Proteins as potential bacterial growth inhibitors in foods: suppression of the activity of water by proteins as determined by NMR relaxation methods

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SUMMARY

Food microbiologists have long known that suppression of the activity of water, a_w, can retard microbial growth in food systems. Traditionally, a_w suppression has been achieved by addition of salts or humectants to foods. To limit the amount of preservatives added to food products, studies were initiated to assess the feasibility of using proteins to suppress a_w to a practical value for retarding bacterial growth and to determine the optimum environmental condition for maximizing this effect for milk proteins. New expressions were developed relating observed longitudinal and transverse NMR relaxation rates, in the absence of cross-relaxation, to protein hydration, $\bar{v}_w$, to the protein activity coefficient, γ_p, and to the correlation time of the bound water, τ_c. From γ_p, the second virial coefficient of the protein, B_o, can be found. By use of $\bar{v}_w$ and B_o, a_w could then be directly evaluated at any protein concentration. Resulting expressions were tested by ^{2}H-NMR relaxation measurements made as a function of protein concentration for: β-lactoglobulin A (the major whey protein) under nonassociating (pH 6.0) and associating (pH 4.65) conditions; and for casein (the major milk protein) in the micellar (with added Ca^{2+}) and submicellar (without Ca^{2+}) forms. Values of a_w calculated from these ^{2}H-NMR data show that casein, at all the concentrations and temperatures examined, suppresses a_w more than does β-lactoglobulin A because of a larger B_o. In turn, micellar casein suppresses a_w to a larger extent than does submicellar casein because of a larger $\bar{v}_w$. Extrapolation of a_w at 4°C to a concentration ten times that in normal milk yields a value of a_w of less than 0.95, at which *Salmonella* and some strains of *Clostridium botulinum* no longer grow. These results are in agreement with what is known about storageability of condensed milk. Generalizations regarding the types of proteins and cosolutes to be used for suppressing a_w will be discussed. Structural information on these proteins calculated from τ_c will also be presented.

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INTRODUCTION

In recent years there has been emphasis on decreasing the amounts of certain preservatives in foods, mainly for health reasons. Traditionally, NaCl has been used to suppress the activity of water, a_w, to retard bacterial growth. However, even this practice is now considered deleterious because of the role of salt in hypertension. For these reasons, our laboratory has investigated the possibility of suppressing a_w in foods adequately by the use of proteins or, if this is not possible, to find the most favorable environemental conditions under which the protein contribution to the suppression of the activity of water is maximized.

Instead of using conventional methodologies for studying protein-water interactions (e.g., vapor pressure, differential thermal analysis, osmometry, etc.) we have treated this system in terms of a ligand-binding phenomenon, namely water binding to protein sites (hydration). Such bound water would be part of the protein component in a one-phase three-component system and thus would decrease the activity of water. The amount of this contribution to a_w is in question, and represents a controversial area of research.

A major problem with this determination is that the concentration of water in any protein solution will be in great excess compared to that of the dissolved protein (i.e., nearly 55.6 M H_2O versus at most 0.01 M protein, or a ratio of more than 5000:1). Thus, only the maximum ratio of bound water to protein is obtainable. Also, any physical property of water, to be sufficiently sensitive to be used as a measure of binding, would need to undergo about a 1000-fold change. Such a property is the rotatory diffusion coefficient of water (or its equivalent, the correlation time, τ_c). In its free state a H_2O molecule can turn around on its own axis in $\approx$3.5 ps [13], which is a working definition of τ_c. When bound to a protein, water should have a τ_c equal to that of the protein (10 ns for a globular protein of molecular weight 35000) [8,11]. Such changes can be detected by NMR relaxation (spin-lattice and spin-spin) measurements, because the relaxation rate of H_2O bound to protein will be so greatly different from that of the free water that it will influence the measured relaxation of the whole system, which is what is observed in these studies. The change in water NMR relaxation as a function of added protein concentration – defined as the water relaxation increment – is evaluated and, after eliminating or minimizing such anomalous effects as cross-relaxation and intermediate asymmetry, the correlation time as well as the hydration can be obtained.

