Investigation of κ–α_{s1}-Casein Interaction by Fluorescence Polarization*

Reginald Clarke and Shuryo Nakai†

ABSTRACT: The interaction between α_{s1}- and κ-casein was studied by the fluorescence polarization technique. The entropy and enthalpy changes, $\Delta S°$ and $\Delta H°$, were positive suggesting that the ease or spontaneity of interaction increases with temperature, and that the interaction is probably hydrophobic in nature. The results at 40 and 50° demonstrate a unit molar ratio for the two interacting proteins. Restricting electrostatic interactions by masking the negative charge on the α_{s1}-casein with polyethylenimine did not inhibit the reaction or affect the interaction with κ-casein. It is, therefore, suggested that α_{s1}- and κ-caseins interact principally through hydrophobic interaction, in a unit molar ratio.

Since the fractionation of α-casein into fractions α_s and κ, calcium sensitive and insensitive, respectively (Waugh and von Hippel, 1956), there has been a great interest in the κ-casein fraction because of its unique stabilizing ability toward the other caseins.

Studies have been done on the interaction between κ- and α_{s1}-caseins, which stabilizes α_{s1}-casein against calcium precipitation, and could act as precursor to stable casein micelles (Noble and Waugh, 1965; Waugh and Noble, 1965).

The techniques used and the results obtained were varied regarding the interacting ratio of α_{s1}- to κ-casein and the type of interaction, *i.e.*, hydrophilic, hydrophobic, or electrostatic. Waugh and von Hippel (1956) studied the interaction between α_{s1}- and κ-casein and found a variable interaction ratio, but found that the α_{s1}:κ ratio was predominantly 4.

Noble and Waugh (1965) found that the α_{s1}:κ ratio was low and close to unity. Garnier (1967) found an interaction ratio of 1 mole of α_{s1}- to 3 moles of κ-casein. However, in an earlier paper, Garnier *et al.* (1964a) reported an interaction ratio of unity. Parry *et al.* (1969) found the interaction ratio to be unity, using gel filtration.

Kenkare and Hansen (1967) found that the interaction product was of variable composition, and suggested that the interaction between the α_{s1}- and κ-casein was hydrophilic in nature. This is in contrast with the work of Garnier *et al.* (1964b) who found a positive entropy for the reaction, suggestive of hydrophobic interaction.

This postulation seems to be feasible as the interaction was inhibited at 2–6° and spontaneous above 37° (Noble and Waugh, 1965).

Payens (1966) suggested that the main interacting force between the caseins, in maintaining the micelle structure, is hydrophobic in nature within the micelle. This concept was based on the amino acid composition of the caseins, the temperature dependence of aggregation of the caseins and the established hydrophobic nature of aggregation of α_{s1}- and β-caseins.

Hill and Wake (1969) discussed the amphiphilic nature of κ-casein and suggested that the α_{s1}–κ-casein interaction is significantly hydrophobic in nature.

However, in addition to the suggestion of hydrophilic and hydrophobic interactions, there have recently been suggestions of electrostatic interactions. Woychik (1969), Pepper *et al.* (1970), and Talbot and Waugh (1970) have modified lysine residues in κ-casein using various techniques and found that the modification of increasing numbers of lysine residues reduced the stabilizing ability toward α_{s1}-casein until it was lost completely after five or more residues were modified.

Hill and Laing (1965) found that the modification by photooxidation of histidine residues in κ-casein reduced its stabilizing ability for α_{s1}-casein. Nakai *et al.* (1967) noted that the modification of histidine in κ-casein caused decreased stabilizing ability and aggregation.

It is obvious that the interactions between α_{s1}- and κ-casein are far from clear. Varying interaction ratios have been reported, and mechanisms involving hydrophilic, hydrophobic, and electrostatic interactions have been postulated.

In this paper are reported the results of a study on the interaction of α_{s1}- and κ-caseins, using fluorescence polarization. These results support the concept of hydrophobic interaction between α_{s1}- and κ-caseins.

Materials and Methods

κ- and α_{s1}-caseins were prepared according to the method of Zittle and Custer (1963).

