containers. Each sample was usually a 0.99% protein solution in a total volume of nine milliliter. Protein concentration was determined by optical density measurements at 280 nm using a 0.05 M Tris - 0.05 M potassium citrate buffer adjusted to pH 7.3. The extinction coefficient, $E_{1\%}^{1\text{cm}}$, is 10.2. During addition of reagents and until shortly before electrode readings are taken, the contents of each dish are stirred using a 5/8 inch teflon coated bar magnet and a multiple magnetic stirrer. Any precipitate present is allowed to settle before e.m.f. measurements are taken.
The protein is delivered using a Gilmont type syringe in conjunction with a micrometer. This syringe has a nylon plunger whose only contact with the rest of the syringe is with a teflon or nylon washer which fits snugly on the plunger. The syringe was calibrated with both mercury and water and delivers approximately ten milliliter per plunger inch. Delivery was corrected for the very small irregularity of plunger diameter. Reproducibility was within 99.9%. It is necessary to use a Gilmont type syringe rather than a regular type syringe which has large plunger-barrel contact because the regular syringe freezes when delivering alpha-s casein even if the plunger is made of teflon. The protein will also precipitate in the glass delivery tip of the Gilmont syringe when applying pressure to the plunger if the tip orifice is too small.

Water, calcium chloride, calcium hydroxide, and hydrochloric acid are added to the protein solutions using syringes equipped with a micrometer. These syringes were calibrated with water and had precisions of 99.9% or greater. The approximate reagent concentrations of calcium chloride, calcium hydroxide, and hydrochloric acid are 0.1 M, 0.02 M, and 0.1 M, respectively. Because of the low solubility of calcium hydroxide, it is necessary to add the calcium chloride and calcium hydroxide separately. When not testing for reversibility of the system, the time lapse between reagent additions was less than five minutes. In all cases the protein and water were delivered to the plexiglass dishes before the above reagents. Some studies were done with tetraethylammonium chloride as a supporting
electrolyte.

Reversibility of experimental points is tested in a manner similar to that used by Tanford (65). By addition of calcium hydroxide the pH of the protein solution is raised to 10 and after 15 to 20 minutes the appropriate amount of hydrochloric acid is added. If the final pH and bound calcium agree with that obtained without prior pH 10 treatment, equilibrium with both hydrogen and calcium ions has been attained.

**Number of Hydrogen Ions Bound.**

A separate acid-base titration is done in the absence of alpha-s protein but otherwise at the same ionic strength as the protein titration. The difference in the amounts of acid or base required to change the pH in the presence and absence of protein is the amount of acid or base which is bound to the protein in going from the reference pH to the final pH (66). Hydroxyl ions bound are equal to hydrogen ions dissociated.

**Determination of Calcium and Chloride Activities.**

The Calcium Activity Electrode of Orion Research, Incorporated was used in conjunction with a calomel electrode for determination of calcium ion activity. The electrode was calibrated by using calcium chloride standards over the range of $2.5 \times 10^{-4}$ to $1 \times 10^{-1}$M. Both calcium and chloride ion activity coefficients were calculated from the equation developed by Shedlovsky (67) for the mean activity coefficient of calcium chloride solutions up to 0.1 M concentration.
The activity coefficient for calcium is the square and that for chloride ion, the square root of the mean activity coefficient.

A typical calibration curve for calcium ion is shown in Figure 2. The millivolt versus log calcium activity plot does not fall on a straight line but one that is curved slightly upward as $1 \times 10^{-1}$ M is approached. The calcium activity usually was within the range of $1 \times 10^{-3}$ to $1 \times 10^{-2}$ for the present studies.

The calcium electrode is stated to be insensitive to hydrogen ion over the pH range 5.5 to 11 but to respond to hydrogen ion below pH 4. Our results in the lower pH range, see Figure 3, indicate that the electrode can be used down to pH 4.5.

For determination of chloride ion activities, a silver, silver chloride electrode was standardized with the calcium chloride standards. The electrode had approximately a 10 to 15 minute response time.

**Calculation of Bound Calcium Ion and Chloride Ion.**

From the experimentally determined calcium and chloride activities of the sample, a first approximation of the ionic strength is made using the total calcium and total chloride concentration. The ionic strength was assumed independent of protein change and concentration. The hydrogen and hydroxyl concentrations were usually of little importance in the ionic strength calculation. Using the equation of Shedlovsky, first approximations for the calcium and chloride activity coefficients could be calculated. These could in turn be used to calculate molarity and a second approximation to the
Fig. 2.—Electrode potential as a function of calcium concentration, open circle, and calcium activity, x, for calcium chloride solutions.
Fig. 3.--Electrode potential as a function of solution pH for $10^{-1}$, $10^{-2}$, and $10^{-3}$ M calcium chloride solutions.
 Ionic strength. The process was continued until the calcium molarity agreed to within $2 \times 10^{-5}$ of the previous approximation.

The difference between the initial and final ion concentration divided by the protein concentration gives $5$, the number of ions bound per alpha-s casein molecule for each type of ion. The calculations are based on a molecular weight of 27,000 gm for alpha-s casein.
RESULTS

Casein Fractionation and Preparation of Purified Alpha-s Casein.

Our preparation procedure yields approximately 34 gm of alpha-s casein from 200 gm of crude air-dried casein. The flow chart for the fractionation has been given in Figure 1. The electrophoretic results are given in Figure 4. The electrophoretic pattern of the final alpha-s product indicates little if any contamination is present. The electrophoretic mobility indicates our preparation is probably on the genetic variant B.

The supernatant from Fraction A-1 may be further treated as indicated in Figure 5. The electrophoretic patterns of Fractions A-1, B and C are given in Figure 6. Fraction C contains most of the beta casein with slight kappa and gamma casein contamination. Also in Figure 6 are shown two patterns resulting from treatment of Fraction B in an attempt to prepare purified kappa casein after the procedure of Zittle (68).

Amino Acid Composition of Alpha-s Casein.

Hydrolysis of the protein was carried out for 20, 40, 70 and 140 hours. The recovery of some of the amino acids is dependent on hydrolysis time. Valine, isoleucine, glycine and alanine reached
Fig. 4. Polyacrylamide-gel electrophoresis patterns at pH 9 and 4.5 M urea. Patterns 1-4 are whole casein, and alpha-s casein Fraction A-1, Fraction A-II, and Fraction A-IV.
Fig. 5.—Outline of Fractionation of whole casein as a suggested initial step in purification of other caseins in addition to alpha-s casein.
Fig. 6.--Polyacrylamide-gel electrophoresis patterns at pH 9 and 4.5 M urea. Patterns 1-4 are whole casein, Fraction A-I, Fraction B, Fraction C. The last two fractions, 5 and 6, are from treating Fraction B by Zittle's procedure to obtain pure kappa casein.
maximal values only after 40 hours and thereafter remained constant within experimental error up to 140 hours. Serine and threonine were degraded during hydrolysis in 0.1 N HCl at 110°C and initial values were estimated by extrapolating to zero time. The remainder of amino acids including tyrosine, appeared to be stable up to 140 hours of hydrolysis. Figure 7 shows the hydrolysis time dependence for the above mentioned amino acids.

For amide nitrogen analysis, there was good agreement after 25 and 40 hours of hydrolysis time and so it was not necessary to extrapolate back to zero time. Stegemann (58) indicated that under the conditions of hydrolysis degradation of amino acid does not take place if cysteine is absent as is the case with alpha-s casein.

Table 1 gives a summary of the amino acid analysis of alpha-s casein. The results are expressed as numbers of amino acid residues per 27,000 grams of alpha-s casein. The total nitrogen percentage recovery based on amino acid analysis is 103 of the chemically determined value. Total grams of amino acid residues is 99.8 per 100 grams of protein. The value for phosphorus gives 9.5 phosphate groups per 27,000 grams of protein.

Table 2 given by McKenzie (11), gives a comparison of various amino acid analyses of alpha-s casein as determined by various workers. Our analysis appears to be in good agreement except for the number of glutamic acid residues recovered.
Fig. 7.—Amino acid recoveries from alpha-s casein as a function of hydrolysis time. The ordinate gives the recoveries in micromoles of amino acid per 2.79 gm of protein.
MICROMOLES AMINO ACID

h i

s e r i n e

v a l i n e

i s o l e u c i n e

g l y c i n e

a l a n i n e

a l a n i n e

t h r o n i n e

0.8

20 60 100 140

HOURS OF HYDROLYSIS
### TABLE 1

**AMINO ACID COMPOSITION OF OUR ALPHA-S CASEIN**

<table>
<thead>
<tr>
<th>Residues per 27,000 Grams</th>
<th>Gly</th>
<th>Cys/2</th>
<th>Ala</th>
<th>Met</th>
<th>Ser</th>
<th>Asp</th>
<th>Thr</th>
<th>Glu</th>
<th>Pro</th>
<th>Arg</th>
<th>Val</th>
<th>His</th>
<th>Ileu</th>
<th>Leu</th>
<th>Phe</th>
<th>Tyr</th>
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<td></td>
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<td>10.8</td>
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<td>15.5</td>
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<td>7.0</td>
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</table>

### TABLE 2

**AMINO ACID COMPOSITION OF ALPHA-S CASEIN: COMPARISON OF VARIOUS WORKERS' RESULTS**

(Residues per 27,000 grams)

<table>
<thead>
<tr>
<th>Variant:</th>
<th>a_1</th>
<th>a_{a_1 - BC}</th>
<th>a_{a_1 - A}</th>
<th>a_{a_1 - B}</th>
<th>a_{a_1 - C}</th>
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</thead>
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<td>(HBG)</td>
<td>(IW)</td>
<td>(KR)</td>
<td>(GBT)</td>
<td>(KR)</td>
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<tr>
<td>Gly</td>
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<td>17.8</td>
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<td>Total N (%)</td>
<td>14.1</td>
<td>14.7</td>
<td>15.01</td>
<td>15.10</td>
<td>14.37</td>
</tr>
<tr>
<td>Phosphorus (%)</td>
<td>0.85</td>
<td>1.0</td>
<td>1.12</td>
<td>1.01</td>
<td>1.12</td>
</tr>
</tbody>
</table>

* See McKinzie (11).
Isoionic Alpha-s Casein.

The deionized alpha-s casein is a milky-looking colloid which appears to be stable at room temperature for several hours and at 4°C up to approximately one week. In Table 3 is shown the change in pH observed upon dilution with water of an isoionic alpha-s casein preparation. The value of \( Z \), the charge per 27,000 grams alpha-s casein, has been calculated for each pH as shown later. For a 1% protein concentration the isoionic pH of our preparations is 5.06 ± 0.03. Ho and Waugh (47) reported a value of 5.16 ± 0.09 at 20°C. His protein concentration varied from approximately 0.25 -1%.

<table>
<thead>
<tr>
<th>Protein Percentage</th>
<th>pH</th>
<th>Charge, ( Z )</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.82</td>
<td>4.977</td>
<td>-0.009</td>
</tr>
<tr>
<td>1.47</td>
<td>5.015</td>
<td>-0.010</td>
</tr>
<tr>
<td>1.34</td>
<td>5.030</td>
<td>-0.010</td>
</tr>
<tr>
<td>1.24</td>
<td>5.043</td>
<td>-0.011</td>
</tr>
<tr>
<td>1.14</td>
<td>5.057</td>
<td>-0.011</td>
</tr>
<tr>
<td>0.99</td>
<td>5.080</td>
<td>-0.012</td>
</tr>
<tr>
<td>0.93</td>
<td>5.092</td>
<td>-0.013</td>
</tr>
<tr>
<td>0.88</td>
<td>5.105</td>
<td>-0.013</td>
</tr>
<tr>
<td>0.83</td>
<td>5.115</td>
<td>-0.014</td>
</tr>
</tbody>
</table>

Binding of Calcium Ions to Alpha-s Casein.

