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INTERACTION SYSTEMS.

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INTERACTION SYSTEMS

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

By
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* * * * *

The Ohio State University
1970

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This investigation was supported in part by Public Health Service Research Grant No. FD-00108 from the Food and Drug Administration, Washington, D. C.
This work is dedicated to my parents, my wife Radha, and our daughters Rina and Hena.
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INTRODUCTION

This work represents an inquiry into the microstructure of interacting food components to develop an understanding of complex food systems. In the simplest sense, food represents varying combinations of carbohydrates, lipids, proteins and electrolytes in water, and exhibits a complex array of body, texture and consistency characteristics. In any given food system, these properties are intimately related to the organization of the constituent macromolecules.

Macromolecules of naturally occurring food components exist in varying degrees of association, from the relatively simple starch granules in grains and tubers, to the intricate protein bodies in seeds and in milk. The nature and stability of these different complexes are largely determined by the properties of the components and the environmental conditions involved in the association phenomena.

Modern practices in industrial food manufacturing have to a great extent been developed with an awareness of the importance of food component interactions. This has been particularly so in the area of protein stability in processed foods and has directly or indirectly led to the development of stabilizer technology which now serves an important function in food science and technology.
The use of many stabilizer gums as food modifiers has become an accepted practice and their selection has traditionally been based on their thickening ability or viscosity effects. However, the demonstrated protein-stabilizing ability of certain hydrocolloids has added to the growing awareness that these polysaccharides also function by interacting with other food components. The mechanism by which these additives confer stability can therefore not be explained merely in terms of viscosity effects without careful consideration of the nature of such complex formation. The underlying thought of this study has been to obtain information about the food protein stability as it relates to major interactions with polysaccharides.

In the present investigation the microstructure of complexes prepared from selected proteins and hydrocolloids were observed in the electron microscope. The study has shown that the physical stability of the protein hydrocolloid complex is functionally related to the structural features of the interacting macromolecules. The protein stabilizing property of polysaccharides rests in part on their ability to provide functional groups with high protein reactivity but also on their characteristic conformation in the system which promotes localization of such protein reactivity into a relatively few interaction centres.
TERMINOLOGY

A. Contamination - Special artifacts formed due to the interaction of the electron beam with organic molecules resulting in the deposition of an amorphous, carbonaceous layer of contaminant on the specimen. Contamination reduces image resolution, induces configurational changes and causes the formation of pebble-like features, obscuring structures of interest. Contamination may be minimized by using special decontamination devices, the application of only minimum amount of vacuum grease in the gaskets and valves and also by the examination of specimens with a beam of reduced intensity.

B. Contrast - Contrast is produced by the differential loss of intensity of the incident electron beam during its transmission through the specimen and support material. Poor specimen visibility occurs unless such loss in intensity is more than 5% of the intensity of incident beam. Under a given set of accelerating voltage and objective aperture, contrast depends on the specimen density and is therefore related to atomic number of elements. Consequently, heavy metals and their compounds are used for their deposition on specimen surface by evaporation and also as staining reagents. Desired contrast may be achieved by the interaction of the
staining reagents with the specimen, in POSITIVE STAINING, or with the background surrounding the specimen, as in NEGATIVE STAINING.

C. Electron Density - The specimen contrast depends on the inherent electron density of specimens; the higher the electron density, the greater the loss in the intensity of the incidental beam. Relative electron density of specimen enables its visualization and also differentiation of structural elements that vary considerably in this regard.

D. Fixation - of biological preparations is often necessary to impart stability to specimens to be examined with electron beam in the microscope. The fixative action is largely due to the inter- and intra-molecular cross-linking of the reactive organic functional groups. The commonly used fixatives in electron microscopy are osmium tetroxide and aldehydes such as formaldehyde and glutaraldehyde.

E. Focusing - Specimen image is focused by regulating the objective lense current of the electron microscope. Biological specimens with poor inherent contrast are often better observed with slight underfocusing. However, excessive under- or over-focusing is undesirable in high resolution electron microscopy, because image artefacts in the form of granularity may arise due to phase contrast effects and because dimensional measurements become less dependable.
F. Resolution - The smallest structural elements that can be distinctly observed in the electron microscope is determined by the image resolution, often expressed in terms of the shortest distance between the clearly distinguishable features in the electron micrographs of known magnification. Magnification does not improve inherent resolution; however, practical means of increasing resolution consist of using higher acceleration voltage with reduced electron noise, increasing specimen contrast and reducing specimen thickness.
REVIEW OF LITERATURE

Macromolecules are capable of interacting with a wide variety of substances ranging from small inorganic metal ions to large colloidal particles and the resulting complexes possess physico-chemical characteristics which are different from the reactants. An appreciation of the fundamental nature of the formation of these complexes is essential to the understanding of the colloidal behaviour of a variety of biological and industrial systems. For example, the structure and function of biological membranes can be explained in terms of macromolecular complexes and have been studied in intact and in model systems by several means including the electron microscope (47). The physical properties of many biological fluids depend also upon macromolecular complexes. For example, the relatively low viscosity of milk is a consequence of the structural organisation of milk proteins into a micellar state, which has distinct practical advantages for their utilization by the new-born offspring. Likewise, the colloidal complex formation has direct bearing on the industrial practices used to achieve physical stability of foods in general, and food proteins and food emulsions, in particular (13,170).

The present review is concerned specifically with the
interactions between proteins and polysaccharides and the electron microscopic investigations of the resulting macromolecular complexes as well as their constituents. In this context, it is however important also to consider the current status of knowledge of the structures for fat globule particles and the protein micelles in milk in the native state and processed forms.

A. Protein Polysaccharide Interactions in Foods

The chemical structure and reactivity of the interacting molecules closely influence the ultrastructure and the properties of the resultant system (104). Proteins, polysaccharides and many lipids differ from each other in the nature of constituent groups, the type of their linkages and the extent of polymerization, but share a common property in their colloidal dimensions, and their physical and chemical behaviour relates to their colloidal state.

Unlike the hydrophobic sols of gold or sulfur, the colloidal phase of biopolymers principally represents an equilibrium state (18). Biocolloids have been classified as MACROMOLECULAR SOLS, representing molecular dispersion of the product, and ASSOCIATION COLLOIDS, represented by molecular aggregates of the smallest possible kinetic units. Aqueous solutions of hydrophilic polysaccharides typically illustrate macromolecular sols (131). A familiar example of association colloids to Dairy Technology is of course the casein micelles
in milk, the structure of which has been recently reviewed by Rose (138).

The striking differences in the overall properties of the small organic monomer molecules and their large, polymeric, colloidal counterparts arise largely due to the influence of secondary forces (129). In the category of secondary forces are included the electrostatic attractions, dipole-dipole interactions, London-van der Waals type dispersions, H-bonding and hydrophobic interactions (36). As these forces are generally much weaker than the primary valence bonds, their presence in small organic molecules is of minor concern. However, they assume considerable magnitude due to their abundance in polymers (129), and therefore exert great influence on the macromolecular conformation and reactivity (131).

The conformation of a macromolecule relates to the position of constituent atoms in space (40). Under any given set of environmental conditions, the conformational state of macromolecules is usually a limited set of possible structures and not an infinite number of interatomic arrangements. The reason for the predominance of a certain conformational state is that the rotational freedom of atoms around their bond axis is restricted to varying extent depending upon the type of bonds and van der Waals atomic radii. The number of possible conformations is further restricted to a few stable ones due to the interaction of secondary forces and thermodynamic parameters favoring entropy gain (166).
The delineation of the \( \alpha \)-helix structure in certain fibrous proteins (117) has provided tremendous impetus to the conformational studies of macromolecules and led to the discovery of the double helix structure in DNA (177). However, the existence of helical structures is by no means restricted to a few specific biopolymers but appear to be a rather common characteristic of linear polymers that have inherent structural periodicity (176). To date, such ordered helical structures have been demonstrated in a variety of natural polymers including xylan and amylose (127), and in synthetic polymers such as carboxymethyl amylose (128) and polyisobutene (29). The X-ray diffraction studies of Anderson et al. (4) have recently indicated the existence of H-bonded double-helix structure in several fractions of carrageenans, the sulfated polysaccharides extracted from seaweeds.

The reactivity of many of the polymers naturally present or added to food is mainly due to the steric factors imposed by the macromolecular conformation. These factors affect the relative availability of various functional groups and influence the reactivity of these groups due to the presence or absence of varying amount of cooperativity of other functional groups in the vicinity (166). This is why the considerations of macromolecular conformation and reactivity are so interrelated. The approaches to studying molecular interactions have included the quantitative determination of
helicity by optical rotary dispersion, by titration, by duterium exchange methods, by x-ray diffraction, space-filling models and lately, by computer simulation techniques (4,76,165). These studies have led to the realisation that an understanding of the macromolecular morphology of food constituents and their interaction products would be essential to the development of plausible theories that would not only account for the observed properties of the conventional foods, but also predict the overall behaviour of fabricated food systems.

1. Interactions in the Native State - Protein Micelles.

Macromolecular interactions occur during the synthesis and storage of many biological compounds in their native state. These include, for example, the storage of energy in the form of starch granules in root tubers (189), protein-bodies in cereals (188), fat in adipose tissues (186), and fatglobules (124) and casein micelles in milk (138). Many food processing techniques utilize similar principles for achieving desired product characteristics. The forewarming treatment of milk is used to improve the heat stability of the milk proteins and relies upon protein-protein interaction (135). The stabilization of evaporated milk against age gelation is achieved by the incorporation of small amount of the polysaccharide carrageenan (54). Many times, however,
Macromolecular interactions may cause technological problems as experienced in heat and age gelation of milk and in feathering and related effects in homogenized products (81). In this regard, the colloidal associations of milk proteins occupy a prominent place as models for major types of interaction in food chemistry (102).

Approximately 97% of casein occur in the form of micelles in strong association with calcium citrate and calcium phosphate complexes (42). These high-molecular-weight, submicroscopic aggregates are essentially association colloids of individual monodisperse polymers. Their structural integrity is maintained by their noninteracting surfaces. The association phenomena relating to the native caseins may be considered in terms of (a) the nature of the protein and non-protein components of micelles, (b) their association behaviour in model systems, (c) the behaviour of the natural micelles under controlled experimental conditions and (d) the proposed models for casein micelles that would account for their observed properties. The subject has been comprehensively reviewed by McKenzie (102) and more recently by Rose (138).

a) Constituents of Micelles:

The normal composition of micellar protein is 55% $\alpha_S$-casein, 30% $\beta$-casein, and 15% $\kappa$-casein (175). The molecular weights of $\alpha_S$- and $\beta$-caseins are known to be 27,000 and 25,000, respectively (102). The molecular weight of $\kappa$-casein
remains uncertain and ranges from about 20,000 (102) to 28,000 (163). Available information on the conformational states of these caseins in model systems indicates essentially random coil structure for $\alpha_s$-casein (93), and rod or coiled polymers for $\beta$-casein (120). The ability of $k$-casein to stabilize the micelle is known (182) but its mechanism is poorly understood.

The micelle size distribution and size memory (37) appear to be correlated to the $k$-casein content of the micelles and the carbohydrate content of the $k$-fraction (162). Rose et al. (140) have recently reported that large micelles are characterized by a low $k$-casein content. Furthermore the carbohydrate concentration of the involved $k$-casein appears to be below average, however, the significance of the carbohydrate moiety in the micelle stabilizing function remains debatable (102). Mackinlay and Wake (99) observed no significant difference in the micelle stabilizing ability of modified $k$-casein fractions that varied in their carbohydrate content from 0 to 10%. However, the fractionation involved modification by the creation of their carboxymethylated derivatives and this treatment may perhaps have introduced other effects. Removal of sialic acid (70%) by neuraminidase, on the other hand, decreased the stabilizing ability of $k$-casein somewhat (30%) as reported by Thompson and Pepper (167).

A detailed knowledge of the molecular nature of the
\( \kappa \)-casein moiety appears to be the key to the understanding of the structure and the stability of casein micelles. However, several attempts in this direction have resulted in even more controversy among various workers (14, 53, 99). Although \( \kappa \)-casein is believed to represent a trimeric aggregate, there is considerable disagreement as to the equivalence of the subunits in terms of composition and function. Mackinlay and Wake (99) reported chemical heterogeneity in the \( \kappa \)-casein with respect to carbohydrate content but observed no functional differences in terms of micelle stabilizing ability and rennet sensitivity. However, according to Beeby (14), functional as well as chemical heterogeneity is inherent in the \( \kappa \)-casein subunits. One of these units contains the rennet-sensitive, sialic acid rich fraction, the other contains all the -SH groups while the third is devoid of both sialic acid and -SH groups. Garnier and Dumas (53), on the other hand, assumed no chemical heterogeneity in the subunits and indicated that functional heterogeneity could arise as a result of asymmetric associations of the subunits in the quarternary structure. McKenzie (102) suggested that lack of agreements among various observations may be due in part to the variations in experimental conditions, such as in the purity of the protein preparations.

In addition to protein, casein micelles contain varying amount of calcium, phosphorus and citrate, and these are known to exist in an apatite type of colloidal calcium
citrate/phosphate complex (19,125). On the basis of sephadex-10 gel filtration studies of Morr (105), the ultrafilterable colloidal calcium phosphate appears to have a molecular weight of about 700. Earlier studies by Ford et al. (50) and by Hostettler and Rychener (71) have indicated that the calcium phosphate content varies directly with the particle size of the micellar casein. The removal of colloidal calcium phosphate (75), calcium ions (106), disintegrate the casein micelles. There is a general agreement that the colloidal calcium phosphate contributes towards the structural integrity of the micelle by forming crosslinkages with proteins involving phosphoserine, ε-aminogroups of lysine and carboxyl groups (136). However, in colloidal phosphate free milk, (CPF milk), the ability of ionic calcium to restore the structural integrity and other physicochemical properties typical of micelles suggests a nonspecific aggregating role for the colloidal calcium citrate phosphate complex (51).

b) Association Behaviour of Micellar Proteins:

Many studies have been reported on the nature of ion and temperature sensitivity of the inter- and intra-species associations of the αₜ-, β- and κ-caseins in model buffers (113,119,120,193). These studies indicate that the constituent proteins of the micelle can undergo reversible association (136).

