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Stabilization of Casein Micelles by Carrageenan[1]

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ABSTRACT: Several naturally occurring, sulfated polysaccharides are capable of interacting with α_s-casein or β-casein, forming complexes which are stable against precipitation by calcium ions. The action appears to be an exclusive property of carrageenan, a group of sulfated galactans with a common structural feature of a sequence of alternating α-1,3- and β-1,4-glycosidic linkages. Within this group the micelle-building properties depend upon the location of the ester sulfate groups in the pyranose units and on the molecular size of the polymer.

The physical stability of the caseins in milk has considerable industrial significance and has been a subject for much study.[2] The casein system is composed of a heterogeneous group of phosphoproteins which in their native state interact to form micelles in strong association with inorganic salts, particularly calcium phosphate. The calcium salts of the two major proteins of this complex, α_s-casein and β-casein, are insoluble at physiological temperature but in the casein micelle they are stabilized against calcium precipitation, primarily through the protective action of the calcium-insensitive κ-casein.

Various models have been proposed over recent years[2a] attempting to explain the structure of the complex casein particles which attain molecular weights of several millions. A current concept[2b] describes the micelles as consisting of elongated, randomly coiled threads of polymerized β-casein to which α_s-casein is bound, possibly through hydrophobic forces. This central portion is sensitive to calcium ions but is stabilized by κ-casein which is attached to some of the α_s-casein residues. Rigidity and resistance toward dissociation is conferred to this structure by numerous bridges of colloidal calcium phosphate. Aggregation of these subunits is mediated by calcium ions which form crosslinks between exposed α_s-casein molecules. The particle size which can be attained, however, is apparently limited by the amount of available κ-casein.

The fundamental role of κ-casein in this as well as other models is that of a protective colloid or stabilizer for the other proteins against precipitation by calcium ions. The existence of a complex involving α_s- and κ-casein in the absence of calcium ions has long been recognized, although conflicting reports have been made about the relative proportions of the two proteins.[3] The stable micelle system is created from this complex upon the addition of calcium ions.

We have previously reported[4,5] that a number of highly water-soluble polysaccharides (hydrocolloids) are also capable of stabilizing α_s-casein against calcium precipitation by an interaction which involves micelle formation. The findings suggested that the reaction was limited to the strongly sulfated carrageenan-type polysaccharides; however, it was not possible to explain the reason for the specificity.

Carrageenan is the sulfated galactan extracted from red seaweeds.[6] Earlier work[7,8] demonstrated that carrageenan was polydisperse and could be separated into two fractions by potassium chloride. Structural analyses[9,10] have shown that the potassium-insensitive fraction (λ-carrageenan) was composed mainly of sulfated D-galactose, whereas the potassium-gelling fraction (κ-carrageenan) contained also 3,6-anhydrogalactose. A recent nomenclature[11] has been suggested for four basic galactan structures found within the carrageenan family; these are κ-, λ-, μ-, and ι-carrageenan. Although these different carrageenan fractions vary considerably in their chemical and physical properties, evidence has been presented[12] that they

(1) Article No. 3-69, Department of Dairy Technology.
(2) (a) H. A. McKenzie, *Advan. Protein Chem.*, **22**, 55 (1967); (b) D. Rose, *Dairy Sci., Abstr.*, **31** (4), 171 (1969).
(3) R. M. Parry, Jr., L. W. Ford, and R. J. Carroll, *J. Dairy Sci.*, **52** (6), 902 (1969).
(4) P. M. T. Hansen, *ibid.*, **51**, 192 (1968).
(5) C. F. Lin and P. M. T. Hansen, *ibid.*, **51**, 945 (1968).
(6) C. K. Tseng, *Science*, **101**, 597 (1945).
(7) D. B. Smith and W. H. Cook, *Arch. Biochem. Biophys.*, **45**, 232 (1953).
(8) D. B. Smith, W. H. Cook, and J. L. Neal, *ibid.*, **53**, 192 (1954).
(9) A. N. O'Neill, *J. Amer. Chem. Soc.*, **77**, 2837, 6324 (1955).
(10) D. B. Smith, A. N. O'Neill, and A. S. Perlin, *Can J. Chem.*, **33**, 1352 (1955).
(11) G. P. Mueller and D. A. Rees, "Drugs from the Sea Conference," University of Rhode Island, 1967.
(12) N. S. Anderson and D. A. Rees, Proceedings of the Fifth International Seaweed Symposium, Halifax, Canada, 1965, E. G. Young and J. L. McLachlan, Ed., Pergamon Press Ltd., London, 1966, p 243.