The calcium binding on deionized alpha-s casein was determined as a function of pH at a total ionic strength of 2 x 10^{-2}, assuming no binding of ions to the protein. The experimental results are summarized in Table 4. The total calcium and protein concentrations...
### TABLE 4

SUMMARY OF BINDING OF CALCIUM AND HYDROXYL IONS TO DEIONIZED ALPHA-S CASEIN IN CALCIUM CHLORIDE AT AN INITIAL IONIC STRENGTH OF 0.02.

<table>
<thead>
<tr>
<th>No.</th>
<th>pH</th>
<th>Total OH(^-) Added M x 10(^4)</th>
<th>Total Cl(^-) M x 10(^3)</th>
<th>Free Ca M x 10(^3)</th>
<th>Calcium Activity x 10(^3)</th>
<th>Per 27,000 grams alpha-s Casein</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>(\overline{V}_{\text{OH}})</td>
</tr>
<tr>
<td>1</td>
<td>4.749</td>
<td>8.78</td>
<td>12.45</td>
<td>6.37</td>
<td>3.76</td>
<td>2.40</td>
</tr>
<tr>
<td>2</td>
<td>4.968</td>
<td>17.57</td>
<td>11.55</td>
<td>6.08</td>
<td>3.63</td>
<td>4.80</td>
</tr>
<tr>
<td>3</td>
<td>5.143</td>
<td>26.35</td>
<td>10.66</td>
<td>5.75</td>
<td>3.48</td>
<td>7.18</td>
</tr>
<tr>
<td>4</td>
<td>5.313</td>
<td>35.13</td>
<td>9.88</td>
<td>5.41</td>
<td>3.32</td>
<td>9.55</td>
</tr>
<tr>
<td>5</td>
<td>5.417</td>
<td>43.92</td>
<td>8.95</td>
<td>5.15</td>
<td>3.18</td>
<td>11.93</td>
</tr>
<tr>
<td>6</td>
<td>5.598</td>
<td>52.70</td>
<td>8.06</td>
<td>4.80</td>
<td>3.03</td>
<td>14.32</td>
</tr>
<tr>
<td>7</td>
<td>5.758</td>
<td>61.48</td>
<td>7.16</td>
<td>4.24</td>
<td>2.74</td>
<td>16.70</td>
</tr>
<tr>
<td>8</td>
<td>5.928</td>
<td>70.27</td>
<td>6.36</td>
<td>3.81</td>
<td>2.51</td>
<td>19.07</td>
</tr>
<tr>
<td>9</td>
<td>6.209</td>
<td>79.05</td>
<td>5.46</td>
<td>3.28</td>
<td>2.04</td>
<td>21.4</td>
</tr>
<tr>
<td>10</td>
<td>6.418</td>
<td>83.41</td>
<td>5.04</td>
<td>2.72</td>
<td>1.90</td>
<td>22.6</td>
</tr>
<tr>
<td>11</td>
<td>6.508</td>
<td>87.83</td>
<td>4.57</td>
<td>2.50</td>
<td>1.76</td>
<td>23.8</td>
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<tr>
<td>12</td>
<td>6.798</td>
<td>92.16</td>
<td>4.15</td>
<td>2.28</td>
<td>1.64</td>
<td>25.0</td>
</tr>
<tr>
<td>13</td>
<td>7.011</td>
<td>96.62</td>
<td>3.67</td>
<td>2.08</td>
<td>1.51</td>
<td>26.2</td>
</tr>
<tr>
<td>14</td>
<td>7.301</td>
<td>100.9</td>
<td>3.27</td>
<td>1.91</td>
<td>1.42</td>
<td>27.3</td>
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<tr>
<td>15</td>
<td>7.518</td>
<td>105.4</td>
<td>2.78</td>
<td>1.74</td>
<td>1.30</td>
<td>28.5</td>
</tr>
<tr>
<td>16</td>
<td>8.920</td>
<td>114.2</td>
<td>1.97</td>
<td>1.73</td>
<td>1.32</td>
<td>30.8</td>
</tr>
<tr>
<td>17</td>
<td>9.648</td>
<td>123.0</td>
<td>1.07</td>
<td>1.73</td>
<td>1.32</td>
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<tr>
<td>18</td>
<td>10.102</td>
<td>133.6</td>
<td>0.0</td>
<td>1.33</td>
<td>0.95</td>
<td>35.5</td>
</tr>
</tbody>
</table>
for these studies are $6.68 \times 10^{-3}$ M and 0.99%, respectively. However, the calcium and hydroxide bound per 27,000 grams alpha-s casein were not found to be dependent on protein concentrations between 0.5 and 1.2%. Furthermore, there was no evidence of chloride binding in the pH range of this study. The chloride concentration calculated from the chloride activity agreed with the total chloride concentration.

The precision of the e.m.f. measurements is estimated to be about 0.2 mv. For the above studies when $\overline{v}_{Ca}$ is greater than 2 this corresponds to an error of less than 15% in $\overline{v}_{Ca}$. In other studies at higher calcium concentration, this may correspond to an error of 20%.

In Figure 8 is shown the titration curve of alpha-s casein using the data of Table 4 and, in addition, experimental points obtained by addition of acid to the calcium chloride-protein system. The total initial ionic strength was kept constant at $2 \times 10^{-2}$.

Below about pH 3.5 and at pH 9.6 and above there is no protein precipitation and the protein solution is clear. However, starting about pH 4 and going up to pH 5.6, over 98% of the protein precipitates. Above pH 5.6 the solubility increases, being approximately 70% in the pH range 7 to 7.5, and a 'milky' colloid is present. See Figure 9.

Alpha-s casein was found to be reversible to hydrogen and calcium ions as judged by the fact that after exposure for 15-20 minutes at pH 10, upon addition of acid the samples returned to points on the titration curve obtained without prior pH 10 treatment. See Figure 4.
Fig. 8.—Hydrogen ion titration curve of alpha-s casein at 25°C, initial ionic strength of calcium chloride equal to 0.02. Open circles are for direct titration points, x for alkaline reversed points.
MOLES BOUND \( \text{OH}^- \) PER 27000 GM \( \alpha_3 \) CASEIN
Fig. 9.—The percentage alpha-s casein precipitated as a function of pH at 25°C and at an initial ionic strength of calcium chloride equal to 0.02.
In the pH region from 6 to 8 there is a time dependent change in the titration curve, the change being complete in approximately 8 hours. All experimental values, unless stated otherwise, are those recorded approximately 24 hours after onset of the experiment and are reproducible. However, the rate of approach to equilibrium change is not reproducible and probably is dependent on the specifics of reagent addition. The above was the case when calcium hydroxide was added before the calcium chloride. If calcium chloride is added first, precipitate forms immediately and the pH and calcium activity are erratic for the first hour. In 24 hours the samples are identical regardless of whether calcium chloride or calcium hydroxide has been added first.

Figure 10 shows the relationship between $\bar{\nu}_\text{Ca}$ and pH using the data from Table 4. Most of the calcium binding takes place between pH 5 and 7, there being no increase in binding between pH 7.5 and 9.6. There is very little calcium binding below pH 5. When calcium chloride is added to isoionic alpha-s casein, the pH drops to 4.475 at 6.68 x $10^{-3}$ M CaCl$_2$ and 0.99% protein. The experimental $\bar{\nu}_\text{Ca}$ was 0.6 but this is so low that the error may be 50% or greater. On addition of calcium chloride to 2.24 or 3.40 x $10^{-2}$ M, the pH dropped to 4.39.

Studies were done to determine the maximum number of calcium binding sites. The results are shown in Figure 11. On the lower curve at pH 5.34-5.48 a maximum value of 9.5 for $\bar{\nu}_\text{Ca}$ is attained. The point on the upper curve at the lowest calcium concentration is #15 of Table 4 at pH 7.5 and is representative of the plateau region in Figure 9. The rightmost point on this same curve gives $\bar{\nu}_\text{Ca}$ equal
to 14.6 at pH 6.9. The middle line represents an intermediate pH, 5.86-5.98. The slope is much greater than the initial slope at pH 5.4. At pH 6.08 and 10.8 x 10^{-3} M CaCl_2, \( \overline{v}_{Ca} \), designated by x in the figure, falls near the upper curve. Binding studies at calcium chloride concentrations higher than 6.68 x 10^{-3} M are summarized in Table 5.

In experiments where 0.1 M tetraethylammonium chloride is used as a supporting electrolyte, at pH 5.37 protein concentration of .85\% and a total calcium concentration of 2.36 x 10^{-2} M, there is only 0.9 moles of calcium bound per 27,000 grams of alpha-s casein. This in contrast with a value of 3.43 for \( \overline{v}_{Ca} \) at pH 5.313 when only calcium is added to a total concentration of 6.68 x 10^{-3} M. At pH 9.0, a calcium concentration of 2.74 x 10^{-2} M, and protein concentration of 0.68\%, \( \overline{v}_{Ca} \) is 12.0. On addition of only the tetraethylammonium chloride to the alpha-s casein, the protein precipitates. However, \( \overline{v}_{Ca} \) is not dependent on order of reagent addition.
Fig. 10.--pH dependence of the binding of calcium ions to alpha-s casein at a total calcium concentration of $6.68 \times 10^{-3}$ M.
MOLES BOUND CALCIUM PER 27000 GM ALPHA-S CASEIN
Fig. 11.—Calcium binding of alpha-s casein as a function of calcium concentration and pH. Upper curve, pH 7.5-6.9; intermediate curve, pH 5.86-5.98; lower curve, pH 5.34-5.48.
<table>
<thead>
<tr>
<th>Number</th>
<th>Total Calcium Ion Added M x 10^2</th>
<th>Experimental</th>
<th>Calculated</th>
<th>Per 27,000 grams Alpha-s Casein</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>pH</td>
<td>Calcium Ion Activity x 10^3</td>
<td>Chloride Ion Activity x 10^2</td>
</tr>
<tr>
<td>19</td>
<td>0.942</td>
<td>5.726</td>
<td>3.55</td>
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<tr>
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<td>5.448</td>
<td>5.90</td>
<td>2.04</td>
</tr>
<tr>
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<td>5.421</td>
<td>7.40</td>
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</tr>
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<td>34</td>
<td>3.56</td>
<td>5.339</td>
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</tr>
<tr>
<td>35</td>
<td>3.52</td>
<td>6.833</td>
<td>11.7</td>
<td>..</td>
</tr>
</tbody>
</table>
DISCUSSION

The Average Net Charge per 27,000 Grams Alpha-s Casein.

An isoionic protein solution is defined (51) as a solution that contains, in addition to the protein, only those ions arising from dissociation of the solvent. In an isoionic solution then, the condition of neutrality is

\[ C_P \bar{Z} + C_{H^+} = C_{OH^-} \]  

(1)

where \( C_P \) is the molar concentration of the protein and \( C_{H^+} \) and \( C_{OH^-} \) are the concentration of hydrogen and hydroxyl ions, respectively. At low salt concentration activity and concentration should be almost identical. The charge, \( \bar{Z} \), of the protein is a function of pH since the equilibrium between the protein and hydrogen is maintained. Thus the isoionic pH must be dependent on protein concentration. In Table 3, the calculated charge of the isoionic protein is shown to be -0.01 using equation 1, the pH and the protein concentration.