Dansylated α_{s1}-casein was prepared by reacting α_{s1}-casein (280 mg/40 ml) in carbonate buffer (0.4 M $NaHCO_3$–0.1 M Na_2CO_3, pH 9.2) with a suspension of 6 mg dimethylaminonaphthalenesulfonyl chloride, in 1 ml of the same buffer. The reaction was carried out in an ice bath for 20 min with constant stirring. The reaction mixture was then centrifuged at 4°, and the supernatant dialyzed against distilled water at 4° for 48 hr and lyophilized. One residue of dansyl chloride was introduced per mole of α_{s1}-casein (mol wt 27,000).

Imidazole buffer (0.08 M, pH 6.8) was prepared by mixing 0.01 M imidazole and 0.01 N HCl in a ratio of 6:4, with 0.07 M NaCl added thereafter.

Samples of κ- and dansylated α_{s1}-caseins were prepared in 0.08 M imidazole buffer (pH 6.8).

Temperatures above and below ambient were maintained by a Sargent thermonitor and a Blue M constant-flow cooling unit, respectively.

Polarization was measured using an Aminco-Bowman spectrophotofluorometer 4-8202 with Zenon lamp and blank-

* From the Department of Food Science, University of British Columbia, Vancouver 8, B.C., Canada. *Received April 14, 1971.* This work was supported by Operating Grant A-3641 from the National Research Council of Canada.

† To whom correspondence should be addressed.

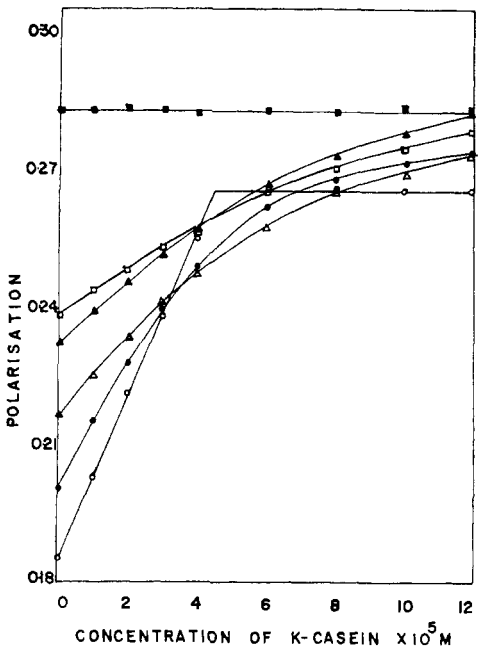

FIGURE 1: Polarization of dansylated α_{s1}-casein (4.0×10^{-5} M) titrated with κ-casein at various temperatures; 0.08 M imidazole buffer, pH 6.8. Each sample was maintained at different temperatures for 1 hr before polarization measurement. (0) 40°, (●) 35°, (△) 30°, (▲) 24°, (□) 17°, and (■) 4°.

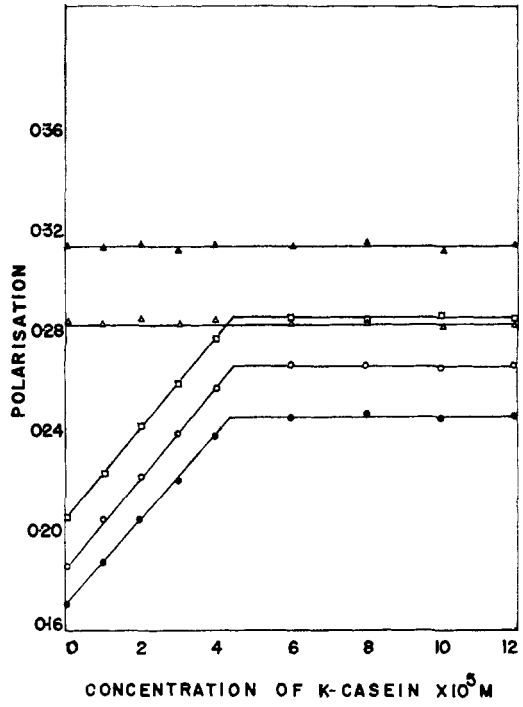

FIGURE 2: Polarization of dansylated α_{s1}-casein (4.0×10^{-5} M) with and without 0.04 mg of PEI/ml titrated with κ-casein at various temperatures. (●) 50°, no PEI; (0) 40°, no PEI; (□) 40°, PEI added; (△) 4°, no PEI; (▲) 4°, PEI added.

subtract photomultiplier. Slits were as follows: no. 2, 5/4 mm; no. 3, 4/2 mm; no. 7/1 mm. Polacoat UV-105 and Polaroid HN38 were used for the polarizer and the analyzer, respectively. Excitation and emission wavelengths were 350 and 508 nm, respectively.

