Dansylation of Casein Micelles and Purified Fractions

Functional Group Analysis and Effect on Micelle Formation

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Functional groups of bovine casein micelles and of κ-, β-, and α_s-caseins were determined by use of fluorescent reagent 1-dimethylaminonaphthalene-5-sulfonyl chloride (DNS–Cl). Reactive groups detected in β- and α_s-caseins were the α-amino group of arginine, ε-amino group of lysine, and hydroxyl group of tyrosine. Groups reactive with DNS–Cl in κ-casein were the ε-amino group of lysine, hydroxyl group of tyrosine, and amino group of galactosamine. DNS derivatives formed by treating casein micelles with DNS–Cl for 10 min and 5 hr at pH values of 6.6, 7.5, and 9.5 were α-DNS-arginine, ε-DNS-lysine, *O*-DNS-tyrosine, and DNS-galactosamine. Moles of each DNS derivative formed during treatment of micelles and solubilized casein at pH 6.6 were determined. Blocking a portion of the functional groups of casein fractions with DNS–Cl did not alter the normal stability of fractions against Ca^{2+}, but the ability of κ-casein to stabilize β- and α_s-caseins was significantly reduced after dansylation. All three major casein fractions were labeled during a 5-min treatment of casein micelles, indicating that these fractions were readily available for reaction in native micelles.

Functional groups of casein micelles and casein fractions appear to play an important role in rennin coagulation, in the stability of casein fractions against Ca^{2+}, and in the interaction of casein fractions to form stable micelles. For example, nitration of tyrosyl residues did not affect normal solubility properties of κ- and α_s-caseins in the presence of Ca^{2+}, but caused aggregation of both proteins as evidenced by their inability to penetrate 8% acrylamide gel pores (Woychik and Wondolowski, 1969). Stable micelles of κ- and α_s-caseins could not be formed when either of the fractions was nitrated. Increasing the net negative charge of β-casein by acylating lysyl amino groups increased its stability against Ca^{2+} above pH 6 (Hoagland, 1968). It has been shown that altering histidine side chains by photooxidation and ε-amino groups by treatment with 1-dimethylaminonaphthalene-5-sulfonyl chloride (DNS–Cl) interfered with action of rennin on casein (Hill and Craker, 1968; Hill and Laing, 1965). In contrast to these findings, blocking amino groups of κ-casein by trifluoroacetylation did not hinder rennin cleavage of this fraction but the TFA-*para*-κ-casein released did not form a visible clot (Woychik, 1969). Modification of free amino groups of κ-casein by trifluoroacetylation or carbamylation markedly reduced the ability of κ-casein to stabilize α_s-casein against Ca^{2+} (Pepper *et al.*, 1970; Woychik, 1969). Pepper *et al.* (1970) found that the capacity of κ-casein B for stabilizing α_s-casein C in the presence of Ca^{2+} was lost after five of nine positively charged lysyl residues per mole were converted to uncharged homocitrulline residues by carbamylation.

Information on identification of reactive functional groups of casein micelles and isolated casein fractions is limited and appears to be contradictory. Hill and Craker (1968) reported the ε-amino groups as the principal group which was reactive with DNS–Cl in soluble casein. When 1-fluoro-2,4-dinitrobenzene (FDNB) was used in end-group analysis, α-dinitrophenyl (DNP)-arginine, di-DNP-lysine, and ε-DNP-lysine were formed in α- and β-caseins (Mellon *et al.*, 1953; Wissman and Nitschmann, 1957). In other studies, ε-DNP-lysine has also been detected but α-DNP-arginine was the only N-terminal residue found in these fractions (Kalan *et al.*, 1964, 1965; Kupfler *et al.*, 1969; Manson, 1961; Schmidt and Payens, 1963; Thompson and Kiddy, 1964). Wake (1959) reported α-DNP-aspartic and α-DNP-glutamic acids in addition to ε-DNP-lysine as the derivatives formed in κ-casein. However, Jollès *et al.* (1962) could not detect any N-terminal residue in κ-casein and indicated that ε-DNP-lysine and *O*-DNP-tyrosine were formed upon dinitrophenylation of κ-casein.

