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Dimethylolurea as a Tyrosine Reagent and Protein Protectant against Ruminal Degradation

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Amino acid analysis of hydrolyzed proteins by ion-exchange chromatography was used to show apparent selective reaction of tyrosine side chains in bovine serum albumin (BSA), lysozyme, casein, soy protein, and wheat gluten with dimethylolurea (DMU). A plot of pH of the reaction medium vs. extent of tyrosine modification for BSA is biphasic, with maxima below pH 3.5 and above pH 10 and a minimum near pH 5. The tyrosine modification increased with the ratio of DMU to protein but not with BSA or casein concentration when the ratio of DMU was constant. These observations are the basis for a proposed mechanism of acid and base catalysis of tyrosine modification by DMU to form modified proteins. In vitro evaluation of degradation of DMU-treated casein and other DMU-treated proteins by ruminal microorganisms suggests that DMU is a potentially useful compound for protecting feed proteins against degradation by microorganisms in the rumen of sheep and cattle.

As part of a program to develop new treatments for feed proteins to increase their nutritional quality by decreasing their microbial degradation in the rumen (Friedman and Broderick, 1977), we evaluated the utility of dimethylolurea-treated casein. Amino acid analysis of DMU-treated casein revealed that only tyrosine residues gave derivatives stable to acid hydrolysis. To establish the generality of this reaction, we investigated several variables expected to govern the interaction of DMU with aromatic (phenolic) groups on tyrosine side chains. Conditions were devised to define the reactivity of tyrosine side chains in several proteins. In addition, we evaluated the degradability of DMU-treated casein by ruminal microorganisms. The results demonstrate the usefulness of dimethylolurea as a reagent for modifying tyrosine residues in proteins and its potential as a protein protectant against ruminal degradation.

MATERIALS AND METHODS

Dimethylolurea was synthesized by the method of Dixon (1918), but most of the experiments were carried out with a commercial sample obtained from Brochem, West Germany. Casein was obtained from International Casein Corp., San Francisco, CA, wheat gluten was obtained from Nutritional Biochemical Corp., Cleveland, OH, bovin serum albumin and lysozyme were obtained from Sigma Chemical Co., St. Louis, MO, and soy protein (Promine-D) was a gift from Central Soya, Chicago, IL.

Chemical Modification. The following is a typical experiment. Dimethylolurea (0.25 g) and bovine serum albumin (0.5 g) were dissolved in 10 cm^3 of buffer of appropriate pH (citrate buffer for pH 1.5–3.5; acetate buffer for pH 5; phosphate buffer for pH 7–8; borate buffer for pH 9–11). the reaction mixture was left standing for 24 h at room temperature and the final pH was measured. The mixture was then dialyzed against water for 3 days and lyophilized. In some cases, the reaction mixutre became gellike, so its pH was measured less accurately. Control experiments without DMU were done in all instances.

Amino Acid Analyses. A weighed sample of protein (about 5 mg) was dissolved in 15 cm^3 of 6 N HCl in a commercial hydrolysis tube. The tube was evacuated, placed in an acetone-dry ice bath, evacuated, and refilled with oxygen-free nitrogen twice before being placed in an oven at 100 °C for 24 h. The cooled hydrolysate was filtered through a sintered disk funnel and evaporated to dryness at 40 °C with the aid of an aspirator, and the residue was twice resuspended in water and evaporated to dryness. Amino acid analysis of an aliquot of the residue was carried out on a Durrum amino acid analyzer, Model D-500, under the following conditions: single-column Moore and Stein ion-exchange chromatography; resin, Durrum DC-4A; buffer pH, 3.25, 4.25, 7.90; photometer, 440 and 590 nm; column, 1.75 mm × 48 cm; analysis time, 105 min. Norleucine was used as an added internal standard. The reproductibility is estimated to be ±3% or better (Friedman et al., 1979).