Our model protein systems, β-lactoglobulin A (β-Lg A) and the caseins, are the primary milk proteins and serve as major food supplements. β-Lg A, a genetic variant of the major whey protein β-Lg, exists at pH 6.2 as a dimer of molecular weight 36730. At pH 4.65 it undergoes a temperature-dependent reversible dimer-to-octamer association [15–19,23] (which will be referred to as tetramerization); the amount of octamer formation increases with decreasing temperature, indicating hydrophilic involvement in the association process. It was one of our objectives to find the role of hydration in the process of octamer formation. The caseins (α_s, β, and κ) exist in two forms. In the presence of calcium, caseins occur in large aggregates called casein micelles; in the absence of calcium, as particles termed submicelles [1]. In addition to the previously mentioned objectives, our research has allowed us to elucidate more fully the process of casein micelle formation and the role of hydration in this process. Finally, the activity of water was calculated for these proteins under various conditions in order to elucidate which proteins, and what conditions, are needed to effectively suppress the activity of water to retard bacterial growth.

MATERIALS AND METHODS

Sample preparations. β-Lg A was the recrystallized lyophilized product, prepared in this laboratory from the milk of homozygous A/A cows [8]. Solutions were made up from a stock solution prepared by partial deuterium exchange. A suitable amount of crystalline protein was allowed to equilibrate repeatedly for 24 h periods as a slurry with

a small quantity of D_2O in a stoppered vial at 4°C, followed by high-speed centrifugation and addition of fresh D_2O, for a total of five times. The solutions were buffered by direct addition of solid potassium phosphate, and the pH was adjusted by addition of 0.1 N NaOD in D_2O. Concentrations of β-Lg were determined spectrophotometrically from an absorption coefficient of 0.96 ml $\cdot$ mg^{-1} $\cdot$ cm^{-1} at 278 nm [8]. Casein micelles were isolated from 2 liters of fresh warm milk to which 1 g of phenylmethylsulfonyl fluoride had been added to retard proteolysis. The milk was centrifuged at 4000 $\times$ g for 10 min to remove the cream fraction. 400 ml of this skim milk was centrifuged for 1 h at 88000 $\times$ g (37°C). The casein pellets were washed twice in D_2O containing 25 mM piperazine-*N,N'*-bis-(2-ethanesulfonic acid) (PIPES, pH 6.75), 20 mM $CaCl_2$, and 80 mM KCl. The final protein concentration was fixed at about 100 mg/ml (total volume of 5 ml). Subsequent dilutions were made with the same buffer. To produce submicelles, sodium caseinate prepared from the same skim milk was dialyzed and lyophilized at pH 7.2; the lyophilized protein was dissolved in D_2O, in the same PIPES-KCl buffer without $CaCl_2$, but with added dithiothreitol to promote self-association of κ-casein [10]. These procedures were designed to minimize the concentration of H_2O in the D_2O solutions and thus to eliminate any significant contribution from deuterium exchange to the relaxation rates. Casein concentrations were determined spectrophotometrically on samples diluted 1/50 to 1/100 in 0.1 N NaOH; an absorption coefficient of 0.850 ml $\cdot$ mg^{-1} $\cdot$ cm^{-1} at 280 nm was used for whole casein [10].

Relaxation measurements. Resonance relaxation spectra were obtained by Pulse Fourier Transform spectroscopy with a JEOL FX60Q spectrometer operating at a nominal proton frequency of 60 MHz. The ^{2}H-NMR frequency of observation was 9.17 MHz. Raw data were in the form of relative intensities as calculated by the JEOL 980B computer.

Since the high concentration of water in a dilute solution produces an intense signal, a single accumulation at the particular sample temperature (2, 15, or 30 $\pm$ 1°C) was sufficient for each spectrum. Even then, care was necessary to avoid exceeding the dynamic range of the computer with consequent truncation. To this end, small sample volumes were employed by use of a microcell assembly with an expendable 35-μl sample bulb, available from Wilmad Glass Co., Inc.

