stainless steel sample holder, uniform pressure is applied that allows for maximum contact between the KRS-5 plate and the sample and produces the spectrum of encapsulated GDL presented in Figure 5a. On the other hand, with a Teflon holder, the sample contact with the plate depends on adhesive characteristics of the sample which, therefore, limits the usefulness of this type of holder. Figure 5b shows the spectrum of encapsulated GDL using the Teflon holder. Figure 5a,b illustrates that the stainless steel holder permits closer contact between the sample and the KRS-5 plate than does the Teflon holder since the intensities of the observed reflections were greater when the stainless steel holder was used.

Second, the amount of pressure applied to the sample in the stainless steel holder affects its usefulness in surface analysis. When excessive pressure is applied to the sample, vibrational frequencies characteristic of crystalline GDL (Figure 5c) are observed in the encapsulated sample (Figure 5d). In this instance, the excess pressure caused the fat to flow exposing the underlying GDL.

Third, in cases where pressure cannot be applied to the sample, a Teflon holder is more applicable to surface analysis. For example, the multiple internal reflection spectrum of encapsulated GDL using the Teflon holder (Figure 5b) has essentially the same spectrum as the extracted fat (Figure 5e) when one allows for expected differences in intensities. Functional groups characteristic of GDL (Figure 5c) are not observed in that spectrum as they are in Figure 5a, indicating that the electromagnetic field penetrated only the sample's surface.

From the above discussion, it is obvious that the characteristics of the food material will dictate the conditions to use for surface analysis. Optimum conditions can only be defined through experimentation.

Although only a few examples showing the possible application of multiple internal reflection to the study of food materials were given, numerous other possibilities exist. Solid or liquid food substances can be easily studied since the only sample preparation necessary is bringing the substance in contact with the reflecting surface. Large particles as well as very thin films can be investigated with ease in their natural state, whereas in conventional spectroscopy few samples can be studied without elaborate sample preparation.

LITERATURE CITED

Allen, R. M. "Practical Refractometry by Means of the Microscope", 2nd ed.; Cargille Labs., Inc.: New York, 1962.
Association of Official Analytical Chemists "Official Methods of Analysis", 11th ed.; AOAC: Washington, DC, 1975.
Collison, R. "Starch and Its Derivatives"; Radley, J. A., Ed.; Chapman and Hall: London, 1968; Chapter 5.
Fahrenfort, J. Spectrochim. Acta 1961, 17, 698.
Harrick, N. J. J. Phys. Chem. 1960, 64, 1110.
Harrick, N. J. "Internal Reflection Spectroscopy"; Interscience: New York, 1967.
Varriano-Marston, E.; Ke, V.; Huang, G.; Ponte, J., Jr. Cereal Chem. 1980, 57, 242.

In Vitro Digestibility and Functional Properties of Chemically Modified Casein

Lourminia C. Sen, Honson S. Lee,[1] Robert E. Feeney, and John R. Whitaker*

Casein amino groups were modified by reaction with aldehydes and ketones via reductive alkylation at pH 9.0 to give stable, non-cross-linked lysine-modified derivatives. The degree of alkylation was controlled by the amount of alkylating reagent. There was a pronounced decrease in the initial rates of α-chymotrypsin-catalyzed hydrolysis of alkylated caseins. The initial rates decreased as the size of the modifying group increased and as the degree of modification increased. This decreased rate of hydrolysis was not due to product inhibition. Extents of hydrolysis after 48 h of the alkylated caseins were essentially independent of the degree of alkylation and the nature of the alkyl group. The conformation of alkylated casein was different from the native casein. Solubilities of methylcasein and isopropylcasein were increased slightly over that of native casein; with bulkier alkyl groups, the solubilities were significantly lower than that of native casein. Emulsifying activities of alkylated caseins, except for butylcasein, were higher than that of native casein.

Increased attention has been directed toward the development of low-cost protein foods (Altschul, 1974; Forsythe and Briskey, 1977; Friedman, 1978). Although there are a multitude of alternative sources of proteins (e.g., trash fish, grain, microbial, and leaf), the feasibility of using them as food proteins has been limited due to their low biological value, undesirable organoleptic properties, toxic contamination, and poor functional properties. The above problems may be overcome by one or more of the following methods: physical or mechanical treatment (Huang and Rha, 1974), enzyme modification (Spinelli et al., 1972; Kuehler and Stine, 1974; Hermansson et al., 1974; Liener, 1977; Miller and Groninger, 1976; Phaff, 1977; Richardson, 1977; Whitaker, 1977; Fujimaki, 1978; Zakaria and McFeeters, 1978), microbial modification (Whitaker, 1978; Beuchat, 1978), chemical modification (Franzen and Kinsella, 1976; Barman et al., 1977; Feeney, 1977; Meyer and Williams, 1977; Puigserver et al., 1979a,b).

We have studied chemical procedures to overcome some of the above limitations. Reductive alkylation was used because it has been shown that under mildly alkaline conditions, the amino groups in protein can be reductively

Department of Food Science and Technology, University of California, Davis, Davis, California 95616.

[1]Present address: The Quaker Oats Company, Barrington, IL 60010.

Digestibility and Properties of Modified Casein

J. Agric. Food Chem., Vol. 29, No. 2, 1981 **349**

methylated, resulting in stable, non-cross-linked lysine derivatives (Means and Feeney, 1968). Since partially methylated casein supported normal rat growth without apparent toxicity (Lee et al., 1978), limited alkylation of food proteins may be useful for the protection of lysyl residues against deteriorative reactions such as nonenzymatic browning.