Use of fluorescent DNS–Cl for terminal group analysis of proteins has been described by Gray and Hartley (1963). This reagent reacts with primary and secondary amines, phenolic hydroxyls, thiols, and imidazoles. Its high sensitivity and the greater stability of DNS derivatives make this method superior to other methods of terminal group determination (Gray, 1967). In addition, several authors (Deyl and Rosmus, 1966; Hill and Laing, 1967; Morse and Horecker, 1966) reported separation of DNS derivatives successfully by thin-layer chromatography on silica gel.

Dansyl chloride has been used to label casein fractions in micelles reconstituted from soluble casein by addition of Ca^{2+} (Ribadeau-Dumas and Garnier, 1970). Binding of DNS–Cl occurred in all the major fractions. It was also observed that uptake of DNS–Cl was more rapid by casein in the reconstituted micelle form than by solubilized whole casein.

In the present study native casein micelles in skim milk or micelles centrifuged from milk were treated with DNS–Cl to determine whether the major casein fractions were labeled. In addition, DNS–Cl-reactive groups of casein micelles and casein fractions were identified and quantified, and the effect of blocking these groups with DNS–Cl on the stability of casein fractions and their ability to form stable micelles in the presence of Ca^{2+} was determined.

EXPERIMENTAL

Amino acids and DNS–Cl were purchased from Nutritional Biochemical Corp., Cleveland, Ohio. D-(+)-Galactosamine, *O*-DNS-L-tyrosine, and ε-DNS-L-lysine were purchased from California Corp. for Biochemical Research, Los Angeles, Calif. Silica gel G, prepared by E. Merck, and thin-layer chromatography equipment were obtained from Brinkmann Instruments, Inc., Westbury, N.Y. Itlc

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chromatography medium type SG (silica gel) was purchased from Gelman Instrument Co., Ann Arbor, Mich. Chemicals required for polyacrylamide gel electrophoresis described by Davis (1964) were purchased from Eastman Organic Chemicals, Rochester, N.Y.

Dansylation of Micelles. Milk samples used in the study were blends from one to five Holstein–Friesian cows of the University dairy herd. The samples were obtained immediately after milking and were never cooled below 22° C during subsequent treatments. The effect of treatment conditions on labeling of casein micelles with DNS–Cl was investigated by treating skim milk at pH values of 6.6 and 7.5 and casein micelles at pH 7.5 for 5 hr. The micelles were separated from skim milk by centrifugation at 55,000 × *g* for 90 min at 25° C after treatment with DNS–Cl. Micelles were recovered from untreated skim milk by the same method. This treatment would not sediment the small particles observed by Parry and Carroll (1969) nor the smaller micelles. The untreated micelle pellet was resuspended in water and the pH adjusted if necessary to 7.5 with 1 *N* NaOH before treatment with DNS–Cl.

A colloidal suspension of DNS–Cl was prepared for treatment of casein samples by dissolving DNS–Cl in acetone and adding the acetone solution to a fivefold excess of water. Sufficient DNS–Cl–acetone–water mixture was added to give a DNS–Cl concentration equal to 1 to 2% of the weight of protein in the sample. This required 5.0 ml of DNS–Cl suspension for 50 ml of skim milk and 2.5 ml for 20 ml of micelle suspension. No visible flocculation of the protein was observed after adding the DNS–Cl–acetone–water mixture. After treatment the suspended DNS–Cl was removed by centrifuging at 3000 × *g* for 30 min. The remaining unreacted DNS–Cl and its hydrolysis product, 1-dimethylaminonaphthalene-5-sulfonic acid (DNS–OH), were removed by washing several times with ethyl ether and dialyzing against 0.2 *M* KCl at room temperature until no fluorescence was observed in the KCl solution. This method as well as direct dialysis against 0.2 *M* KCl was effective in removing the excess reagents. After either of these treatments, the pH of the protein solution was adjusted to 7.5 and the solution was freeze-dried.