In Vitro Incubations. Samples containing 180 mg of casein or isonitrogenous amounts of other proteins were weighed exactly into 50 mL of polyethylene centrifuge tubes. For Michaelis–Menten incubations, 25, 50, 100, 150, and 250 mg of casein were added to separate tubes. Five milliliters of McDougall's (1948) buffer was added to each tube. Protein sources plus buffer were allowed to soak overnight at 4 °C. The next morning the tubes were put in a water bath to warm to 39 °C, and 10 mL of incubation mixture was added to each tube; tubes were rapidly capped with stoppers with Bunsen valves and incubated for 2 h at 39 °C in a shaker water bath. Digestion was stopped

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Table I. Effect of Reaction Time on the Amino Acid Composition of BSA and DMU-BSA[a]

amino acid	time of reaction: 1 h A	1 h B	4 h A	4 h B	8 h A	8 h B	24 h A	24 h B
Asp	9.61	9.67	9.63	9.68	9.58	9.73	9.56	9.81
Thr	5.95	6.03	5.95	5.96	5.93	6.08	5.87	6.07
Ser	4.80	4.92	4.80	4.95	4.80	4.89	4.72	4.92
Glu	13.42	13.82	13.54	13.68	13.73	13.96	13.71	14.08
Pro	4.99	5.14	5.02	5.06	5.11	5.19	5.13	5.19
Gly	2.94	3.02	2.92	2.95	2.91	2.99	2.87	2.99
Ala	8.27	8.50	8.38	8.40	8.26	8.49	8.23	8.44
Cys[b]	5.01	3.81	4.56	4.77	5.06	4.03	5.18	5.34
Val	6.05	6.11	6.10	6.10	6.04	6.20	5.98	6.23
Met	0.57	0.52	0.54	0.49	0.62	0.48	0.68	0.61
Ile	2.32	2.32	2.35	2.39	2.29	2.43	2.31	2.37
Leu	10.97	11.03	11.07	11.15	10.92	11.31	10.91	11.25
Tyr	3.16	*3.10*	3.09	*2.65*	3.04	*2.10*	3.21	*0.96*
Phe	4.73	4.79	4.73	4.78	4.70	4.87	4.68	4.81
His	2.97	2.95	2.96	2.93	2.95	3.05	2.91	3.00
Lys	10.23	10.21	10.37	9.98	10.06	10.16	10.07	9.79
Arg	4.03	4.07	3.99	4.07	4.00	4.07	3.98	4.15

[a] Conditions: 0.2 g of DMU; 0.5 g of BSA; room temperature; 10 cm³ of pH 9.1 borate buffer; 24 h. Numbers of each amino acid in mole percent. A values are for BSA carried through the procedure without DMU. B values are for DMU-treated BSA. [b] Direct cystine analyses are not reliable.

Table II. Effect of pH on Tyr, Phe, and Lys (in Mole Percent) of BSA (A Columns) and DMU-BSA (B Columns)[a]

amino acid	A	B	A	B	A	B	A	B	A	B
Tyr	*3.04*	*0.00*	*3.25*	*1.16*	*2.91*	*0.91*	*3.10*	*0.00*	*3.22*	*0.00*
Phe	4.65	4.88	4.74	4.71	4.53	4.62	4.87	4.86	10.48	10.13
Lys	10.12	10.67	10.15	10.40	10.37	10.29	10.34	10.20	10.48	10.13
initial pH:	1.45	1.45	7.92	7.97	9.04	9.05	10.00	10.00	11.18	11.08
final pH:	1.60	1.55	7.98	7.76	9.08	8.87	10.03	9.63	11.10	10.41

[a] Only tyrosine was altered by the treatment. Conditions: 0.5 g of BSA plus 0.2 g of DMU in 10 cm³ of pH 9.1 borate buffer; 24 h; room temperature. Numbers for each amino acid in mole percent.

by adding 1 mL of 50% w/v trichloroacetic acid (Cl_3AcOH) to each tube.

The incubation mixture contained 50% v/v McDougall's buffer, 50% v/v strained rumen liquor (SRL), 1% w/v maltose, and 1.5 mM dithiothreitol (DTT). In most experiments, 1.5 mM hydrazine sulfate (HS) was also added to inhibit microbial removal of NH_3 released during protein degradation (Broderick, 1978). Maltose and HS were dissolved in an appropriate amount of McDougall's buffer freshly saturated with CO_2. This mixture was warmed to 39 °C with continued CO_2 gassing until the SRL was brought to the laboratory. Just before the appropriate volume of SRL was added, the DTT was weighed and dissolved in the warmed, gassed McDougall's solution. SRL was mixed with the buffer, and the incubation mixture was rapidly dispensed to the incubation tubes. Thus each tube contained 15 mL total (10 mL of buffer + 5 mL of SRL), 1 mM DTT, and 1 mM HS, and 0.67% w/v maltose (0.10 g of maltose/tube).