Studies on the correlation of chemical properties of proteins with functional properties in a food use context can provide insight into ways of tailoring properties of protein for specific food uses. Such studies have been quite limited, and the existing studies are difficult to compare because of lack of standardized methods of measuring functional properties.

The purpose of this work was to determine systematically the effects of the size of alkylating reagent and the degree of alkylation on the in vitro digestibility and functional properties of the modified protein. Casein was chosen as the model protein since it can be easily methylated (Galembeck et al., 1977; Lee et al., 1978).

MATERIALS AND METHODS

Materials. Hammarsten quality casein was obtained from ICN Pharmaceutical Inc., Cleveland, OH. Bovine serum albumin, sodium borohydride, and 2,4,6-trinitrobenzenesulfonic acid were obtained from Sigma Chemical Co., St. Louis, MO. Bovine α-chymotrypsin (3 times crystallized lot no. 58C-8135) was from Worthington Biochemical Corp., Freehold, NJ. All other reagents and chemicals were of analytical grade.

Preparation of Alkylated Casein. The procedures for alkylation were in general according to Means and Feeney (1968) as modified by Lee et al. (1978 and Fretheim et al. (1979). All alkylations were done at room temperature (25 °C) except for methylation at 0 °C. A 1% solution (250 mL) of casein in 0.2 M borate buffer, pH 9.0, containing 20% dioxane was prepared by warming to $\sim$40 °C. The solution was then cooled to room temperature or 0 °C. To prevent cross-linking, we added 0.012 mol of $NaBH_4$ first. This was followed by 0.037 mol of carbonyl reagent in one addition, except for methylation. For reductive methylation four increments of $NaBH_4$ and formaldehyde were added alternately over a period of 15 min. 2-Octanol was added dropwise to control foaming. The reaction was stirred for 15 min and then dialyzed against water and lyophilized. The amount of carbonyl reagent was varied according to Table I in order to produce various degrees of modification. Appropriate controls were prepared in the same manner in the absence of the carbonyl reagent. The overall weight yields of all control and alkylated casein preparations were $\sim$70%.

Analysis of Alkylated Casein. The degree of alkylation was determined by quantitating the decrease in free amino groups with 2,4,6-trinitrobenzenesulfonic acid (Fields, 1972) and by measuring the loss in lysine by amino acid analysis (Means and Feeney, 1968; Lee et al., 1978; Fretheim et al., 1979).

In Vitro Hydrolysis. Hydrolyses of casein and alkylated casein were determined by measuring the amount of amino groups liberated with 2,4,6-trinitrobenzenesulfonic acid (Fields, 1972). The reaction mixtures contained 0.1% native casein or alkylated casein in 0.02 M borate buffer, pH 8.2, and the reaction was performed at 38 °C. For determination of initial rates of hydrolysis, assays were done with 7.45 nM α-chymotrypsin. Aliquots removed at 5-min intervals for a period of 30 min were quenched by 0.05 M HCl. For determination of the extents of hydrolysis, assays were done for 48 h at 38 °C, with 115 nM α-chymotrypsin added twice at 24-h intervals. The

reaction was quenched as above.

Peptides from chymotryptic hydrolysates of cyclohexyl- and benzylcaseins were prepared by incubating 0.1% cyclohexyl- or benzylcasein with 115 nM α-chymotrypsin under similar conditions as above. The reaction was quenched by boiling the solution for 10 min. At the time of quenching, $\sim$77% of susceptible bonds had been hydrolyzed.

Viscosity. A Cannon-Ubbelohde (semimicro size 50) viscometer was used to measure the viscosity of 0.1% protein solutions in 0.02 M borate buffer, pH 8.2, at 25 °C (maintained in a water bath).

Protein Solubility. Samples containing 100 mg of protein/mL in the appropriate buffer were incubated for 1 h at 60 °C and then for 48 h at 4 °C. Following centrifugation at 27000g for 1 h at 4 °C, the amount of protein in the supernatant was determined by the Lowry method (Lowry et al., 1951). The following buffers (0.1 M) were used: sodium phosphate, pH 7.0 and 8.0; sodium citrate, pH 3.0, 4.0, 5.0, and 6.0; sodium bicarbonate, pH 9.0 and 10.0. The ionic strength of the buffers was adjusted to 0.55 by adding appropriate amounts of NaCl.

Ultraviolet Difference Spectra. Equal concentration (0.1% protein) solutions of native and alkylated caseins were prepared in 0.02 M borate buffer, pH 8.2. Difference spectra between alkylated casein and native casein were measured at 20 °C with a Cary 118C double-beam spectrophotometer.

Emulsifying Activity. As defined by Miller and Groninger (1976), the emulsifying activity indicates whether a protein has any emulsifying ability and, if it does, the percent emulsion for a given quantity of protein. In contrast, the emulsifying capacity measures the quantity of oil a protein can absorb before the emulsion breaks. The methods for determining the end point of emulsion are usually visual; however, we chose to determine emulsifying activity as measured by turbidity at 600 nm (Pearce and Kinsella, 1978).

For native casein, the emulsifying activity was determined at five protein concentrations ranging from 0.4 to 2.0%. For cyclohexylcasein, the emulsifying activity was determined at three protein concentrations ranging from 0.4 to 2.0%. For other highly alkylated caseins, the emulsifying activity was measured only at 0.4%.

Ten milliliters of protein solution in 0.1 M sodium phosphate buffer, pH 7.0, was blended with 3.0 mL of Planters peanut oil in a monel-jacketed stainless steel container (Eberback Model No. 8580, semimicro) at the top speed of a Waring Blendor for 60 s at 25 °C. A 10-μL sample of the emulsion was diluted to 10 mL with an aqueous solution of sodium dodecyl sulfate (final concentration of $NaDodSO_4$ of 1%). Absorbance at 600 nm was measured immediately.