The κ-casein differs from the other two in its ability to bind calcium without its precipitation at temperature and
ionic strength normal to milk (168). While Buckley (28) reported a characteristic discontinuity in the calcium binding behaviour for the \( \alpha_s \)-casein that undergoes rapid polymerisation (119), a relatively slow, temperature sensitive interaction has been noted in the predominantly hydrophobic \( \beta \)-caseins that exist as monomers at 0° - 4° C and polymers at 20° - 30° C (120).

The calcium sensitive \( \alpha_s \)- and \( \beta \)-caseins apparently undergo interspecies association according to the limited data on the composition of the fractionated casein micelles (138). The \( \kappa \)-Casein can associate with both of the fractions in the model systems, and the resultant stability depends on the ratio of the protective and the sensitive fractions at a given ionic environment (182,193). These model systems exhibit turbidity and other colloidal characteristics typical of the native micellar state (181).

Studies on milk protein interactions in model systems have essentially led to the conclusion that apolar forces may be as important as the ionic bonding. It is quite apparent, nevertheless, that their relative importance in the formation of micellar stability can not be assessed without considering the native environment.

c) Behaviour of Natural Micelles:

The native state of casein micelles in milk represents a broad and continuous spectrum of size (107,111,162). The dilution of skim milk with milk ultrafiltrate and the analysis
of the casein composition in different fractions revealed no
equilibrium between the serum and micellar caseins (137); this has been confirmed by Dumas and Garnier (43). The lack of a rapid attainment of equilibrium is contrary to the findings from model studies of $\alpha_s$-k-casein by Waugh (179) and illustrates the inherent difficulties in extrapolating the behaviour observed in model studies to the intact systems.

From the interaction and stability studies on the micellar caseins in model systems, the $\alpha_s$- and $\beta$-caseins have been thought to be the only two necessary and sufficient proteins, required to form micelles and the $\beta$-caseins have been assigned no definite role (179). However, Rose (138) pointed out that even the smaller fragments of disaggregated casein micelles contain $\beta$-casein and do not merely represent an $\alpha_s$-$\kappa$-interaction product. The proteolysis of $\beta$-casein by rennin in intact and disrupted micelles indicates that in the native system $\beta$-casein may be present in more than one functional form that differs in the accessibility to outside environment in response to temperature changes (51).

Similar functional differences have recently been reported for the $\kappa$-casein in the micelle in terms of its accessibility to rennet coagulation (115). Rennet coagulation of skim milk micelles could be markedly delayed due to the repeated washings of the micellar fraction with isotonic buffer resulting in the migration of 30% $\kappa$-casein to the serum fraction. This serum $\kappa$-casein is believed to be a non-
micellar component and has been suggested as the primary component attacked by rennin. The remaining λ-casein (70\%) is situated at the micellar core.

The relative accessibility of the casein micelle to various chemical reagents and enzymic treatments has been recently used in the structural investigations of micellar caseins (43,51,184). Fox observed that in intact micelles rennet proteolysis of αs- and β-casein was considerably slower than that in the non-micellar sodium caseinates (51). However, the enzyme activity of carboxypeptidase-A in natural micelles (43) and of wheat germ acid phosphatase in the synthetic αs-λ-casein micelles (184) was not significantly different from their non-micellar controls.

The lack of agreement between the rennet experiments and those with the two other enzymes is difficult to reconcile but may perhaps be explained by a physical barrier imposed due to the coagulation effects. Such a phase change might restrict the mobility of enzyme and limit the enzyme activity to the immediate surrounding only. Therefore, the valid experiments by Fox notwithstanding, it seems that the casein micelles are likely to be of a porous and spongy nature as suggested by Dumas and Garnier (43), Parry and Carroll (115) and Whikehart and Rafter (184). This, of course, does not include the approximately 3\% of soluble, non-micellar caseins (42).

d) Models of Casein Micelles:
In general, a great deal is known about the major constituents of the casein micelle and the possible mode of interaction, but very little information is available regarding the molecular organization of these constituents in the micellar unit and the importance of such arrangements to the behaviour of the micellar state observed in milk. Adequate morphological differentiation of the constituent proteins has not been possible even in the best reported ultrastructure studies on casein micelles (3,46,151).

Several models of casein micelles have been proposed to account for the known facts about the caseins and their micellar state. Initially, these models began with a set of simple assumptions. Over the years, however, their complexity as well as usefulness have increased as more knowledge has accumulated on the nature of the micellar proteins. Rose (138) has critically reviewed the important micellar models, currently proposed.

The earliest model of micellar casein was proposed by Waugh (179) to account for the 1:4 stoichiometry of $\kappa$- and $\alpha_s$-caseins, respectively, in the stable complex formed by adding ionic calcium. The molecular arrangement representing four rod-like $\alpha_s$-macromolecules around the partially extended $\kappa$-casein molecule was later modified to a "rosette" pattern as partial hydrophobicity was recognized in the $\alpha_s$-casein (138). These models could account for the physical stability and the rennet accessibility of the $\alpha_s$-$\kappa$-complex
but assigned no major role to the $\beta$-casein. In Payen's model (118), however, $\beta$-casein was considered to exist as loose threads containing apolarly bonded $\alpha_s$-casein and stabilized by a coat of $\kappa$-casein. The location of calcium phosphate was also believed to be on the surface of the micelle.

The occurrence of $\kappa$-casein on the surface, considered to be essential for rennet action, has been denied in two
recently proposed models. According to the concept of Parry and Carroll (115), a central core of high molecular weight k-fraction is surrounded by irregularly placed aggregates of \( \alpha_\text{s} \)-casein and branched-chain or rod-like association products of \( \beta \)-casein. The hydrophobic interaction between the \( \alpha_\text{s} \) and \( \beta \)-caseins has not been considered. The resultant micelle has a porous structure stabilized due to the salt-bridges formed by the colloidal calcium phosphate at different places in the protein network. In the other micellar model by Garnier and Dumas (Adapted from Ref. 53).
Dumas (53) porosity has also been emphasized. However, this has been achieved in their model by assigning nodal positions to κ-casein trimers that are connected to each other by varying lengths of rod-like structures, copolymers of the αs- and β-caseins. Such structures permit 100-120 Å diameter network spacings accessible to rennin. In the model by Parry and Carroll, the rennet susceptibility had been assigned to the non-micellar, serum κ-fraction (115). Either of these two models can account for the observed correlation between the micelle size and the concentration of κ-casein and colloidal calcium phosphate. However the ability of native casein micelles to remain within a size group and to maintain their structural integrity is poorly understood in terms of these models, as they do not appear to have any specific non-interacting surface characteristics. They further fail to account for the fact that in the absence of colloidal calcium phosphate, micelles can be fractionated to smaller aggregates containing a definite proportion of αs-, and β-caseins (138).

The role of subunit structures as the building blocks for casein micelles was originally suggested by Shimmin and Hill (151). The subunit structures formed the basis of the casein micelle model proposed by Morr (106). Morr's submicelles were in essence the miniature micelles of Waugh (180), composed of an inner core of αs-β-casein complex and outer core of αs-κ-casein complex. Colloidal calcium phosphate aggregated these submicelles into larger casein
micelles. The overall surface of these micelles would be rich in $\kappa$-casein content and would therefore possess surface charges that could account for the noninteracting surface character.

Rose (138) added further molecular details to the submicelle structure by proposing end to end aggregation of $\beta$-caseins at the hydrophobic centres where hydrophobic ends of $\alpha_S$ as well as $\alpha_S$-$\kappa$-complex would also associate. Such
threaded structures could be folded, twisted and turned to assume a spherical conformation with \( \kappa \)-caseins peripherally located. Colloidal calcium phosphates are involved in providing rigidity to such structural arrangements and in promoting agglomeration of subunits into larger micelles. Aggregation of submicelles continues unless the micelles have acquired enough surface \( \kappa \)-casein to permit electrostatic repulsion between the adjacent micelles. According to Rose (136), the lower the carbohydrate content of \( \kappa \)-casein, the greater will be the agglomeration of subunits. Thus, the functional relationship between the nature of \( \kappa \)-casein and size memory of micelles is well accounted for by these two models.

The currently proposed models of casein micelles are speculative to varying degree and many of their inherent assumptions have not yet been tested for validity. Although casein micelles are essentially the products of biological origin and although the biosynthetic pathways for the constituent proteins are not known to be different from the accepted classical schemes for protein biosynthesis (94), it has not yet been demonstrated if the assemblage of these proteins into micellar aggregate is biologically mediated. In the absence of such knowledge, the micelle models are, at best, physicochemical rationales for their observed behaviour in milk. The choice of such rationales appear to be more than one, as satisfactory morphological evidence is not
2. Interactions During Processing.

The colloidal complex formation, important in the native structure and stability of natural food components including casein micelles, assumes further significance in the subsequent processing and handling of foods. In the colloidal phase of milk, such interactions are typified by the heat induced complex formation between the natural constituents of caseins and wheyproteins and by the associations of hydrophilic stabilizer additives with milk proteins.

a) β-Lactoglobulin-κ-Casein Interaction:

When milk is heated to 65°C and above, the micellar and the serum proteins interact (156,169) and as a result, influence the heat stability (171) and rennet coagulation of milk (77). The specific proteins involved are κ-casein and β-Lactoglobulin and their interaction has been recently reviewed by Sawyer (143).

From the earlier work of Pederson (122) and the recent observations of McKenzie and Sawyer (103), β-Lactoglobulin in milk appears to possess a quarternary structure composed of two identical subunits of 18,000 molecular weight. The conformational states of β-lactoglobulin, near pH 5, possess elements of helix, pleated sheet and random coil (102). However, at the neutral pH regions in milk, conformation may be disordered due to the oxidation of -SH groups and the rupture
of S-S bond of the cysteine residues.

\( \beta \)-Lactoglobulin exhibits unique intermolecular association-dissociation phenomena that are influenced by pH, ionic strength and heat (22,168). The heat induced interaction of \( \beta \)-Lactoglobulin and \( \kappa \)-casein can not be ignored in searching for an understanding of the relationship between macromolecular associations and physical stability of food systems since such stability achieved in evaporated milk due to the forewarming treatment is in many ways similar to the effects imparted by the addition of chemical stabilizers, whether these be inorganic phosphate salts or hydrophilic colloids.

Even though the understanding of the \( \beta \)-lactoglobulin-\( \kappa \)-casein complex formation in milk is incomplete, its industrial importance for stabilizing milk has long been recognized (135). Trautman and Swanson (171) demonstrated that interference with this interaction during forewarming treatment resulted in the failure of these milks to withstand subsequent sterilization temperatures during the manufacture of evaporated milks.

The possible interrelationships between the heat induced macromolecular interaction among the milk proteins and the beneficial effects of the forewarming treatment of milk have been reviewed by Rose (135), who has suggested that the SALT BALANCE THEORY (158) in its classical form, is inadequate in accounting for the altered physicochemical properties of forewarmed milk. The heat-induced \( \beta \)-lactoglobulin-
κ-casein complex changes the surface characters of the casein micelles, and this in turn results in an increased heat resistance of the milk. Since the α-lactoglobulin interaction is known to be ion sensitive, any unfavorable shift in salt balance may influence the heat-induced complex formation between α-lactoglobulin and κ-casein and thus indirectly influence the stability characters of milk. However, on the basis of evidence presented by Morr and his associates (80, 108), it is apparent that in addition to the micellar state, the serum phase of the colloidal system in milk is also influenced by the casein-whey protein complexes. In the micellar phase the complex formation may occur preferentially between α-lactoglobulin and κ-casein, while in the serum phase, such interaction may involve nonspecific, chemical as well as physical association of soluble caseins and whey proteins.

b) Protein Polysaccharide Interactions in Milk:

The interaction of proteins with food stabilizers or gums is important in the industrial stabilization of food products. These additives are essentially natural plant polysaccharides or their derivatives. The major supply of food gums is furnished from the extracts of marine algae, the extracts and exudates of land plants and plant seeds and from a variety of cellulose derivatives. The structure, property and important food uses of many useful stabilizers have been compiled by Klose and Glicksman (84).
(i) The basic nature of polysaccharides - Considerable research effort has been devoted to the understanding of the chemical nature of the polysaccharides (9), their conformational characters (161), and the mechanisms of their action in food systems (131). In general, polysaccharides exhibit polydispersity in molecular weights and constituent monomers differ in the type of sugar units and in their glycosidic linkages resulting in the formation of linear as well as branched macromolecules with characteristic conformational features (185). Further differences among these macromolecules arise due to the varying degree of substitution of the available sugar OH-groups by different substituents including carboxyl and sulfate groups. Due to the presence of these ionic groups, the hydrophilic colloids exhibit a varying degree of acidic character and may be grouped as NEUTRAL, WEAKLY ACIDIC, and STRONGLY ACIDIC polysaccharides (45).

Stabilizers differ markedly in terms of their effect in food systems. Such differences are not only exhibited by stabilizers belonging to different classes, but may often be pronounced amongst the stabilizers that are closely related with respect to chemical composition. A possible explanation for this may be sought in terms of the conformational characters of these hydrocolloids rather than in their gross chemical composition.

Considerable progress has been made in the elucidation of the structure and conformation of several linear poly-
saccharides (161). The basic conformational feature of linear polysaccharides may be staggered or helical, depending on the nature of the glycosidic linkage. In the absence of steric hindrance by bulky substituents and due to the presence of H-bonds staggered conformations may be stabilized in the form of flat ribbon like chains, such as occurs in cellulose (126). Similar factors may also determine the compactness of helix structure or its degree of extended non-helical state as observed in amylose and its derivatives (128).