To measure the number of DNS residues formed during treatment with DNS–Cl, micelles obtained by centrifugation of uncooled skim milk were resuspended in Jenness and Koops' (1962) solution at pH 6.6. One-half of the micelle suspension was solubilized by adding potassium citrate to attain a final concentration of 0.04 *M* while maintaining the pH of the solution at 6.6. The solubilized casein and the native micelle suspension were treated with DNS–Cl (equal to 3% of protein weight) at pH 6.6 for 10 min. The excess DNS–Cl and DNS–OH was removed and the treated samples were hydrolyzed and fractionated by thin-layer chromatography.

Treatment Duration. The effect of duration of treatment with DNS–Cl was determined by treating isolated micelles at pH 7.5 for 5, 10, and 30 min, and 1, 2, 3, 4, 5, 6, and 10 hr at room temperature. At the end of each treatment interval, the pH of the micelle suspension was adjusted quickly to approximately 2 to prevent further reaction with DNS–Cl. The solution was washed with ethyl ether until the washings showed no fluorescence. The pH was raised to 4.6 with 1 *N* NaOH and the precipitated casein was washed several times with water. The pH of the final casein suspension was raised to pH 7.5 and was dialyzed against 0.2 *M* KCl for 24 to 36 hr at room temperature.

Casein Fractions. κ-Casein was prepared by the method of Zittle and Custer (1963); α_s- and β-caseins were prepared by the method of Zittle *et al.* (1959). The casein fractions were resuspended in water by adjusting the pH to 7.5, freeze-dried, and stored at −20° C. A 2% solution of each fraction was treated with DNS–Cl at pH 7.5 for 5 hr at room temperature. Excess reagents were removed by dialysis against 0.2 *M* KCl.

Gel Electrophoresis. Treated casein samples were prepared for electrophoresis by combining 1 volume of 2% solutions of casein or casein fractions with 2 volumes of tris buffer of pH 8.3. The urea concentration was adjusted to 7 *M* and the sucrose concentration to 10% in the casein solution, which was then held for 2 hr at room temperature before application to the gels. Volumes of solubilized casein micelles and of casein fractions applied to gels contained 500 to 700 μg and 150 to 200 μg of protein, respectively. Polyacrylamide gels were prepared by a modification of the method of Davis (1964). Ammonium persulfate was dissolved in 9 *M* urea rather than water so that the finished gels contained 4.5 *M* urea except in experiments evaluating effects of urea in gels. The gel was composed of two layers, the spacer gel and small-pore gel. The prepared casein samples in sucrose were layered on the spacer gel and tris buffer was added carefully on the sample layer to avoid mixing. Electrophoresis was done at room temperature by applying 2 to 3 mA per tube for about 1 hr. The gels were exposed to uv light to locate the fluorescent bands and then stained with amido black 10B dye. Destaining was done electrophoretically as described by Davis (1964).

DNS–Casein and DNS–Casein Fraction Hydrolysates. Isolated casein micelles and κ-, β-, and α_s-caseins were prepared and treated with DNS–Cl for 10 min and 5 hr at pH values of 6.6, 7.5, and 9.5. The treated proteins were hydrolyzed with 6 *N* HCl for 24 hr at 110° C in evacuated, sealed borosilicate glass tubes, and the hydrolysates were evaporated to dryness under vacuum at room temperature. The dried hydrolysates were redissolved in 0.5 ml of 0.1 *M* $NaHCO_3$–95% ethanol mixture (4 to 1 v/v); ethanol was necessary to dissolve completely the *O*-DNS-tyrosine.

DNS Derivatives. An amount of amino acid equivalent to 1 μmol was dissolved in 0.5 ml of 0.1 *M* $NaHCO_3$ before addition of 0.5 ml of DNS–Cl acetone solution (3 mg per ml). After thorough mixing, the solution was left at room temperature for 2 to 3 hr. DNS–galactosamine was prepared in the same manner; DNS–NH_2 and DNS–OH were prepared by substituting NH_4OH and water, respectively, for the amino acid. Drying and resolubilization were carried out as described for hydrolysates.