Water Binding Studies. Weighed samples of protein were kept in desiccators equilibrated to 18.8% relative humidity (60.1% H_2SO_4). After 72 h, the samples were reweighed.

RESULTS

Analyses of Alkylated Casein. The extents of alkylation of casein by several carbonyl reagents, determined by amino acid analysis and the TNBS method, are shown in Table I. By varying the ratio of the carbonyl compound to the amount of available amino groups, we obtained different extents of alkylation. The results based on the TNBS analyses are in general agreement with the results by amino acid analyses. For proteins with tertiary structure, the TNBS method usually gives values lower than the true value, probably because the amino groups are

Table I. Extents of Alkylation[a] of 12 mM Casein with Different Carbonyl Compounds

| reagent	molar ratio of carbonyl reagent to amino groups	% lysine alkylated as measured by	
		TNBS	amino acid analysis
formaldehyde	267	97	98
	27	88	93
	18	41	46
acetone	533	83	91
	133	20	28
n-butyraldehyde	267	95	97
	44	82	88
	22	58	67
cyclopentanone	533	66	71
	267	22	27
	44	17	10
cyclohexanone	533	92	94
	267	72	83
	133	38	43
	27	3	4
benzaldehyde	533	97	96
	133	60	68
	22	10	16

[a] Alkylation was performed with 12 mM casein and varying concentrations of carbonyl reagent at pH 9.0 for 30 min in the presence of 353 mM $NaBH_4$.
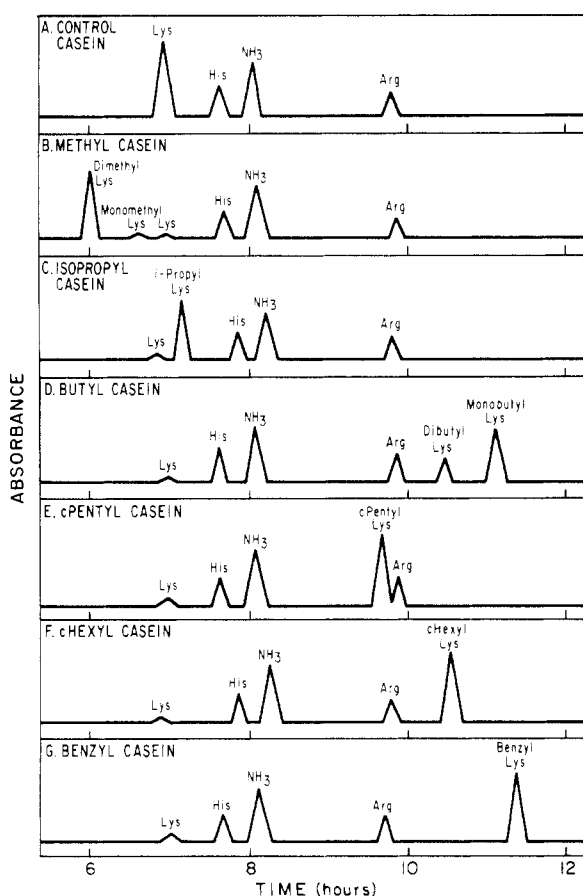
Figure 1. Separation of the basic amino acids and derivatives by ion-exchange chromatography following acid hydrolysis of the native and alkylated caseins.

buried and not very accessible to the reagent (Osuga, 1978). For casein, which exists in a random coil (Slattery, 1976), the amino groups are exposed and readily react with TNBS.

The basic amino acid composition and derivatives of lysine of the alkylated casein determined by amino acid analysis are shown in Figure 1. For all the alkylated
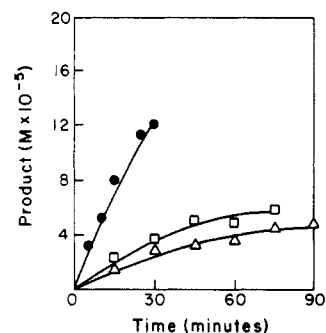
Figure 2. Increase in free amino groups measured as a function of time during chymotryptic hydrolysis of native casein (●), methylcasein (□), and isopropylcasein (△).

Table II. Effect of Size of Alkyl Group on Initial Rates of Hydrolysis of Alkylated Caseins by α-Chymotrypsin[a]

protein	deg of alkylation,[b] %	rel initial rates
native casein	0	100
alkylated casein		
methyl	97	57
isopropyl	83	42
butyl	95	12
cyclohexyl	92	2
benzyl	97	2

[a] 7.45 nM α-chymotrypsin and 0.1% substrate in 0.02 M borate buffer at pH 8.2, 38 °C. [b] Measured by TNBS.

Table III. Effect of Degree of Alkylation on Initial Rates of Hydrolysis of Alkylated Casein by α-Chymotrypsin[a]

protein	deg of alkylation,[b] %	rel initial rates
native casein	0	100
alkylated casein		
methyl	41	66
	97	57
butyl	58	35
	82	23
	95	12
cyclopentyl	3	70
	66	40
cyclohexyl	3	70
	38	37
	72	4
	92	2
benzyl	10	6
	60	3
	97	2

[a] 7.45 nM α-chymotrypsin and 0.1% substrate in 0.02 M borate buffer, pH 8.2, 38 °C. [b] Measured by TNBS.

caseins, the losses in lysine were accompanied by a corresponding increase in the respective alkyllysine derivative. Except for methyl- and butylcaseins whose principal products were the disubstituted derivatives, the other alkylated lysines were monoalkyl derivatives. There were no significant changes in other amino acids of the alkylated casein.