Each incubation experiment consisted of two blank tubes, two tubes with each casein preparation, and two special blank tubes that were stopped with exactly 1 mL of 50% w/v Cl_3AcOH solution containing 32 mM leucine (amino acid standard) and 32 mM NH_3 [ammonia standard added as $(NH_4)_2SO_4$]. This gave added leucine and ammonia concentrations of 2.0 mmol/mL. Ammonia and corrected amino acid (non-NH_3 ninhydrin positive material) concentrations were determined with a Technicon auto analyzer system (Broderick and Kang, 1980). Net NH_3 and net amino acid release were calculated by subtracting concentrations of NH_3 and amino acids in blank tubes from those in tubes receiving casein preparations. Net release values were adjusted according to recoveries of added NH_3 and leucine in the special blank tubes.

Protein Efficiency Ratio. The protein efficiency ratio (PER) was determined by the standard AOAC (1975) method except that rats were fed both untreated and DMU-treated casein for 24 instead of 28 days.

RESULTS AND DISCUSSION

To develop conditions for optimum modification of tyrosine residues in protein by dimethylolurea, we examined the influence of the following variables on the amino acid composition of untreated and treated proteins. Note, however, that the usual amino acid analysis will not reveal derivatives from which the original amino acids are regenerated by acid hydrolysis.

Time of Reaction. Results in Table I show that exposure of 0.5 g of BSA to 0.2 g of DMU in a 10 cm³ of pH 9.0 borate buffer for 1 hr had a negligible effect on the tyrosine content of the protein. When the reaction time was increased to 4 h, however, about 14% of the tyrosine was modified. The extent of modification increased to 31% after 8 h and to 70% after 24 h. None of the other amino acids listed in the Table I appears to have formed a derivative stable to acid hydrolysis.

Effect of pH. The effect of pH on the amino acid composition of BSA treated with DMU in a 2:5 weight ratio at room temperature for 24 h shows that tyrosine residues can be completely modified in either acid (pH 1.5–3.0) or alkaline media (pH 10–11), whereas at intermediate pHs modification is less (Table II). Again, no other amino acid residue appears to be affected. Figure 1 shows this biphasic reaction of tyrosine vs. pH, with maxima in acidic and basic regions (Figure 1 contains several points in the acid range not listed in Table II).

Table III. Effect of Varying Protein Concentration at Constant (2:5) DMU to BSA Weight Ratio on Extent of Tyrosine Modification

	BSA concentration							
	1 wt %		5 wt %		10 wt %		20 wt %	
amino acid, mol %	BSA only	BSA plus DMU[a]	BSA only	BSA plus DMU[b]	BSA only	BSA plus DMU[c]	BSA only	BSA plus DMU[d]
Tyr	3.20	1.27	2.97	1.07	3.16	0.98	2.89	0.98
initial pH:	9.16	9.16	9.08	9.08	8.96	8.97	8.67	8.68
final pH:	9.16	9.13	9.06	8.88	8.93	8.55[e]	8.75	7.93[e]

[a] 100 mg of BSA plus 40 mg of DMU; 10 cm³ of buffer; room temperature; 24 h. [b] 500 mg of BSA plus 200 mg of DMU; 10 cm³ of buffer; room temperature; 24 h. [c] 1000 mg of BSA plus 400 mg of DMU; 10 cm³ of buffer; room temperature; 24 h. [d] 2000 mg of BSA plus 800 mg of DMU; 10 cm³ of buffer; room temperature; 24 h. [e] Product was a gel.

Table IV. Effect of Varying Weight Ratio of DMU to BSA on Tyrosine Content (in Mole Percent)

amino acid	BSA control	DMU to BSA weight ratios 1:10[a]	1:5[b]	1:2[c]	1:1[d]
Tyr	3.16	1.74	0.74	0.48	0.13
initial pH:	9.10	9.10	9.10	9.10	9.10
final pH:	9.06	8.98	8.83	8.78	8.68

[a] 50 mg of DMU; 500 mg of BSA; 10 cm³ of buffer; room temperature; 24 h. [b] 100 mg of DMU; 500 mg of BSA; 10 cm³ of buffer; room temperature; 24 h. [c] 250 mg of DMU; 500 mg of BSA; 10 cm³ of buffer; room temperature; 24 h. [d] 500 mg of DMU; 500 mg of BSA; 10 cm³ of buffer; room temperature; 24 h.