Thin-Layer Chromatography. Preliminary experiments indicated that DNS derivatives could be separated most successfully on glass plates coated with 0.25 mm of silica gel G using the following solvent: butanol:water:acetic acid, 100:25:5 (v/v). Samples (3 to 5 μl) were applied in small spots and the solvents were allowed to travel 10 to 12 cm after the plates had been equilibrated for 20 min in the closed chambers. Fluorescent spots were detected under uv light; DNS derivatives of amino acids and galactosamine gave yellow spots, whereas the DNS–OH spot was blue. The DNS derivatives formed by treatment of casein micelles and purified casein fractions were identified by comparison with R_f values of standard DNS derivatives obtained under identical operating conditions.

Fluorescence of spots was measured by scanning developed plates using an Aminco-Bowman spectrofluorometer employ-

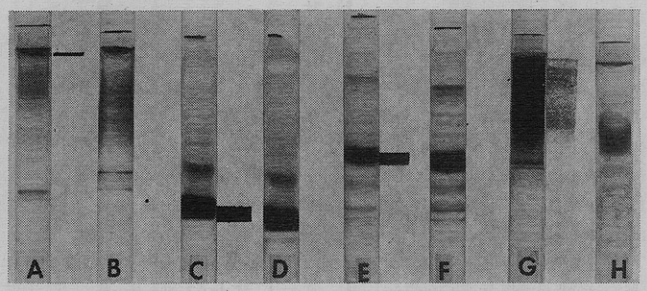

Figure 1. Electrophoretograms of DNS–Cl treated and control casein fractions: (A) DNS–Cl treated κ-casein; (B) κ-casein; (C) DNS–Cl treated α_s-casein; (D) α_s-casein; (E) DNS–Cl treated β-casein; (F) β-casein; (G) DNS–Cl treated β-casein, gel did not contain urea; and (H) β-casein, gel did not contain urea. The solid lines and dotted area to the right of each gel indicate the location of fluorescent bands seen under uv light before staining gels with amido black dye

Table I. Identification of DNS Derivatives from Casein Micelles and Casein Fractions Using Silica Gel G and a Solvent System of Butanol: Water: Acetic Acid in a Ratio of 100:25:5 (v/v)

DNS Treated samples	R_f of unknown	Corresponding DNS derivative
κ-Casein	0.31	ε-Lysine
	0.38	DNS–OH[a]
	0.40	*O*-Tyrosine
	0.55	Galactosamine
β-Casein	0.25	Arginine
	0.31	ε-Lysine
	0.38	DNS–OH
	0.40	*O*-Tyrosine
α_s-Casein	0.25	Arginine
	0.31	ε-Lysine
	0.38	DNS–OH
	0.40	*O*-Tyrosine
Casein micelles[b]	0.25	Arginine
	0.31	ε-Lysine
	0.38	DNS–OH
	0.40	*O*-Tyrosine
	0.55	Galactosamine

[a] DNS–OH gives a blue spot under uv; other DNS derivatives give yellow spots. [b] The four DNS-casein hydrolysates obtained after treating casein micelles with DNS–Cl for 10 min and 5 hr at pH 7.5 and 10 min and 5 hr at pH 9.5 gave the same number of DNS derivatives.

ing wavelengths of 350 nm for activation and 505 nm for measurement of fluorescence. The moles of identified DNS residues were derived from constructed standard curves of known DNS derivatives. The total number of DNS residues in each of the treated casein samples was determined also by the method of Chen (1968). Numbers of residues were calculated from absorbance measured at 339 nm with a Perkin-Elmer spectrophotometer model 139 and using an average molar absorbancy index of $3.4 \times 10^3\ M^{-1}\ cm^{-1}$ which was proposed by Chen.

Determination of Proteins. The amount of protein in casein fraction solutions was determined by the semimicro Kjeldahl procedure.