The value of \( \bar{Z} \) for the experimental points of Table 4 has not been corrected for the residual charge of the isoionic protein since it is within the experimental error of the binding data. The
charge per 27,000 grams alpha-s casein was calculated from $\bar{v}_{OH}$ and $\bar{v}_{Ca}$ as follows:

$$Z = -\bar{v}_{OH} + 2\bar{v}_{Ca}$$

(2)

In figure 12 is shown the pH dependence of the charge on the alpha-s casein for the data in Table 4. In the region between pH 4.75 and 5.60 the charge becomes more negative as the pH increases since the dissociation of hydrogens is more than twice the binding of calcium. From pH 5.6 to 6.2 the additional binding of calcium is approximately equal to the dissociation of hydrogen and so the average net charge of the protein must become less negative. From pH 6.2 to 7.5 the binding of calcium nearly equals one half the dissociation of hydrogen and from pH 7.5 to 9.65 only dissociation of hydrogen takes place.

**Determination of the Total Number of Carboxyl Groups.**

The charge on the protein together with the amino acid results can be used to estimate the number of carboxyl groups titrated. The charge on the protein at any pH equals the total number of positive groups minus the total number of negative groups. At pH 8.920, assuming that the terminal amine, the histidines and the hydrogens from the second dissociation of the phosphate groups have dissociated,

$$\bar{Z} = -4.0 = -(COO^-) - 2 \times 9.5 \text{ phosphates} + 15.09 \text{ lys} + 6.97 \text{ arg}$$

$$+ 2 \times \bar{v}_{Ca}$$

Thus $$(COO^-) = 33.9$$
Fig. 12.—The dependence of the average net charge per 27,000 grams alpha-s casein as a function of pH at 25°C and at an initial ionic strength equal to 0.02 in calcium chloride.
AVERAGE NET CHARGE PER 27000 GM $\alpha_s$ CASEIN

![Graph showing the average net charge per 27000 GM $\alpha_s$ casein with pH values on the vertical axis and concentration values on the horizontal axis.]
The number of carboxyl groups titrated can also be determined by first considering the data of Figure 8 at low pH. At pH near 2, one would expect most of the first dissociation of the phosphate groups to be completed. There is a slight inflection of the titration curve at pH 2.4 which suggests that there are 2 phosphate groups that titrate between this pH and pH 2. Thus, it was estimated that 19.0 hydrogens, in addition to those involved in the first dissociation of the phosphate groups, are dissociated from the protein at the isoionic point. These 19.0 hydrogens would be dissociated mostly from the carboxyl groups and to a lesser extent from some phosphate groups that have lost a second hydrogen at the isoionic point.

If the assumptions are correct and 19.0 does not include any hydrogen from the first dissociation of phosphate, at pH 8.920 the carboxyls dissociated should also equal

\[
(COO^-) = 19.0H^+ + \sum OH - 5.6 \text{ hist} - 1 \text{ terminal NH}_2 - 9.5 \text{ phosphate (second dissociation)}
\]

\[= 33.7\]

At this high a pH, since the pK of the carboxyl group is usually near 4.6, it can be assumed all the carboxyl groups are dissociated and that alpha-s casein therefore has a total of 34 titrable carboxyls.
To check on the figure of 19, consider at the isoionic point,

\[
(\text{COO}^- + \text{phosphates dissociated second time}) = 5.6 \text{ hist} + \\
15.09 \text{ lys} + 6.97 \text{ arg} + 1 \text{ terminal NH}_2 - 9.5 \text{ (phosphates, first dissociation)} - Z(=0.0) \\
= 19.2
\]

and

\[
\overline{v}_{\text{OH}} = 19 + Z(=0.0)
\]

Similar calculations on other experimental points show similar agreement. The above calculations have been carried out on the assumption that the phosphates are all of the monoester form. If this were not the case, one would expect less agreement between the two methods of calculating the total number of titrable carboxyl groups.

From the amino acid data the total number of carboxyl groups should be

\[
47.90 \text{ Glu} + 17.13 \text{ Asp} - 27.3 \text{ Amide} = 37.7 \text{ Carboxyl}
\]

which is almost four more than we find by titration. As mentioned before our value for glutamic acid is about this much higher than results by other workers. However, Ho and Waugh (47) were not able to titrate, in the presence of KCl, all the carboxyl groups they had determined by amino acid analysis. Since we will be considering the titration data, 34 will be considered the total number of carboxyl groups.
Hydrogen Ion Equilibrium.

For proteins which may have many sites per molecule for binding ions, including hydrogen ion, instead of determining the $2^n - 1$ stepwise association constants for the $n$ binding sites as would be the approach on a much smaller molecule, an alternative representation of the equilibrium is usually used (51). If $n$ combining sites are identical and have no interaction, $n$ conventional equilibrium constants can be related to a single equilibrium constant, $k$. The assumption is usually made that all ionizable groups of a given type are intrinsically identical and the electrostatic interaction between charged sites can be considered as being dependent on the net average charge, $\overline{Z}$, of the protein molecule. Thus, at the isoelectric point where the average net charge is zero, the apparent hydrogen dissociation constant, $K_H$, is considered to be equal to the intrinsic dissociation constant, $K_{H}^{0}$. When the pH departs from the isoelectric point $K_H$ no longer equals $K_{H}^{0}$ but depends on the pH and an electrostatic interaction term must be used. The hydrogen dissociation of a group may then be analyzed by equation 3 (51)

$$\text{pH} - \log (\alpha_i / 1 - \alpha_i) = pK_{i}^{0} - 0.868 \times \overline{Z}$$

where $\alpha_i$ is the degree of dissociation of the hydrogen from the $i^{th}$ type of group and $\overline{Z}$ is the average net charge per macroion taking into account the binding of cations and anions. The value of $\omega$, the electrostatic interaction factor, is dependent on temperature, ionic strength, and size and conformation of the protein molecule and is
usually determined from the protein titration curve. Actually, according to the Debye-Hückel theory $e^{-2wZ_{\text{m}}}$ is a correction term for the bulk concentration or activity of an ion on close approach to the macromolecule. The quantity $2RTZ^2$ is the electrostatic work necessary to remove hydrogen ion from the surface of a protein molecule to infinity.

When all groups are considered to be smeared evenly over the surface of an impenetrable sphere

$$w = \frac{N \varepsilon^2}{2DRT} \left( \frac{1}{b} - \frac{1}{1-\kappa a} \right)$$

where $D$ is the dielectric constant of the solvent, $\varepsilon$ is the electronic charge, $\kappa$ is the Debye-Hückel parameter proportional to the square root of the ionic strength, and $a$ and $b$ are the hypothetical radii of ion exclusion and of the molecule, respectively. Other models have been mathematically derived for calculation of $w$ such as for a solvent-permeated spherical protein which results in a much lower value of $w$. Usually, however, $w$ is treated as an experimental electrostatic parameter required to fit the data (69) and for the above models should be independent of the group binding the ion.

The application of equation 3 to alpha-s casein is complicated by the fact that the degree of association of the protein changes with pH and that precipitation occurs over a portion of the titration curve. However, the equation can be applied if $\frac{Z}{Z}$ is considered to be the average charge per 27,000 grams alpha-s casein or per alpha-s monomer using 27,000 grams as the molecular weight, and the degree
of polymerization is incorporated into \( \omega \). Hydrogen ion equilibrium is assumed for alpha-s casein since the titration curve was found to be alkaline reversible. Under our experimental conditions, alpha-s casein was reversible to calcium ion also. This would suggest that the precipitate is highly hydrated and allows free passage of hydrogen, hydroxyl and unbound calcium ions.

If there is no ion binding of a group by ions other than hydrogen, the degree of dissociation, \( \alpha_1 \), in equation 3 equals the average number, \( \bar{F} \), of hydrogen ions dissociated divided by \( n_1 \), the total number of groups of a particular type. If part of a particular type of groups are bound, for instance by calcium, then the number bound to calcium are in effect removed from the hydrogen equilibrium and are not included in the calculation of \( \alpha_1 \) in equation 3.

According to equation 3 a plot of \( (\text{pH} - \log (\frac{\alpha}{1-\alpha})_1) \) vs. \( \bar{Z} \) will give \( pK_1 \) at \( \bar{Z} = 0 \) and the slope of the line at any pH within approximately 1 pH unit of the \( pK (70) \) will be equal to \(-0.868\omega\). If the particles are of constant degree of polymerization and conformation and over a pH range, are at constant ionic strength, the plot is expected to be linear in this range.

**First Approximation of the Electrostatic Factor in the pH Range 4.7 to 5.6.**

If equation 3 could be applied to the carboxyl groups of alpha-s casein, not distinguishing between alpha and side chain carboxyls, the value of \( \omega \) could be evaluated in the region where precipitate forma-
tion is 98% or greater when the total calcium concentration is $6.68 \times 10^{-3}$ M.

In order to use the carboxyls to find an estimate of $w$ an assumption is made that the carboxyl groups do not bind calcium to any extent. This assumption is supported by the experimental results shown in Figure 11. These results suggest that

1) competitive hydrogen binding appears to be important indicating that the groups involved must lose hydrogen before binding calcium.

2) there are at least two types of binding sites for calcium available in the pH region from 5 to 7.5.

3) the first group to ionize above pH 5 and to bind calcium appears to have a maximum of 9.5 sites available and may thus be assumed to the phosphate monoesters since chemical analysis gives 9.5 moles of phosphorus per 27,000 grams of protein.

These results are consistent with those of Yamauchi et al. (2) who dephosphorylated whole and alpha-s caseins. His results indicate that the phosphate groups begin to bind calcium as the pH is increased above 5 and, at pH 6 and above, that one or more additional types of groups are involved in calcium binding in addition to that due to phosphate esters.

The above evidence does not rule out the possibility that the carboxyl group may form a chelate of calcium with some other type of group ionizing at pH 6 or higher. In fact, salts of amino acid in-
Eluding glutamate will bind calcium by formation probably of a chelate structure (71)

\[
\text{CHR}_1 - \text{NH}_2 \text{R}_2 \\
\quad / \quad \text{Ca} \\
\text{CO} - 0
\]

The tendency to bind calcium is rapidly reduced by loading the glycine molecule with extraneous groups R1 and R2. Since the chelate carboxyl and N would be involved in peptide bonds in a protein molecule, the above chelate type would not, as such, be applicable. Saroff and Lewis (53) have proposed a carboxyl ion - imidazole or amino chelate for calcium ion but this would not be applicable in the pH range 4.7 to 5.4 since neither the imidazole or amino group would be expected to have lost their positive charge.

Considering the pH range 4.7 to 5.6 where alpha-s casein is over 98% precipitated when the total calcium concentration is \(6.68 \times 10^{-3}\) M, a first approximation for the dissociation, \(\alpha\), of the carboxyl groups was estimated as

\[
\alpha = (19 + \overline{\nu}_{\text{OH}} - \overline{\nu}_{\text{Ca}})/3^4
\]

This assumes no binding of calcium by carboxyl groups and only one calcium bound per binding site. The calculation of \(\alpha\) was done for the first six experimental points of Table 1 and an additional point obtained by the addition of isoionic casein of calcium chloride to a total concentration of \(6.68 \times 10^{-3}\) M. These values, in turn, were applied in equation (3), not correcting them for hydrogen dissociated
from other groups not bound to calcium. Figure 13 is a plot of

\[(\text{pH} - \log(\alpha/1-\alpha))\] versus \(Z\) for a first approximation of the pK of

the carboxyl groups and of \(w\), the electrostatic factor. Least

squares treatment gives values of 0.113 for \(w\) and 4.53 for the pK

of the carboxyl group. The experimental or final ionic strength has

not remained constant, but for the points one through six of Table

4 has ranged from \(1.9 \times 10^{-2}\) to \(1.4 \times 10^{-2}\). From examination of

Figure 13, however, the data are not sufficiently accurate to dif­

ferentiate in this range of ionic strength.

Consideration of Competitive Hydrogen and
Calcium Ion Binding at pH 4.7 to 5.6 and
Total Calcium of \(6.68 \times 10^{-3}\) M.