According to Rees and his coworkers (4,132), the helical chain may also be the basic feature of many polygalactans containing an alternating sequence of\(\alpha\)-3, \(\beta\)-4 linked units. Since considerable heterogeneity is known to exist in many of these biological preparations (98), such assumptions may however be regarded as broad generalizations based on a relatively few idealised structures (131).

The conformational states of the hydrocolloids in solution may be further influenced by solvent effects, binding of ions and interactions with other macromolecules. According to Rees (131), these considerations are important in understanding the functional role of the polysaccharides in biological systems, including foods.

(ii) The nature of interactions in the gel-forming polysaccharides - The important functional properties of the hydrocolloids used in foods include increased water binding capacity, suspension of insoluble particles, stabilization
of foams and emulsions, and regulation of rheological properties (48). The functional versatility of many polysaccharides may be attributed directly to their gel forming property. Polysaccharide gels are represented by the interconnected network of the polymerchains containing solvents and other materials in the interstices (131). The physical nature of the gels depends largely on the relative proportion of the solvated chains that do not associate with each other and the JUNCTION ZONES formed due to the interaction of the chains resulting in a three-dimensional network structure.

The significance of junction zone formation in polysaccharide gels and networks has been reviewed by Rees (131) with special reference to the chemical nature of the macromolecules involved. Among the several types of polymer-polymer interaction recognized in junction zones, the phenomena particularly important to the food industry are the formation of DOUBLE HELIX junctions by the carrageenans, the MICROCRYSTALLITES by the algin and pectins, and the ENTANGLEMENT & SHARED COUNTERIONS by carboxymethyl cellulose gels.

The gelforming ability of the potassium sensitive carrageenans has been explained in terms of the double-helix junction zones model developed on the basis of the H-bonded double helix fibre structures recently proposed for the \( \iota \)- and \( \kappa \)-carrageenans (4). According to this model, isolated double helices are crosslinked in the first stage of gel-formation by sharing different portions of the same polymer-
Fig. 7. Double-helix Junction Zones (adapted from Ref. 131).

chain. In the second stage, a varying amount of aggregation of the double helix fibres occurs. The impairment of the gel formation by certain structural modifications and polymer degradations of the carrageenans can be explained by the double-helix model. The alternation of double-helix content due to the chemical modification of the glycosidic units affects the formation of junction zones; for example, extensive polymer degradation affects the cross-linking ability of the polymers. Marked differences in the gel forming ability of the carrageenans obtained from various sources may therefore be understood in terms of the considerable variations reported in their molecular weights and in the location of certain functionally critical groups like C-6 sulfate or 3-6 anhydroglucose units (132).

The junction zones in alginate and carboxymethylcellulose gels have points of similarity and are thought to be
characterized by partly crystalline regions (paracrystalline or microcrystalline) within a randomly entangled polymer network. The formation of MICROCRYSTALLITE junction zones in alginate and pectin gels are mediated by divalent cations like Ca$^{++}$. On the other hand, the ENTANGLEMENT & SHARED COUNTERIONS type of junction zones of CMC gels are formed by the highly solvated carboxymethylated portions of the cellulose chains that undergo random entanglement and are stabilized by counter ion sharing. Therefore, crystalline structures in the carboxymethylcellulose gels represent H-bonded regions of the native cellulose chains resistant to carboxymethylation (44). The carboxymethylation of cellulose throughout the entire chain length affects the crosslinking ability of the polymers due to the absence of crystalline regions and as a result, gel forming characters are altered (131). It is also possible to interfere with the formation
of microcrystallite structures in alginates (148) and pectates (149) by inducing uneven acetylation; gel formation in the presence of calcium is consequently affected.

Although further work is necessary to firmly establish the nature of the intermolecular associations of gel-forming stabilizers, the current concepts on the formation of junction zones emphasize the role of structure and conformation on the functional properties of these additives. The recently proposed gel forming mechanisms may be important in the food stabilizer selection which has been based traditionally upon the empirical observation rather than the fundamental knowledge of the polysaccharide interactions in foods.

(iii) Interactions with milk proteins - A variety of vegetable and industrial gums has long been used in various milk-
based beverages, concentrated products and frozen desserts (54). However, no comprehensive work is available on the manner in which these additives impart desirable qualities in the dairy products. Recent investigations in several laboratories have indicated that various types of interactions occur between the milk proteins and polysaccharides under different reaction conditions and at different combinations of reactants (38, 55, 57, 59, 55).

Upon the addition of 0.1 to 0.5% hydrocolloids in skim milk preparations, considerable physical destabilization may be observed (55, 59). Analysis for ester sulfate content (57) and zonal electrophoretic patterns (38, 55) indicated that under the normal pH and ionic conditions in milk, the micellar caseins are mainly involved in this interaction. Since the neutral hydrocolloids, locust bean gum and guar gum and the ionic polysaccharides, carrageenan and CMC can cause aggregation of micellar caseins, the complex formation between the stabilizers and casein micelles appears to be a general type of macromolecular interaction involving secondary forces in addition to the ionic type.

On the basis of moving boundary electrophoresis and turbidimetric measurements, Asano (7) demonstrated that under the acidic pH conditions of fruit flavored milks, the interaction of CMC may also take place with the whey protein, \( \alpha \)-lactoglobulin. Ebel (45) studied the interactions of hydrocolloids with serum proteins and concluded that \( \alpha \)-lacto-
globulin as well as α-Lacalbumin undergoes nonspecific ionic interactions with the anionic polysaccharides, CMC and carrageenans under favorable pH and ionic conditions. For a given protein and polysaccharide, the complex formation reaches a peak at a characteristic pH value. The solubility of the complex can be influenced by the salt concentration as well as the combining ratio of the components.

Hansen (58) reported a specific type of interaction by which carrageenan stabilized αs-casein against precipitation by ionic calcium. Further studies on the stabilization of the calcium sensitive caseins by Lin and Hansen (98) established that only the sulfated polygalactans possessing an alternating α1-3, β1-4 linkage, typical of carrageenans were effective. The effectiveness of the polysaccharides increased in the absence of C-6 sulfates and was apparently required in an optimum molecular size. However, exceptions to these generalizations was noted in the casein stabilizing ability of sulfated locust bean gum, that possessing a β1-4 linked structure, branched at the α1-6 positions.

Although the significance of various polysaccharide-protein interactions in food systems may not yet be completely realized, their role in other biological systems has long been recognized; for example, the sulfated polysaccharide heparin exhibits blood anticoagulant activity. Similar interactions between the anionic polysaccharides and blood lipoproteins have been extensively studied (16) and
these principles have been utilized in the characterization of various lipoproteins (39).

As the potential applications of hydrophilic colloids in food become apparent, a clear understanding of the stabilizer action in food systems will be required. However, such information is not likely to emerge until stabilizers are better characterized in terms of molecular weights, conformations, reactivity and intermolecular forces.

B. Electron Microscopy of Food Components

A detailed knowledge of food microstructure is helpful for understanding how the macromolecular orientation of interacting food components relates to the observed behaviour of the intact food systems. An effective way of obtaining such knowledge is through the morphological studies of food systems as well as of their constituents. The physical dimensions of food colloids are usually too small for effective resolution of microstructure by light microscopy. The electron microscope, on the other hand, offers much higher resolution and has therefore been considered a valuable tool for ultrastructure studies (49). However, at the present state of development, the science and art of electron-microscopy are such that improved resolution of biological ultrastructure is not achieved without sacrificing some informational quality of macromolecular morphology (155). The ultimate and ideal objective of the ultrastructure studies is to
obtain sufficient morphological details, at the molecular level, to permit the visualization of structure in three dimension and enable identification of all individual structural elements, without distorting the dimensions, geometry and the state of existence of the native systems. Considerable progress towards this goal is evident from recent treaties on the fundamentals of electron microscopy (eg. 78, 153,154,191).


A basic appreciation of the effect of the instrumentation and methodology in electron microscopy is necessary for the interpretation of ultrastructure. For a scatter-free, efficient operation of the electron microscope, a high degree of vacuum is required (178). However, the mainenance of vacuum not only rules out the possibilities of observing the biological specimens in their aqueous solution phase, but also distorts the macromolecular structures during their dehydration by the gradual evaporation of the liquid phase. The extent of distortion may be negligible for the relatively rigid molecules in the case of synthetic polymers (174) or of considerable magnitude for soft globular proteins in the case of casein micelles (151). Such dehydration artifacts arise due to the surface tension forces, and may be minimized by the critical point method of evaporation (121), the freeze drying techniques (190) or the use of water
soluble dehydrating-embedding media (96). Details on these methods are available from Anderson (5) and Wischnitzer (187).

In the electron microscope, it is the image of the specimen and not the specimen itself that is under view. This is achieved in the SCANNING ELECTRON MICROSCOPE (62) by utilizing the electron beams diffracted from the surface of the specimen. In the TRANSMISSION ELECTRON MICROSCOPE (2), the electron beam is transmitted through the thickness of specimen and received on the viewing screen or photosensitive surface.

The bulk of the biological ultrastructure studies rely on the use of the electron transmission system developed in the earliest phase of electron microscopy. In this system, satisfactory image formation as determined by the presence of sufficient structural details and the adequate resolution of the structural elements depends not only on the accelerating potential of the electron beam, but also on the thickness of specimen and the specimen contrast (155). The inherent contrast of proteins, lipids and polysaccharides is rather poor (194), however by lowering the beam accelerating potential, the contrast can be increased (172), but the image intensity and resolution is lost. By increasing the beam voltage on the other hand, an improved image intensity and resolution can be achieved at the cost of contrast. Since high contrast is as desirable as high resolution, the elec-
tronmicroscopist may resort to increasing contrast by incorporating electron dense materials in the preparations; at the same time, he can improve the resolution by reducing the specimen thickness without losing contrast. Artifacts are nevertheless not ruled out by the processes currently available for contrast improvement and for the preparation of thin sections. Consequently, a great deal of effort is being devoted to the improvement of vacuum evaporating methods, the staining techniques, ultramicrotomy and the freeze-etching techniques, which have been reviewed by several authors in terms of their basic principles and practical instrumentation (2,92,194).

Proteins, lipids and polysaccharides do not markedly differ in contrast, and the problem of their differentiation in the multicomponent biological systems has received considerable attention (15,85,194,195). Towards this goal, only partial success has been achieved in the use of electron dense staining compounds that preferentially act either on proteins (195) or nucleic acids (194). Further development in this direction has been attempted in terms of using chemical modifications (15), enzyme treatments (30) and immunochemical reagents (115) to promote binding of electron dense material to specific components for the purpose of differentiation. The application of autoradiographic techniques to electron microscopy has also provided means of detecting individual, labelled, components in the specimen (147). However, these
techniques generally suffer from a relatively limited application, lack of sufficient resolution, or introduction of further preparatory artifacts.

Fixation of biological samples is often required to avoid specimen unstability during exposure to the intense electron beam in the microscope. The fixative traditionally used for this purpose is osmium tetroxide; in addition several aldehydes including formaldehyde and glutaraldehyde are also used (141). The fixative action is achieved mainly by establishing intra- and inter- molecular crosslinking of the numerous reactive groups of macromolecules, and may therefore introduce artifacts in the state of molecular aggregation and/or arrangement. The fixation of unstable, particulate preparations can, however, be avoided by examining sample replicas instead of samples (21). Although replica techniques are particularly suitable for studying surface structures of particles, they do not permit differentiation nor the examination of the internal structures of the particles (178).

It is thus apparent that no single instrumentation or methodology is completely satisfactory for the electron microscopist in terms of highest structural resolution, adequate component differentiation, and absence of artifact. However, useful information can often be gathered by a suitable combination of several techniques. Currently, detailed methodologies are available for electronmicroscopy in the
many specialized areas of biomedicine (187), virology (68), enzymology (61), and protein biochemistry (157). However, the study of food ultrastructure is at its infancy and has to lean heavily for its development on the progress made in other related biological fields.


Electron microscopy has been used in the various phases of investigations pertaining to the biochemical cytology of mammary glands (101), the morphology of the particulate structures of fatglobules (24) and casein micelles (152), and the microstructure of milk and dairy products at various stages of processing and storage (25). Earlier work in this area was largely the histological studies of mammary glands and has been reviewed by Mayer and Klein (101).

a) Fat Globules:

Fat globules represent association of proteins and lipids which are particularly important for the emulsion stability of milk.

The electron microscopy of lactating mammary gland has yielded valuable information regarding the site of the synthesis of the fat globules and the mode of their secretion into the lumen or milk cavity. Hollman (66) detected the presence of intra vacuolar protein granules and large lipid droplets in the lactating cells and thought these to be the precursors of the casein particles and fatglobules in milk.
Since then, further elaboration of those processes has been attempted in several investigations.

The electron microscopic radioautography with tritiated fatty acids demonstrated that the esterification of fatty acids occur at the rough endoplasmic reticulum (ER). In situ aggregation of lipids gives rise to the formation of fat globules and these globules are directly discharged into the lumen (66). Helminen and Ericsson (63) observed an increase in size of the fatglobules approaching the apical portion of the cell, prior to their discharge into the lumen. The electronmicroscopy of Bargmann and Knoop (12) suggested that at the apex of the lactating cells, fat droplets are progressively enveloped with plasma membrane material and are eventually pinched off as they are discharged into lumen. Several electron microscopic investigations of the lactating mammary tissues of rats have recently confirmed these observations (63,159). These studies have further established that the lactating cells differ from other secretory cells due to the non-involvement of the Golgi bodies in the lipid transport (159).