Stabilization Tests. Casein fractions and calcium chloride solutions were tempered in a water bath at 30° C for 15 min prior to mixing. Casein fractions were then mixed as shown in Table III, and the final volume was adjusted, if necessary, to 4 ml with water before 1 ml of 0.1 *M* calcium chloride solution was added to obtain a Ca^{2+} concentration of 0.02 *M*. The amounts of κ-, β-, and α_s-caseins added to the 4-ml volumes were 4, 10, and 16 mg. The solutions were mixed thoroughly and held at 30° C for 15 min to observe any formation of a precipitate. In the absence of a precipitate, the absorbance was measured at 600 nm using a Perkin-Elmer spectrophotometer model 139. The mixtures were then centrifuged at 3000 × *g* for 10 min and the absorbance was measured. Absorbances and ratios between absorbances before and after centrifugation were considered as measures of stability of the mixtures against Ca^{2+}.

In a second stability experiment, mixtures of casein fractions were constituted again as shown in Table III following the procedure mentioned above. The supernatants resulting from centrifugation at 3000 × *g* for 10 min were decanted and further centrifuged at 30,000 × *g* for 1 hr. Precipitates resulting from low speed and high speed centrifugation were subjected to polyacrylamide gel electrophoresis; fractions observed in each of the pellets are shown in Table III.

RESULTS

Dansylation of Casein Fractions. Each of the isolated casein fractions was capable of reacting with DNS–Cl as indicated by electrophoretic patterns of untreated and DNS–Cl treated κ-, β-, α_s-caseins in Figure 1. Electrophoretic mobilities of the treated fractions were the same as controls when gels contained 4.5 *M* urea (gels A–F). Abolishing the charge on the ε-amino group of a limited number of lysine residues by dansylation (Klotz and Fiess, 1960) apparently was not sufficient to affect the electrophoretic mobility of the fractions. Treatment of casein micelles with DNS–Cl yielded only a limited number of DNS residues, as shown later in this paper. The change in charge caused by this level of labeling should not alter the apparent electrophoretic mobility of fractions since the system used in this study did not resolve genetic polymorphs.

When treated β-casein was subjected to electrophoresis in gels not containing urea (gel G) it migrated slower than untreated β-casein (gel H) and also showed considerable spreading. The more discrete β-casein band obtained in gels containing urea suggests that the spreading in the absence of urea resulted from aggregation of β-casein through hydrophobic and hydrogen bonding. Hydrophobic bonding probably played a more important role because of the high level of hydrophobic amino acids in this fraction (von Hippel and Waugh, 1955). Hoagland (1968) found that acrylation of β-casein A reduced aggregation in the presence of calcium ions by increasing the net negative charge of the modified casein. Using an acylating agent with a longer alkyl group increased the tendency to aggregate. Hoagland attributed this effect to increased hydrophobic bonding by the larger alkyl groups. Dansyl chloride should also increase the hydrophobicity of the casein fractions. This increase apparently was sufficient to cause aggregation of β-casein but not great enough to affect κ- or α_s-caseins containing lesser amounts of hydrophobic amino acids. The relative electrophoretic mobilities of treated and untreated κ- and α_s-caseins were not affected by the absence of urea in gels. Spreading of κ- and α_s-caseins did not occur in gels without urea, whereas unlabeled β-casein exhibited slight spreading when urea was not added to the gel.

Dansylation of Micelles. Treatment of skim milk at pH 6.6 and 7.5 and casein micelles at pH 7.5 with DNS–Cl at room temperature for 5 hr yielded three fluorescent bands in polyacrylamide gels. These bands corresponded to κ-, β-, and α_s-caseins. The duration of treating micelles produced no qualitative differences in labeling of the major casein

fractions. All three major casein fractions were labeled during the 5-min treatment; the intensity of fluorescence increased with increases in treatment time.

Functional Groups. ε-DNS–Lysine and *O*-DNS–tyrosine were detected in all DNS-treated casein fractions and micelles (Table I). Arginine was the only N-terminal amino acid in β-casein, α_s-casein, and casein micelles, whereas no terminal amino acid was detected in κ-casein. DNS–Galactosamine was formed by dansylation of κ-casein and casein micelles. The same DNS derivatives, DNS–arginine, ε-DNS–lysine, *O*-DNS–tyrosine, and DNS–galactosamine were formed when casein micelles were treated with DNS–Cl for 10 min and 5 hr and at pH values of 7.5 and 9.5. The only effect of these treatment variations was an increase in fluorescence of the derivatives with increased time and at the higher pH value.