Polyethylenimine (PEI-1000)[1] from Dow Chemicals in 0.08 M imidazole buffer (pH 6.8) was added to the α_{s1}-casein solution (4×10^{-5} M) to give a final concentration of PEI of 0.04 mg/ml.

Theory

Fluorescence polarization of a macromolecule is mainly dependent on the geometry and rigidity of the molecular structure. Changes in molecular conformation due to aggregation and interaction will, therefore, result in changes in the polarization, because these changes invite a modification of molecular geometry and volume in the macromolecule (Weber, 1952; Steiner, 1954; Haber and Bennett, 1962; Kierszenbaum *et al.*, 1969).

Thus the polarization technique will be a useful tool for the study of the interaction between α_{s1}- and κ-casein. By labeling one of the caseins with dansyl chloride, in our case α_{s1}-casein, it is possible to follow the interaction between the two caseins by measuring changes in polarization.

Weber (1952) demonstrated that if more than one oscillator, corresponding to more than one macromolecule in solution, are simultaneously excited, the observed polarization, $\bar{P}$, and the fluorescent intensity, F, emitted by the components, are represented by

$$\bar{P} = \frac{\sum_i F_i P_i}{\sum_i F_i} \tag{1}$$

[1] Abbreviation used is: PEI, polyethylenimine PEI-1000.

The observed polarization of the interacting system α_{s1}- and κ-caseins, would therefore be

$$\bar{P} = \frac{F_1P_1 + F_2P_2 + F_3P_3}{F_1 + F_2 + F_3} \tag{2}$$

where the subscripts 1, 2, 3, refer to α_{s1}-, κ-caseins, and their interaction product.

In our study the concentration of dansylated α_{s1}-casein was maintained constant and the fluorescent intensity did not change by the interaction. Therefore, eq 2 can be simplified to

$$\bar{P} = \frac{(F_0 - F_3)P_1 + F_3P_3}{F_0} \tag{3}$$

where F_0 is the fluorescent intensity of original dansylated α_{s1}-casein, as the unreacted κ-casein would not contribute to the fluorescence and polarization of the mixture.

Rearranging eq 3 we obtain

$$\frac{\bar{P} - P_1}{P_3 - P_1} = \frac{F_3}{F_0} \tag{4}$$

This equation is eventually the same as the eq 9 by Dandliker *et al.* (1964).

Since the interaction between α_{s1}- and κ-casein is known to be negligible at 0–6° and to be spontaneous above 37° (Noble and Waugh, 1965) it is possible, using eq 4, to predict the polarization changes of dansylated α_{s1}-casein during titration with nonfluorescent κ-casein at these and intermediate temperatures.

Since there is almost no reaction, *i.e.*, the equilibrium constant is very small at 2–6°, the concentration of the α_{s1}-κ-

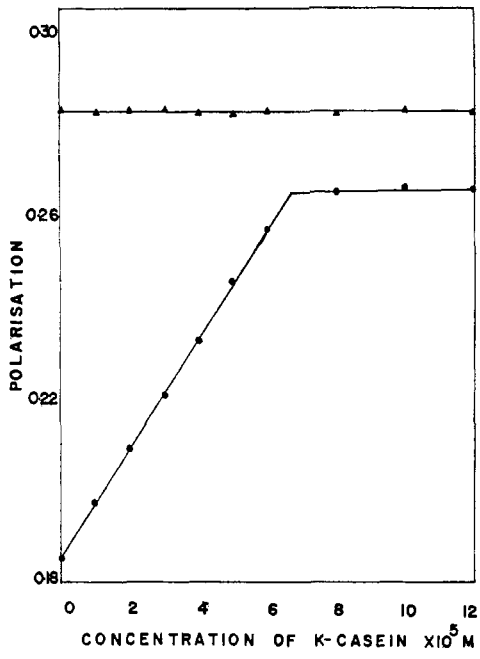

FIGURE 3: Polarization of dansylated α_{s1}-casein (6.0×10^{-5} M) titrated with κ-casein at 40° (●) and 4° (▲).