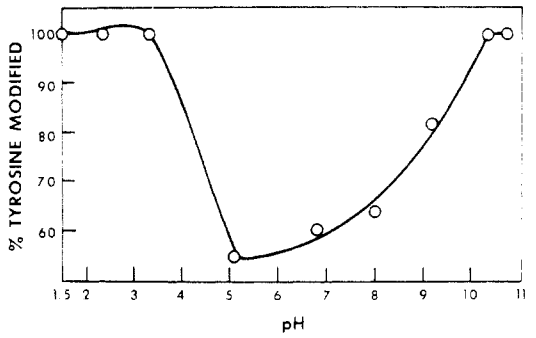

Figure 1. Effect of pH on the extent of modification of tyrosine residues of bovine serum albumin by dimethylolurea.

Concentration Effects. Two sets of experiments were carried out. In the first experiment, protein concentrations were progressively increased from 1 to 20%, while the DMU to protein weight ratio was kept constant at 2:5. This increase in concentration appears to have no significant effect on the extent of tyrosine modification (Table III). In the second, 5% solutions of BSA (0.5/10 cm³) in pH 9 borate buffer were treated with varying amounts of DMU for 24 h at room temperature. The results (Table IV) show that increases in the weight ratio of DMU to BSA produce corresponding decreases in the tyrosine content of the treated BSA. Results from a parallel study with casein (Table V) reinforce the conclusion that tyrosine modification increases with DMU concentration (Table V). No other amino acid appears to have been altered with the possible exception of lysine, which decreased 5–10% at the higher DMU concentrations. Note, however, that these cited results cannot reveal the possible formation of derivatives that liberate the free amino acids during acid hydrolysis.

Several other proteins gave similar trends, as illustrated in Table VI.

Reaction Mechanisms. Reaction mechanisms for the observed acid- and base-catalyzed modification of tyrosine residues are shown in Figures 2 and 3, respectively. Acid catalysis is postulated to arise from protonation of the methylol OH to OH_2^+ groups, as shown in Figure 2. The OH_2^+ groups are better leaving groups than the unprotonated forms in nucleophilic displacements by tyrosine side chains leading to the formation of cross-linked proteins. On the other hand, base catalysis is postulated to

Table V. Effect of DMU to Casein Weight Ratio on Amino Acid Composition (in Mole Percent) of Hydrolyzed Proteins[a]

amino acid	casein control	DMU/casein weight ratios 1:100	1:20	1:10	1:5	1:2
Asp	6.91	6.90	7.06	7.09	7.08	7.02
Thr	4.64	4.39	4.56	4.65	4.55	4.72
Ser	7.14	7.46	7.66	7.71	7.74	7.27
Glu	18.69	18.10	17.88	19.18	19.66	19.72
Pro	11.63	12.09	12.51	12.47	12.59	12.61
Gly	3.29	3.13	3.24	3.13	3.25	3.33
Ala	4.43	4.43	4.56	4.51	4.43	4.43
Val	6.68	6.55	6.75	6.66	7.01	7.03
Met	2.52	2.20	2.05	2.26	2.20	2.64
Ile	4.80	4.73	4.82	4.64	4.98	4.97
Leu	9.42	9.21	9.38	9.51	9.71	9.74
Tyr	*3.99*	*3.90*	*3.62*	*2.28*	*1.15*	*0.93*
Phe	4.17	4.01	4.05	4.04	4.07	4.20
His	2.40	2.37	2.43	2.51	2.56	2.48
Lys	6.70	6.84	6.84	6.75	6.41	6.18
Arg	2.58	2.52	2.49	2.61	2.63	2.71

[a] Conditions: 0.5 g of casein in 10 cm³ of pH 9.2 borate buffer; room temperature; 24 h.