At calcium concentrations of \(2.5 \times 10^{-2}\) and higher and at pH

5.4, 9.5 moles of calcium is bound to 27,000 grams of alpha-s casein.
However, this does not rule out the possibility that calcium might
react with two phosphate groups at low calcium concentrations. This
possibility of intra- or inter- molecular phosphate - Ca - phosphate
salt bridges has been suggested repeatedly. This type of binding
takes place for zinc binding of insulin (72), at least at low zinc
concentrations. A calcium concentration of \(6.68 \times 10^{-3}\) M is just
above that reported by Noble and Waugh (27) as the limiting concen­
tration for alpha-s casein solubility in a 1\% protein concentration.

If the above type of binding applies to alpha-s casein and
there are \(n\) binding sites for the calcium at low calcium concen­
trations, each binding site consisting of a pair of phosphate groups
both of which can bind one hydrogen above pH 2-3, a fraction of these
Fig. 13--A plot of equation 3 for the first approximation of $w$ and $pK$ for the carboxyl groups. The intercept value is 4.53 and the slope gives a value of 0.113 for $w$. 

$\text{pH} = \text{LOG}(\alpha/(1-\alpha))$, for carboxyl groups.
sites, \( \overline{v}_{Ca}/n \), are occupied by calcium ions. The number of hydrogen binding sites is twice the number of metal binding sites and each bound metal removes two groups from the acid-base equilibrium assuming that each phosphate group must have a -2 charge for the metal binding site. This is a safe assumption since there is little binding of calcium below pH 5. The following equation would then apply (51)

\[
\frac{\overline{v}_{Ca}/(n - \overline{v}_{Ca})}{k} = ke^{-4wZ} a_{Ca}^{\alpha^2}
\]

where \( k \) is the intrinsic calcium binding constant, \( \overline{v}_{Ca} \), is the number of calcium ions bound to this type of site per molecule, \( a_{Ca} \) is the activity of the calcium ion and

\[
\alpha = \left( K_H^0 e^{2wZ/a_H} \right) / \left( 1 + K_H^0 e^{2wZ/a_H} \right)
\]

where \( K_H^0 \) is the second hydrogen intrinsic dissociation constant for the phosphate group and \( a_H \) is the activity if hydrogen ion which is assumed to be equal to \( 10^{-pH} \).

The applicability of this type of binding to the data of Table 4 for experimental points 2 through 6 can be tested if a pK for the second dissociation of the phosphate group and a value for \( \alpha \) is assumed. Österberg has determined an intrinsic dissociation of 5.3 for this phosphopeptide (40). This becomes 5.2 if pH is used rather than pch.

Calculations with equation 4 using \( pK_H^0 \) equal to 5.2 and \( \alpha \) equal to 0.113 gave values of \( k \) which were not constant but increased approximately 5-fold in the pH range 4.7 to 5.6. Thus at this and
higher calcium concentrations it can be concluded that calcium ion
does not form salt bridges between two phosphate groups.

Equation 6 applied to competitive hydrogen and calcium binding
when there is only one calcium bound to one phosphate group at each
site (51).

$$\frac{\bar{v}_{Ca}}{(n - \bar{v}_{Ca})} = \frac{k e^{-\frac{1}{2}wZ}}{(1 + \frac{a_{H}}{K_{H}^0} e^{2wZ})}$$

(6)

where n is the number of binding sites, in this case phosphate
groups which can bind calcium, and $K_{H}^0$ is the second intrinsic disso­
ciation constant of the phosphate group. This equation can be re­
arranged:

$$\bar{v}_{Ca} / (1 + \frac{a_{H}}{K_{H}^0} e^{2wZ}) = \frac{n k - k \bar{v}_{Ca}}{a_{Ca} e^{-\frac{1}{2}wZ}}$$

(7)

If the left hand side of the equation is plotted versus $\bar{v}_{Ca}$ the
resulting slope is equal to minus k and the intercept should equal
nk providing the equation is applicable to the data.

Österberg's value of the second intrinsic dissociation constant
of the phosphate groups as determined in 0.15 M KCl is lower than
expected. He assumed no binding of potassium ion by the phosphate
groups of the peptide but if this assumption is not correct, this
could lead to a low value. However, his data plotted according to
equation 3 using pH instead of pH, did not give evidence of potas­
sium binding. Fölsch and Österberg (73) have also determined at
0.15 M KCl the apparent second dissociation constant of the phosphate
group in O-phosphorylated peptides and related compounds. For the
peptide Gly-SerP-Gly the apparent second dissociation constant is $5.76 \pm 0.01$. This is approximately the mean of their values for Gly-SerP, 6.02, and SerP-Gly, 5.41. Their value for SerP is 5.67 also. They conclude that in phosphorylserine peptides second ionization of the phosphate group occurs at lower pH than that of other monophosphate esters. Apparently the phosphate groups of alpha-s casein ionize at an even lower pH than these simple di- and tri-peptides containing phosphorylserine.

For a better approximation of $w$ for the carboxyl groups, values of $w$ were calculated by successive approximation using equation 3 for the dissociation of hydrogens for the phosphate groups not bound to calcium and, of course, for the carboxyl groups, assuming no calcium binding to the latter. The carboxyl dissociation was also corrected for histidine dissociation using a $pK$ of 6.15 (66) for this group. This correction amounted at most to less than 0.5 hydrogens. Some results of least squares calculations are given in Table 6.

Since equation 6 gave reasonably good fit using first approximations for $w$ and $pK$ for the phosphate groups, it was further employed to calculate, by method of least squares, $n$ and $k$ at various $w$ and $pK$ values for the phosphate groups. Results of this type calculation are given in Table 7 for values of $n$ equal to 9.5.
TABLE 6

ESTIMATION OF \( w \) AND \( \text{pK}_H^\circ \) FOR THE CARBOXYL GROUP AT VARIOUS VALUES FOR THE SECOND HYDROGEN DISSOCIATION CONSTANT OF THE PHOSPHATE GROUPS.

<table>
<thead>
<tr>
<th>Second ( \text{pK}_H^\circ ) Phosphate Group</th>
<th>Calculated</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>( \text{pK}_H^\circ ) Carboxyl Group</td>
</tr>
<tr>
<td>5.00</td>
<td>4.68</td>
</tr>
<tr>
<td>5.15</td>
<td>4.65</td>
</tr>
<tr>
<td>5.20</td>
<td>4.64</td>
</tr>
<tr>
<td>5.25</td>
<td>4.63</td>
</tr>
<tr>
<td>5.31</td>
<td>4.62</td>
</tr>
<tr>
<td>5.34</td>
<td>4.62</td>
</tr>
<tr>
<td>5.46</td>
<td>4.63</td>
</tr>
</tbody>
</table>

TABLE 7

VALUES OF \( w \) AND \( k \) FOR THE BINDING OF CALCIUM IONS TO 9.5 PHOSPHATE GROUPS ASSUMING DIFFERENT DISSOCIATION CONSTANT FOR THE PHOSPHATES.

<table>
<thead>
<tr>
<th>Second Hydrogen Dissociation Constant, Phosphates Groups</th>
<th>Calculated</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>( w )</td>
</tr>
<tr>
<td>4.93</td>
<td>0.147</td>
</tr>
<tr>
<td>5.15</td>
<td>0.137</td>
</tr>
<tr>
<td>5.21</td>
<td>0.135</td>
</tr>
<tr>
<td>5.25</td>
<td>0.133</td>
</tr>
<tr>
<td>5.27</td>
<td>0.132</td>
</tr>
<tr>
<td>5.31</td>
<td>0.130</td>
</tr>
<tr>
<td>5.40</td>
<td>0.126</td>
</tr>
<tr>
<td>5.52</td>
<td>0.121</td>
</tr>
<tr>
<td>5.58</td>
<td>0.119</td>
</tr>
<tr>
<td></td>
<td>( k )</td>
</tr>
<tr>
<td></td>
<td>60</td>
</tr>
<tr>
<td></td>
<td>86</td>
</tr>
<tr>
<td></td>
<td>96</td>
</tr>
<tr>
<td></td>
<td>103</td>
</tr>
<tr>
<td></td>
<td>107</td>
</tr>
<tr>
<td></td>
<td>116</td>
</tr>
<tr>
<td></td>
<td>138</td>
</tr>
<tr>
<td></td>
<td>176</td>
</tr>
<tr>
<td></td>
<td>200</td>
</tr>
</tbody>
</table>
Österberg (2) calculated for data at 0.15 M KCl of the phosphopeptide of alpha casein a higher \( w \) factor for the phosphate groups, 0.071, than for the carboxyl groups, 0.044. If it is assumed in the protein molecule that \( w \) is the same for both types of groups, from inspection of Tables 6 and 7, \( w \) equals 0.133 and the second intrinsic dissociation constant for the phosphates is 5.25. If \( w \) is assumed to be higher for the phosphate groups then the second dissociation constant would be lower than 5.25. Since 5.25 is in close agreement with the value determined by Österberg (5.3 - 0.1 = 5.2) this value was used in subsequent calculations.

The plot of equation 6 for experimental points 2 through 6 of Table 4 using a \( pK^0 \) of 5.25 and \( w \) equal to 0.133, is shown in Figure 14. The data give a good fit. If another type of binding group was also involved in this pH range or, for example, there were 6 or 7 groups acting as binding sites, the data would give a very poor fit to the least squares line.

If no assumption had been made as to the value of \( n \) but a \( pK^0 \) of 5.20 (5.30) was assumed on the basis of Österberg's results, using a value of 0.133 for \( w \) from Table 6, the calculated values for \( n \) and \( k \) would be 9.7 (9.3) and 93 (115), respectively. The results indicate that all of the phosphate esters of alpha-s casein enter equally into the equilibrium binding of calcium at least up to pH 5.6 when the total calcium concentration is 6.68 x 10^{-3} M.

Values for \( \overline{V}_{Ca} \) were calculated for the first six experimental points of Table 4 and are compared with the experimental values in
Fig. 14.--A plot of equation 6 for determination of calcium bonding constant, $k$, to 0.133. Experimental points 2 through 6 of Table 4.
\[ \frac{W_{Ca}(1+10^{-pH} e^{-2wZ})/a_{Ca} e^{-4wZ}}{10^2} \]
Table 8. The calculations were done using $w$ equal to 0.133, and $n$ equal to 9.5. The agreement is within experimental error.

**TABLE 8**

**CALCIUM BINDING BY PHOSPHATE GROUPS OF ALPHA-S CASEIN**

<table>
<thead>
<tr>
<th>Number</th>
<th>pH</th>
<th>$\overline{v}_{Ca}$ experimental</th>
<th>$\overline{v}_{Ca}$ calculated</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>4.749</td>
<td>0.83</td>
<td>1.00</td>
</tr>
<tr>
<td>2</td>
<td>4.968</td>
<td>1.64</td>
<td>1.69</td>
</tr>
<tr>
<td>3</td>
<td>5.143</td>
<td>2.50</td>
<td>2.45</td>
</tr>
<tr>
<td>4</td>
<td>5.313</td>
<td>3.43</td>
<td>3.24</td>
</tr>
<tr>
<td>5</td>
<td>5.417</td>
<td>4.18</td>
<td>4.20</td>
</tr>
<tr>
<td>6</td>
<td>5.598</td>
<td>5.08</td>
<td>5.20</td>
</tr>
</tbody>
</table>

Österberg has determined the calcium binding constant for $\beta$-phosphorylserine at 25°C and an ionic strength of 0.15 in KCl. For amino group positively charged and uncharged the binding constants are 27 and 155, respectively. In both of these cases the carboxyl group is negatively charged. In 0.1 M KCl at 29°C values for glycerophosphate and fructose phosphate were 46 and 30, respectively. At the same experimental conditions values for AMP, ADP and ATP were 26, 600 and 4000 respectively (75). Values for AMP$^-2$, ADP$^-3$ and ATP$^-4$ at 25°C and 0.2 M $(n - \text{C}_3H_7)_4N^+$ are reported as 27, 642 and 1970, respectively (76). The ADP and ATP probably may be considered as bidentate and tridentate ligands.
The Effect of Tetraethylammonium Chloride on the Binding of Alpha-s Casein.