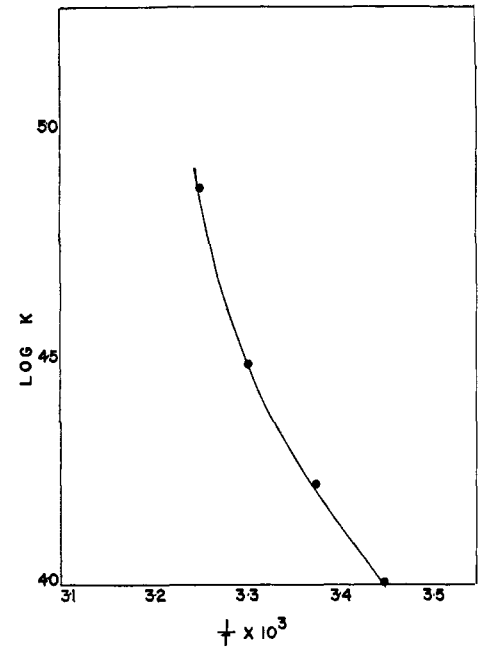

FIGURE 4: Log K for the association equilibrium plotted against the equilibrium temperatures.

casein interaction product and F_3, accordingly, tends to 0, therefore eq 4 simplifies to: $\bar{P} - P_1 = 0$, which means $\bar{P} = P_1$.

There should, therefore, be no change in the polarization of α_{s1}-casein titrated with κ-casein in this temperature range.

At 37°, the association constant is very large and there is spontaneous conversion of α_{s1}-casein into α_{s1}-κ-casein complex. If enough κ-casein is added to the α_{s1}-casein, such that all the α_{s1}-casein is reacted, the only fluorescent species present in the mixture would be α_{s1}-κ-casein complex. In eq 4 $F_3 = F_0$ and the equation simplifies to $\bar{P} = P_3$, *i.e.*, the observed polarization is due only to the α_{s1}-κ-casein complex. Further addition of κ-casein to the reaction mixture, after all the fluorescent-labeled α_{s1}-casein has reacted, should not affect the observed fluorescent yield as the unreacted κ-casein is nonfluorescent.

By eq 4 F_3 can be calculated from the observed polarization, $\bar{P}$, since P_1 and P_3 are constant for a given temperature and viscosity, and F_0 is constant if the concentration of α_{s1}-casein is kept constant during the experiment, and there is no change in fluorescent intensity with interaction. The observed polarization should, therefore, increase depending on the amount of κ-casein interacted with dansylated α_{s1}-casein.

For conditions of complete interaction, *i.e.*, 37° or above, $\bar{P}$ should increase linearly with added κ-casein, as all would be converted into the α_{s1}-κ-casein complex. As stated previously, after all of the α_{s1}-casein has reacted, there is no further increase in observed polarization.

For conditions of incomplete interaction, between 6 and 37°, because of the equilibrium conditions existing between reactants and products, all the κ-casein added is not converted into α_{s1}-κ-casein complex; therefore the increase in polarization with added κ-casein would be nonlinear and less than that for the complete interaction. As the equilibrium constant decreases at lower temperatures (Noble and Waugh, 1965) a fixed amount of κ-casein added to α_{s1}-casein would result in less interaction at reduced temperatures, and, as a result, the increase in observed polarization should decrease.

These theoretical considerations were supported by the results indicated in Figures 1 and 2. There was no change in polarization of dansylated α_{s1}-casein when titrated with κ-casein, at 4°. At 40 and 50° there was a linear relationship between observed polarization and concentration of κ-casein added to dansylated α_{s1}-casein. At intermediate temperatures the slope of the curve, relating observed polarization to κ-casein added, diminished with decreasing temperature.

From eq 4 it is possible to calculate the equilibrium constant at any temperature and concentration of added κ-casein, since F_3/F_0 is equivalent to the fraction of α_{s1}-casein reacted, and the reacting molar ratio is 1:1 (Figures 1 and 3).

The value of P_3 at intermediate temperatures can be obtained from the Perrin plot (1926) for α_{s1}-κ-casein complex. Polarization values for the complex were determined between the temperature range 37–50°. Extrapolation makes it possible to calculate the polarization of the complex (P_3) if the Perrin equation is valid at lower temperatures.