Amounts of DNS residues formed by dansylation of micelles and solubilized casein in Jenness and Koops' solution at pH 6.6 were quantified and are shown in Table II. Solubilizing casein micelles with citrate did not increase the number of functional groups available for dansylation significantly as indicated by the similar amounts of DNS residues formed in soluble and micellar casein samples. These data support the suggestions by Ribadeau-Dumas and Garnier (1970) that DNS–Cl can readily penetrate the casein micelle.

The fluorescent scanning technique was found to be a very sensitive method of quantifying DNS residues separated on tlc plates. As low as 1×10^{-5} μmol of the standard DNS residues listed in Table II could be measured by this technique.

The total number of DNS residues in each of the samples shown in Table II were determined also by the spectrophotometric procedure of Chen (1968). The numbers of DNS residues/10^5 g casein estimated by this method in the micellar sample and in the solubilized casein sample were 0.91 and 1.25, respectively. These values agree closely with total numbers of residues shown in Table II obtained by fluorescent scanning techniques. This corroborates data obtained by the scanning method and suggests that the average molar absorbancy index of $3.4 \times 10^3\ M^{-1}\ cm^{-1}$ proposed by Chen is suitable for estimating low levels of DNS residues in casein.

Micelle Stabilization. Dansylation of casein fractions significantly affected their ability to form stable micelles in the presence of Ca^{2+} (Table III). Control samples (mixtures 1, 7, and 12) formed stable micelles with only small amounts of the total α_s- and β-caseins aggregating in the presence of Ca^{2+}. Apparently the amount of κ-casein or the conditions used to form micelles did not allow complete stabilization of the Ca^{2+}-sensitive fractions. Stabilization of α_s- and β-caseins by κ-casein was reduced markedly by dansylation of this fraction. The absorbance of the centrifuged dansylated κ-casein and untreated α_s-casein mixture (mixture 2) was only 29% of that prior to centrifugation at 3000 × *g*. Similar effects were noted for the dansylated κ-casein and untreated β-casein mixture (mixture 8); low speed centrifugation removed sufficient Ca^{2+}-sensitive protein to lower the absorbance to 49% of the initial absorbance of the mixture. Dansylation of α_s- and β-caseins reduced the ability of untreated κ-casein to protect the two dansylated fractions against Ca^{2+} only slightly (mixtures 3 and 9). Likewise, mixing treated α_s- or β-caseins with treated κ-casein produced systems that were slightly less stable than those in which κ-casein was the only dansylated fraction (mixtures 4 and 10 *vs.* 2 and 8).

Effects of dansylation on calcium sensitivity of the casein fractions were different when all three fractions were mixed. Inclusion of either treated α_s- or β-caseins markedly reduced the protective action of untreated κ-casein on the ternary system (mixtures 13 and 14). When all fractions were dansylated, the Ca^{2+} sensitive fractions were completely unstable and precipitated out (mixture 15). Treatment of κ-, α_s-, or β-caseins did not alter their individual sensitivities toward Ca^{2+} (mixtures 5, 6, and 11).

Table II. Moles of DNS Residues Formed in Micellar and Solubilized Whole Casein Samples Treated with 3% DNS–Cl for 10 min at pH 6.6

Sample	Moles of DNS residues formed per 10^5 g of casein				
	Arginine	ε-Lysine	*O*-Tyrosine	Galactosamine	Total
Micellar casein	0.05	0.24	0.48	0.02	0.79
Soluble casein	0.08	0.41	0.74	0.04	1.27

Table III. Absorbances at 600 nm of Casein Fraction Mixtures Used in Stabilization Tests and Identification of Casein Fractions in Aggregates Centrifuged Out at 3000 × *g* for 10 Min and in Micelles Centrifuged at 30,000 × *g* for 1 Hr