Tetraethylammonium chloride at a concentration of 0.1 M was added as a supporting electrolyte in one series of experiments in order that the ionic strength remain fairly constant. This higher ionic strength should lower the electrostatic factor and result in greater hydrogen dissociation at a given pH. At pH 5.3 and a total calcium concentration of $2.47 \times 10^{-2}$ M if 1) the calcium activity is $8.9 \times 10^{-3}$, 2) $\nu$ equals approximately 0.03 and 3) there is no binding of tetraethylammonium ions to phosphate groups, then $\bar{v}_{Ca}$ by calculation should be 7.5 which is almost an order higher than observed. With no binding of tetraethylammonium ion $\bar{Z}$ would be -27 at this pH. With this magnitude of charge, precipitation of the protein would not be expected. This result at pH 5.3 and that at pH 9 suggest that some tetraethylammonium ion may be bound by some of the phosphate with additional binding perhaps by carboxyl groups.

There is some support for this type of binding in the literature. Smith and Alberty (32) report that the $pK_a$ for adenosine phosphate was higher in the presence of $(n-C_3H_7)_4N^+$ than in the presence of either $(CH_3)_4N^+$ or $(C_2H_5)_4N^+$ which suggests that there is some binding by the phosphate group of at least the latter two ions. In addition, Strauss and Ross (77) have reported competitive binding of alkali and tetramethylammonium ion by long chain polyphosphates.
Application of the Smeared Charge Model at Total Calcium Concentrations Above 6.68 x 10^{-3}.

Experimental data, numbers 1 through 6 of Table 4 and 19 through 22 of Table 5 are all at a final calcium activity between 3.76 x 10^{-3} and 3.18 x 10^{-3}. Since the electrostatic factor, \( w \) equal to 0.133, was able to be applied in this range of activity, \( \overline{\nu}_{Ca} \) for phosphate groups was calculated for numbers 19 through 22 which are in a pH range 5.7-6.1. The protein is precipitated in this region as it is in the pH range 4.7 to 5.6. Thus if there is no configuration change and the theory is applicable, calculated values of \( \overline{\nu}_{Ca} \) for the phosphate groups should give reasonable values. The calculated calcium bound to phosphate groups drops from 5.08 at pH 5.598 to approximately 3.3 to 4.3 in the pH range 5.7 to 6.1. These low calculated values are due to the less negative values of the net charge of the protein in this pH range. As a rough guess values for calcium bound to phosphate for numbers 20, 21, and 22 might be about 7, 8 and 9, respectively.

### Table 9

<table>
<thead>
<tr>
<th>Number</th>
<th>Activity Calcium Ion x 10^3</th>
<th>pH</th>
<th>( \overline{Z} )</th>
<th>Experimental ( \overline{\nu}_{Ca} )</th>
<th>Calculated ( \overline{\nu}_{Ca} )</th>
</tr>
</thead>
<tbody>
<tr>
<td>19</td>
<td>3.55</td>
<td>5.726</td>
<td>-2.1</td>
<td>9.7</td>
<td>3.9</td>
</tr>
<tr>
<td>20</td>
<td>3.50</td>
<td>5.860</td>
<td>-1.5</td>
<td>11.2</td>
<td>3.5</td>
</tr>
<tr>
<td>21</td>
<td>3.59</td>
<td>5.984</td>
<td>-2.1</td>
<td>12.1</td>
<td>4.3</td>
</tr>
<tr>
<td>22</td>
<td>3.49</td>
<td>6.084</td>
<td>-1.1</td>
<td>13.8</td>
<td>3.3</td>
</tr>
</tbody>
</table>
The smeared charge model also fails, as might be expected for the rest of the experimental data of Table 5 most of which have a positive charge. The calculated values of calcium bound to phosphate range in value from 1.7 for number 23 to 5.1 for number 35.

**Binding of Calcium by Group(s) other than Phosphate Esters.**

The binding of calcium by a group or groups other than the phosphate esters appears to be quite strong above pH 5.4. See Figure 11. The number of groups involved \((14.6 - 9.5 = 5.1)\) is approximately equal to the number of histidine groups in alpha-s casein as determined by amino acid analysis. The pH region of calcium binding is also in the range where histidine should be ionizing. However, imidazole \((50)\) has a low affinity for calcium ion but a tri- or tetradentate chelate such as Baker and Saroff \((50)\) have proposed for the calcium binding by \(\beta\)-lactoglobulin and myosin may be an appropriate model for the binding by groups other than the 9.5 phosphate groups. However, further consideration of this second type binding will not be given here due to the failure of the smeared charge model binding theory to account for the phosphate binding at calcium concentrations above \(6.68 \times 10^{-3}\) M and pH above 5.6.

**Local Electrostatic Interactions**

Failure of the smeared charge model might be due to lack of consideration of local electrostatic interactions. The partial
structure (40) of two of the peptides in the amino half of Österberg's original trypsin phosphopeptide are as follows:

P1

Asp-(SerP, Ileu)-(Asp, ThrP, Gly, Glu2)-(SerP, Glu)

P2

Glu-Ala-SerP-SerP-(SerP₂, Ileu₂, Glu₂, Asp, Val₂, Lys, Pro)-GluNH₂

These two peptides contain seven phosphoamino acid residues.

In accord with results on synthetic phosphorylpeptides, the amino terminal sequence of P1 is believed to be Asp-SerP-Ileu rather than Asp-Ileu-SerP. Thus each of the three phosphoamino groups of P1 is probably adjacent to either one or two glutamic or aspartic residues. The sequence of two of the phosphoserine groups of P2 is uncertain. At least two of the phosphoserine residues are adjacent to each other. The remaining two may be arranged such that there are four consecutive phosphoserine groups. There is also the possibility that P2 contains two pairs of phosphoserine groups or that two of the groups are adjacent to aspartic or glutamic acid residues.

Hill (78) has considered the problem of intermolecular and intramolecular interactions between independent pairs of binding sites in proteins or other molecules. He states that his results suggest that the usual discussion of electrostatic effects on titration curves of proteins in terms only of the net charge of the entire molecule may overlook important local electrostatic interactions. In his treatment he has considered binding of only one type of ion.
In the following treatment his approach is followed and enlarged to include binding of two different kinds of ions.

Consider a pair of residues composed of a phosphomonoester, P, and a carboxyl group, C. Let o, h, and c refer to unbound residue, hydrogen ion bound residue and calcium ion bound residue, respectively. For example, CoPc refers to a pair of residues with the carboxyl group unbound (R-COO⁻) and calcium bound to the phosphate group (R'-OPO₄²⁻Ca⁺³). It will be assumed that only hydrogen ion can bind to the carboxyl group and either hydrogen ion or calcium ion can bind to the phosphate group. Let W refer to the free energy of interaction between the two residues which are separated by a distance r. At r equal to infinity, W equals zero. Let the first lower case letter following W indicate the bound state of the carboxyl group and the second lower case letter indicate the bound state of the phosphate residue. Then the free energy of interaction for the CoPc pair is Woe. The resulting possibilities for the types of pairs, the free energy of interaction of the pair and the charges per residue are as follows:

<table>
<thead>
<tr>
<th>pair</th>
<th>free energy of interaction</th>
<th>charges</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>C</td>
</tr>
<tr>
<td>CoPo</td>
<td>Woo</td>
<td>-1</td>
</tr>
<tr>
<td>CoPh</td>
<td>Woh</td>
<td>-1</td>
</tr>
<tr>
<td>ChPo</td>
<td>Who</td>
<td>0</td>
</tr>
<tr>
<td>ChPh</td>
<td>Whh</td>
<td>0</td>
</tr>
<tr>
<td>CoPc</td>
<td>Woc</td>
<td>-1</td>
</tr>
<tr>
<td>ChPc</td>
<td>Whc</td>
<td>0</td>
</tr>
</tbody>
</table>

It is assumed that Who = Whh = Woc = Whc = 0, Woo = 2 Woh and that $W_{ij}$ is the product of the charges at the two residues divided by the
product of $D$, the effective dielectric constant, and $r$, the distance
between the charges. This is treating the charges as point charges
and not taking into account the ion dipole interactions.

Each pair of sites is considered as a system in a grand ensem­
ble. The ensemble consists of a very large number of pairs or systems.
Let $J_{lh}$ be the partition function including the binding energy for
the binding of hydrogen ion at a carboxyl residue and similarly, $J_{2h}$
and $J_{2c}$ refer, respectively, to hydrogen ion and to calcium ion
bound at the phosphate group. Then the cannonical ensemble partition
functions for the pairs are

\[ Q_{oo} = e^{-W_{oo}/kt} = e^{-2W_{oh}/kt} \]
\[ Q_{hh} = J_{lh}J_{2h} e^{-W_{hh}/kt} = J_{lh}J_{2h} \]
\[ Q_{oh} = J_{2h} e^{-W_{oh}/kt} \]
\[ Q_{ho} = J_{lh} e^{-W_{ho}/kt} = J_{lh} \]
\[ Q_{oc} = J_{2c} e^{-W_{oc}/kt} = J_{2c} \]
\[ Q_{hc} = J_{lh}J_{2c} e^{-W_{hc}/kt} = J_{lh}J_{2c} \]

where the lower case letters following $Q$ are assigned as for $W$, the
free energy of interaction.

The grand partition function is thus

\[ \Xi = Q_{oo} + Q_{hh} + Q_{oh} + Q_{ho} + Q_{oc} + Q_{hc} \]

where $\lambda_c = e^{\mu_c/kT}$ and $\lambda_h = e^{\mu_h/kT}$ and $\mu$ is the chemical potential
of the bound ion. If $a$ is the activity of the ion then

\[ \mu = \mu_{\text{SoLn}} = \mu^* + kT \ln a \]
Using the lower case letters in the same manner as for $Q$, from the fundamental distribution law of the grand ensemble

$$
\theta_{oo} = e^{-2W_{oh}/kT} / \Xi
$$

$$
\theta_{oh} = J_{2h} e^{-W_{oh}/kT} \lambda_{h} / \Xi
$$

$$
\theta_{hh} = J_{1h} J_{2h} \lambda_{h}^2 / \Xi
$$

$$
\theta_{ho} = J_{1h} \lambda_{h} / \Xi
$$

$$
\theta_{oc} = J_{2c} \lambda_{c} / \Xi
$$

$$
\theta_{hc} = J_{1h} J_{2c} \lambda_{h} \lambda_{c} / \Xi
$$

where $\theta$ is the probability for the particular type binding designated by the lower case letters. Then the total probability, $\theta_{1}$, that the carboxyl group of the pair is occupied by a hydrogen is thus

$$
\theta_{1} = \theta_{hh} + \theta_{ho} + \theta_{hc} = (J_{1h} J_{2h} \lambda_{h}^2 + J_{1h} \lambda_{h} + J_{1h} J_{2c} \lambda_{h} \lambda_{c}) / \Xi
$$

and, for the phosphate group, the probability that it is occupied by hydrogen is

$$
\theta_{2h} = \theta_{hh} + \theta_{oh} = (J_{1h} J_{2h} \lambda_{h}^2 + J_{2h} e^{-W_{oh}/kT} \lambda_{h}) / \Xi
$$

or by calcium is

$$
\theta_{2c} = \theta_{oc} + \theta_{hc} = (J_{2c} \lambda_{c} + J_{1h} J_{2c} \lambda_{h} \lambda_{c}) / \Xi
$$

The carboxyl intrinsic dissociation constant, $K_{1}$, for hydrogen is

$$
K_{1} = a_{H} (1 - \theta_{1}) / \theta_{1} = a_{H} / J_{1h} \lambda_{h} = e^{-\mu_{H}^{*}/kT} / J_{1h}
$$
where the superscript on $\theta_1^0$ refers to $r = \infty$ ($w's = 0$) and $a_\text{H}$ is the activity of the hydrogen ion. Similarly for the phosphate group, the second intrinsic dissociation constant, $K_2^h$, for hydrogen is

$$K_2^h = a_\text{H} \left(1 - \theta_2^0 - \theta_2^c\right)/\theta_2^0 = a_\text{H}/J_2^h \lambda_\text{H} = e^{-\mu_\text{H}/kT}/J_2^h$$

and

$$K_1/K_2^h = J_2^h/J_1^h$$

The phosphate intrinsic binding constant, $k$, for calcium is

$$k = \theta_2^c/a_\text{Ca} \left(1 - \theta_2^c - \theta_2^h\right) = J_2^h \lambda_\text{Ca}/a_\text{Ca}$$

where $a_\text{Ca}$ is the activity of the calcium ion.