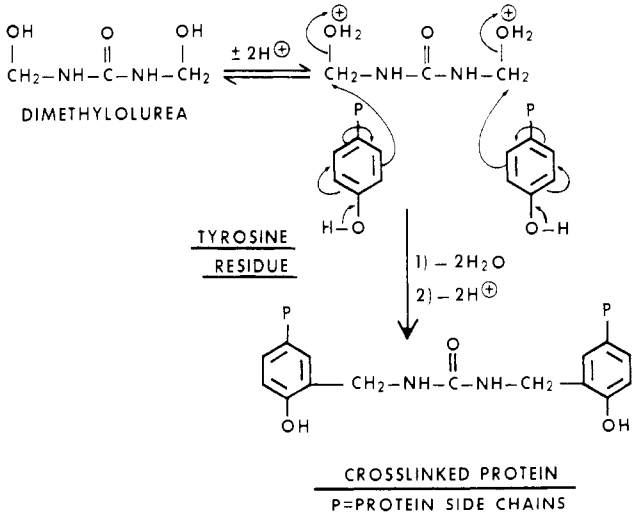

Figure 2. Postulated mechanism of reaction of tyrosine residues with dimethylolurea in acid media. Protonation of a DMU hydroxyl group facilitates the indicated nucleophilic displacement. Attack by tyrosine can take place at both ends of the DMU molecule to produce a cross-linked protein.

Table VI. Effect of Protein Type on Extent of Reaction with Dimethylolurea at the Indicated DMU/Protein Weight Ratios

amino acid	casein control[a]	2 DMU:5 casein[b]	lysozyme control[a]	2 DMU:5 lysozyme[c]	Promine-D control[a]	1 DMU:2 Promine-D[d]	wheat gluten control[a]	1 DMU:2 wheat[e]	BSA control[a]	1 DMU:2 BSA[f]
Asp	7.25	7.30	15.07	18.78	11.89	11.65	3.23	3.15	9.57	10.18
Thr	4.77	4.94	6.24	6.46	4.28	4.49	2.92	2.87	5.77	6.12
Ser	7.51	7.85	8.19	8.33	7.20	7.59	6.64	6.52	4.97	5.36
Glu	18.57	18.84	4.48	4.38	17.31	18.07	33.41	33.04	13.65	14.12
Pro	12.27	12.40	1.92	1.75	6.30	6.44	14.01	14.79	5.10	5.34
Gly	3.38	3.46	10.48	10.84	7.18	7.46	5.93	5.63	2.83	3.04
Ala	4.61	4.51	10.50	10.73	6.25	6.56	3.85	3.63	8.06	8.67
Cys	0.00	0.00	5.62	3.89	0.29	0.37	0.57	0.83	5.03	3.26
Val	6.46	6.88	4.79	4.62	5.02	5.03	4.11	3.85	5.89	6.43
Met	2.26	2.44	1.59	0.48	0.76	0.87	1.23	0.13	0.52	0.58
Ile	4.78	4.85	4.40	4.63	4.63	4.67	3.52	3.56	2.39	2.51
Leu	9.02	9.57	6.88	7.25	8.21	8.80	7.09	7.22	10.94	11.66
Tyr	*3.47*	*1.17*	*2.20*	*1.07*	*2.77*	*0.92*	*2.66*	*0.98*	*3.34*	*0.00*
Phe	3.65	3.75	2.60	2.25	4.50	4.57	4.25	4.39	4.88	5.06
His	2.35	2.34	0.81	0.77	2.19	2.30	1.82	1.77	3.88	3.03
Lys	7.06	6.90	4.98	4.11	5.56	5.05	1.46	1.10	10.25	10.48
Arg	2.59	2.78	9.28	9.54	5.55	5.78	2.58	2.55	3.88	4.11
initial pH:	9.22	9.22	9.58	9.58	9.45	9.15	9.40	9.12	9.34	9.10
final pH:	8.94	8.78	9.38	9.26		9.01		9.11		

[a] Control: protein carried through treatment conditions but without DMU. [b] 500 mg of casein; 200 mg of DMU; 10 cm³ of buffer; room temperature; 24 h. [c] 500 mg of lysozyme; 200 mg of DMU; 10 cm³ of buffer; room temperature; 24 h. [d] 500 mg of Promine-D, 250 mg of DMU; 10 cm³ of buffer; room temperature; 24 h. [e] 500 mg of wheat gluten; 250 mg of DMU; 10 cm³ of buffer; room temperature; 24 h. [f] 500 mg of BSA; 250 mg of DMU; 10 cm³ of buffer; room temperature; 24 h.