Mixture	Components[a]	Absorbance of mixtures before (A_1) and after (A_2) centrifugation at 3000 × *g* for 10 min			Identified casein fractions centrifuged out at	
		A_1	A_2	A_2/A_1	3000 × *g*	30,000 × *g*
1	κ, α_s	0.82	0.68	0.83	α_s	α_s, κ
2	$\kappa_{(T)}$, α_s	1.20	0.35	0.29	α_s	α_s, κ
3	κ, $\alpha_{s(T)}$	0.86	0.60	0.70	α_s	α_s, κ
4	$\kappa_{(T)}$, $\alpha_{s(T)}$	1.32	0.28	0.21	α_s	α_s, κ
5	$\kappa_{(T)}$	...	...	...		
6	$\alpha_{s(T)}$	ppt	...	...		
7	κ, β	0.71	0.62	0.88	β	β, κ
8	$\kappa_{(T)}$, β	0.78	0.38	0.49	β	β, κ
9	κ, $\beta_{(T)}$	0.72	0.56	0.78	β	β, κ
10	$\kappa_{(T)}$, $\beta_{(T)}$	1.10	0.40	0.36	β	β, κ
11	$\beta_{(T)}$	ppt	...	...		
12	κ, β, α_s	0.98	0.75	0.77	α_s, β	κ, β, α_s
13	κ, $\beta_{(T)}$, α_s	1.10	0.50	0.46	α_s, β	κ, β, α_s
14	κ, β, $\alpha_{s(T)}$	1.30	0.38	0.30	α_s, β	κ, β, α_s
15	$\kappa_{(T)}$, $\beta_{(T)}$, $\alpha_{s(T)}$	ppt	...	...		

[a] κ, β, α_s correspond to κ-, β-, and α_s-caseins, respectively; (T) indicates treated with DNS–Cl (see text).

Identification of casein fractions in the Ca^{2+}-induced aggregates centrifuged out at 3000 × *g* and in micelles centrifuged out at 30,000 × *g* yielded expected results. The aggregates contained α_s- and β-caseins; micelles were composed of κ- and α_s- and/or β-caseins.

DISCUSSION

The three major fractions, κ-, β-, and α_s-caseins, were labeled during treatment of native micelles in skim milk with DNS–Cl. Ribadeau-Dumas and Garnier (1970) also labeled these three fractions during treatment of micelles formed from soluble whole casein by addition of Ca^{2+}. Reaggregation of the fractions from soluble casein apparently produced a micelle similar to native micelles from the standpoint of having some reactive functional groups exposed in all fractions.

Dansyl chloride was found to be a very effective reagent for functional group analysis of casein micelles and casein fractions. Because of its high sensitivity compared to FDNB reagent, additional functional groups reactive with DNS–Cl such as hydroxyl group of tyrosine in both β- and α_s-caseins and amino group of galactosamine in κ-casein were detected.

The results also confirmed that arginine is the only terminal amino acid of both β- and α_s-caseins since ε-DNS–lysine and not bis-DNS–lysine was detected. Functional group analysis of κ-casein showed no evidence of an N-terminal amino acid. It is not clear why dinitrophenylation of κ-casein yielded measurable amounts of two N-terminal amino acids, aspartic and glutamic, as reported by Wake (1959). We could not detect either of these amino acids with the more sensitive DNS–Cl. Our results, however, are in agreement with Jollès *et al.* (1962), although they did not detect the amino group of galactosamine with FDNB.

The lack of effect of dansylation of isolated fractions on their stability against Ca^{2+} agrees with the findings of Hill and Craker (1968). Formation of DNS derivatives in β-casein did not protect this fraction against calcium precipitation as did acylation (Hoagland, 1968). The protective effect of acylation was attributed to an increase in the net negative charge of β-casein, since acylation of amino groups was practically complete. The lack of effect of dansylation in the present study may have been caused, in part, by the low level of DNS derivatives formed. In addition, formation of DNS derivatives should increase the hydrophobicity of β-casein and partially negate effects of the increased charge.