Longitudinal (spin-lattice) relaxation rates R_1 were measured by the inversion-recovery method [13], where the repetition time T in the pulse sequence $[...T...\pi...\tau...\pi/2...]$ was chosen to be at least five times T_1 ($\equiv R_1^{-1}$) and the values of the variable delay time τ ranged from 10 ms to 3 s, for a total of between 5 and 20 τ values, depending on the detail desired. From the Bloch equations [13] under the conditions of this method, the relation of the peak intensity A_τ to the pulse delay time τ becomes

$$A_\tau = A_\infty [1 - 2 \exp(-R_1\tau)] \quad (1)$$

where A_∞ is the limiting peak intensity for $\tau \rightarrow \infty$. Independent measurement of A_∞, a source of irreducible error, can be dispensed with, and the problem of weighting the data points in the conventional linear plot of the logarithm of a function of relative peak heights can be eliminated, by fitting directly to the data points (τ, A_τ) by least squares an exponential of the form of Eqn. 1, from which the two parameters A_∞ and R_1 can be obtained (Fig. 1, curve A). The fitting of this two-parameter exponential was carried out by means of an iterative computer program. For each sample R_1 was determined at least four times, and the results were averaged; relative standard errors amounted to 1–2%. This procedure was repeated at each concentration; a minimum of six concentrations were used under each set of temperature conditions at which the resonance relaxation of the nuclide was examined.

Transverse (spin-spin) relaxation rates R_2 were determined by spin-locking measurement [13] of $R_{1\rho}$, the longitudinal relaxation rate in the rotating frame. $R_{1\rho}$ equals R_2 in dilute solutions of low viscosity whenever the magnitude of $R_{1\rho}$ is independent of $H_{1\rho}$, the spin-locking radio-frequency field in the rotating frame; this was the case, within the limits of experimental error, in the present work. R_2 was evaluated as described above for R_1, except

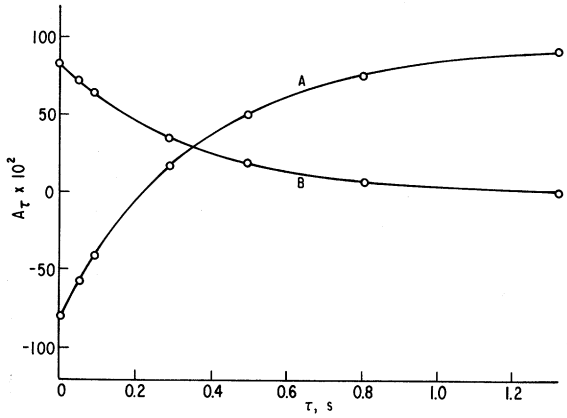

Fig. 1. Deuteron resonance peak intensities, A_τ, as a function of time, τ, for casein micelle solution at pH 6.75, 15°C, in D_2O (78.5 g/ml) and in PIPES-KCl-$CaCl_2$ buffer. (A) Spin-lattice relaxation measurements from inversion-recovery method. (B) Spin-spin relaxation measurements from spin-locking measurements of $T_{1\rho}$. Solid lines are best fit (F-test) of corresponding equations in text (Eqns. 1 and 2) to experimental intensities by nonlinear regression.

that the relation between peak intensity A_τ and decay time τ derived from the Bloch equations [13] in this case becomes

$$A_\tau = A_o \exp(-R_2\tau) \tag{2}$$

where the initial intensity A_o replaces A_∞ as the maximum peak intensity. Again, a least-squares two-parameter exponential fit to the data points was performed by an iterative computer program, from which A_o and R_2 were obtained (Fig. 1, curve B).

For each sample, R_2 was determined with the same number of replications as R_1; relative standard errors amounted to 2–3%. Measurements of one mode of relaxation were made on the identical sample and immediately following the completion of measurements of the other mode.

Analysis. For a two-state model (bound and free water), Kumosinski and Pessen [8,11] have shown that the increase in R_{obs}, the observed spin-lattice or spin-spin relaxation rate of water in the presence of varying protein concentration, c, is

$$R_{obs} - R_f = (R_b - R_f)\,\bar{\upsilon}_w\, a_p/W \tag{3}$$

Here R_f is the appropriate relaxation rate (R_1 or R_2) of free water, R_b is the corresponding relaxation rate of bound water, $\bar{\upsilon}_w$ is the degree of hydration, W is the total concentration of water, and a_p is the activity of the protein. For the latter, $a_p = c\gamma_p$, where γ_p is the activity coefficient of the protein or, more usefully,

$$a_p = c \exp(2B_o c + \ldots) \tag{4}$$

where B_o is the second virial coefficient of the protein.