Initial Rates of Hydrolysis by α-Chymotrypsin. Increases in TNBS reaction of amino groups were used to determine the initial rates of hydrolysis of casein and alkylated caseins by chymotrypsin (Figure 2).

As the size of the alkylating group increased, the relative initial rates of hydrolysis of the alkylated casein by α-chymotrypsin decreased (Table II).

The increase in the degree of alkylation was accompanied by a corresponding decrease in the rates of hydrolysis by α-chymotrypsin as shown in Table III. For example,

Table IV. Extent of Hydrolysis of Alkylated Caseins by α-Chymotrypsin after 48 h of Incubation[a]

protein	deg of alkylation, %	rel extent	deg of hydrolysis of susceptible bonds, %
native casein	0	100	92–98
alkylated casein			
methyl	41	79	73
	97	79	73
isopropyl	20	77	71
	83	73	68
butyl	58	79	73
	95	82	76
cyclopentyl	22	72	67
	66	69	64
cyclohexyl	38	80	74
	92	79	73
benzyl	10	85	78
	60	83	77

[a] 0.1% substrate in 0.02 M borate buffer, pH 8.2, 38 °C, and 115 nM α-chymotrypsin added at 0 time and after 24 h.

Table V. Effects of Hydrolysis Product of Highly Alkylated Caseins on Hydrolysis of Native Casein[a]

native casein:hydrolysis products of alkylated casein, mg:mg	rel initial rate
1:0	1.0
Products of Cyclohexylcasein[b]	
1:0.1	1.0
1:0.5	1.0
1:1	1.0
Products of Benzylcasein[c]	
1:0.1	1.0
1:0.5	1.0
1:1	1.0

[a] 0.1% native casein in 0.02 M borate buffer, pH 8.2, 38 °C, and 7.45 nM α-chymotrypsin. [b] 92% alkylated. [c] 97% alkylated.

butylcasein gave the following results: 58% modified, 35% relative initial rate; 82% modified, 23% relative initial rate; 95% modified, 12% relative initial rate.

Extent of Hydrolysis after 48 h by α-Chymotrypsin. Table IV shows that the extent of hydrolysis of chymotrypsin-susceptible bonds in alkylated caseins was significantly lower than that of native casein. The degree of alkylation did not appear to affect the extent of hydrolysis.

Effect of Hydrolysis Products of Highly Alkylated Caseins and of Alkylated Caseins on Initial Rates of Hydrolysis of Native Casein. The hydrolysis products of chymotrypsin treatment of cyclohexylcasein or of benzylcasein did not affect the rates of hydrolysis of native casein (Table V).

In the presence of either cyclohexylcasein or benzylcasein, there was a significant decrease in initial rates of hydrolysis of native casein (Table VI).

UV Difference Spectra. The alkylated caseins had higher absorbance in the 290–330- and 245–270-nm regions than native casein (Figures 3 and 4) but lower absorbances in the 270–290-nm region. In general, the extent of deviation from that of native casein correlated with the extent of modification and/or the size of the alkyl group. The larger the alkyl group, the more the deviation from the spectrum of native casein.

Functional Properties of Alkylated Casein. *Solubility.* The solubility patterns of highly alkylated caseins at different pH values and at constant ionic strength are shown in Figures 5 and 6. Alkylation leads to retention

Table VI. Effect of Highly Alkylated Casein on Initial Rates of Hydrolysis of Native Casein[a]

substrate, %		rel initial rates
native	modified	
0.1	0	100
Cyclohexylcasein[b]		
0.1	0.003	82
0.1	0.007	74
0.1	0.010	49
Benzylcasein[c]		
0.1	0.003	97
0.1	0.007	74
0.1	0.013	65

[a] pH 8.2, 38 °C, 7.45 nM α-chymotrypsin. [b] 92% alkylated. [c] 97% alkylated.
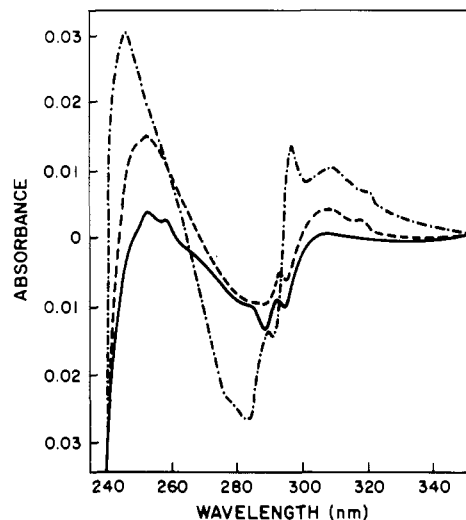

Figure 3. Ultraviolet difference spectra of reductively alkylated caseins with native casein as the reference at 20 °C. Methylcasein (—); isopropylcasein (-·-); butylcasein (···).
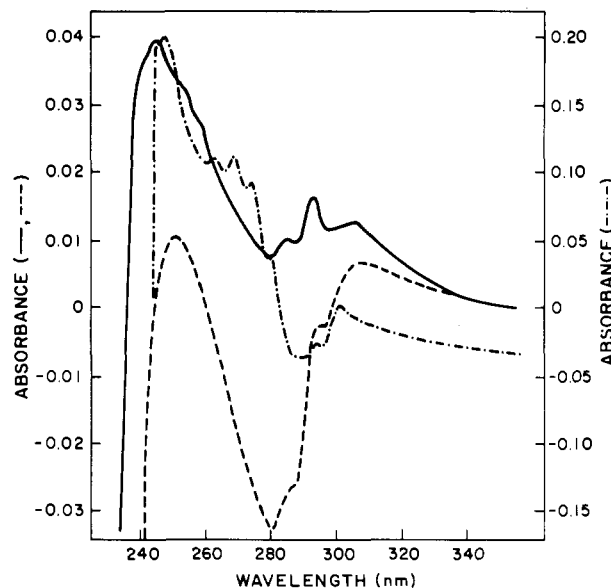