The grand partition function and also $\theta_2^c$ may then be expressed in terms of $K_1$, $K_2^h$, $a_\text{H}$, $a_\text{Ca}$, and $k$.

$$\theta_2^c = ka_\text{Ca} \left(1 + a_\text{H}/K_1\right)/\Xi$$

$$\theta_2^c = ka_\text{Ca} \left(1 + a_\text{H}/K_1\right)/(e^{-2W_\text{oh}/kT} + a_\text{H}/K_1K_2^h + a_\text{H}e^{-W_\text{oh}/kT}/K_2^h + a_\text{H}/K_1 + ka_\text{Ca} \left(1 + a_\text{H}/K_1\right))$$

Since $\theta_2^c$ equals $\bar{\nu}_\text{Ca}/n$ where $\bar{\nu}_\text{Ca}$ and $n$ are the average number of bound calcium ions and the total number of calcium binding sites per molecule, respectively,

$$\bar{\nu}_\text{Ca}/(n - \bar{\nu}_\text{Ca}) = ka_\text{Ca} \left(1 + a_\text{H}/K_1\right)/(e^{-2W_\text{oh}/kT} + (a_\text{H}/K_1K_2^h)

+ a_\text{H}e^{-W_\text{oh}/kT}/K_2^h + a_\text{H}/K_1)$$
If the activity of calcium and hydrogen ion is multiplied by $e^{-4wZ}$ and $e^{-2wZ}$, respectively, the equation reduces, as it should, to the relationship

$$\bar{v}_{Ca}/(n - \bar{v}_{Ca}) = k_{Ca} e^{-4wZ}/(1 + a_{He} e^{-2wZ}/K)$$

for the binding of calcium ion to the phosphate ester when local electrostatic free energy is not considered but instead it is assumed that the electrostatic free energy of the macromolecule may be expressed in terms of the net average charge, $\bar{Z}$, of the macromolecule and $w$, the electrostatic factor. $K$ is the second hydrogen ion dissociation constant for the phosphate group.

Pairs of Interacting Phosphate Groups.

A treatment similar to the above one can be made for a grand ensemble in which a system consists of a pair of phosphate groups. The two phosphate groups of the pair are distinguished on the basis of position. Lower case letters are used as in the previous treatment.

<table>
<thead>
<tr>
<th>Pair</th>
<th>Free Energy of Interaction</th>
<th>Charges</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>$P$</td>
</tr>
<tr>
<td>PoPo</td>
<td>$W_{oo} = 4W_{hh}$</td>
<td>-2</td>
</tr>
<tr>
<td>PoPh</td>
<td>$W_{oh} = 2W_{hh}$</td>
<td>-2</td>
</tr>
<tr>
<td>PhPo</td>
<td>$W_{ho} = W_{oh}$</td>
<td>-1</td>
</tr>
<tr>
<td>PhPh</td>
<td>$W_{hh}$</td>
<td>-1</td>
</tr>
<tr>
<td>PhPc</td>
<td>$W_{ch} = 0$</td>
<td>0</td>
</tr>
<tr>
<td>PcPh</td>
<td>$W_{hc} = 0$</td>
<td>0</td>
</tr>
<tr>
<td>PcPo</td>
<td>$W_{co} = 0$</td>
<td>0</td>
</tr>
<tr>
<td>PoPc</td>
<td>$W_{oc} = 0$</td>
<td>-2</td>
</tr>
<tr>
<td>PcPc</td>
<td>$W_{cc} = 0$</td>
<td>0</td>
</tr>
</tbody>
</table>
The canonical partition functions for the pairs are

\[
\begin{align*}
Q_{oo} &= e^{-\frac{W_{hh}}{kT}} \\
Q_{oh} &= \beta_{2h} e^{-\frac{W_{hh}}{kT}} = \beta_{h} e^{-\frac{2W_{hh}}{kT}} \\
Q_{ho} &= \beta_{1h} e^{-\frac{2W_{hh}}{kT}} = \beta_{h} e^{-\frac{2W_{hh}}{kT}} = Q_{oh} \\
Q_{hh} &= \beta_{1h} \beta_{2h} e^{-\frac{W_{hh}}{kT}} = \beta_{h}^2 e^{-\frac{W_{hh}}{kT}} \\
Q_{hc} &= \beta_{1h} \beta_{2c} = \beta_{hc} \\
Q_{ch} &= \beta_{2h} \beta_{1c} = \beta_{ch} \\
Q_{oc} &= \beta_{2c} = \beta_{o} \\
Q_{co} &= \beta_{2c} = \beta_{oc} \\
Q_{cc} &= \beta_{1c} \beta_{2c} = \beta_{c}^2
\end{align*}
\]

where \( \beta_{1h} = \beta_{2h} = \beta_{h} \) and \( \beta_{1c} = \beta_{2c} = \beta_{c} \). The grand partition function is thus

\[
\begin{align*}
\mathcal{Z} &= Q_{oo} + 2Q_{oh} \lambda_{h} + Q_{hh} \lambda_{h}^2 + 2Q_{hc} \lambda_{c} + 2Q_{oc} \lambda_{c}^2 + Q_{cc} \lambda_{c}^2 \\
&= e^{-\frac{W_{hh}}{kT}} + 2 \beta_{h} \lambda_{h} e^{-\frac{2W_{hh}}{kT}} + \beta_{h}^2 \lambda_{h}^2 e^{-\frac{W_{hh}}{kT}} + t + 2 \beta_{h} \lambda_{c} \lambda_{c} + 2 \beta_{c} \lambda_{c}^2 + \beta_{c}^2 \lambda_{c}^2
\end{align*}
\]

From the fundamental distribution law of the grand ensemble

\[
\begin{align*}
\theta_{oo} &= e^{-\frac{W_{hh}}{kT}} / \Xi \\
\theta_{oh} &= \theta_{ho} = \beta_{h} \lambda_{h} e^{-\frac{2W_{hh}}{kT}} / \Xi \\
\theta_{hh} &= \beta_{h}^2 \lambda_{h}^2 e^{-\frac{W_{hh}}{kT}} / \Xi \\
\theta_{ch} &= \theta_{hc} = \beta_{h} \lambda_{c} / \Xi \\
\theta_{oc} &= \theta_{co} = \beta_{c} \lambda_{c} / \Xi
\end{align*}
\]
The probability, $\theta_h$, that a given phosphate is occupied by a hydrogen ion is

$$\theta_h = \theta_{ho} + \theta_{hh} + \theta_{hc} = \theta_{oh} + \theta_{hh} + \theta_{ch}$$

$$= (J_n\lambda_h \frac{e^{-2Whh/kt}}{\lambda} + J_h\lambda_h^2 \frac{e^{-Whh/kt + J_hJ_c\lambda_h^c}}{\lambda}) / \Xi$$

Similarly the probability, $\theta_c$, that a given phosphate is occupied by a calcium ion is

$$\theta_c = \theta_{oc} + \theta_{hc} + \theta_{cc} = \theta_{co} + \theta_{ch} + \theta_{cc}$$

$$= (J_c\lambda_c + J_hJ_c\lambda_h^c + J_c^2\lambda_c^c) / \Xi$$

In addition, the phosphate secondary dissociation constant, $K$, for hydrogen ion is

$$K = a_h (1 - \theta_h^c - \theta_h^c) / \theta_h^c = a_h / J_h\lambda_h$$

and the calcium intrinsic binding constant equals

$$k = \theta_c^c / (1 - \theta_c^c - \theta_c^c) a_{Ca} = (J_c\lambda_c + J_hJ_c\lambda_h^c + J_c^2\lambda_c^c) / (1 + J_h\lambda_h + J_c\lambda_c) a_{Ca}$$

or

$$a_{Ca} \cdot k = J_c\lambda_c$$

The grand partition function and also $\theta_h$ and $\theta_c$ may then be
expressed in terms of the activities of the ions, K and k.

\[ \mu = e^{-\frac{Whh}{kt}} + 2a_H e^{-\frac{2Whh}{kt}}/K + a_H^2 e^{-\frac{Whh}{kt}}/K^2 + \\
2a_H a_{Ca} K/K + 2ka_{Ca} + k^2 a_{Ca}^2 \]

\[ \theta h = (a_H e^{-\frac{2Whh}{kt}}/K + a_H^2 e^{-\frac{Whh}{kt}}/K^2 + a_H^2 a_{Ca}/K)/\mu \]

\[ \theta c = \frac{\bar{v}}{n} = ka_{Ca} (1 + a_H/K + ka_{Ca})/\mu \]

On rearrangement of \( \theta c \),

\[ k = \frac{(-H + (H^2 + lB)\frac{1}{2})}{2} \]

where \( H = (1 + a_H/K) \frac{(1 - \bar{v}/(n - \bar{v}))}{a_{Ca}} \)

and

\[ B = (e^{-\frac{Whh}{kt}} + 2a_H e^{-\frac{2Whh}{kt}}/K + a_H^2 e^{-\frac{Whh}{kt}}/K^2) \cdot \\
(\bar{v}/(n - \bar{v})) \cdot (1/a_{Ca}^2) \]

In the absence of local electrostatic interaction (Whh = 0), rearranging \( \theta c \) yields

\[ \frac{\bar{v}}{n - \bar{v}} = \frac{ka_{Ca} (1 + a_H/K + ka_{Ca})}{(1 + 2a_H/K + a_H^2/K^2 + \\
a_H^2 a_{Ca}/K + ka_{Ca})} \\
= \frac{ka_{Ca}}{(1 + a_H/K)} \]

Thus is the activity of calcium ion is multiplied by \( e^{-\frac{WhZ}{2}} \) and that of hydrogen ion by \( e^{-2wZ} \), the above equation is identical to the relationship for the binding of calcium ion when it is assumed that the electrostatic free energy of the macromolecule may be expressed in terms of \( Z \) and \( w \).
The carboxyl-phosphate pair and the phosphate-phosphate pair may be considered as independent subsystems which can exchange energy, calcium and hydrogen ions. Then at equilibrium the chemical potential of the calcium ion or the hydrogen ion is the same in all subsystems and the distributions developed above for the carboxyl-phosphate pair and the phosphate-phosphate pair are still applicable.

For example, suppose that there are six carboxyl-phosphate pairs and two phosphate-phosphate pairs per alpha-s molecule. Then

\[ \bar{\nu}_{\text{Ca}} = 6\, \theta_{\text{nc}} \text{(carboxyl-phosphate pair)} + 4\theta_{\text{c}} \text{(phosphate-phosphate pair)} \]

In Appendix I a similar derivation is made for a cluster of four phosphate groups.