Data points of the observed relaxation rate (spin-spin and spin-lattice) vs. protein concentration were fitted with a combined function of Eqns. 3 and 4 via an iterative Gauss-Newton nonlinear regression program developed at this laboratory. Analysis by this program produced values for B_o, $(R_b - R_f)\,\bar{\upsilon}_w$, and R_f. R_{1b} or R_{2b}, $\bar{\upsilon}_w$, and τ_c values were obtained by simultaneous solution of the Kubo-Tomita-Solomon equations [7,14],

$$R_{1b} = 2K\tau_c[(1 + \omega_o^2\tau_c^2)^{-1} + 4(1 + 4\,\omega_o^2\tau_c^2)^{-1}] \tag{5}$$

and

$$R_{2b} = K\tau_c[3 + 5(1 + \omega_o^2\tau_c^2)^{-1} + 2(1 + 4\,\omega_o^2\tau_c^2)^{-1}] \tag{6}$$

where R_{1b} and R_{2b} are the spin-lattice and spin-spin relaxation rates of bound water, respectively, τ_c is the correlation time of the bound water, $\omega_0 = 2\pi\upsilon_o$ is the nuclear angular precession frequency in radians per second for the observed nuclide, υ_o is the Larmor frequency in Hertz, and K is a measure of the strength of the nuclear interaction, i.e.,

$$K = (3/80)(e^2qQ/h)^2(\eta^2/3 + 1)^{-1}S^2 \tag{7}$$

Here e is the electronic charge, 1.6022×10^{-19} coulomb, q is the electric field gradient, Q is the nuclear electric quadrapole moment, h is Planck's constant divided by 2π, 1.056×10^{-27} erg, η is a dimensionless parameter measuring the deviation from axial symmetry [8,11], and S is the order parameter for intermediate asymmetry of the motion of the bound water [8,11]. Hence, this thermodynamic theory can be used whether isotropic ($S = 1$) or anisotropic motion ($S < 1$) is hypothesized, where

in the latter case the 'bound' should be understood in the sense of 'hydrodynamically influenced layers' or 'surface-induced probability distribution of water molecules'. For these experiments η is assumed to be zero, υ_o = 9.17 MHz and e^2qQ/h = 215.6 kHz [22].

When evaluated from the above analysis, a_w may be determined at each protein concentration. Since the total concentration of water (protein-bound + free) must equal unity, and $\bar{\upsilon}_w a_p$ is a measure of bound water,

$$a_w = 1.0 - \bar{\upsilon}_w a_p \quad (8)$$

where a_p is as defined by Eqn. 4.

RESULTS AND DISCUSSION

β-Lg A

Dynamics of dimer. From Eqns. 3–7 and the deuteron NMR spin-spin and spin-lattice data of Fig. 2, τ_c values can be calculated; from these values and the Stokes-Einstein relation [13] with the assumption of a spherical model, a Stokes radius (r_{NMR}) corresponding to this τ_c can be obtained (Table 1). At pH 6.2, where β-Lg A exists as the unassociated dimer, r_{NMR} is slightly lower than the value of 27 Å for the protein itself, derived from hydrodynamic data [12]. This discrepancy could be due to the spherical approximation inherent in the use of the Stokes-Einstein equation, since the β-Lg A dimer has an axial ratio of approximately 2:1 [23]. Moreover, the Stokes radius of the protein obtained from sedimentation includes the water of hydration (the sweep-out volume of the particle) and should therefore be larger than the r_{NMR} calculated from the ^{2}H-NMR relaxation data. What should be compared with the τ_c of the bound water is the τ_c of the protein without any contribution from hydrodynamic hydration. For the latter, values of 10.2 ns at 30°C and 22.5 ns at 2°C (Table 1) can be calculated for the protein with the use of its partial specific volume, 0.751 mg/g, and an asymmetry factor of

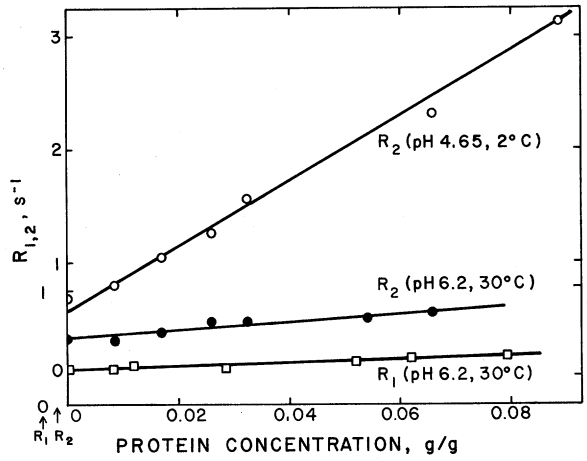

Fig. 2. Dependence of deuteron NMR relaxation rates on β-Lg A concentrations (g protein/g water) in D_2O. Transverse relaxation rates R_2 at pH 4.65, 2°C (○) and at pH 6.2, 30°C (●). Longitudinal relaxation rates R_1 (□) at pH 6.2, 30°C. (R_1 data at pH 4.65 overlay the pH 6.2 data.) Points represent experimental values; lines represent least-square fits. Points at all concentrations show linear relationship of relaxation rates to concentration, at both sets of pH and temperature conditions, and for both modes of relaxation.