Figure 4. Ultraviolet difference spectra of reductively alkylated caseins with native casein as the reference at 20 °C. Cyclopentylcasein (—); cyclohexylcasein (-·-); benzylcasein (···).

in the number of positive charges in the protein, and a small decrease (~0.4–0.6 unit) in the pK_a of the amino group (Means and Feeney, 1968). Methylcasein was more soluble than native casein in the pH range of 3–9 while isopropylcasein was more soluble in the pH range of 3–6.
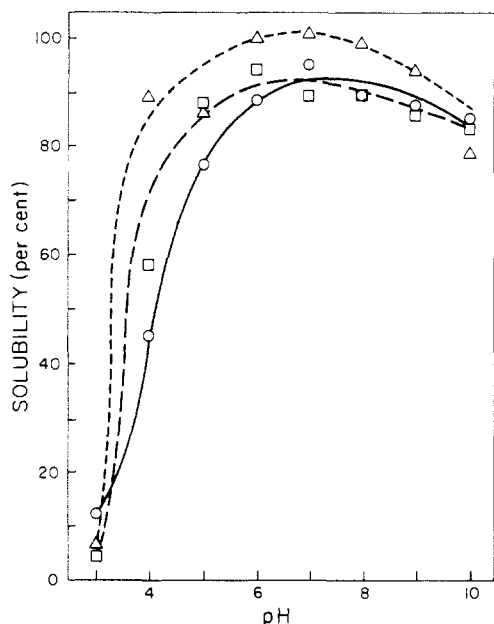
352 J. Agric. Food Chem., Vol. 29, No. 2, 1981

Sen, Lee, Feeney, and Whitaker

Figure 5. Solubilities of highly alkylated caseins at different pH values. Native casein (O); methylcasein (△); isopropylcasein (□).

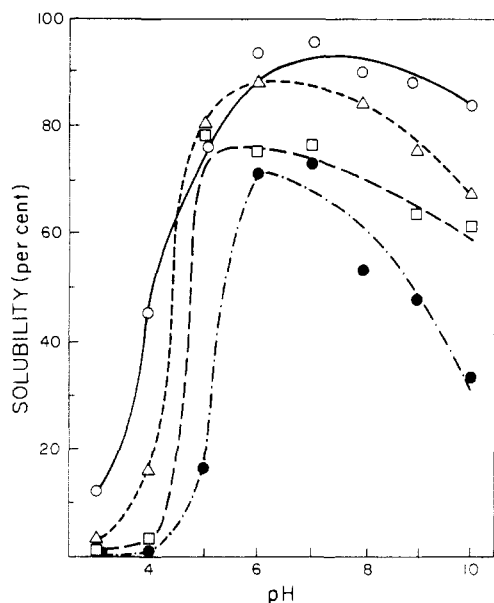**Figure 6.** Solubilities of highly alkylated caseins at different pH values. Native casein (O); butylcasein (△); cyclohexylcasein (□); benzylcasein (●).

Above pH 6, there was no significant difference between isopropylcasein and native casein. With butyl-, cyclo-hexyl-, and benzylcaseins, there was an overall decrease in solubility from pH 3 to 10 (Figure 6) due to the presence of these much larger hydrophobic groups.

Viscosity. Alkylated caseins had significantly lower relative viscosity than native casein (Table VII). More or less uniform distribution of proline along the chain of casein results in a random coil configuration with very little helical structure (Slattery, 1976). The attachment of numerous (~16 residues/mol) hydrophobic residues may have caused the chain to fold to allow for hydrophobic interaction. Due to the electrostatic repulsion between positively charged nitrogens, there is no maximum overlap between hydrophobic residues resulting in formation of weaker hydrophobic bonds. All the alkyl caseins may have folded to the same degree with the bulkier groups sticking out contributing more drag than the smaller groups. The decrease in relative viscosity upon introduction of alkyl

Table VII. Functional Properties of Highly Alkylated Caseins

sample	rel viscosity[a]	moisture absorbed,[b] %	rel emulsifying act.[c]
native casein	1.0589	0.48	100
alkylated casein			
methyl	1.0171	0.71	164
isopropyl	1.0367	0.84	181
butyl	1.0496	0.40	100
cyclopentyl		0.39	148
cyclohexyl	1.0525	0.97	133
benzyl		0.67	153

[a] 0.1% protein solution in 0.02 M borate, pH 8.2, at 25 °C, relative to dissolving buffer. [b] At 25 °C in 18.8% relative humidity. [c] 0.31% protein in 0.1 M phosphate buffer, pH 7.0, at 25 °C.

groups became increasingly smaller as the size of the alkyl group increased.

Water Binding. Except for butyl- and cyclopentyl-caseins, all highly alkylated caseins absorbed more moisture than native casein (Table VII). In most systems, the amount of water absorbed depends primarily on the number and availability of hydrophilic groups which are capable of hydrogen bonding with water. In proteins, such hydrophilic groups are the polar side chains and the carboxyl and imido groups of peptide bonds. The greater amount of moisture absorbed is unexpected in light of the hydrophobicity of the alkyl groups and of the retention of the positive charge of amino groups upon alkylation.