The electron microscopy of Hansson (60), presented an elementary concept of fat globules absorbing on their surface a fat globule membrane. The role of fat globule membranes has long been recognized in various phenomena in milk and cream related to phase stability, including the formation of cream layer, its prevention by homogenization, and breaking
stage during butter making (23). Consequently, the investigation of the physical and chemical nature of this membrane material has been pursued with great interest (24). Based on these studies, King (81) proposed a model of fat globule membrane consisting mainly of an orderly array of polar phosphopholipids that radially oriented their apolar groups towards the triglyceride phase and the polar groups towards a layer of uncoiled protein material. However, the electron microscopic studies of Bargmann and Knoop (12), on the secretion of milk fat globules raised doubts as to the validity of King's proposed model and triggered renewed attack on this problem with radioisotope (116) and immunologic techniques (41) in addition to the electronmicroscopy. Brunner (24) has comprehensively reviewed the work in this area, and Prentice (124) has presented the current concepts of the fat globule membrane that emerged from these studies.

Knoop, Wortmann and Knoop (91) observed that the surface of the fat globule membranes in freshly drawn milk appeared rough and became smoother after vigorous agitation of milk. Dowben et al. (41) established with electronmicroscopy and antisera-agglutination tests that the membranes of fat globules and erythrocytes are similar in structure and biological origin. A comparison of the fatglobule membrane structure in the lactating mammary gland of cow and in freshly drawn milk revealed that in the lumen, the fat globules are surrounded by two membrane layers (160). An
electron dense, 150-250 Å thick, intracellular, cytoplasmic membrane was surrounded by a typical unit membrane structure that showed disintegration in places. In the freshly secreted milk this outer plasma membrane appeared to be completely disintegrated and lost. Keenan et al. (79) also observed distinct morphological differences between plasma membranes and fat globule membranes and suggested that fat globule membranes are derived from plasma membrane by structural rearrangements. On the basis of chemical characterization and electronmicroscopic morphology of the fractionated fat globule membranes, Swope and Brunner (164) have recently proposed a two-layer fat globule membrane structure consisting of an inner layer of protein or lipoprotein matrix to which varying amounts of lipid-rich, lipoprotein particulate structures may adhere by adsorption. Adjacent to the inner membrane, a radially arranged 50 Å thick monomolecular layer of triglyceride has been detected in the lipid phase of fat globule by Buchheim (26,27) on the basis of polarization microscopy and electron microscopy. Prentice (124) concluded that the ultrastructure studies on the fat globules have established the bilayer nature of the enveloping membrane and the biological nature of the inner membrane. However, the nature of the outer layer and the details of the macromolecular orientation in the bilayer structure remain to be worked out.

b) Casein Micelles:
The role of the Golgi apparatus in the formation and transport of micellar caseins, has been firmly established in the lactating tissues of rat. Welling and Deome (183) originally outlined a three step process consisting of the synthesis of the milk proteins at the rough ER, the formation of micellar structure in the Golgi vacuoles, and the discharge of the mature protein particles by the rupture of the vacuoles. However, the electron microscopic evidence of the synthesis of milk protein at the rough ER was not available at that time and the ultrastructural details of the other steps were lacking. Nevertheless, their basic scheme was later confirmed by Helminen and Ericsson (63). In the secretory cells, milk proteins are visible as 80-90 Å thick, branched fibrils in the lumens of the rough ER and Golgi vacuoles. As the fibrillar milk proteins mature into more compact globular forms in the Golgi bodies, the 70 Å thick membranes of the protein containing Golgi vacuoles develop into 100 Å thick triple layered structures. The mature milk proteins exhibit typical mosaic substructure and are released into the lumen by the rupture of the Golgi vacuole membranes. The partial replenishment of the epithelial plasma membrane bordering the lumen by the ruptured triple layered membranes may be facilitated due to their structural kinship (63). As these membrane materials envelope the fat globules, Patton and Fowkes (116) considered a possible relationship between the secretion of fat globules and casein micelles. Carroll,
Thompson and Farrell (34) have recently speculated on the nature of the fibrillar proteins seen in the ER lumens and the possible mechanism of their compaction into the micellar state inside the Golgi vacuoles: (a) the freshly synthesized protein fibrils represent macrocasein molecules of about 150,000 daltons, equivalent to 1 unit of k-caseins, 2 units of β-caseins, and 3 units of αs-caseins. (b) In the Golgi vacuoles, the macrocaseins undergo phosphorylation and carbohydation and are partially hydrolyzed due to the splitting action of an enzyme specific for arginine-hydrophobic aminoacid sequence. (c) In the Golgi vacuoles these partially hydrolyzed macrocaseins form thread-like structures due to the hydrophobic interaction, and eventually roll up into porous, solvated, micellar aggregates as the vacuoles migrate towards the cell apex. (d) Further organization of the internal structure and the stabilization may be attained by the ionic interaction with calcium.

So far, the isolation and characterization of the macrocaseins and their partially hydrolysed forms have not yet been possible and not much is known about the enzymes that split the arginine-hydrophobic aminoacid peptide link.

After the initial electron microscopy of Nitschmann (112), several workers used the electron microscope for observing the casein micelles in skim milk (1,69,142,151). Various preparatory techniques used in these studies included high dilution of skim milk (112), replica and spraying meth-
ods (142), and use of fixatives other than OsO₄ (35). Casein micelles in skim milk were shown to be roughly spherical particles ranging in diameters of less than 200 Å to more than 2000 Å. Van Winkle et al. (173) observed that micelles from whole casein appeared to be softer and fluffier than those prepared from skim milk by the identical OsO₄-fixation and uranylacetate staining procedures, and attributed this difference to the absence of colloidal calcium apatite from the reconstituted micelles. Knoop and Wortmann (90) used sections of methacrylate embedded specimens for studying the size distribution of casein micelles in cow, goat and human milks, and reported the most frequently occurring average diameters to be 930 Å, 1330 Å and 420 Å, respectively, for these three species. Shimmin and Hill (151) considerably improved the structural resolution of casein micelles by using sections of araldite-embedded specimens as thin as 100 Å and demonstrated that the internal structure of casein micelles consists of 90-100 Å electrondense subunits. The evidence of subunit structures in casein micelles was also presented by them in the platinum-palladium shadowed specimens and in those, negatively stained with uranylacetate (152). The electronmicroscopy of Calapaj (31) and the freeze-etching techniques of Eggmann (46) have confirmed that these aggregates may be 80 to 100 Å in cow milk and 60 to 75 Å in human milk. Calapaj estimated that about 1200 to 1600 subunits would constitute a micelle of average size. Although
the proteinaceous nature of these electron dense substructures was initially questioned by Rose and Colvin (139), these particles are now believed to represent the aggregation of αs-, β- and κ-caseins (31,53,138).

The identification of the constituent proteins in the subunit structures has not yet been possible. The use of specific antibodies for the localization of the individual casein has been attempted in a limited scale by Parry and Carroll (115) but has been met with only partial success. Further delineation of the subunit structures of the casein micelles would be possible as better techniques of ultra-structure studies become available.

The electronmicroscopy of secretory tissues has revealed, in general, varying degree of biological mediation in the structural organization of the fat globules and casein micelles in milk. Consequently, in the absence of adequate information on the native microstructures of these particulate phases, an approach to the building of working models largely on the basis of known properties of the components may no longer be considered satisfactory in spite of their ability to account for the overall behaviour of the system.

c) Process Induced Particulate Structures:

The lack of the structural resolution at the molecular level has by no means discouraged the use of the electronmicroscope in the investigation of food systems. For example, electronmicroscopy was used by Badekas and Vakaleris (11) in
an attempt to correlate the microstructure of synthetic fluid foods with the emulsifying ability of caseinates and polysaccharide gums. The effect of processing conditions on milk and milk products has been investigated by the electronmicroscope in order to gain better understanding of involved mechanisms.

Hostettler and Imhof (70) detected casein agglomerates in diameters exceeding 1μ in UHT milks that was homogenized prior to the steam injection at 150° C. Such aggregations, presumably resulted from a fat-casein complex formation and created a mealy-chalky defect in the product and could be eliminated by UHT-treatment before homogenization, preventing the formation of the complex.

Carroll et al. (33) reported an increase in the size of casein micelles by 2 to 2.5 times as skim milk was concentrated by high-temperature short-time processing. Schmidt studied the gelation of UHT sterile concentrates (144,145, 146) and demonstrated with electronmicroscopy that the casein micelles began to coalesce with each other prior to the formation of a three dimensional gel structure. The process of coalescence was initiated at the surface of the micelle but later on proceeded to the micellar interior. Upon the addition of excess k-casein (50%) and αs-+ β-casein (20%) before the concentration process a corresponding decrease and increase were observable in the micelle-size of the concentrates, which however gelled at the same time. No
significant change in the age thickening ability of different caseins was attributed to the surface localization of \( \kappa \)-caseins. Numerous 400 Å diameter, glycoprotein rich particles were detected in the samples and larger micelles were thought to be the aggregates of these particles.

Roelofsen and Salomé (134) studied the microstructure of whole and skim milk powder and detected the presence of 100 Å thick membrane in fat globules but failed to notice any subunit structure in the casein micelles using 500 Å thick sections. According to Eggmann (46) freeze dried milk powders were not structurally different from the spray dried preparations.

The rennet action on casein micelles has been studied with the electron microscope by several workers (69,82,115, 123). All these studies have shown that the casein micelles do not lose their structural integrity immediately after rennet addition and appear to coalesce with each other before visible coagulation of milk occurs. Kiyosawa et al. (82) noticed the disappearance of surface roughness of the casein micelles before initial agglomeration started. The electron micrographs of Schmidt (144) and Parry and Carroll (115) would indicate that the process of coalescence is initiated at the micelle surface by para \( \kappa \)-casein and later on penetrates the internal structure of the micelles, due to which globular structure is lost and three dimensional gel network typical of rennet curd is formed. Electronmicroscopy of edam
cheese by Fricker and Meyer (52) has suggested embedding of fat globules in a continuous phase of a protein matrix.

Advanced electronmicroscopic methodologies, such as replica techniques, freeze etching and stereophotography have been used for studying the effects of processing conditions on the lipid phase of milk, butter and ice cream (26, 82, 88). Eggmann (46) and Henstra and Schmidt (64) have presented electronmicrographs that have confirmed the presence of partly spread out, denatured casein on the surface of fat globules, originally demonstrated by Jackson and Brunner (74) on the basis of electrophoresis. Freeze-etching techniques of Buchheim (26) demonstrated that unlike the radially oriented 50 Å thick macromolecular triglyceride layers, bound tenaciously to the fat globule membrane, crystals in the bulk lipid phase are not distinctly layered. The fat inside the globule may remain liquid for hours when milk is quickly cooled to 20° C, or largely crystalline when heated to the same temperature from 10° C.

The relationship between the microstructure of butter and its consistency has been studied by Knoop and co-workers (86, 87, 88, 89) using carbon replicas and stereoscopic viewing. Depending on the manufacturing methods, the microstructure of a given sample of butter could be represented by a position anywhere between the two extremes, granular at one end and homogenous, at the other. A clear correlation existed between the structural state observed in the elec-
tronmicroscope and the spreadability of butter. The electronmicroscopy of ice cream by Alsafe and Wood (3) has supported the role of the emulsifiers in controlled fat-agglomeration during freezing and agitation of ice cream mixes, previously observed in the light microscope (6).

Extent of membrane disintegration was higher in the cream containing mixes than in the butter oil formulated ones, indicating the inherent differences between the natural membrane material and lipoprotein complexes formed out of milk proteins during the homogenization of mixes.

Although electronmicroscopy is a powerful tool for ultrastructure studies its usefulness is limited by the considerations of preparatory artifacts. In the absence of complete knowledge of the system under study, the exact nature of artifacts are not known, and their extent of occurrence is difficult to predict. Consequently, informations obtained by studying macromolecular morphology with electronmicroscopy are frequently used as evidence that would support or discard previously established ideas and also for developing working hypothesis to be verified by some other technique. The contribution of electronmicroscopy in the replacement of King's model of fat globule membrane with the establishment of its identity with other biological membrane (124) or its role in generating the new concept of macrocasein (34) highlights the benefits to be derived from either approaches in the studies of macromolecular interactions.
in food systems with electron microscope.
SCOPE OF INVESTIGATION

The aim of this study was to explore the relationship between the physicochemical properties of food hydrocolloids and the microstructure of their interaction products with proteins and to explain on this basis elements of stabilizer action in complex food systems. The specific objectives of this study were:

1. to develop suitable electron microscopic procedures for the examination of the food proteins and polysaccharides and their complexes.

2. to observe the morphology of the ionic interaction products formed between soluble proteins and anionic polysaccharides as represented by the $\beta$-lactoglobulin/carboxymethyl cellulose complexes.

3. to study interactions between calcium-sensitive proteins and hydrocolloids as typified by the complexes of the micellar caseins with k-carrageenan, and

4. to investigate specific interactions of the calcium sensitive $\alpha_s$-casein with the weakly acidic, strongly acidic and neutral polysaccharides such as carboxymethyl cellulose carrageenans and locust bean gums.
EXPERIMENTAL PROCEDURES

A. Proteins

1. Milk Proteins.

In this study the milk proteins, α_{s}-casein, and β-lactoglobulin were used. These proteins were isolated from fresh raw milk of individual Ayrshire cows at the Ohio State University Dairy Farm. This breed is homozygous with respect to α_{s}-casein variant B. α_{s}-Casein was prepared according to the method of Zittle and Custer (192). Polyacrylamide gel electrophoresis was used to check the purity of this preparation (Fig. 10). β-Lactoglobulin (A variant) was isolated by the method of Aschaffenburg and Drewry (8) from the milk of individual phenotyped cows. The purity of this preparation was also checked by polyacrylamide gel electrophoresis (Fig. 10).

Protein preparations were stored as frozen solutions at -15° C.

2. Soy Proteins Isolate.

Edi-pro N (Ralston Purina Co., Missouri) was the soy protein used in this study. The protein solution was prepared by suspending a weighed amount of soy protein in water, adjusting the pH to 7.8 by 0.1N NaOH, warming to 95° C with
Fig. 10. POLYACRYLAMIDE GEL ELECTROPHORETIC PATTERNS OF PROTEINS AND THEIR COMPLEXES. (7.5% polyacrylamide in Tris-Borate Buffer.)