share a common structural arrangement of galactose units linked in a linear polymer chain by a sequence of alternating α-1,3- and β-1,4-glycosidic bonds.

Since the creation of artificial casein micelles through stabilization with food-grade additives would be potentially significant, further studies were undertaken to investigate the relationship between the micelle-building properties and the molecular structure of carrageenan polysaccharides.

Procedures

1. Protein Samples. α_s-Casein, β-casein, and κ-casein were prepared from unheated skim milk according to the procedures by Zittle and Custer.[13] The preparations were stored in small frozen portions at $-15°$. The specific absorbance of 1.02 for α_s-casein, 0.56 for β-casein, and 1.22 for κ-casein were estimated from the slopes obtained by plotting the absorbance values *vs.* the protein content by micro-Kjeldahl analysis. These values were used in the estimation of the protein concentration in the stabilization test.

2. Hydrocolloid Samples. Selected carrageenan samples were supplied by Marine Colloids, Inc., Rockland, Maine, and by Arthur D. Little Research Institute, Midlothian, Scotland. Fucodian was obtained from Kelco Co., San Diego, Calif. Others were of commercial origin.

3. Alkaline Modifications. Alkaline modification was performed according to the procedures by Rees.[14]

4. Infrared Study. Approximately 3–4 ml of 0.4% sample solution was pipetted into a 2 in. plastic ring placed on a glass plate covered with a thin layer of polyethylene (Dow kitchen wrap). After 16 hr evaporation at 40°, the samples were transferred to a desiccator and the evaporation was continued under vacuum for at least 1 hr. The dry films were then carefully removed from the support and placed securely in a cardboard film holder. A Perkin-Elmer IR-237B spectrophotometer was used to scan the films against air as reference.

5. Stabilization Test. The stabilization test was performed according to the procedures previously described.[4]

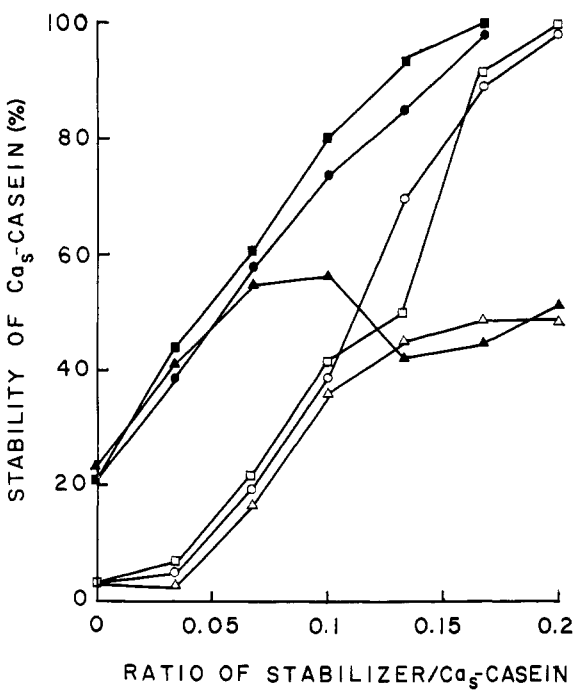

Figure 1. Stabilization of Ca_s-casein (calcium-sensitive casein) by stabilizers: □, α_s-casein/κ-casein (α_s-casein stabilized by κ-casein); ○, α_s-casein/κ-carrageenan; △, α_s-casein/λ-carrageenan; ■, β-casein/κ-casein; ●, β-casein/κ-carrageenan; ▲, β-casein/λ-carrageenan.