Emulsifying Activity. Our results with native casein and cyclohexylcasein indicate that at 0.4% protein, the degree of turbidity is not limited by the amount of oil present during emulsification but is dependent on the amount of protein. The highly alkylated caseins, with the exception of butylcasein, have higher emulsifying activity than native casein (Table VII).

DISCUSSION

Chemical modification of proteins in order to elucidate the mechanism of action has been a very popular and fruitful technique (Hirs, 1967; Means and Feeney, 1971; Hirs and Timasheff, 1977). Only recently have food scientists taken advantage of the vast basic information on chemical modification and applied it to food proteins (Gandhi et al., 1967; Childs and Park, 1976; Miller and Groninger, 1976; Barman et al., 1977; Aoki et al., 1978; Lee et al., 1978, 1979; Matoba and Doi, 1979; Puigserver et al., 1979a,b).

Nonenzymatic browning, also known as the Maillard reaction (Finot, 1973), results in the development of the certain taste, color, and odor (both desirable and undesirable) of some food. The Maillard reaction leads to the unavailability of lysine because of reaction of the ε-amino group of lysines with reducing sugars. Bjarnasson and Carpenter (1969) reported that there was less heat damage to formylated and acetylated proteins than with unmodified proteins. The blocking of the ε-amino group of lysine may then serve as a means of protection for food proteins.

Although the amino group can be protected from the Maillard reaction by alkylation, this could also prevent its hydrolysis by proteolytic enzymes. Chymotrypsin was chosen to test this as its specificity is not directed toward the modified amino acid, lysine. All alkylated caseins gave lower initial rates of hydrolysis with chymotrypsin. The bulkier the alkyl group and/or the higher the degree of alkylation, the lower was the initial rate of hydrolysis.

The extent of hydrolysis of the alkylated caseins were significantly lower than that of the native casein. A pos-

Digestibility and Properties of Modified Casein

J. Agric. Food Chem., Vol. 29, No. 2, 1981 **353**

sible explanation could be the more ordered structure in the alkylated caseins. Proteins with ordered tertiary structure (native state), such as globular proteins, are not susceptible to proteolytic hydrolysis. Only after the protein has been denatured can the proteolytic enzyme hydrolyze the potentially susceptible bonds. Since it has very little tertiary structure (Slattery, 1976), native casein serves as an ideal substrate for proteolytic enzymes. The covalent attachment of numerous alkyl groups (~15–16) may have caused casein to fold more compactly to allow for hydrophobic interactions between the alkyl groups. This folding may have hindered the approach of chymotrypsin to the susceptible bonds, which in turn led to an overall decrease of hydrolysis of the substrate. Difference spectra also support this concept of folding of the alkylated caseins (Figures 3 and 4). Samples which are alkylated to the same degree but with different alkyl groups (Table II) showed significant differences in hydrolysis by chymotrypsin. This indicates that, in addition to the degree of folding, there may be other factors which affect the rate of hydrolysis. That other major factor could be the size of the alkyl group. The greater the size of the alkyl groups, the greater is the steric hindrance for the enzyme.

Galembeck et al. (1977) reported low rates of chymotryptic hydrolysis of reductively methylated bovine serum albumin. They interpreted these low rates as due to product inhibition. This hypothesis was tested with the chymotrypsin hydrolysis products of cyclohexyl- and benzylcaseins, because these two alkylated caseins had virtually zero rates of hydrolysis. As shown in Table V, chymotryptic hydrolysis products of either cyclohexylcasein or benzylcasein, in equal amounts to native casein, had no effect on the initial rate of hydrolysis of native casein. It should be noted that initial rates of hydrolysis were used in the present investigations; therefore, product inhibition would not be expected (in the absence of added products).

Nonproductive binding of substrates occurs with chymotrypsin, as with many enzymes. This becomes more prevalent in the presence of aromatic leaving groups and *N*-acyl groups (Coll, 1977). Our results (Table VI) show that benzylcasein and cyclohexylcasein inhibit the hydrolysis of native casein. These data suggest that the decreased rate of hydrolysis of alkylated caseins may be due to nonproductive binding of enzyme and alkylated substrate.

With proteins lacking tertiary structure, it is expected that only extensive conformational changes can induce modification of the physical properties studied in this work. For this reason, we examined the physical and functional properties of only the highly alkylated caseins. Attachment of various sizes of hydrophobic groups to casein by reductive alkylation changed the conformation of casein as shown by the ultraviolet difference spectra. Solubilities of methylcasein and isopropylcasein were increased slightly over that of native casein; however, with bulkier alkyl groups, the solubilities were significantly lower than that of native casein in the pH 3–4 and 6–10 range as might be expected. Fretheim et al. (1979) also have recently reported that solubilities were greatly lowered on reductive benzylation or cyclohexylation of two globular proteins, lysozyme and ovotransferrin.

Covalent attachment of hydrophobic groups to proteins may be useful for increasing emulsifying activities of food proteins.

Reductive alkylation, because of its ease and specificity, may be useful as a means for protecting the lysyl residues against deteriorative reactions during food processing and

storage as well as for changing physical properties. However, one must be aware that the larger the size of the alkyl group and the greater the degree of alkylation, the lower may be the digestibility of the alkylated casein. If the alkylated protein constituted only a small fraction of the total proteins, some lowering of digestibility could be of little consequence. The protein may be able to regain its complete nutritional value if the modification can be reversed at the digestive or absorption level. Nevertheless, the data reported in this paper illustrate that one must be judicious in the choice of methods of chemical modification in food systems.