A. 1) \( \beta \)-Lactoglobulin-A.
   ii) \( \beta \)-Lactoglobulin-A/CMC complex at pH 4.0, fixed with Glutaraldehyde.
   iii) \( \beta \)-Lactoglobulin/CMC complex at pH 4.0.
   iv) \( \beta \)-Lactoglobulin-A.

B. 1) \( \alpha_s \)-casein-B.
   ii) \( \alpha_s \)-casein-B, fixed with Glutaraldehyde
   iii) \( \alpha_s \)-casein-B/\( \kappa \)-carrageenan complex.
   iv) \( \alpha_s \)-casein-B/\( \kappa \)-carrageenan complex, fixed with Glutaraldehyde.
   v) \( \alpha_s \)-casein-B.
   vi) \( \beta \)-Lactoglobulin-A.
   vii) \( \beta \)-Lactoglobulin-A, fixed with Glutaraldehyde.
   viii) \( \beta \)-Lactoglobulin-A.

Note the absence of any protein band due to the crosslinking action of glutaraldehyde in the samples shown in A(ii) and in B(ii), (iv), and (vii).
continuous agitation, cooling and diluting the sample with water after the final pH adjustment to 7.0. A small amount of insoluble material was removed by centrifugation at 3000x G for 5 min.

B. Polysaccharides

1. Sodium-Carboxymethylcellulose (CMC-7HP).

The samples of CMC-7HP obtained from Hercules Powder Co., Inc., (Wilmington, Delaware) had a designated substitution range of 0.65 to 0.85 and a high viscosity profile.

2. Carrageenans.

All the carrageenan samples used in this study were obtained from Marine Colloids, Inc. (Springfield, N. J.) A purified preparation of λ-carrageenan (RENJ 5519) was obtained as the sodium salt extracted from Chondrus crispus. The samples of λ-carrageenans were extracted from C. crispus, Gigartina Acicularis and G. pistillata. The alkali modified samples of λ-carrageenan from C. crispus were prepared according to Rees (130).

3. Locust Bean Gums.

Commercial-grade locust bean gums were obtained from Stein, Hall & Co., Inc., (New York). The sulfated locust bean gum samples were supplied by Marine Colloids, Inc. (Springfield, N. J.).
C. Preparation of Protein-Hydrocolloid Complexes


Complexes of β-lactoglobulin and carboxymethylcellulose were prepared according to Hidalgo and Hansen (65). The insoluble complex formed at pH 4.0 and at a protein to hydrocolloid ratio of 3 was isolated by allowing the precipitate to slowly settle to the bottom. The peptization of the insoluble complex was attained by the addition of excess CMC to reduce the ratio from 3 to 1.25. The content was agitated to facilitate dissolution and was then centrifuged at 3000x G for 5 min. to remove the sediments.

2. Complexes of αs-Casein with Stabilizers.

These complexes were prepared for electron microscopy according to the procedure of Lin and Hansen (98). The complex formulation consisted of protein and hydrocolloid in the ratio of 5 to 1 at a 0.15% protein level in the final solution.

D. Reagents for Electron Microscopy

1. Support Membrane.

For the casting of support membrane a 2% solution of Parloidon (purified pyroxylin from Mallinckrodt Chemical
Works, New York) was used. Measured quantity of parlloidon strips was allowed to dissolve for 24 hrs. in amyl acetate to yield a 2% solution. The filtered solution was stored in air-tight stoppered bottle at room temperature.

2. Fixative.

Glutaraldehyde solution was prepared as a fixative agent according to the method of Hopps and Strano (67). Commercially available, 25% glutaraldehyde solutions were treated with activated charcoal (10g./100 ml. of solution) for several days at cold room temperature until the pH of the preparation was between 4 and 5. This preparation was then diluted to give a 3% glutaraldehyde solution in 0.1 M phosphate buffer with pH 7.2. The charcoal treatment of glutaraldehyde was avoided by using highly purified electronic microscopy grade samples from Polyscience Inc. (Warrington, Pennsylvania). The solution was stored at 0-5°C and used within a week.


Stains used for electronmicroscopy, were uranyl acetate, potassium permanganate and phosphotungstic acid.

One gram of uranyl acetate (UA) was gently stirred into 100 ml. absolute methanol and the preparation was filtered through Whatman No. 1 after settling for 10-15 minutes. The filtered stain was stored in dark bottles at 0°-4°C and was
used within a week.

Potassium permanganate (K\textsubscript{2}MnO\textsubscript{4}) solution was prepared by dissolving the permanganate crystals (1 g./100 ml.) in water for 24 hours at the cold room temperature. The stain was stored in the cold room and used within a week.

A 2% solution of phosphotungstic acid (PTA) in distilled water was used as a heavy metal stain, that was stored at 0-5°C.

E. Electron Microscopic Techniques

1. Glutaraldehyde Fixation.

Aqueous solutions or suspensions of proteins, polysaccharides and complexes were mixed in 0.2 ml. amounts with 0.6 ml. of 3% glutaraldehyde and stored for 12-18 hours at 0-5°C, before diluting to 4 ml. by the addition of double distilled water. The fixed samples, stored in cold room were used within a week.

2. Preparation of Grids.

This stage consisted of three main steps, the cleaning of grids, the casting of support membrane and finally, the application of fixed specimens on the grid (20).
a) Cleaning Procedure:

The cleaning and washing of 300 mesh copper grids consisted of 15 minutes sonication in acetone at 80 KC frequency
(Balsonic-I, Bausch & Lomb, Rochester, N.Y.), followed by two washings with 95% ethyl alcohol and a final rinse with double distilled water. The grids were dried on filter paper in a covered petri dish.

b) Casting of Support Membrane:

The parlodion support membrane was deposited on the matt surface of the cleaned and dried grids in an apparatus shown schematically in Fig. 11. The set up essentially consisted of a perforated plastic petri dish fitted on the top of a glass funnel, connected to a water reservoir and drain tube through tubings and stopcocks. The water in the petri dish could be raised to any desired level by opening the stopcock below the water reservoir and could be lowered or drained by opening the stopcock above the drain tube. The grids were carefully placed, matt side up, on a filter paper at the bottom of the petri dish, previously filled with

Fig. 11. Apparatus for Casting Support Membrane on Grid.
double distilled water. A drop of 2% parloidon solution was gently dispensed from the tip of a medicine dropper, and after evaporation of amyl acetate the parloidon formed a thin membrane over the water surface. The first formed membrane was routinely discarded due to the presence of surface contamination. The step was repeated and the membrane subsequently formed on the cleaned water surface would be gently deposited on the grids by slowly lowering the water level below the bottom of the petri dish. The filter paper with the grids were dried overnight in a dust free atmosphere. When required, carbon films of 50-70 Å thickness was deposited on the support membrane in a shadow-casting apparatus according to Bradley (21).

c) Application of Specimen:

With the help of previously cleaned and dried fine-tipped Pasteur pipettes, minute drops of specimen preparation already fixed in glutaraldehyde were dispensed on the support membrane. Precaution was taken to avoid injuring the support membrane by the tip of the pipette during the spreading of the specimen drop on the grid. The film thus spread would be allowed to dry within a few minutes by placing the grids in a dust-free atmosphere. Grids thus prepared could subsequently be used for staining or shadowing with metal.

3. Staining.

In general, a small drop of stain was spread over the
dried specimen film on the grid, and was washed away with distilled water after allowing sufficient reaction time. For PTA and UA, the reaction time of 2-3 minutes was adequate. For KMnO₄, the period was extended to 10-15 minutes (95). Excess stain was removed by suction with a medicine dropper and grids were washed with distilled water. For UA, this washing had to be repeated several times for removing small deposits of UA particles. For KMnO₄ staining, water washing was followed by the addition of a few drops of solution containing a drop of 5% citric acid in 1 ml. water. After 30 sec. the dilute citric acid solution was removed by suction and the grids were given a final distilled water wash. Stained grids were air-dried in a dust-free atmosphere after the final distilled water-wash and before observation in the electron microscope.

4. Observation and Photography.

The stained or shadowed specimens were examined by transmission electron microscopy in a Hitachi HU-10 Electron Microscope using 50 KV accelerating voltage.

Preliminary visual observations were made on several grids prepared from the same specimen. Several areas of the grids were examined to ensure that structural characteristics observed in different fields were representative of the specimen, and not some isolated artifacts or contaminants. Representative areas of the grids were then more closely
observed for finer structural details, using different ranges of magnification. Electron micrographs of representative fields were photographed on 2" x 2" contrast grade projection slide plates (Eastman Kodak Co., Rochester, N.Y.).

The development of negative plates and their photographic enlargement and printing were based on recommended black and white photographic techniques along with the manufacturers' instructions, whenever applicable.

Additional Electron micrographs of several selected grids were recorded with a Philips 200 electron microscope at an accelerating voltage of 60 KV.
RESULTS

In this study, the macromolecular morphology of protein-hydrocolloid interaction systems was examined with electron microscopy. Appropriate methodology was developed for the examination of the selected complexes representing general and specific interactions of food proteins and stabilizers. The application of the methodology involved the examination of the general interactions of the calcium-insensitive proteins, the general interactions of casein micelles in milk and the specific interactions of the calcium-sensitive proteins in model systems.

A. Development of Methodology

The successful application of electron microscopy depends on the proper choice of instrumentation and methodology. For any given system of instrumentation it is necessary to develop satisfactory methods of sample preparation to minimize artifacts without impairing simplicity. Experiments were carried out to develop a suitable method of fixation, to choose between shadow casting and staining for the development of specimen contrast, and to select proper stains for different systems to be studied.
1. Glutaraldehyde Fixation Procedure.

To test the suitability of the glutaraldehyde-fixation procedure of Carroll, Thompson and Nutting (35), 0.2 ml. samples of skim milk and 0.1% soy protein solutions were mixed with 2 ml. of 1.0% glutaraldehyde and kept for 15 min. at room temperature. The preparations were diluted to a final volume of 40 ml., applied on carbon coated grids and shadowed with germanium. The electron micrographs of casein micelles and soy proteins are presented in Fig 12 and Fig 13, respectively.

In Fig 12 the casein micelles appear as spherical particles of varying sizes ranging from 400 Å to 1500 Å in diameter. The rough, golf ball like surface appearance suggests the presence of submicellar units of about 200 Å diameter. Similar surface features have also been observed in casein preparations shadowed with platinum-palladium (152). The ratio of the particle diameter to the length of the white shadow area in the larger micelles was approximately 0.90 and in the smaller ones, 0.80. Therefore the micelles larger than 1000 Å diameter appear to have been more deformed due to flattening than the smaller ones. The presence of excessive background structure in the micrograph shows that the germanium shadowing is not suitable for high resolution microscopy, even though its low melting point would cause minimum heat induced artifacts (174).

Fig 13A and B represent two micrographs taken from the
Fig. 12. CASEIN MICELLES. (Glutaraldehyde fixed and germanium shadowed; instrument - Philips - 200 Electron Microscope; magnification - 117,500x.)

(Note the golf-ball like surface features in large micelles)
Fig. 13. EFFECT OF GLUTARALDEHYDE FIXATION PROCEDURES ON SPECIMEN STABILITY. (Soyprotein preparations germanium shadowed.)

A. 15 min. fixation with 1\% glutaraldehyde at room temperature; initial appearance.

B. 15 min. fixation with 1\% glutaraldehyde at room temperature; after 2-3 min. of exposure to the electron beam. (Note "Cell"-like artifacts)

C. 15-18 hrs. fixation with 3\% glutaraldehyde at 0-4°C; after 5 minutes of exposure to the electron beam.
same protein specimen grid (soy protein) only a few minutes apart. Approximately spherical bodies of 1000-1500 Å diameter were apparent in both cases. The amount of germanium shadow in the specimen was not heavy enough to create appreciable shadow effect, but sufficient for adequate visualization of the particles. The uniformly electron dense round bodies, observed initially (Fig 13A), rapidly attained the appearance of "cellular" structures (Fig 13B) under the influence of the electron beam. Badekas (10) has reported similar artifacts in unfixed preparations of CMC. Such bubbling effect may perhaps result from sample instability due to the absence of or incomplete fixation.

To eliminate the sample instability observed in the soy protein preparations, the concentration of glutaraldehyde was increased from 1 to 3% and the period of fixation was extended from 15 min. at room temperature to 15-18 hours at cold room temperature. A similar treatment has been successfully used by Schmidt (144). The effectiveness of such modification is apparent from a typical micrograph presented in Fig 13C. Globular particles of 3000-6000 Å diameter appear as uniformly electron dense material casting sharp, well defined shadow areas. Surface details of these large particles appear quite clearly as roughness in the border regions of the shadow areas. Though the carbon-coated support membrane is still somewhat coarse, smaller protein particles of 150 to 250 Å diameter can readily be observed in the background.
2. Shadowing Vs. Staining in Specimen Contrast.

For the selection of a suitable method of importing specimen contrast, complexes of $\alpha_S$-casein and $\kappa$-carrageenan were subjected to shadow casting as well as staining treatment. The samples were fixed with a 3% glutaraldehyde solution for 15-18 hrs. The fixed samples were air dried on two separate sets of grids, one of which was germanium shadowed and the other stained with KMnO$_4$.

A typical view of a germanium shadowed casein-carrageenan complex is presented in Fig 14A. Several round bodies of 1000-3000 Å diameter appear to be well separated from each other. However, a few smaller particles of 250-300 Å diameter seemed to be clustered in a somewhat fibre-like fashion. From the micrographs, it is not possible to distinguish the protein moieties from the polysaccharide entities in the complex.