arise from the enhanced nucleophilic reactivity of ionized tyrosine hydroxyl groups in comparison with the un-ionized forms, as illustrated in Figure 3. Since the ionization constant (pK_a) for tyrosine OH groups is near 10 (Friedman, 1966), base catalysis is expected to occur above pH 8. Attack of two phenoxide ions at both ends of DMU leads to the formation of the same cross-linked protein inferred for the acid-catalyzed reaction. Tyrosine modification is expected to proceed at slower rates at intermediate pH values, presumably because both protonation of aliphatic hydroxyl groups of dimethylolurea and ionization of the phenolic group of tyrosine are minimal in the pH range 5–8. Furthermore, the pH in the range 2–10 is unlikely to affect the stability of the cross-linked proteins because amide (urea) bonds are generally expected to be stable at these pHs.

As noted earlier, the extent of tyrosine modification increases with the proportion of DMU, as expected, but not with protein concentration when the reagent is kept constant. This result can also be explained. Evidently, the major factor controlling reaction of tyrosine residues at a particular DMU concentration may be the conformation of the peptide chains. Once a small fraction of these is modified by cross-linking, the remainder are locked into rigid, relatively nonflexible conformations in which the rate of further reaction may be a function of the ability of DMU to diffuse to immobilized tyrosine residues. Additional physicochemical studies are needed to prove this unequivocally.

Similar arguments have previously been offered to explain the apparent nondependence of alkali-induced lysinoalanine cross-link formation in wheat gluten and soy protein on protein concentration (Friedman, 1978a,b).

Efforts to locate the postulated 3-(aminoethyl)tyrosine (Figure 3) on amino acid chromatograms of hydrolysates of DMU-modified casein and poly-L-tyrosine were not successful, possibly because the compound is too polar to be eluted under standard amino acid analytical conditions.

In Vitro Ruminal Digestibility. The digestion of nitrogenous compounds by ruminant animals is complex, occurring in two interrelated stages. The first stage is

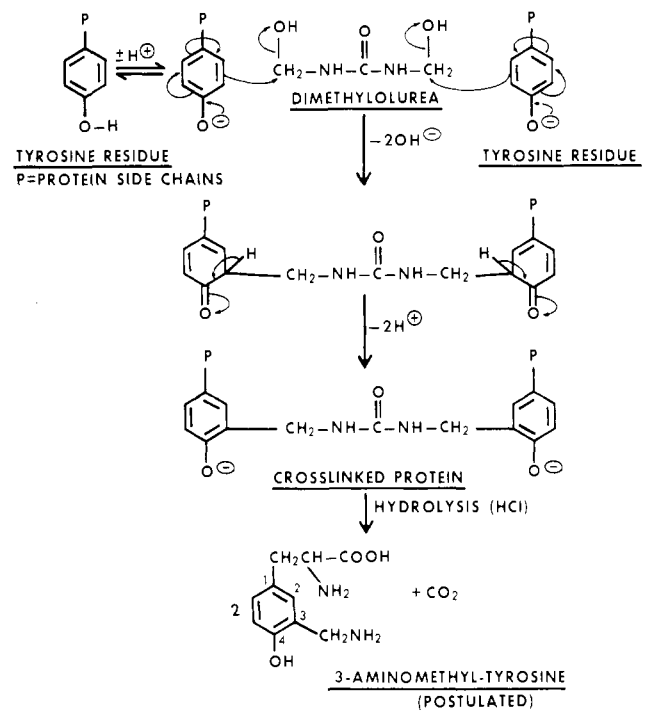

Figure 3. Postulated mechanism of reaction of tyrosine residues with dimethylolurea in basic media. Ionization of the phenolic OH group of tyrosine to a phenoxide ion enhances its nucleophilic reactivity.

microbial digestion in the reticulum, rumen, and omasum; in the second, hydrolysis by secreted enzymes occurs in the abomasum and intestines.