LITERATURE CITED

Altschul, A., Ed. "New Protein Foods"; Academic Press: New York, 1974; Vol. 1A.

Aoki, H.; Orimo, N.; Shimazu, R.; Wakabayashi, K. *Nippon Shokuhin Kogyo Gakkaishi* 1978, *25*, 668.

Barman, B. G.; Hansen, J. R.; Mossey, A. R. *J. Agric. Food Chem.* 1977, *25*, 638.

Beuchat, L. R. *Food Technol. (Chicago)* 1978, *32*, 193.

Bjarnasson, J.; Carpenter, K. J. *Br. J. Nutr.* 1969, *23*, 859.

Childs, E. A.; Park, K. K. *J. Food Sci.* 1976, *41*, 713.

Coll, R. C. Ph.D. Thesis, University of California, Davis, 1977.

Feeney, R. E. *Adv. Chem. Ser.* 1977, *No. 160*, 3.

Fields, R. *Methods Enzymol.* 1972, *25B*, 464.

Finot, P. A. In "Proteins In Human Nutrition"; Porter, J. W. G.; Rolls, B. A., Eds.; Academic Press: New York, 1973; p 501.

Forsythe, R. H.; Briskey, E. J. In "Food Proteins"; Whitaker, J. R.; Tannenbaum, S. R., Eds.; Avi Publishing Co.: Westport, CT, 1977; p 554.

Franzen, K. L.; Kinsella, J. E. *J. Agric. Food Chem.* 1976, *24*, 914.

Fretheim, K.; Iwai, S.; Feeney, R. E. *Int. J. Pept. Protein Res.* 1979, *14*, 451.

Friedman, M., Ed. *Adv. Exp. Med. Biol.* 1978, *105*.

Fujimaki, M. *Ann. Nutr. Aliment.* 1978, *32*, 233.

Galembeck, F.; Ryan, D. S.; Whitaker, J. R.; Feeney, R. E. *J. Agric. Food Chem.* 1977, *25*, 238.

Gandhi, S. K.; Schultz, J. R.; Boughey, F. W.; Forsythe, R. H. *J. Food Sci.* 1967, *33*, 163.

Hermansson, A. M.; Olson, D.; Holmberg, B. *Lebensm.-Wiss. Technol.* 1974, *7*, 176.

Hirs, C. H. W., Ed. *Methods Enzymol.* 1967, *11*, Section VIII.

Hirs, C. H. W.; Timasheff, S. N., Eds. *Methods Enzymol.* 1977, *47E*, Section IX.

Huang, F.; Rha, C. *Polym. Eng. Sci.* 1974, *14*, 81.

Kuehler, C. A.; Stine, C. M. *J. Food Sci.* 1974, *39*, 379

Lee, H. S.; Sen, L. C.; Clifford, A. J.; Whitaker, J. R.; Feeney, R. E. *J. Nutr.* 1978, *108*, 687.

Lee, H. S.; Sen, L. C.; Clifford, A. J.; Whitaker, J. R.; Feeney, R. E. *J. Agric. Food Chem.* 1979, *27*, 1094.

Liener, I. E. *Adv. Chem. Ser.* 1977, *No. 160*, 283.

Lowry, O. H.; Rosebrough, N. J.; Farr, A. L.; Randall, R. J. *J. Biol. Chem.* 1951, *193*, 265.

Matoba, T.; Doi, E. *J. Food Sci.* 1979, *44*, 537.

Means, G. E.; Feeney, R. E. *Biochemistry* 1968, *7*, 2192.

Means, G. E.; Feeney, R. E. "Chemical Modification of Proteins"; Holden-Day: San Francisco, 1971.

Meyer, E. W.; Williams, L. D. *Adv. Chem. Ser.* 1977, *No. 160*, 52.

Miller, R.; Groninger, H. S., Jr. *J. Food Sci.* 1976, *41*, 268.

Osuga, D. T., personal communication, 1978.

Pearce, K. N.; Kinsella, J. E. *J. Agric. Food Chem.* 1978, *26*, 716.

Phaff, H. J. *Adv. Chem. Ser.* 1977, *No. 160*, 244.

Puigserver, A. J.; Sen, L. C.; Clifford, A. J.; Feeney, R. E.; Whitaker, J. R. *J. Agric. Food Chem.* 1979a, *27*, 1286.

Puigserver, A. J.; Sen, L. C.; Gonzales-Flores, E.; Feeney, R. E.; Whitaker, J. R. *J. Agric. Food Chem.* 1979b, *27*, 1099.

Richardson, T. *Adv. Chem. Ser.* 1977, *No. 160*, 67.

Slattery, C. W. *J. Dairy Sci.* 1976, *59*, 1547.

Spinelli, J.; Koury, B.; Miller, R. *J. Food Sci.* 1972, *37*, 604.

Whitaker, J. R. *Adv. Chem. Ser.* 1977, *No. 160*, 95.

Whitaker, J. R. *Food Technol. (Chicago)* **1978,** *32,* 175.
Zakaria, F.; McFeeter, R. F. *Lebensm.-Wiss. Technol.* **1978,** *11,*
     42.