Results

The changes in polarization of a 4.0×10^{-5} M solution of dansylated α_{s1}-casein due to titration with κ-casein at 4, 17, 24, 30, 35, and 40° are presented in Figure 1.

The effect of PEI on the polarization of dansylated α_{s1}-casein titrated with κ-casein at 4 and 40° is presented in Figure 2. Also in Figure 2 is the result of titration of dansylated α_{s1}-casein with κ-casein at 4, 40, and 50°.

The changes in polarization of a 6.0×10^{-5} M solution of dansylated α_{s1}-casein due to titration with κ-casein at 4 and 40° are presented in Figure 3.

Figure 4 shows a plot of log K *vs.* $1/T$ from which $\Delta H°$ was calculated from the slope at different temperatures.

Figure 5 shows the Perrin plot of the α_{s1}-κ-casein complex. Temperature ranges 37–50°.

Table I shows the variation in polarization of dansylated α_{s1}-casein and polarization of dansylated α_{s1}-κ-casein complex with temperature.

Table II shows the variation in association constant as

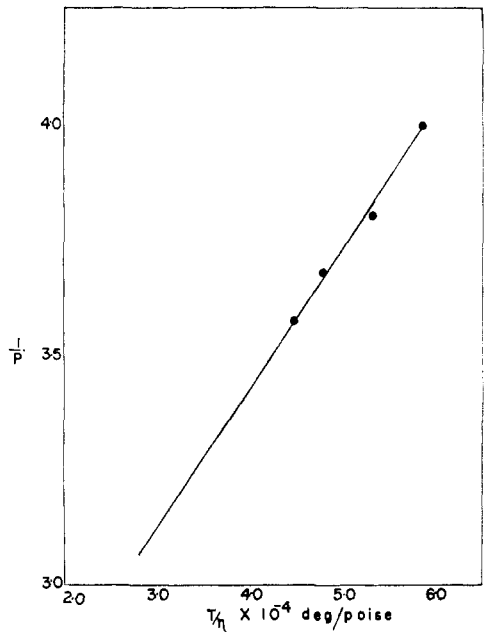

FIGURE 5: Perrin plot for 4.0×10^{-5} M α_{s1}-κ-casein complex. Temperature range, 37–50°. Viscosity determined by Ostwald capillary viscometer.

calculated with the aid of eq 4 and data presented in Table I and Figure 1, with temperature. Concentration of dansylated α_{s1}-casein was 4×10^{-5} M.

$\Delta F°$ and $\Delta S°$ were calculated from the following equations and are listed in Table III.

$$\Delta F° = -RT \ln K$$

$$\Delta S° = \frac{\Delta H° - \Delta F°}{T}$$

Discussion

Dandliker *et al.* (1964) and Kierszenbaum *et al.* (1969) reported a method for estimation of the association constant between antigen and antibody by fluorescence polarization. The basic equation in those papers and ours are the same.

TABLE I: Fluorescence Polarization of Dansylated α_{s1}-Casein and α_{s1}-κ-Casein Complex.[a]

Temp (°C)	Polarization of Caseins	
	α_{s1}-Casein	α_{s1}-κ Complex
4	0.283	
17	0.238	0.322
24	0.232	0.305
30	0.216	0.292
35	0.200	0.288
40	0.185	0.268
50	0.170	0.246

[a] Concentration, 4.0×10^{-5} M. Polarization of α_{s1}-κ complex (P_3) at 17, 24, 30, and 35° was calculated from the Perrin plot in Figure 5.

TABLE II: Association Constant for the Interaction between Dansylated α_{s1}-Casein and κ-Casein.[a]

Concn of κ-Casein $\times 10^5$ M	Concn of κ-α_{s1}-Casein Complex $\times 10^5$ M				Association Constant $\times 10^{-4}$			
	17°	24°	30°	35°	17°	24°	30°	35°
1.0	0.27	0.39	0.49	0.69	0.99	1.71	2.81	6.80
2.0	0.49	0.70	0.94	1.29	0.96	1.63	2.88	6.81
3.0	0.74	1.01	1.32	1.86	1.02	1.68	2.93	7.61
4.0	0.94	1.25	1.65	2.40	1.00	1.61	3.00	7.51
6.0	1.29	1.64	2.16	2.81	1.02	1.60	3.05	7.40
8.0	1.56	1.99	2.49	3.14	1.06	1.63	3.05	7.27
10.0	1.79	2.55	2.77	3.28	0.97	1.74	3.09	6.82
12.0	1.99	2.49	2.93	3.42	0.97	1.70	3.00	6.87
				Mean	1.00	1.66	2.97	7.16

[a] α_{s1}-Casein concentration was constant at 4.0×10^{-5} M. Concentration of κ-α_{s1}-casein complex was calculated as $4.0 \times F_3/F_0 \times 10^{-5}$ M. F_3/F_0 was obtained from eq 4.