Comparison of Models for Calcium Binding by Phosphate Esters at Low Calcium Concentration.

Various models for localized electrostatic interaction were considered. Calculations were made on the assumption that there were ten phosphate groups per molecule and the calculated results were then multiplied by 0.95 so that they could be compared with the experimental values. For calculation of the interaction energy in the localized electrostatic theory, a value for the dielectric constant, \(\varepsilon\), must be assigned and also a value for \(r\), the distances between charges. In the calculations no assumption was made about an effective dielectric constant. Instead 78.5 was used for \(\varepsilon\) with \(r\) equal to 3.6 Å, giving a product of 282.6.

Comparison of models with experimental data at low salt concen-
trations in the calcium activity range of $3.03 \times 10^{-3}$ to $3.76 \times 10^{-3}$ are shown in Table 10. The first localized model considered, C-P10, is a set of ten carboxyl-phosphate pairs for the molecule. A binding constant, $k$, equal to 35 has been used since it gives a fairly good fit below pH 5.7. For the other models C-P8P2 refers to 8 carboxyl-phosphate pairs plus one phosphate-phosphate pair for the molecule. Similarly C-P6P2 refers to 6 carboxyl-phosphate pairs plus two phosphate-phosphate pairs and C-P6Cl4 refers again to six carboxyl-phosphate pairs and one cluster of four phosphates. In the lower part of the table is given the fraction of phosphate ester binding calcium, using a binding constant of 35 for comparison. In the statistical thermodynamic equations for the models, calcium activity has been multiplied by $e^{-\mu_{\text{Ca}}}^{-\mu Z}$ and hydrogen activity by $e^{-2\mu Z}$ using a value of 0.133 for $w$ for the calculations in Table 10.

The first three localized models give fairly good fit for the experimental points below pH 5.7. At pH 5.7 and above they give low values for $\tilde{v}_{\text{Ca}}$ bound to phosphate. If it is assumed that the other calcium binding group(s) has become saturated with calcium for number 22 then 8.6 calcium ions should be bound to the phosphate ester groups. Thus 8.6 can be considered a minimum value. For number 22 the highest value for the models is 6.65 for C-P10 while the C-P8P2 model gives 6.35. Both of these results are improvement over the smeared charge model which gives only 3.29. Values for number 21 for the models are all higher than for 22. This is because of the activity correction terms used which are functions of the charge $\overline{Z}$. 
**TABLE 10**

**COMPARISON OF MODELS FOR CALCIUM BINDING BY PHOSPHATE ESTERS AT CALCIUM ACTIVITY LEVELS OF 3.03-3.76 \times 10^{-3}**

<table>
<thead>
<tr>
<th>Number</th>
<th>pH</th>
<th>$Z$</th>
<th>Activity Calcium Ion ( \times 10^3 )</th>
<th>Experimental $\tilde{V}_{Ca}$</th>
<th>Calcium Bound, Calculated</th>
<th>Smeared Charge ( k = 103 )</th>
<th>Local Electrostatic Interaction</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>C-P 10 ( k = 35 )</td>
<td>C-P6P2 ( k = 35 )</td>
</tr>
<tr>
<td>1</td>
<td>4.749</td>
<td>-0.74</td>
<td>3.76</td>
<td>0.83</td>
<td>1.00</td>
<td>...</td>
<td>...</td>
</tr>
<tr>
<td>2</td>
<td>4.968</td>
<td>-1.52</td>
<td>3.63</td>
<td>1.64</td>
<td>1.69</td>
<td>1.32</td>
<td>1.72</td>
</tr>
<tr>
<td>3</td>
<td>5.143</td>
<td>-2.18</td>
<td>3.48</td>
<td>2.50</td>
<td>2.45</td>
<td>2.18</td>
<td>2.52</td>
</tr>
<tr>
<td>4</td>
<td>5.313</td>
<td>-2.69</td>
<td>3.32</td>
<td>3.43</td>
<td>3.24</td>
<td>3.23</td>
<td>3.45</td>
</tr>
<tr>
<td>5</td>
<td>5.417</td>
<td>-3.57</td>
<td>3.18</td>
<td>4.18</td>
<td>4.20</td>
<td>4.18</td>
<td>4.31</td>
</tr>
<tr>
<td>6</td>
<td>5.598</td>
<td>-4.16</td>
<td>3.03</td>
<td>5.08</td>
<td>5.20</td>
<td>5.51</td>
<td>5.50</td>
</tr>
<tr>
<td>19</td>
<td>5.726</td>
<td>-2.1</td>
<td>3.55</td>
<td>9.7</td>
<td>3.92</td>
<td>5.60</td>
<td>5.48</td>
</tr>
<tr>
<td>20</td>
<td>5.860</td>
<td>-1.5</td>
<td>3.50</td>
<td>11.2</td>
<td>3.51</td>
<td>5.89</td>
<td>5.75</td>
</tr>
<tr>
<td>21</td>
<td>5.984</td>
<td>-2.1</td>
<td>3.59</td>
<td>12.1</td>
<td>4.34</td>
<td>6.84</td>
<td>6.50</td>
</tr>
<tr>
<td>22</td>
<td>6.084</td>
<td>-1.1</td>
<td>3.49</td>
<td>13.8</td>
<td>3.29</td>
<td>6.65</td>
<td>6.35</td>
</tr>
</tbody>
</table>

**Fraction of Phosphate Ester Binding Calcium for k = 35**

<table>
<thead>
<tr>
<th>Cluster of Four Phosphates</th>
<th>Pair of Phosphate Esters</th>
<th>Carboxyl-Phosphate Pair</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>0.39</td>
<td>0.34</td>
</tr>
<tr>
<td>3</td>
<td>0.53</td>
<td>0.41</td>
</tr>
<tr>
<td>4</td>
<td>0.58</td>
<td>0.46</td>
</tr>
<tr>
<td>Number</td>
<td>Cluster of Four Phosphates</td>
<td>Pair of Phosphate Esters</td>
</tr>
<tr>
<td>--------</td>
<td>----------------------------</td>
<td>--------------------------</td>
</tr>
<tr>
<td>5</td>
<td>0.63</td>
<td>0.51</td>
</tr>
<tr>
<td>6</td>
<td>0.68</td>
<td>0.55</td>
</tr>
<tr>
<td>19</td>
<td>0.63</td>
<td>0.53</td>
</tr>
<tr>
<td>20</td>
<td>0.60</td>
<td>0.52</td>
</tr>
<tr>
<td>21</td>
<td>0.65</td>
<td>0.55</td>
</tr>
<tr>
<td>22</td>
<td>0.56</td>
<td>0.53</td>
</tr>
</tbody>
</table>
The behavior of the carboxyl-phosphate pair in contrast to the cluster or pair of phosphates is interesting. When only phosphate groups are involved a larger fraction of phosphate ester binds at a lower pH and a smaller fraction at a higher pH as compared to the carboxyl-phosphate pair. The experimental pH dependence of calcium binding suggests that an improved model would be one with one phosphate pair plus clusters of phosphate-carboxyls which might have more pronounced but similar C-P pair characteristics. Examination of Österberg's phosphopeptide PI indicates that the C-P pair can only be a poor approximation to the actual electrostatic interaction in this group of ten amino acid residues, eight of which can be negatively charged.

Comparison of Models for Calcium Binding by Phosphate Esters at Higher Calcium Concentration.

In Table 11 are given results of calculations similar to those in Table 10 but for higher calcium concentrations. For the smeared charge model $w$ has been set equal to zero in order to give the highest possible calculated values for $k$ equal to 103. For the rest of the calculations $k$ has been adjusted for fit and $w$ has been arbitrarily set to 0.05 and 0.01, the latter for the higher ionic strengths. On the basis of ionic strength alone $w$ would be expected to fall below 0.133. However, Ho and Waugh (47) determined values of $w$ ranging from 0.20 to approximately 0.02 at ionic strengths of 0.05 and 0.4 (KCl) in the range where precipitate formation took place and where the charge varied from zero to four. The carboxyl groups for this
<table>
<thead>
<tr>
<th>Number</th>
<th>Activity Calcium Ion x 10^3</th>
<th>pH</th>
<th>Z</th>
<th>Experimental U_Ca</th>
<th>Calcium Bound, Calculated</th>
<th>Local Electrostatic Interaction</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Smeared Charge</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>C-P 10</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>k = 103</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>w</td>
</tr>
<tr>
<td>23</td>
<td>6.93</td>
<td>4.895</td>
<td>4.0</td>
<td>4.3</td>
<td>0.0</td>
<td>1.7</td>
</tr>
<tr>
<td>24</td>
<td>6.80</td>
<td>5.101</td>
<td>5.1</td>
<td>5.1</td>
<td>2.1</td>
<td>6.2</td>
</tr>
<tr>
<td>26</td>
<td>6.18</td>
<td>5.760</td>
<td>2.4</td>
<td>10.0</td>
<td>3.1</td>
<td>6.2</td>
</tr>
<tr>
<td>27</td>
<td>4.38</td>
<td>5.549</td>
<td>0.8</td>
<td>7.6</td>
<td>2.2</td>
<td>6.2</td>
</tr>
<tr>
<td>28</td>
<td>5.41</td>
<td>5.466</td>
<td>0.7</td>
<td>7.5</td>
<td>2.4</td>
<td>6.6</td>
</tr>
<tr>
<td>29</td>
<td>5.90</td>
<td>5.448</td>
<td>1.5</td>
<td>7.9</td>
<td>2.6</td>
<td>6.5</td>
</tr>
<tr>
<td>31</td>
<td>9.25</td>
<td>5.424</td>
<td>4.5</td>
<td>9.4</td>
<td>3.4</td>
<td>7.4</td>
</tr>
<tr>
<td>32</td>
<td>9.00</td>
<td>7.362</td>
<td>-0.4</td>
<td>14.0</td>
<td>4.6</td>
<td>7.6</td>
</tr>
<tr>
<td>33</td>
<td>11.4</td>
<td>5.370</td>
<td>4.5</td>
<td>9.4</td>
<td>3.8</td>
<td>7.7</td>
</tr>
<tr>
<td>34</td>
<td>12.2</td>
<td>5.339</td>
<td>4.7</td>
<td>9.5</td>
<td>3.9</td>
<td>7.8</td>
</tr>
<tr>
<td>35</td>
<td>11.7</td>
<td>6.833</td>
<td>0.6</td>
<td>14.6</td>
<td>5.1</td>
<td>8.0</td>
</tr>
</tbody>
</table>
study appeared to be completely dissociated at the higher calcium concentrations so there was no basis for estimating w.

The protein under experimental conditions of those in Table 11 carries a near zero or positive charge, but nevertheless strongly binds calcium as is evident by the much larger binding constants needed to give a reasonable fit of the models to the data. At as low a pH as 5, points 23 and 24, half of the phosphate esters bind calcium ion in spite of the fact that the protein has a 4-5 positive charge. The binding constants for the models in Table 11 are about 6-fold higher than those used in Table 10.

This abrupt change in binding intensity suggests that possibly binding sites have, in concerted action, changed from a unidentate to a bidentate structure which, however, does not allow any increase in binding capacity. Baker and Saroff (50) have suggested as explanation for increased binding constants of myosin and β-lactoglobulin as compared to serum albumin, an increase from bidentate to tetradentate structure for their chelation site. The above suggested transition from one type binding to another involving steric or conformational changes could be classified as an allosteric transition (79, 80).
SUMMARY

A low temperature (0-4°C) urea fractionation of whole casein has been developed for the isolation and purification of alpha-s casein. Acrylamide gel electrophoresis indicates little or no contamination of the protein. Amino acid analysis was done.