Interaction of Sunset Yellow with Copper(II) Ion

Eleanor A. Frazier[1] and Richard A. Bernhard*

Reactions between Sunset Yellow (FD&C No. 6) and cupric ion were examined. When the dye combined
with cupric ion, a shift in the visible absorption spectrum occurred and hydrogen ion was liberated. The
hydrogen ion liberated per dye molecule was determined by potentiometric and spectrophotometric
titration. The dye–Cu(II) complex ratio was determined from spectrophotometric data by means of
the Scatchard plot, Job's method, and the slope–ratio method. Sunset Yellow reacted with cupric ion
in a ratio of 2:1 with the liberation of 0.5 equiv of hydrogen ion and a ratio of 1:1 with the liberation
of one hydrogen ion. Earlier reports of a 1:2 complex could not be confirmed. The dissociation constants
for the complexes are 8.87×10^{-4} and 5.37×10^{-4}, respectively.

Banerjee et al. (1977) reported that the food dyes, FD&C
Red No. 2, FD&C Red No. 4, and FD&C Yellow No. 6
formed complexes with cupric ion. The authors presented
arguments for their conclusions that the dyes reacted with
cupric ion in the ratios of 1:1 and 1:2 dye to cupric ion. The
1:1 dye–Cu(II) complex is consistent with the findings of
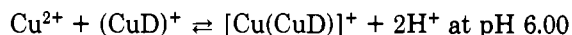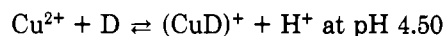other authors, while the 1:2 dye–Cu(II) complex had not
been previously reported.

It is our view that the evidence for the formation of a
1:2 dye–Cu(II) complex is subject to other interpretation.
Indeed, Banerjee et al. (1977) expressed the hope that their
findings would stimulate further investigation. It is in
keeping with this hope that we reexamined this com-
plexation reaction.

In solution the azo dye is involved in three equilibriums:
acid–base equilibrium, tautomeric equilibrium, and poly-
meric equilibrium.

The acid–base equilibriums (Figure 1) involve the sul-
fonic acid groups and the *o*-hydroxyazo group. The sul-
fonic acid groups are strong acids and dissociate around
pH 2.00 (Jablonski, 1951). The *o*-hydroxyazo hydrogen
is usually more basic than a phenolic group and dissociates
around pH 12.00 (Zollinger, 1961). Its more basic character
is attributed to proton tautomerism.

Historically, the theory of proton tautomerism was first
demonstrated by using a hydroxyazo–hydrazone equilib-
rium (Zollinger, 1961). The equilibrium is a transfer of
a proton (from the *o*-hydroxyl group) between the oxygen
and the *β*-nitrogen (Figure 1). The change in the electronic
configuration of the azo chromophore changes the absor-
bance spectrum. Likewise, the removal of the hydrogen
in the acid–base equilibrium will also change the spectrum
(Mason, 1970). The position of the tautomeric equilibrium
is solvent dependent. The hydroxyazo form dominates in
organic, nonpolar solvents. The hydrazone form is favored
in polar solvents which undergo hydrogen bonding. In
aqueous solution essentially 100% of the dye is present
as the hydrazone (Krueger, 1975). Both forms aggregate
(Zollinger, 1961).

Department of Food Science and Technology, University
of California, Davis, Davis, California 95616.

[1]Present address: Day-Glo Color Corporation, El Monte,
CA 91733.

Banerjee et al. (1977) indicated the formation of 1:1 and
1:2 dye–Cu(II) complexes:

$$Cu^{2+} + D \rightleftarrows (CuD)^+ + H^+ \text{ at pH 4.50}$$

$$Cu^{2+} + (CuD)^+ \rightleftarrows [Cu(CuD)]^+ + 2H^+ \text{ at pH 6.00}$$

They propose that at pH 4.50 the *o*-hydroxyazo hydro-
gen is replaced. The reaction, because it involves the
chromophore of the dye molecule, would produce a spectral
shift and such a shift was observed. They proposed that
at pH 6.00 the cupric ion neutralizes the two sulfonic acid
groups via a "salt formation equilibrium" with the exact
location of the second cupric ion being unknown. The
reaction would produce little or no spectral change. Be-
cause of their location, the sulfonic acid groups exert little
or no influence on the chromophore of the dye (Zollinger,
1961; Blumberger, 1940). At pH 6.00 only a slight spectral
change was observed with the production of two protons.
The sulfonic acid groups being strong acids should be
dissociated at pH 4.50 and certainly at pH 6.00. A
"salt-forming equilibrium" between cupric ion and the acid
groups would be expected at a lower pH and the reaction
with the basic hydroxyazo hydrogen at a higher pH, rather
than the reverse as suggested by the authors. The reverse
order could be explained if the salt-forming equilibrium
could only occur after the 1:1 complex had formed. Such
a mechanism is not indicated in this case, because the
sulfonic acid groups and the *o*-hydroxyazo group have little
or no influence on each other (Zollinger, 1961; Blumberger,
1940). This is also confirmed by the slight spectral shift
(which we calculated to be statistically insignificant) that
Banerjee et al. observed. Therefore, the ordered mecha-
nism suggested by Banerjee et al. appears questionable.

Figure 2 illustrates previously reported dye–Cu(II)
complexes (Jarvis, 1961; Krueger, 1975; Zollinger, 1961).
The 1:1 complex presented by Banerjee et al. (1977) as-
sociates the cupric ion with the *α*-nitrogen. The interaction
between the azo group and cupric ion should be with the
β-nitrogen (Jarvis, 1961; Krueger, 1975; Zollinger, 1961).
The *β*-nitrogen is slightly more electronegative than the
α-nitrogen because the oxygen ortho to the *α*-nitrogen
tends to draw electrons from the *α*-nitrogen. Also, the
six-membered ring formed by the interaction of the *β*-
nitrogen with cupric ion is more stable than a five-mem-
bered ring would be (Krueger, 1975).