The equilibrium constants described in this paper are average association constants for the polydisperse system. These constants are for the association between dansylated α_{s1}-casein and unlabeled κ-casein which should not be significantly different from those between unlabeled α_{s1}- and κ-caseins, as demonstrated for bovine serum albumin and its antibody by Kierszenbaum *et al.* (1969), since the level of dansylation was one molecule per monomer unit of α_{s1}-casein. Such a low level of modification would preferably be at the terminal amino group, and as such should not significantly interfere with the interaction.

In the calculation of the association constant, concentrations of α_{s1}- and κ-caseins were determined as monomer units. If n-mers of both species are interacting and the interaction ratio being 1:1, the equilibrium constant would increase n times, as the molar concentrations of the species would be decreased by the factor n. Values of n for α_{s1}-casein range from 3 to 5 (Payens, 1966). Such a value would have a very slight effect upon the values of the thermodynamic parameters and would not change the direction of variation of the parameters with temperature.

The stability of casein micelles is probably a reflection of many parameters, one of which is undoubtedly the interaction

TABLE III: Changes in Free Energy, Enthalpy, and Entropy for the Interaction between Dansylated α_{s1}-Casein and κ-Casein.[a]

Temp (°C)	Equil Constant $\times 10^{-4}$	$\Delta F°$ (kcal/Mole)	$\Delta H°$ (kcal/Mole)	$\Delta S°$ (eu)
17	1.00	−5.29		
24	1.66	−5.71	3.33	30.4
30	2.97	−6.19	4.67	35.8
35	7.16	−6.82		

[a] $\Delta H°$ was calculated from the slope of the curve, log K *vs.* $1/T$ in Figure 4, at different temperatures.

between the caseins. The endothermic nature of interaction of the casein has been known for sometime. This endothermic nature of associations of the caseins has been suggested as being due to conformational change in the protein and subsequent changes in the calcium ion binding (Waugh, 1961). However, Nemethy and Scheraga (1962) have postulated that the endothermic nature of interactions could be due to hydrophobic interactions in the proteins, with a subsequent increase in both the enthalpy and entropy of the system.

The results presented in Table III clearly demonstrate the endothermic nature of the interaction between α_{s1}- and κ-casein. The association constant increases with temperature, and at 40° the interaction is almost complete. The increasing ease of reaction is also reflected in the lower level of free energy with temperature. In agreement with the theory of hydrophobic interactions (Nemethy and Scheraga, 1962) there is a positive enthalpy and entropy. These parameters both increased with temperature, indicative of the hydrophobic nature of the interaction.

These results confirm the suggestions of Garnier *et al.* (1964b) who found a positive entropy for the α_{s1}-κ-casein interaction, which indicated the hydrophobic nature of the interaction, and also Payens (1966) who suggested that the interaction would be hydrophobic in nature.

From the 40 or 50° curves in Figures 1, 2, and 3 the interaction ratio α_{s1}:κ is 0.93 on a molar basis as evidenced by the linear increase in polarization of α_{s1}- with added κ-casein, until the above ratio was reached; then there was no further increase in polarization, *i.e.*, no further interaction.

That the relationship between polarization and added κ-casein is linear, is indicative of only one reaction product. If there were an interaction product or products with a higher ratio, such a linear relationship would not exist, but on the contrary, the first addition of κ-casein would lead to a maximum increase in polarization, corresponding to the highest possible ratio of α_{s1}:κ under the experimental conditions; as this would represent the largest possible molecule. Further addition of κ-casein would decrease the α_{s1}:κ ratio; therefore the molecular weight of the interaction product, and hence the polarization would also decrease. That such results were not obtained denies the possibility of multiple interaction products. This reaction ratio is independent of concentration (Figures 1–3).