Table 1

Parameters calculated for β-lactoglobulin in solution from ^{2}H-NMR relaxation measurements[a]

pH	Temp. (°C)	τ_c (ns)	r_{NMR} (Å)	$\bar{\upsilon}_w$ (g/g)	R_{1b} (ms^{-1})	R_{2b} (ms^{-1})	a_w, at 300 mg/ml
6.2	30	10.0	23.3	0.0063	3.29	5.06	0.997
	2	25.6	24.0	0.0072	2.49	8.18	0.997
4.65	30	22.5	30.4	0.0095	2.67	7.71	0.995
	2	32.2	25.9	0.0301	2.12	9.11	0.985

[a] Definitions of terms as described in Materials and Methods; B_o was 0.0009 ml/mg for all combinations of pH and temperature studied.

1.168 [21] to take into account the dimer axial ratio of 2:1. These values are in excellent agreement with the experimental τ_c of the bound water at pH 6.2, 30°C and 2°C (Table 1).

Hydration and dynamics of octamer. At pH 4.65, where at concentrations above 0.01 g/ml β-Lg A exists to a considerable extent as an octamer even at 30°C [18], r_{NMR} is about 30% lower than the Stokes radius of the octamer itself, i.e., 43.3 Å [12]. However, this value is still much closer to the theoretical value than those obtained by other investigators for other proteins [2–6,9]. Furthermore, the 422-symmetry model for the octamer according to Timasheff and Townend [17] possesses a large central cavity which could accommodate water restricted in its motion; if the NMR experiment observed this 'trapped' water, the τ_c value found would be less than that of the protein. This is an indication also that the local motion of bound water does not contribute to τ_c.

If the assumption is made that the NMR hydration of the octamer itself at 2°C equals $(\bar{\upsilon}_w)_{pH4.65} - (\bar{\upsilon}_w)_{pH6.2}$, where $(\bar{\upsilon}_w)_{pH4.65}$ and $(\bar{\upsilon}_w)_{pH6.2}$ are the NMR hydration values at pH 4.65 and 6.2, respectively, hydrations from 0.019 to 0.028 g H_2O/g protein can be calculated by the methods described here. The total volume of the cavity, approximated by an internal sphere tangent to the subunits on the basis of known structural parameters [17], amounts to about 6500 Å^3. Taking the specific volume of water as unity and thus its molecular volume as 30 Å^3/molecule, this would correspond to about 220 mol H_2O/mol of octamer, or 0.027 g H_2O/g protein, which is within range of the NMR-derived hydration values for the octamer.

Since the derived NMR correlation times are number-average values, the hypothesis that the increase in hydration accompanying octamer formation is largely due to water restricted in the central cavity (trapped) may be tested by calculating a number-average correlation time from the following relationship: $(\bar{\upsilon}_w)_{pH4.65}\,(\tau_c) = (\bar{\upsilon}_w)_{pH6.2}\,(\tau_c)_o + [(\bar{\upsilon}_w)_{pH4.65} - (\bar{\upsilon}_w)_{pH6.2}]\,(\tau_c)_{cc}$, where $(\tau_c)_o$ is the correlation time of the octamer at 2°C (i.e., 145 ns) and $(\tau_c)_{cc}$ is the correlation time of the central cavity of volume 6500 Å^3 (i.e., 1.4 ns). τ_c calculated from ^{2}H-NMR hydrations at 2°C is 36 ns, in fair agreement with the ^{2}H-NMR experimental value of 32.2 ± 4.6 ns at pH 4.65 and 2°C.