Results

The stabilization of calcium-sensitive caseins by κ-casein and by carrageenan is shown in Figure 1. On a weight-for-weight basis κ-carrageenan was as effective as κ-casein, while λ-carrageenan was less effective for this purpose. It is noteworthy that the action of the polysaccharides in this scheme resembled the behavior of κ-casein quite closely. Both systems produced characteristic, opaque solutions at pH 6.5–7.0 which remained physically stable at room temperature. An increase in alkalinity above pH 8 caused a dissociation of the micelles. However, stabilization of casein by the polysaccharides prevented the usual isoelectric precipitation at pH 4.6.

The results in Table I were obtained by studying a variety of hydrocolloids in respect to their stabilizing capacity for α_s-casein. The data confirmed the previous findings[4] that only the sulfated polysaccharides of the carrageenan family possessed this property. None of

TABLE I
STABILIZATION OF α_s-CASEIN (0.15%) BY SOME HYDROCOLLOIDS AT pH 6.7
(HYDROCOLLOID/α_s-CASEIN = 1/4)

Groups	Glycosidic linkages	Stabilized α_s-casein, %
Neutral		
Guar gum	β-1,4 (branch α-1,6)	0
Locust bean gum	β-1,4 (branch α-1,6)	0–0.1
Agarose	β-1,4 and β-1,3	0–1.5
Carboxylated		
CMC	β-1,4	0–0.1
Algin	β-1,4	0
Pectin	α-1,4	0–2.8
Gum arabic	β-1,3 and α-1,6	0–1.3
Hyaluronic acid	β-1,3 and β-1,4	0
Mixed carboxylated and sulfated		
Heparin	α-1,3 and α-1,4	0–6.0
Chondroitin sulfate A (4-sulfate)	β-1,3 and β-1,4	0–10.8
Chondroitin sulfate C (6-sulfate)	β-1,3 and β-1,4	0–1.5
Chondroitin sulfate D	β-1,3 and β-1,4	0–0.5
Sulfated		
Sulfated cellulose	β-1,4	6–15.4
Fucoidan	α-1,2 and α-1,4	0
λ-Carrageenan	α-1,3 and β-1,4	40.0–50.0
ι-Carrageenan	α-1,3 and β-1,4	92.0–100
κ-Carrageenan	α-1,3 and β-1,4	90.0–100

(13) C. A. Zittle and J. H. Custer, *J. Dairy Sci.*, **46**, 1183 (1963).
(14) D. A. Rees, *J. Chem. Soc.*, 1821 (1963).

TABLE II
STABILIZING ABILITY OF SEVEN CARRAGEENAN SAMPLES IN COMPARISON WITH THEIR ANALYTICAL DATA AS REPORTED BY BLACK, *et al.*[a]

Stabilizing strength in descending order	$[\alpha]_D$	Potassium sensitivity	η_{inh}	Galactose, %	SO_3Na, %	3,6-AG, %	Molar ratio Gal:SO_3Na:3,6-AG
C. crispus (κ fraction)	50	+++	11.6	28.6	29.6	22.9	1:1.63:0.90
G. stellata (unfractionated)	55	+	14.4	27.7	33.8	19.7	1:1.23:0.60
E. spinosum (ι fraction)	35	+−	7.2	30.3	37.8	19.0	1:1.97:0.71
G. pistillata (κ fraction)	88	++	10.5	35.0	35.0	12.8	1:1.57:0.41
C. crispus (λ fraction)	38	−	14.4	39.4	34.6	4.7	1:1.38:0.13
G. pistillata (λ fraction)	83	−	20.6	43.6	41.1	4.2	1:1.48:0.11
P. rotundus (unfractionated)	23	−	5.1	25.4	36.3	2.8	1:2.25:0.12

[a] See ref 15.

the types represented in the neutral group, carboxylated group, or mixed carboxylated–sulfated group provided any comparable protective effect against the precipitation of the protein by calcium.