The stained preparations of the complex, presented in Fig 14B, reveals a number of electron-dense particles en-meshed in a relatively electron transparent fibre-like network. On the basis of structural studies on polysaccharides (131) carrageenan would be expected to exhibit this particular fibrous character not usually observed in the caseins. Furthermore, since KMnO$_4$ is generally recognized as a protein stain (194), the dark bodies were considered to be the casein-rich area and the relatively electron-transparent network structure to be the carrageenan fibres.
Fig. 14. EFFECT OF SHADOW CASTING AND STAINING ON THE VISUALIZATION OF THE PROTEIN AND POLYSACCHARIDE STRUCTURES. (α₆-casein/k-carrageenan complex gluteraldehyde fixed; magnification - 40,000x.)

A. Germanium shadowed.

B. KMnO₄ stained.

C. PTA stained.
A comparison of shadow-casting and staining has, therefore, established the merit of the latter approach in the visualization of the protein-hydrocolloid complex. Although with shadowing a three dimensional effect can be created and informations on the size and shape of the particles may be obtained, the staining techniques appear to be particularly suitable in the differentiation of the protein and hydrocolloid areas in the complex.

3. Choice of Stains for the Protein-Hydrocolloid Complexes.

A comparison was made of the staining ability of UA, PTA and KMnO₄. UA was selected for the microscopy of polysaccharides alone as it produced the most intensive contrast with the hydrocolloids. PTA and KMnO₄ were relatively ineffective with the stabilizers. Due to the preferential protein staining tendency of PTA and KMnO₄, these were considered particularly useful in the differentiation of proteins in their complexes with polysaccharides.

A comparison of the PTA-stained material Fig 14C with the KMnO₄-stained complex in Fig 14B shows essentially the same structural features without any marked difference in the development of contrast. The differentiation of the proteins and the polysaccharides in the complex was about the same in both cases.

However, for the staining of the complexes, and
constituent proteins, KMnO₄ was preferred to PTA because it could be used at neutral pH, a requirement for many of the protein-hydrocolloid complexes considered in this study.

On the basis of these experiments on the development of methodology, most samples were satisfactorily prepared for electron microscopy by 15-18 hrs. fixation with 3% glutaraldehyde, followed by the KMnO₄ staining of the proteins and complexes or Uranyl acetate staining of the polysaccharide preparations.

B. Ionic Complexes of β-Lactoglobulin and Carboxymethyl Cellulose

Hidalgo and Hansen (65) reported that β-lactoglobulin interacts with anionic polysaccharides such as carboxymethyl cellulose at acidic pH and low ionic strength. While heavy precipitation of the complex occurred at pH 4.0, physical stability could be achieved by peptization as well as by shifting pH to 5.2 or above. Samples of complex which were precipitated at pH 4.0 were examined in the electronmicroscope along with those formed by peptization and at pH 5.2. Preparations of β-lactoglobulin and CMC solutions were also examined.

1. β-Lactoglobulin and CMC Samples.

In Fig 15A and B, the electron microscopic views of the
Fig. 15. WHEY PROTEINS, CMC, AND THEIR COMPLEXES PREPARED BY VARIOUS METHODS. (CMC stained with UA, others with KMnO₄.)

A. β-Lactoglobulin; magnification - 40,000x.
B. CMC; magnification - 40,000x.
C. β-Lactoglobulin/CMC complex at pH 4.0; Philips 200 E.M., magnification 117,500x. (Arrows indicate a few dark particles of about 40 Å diameter. Note numerous dark particles of similar dimensions.)
D. Peptized complex of β-lactoglobulin-CMC at pH 4.0; Philips 200 E.M., magnification 235,000x. (Note dark particles are fewer in number.)
E. Whey protein/CMC complex at pH 4.0; magnification 40,000x. (Note dark whey protein aggregates indicated by arrow.)
F. Whey protein/CMC complex at pH 5.2; magnification 40,000x. (Arrows indicate whey protein aggregates.)
protein \( \beta \)-lactoglobulin and the stabilizer CMC are presented. Both of these preparations were fixed with glutaraldehyde; the protein was stained with \( \text{KMnO}_4 \) and the hydrocolloid with UA.

\( \beta \)-Lactoglobulin appeared (Fig 15A) as a mass of amorphous material without any particular structural feature. The mass of protein is represented by dark material on a lighter background and appear to be aggregated in a non-specific manner. The carboxymethyl cellulose (Fig 15B), on the other hand, could be seen as mass of fibrous structures, bundled and entangled to varying degree. At the present level of magnification (40,000) no further structural details of the CMC chains could be seen. Badekas (10) attempted to observe CMC preparations but failed to observe any similar structural features. However, Rees (131) indicated that the appearance of CMC would be similar to the parent cellulose molecules. The successful visualization of the fibrous structure in CMC preparations appears to be due to the improved glutaraldehyde fixation procedures.

2. Complexes of \( \beta \)-Lactoglobulin and CMC.

The electron micrographs of precipitated and peptized complexes prepared at pH 4.0 and stained with \( \text{KMnO}_4 \) are presented in Fig 15C and D.

In the complex precipitated at pH 4.0 (Fig 15C) numerous, 40-50 Å diameter, dark, round spots were scattered
uniformly between the electron transparent CMC chains. The apparent order and periodicity of these spots suggest that these may possibly correspond to the negatively charged regions of the CMC chains where β-lactoglobulin interacted to yield the dark globular spots. The structure of native cellulose chains are known to consist of 35-40 Å wide microfibrillar subunits (100). According to Mühlethal (110) this would correspond to about 40 monomer units of cellulose. From the known dimensional features of β-lactoglobulin (168) an area corresponding to 40-50 Å diameter may be adequate for a dimer of β-lactoglobulin molecule.

The soluble peptized complex of β-lactoglobulin and CMC (Fig 15D) differs from the precipitate in the extent of protein interaction with the CMC chains. The CMC chains in the peptized complex appear much lighter than those in the precipitated complex and the number of dark, presumably protein-rich, spots are fewer. Although the micrograph was recorded at a magnification different than the one in Fig 15C, the dark spots still appeared as 40-50 Å diameter areas in the precipitated complex. The morphology of the peptized complex supports the earlier hypothesis of Hidalgo and Hansen (65) that the redistribution of protein in the excess CMC was the possible reason for solubility due to the peptization process.

3. Complexes of Wheyproteins and CMC.
The interaction products of CMC prepared in cheese whey at pH 4.0 and at pH 5.2 were also examined after KMnO₄ staining. The precipitated complex of whey proteins and CMC, presented in Fig 15E, indicates the structural similarity of this preparation with that of CMC with β-lactoglobulin alone. In addition to the uniform presence of the whey proteins on the CMC structure, a few dark spherical particles of 250 Å to 1000 Å diameter are visible in the complex macromolecules. These particles may be the particulate aggregates of whey proteins, as observed by Morr et al. (109).

The complex prepared at pH 5.2, the isoelectric point of β-lactoglobulin (Fig 15F) also exhibited the presence of whey proteins over the entire CMC chain, along with a few large agglomerates of whey proteins entrapped in the network. However, the CMC network appeared to be of shorter chain length and not massively aggregated as in the case of the complex precipitated at pH 4.0.

In general, the macromolecular morphology of the precipitated complexes of CMC in whey as well as in β-lactoglobulin solutions revealed a relationship of complex solubility with the extent of hydrophilic sites available for solvation and the tendency of aggregation of the complex macromolecules. The process of peptization promoted redistribution of protein molecules in a larger amount of hydrophilic colloids, resulting in the solubility of the system due to more solvation. Unlike the peptized complex, the soluble interaction products
of β-lactoglobulin and CMC formed at pH 5.2 did not exhibit any marked dissociation of protein when compared to those prepared at pH 4.0. The pH shift of the environment improved solubility characters of the complexes by preventing excessive aggregation between the macromolecules.

C. κ-Carrageenan Interactions in Milk

When carrageenan is added to skim milk, the resultant system remains stable at pH 6.7, provided the stabilizer concentration does not exceed 0.02-0.03%. Above this concentration, spontaneous separation of coagulated protein and serum phase occurs (59). On the basis of the relative distribution of ester sulfate in the supernatant, sediment and whey fractions it has been shown (57) that both λ- and κ-carrageenan interact with casein micelles.

In the present study it was further observed that at the neutral pH ranges, the physical stability of the system also depended on the temperature at which the stabilizer was incorporated. The stabilized system containing carrageenan could, however, be precipitated at pH 4.6.

For the purpose of electron microscopy, κ-carrageenan systems were prepared at 0.1% concentration in skim milk at 0° and 60°C to provide fluid and gelled systems and the interaction products were examined along with the sediments and the whey fractions at pH 4.6. Carrageenan-free skim milk preparations were examined at pH 6.7 and 4.6 and used as
controls.

1. Skim Milk and Acid Curd without Carrageenan.

Skim milk at pH 6.7 and the curd prepared by acid precipitation at pH 4.6 were glutaraldehyde fixed and KMnO$_4$ stained. The casein micelles in skim milk (Fig 16A) appeared as spherical structures of 1000-1500 Å diameter, with many particles in larger as well as smaller sizes.

In the specimens prepared from the acid curds of the same skim milk, the aggregation of micelles is evident (Fig 16B). Although the aggregation was generally massive, however, a relatively clear area was chosen for presentation in the micrograph to show the lateral coalescence of aggregating casein micelles, observed in many other studies (31,115,144). Although the structural integrity of a few micelles appeared well preserved, the major portion of the micelles had lost the individual globular structure.

2. Skim Milk with Carrageenans, pH 6.7.

A typical view of the interaction products prepared at 0°C is presented in Fig 17A. These systems remained fluid. Many dark spherical bodies of 500-1500 Å diameter appeared to be interconnected by electron transparent network structures. These heavily stained particles were presumed to be the micellar caseins entrapped in the carrageenan net work. On the basis of these micrographs the presence or absence of whey
Fig. 16. CASEIN MICELLES IN SKIM MILK AND IN ACID CURD.
(Glutaraldehyde fixed and KMnO₄ stained; magnification - 40,000x.)

A. at pH 6.7.

B. at pH 4.6. (Arrows indicate lateral coalescence of the micelles.)
Fig. 17. EFFECT OF TEMPERATURE AND pH ON THE MORPHOLOGY OF k-CARRAGEENAN-CONTAINING SKIM MILK. 
(Glutaraldehyde fixed, KMnO₄ stained and 40,000x magnification.)

A. Skim milk containing k-carrageenan at pH 6.7 and temperature - 0°C.
B. Skim milk containing k-carrageenan at pH 6.7 and temperature 60°C.
C. Sediment at pH 4.6 and 0°C. (Arrows indicate smaller dark particles entrapped in large aggregates as well as free.)
D. Sediment at pH 4.6 and 60°C. (Arrows indicate smaller dark particles. Note the interlinking between large particles.)
E. Whey at pH 4.6 and 0°C. (Note compact fibre structure.)
F. Whey at pH 4.6 and 60°C. (Note intensive interlinking in the network.)
proteins could not be detected in these structures.

The systems prepared by heating to 60°C gelled upon cooling to 4°C. The micrograph of this system is presented in Fig 17B. The basic morphology of this material was similar to those observed in the nongelled materials in terms of the presence of casein micelles entrapped in a carrageenan network. However, the network structure appeared to be more completely interlinked in a massive continuous structure in contrast to the relatively isolated aggregates in the nongelled sample (17A). Furthermore, the polysaccharide materials in the gelled system appeared to be spread out, and lacked the compactness observed in the fluid products. The differences in the polysaccharide fractions in the gelled and non-gelled skim milks indicate that the morphology of this system is altered largely due to the temperature sensitive gelling behavior of κ-carrageenan.

3. Skim Milk with Carrageenan at pH 4.6.

The pH of the carrageenan/skim milk preparations was reduced from 6.7 to 4.6 with dilute HCl. Immediate precipitation occurred in both of these samples and the precipitates as well as the supernatant were examined.

The typical appearance of the sedimented fractions are presented in Fig 17C and D. Both the micrographs show the presence of large, irregularly shaped, particulate structures of dimensions in excess of several thousands angstrom in
addition to many small dark round particles of 250-500 Å diameter. The gelled samples exhibited a certain amount of interlinking between the large particulate structures but in neither case was a differentiation between the protein-rich zones and the polysaccharide materials clearly observable. The dark bodies observed in the controls as well as in samples at neutral pH were conspicuously absent from these sediment fractions. Instead, the presence of numerous smaller particles, free as well as entrapped in large particulate structures, suggested some structural degradation of the micellar subunits at pH 4.6 due to carrageenan. Such dissociation may in part be due to the shift in the isoelectric point of the complex as a result of interaction between the protein and the sulfate groups of the stabilizer, or it may perhaps be due to the destruction of the colloidal calcium phosphate system at the reduced pH and the subsequent entrapment of the disaggregated subunit micelles in the carrageenan network. This observation may be important in considering the mechanism of stabilization of acidified products such as cultured milk, creams and dips.

The electron micrographs of the whey fractions from the gelled and non-gelled product are presented in Fig 17E and F. The presence of characteristic fibrous networks due to carrageenans can be noticed in both cases. The development of weaker contrast in these specimens indicates the presence of only the smaller amounts of whey proteins.
The specimen from the non-gelled samples (Fig 17E) was different from the gelled samples (Fig 17F) in the extent of network structure. At the 0°-4°C, the carrageenan network appeared to be more or less compact fibrous bundles, which appeared to have been uncoiled during heating to 60°C and reassOCIated with other fibre-structures during cooling to 0°C, creating extensive network structures responsible for the gelled state at the neutral pH ranges.

The detection of carrageenan in the acid whey by electron microscopy is in disagreement with the findings by Hansen (57), who reported only insignificant amounts of carrageenans remaining in the whey by chemical analyses. It is possible that the conflicting results may be due to the use of unfractionated carrageenans in the earlier studies.