As can be seen in Table 1, even at a concentration of 300 mg/ml β-Lg A cannot suppress the activity of water below a value of 0.95 under any of the environmental conditions studied. Although the hydration of β-Lg A at pH 4.65 and 2°C is increased to 0.0301 g H_2O/g protein due to tetramer formation, the low value of the second virial coefficient, namely 0.008 ml/mg, does not allow effective suppression of water. Only if the charge-to-mass ratio of β-Lg A could be increased by changing the pH would the second virial coefficient increase to a useful value. Under those conditions, however, octamer formation would not occur. Hence, at relatively neutral pH values β-Lg A would not be an effective additive for suppression of the activity of water for the purpose of retarding bacterial growth.

The caseins

From the ^{2}H-NMR relaxation increments (Fig. 3), the hydration values $\bar{\upsilon}_w$ shown in Table 2 were obtained for caseins. Over the entire temperature range, the micellar form exhibits substantially greater NMR-hydration than the submicellar form; both forms show increased hydration with decreas-

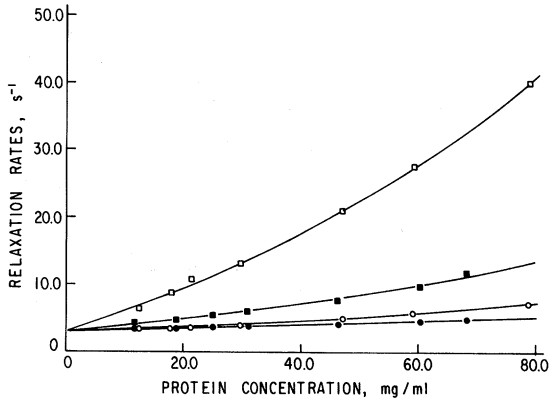

Fig. 3. Dependence of deuteron NMR relaxation rates of water on casein concentrations in D_2O at pH 6.75 in 0.2 M PIPES-KCl buffer at 15°C. ●, R_1 measurements, submicellar form; ■, R_2 measurements, submicellar form; ○, R_1 measurements, micellar form; □, R_2 measurements, micellar form.

Table 2

Parameters calculated for caseins in solution from ^{2}H-NMR relaxation measurements[a]

Species	Temp. (°C)	τ_c (ns)	r_{NMR} (Å)	$\bar{\upsilon}_w$ (g/g)	R_{1b} (ms^{-1})	R_{2b} (ms^{-1})	a_w, at 300 mg/ml
Submicelle	30	38.9	36.4	0.0065	1.90	10.51	0.987
	15	34.7	30.5	0.0082	2.08	9.84	0.983
	2	29.8	25.5	0.0120	2.32	9.07	0.975
Micelle	30	63.5	42.9	0.0165	1.25	14.79	0.966
	15	51.2	34.8	0.0225	1.52	12.57	0.954
	2	45.1	29.3	0.0282	1.69	11.53	0.943

[a] Definitions of terms as described in Materials and Methods; B_o was 0.0032 ml/mg for all temperatures, with and without Ca^{2+}.

ing temperature. The second virial coefficient, B_o, also calculated from the relaxation increment, was found to be 0.032 ml/mg regardless of the conditions or the form of casein.

Although the caseins are self-associating, we need to consider here only the aggregated form. The concentrations used here were high enough so that the association equilibrium was very far to the right, favoring aggregation. For both micelles and submicelles, no differences in hydration would result from any protein concentration-dependent dissociation effect at 30°C.

A Stokes radius of 36.4 Å attributable to bound water at 30°C (see discussion above for β-Lg) is of the order of magnitude of that for a submicelle [10] but somewhat smaller than would be expected from the argument made earlier for β-Lg A. Results for the submicelles show a decreasing hydration value and an increase in the Stokes radius with increasing temperature. This suggests that hydrophobic interactions are involved in the formation of the submicelle, since, as the temperature is raised, water is excluded from the hydrophobic interface during an association process.

Although the absolute value calculated for the Stokes radius of the micelle is of the same order of magnitude as that of the submicelle, it is not as large as expected, because of instrumental limitations. The micelle exhibits the same temperature dependence as the submicelle, showing hydrophobic interactions. This is in agreement also with previous investigators who theorized that micelles are formed by aggregation of submicelles via Ca^{2+} salt bridges [1]. The slight increase in radius from submicelle to micelle is probably the result of competing processes between electrostatic forces, involving Ca^{2+} and phosphate or carboxyl groups on the exterior of the submicelle, and hydrophobic forces within the submicelle as it is incorporated into the larger micelle.