A comparison of the effectiveness of carrageenan extracts obtained from selected botanical species of seaweeds is shown in Figure 2 and revealed a marked difference among the samples. A summary of the chemical properties of these specimens, as reported by Black, *et al.*,[15] is given in Table II. It is apparent from this table that there was a direct relationship between the stabilizing strength and the 3,6-anhydrogalactose (3,6-AG) content and also a possible correlation with the potassium sensitivity. The stabilizing strength was dependent neither on the total sulfate content nor on the viscosity values.

The corresponding infrared spectra obtained from dry films of the carrageenan samples are presented in Figure 3. Such spectra have frequently been used to identify the position of sulfate groups in the pyranose rings[16,17] and as an aid in classifying the carrageenans according to the recently suggested nomenclature.[11] The most effective carrageenans (Figure 3A) were those which contained the typical features of κ- or ι-carrageenan, namely (a) a peak at 930 cm^{-1} due to 3,6-AG, (b) a strong peak at 850 cm^{-1} by sulfate on the C-4 position, and (c) the virtual absence of any absorbance at 820 cm^{-1} from primary sulfate in the C-6 position. In contrast, the relatively ineffective fractions (Figure 3B) all displayed the λ-carrageenan characteristics of very little 3,6-AG, little or no C-4 sulfate, but contained a rather broad sulfate band in the 830-cm^{-1} region reflecting the presence of sulfate in both the C-2 and the C-6 positions.

Additional information about the relationship between the stabilizing mechanism and the polysaccharide structure was obtained by exposing λ-carrageenan from *C. crispus* to an alkaline treatment[14] whereby C-6 sulfate was eliminated by a ring closure leading to 3,6-AG. The infrared spectrum in Figure 4 confirmed that upon alkaline modification the broad sulfate band at 810–850-cm^{-1} had been sharpened. The reduced absorbance at 820 cm^{-1} in the alkaline-modified material was consistent with the removal of C-6 sulfate and the 930-cm^{-1} peak demonstrated the presence of 3,6-AG. The stabilization curves shown in Figure 5 revealed that this treatment was an effective means for improving the stabilizing potential of λ-carrageenan.

For the purpose of exploring the nature of the involvement of 3,6-AG in the observed protein–polysaccharide interaction, attempts were made to secure a

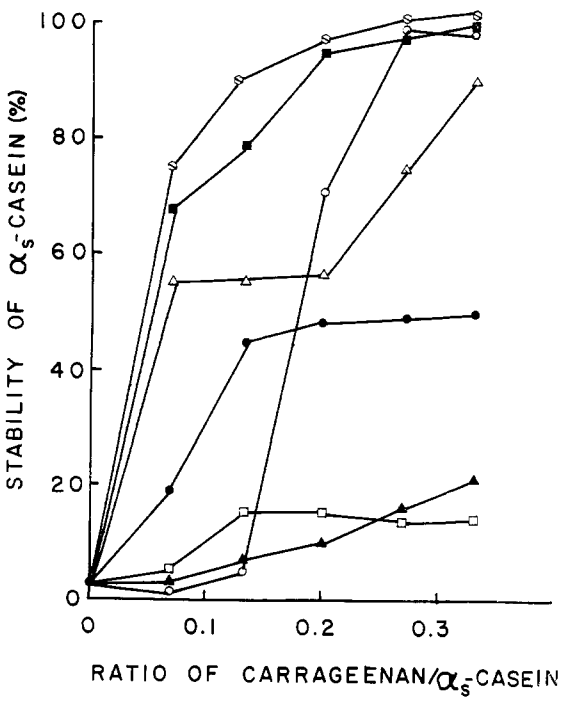

Figure 2. Stabilization of α_s-casein (0.15%) by carrageenans at pH 6.7: ◇, κ-carrageenan from *C. crispus*; ■, unfractionated carrageenan extract from *G. stellata*; ○, ι-carrageenan from *E. spinosum*; △, κ-carrageenan from *G. pistillata*; ●, λ-carrageenan from *C. crispus*; □, λ-carrageenan from *G. pistillata*; ▲, λ-carrageenan from *P. rotundus*.