D. Specific Interactions of $\alpha_b$-Caseins in Model Systems

Stability studies in model systems have demonstrated that the calcium precipitation of $\alpha_b$- and $\beta$-caseins can be prevented by inducing complex formation with the protective colloids such as $\kappa$-casein (182) or carrageenans (58). Stability is indicated by the amount of protein in the supernatant after centrifugation and instability by the amount sedimented. The $\alpha_b$-casein stabilizing ability of several carrageenans is presented in Fig 18.
Fig. 18. STABILIZATION OF $\alpha_s$-CASEIN BY CARRAGEENANS.
(pH 6.7, at 0.15% protein and 0.01M CaCl$_2$)

○ $\kappa$-carrageenan from _C. crispus_.
○ $\lambda$-carrageenan from _G. acicularis_.
□ $\lambda$-carrageenan from _G. pistillata_.
● $\lambda$-carrageenan from _C. crispus_.
■ $\lambda$-carrageenan (alkali modified) from _C. crispus_.

Adapted from Lin and Hansen (98)
1. Stable Supernatant Fractions.

The stable systems develop turbidity typical of skim milk and the particles in the κ-casein containing systems resemble the 180-200 Å subunit structures of the native casein micelles (32). However, nothing is known about the morphology of polysaccharide-stabilized caseins.

Lin and Hansen (98) demonstrated that the physical stability of the casein/polysaccharide interaction products depended on the choice of the stabilizer. For the sulfated polygalactans, the effectiveness depended on the glycosidic linkages, the location of sulfate groups as well as on the polymer size.

Attempts were made in this study to examine the ultrastructure of the interaction products between κ-casein and several polysaccharides of varying stabilizing ability. For this purpose, the supernatant as well as the sediment fractions of the various complex systems at pH 6.7 were obtained by centrifugation at 3000xG.

a) Effect of Ionic Calcium on the Complex:

Figure 19 A and B represent the typical electronmicroscopic view of κ-casein preparations and their aggregation in the presence of ionic calcium. The permanganate-stained, calcium-free κ-casein appeared as loose, amorphous aggregates of 500-1000 Å cross-section. Upon the addition of calcium, these protein particles attained a more compact globular structure of 1000-3000 Å. The globules were appar-
Fig. 19. EFFECT OF IONIC CALCIUM ON THE $\alpha_s$-CASEIN/ k-CARRAGEENAN COMPLEX. (Glutaraldehyde fixed and KMnO$_4$ stained.)

A. Calcium-free $\alpha_s$-casein; Philips 200 E.M.; magnification = 57,500x.

B. $\alpha_s$-casein with 0.01M CaCl$_2$; magnification = 40,000x. (Note compact structure.)

C. $\alpha_s$-casein/k-carrageenan complex without Ca$^{++}$; Philips 200 E.M.; magnification = 57,500x. (Note the network structure of polysaccharide designated by N marked arrow. Areas burnt in the electron beam are designated by B marked arrows.)

D. $\alpha_s$-casein/k-carrageenan complex with 0.01M CaCl$_2$; Philips 200 E.M.; magnification = 57,500x. (Note the compact protein bodies in polysaccharide network structure.)
ently formed by calcium bridging of numerous casein molecules still visible as less compact fibre-like linkages connecting the globules. The fibre materials in the $\alpha_s$-casein samples were apparently formed by a linear association of the protein material.

The calcium-free mixture of casein-carrageenan is presented in Fig 19C. The fibrous mass of the electron-transparent $\kappa$-carrageenan appeared as a network structure interspersed with dark areas, indicating the presence of complexed proteins materials. The polysaccharide network observed in this case was more electron transparent than the fibrous material observed in the stabilizer-free $\alpha_s$-casein samples (Fig 19A & B) and exhibited characteristic interlaced features. The numerous white streaky spots in the micrograph indicate a certain amount of specimen contamination due to a burning effect by the electron beam.

On the basis of free boundary electrophoresis, the interaction of $\alpha_s$-casein with $\kappa$-carrageenan in the absence of calcium could not be demonstrated (58). The presence of the protein moiety interspersed with the stabilizer network, observed in the electron micrographs of the calcium-free preparations, may therefore be due to the physical entanglement or due to the formation of a weak complex dissociating under the conditions of electrophoresis.

The supernatant fractions of the calcium containing $\alpha_s$-casein-$\kappa$-carrageenan complex are presented in Fig 19D.
Upon the addition of ionic calcium, the stable complex appeared as many dark particles entrapped in a carrageenan network structure (Fig 19D). The loose polysaccharide network seen previously in Fig 19C now appeared as a compact fibrous structure linked to the dark, globular protein-polysaccharide bodies. Apparently, it is this interaction which is responsible for stabilizing the system by keeping the protein bodies physically separated. One may notice, however, that the carrageenan stabilized complex bears no resemblance to the casein micelles stabilized by $\kappa$-casein.

b) Effect of Polymer Size of the $\lambda$-Carrageenans.

Lin and Hansen (98) indicated that although the $\lambda$-carrageenans from *Gigartina pistillata* and *G. acicularis* exhibited identical infrared spectra, they varied greatly in their $\alpha_s$-casein stabilizing ability. The relative ineffectivity of the $\lambda$-carrageenan from *G. pistillata* was shown to be due to the high degree of polymerization, since by polymer degradation, the stabilization ability could be improved to a level comparable to that of *G. acicularis*.

The supernatant fractions of the complex of $\alpha_s$-casein and $\lambda$-carrageenans from these two botanical sources are presented in Fig 20A and B. The complex containing the more effective stabilizer from *G. acicularis* (Fig 20A) is shown in three separate fields and presented itself as well separated, individual, spherical particles of about 2000 Å diameter. The heavily stained protein bodies appeared to be
Fig. 20. \( \alpha \)-CASEIN COMPLEX WITH \( \lambda \)-CARRAGEENANS OF DIFFERENT POLYMER SIZE. (Glutaraldehyde fixed, KMnO\(_4\) stained, and instrument magnification - 40,000x.)

A. Complex containing \( \lambda \)-carrageenan from \( G. \) acicularis. (Notice in all the three fields the presence of individual spherical particles. A white halo around the particles in field ii and iii is due to the underfocussing.)

B. Complex containing \( \lambda \)-carrageenan from \( G. \) pistillata.
attached to lighter fibrillar materials presumably of polysaccharide nature. However, the morphology of this complex material was distinctly devoid of any network structure of hydrocolloids, characteristic of many other polysaccharides. The failure to detect any network structure may suggest that the degree of polymerisation of \( \lambda \)-carrageenan was insufficient to promote any entanglement or network formation.

The complex containing \( \lambda \)-carrageenan from \( G. \) Pistillata, on the other hand, exhibited compact, well defined network structures containing separated protein bodies. The general appearance of this stable macromolecular complex was essentially similar to the system prepared with the \( \kappa \)-carrageenan (\( C. \) crispus). However, the stable complex represented only the minor fraction of the system since the stability was merely 20%.

c) Effect of Alkali Modification of the \( \lambda \)-Carrageenans:

The removal of C-6 sulfate by alkali modification greatly improved the casein-stabilizing ability of \( \lambda \)-carrageenan from Chondrus crispus (98). The associated morphological changes in the complexed protein are apparent from the micrographs in Fig 21A and B. The stable portion from the preparation containing the unmodified \( \lambda \)-carrageenan exhibited essentially network-free bundles of fibrous material entrapping a few protein bodies in the structure. The staining character of the fibre material indicated the presence of protein throughout the polysaccharide material.
Fig. 21. \( \alpha_5 \)-CASEIN COMPLEXES OF \( \lambda \)-CARRAGEEENANS. (C. crispus) Before and after alkali modification. (Glutaraldehyde fixation, \( \text{KMnO}_4 \) staining and instrument magnification - 40,000x.)

A. Complex with unmodified \( \lambda \)-carrageenan.

B. Complex with alkali modified \( \lambda \)-carrageenan. (Note similarity with the \( \kappa \)-carrageenan stabilized structure in Fig. 14B.)
The complex with the alkali-modified preparations resembled closely the materials obtained from the $\alpha_s$-casein/\(k\)-carrageenan complexes (Fig 14B, 14C & 21B).

The appearance of complexes prepared with \(\lambda\)-carrageenans before and after the alkali-modification supports the view that the removal of C-6 sulfate group increases the conformational similarity between \(\lambda\)- and \(k\)-carrageenans and would therefore also improve the stabilizing ability.

d) Effect of Sulfation of Locust Bean Gum:

Although the neutral stabilizer locust bean gum, a branched polysaccharide, has no casein stabilizing ability, its sulfated derivative has been shown to be a potent $\alpha_s$-casein stabilizer (97). The electron microscopic view of different fields is shown in Fig 22.

The stable complex containing this modified hydrocolloid exhibited a unique filligre structure with centres of spherical protein bodies surrounded by numerous smaller proteinaceous spots uniformly scattered throughout the branched network of the hydrocolloids. However the complex did not contain the polysaccharide network which was so characteristic of the carrageenan family.

2. Unstable Sediment Fractions.

Varying amount of sediment can be obtained by centrifugation of systems containing $\alpha_s$-casein and polysaccharides. With the effective stabilizers the sediment fractions were very
Fig. 22. \(\alpha_5\)-CASEIN COMPLEX WITH THE SULFATED LOCUST BEAN GUM. (Glutaraldehyde fixed and KMnO\(_4\) stained.)

A. A few complex particles at 40,000x magnification. (Note filligree structure.)

B. Another view of the complex at 40,000x magnification.

C. A portion of complex particle at 545,000x magnification. (Note dark particles of about 100Å diameter in the lightly stained polysaccharide network.)
small. However, for the complex containing CMC, locust bean gum and ineffective \( \lambda \)-carrageenans (from \textit{G. pistillata}), the amount of sediment was of considerable magnitude (58,98).

The electron micrographs of typical sediment preparations are presented in Fig 23. These preparations exhibited the presence of heavily stained materials massively interacted with polysaccharide. The differentiation of the protein and polysaccharide materials in these preparations is not as clear as in the case of the stable supernatants. However, in the sediments obtained from the CMC/casein complex, the remarkable structural resemblance of this system with the CMC-containing whey protein complexes in Fig 15E and F should not be overlooked.

A general comparison of the sediment and supernatant fractions indicates that the protein-polysaccharide interaction is more complete and occurs more uniformly throughout the hydrocolloid structure. Such structures may have poor solvation characters and may be subject for further non-specific interaction resulting in the massive aggregation to a size far beyond the colloidal stability.
Fig. 23. SEDIMENT FRACTIONS OF $\alpha_s$-CASEIN/HYDROCOLLOID COMPLEXES. (Glutaraldehyde fixed and KMnO$_4$ stained; magnification - 40,000.)

A. Complex of $\alpha_s$-casein with $\kappa$-carrageenan.

B. Complex of $\alpha_s$-casein with $\lambda$-carrageenan from $G$. acicularis.

C. Complex of $\alpha_s$-casein with $\lambda$-carrageenan from $G$. pistilliata.

D. Complex of $\alpha_s$-casein with $\lambda$-carrageenan from $C$. crispus (Alkali modified).

E. Complex of $\alpha_s$-casein with locust bean gum.

F. Complex of $\alpha_s$-casein with CMC. (Note the structural similarity with the whey protein/CMC complex in Fig. 15E & F.)
DISCUSSION

This study has revealed that an intimate relationship exists between physical stability and macromolecular morphology of protein-hydrocolloid interaction systems. The examination of the electron micrographs indicated that the structural features of the complexes depended largely on the conformation of the polysaccharide fraction.

Although electron microscopy has been proven to be a powerful tool in these studies, one must bear in mind that careful interpretation of the micrographs is essential in developing any meaningful concept. In particular, it is necessary to consider the extent of artifacts induced due to the experimental conditions. In the present study, such considerations involved the use of glutaraldehyde as fixative, drying of liquid specimen preparations spread as a thin films, and the development of specimen contrast by staining. The choice of glutaraldehyde as a fixative agent for the electron microscopy was based upon the earlier work by Sabatini et al. (141). It has been a preferred reagent due to its nontoxic and electron transparent nature in the electron microscopy of milk proteins (35). Glutaraldehyde has been demonstrated to promote inter-molecular crosslinking via epsilon amino groups in proteins (56) and, secondary OH-groups
in polysaccharides (73). The glutaraldehyde-fixation has been shown to cause minimal molecular disorder in proteins, including β-lactoglobulin (17), because fixed proteins retained immunospecificity (133).

Unlike the proteins, polysaccharides have often been examined in the electron microscope without any fixation. They undergo, by themselves, intensive hydrogen bonding during the specimen drying operation, resulting in a strong fibre-like structure which are stable to the electron beam. However, experience with shadowcast preparations in this study warranted the use of a proper fixative procedure because of frequent specimen instability. In the absence of any fixative procedure, Badekas (70) reported bubbling effects in the CMC preparations and failed to observe the characteristic fibre-like structures revealed in the present study with the glutaraldehyde fixation. From work on textile grade cellulose fibres (73) it is known that the aldehydic carbonyl groups of glutaraldehyde undergo condensation reactions with the C-2 and C-3 OH-groups of the cellulose chains resulting in intermolecular cross-linking amongst chains placed side by side. The effect of such cross-linking would be similar to the H-bonded lateral aggregation in the absence of fixation. Therefore, the characteristic fibre networks, observed in the glutaraldehyde fixed hydrocolloids, in our opinion, reflected the inherent morphology of these polysaccharides.
The possible artifacts from the formation of the complexes between proteins and hydrocolloids must be judged in the context of glutaraldehyde reactions on proteins and polysaccharides, individually. Since the cross-linking reaction of glutaraldehyde is covalent, the hydrogen bonding and the ionic interaction, important in the complex formation, should not be affected to any great extent. This expectation has been substantiated by the electron micrographs of the $\alpha_s$-casein/$\kappa$-carrageenan complex, before and after the addition of calcium. In the calcium-free complex of protein and hydrocolloid, the loose amorphous nature of $\alpha_s$-casein and the characteristic network structure of the polysaccharides were well preserved. In the calcium-containing complexes, glutaraldehyde preserved the typical globular aggregation of $\alpha_s$-casein, observed in the calcium-containing, stabilizer-free preparations. The entangled fibrous network of the polysaccharide, formed due to the H-bonding and counter-ion activity, also remained unaffected. This close agreement between the established chemical reactions of the fixative and the observed morphological features in the micrographs would make it highly improbable that the significant structural features of the protein-hydrocolloid complexes were in any way due to preparatory artifacts from glutaraldehyde.