The drastic effect of dansylation of functional groups of κ-casein on its stabilizing power toward α_s- and β-caseins found in this study was not observed by Hill and Craker (1968). These workers reported that the stabilizing activity of κ-casein decreased only 15% after treatment with 6% DNS–Cl. However, Woychik (1969) reported that κ-casein failed to stabilize α_s-casein against Ca^{2+} after trifluoroacetylation of free amino groups. The reasons for these differences are not clear since similar procedures were used in the three studies, except for the method of preparing κ-casein. The urea–sulfuric acid method of Zittle and Custer (1963) was used in this study and also by Woychik. Hill and Craker used the method of Hill and Hansen (1963).

LITERATURE CITED

Chen, R. F., *Anal. Biochem.* **25,** 412 (1968).
Davis, B. J., *Ann. N.Y. Acad. Sci.* **121,** 404 (1964).
Deyl, Z., Rosmus, J., *Anal. Biochem.* **14,** 429 (1966).
Gray, W. R., *Methods Enzymol.* **11,** 139 (1967).
Gray, W. R., Hartley, B. S., *Biochem. J.* **89,** 59 (1963).
Hill, R. D., Craker, B. A., *J. Dairy Res.* **35,** 13 (1968).
Hill, R. D., Hansen, R. R., *J. Dairy Res.* **30,** 375 (1963).
Hill, R. D., Laing, R. R., *Biochem. Biophys. Acta* **132,** 188 (1967).
Hill, R. D., Laing, R. R., *J. Dairy Res.* **32,** 193 (1965).
Hoagland, P. D., *Biochemistry* **7,** 2542 (1968).
Jenness, R., Koops, J., *Neth. Milk Dairy J.* **16,** 153 (1962).
Jollès, P., Alais, C., Jollès, J., *Arch. Biochem. Biophys.* **98,** 56 (1962).
Kalan, E. B., Thompson, M. P., Greenberg, R., *Arch. Biochem. Biophys.* **107,** 521 (1964).
Kalan, E. B., Thompson, M. P., Greenberg, R., Pepper, L., *J. Dairy Sci.* **48,** 884 (1965).
Klotz, I. M., Fiess, H. A., *Biochim. Biophys. Acta* **38,** 57 (1960).
Kopfler, F. C., Petterson, R. F., Kiddy, C. A., *J. Dairy Sci.* **52,** 1573 (1969).
Manson, W., *Arch. Biochem. Biophys.* **95,** 336 (1961).
Mellon, E. F., Korn, A. H., Hoover, S. R., *J. Amer. Chem. Soc.* **75,** 1675 (1953).
Morse, D., Horecker, B. L., *Anal. Biochem.* **14,** 429 (1966).
Parry, R. M., Carroll, R. J., *Biochim. Biophys. Acta* **194,** 138 (1969).
Pepper, L., Hipp, N. J., Gordon, W. G., *Biochem. Biophys. Acta* **207,** 340 (1970).
Ribadeau-Dumas, B., Garnier, J., *J. Dairy Res.* **37,** 269 (1970).
Schmidt, D. B., Payens, T. A. J., *Biochem. Biophys. Acta* **78,** 492 (1963).
Thompson, M. P., Kiddy, C. A., *J. Dairy Sci.* **47,** 626 (1964).
von Hippel, P. H., Waugh, D. F., *J. Amer. Chem. Soc.* **77,** 4311 (1955).
Wake, R. G., *Aust. J. Biol. Sci.* **12,** 479 (1959).
Woychik, J. H., *J. Dairy Sci.* **52,** 17 (1969).
Woychik, J. H., Wondolowski, M. V., *J. Dairy Sci.* **52,** 1669 (1969).
Wissman, H., Nitschmann, H., *Helv. Chem. Acta* **40,** 356 (1957).
Zittle, C. A., Custer, J. H., *J. Dairy Sci.* **48,** 1183 (1963).
Zittle, C. A., Cerbulis, J., Pepper, L., Della Monica, E. S., *J Dairy Sci.* **42,** 1897 (1959).

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