(15) W. A. P. Black, W. R. Blakemore, J. A. Colquhoun, and E. T. Dewar, *J. Sci. Food Agr.*, **16**, 573 (1965).
(16) A. G. Lloyd, K. S. Dodgson, R. S. Price, and F. A. Rose *Biochim. Biophys. Acta*, **46**, 108 (1961).
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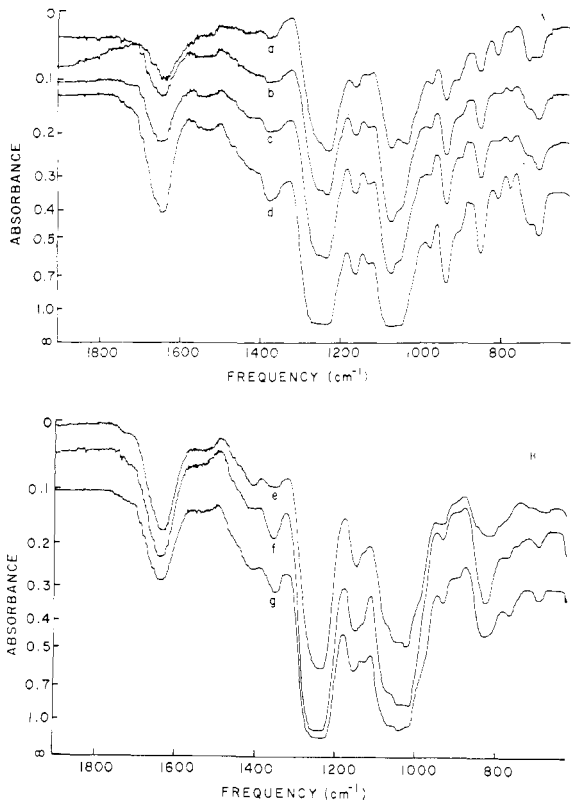

Figure 3. (A) Infrared spectra of carrageenans: (a) extract from *E. spinosum*, (b) extract from *G. stellata*, (c) κ extract from *C. crispus*, (d) κ extract from *G. pistillata;* (B) infrared spectra of carrageenans: (e) λ extract from *C. crispus*, (f) λ extract from *G. pistillata*, (g) λ extract from *P. rotundus*.

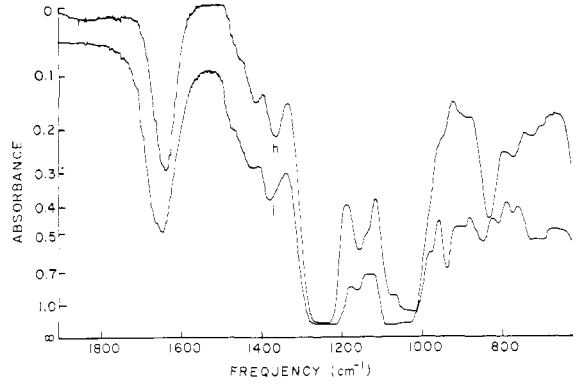

Figure 4. Infrared spectra of λ-carrageenan from *G. acicularis* (h) and alkaline modified λ-carrageenan from *C. crispus* (i).

carrageenan fraction which would possess no 3,6-AG and relatively little sulfate on the C-6 positions, but would otherwise be sulfated on the C-4 or the C-2 position. The infrared spectrum for λ-carrageenan isolated from *G. acicularis* (Figure 4) showed this material met these requirements, and was, therefore, subjected to the stabilization test. The results presented in Figure 6 indicated that this carrageenan material achieved complete stabilization of α_s-casein at the ratio of 2:5 of carrageenan to protein. From these results, it became apparent that 3,6-AG was not essential for the stabilizing ability of the hydrocolloid. This