Our result is in agreement with the results of Noble and Waugh (1965), who found that α_{s1}- and κ-caseins reacted in a low weight ratio and also of Parry *et al.* (1969) who found that the interacting weight ratio of α_{s1}- to κ-casein is unity.

Kason *et al.* (1971) have shown that there is an interaction between α_{s1}-casein and polyethylenimine which is electrostatic in nature. That such an interaction does occur is evident from Figure 2, where the interaction between polyethylenimine-treated α_{s1}-casein and κ-casein was compared to the interaction between α_{s1}- and κ-casein at 4 and 40°. In both cases the slopes at the two temperatures were identical, however, the polarization values for the polyethylenimine-treated α_{s1}-casein was higher than for the untreated α_{s1}-casein. Two conclusions can be drawn from these results. Firstly, the polyethylenimine-treated α_{s1}-casein is a larger molecule, indicative of an interaction product as demonstrated by Kason *et al.* (1971). Secondly, the nonspecific electrostatic nature of the interaction product of polyethylenimine and α_{s1}-casein neither interfere with the α_{s1}-κ-casein interaction, nor affect the interaction ratio. This indicates that the interaction is quite specific and not electrostatic in nature.

In our other paper (R. Clarke and S. Nakai, 1971) it was demonstrated that κ-casein has one hydrophobic region. This is in total agreement with the results presented above, namely that the interaction ratio of α_{s1}:κ is unity and also that the reaction is principally hydrophobic in nature.

References

Dandliker, W. B., Schapiro, H. C., Meduski, J. W., Alonso, R., Feigen, G. A., and Hamrick, J. R., Jr. (1964), *Immunochemistry 1*, 165.

Garnier, J. (1967), *Biopolymers 5*, 473.

Garnier, J., Libadeau-Dumas, B., and Brignon, G. (1964b), *Abstr. 6th Intern. Biochem. Congr., New York, N. Y.*

Garnier, J., Yon, J., and Mocquot, G. (1964a), *Biochim. Biophys. Acta 82*, 481.

Haber, E., and Bennett, C. S. (1962), *Proc. Nat. Acad Sci. U. S. 48*, 1935.

Hill, R. D., and Laing, R. R. (1965), *J. Dairy Res. 23*, 193.

Hill, R. D., and Wake, R. G. (1969), *Nature (London) 221*, 635.

Kason, W. R., Nakai, S., and Bose, R. J. (1971), *J. Dairy Sci. 54*, 461.

Kenkare, D. A., and Hansen, P. M. T. (1967), *J. Dairy Sci. 50*, 135.

Kierszenbaum, F., Dandliker, J., and Dandliker, W. B. (1969), *Immunochemistry 6*, 125.

Nakai, S., Wilson, H. K., and Herreid, E. O. (1967), *J. Dairy Sci. 50*, 456.

Nemethy, G., and Scheraga, H. A. (1962), *J. Phys. Chem. 66*, 1773.

Noble, R. W., Jr., and Waugh, D. F. (1965), *J. Amer. Chem. Soc. 87*, 2236.

Parry, R. M., Jr., Ford, L. W., and Carroll, R. J. (1969), *J. Dairy Sci. 52*, 902.

Payens, T. A. J. (1966), *J. Dairy Sci. 49*, 1317.

Pepper, L., Hipp, N. J., and Gordon, W. G. (1970), *Biochim. Biophys. Acta 207*, 340.

Perrin, F. (1926), *J. Phys. Radium 7*, 390.

Steiner, R. F. (1954), *Arch. Biochem. Biophys. 49*, 71.

Talbot, B., and Waugh, D. F. (1970), *Biochemistry 9*, 2807.

Waugh, D. F. (1961), *J. Phys. Chem. 65*, 1793.

Waugh, D. F., and Noble, R. W., Jr. (1965), *J. Amer. Chem. Soc. 87*, 2246.

Waugh, D. F., and von Hippel, P. H. (1956), *J. Amer. Chem. Soc. 78*, 4576.

Weber, G. (1952), *Biochem. J. 51*, 145, 155.

Woychik, J. H. (1969), *J. Dairy Sci. 52*, 17.

Zittle, C. A., and Custer, J. H. (1963), *J. Dairy Sci. 46*, 1183.