The structures observed in the electron microscope essentially represents the morphology of the macromolecules in their dried, solid state. How closely these features
resemble the colloidal state cannot be predicted accurately. It is, however, necessary to consider if the entangled polysaccharide network truly reflects the colloidal state or was caused by the known H-bonding tendency of the polysaccharides during the specimen drying operation. A comparison of the morphology of complexes containing different polysaccharides revealed that the presence of bundled polysaccharide chains was not common for all (Fig 20A and B), but depended upon the nature of the stabilizers. Furthermore, Rees (131) has argued that the gels formed as a result of polysaccharide networks may be considered rigid or permanent for all practical purpose. In addition, the glutaraldehyde fixation of the complex prior to the specimen drying operation would have stabilized the conformation in the colloidal state. Therefore, the observed morphology of the protein-hydrocolloid complexes largely reflected the inherent properties of these systems, rather than the artifacts induced due to the drying.

The flattening effect on the air dried specimens due to the surface tension forces was observed in the shadow cast preparations. However, this effect could be disregarded in the qualitative appraisal of the micrographs. Similarly, the minor effects of staining chemicals in terms of the increase in the volume of the stained particles could be neglected in the present study.

Although the preparatory techniques used in this study
were selected in order to minimize artifacts, the interpretation of the results must be made with caution. The morphological description of macromolecular ultrastructure is to a large extent influenced by the subjective judgement, experience and personal bias of the investigator, and the measurements are crude as compared to the more precise description of macromolecular conformation in terms of coordinate geometry.

Difficulties in the differentiation of the structural elements, further influences the interpretation of the micrographs. However, the choice of the neutral protein-stain, KMnO₄, has been particularly suitable for studying the protein-polysaccharide complexes, since the protein materials were preferentially stained. By examining the proteins and polysaccharides, stained separately, their identification in the mixture as the heavily stained protein bodies and the lightly stained, polysaccharide fibre would therefore be justified.

An example of the inherent difficulties in interpreting the comparative structures of the complexes and their constituents was encountered early in the study. The question arose with respect to the nature of fibre-like materials observed in the stabilizer-free preparations of α₅-casein. The fibre-like material was observed in the loose, amorphous calcium-free preparations as well as in the calcium-aggregated globular mass of protein. Although these structures were
different from the characteristic network structures of polysaccharides, their low electron density may be indicative of a non-protein nature. Thread-like structures in the embryonic state of casein micelles have been demonstrated by Helminen and Ericsson (63) and also by Carroll, Thompson and Farrell (34). Sharma and Hansen (150) reported considerable electrophoretic and chemical changes in $\alpha_s$-casein by the action of hyaluronidase and $\beta$-glucuronidase and suspected the presence of carbohydrate materials resembling hyaluronic acid. Heparin-like material and hyaluronic acid has in fact been detected recently in milk and in caseins (114). Therefore, the possibility can not be excluded that the fibre-like material in the casein preparations may be of carbohydrate nature. However, since $\alpha_s$-casein is reasonably characterized by chemical analysis, only a small amount of the carbohydrate material can be expected. Further studies would be required to substantiate this possibility.

An unexpected high level of resolution was obtained in some preparations, which appears promising for future work. This was particularly so in the case of the complexes prepared with CMC. In the protein-free preparations, the polysaccharides usually appeared as bundles of fibrous material, branched and entangled to varying extent, and it was difficult to view any portion of an isolated chain with any degree of clarity. However, when the complex formation was induced, the protein-interacted fibre regions could be observed as
single-stranded chains. Furthermore, in the β-lactoglobulin/CMC complexes at pH 4.0, the abundant, 40-50 Å, dark spherical particles were unmistakably β-lactoglobulin molecules. This judgement was based on the dimensions of β-lactoglobulin reported by Timasheff (168) as 34.6 Å for the monomer. The presence of the β-lactoglobulin in the CMC structure was not an isolated case, since the particles were also observed in the peptized samples. One possible explanation for this high resolution may be due to the flat, ribbon-like nature of the cellulose chains of about 5 Å thickness. In isolated areas of the strands, a thickness of that order would promote high resolution without ultrathin sectioning procedures, even in air-dried specimens.

A careful examination of the β-lactoglobulin micrographs revealed an ordered arrangement of many of these molecules, in pairs or in arrays. Such ordered arrangement may be considered in terms of the ultrastructure of native cellulose and may be important in understanding the molecular nature of the β-lactoglobulin/CMC interaction. The widely studied ultrastructure of cellulose fibres has not been established unequivocally, however, but a possible structure of 35-40 Å units of microfibrils, tightly coiled in a helical fashion has been proposed by Manley (100). On the basis of the structural similarity of the native cellulose and its carboxymethylated derivatives, proposed by Rees (131), the β-lactoglobulin molecules may be visualized to interact on
the alternating strips of the ribbon or may be entrapped in the folds of helix, as shown schematically in the following diagram (C & D). The entrappment in the helical folds may

![Diagram of proposed ultrastructure of \( \beta \)-Lactoglobulin/CMC complex](image)

Fig. 24. Proposed Ultrastructure of \( \beta \)-Lactoglobulin/CMC Complex

impart conformational restraint on the part of the protein as well as the CMC chains, and may explain the unique pH and ion sensitivity of this interaction system. A certain amount of interconversion between the forms exhibited in C and D would be possible on the basis of the known dispersion behaviour of these systems observed in this laboratory. The confirmation of these models can only be made after more detailed studies have been conducted on these systems by the use of internal standard for accurate measurement of relative dimensions and the observation of the effect of varying degree of carboxymethylation on the morphology of the complexes.

The general appearances of the highly stable complexes were different from those exhibiting poor stability. Such
differences in morphology were not merely a matter of degree but could be characterized in terms of the conformational features of the constituents. At the neutral pH, the ion-sensitive aggregation of protein into spherical bodies 1000-3000 Å diameter was a common feature of the complexes formed in skim milk as well as in α₅-casein-containing model systems, irrespective of the system stability. On the other hand, the physical separation of the protein bodies in the complex was the characteristic feature of all effective stabilizers. It is therefore obvious that the physical stability of the protein hydrocolloid interaction system depends not so much on the nature of protein or the type of interaction but is very intimately related to the properties of stabilizers.

The electron micrographs of the stable systems demonstrated that the aggregated protein-particles are adequately surrounded by hydrocolloid material, providing solvation and effective physical separation of individual particles that would have otherwise interacted to form large, colloidal unstable precipitates. It was further apparent from the complexes with different λ-carrageenans of identical chemical structures, that although all the fractions would interest with α₅-casein, they differed considerably in their ability to localize the protein bodies in the network structure and consequently in their effectivity as a stabilizer.

A remarkable relationship observed between the casein-stabilizing ability (98) and double helix-forming potential
(4) of the sulfated polygalactans would suggest that the formation of double helix may be more significant in the effective localization of protein aggregates in the polysaccharide network. Rees (132) has proposed the ionic aggregation of double helix strands into the secondary phase of junction zone formation of the gel forming carrageenans (Fig 7). The protein reactivity of such aggregates would be limited to the relatively few sulfate groups available on the surface. The uncoiled helix-free zones, on the other hand, would exhibit high order of protein reactivity, since numerous sulfate groups would be available at random. Consequently, the most intensive ion-protein aggregation would occur at the loose ends of the polysaccharide network, free from ordered structure, and such aggregates would remain isolated by an intervening length of ordered aggregates of double helices schematically presented below. Such ordered aggregates of double helices would appear in the electron micrographs as fibre materials, poorly stained with KMnO₄ due to their low protein reactivity.

![Diagram](image_url)

*Fig. 25. Proposed Ultrastructure of αs-Casein/κ-Carrageenan Complex.*
Their approximate dimensions suggest that a bundle would be composed of several hundred double-helix strands.

It is tempting to suggest that the remarkable stabilizing ability of carrageenan is due to the unique double helix conformation. However, this study indicated that such stabilization was achieved effectively, not by promoting intensive protein aggregation in the ordered regions of double helix, but by a protein interaction in the junction zones by cooperative effects mediated by counter ions.

The study further indicates that for effective physical separation of the protein aggregates, the ordered regions should have an optimum length. If too short, the protein bodies are not efficiently separated; if too long, large portion of polysaccharide would remain unreacted, and only a small amount of protein can be localized in such a structure. It is therefore apparent that for the effective stabilization of proteins, the relative content of ordered double-helix structure may be as important as the ability to form helical structure by the hydrocolloids. On this basis, the protein stabilizing ability of hydrocolloids would be expected to be influenced by the location of sulfate groups and alkali modifications, affecting the double-helix forming ability and by the polymer size and temperature of interaction, affecting the relative content of double helix structures.

For the branched polysaccharide, sulfated locus bean
gum, the branching feature appeared to be adequate to provide localization of the aggregated proteins. Due to the branching, such hydrocolloids may be able to form network structure of different kind, but our data are far too limited to derive a definite conclusion.

The electron microscopy of protein-hydrocolloid interaction systems has firmly established the fact that the hydrocolloid stabilized milk proteins possess unique morphology distinctly different from the native casein micelles. For the casein micelles, physical stability is imparted largely due to the creation of a non-interacting micellar surface by the carbohydrate-rich \( \kappa \)-casein. The stability of the polysaccharide containing caseins is largely due to the effective isolation of protein "precipitates" entrapped in the hydrocolloid network structure. Stabilizing ability of these complexes is dependent upon the conformational features of the hydrocolloids, which in turn are influenced by the chemical nature, polymer size and reaction conditions. For the carrageenans, these characteristics lead to the protein stability on one hand, and gelformation, on the other.

The concept proposed in this study on the mechanism of protein stabilization by the hydrocolloids may have significance far beyond the realms of food stabilizer technology. For example, varying degree of colloidal stability have been reported in the complex formations involving blood lipoproteins with sulfated polysaccharides (16) and the proteins
with synthetic polynucleotides (72). In the polynucleotide studies, the involvement of double-helix structure was suspected. The electron microscopy of the protein-hydrocolloid interaction systems leads to the inescapable conclusion that for the effective protein stabilization, the hydrocolloid macromolecules should possess chemical structure, and polymer size resulting in conformational features that (a) would localize protein interaction to a few reaction centres, (b) cause effective isolation of the precipitated protein bodies and (c) would permit adequate solvation of the interacted complex.
SUMMARY AND CONCLUSIONS

A procedure was developed for the electronmicroscopic observation of food proteins, hydrocolloids and their complexes. The proteins and their complexes with polysaccharides were fixed with a 3% glutaraldehyde solution for 15-18 hrs. at 0-4°C, diluted with water to 40 times the initial volume, air dried as a thin film on parloidon support membrane and stained with a 1% KMnO₄ solution for 15 minutes. Polysaccharide materials were fixed, diluted and air-dried in a similar way, but stained with a 1% uranyl acetate solution.

β-Lactoglobulin (variant-A) appeared as an electron dense mass of amorphous agglomerated protein. The calcium-free preparations of αₛ-casein also exhibited amorphous characteristics that became compacted into globules of 500-1000 Å cross section upon the addition of ionic calcium. The modified polysaccharide, carboxymethyl cellulose (D.S. 75%) appeared as an entangled mass of fibres, a morphology typical for many hydrocolloids. The permanganate stained protein/hydrocolloid complexes were usually characterized by the presence of varying amounts of electron dense protein material in a polysaccharide network of a relatively low electron density. Depending on the type of polysaccharides
chosen, and the conditions of their interaction with the proteins, the physical stability of those complexes were intimately related to the macromolecular morphology of the hydrocolloid.

In the ionic complex between $\beta$-lactoglobulin and CMC at pH 4.0 the presence of 40 Å sized $\beta$-lactoglobulin molecules could be detected and could be schematically represented as $\beta$-lactoglobulin monomers or dimers localized on the surface the microfibril units or in the helical folds of the CMC ribbon. The distribution of $\beta$-lactoglobulin particles of similar size in the peptized complex explained the solubility of this system on the basis of increased solvation and was consistent with a concept of protein redistribution within the excess CMC. The solubility of the whey protein/CMC complexes at pH 5.2 appeared to be primarily due to disaggregation rather than dissociation.

The stable interaction products between carrageenans and $\alpha_s$-casein in the presence of calcium showed features which were different from the typical micellar spheres of the casein system. The carrageenan stabilized $\alpha_s$-casein complexes appeared as 1000-1500 Å electron dense protein bodies entrapped in a polysaccharide network and physically isolated from each other by an intervening length of polysaccharide fibre region with very little protein reactivity. The hydrocolloid regions of low protein reactivity corresponded to the ion-aggregated, double-helix-junction zones. A
correlation existed between the ability of different carrageenans to attain such conformation and their $\alpha_s$-casein stabilizing ability and corresponded to their gel-forming ability.

The temperature sensitive gelformation of skim milk containing $\kappa$-carrageenan was due to the extensive polysaccharide interaction resulting into three dimensional networks. When the pH of this system was reduced to 4.6, a disruption of casein micelles into 200 Å subunit structures was noticed. The electron microscopic detection of carrageenan fibres in the supernatant contradicts the chemical findings.

The stable complex of $\alpha_s$-casein with sulfated locust bean gum exhibited unique filligre structure, where numerous dark protein bodies could be noticed in polysaccharide structure.
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