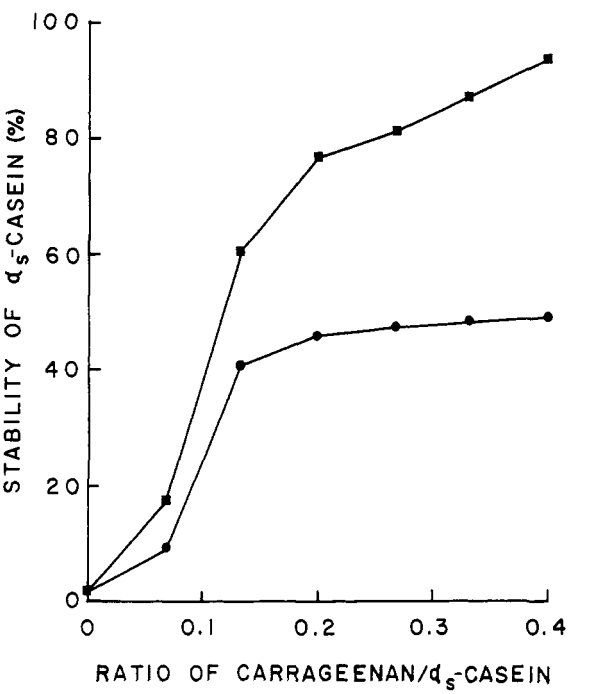

Figure 5. Effect of alkaline treatment on stabilizing ability of λ-carrageenan from *C. crispus:* ■, alkaline treated λ-carrageenan; ●, original λ-carrageenan.

conclusion was also supported by considering the performance of λ-carrageenan from other sources such as from *C. crispus*. This fraction is reported to have a very low content of 3,6-AG[9,10] and although it was a poor stabilizer for α_s-casein in comparison with κ-carrageenan, it was, nevertheless, far superior to any of the natural polysaccharides outside the carrageenan family. The strong correlation between the stabilizing power and the 3,6-AG content cannot, however, be

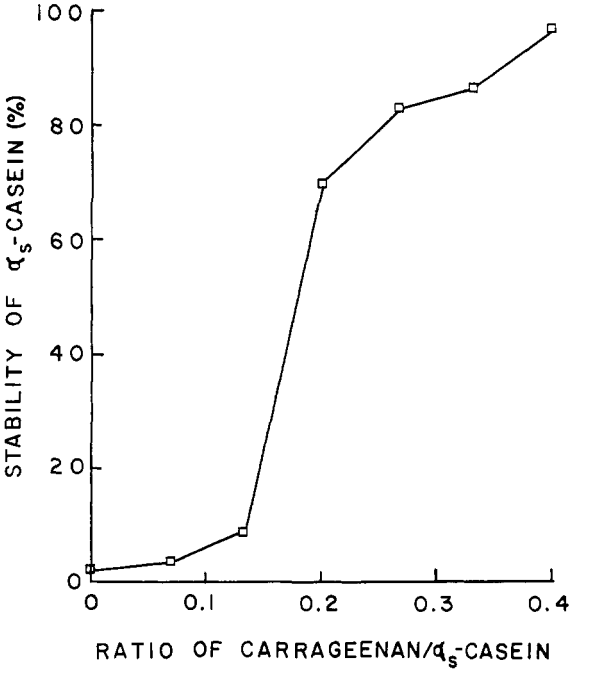

Figure 6. Stabilization of α_s-casein by λ-carrageenan from *G. acicularis*.

ignored and reflects possibly an indirect, antagonistic effect of sulfate in the C-6 position. The improved results by the alkaline modification would be consistent with the elimination of such an inhibitory group. In a similar fashion, any carrageenan material of a high 3,6-AG content would be of a low C-6 sulfate content.

The relationship between the stabilizing activity and polymer size of degraded carrageenan is shown in Figure 7. The depolymerization was accomplished by the acid-autohydrolysis treatment at 60°, previously described by Black, *et al.*[15] According to their data a reduction in the logarithmic viscosity number (η_{inh}) from 7.2 to 0.5 was achieved by a 2-hr treatment and without any significant loss of ester sulfate. From this plot, it was evident that the stabilizing capacity of carrageenan from *E. spinosum* was seriously impaired by even mild degradation of polymer size. In contrast, there was an initial improvement in the effectiveness of λ-carrageenan from *G. pistillata* at low degree of degradation which was then followed by a rapid loss of stabilizing power. Therefore, there appeared to be a definite dependence of this property upon the polymer size of the carrageenan. In this connection it was of interest to observe that the infrared spectrum of λ-carrageenan from *G. pistillata* (Figure 3B) bore a strong resemblance to that from *G. acicularis* (Figure 4). In spite of this similarity, the undegraded product from *G. pistillata* was found to be inferior to the *G. acicularis* fraction in the stability test. According to Table II, the λ-fraction from *G. pistillata* possessed the highest inherent viscosity of all samples tested and may possibly have been of a molecular weight which was too high for optimum performance.