Deionized alpha-s casein was used for calcium binding studies in calcium chloride at 25°C. An ion exchange electrode was used to measure calcium activity. The isoionic pH is 5.06 ± 0.03 at 0.99% protein concentration. The binding of calcium and hydrogen is alkaline reversible and is time dependent from pH 6 to 8. Equilibrium values were attained after eight hours. At 6.68 x 10^{-3} M and higher calcium concentrations only one calcium ion will bind per phosphate ester. There is at least one other type of calcium binding group in alpha-s casein. No chloride binding takes place at least above pH 4.7 even at higher calcium concentrations when the protein becomes positively charged.

At calcium concentrations of 6.68 x 10^{-3} M below pH 5.7 the intrinsic binding constant is 103 and all the phosphate groups appear to be equivalent and in equilibrium. However, local electrostatic interactions appear to be important at higher calcium concentrations or pH values. Electrostatic interaction models are examined. There
is a discontinuity in bonding constant between a calcium ion activity of $3.5 \times 10^{-3}$ and of $4.4 \times 10^{-3}$ and above. A suggested explanation for this approximately six-fold increase in binding intensity is a change in dentate structure of the bonding site.
APPENDIX I

CLUSTER OF FOUR PHOSPHATE ESTERS

Each cluster of four phosphate ester sites is considered as a system in a grand ensemble. The ensemble consists of a very large number of clusters or systems. Let $J_c$ and $J_h$ be the partition function including the binding energy for the binding of calcium ion and hydrogen ion, respectively, to a phosphate ester group. In addition, the nearest neighbor and next to nearest neighbor interaction energies are both designated by $W$ which is followed by two lower case letters indicating the states of the two particular phosphate groups interacting where $o$, $h$ and $x$ refer to unbound, hydrogen ion bound and calcium ion bound residues. An overbar and an underbar on the two lower case letters designate nearest and next to nearest neighbor interactions, respectively. If there is interaction between the first and fourth phosphate residues, the interaction energy is designated by a $T$ followed by two lower case letters as above. As an example, a cluster, oxho, has interaction energies $W_{oo}$, $W_{ho}$ and $W_{oo}$. Let $\bar{r}$ equal twice the spacing between adjacent phosphate residues. Then

$$W_{hh} = W$$

$$W_{oo} = 8 \ W_{hh} = 8W$$
\[
\begin{align*}
W_{hh} &= 2W_{hh} = 2W \\
W_{cc} &= 4W_{cc} = 4W \\
W_{cc} &= 4W \\
W_{cc} &= 2W
\end{align*}
\]

\[
Whh = 2Whh = 2W \\
Wcc = 4Whh = 4W \\
Wcc = 4W \\
Wcc = 2W
\]

\[
Thh = 0.67W \\
Tho = 1.33 W \\
Too = 2.67
\]

In Table 12 are given the canonical partition functions for the various states of the cluster where \( k \) equals the boltzman constant. As before \( Jc = k_a^c \) and \( Jh = a/H^c \), where \( k \) refers to the calcium binding constant.

For the grand ensemble \( N_j \), the average number of particles of type \( j \) per system is

\[
N_j = \frac{\sum_{N_{j,i}} N \lambda_j^N \exp (-E_{N_j}/kT)/Z_i}{\sum_{N_{j,i}} \sum_i \sum_1}
\]

where \( \sum_{N_{j,i}} = \sum_{N_j} \sum_i \sum_1 \) and \( \sum_1 \) is over all states containing a particular number of particles, \( N_j \). When \( j \) equals \( c \) (\( h \)), \( N_j \) equals the number of calcium ions (hydrogen ions) bound per state.

If each of the canonical partition function is multiplied by \( \lambda_c^{N_c} \) and \( \lambda_h^{N_h} \) where \( \lambda_c = e^{\mu_c/kT} \) and \( \lambda_h = e^{\mu_h/kT} \), summation of these terms over all states equals the grand partition function,
TABLE 12

THE CANNONICAL PARTITION FUNCTIONS FOR THE STATES OF A CLUSTER OF
FOUR PHOSPHATE ESTERS.

<table>
<thead>
<tr>
<th>Number of Calciums Bound</th>
<th>State</th>
<th>Canonical Partition Function</th>
</tr>
</thead>
<tbody>
<tr>
<td>none</td>
<td>oooo</td>
<td>exp(- (3Woo + 2Woo)/kT)</td>
</tr>
<tr>
<td></td>
<td>hooo + ooooh</td>
<td>2 Jh exp(-(2Woo + Woo + WTh + WTh + Thh)/kT)</td>
</tr>
<tr>
<td></td>
<td>ooho + ohoo</td>
<td>2 Jh exp(-(Woo + 2Woo + WTh + WTh + Thh)/kT)</td>
</tr>
<tr>
<td></td>
<td>hhoo + ohhh</td>
<td>2 Jh^2 exp(-(WTh + WTh + Woo + 2WTh + Thh)/kT)</td>
</tr>
<tr>
<td></td>
<td>hoho + ohoh</td>
<td>2 Jh^2 exp(-(3Woo + WTh + WTh + Thh)/kT)</td>
</tr>
<tr>
<td></td>
<td>hoooh</td>
<td>Jh exp(-(2WTh + WTh + 2WTh + Thh)/kT)</td>
</tr>
<tr>
<td></td>
<td>hhho + ohhh</td>
<td>2 Jh^3 exp(-(2WTh + WTh + WTh + WTh + Thh)/kT)</td>
</tr>
<tr>
<td></td>
<td>hhhh</td>
<td>Jh^4 exp(-(3WTh + 2WTh + Thh)/kT)</td>
</tr>
<tr>
<td>One</td>
<td>xooo + oox</td>
<td>2 Jc exp(-(2Woo + WTh)/kT)</td>
</tr>
<tr>
<td></td>
<td>xhoo + ooxh</td>
<td>2 Jc Jh exp(-(Woo + WTh + WTh)/kT)</td>
</tr>
<tr>
<td></td>
<td>xoho + oohx</td>
<td>2 Jc Jh exp-(2WTh + WTh)/kT)</td>
</tr>
<tr>
<td></td>
<td>xooh + hoox</td>
<td>2 Jc Jh exp(-(Woo + WTh + WTh)/kT)</td>
</tr>
<tr>
<td></td>
<td>xhho + ohhx</td>
<td>2 Jc Jh^2 exp(-(WTh + WTh + WTh)/kT)</td>
</tr>
<tr>
<td></td>
<td>xhoh + hohx</td>
<td>2 Jc Jh^2 exp(-(2WTh + WTh)/kT)</td>
</tr>
<tr>
<td></td>
<td>xooh + kheox</td>
<td>2 Jc Jh^2 exp(-(Woo + WTh + WTh)/kT)</td>
</tr>
<tr>
<td>Number of Calciums Bound</td>
<td>State</td>
<td>Canonical Partition Function</td>
</tr>
<tr>
<td>--------------------------</td>
<td>------------------------</td>
<td>-------------------------------------------------------</td>
</tr>
<tr>
<td></td>
<td>xhh + hhx</td>
<td>2 $Jc Jh^2 \exp\left(-\frac{(2Whh + Whh)}{kT}\right)$</td>
</tr>
<tr>
<td></td>
<td>oxoo + ooxo</td>
<td>2 $Jc \exp\left(-\frac{(Woo + Wsh + Too)}{kT}\right)$</td>
</tr>
<tr>
<td></td>
<td>hxoo + ooxh</td>
<td>2 $Jc \exp\left(-\frac{(Woo + Whh + Too)}{kT}\right)$</td>
</tr>
<tr>
<td></td>
<td>oxho + ohxo</td>
<td>2 $Jc \exp\left(-\frac{(Woo + Whh + Too)}{kT}\right)$</td>
</tr>
<tr>
<td></td>
<td>oxoh + hoxo</td>
<td>2 $Jc \exp\left(-\frac{(Woo + Whh + Too)}{kT}\right)$</td>
</tr>
<tr>
<td></td>
<td>hxho + ohxh</td>
<td>2 $Jc \exp\left(-\frac{(Woo + Whh + Too)}{kT}\right)$</td>
</tr>
<tr>
<td></td>
<td>oxhh + hhoxh</td>
<td>2 $Jc \exp\left(-\frac{(Woo + Whh + Too)}{kT}\right)$</td>
</tr>
<tr>
<td></td>
<td>hxhh + hhxh</td>
<td>2 $Jc \exp\left(-\frac{(Woo + Whh + Too)}{kT}\right)$</td>
</tr>
<tr>
<td></td>
<td>xhoo + oxox</td>
<td>2 $Jc \exp\left(-\frac{(Woo)}{kT}\right)$</td>
</tr>
<tr>
<td></td>
<td>xxho + ohxx</td>
<td>2 $Jc \exp\left(-\frac{(Woo)}{kT}\right)$</td>
</tr>
<tr>
<td></td>
<td>xoxh + hxxh</td>
<td>2 $Jc \exp\left(-\frac{(Woo)}{kT}\right)$</td>
</tr>
<tr>
<td></td>
<td>xxhh + hhxx</td>
<td>2 $Jc \exp\left(-\frac{(Woo)}{kT}\right)$</td>
</tr>
<tr>
<td></td>
<td>oxox</td>
<td>$2 Jc^2 \exp\left(-\frac{Woo}{kT}\right)$</td>
</tr>
<tr>
<td></td>
<td>xhhx</td>
<td>$2 Jc^2 \exp\left(-\frac{Woo}{kT}\right)$</td>
</tr>
<tr>
<td></td>
<td>xoxh + xohx</td>
<td>2 $Jc^2 \exp\left(-\frac{Woo}{kT}\right)$</td>
</tr>
<tr>
<td></td>
<td>hxox + oxhx</td>
<td>4 $Jc^2 \exp\left(-\frac{Woo}{kT}\right)$</td>
</tr>
<tr>
<td></td>
<td>oxoo</td>
<td>$2 Jc^2 \exp\left(-\frac{Too}{kT}\right)$</td>
</tr>
<tr>
<td></td>
<td>hxxx</td>
<td>$2 Jc^2 \exp\left(-\frac{Toh}{kT}\right)$</td>
</tr>
<tr>
<td></td>
<td>hxox + oxoxh</td>
<td>2 $Jc^2 \exp\left(-\frac{Toh}{kT}\right)$</td>
</tr>
<tr>
<td></td>
<td>oxhh</td>
<td>$2 Jc^2 \exp\left(-\frac{Toh}{kT}\right)$</td>
</tr>
<tr>
<td></td>
<td>hxxh</td>
<td>$2 Jc^2 \exp\left(-\frac{Toh}{kT}\right)$</td>
</tr>
</tbody>
</table>
TABLE 12—Continued

<table>
<thead>
<tr>
<th>Number of Calciums Bound</th>
<th>State</th>
<th>Canonical Partition Function</th>
</tr>
</thead>
<tbody>
<tr>
<td>Three</td>
<td>xxxo + oxxx</td>
<td>$2 \ J_c^3$</td>
</tr>
<tr>
<td></td>
<td>xxxh + hxxx</td>
<td>$2 \ J_c^3 \ J_h$</td>
</tr>
<tr>
<td></td>
<td>xxxx + xxxx</td>
<td>$2 \ J_c^3 \ J_h$</td>
</tr>
<tr>
<td></td>
<td>xxhx + xhxx</td>
<td>$2 \ J_c^3 \ J_h$</td>
</tr>
<tr>
<td>Four</td>
<td>xxxx</td>
<td>$J_c^4$</td>
</tr>
</tbody>
</table>
<pre><code>                      |               | $2 \ J_c^3 \ J_h$           |
                      |               | $2 \ J_c^3 \ J_h$           |
                      |               | $2 \ J_c^3 \ J_h$           |
</code></pre>
LIST OF REFERENCES