As expected, a greater hydration value for the micellar form is accompanied by greater suppression of water activity, as shown in Table 2 for the a_w at 0.3 g/ml (30%) protein solution. Fig. 4 shows micellar casein to exhibit a greater a_w suppression than submicellar casein over the entire range of protein concentrations. By extrapolation of those data, for casein micelle concentrations of 0.3 g/ml (ten times that normally occurring in milk) and a temperature of 2°C, a_w is 0.943. This activity level is well below the minimum needed to support growth of many bacteria under normal conditions prevailing in foods; organisms whose growth is suppressed include *Lactobacillus viridescens,* some types of *Clostridium botulinum, Escherichia coli,* some strains of *Salmonella,* and *Pseudomonas fluorescens* [20]. Theoretically, then, a 10:1 concentrate of skim milk at 4°C would retard bacterial growth by virtue of a_w suppression due only to protein, without even considering suppression resulting from accompanying elevated salt and lactose concentrations. Similar reduction of water activity with the submicelles

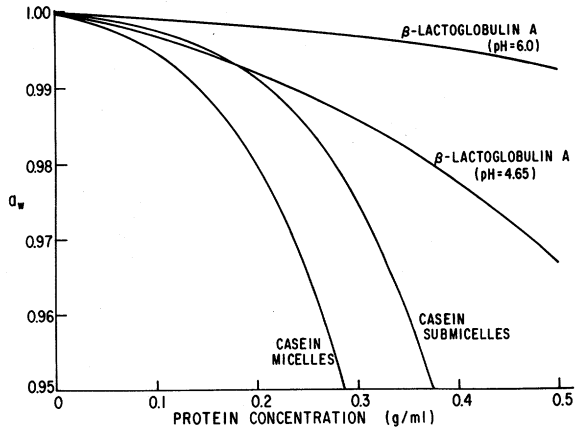

Fig. 4. Theoretical curves for activity of water from 0.95 to 1.0 versus protein concentration in g/ml for milk proteins at 2°C, calculated from the hydration values derived at low concentrations from the NMR relaxation measurements (Tables 1 and 2) and Eqns. 8 and 4, using B_o values indicated in Tables 1 and 2.

alone does not occur. Apparently, then, some forms of protein can suppress water activity, but only under carefully specified conditions, related in this case to the formation of colloidal complexes with the concomitant trapping of water.

CONCLUSION

The curves of Fig. 4 summarize the findings for casein and β-Lg A under the various relevant conditions and allow ready comparison of the relative effectiveness of these proteins in retarding microbial growth. A suppressed a_w value of 0.95 can be obtained for casein submicelles at approximately 0.38 g/ml, while casein micelles require (with the addition of calcium) a concentration of less than 0.3 g/ml. This, of course, arises from the increase in hydration of casein upon addition of calcium. This increase in hydration is presumably caused by water trapped in the micelle. β-Lg A, on the other hand, cannot attain a suppressed a_w value of 0.95 even at pH 4.65 (octamerization conditions, with hydration at a maximum) because of its low second virial coefficient, short of concentrations in excess of 0.6 g/ml. The solubility of β-Lg A, however, is too low to permit such concentrations in a single-phase system, whereas this treatment for measuring the suppression of a_w by proteins has been validated only for proteins in solution [8,11]; therefore changes in phase (solution to solid, or solution to sol) are not encompassed by the concentration-dependent plots used here.

The reason for the smaller value of the second virial coefficient for the β-Lg A compared to the caseins lies in the fact that the β-Lg A octamerization conditions are at pH 4.65, close to the isoelectric point of 5.2, whereas casein was studied at pH 6.8, far from its isoelectric point of 4.6. Under these conditions, β-Lg A would have a lower charge-to-mass ratio than the caseins. The second virial coefficient has been shown to be proportional to the square of the charge, as well as to the molecular weight of the protein and to the molarity of the ionic cosolute added to the solution [11].

Hence, all these environmental conditions (namely, charge of macromolecule, ionic strength, pH, amount of salt added) must be taken into account when attempting to find the optimum protein for suppressing a_w and thereby retarding bacterial growth. It is expected that a computer data base of proteins, with their hydration values and activities under several environmental conditions, can be established from experimental data. From such a data base, food processors could select combinations of proteins that would meet their requirements for manufacturing a product and at the same time maximizing the suppression of a_w, thus limiting the need for other preservatives.

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