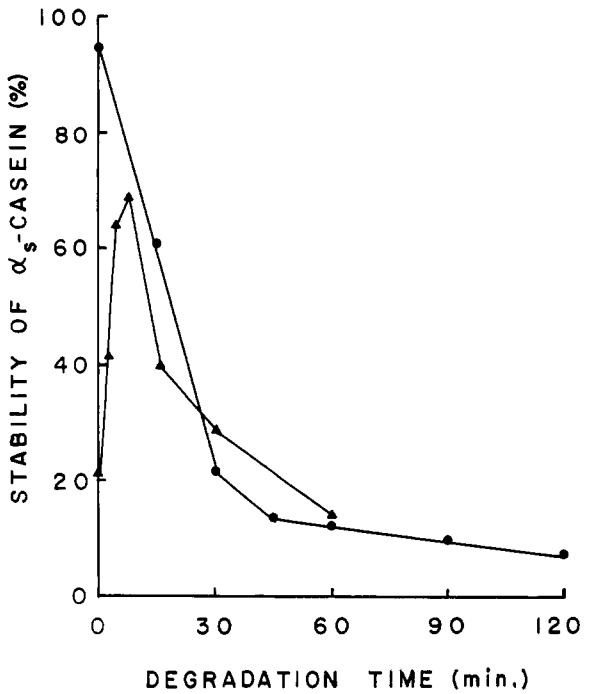

Figure 7. Effect of depolymerization on the stabilizing ability of carrageenan (ratio of carrageenan to protein 1:4): ●, ι extract from *E. spinosum*; ▲, λ extract from *G. pistillata*.

Discussion

This study has revealed that the stabilization of calcium-sensitive caseins by polysaccharides is dependent upon the following factors: (a) the presence of sulfate groups, (b) the location of the sulfate in the pyranose units, and (c) the chain length or molecular weight of the polymer. In addition, it is probable, although not certain that the configuration of the polysaccharide is also important.

By comparing the stabilizing ability of different hydrocolloids, it was found that only sulfated polysaccharides possessed this property. Evidently, the ester sulfate groups serve as binding sites for the protein in this reaction. To some extent the binding may be through calcium-ion bridges, because a complex formation between the two polymers has not been detected in the absence of calcium.[4] However, coulombic attractions must also be important for the system because these micelles are readily dispersed at alkaline pH.

We have previously expressed doubts[4,5] that the α_s-casein stabilizing function of carrageenan could be a consequence of the presence of ester sulfate alone, because of the failure of chondroitin sulfate and heparin to achieve stabilization and because of the decided difference in effectiveness of the various carrageenan fractions, which was not proportional to the total sulfate content (Table II). The suggestion[4] that the micelle-building properties could reside in some minor component associated with carrageenan in varying concentration is probably unfounded, because the stabilizing strength appears to be closely dependent upon the structure of the polysaccharide particularly when viewed in respect to the distribution of the sulfate groups. However, it would be entirely possible that within a given carrageenan product not all of the molecules would be of the optimum size for maximum effect and that careful sizing may yield fractions of greatly varying stabilizing ability.

The requirements with respect to the optimum position of sulfate in the polysaccharide were derived by considering the experimental findings for a number of hydrocolloids, for which some details of structure were already known. The effectiveness of the C-2 and C-4 sulfates in contrast to the antagonistic action of C-6 sulfate suggests steric effects. For example, it is possible that the structure of the calcium-stable complex is one for which only a minimum number of ionic groups are exposed to the external calcium environment. If the C-6 sulfate orientation is such that this group appears in an exposed position this might conceivably contribute to the calcium sensitivity of the complex by furnishing ionic groups for intermolecular calcium bridges.

The evidence accumulated in this study has indicated that the ability to stabilize α_s-casein is limited to the carrageenan-type polysaccharides, suggesting the presence of a common feature in their structure. The only shared characteristics of the carrageenans, which is also unique for this group, is the reported[12] regular sequence of alternating α-1,3- and β-1,4-glycosidic linkages in their backbone structure. However, a recent publication[18] on the conformational analysis of carrageenan and related polysaccharides has suggested

(18) D. A. Rees, *J. Chem. Soc., B*, 217 (1969).

that a remarkable similarity governs the entire group of seaweed and animal polysaccharides. It was pointed out that κ-carrageenan and ι-carrageenan are capable of forming double helices in the aqueous state, a stereochemical property which perhaps is shared by the other structural polysaccharides. However, it may be significant that some of the possible carrageenan conformations were found to be sterically excluded for the same sulfated polysaccharides which we found were incapable of stabilizing casein micelles. Therefore, there is a distinct possibility that the casein stabilizing function of carrageenan may be dependent upon conformational properties.

The importance of the polymer size in respect to the stabilizing ability has been well established in the degradation studies. We do not know the reason for this dependency upon polymer size for a given sample, but note that a similar observation has been made for a correlation between the molecular weight of heparin and its anticoagulant activity. Braswell[19] has pointed out that the correlation can be confirmed only for fractions derived for the same sample but not for samples of different origin. Before the interaction mechanism between α_s-casein and carrageenan can be fully delineated it is clearly necessary to obtain a great deal more information about the purity and the molecular weights of the effective polysaccharides, and to determine to which extent the conformation of the polysaccharides is affected by the degradation treatments.

Finally, the hypothesis that the interaction depends in some way upon the stereochemical properties of carrageenan does not in our opinion preclude that other polysaccharides possessing different structure would also be capable of a similar interaction. Whether or not a polysaccharide exhibits the stabilizing ability may depend only upon the specific characteristics of the backbone structure and the relative location of the functional groups.

Acknowledgment. Appreciation is expressed to Dr. S. D. Upham, Marine Colloids, Inc., Rockland, Maine, and Dr. E. T. Dewar, Arthur D. Little Research Institute, Midlothian, Scotland, for supplying purified carrageenan extracts, and to Dr. D. J. Pettit, Kelco Co., San Diego, Calif., for a sample of fucoidan. Appreciation is also expressed to Dr. E. J. Behrman, Associate Professor of the Department of Biochemistry and Molecular Biology, who extended the use of his laboratory facilities and gave many helpful suggestions in the infrared study. This investigation was supported by Public Health Service Research Grant No. FD-00117 from the Office of Research and Training Grants, Food and Drug Administration, and by a grant from Marine Colloids, Inc., Rockland, Maine.

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Chain Conformation of Syndiotactic Poly(α-methylvinyl methyl ether) in the Crystalline State

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ABSTRACT: The chain structure of poly(α-methylvinyl methyl ether) in the crystalline state was investigated by using X-ray diffraction and conformational energy calculations. Bond angle deformations coupled to preferred internal rotation angles are included in our approach. In agreement with previous nmr results, this polymer is syndiotactic and in the crystalline state forms a 5_2 helix.

Combined application of X-ray diffraction and conformational analysis has been recognized as a powerful route to determining the chain structure of crystalline polymers.[4-7]

Parallel application of these two methods of investigation appears to be particularly useful for those polymers which are subjected to strong intramolecular interactions between nonbonded atoms or groups such as the polymers obtained from α-substituted vinyl monomers. The internal rotation angles defining the chain conformation tend in fact to depart appreciably from the "staggered" values. In addition, bond angle deformations must be considered, as has been suggested in the case of polyisobutene.[8] Only through a detailed analysis of the intramolecular conformational energy is it possible to select a few most probable chain models, which must then be checked against the X-ray data. These, in turn, contain information which can be regarded as distinct at four different levels. First, the measure of the repeat distance along the chain axis can usually be obtained without difficulty from an oriented

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