Most nitrogenous material ingested by ruminants in natural feed consists of proteins. Although dietary proteins are seldom completely degraded in the rumen, these proteins are extensively hydrolyzed by rumen bacteria to their constituent amino acids, which are then rapidly deaminated to produce ammonia. This process wastes valuable amino acids required by the host because the evolved ammonia, although used by microbes in part to resynthesize

Table VII. Ruminal Degradation in Vitro and Protein Efficiency Ratio (PER) of Casein and DMU-Treated Casein[a]

	casein	DMU-casein
ruminal degradation[b]		
V_{max}, mg h^{-1} (mL of SRL)$^{-1}$	0.532 ± 0.028[c]	0.497 ± 0.124
K_m, mg (mL of SRL)$^{-1}$	1.088 ± 0.077	8.02 ± 2.51
K_d (V_{max}/K_m), h^{-1}	0.493 ± 0.060	0.063 ± 0.004
estimated ruminal escape, %	7.5	39.0
protein efficiency ratio[d]		
protein ($N \times 6.25$) consumed, g/rat	34.91	34.46
weight gain, g/rat	98.87	95.55
PER (weight gain/protein consumed)	2.83	2.77

[a] DMU treatment: 300 g of casein plus 30 g of DMU in 3 L of pH 9.1 borate buffer; room temperature; 24 h; dialyzed against water; lyophilized. [b] Ruminal degradation estimated by using the Michaelis-Menten in vitro technique of Broderick (1978), except that the integrated rather than the Lineweaver-Burk variant of the Michaelis-Menten equation was used. Estimated ruminal escape = $[k_r/(k_r + k_d)] \times 100$, where k_r (ruminal turnover rate) is assumed to equal 0.04 h^{-1}. [c] Means ± SEM ($n = 2$). [d] PER determined according to the AOAC (1975) procedure, with 10 rats/group, except the feeding period was 24 rather than 28 days.

Table VIII. Ruminal in Vitro Degradation of Dimethylolurea-Treated Proteins with Excess Substrate[a]

protein source	relative degradation, %[b]
casein control	100.0
casein-DMU (1)[c]	77.3
casein-DMU (2)[d]	53.2
casein-DMU (3)[e]	42.4
casein-DMU (4)[f]	24.9
casein-DMU (5)[g]	22.3
gluten control	54.9
gluten-DMU[h]	42.2
Promine-D control	67.9
Promine-D-DMU[i]	35.8

[a] 180 mg of each protein preparation/tube. Each tube contained 10 mL of McDougall's buffer and 5 mL of strained ruminal liquor (SRL). The medium had 1 mM DTT and 1 mM hydrazine. Means from two incubations. [b] Sum of ammonia and amino acids released [in mol h^{-1} (mL of SRL)$^{-1}$] divided by the corresponding value for casein control times 100. Degradation rate of casein control was 3.82 mg/h. [c] 10 g of casein plus 0.5 g of DMU in 135 mL of pH 9.2 buffer; room temperature; 24 h. [d] 10 g of casein plus 1.0 g of DMU in 135 mL of pH 9.2 buffer; room temperature; 24 h. [e] 10 g of casein plus 2.0 g of DMU in 135 mL of pH 9.2 buffer; room temperature; 24 h. [f] 4 g of casein plus 2.0 g of DMU in 40 mL of pH 9.2 buffer; room temperature; 24 h. [g] Duplicate of (4). [h] 4 g of gluten plus 2.0 g of DMU in 40 mL of pH 9.2 buffer; room temperature; 24 h. [i] 4 g of Promine-D (soy protein isolate) plus 2.0 g of DMU in 40 mL of pH 9.2 buffer; room temperature; 24 h.

protein that is assimilated by the animal, is partly excreted. Therefore, it is desirable to decrease ruminal degradation of protein and thereby increase the net absorption of amino acids in the intestines, so that proteins in feed will be used efficiently. Consequently, body growth, and production of meat, milk, and hair, requiring protein synthesis will be increased.

Formaldehyde-treated casein fed to sheep has been found to increase their weight gain and wool growth. This result is ascribed to temporary cross-linking that protects the protein while it is in the rumen. Since results with formaldehyde have not been reproducible when evaluated by different investigators (Dinius et al., 1974; Faichney, 1974; Clark, 1975; Broderick, 1975; McDonald et al., 1975), we have modified casein with various acid anhydrides, vinyl compounds, and epoxides, including reagents with one and two reactive sites. Treated materials were evaluated for resistance to ruminal degradation in vitro as described under Materials and Methods (Friedman and Broderick, 1977; Broderick, 1978).

The results of the present study show the effectiveness of dimethylolurea as a potential protectant against ruminal degradation in vitro (Tables VII and VIII). Table VII shows that the Michaelis–Menten constant, K_m, for ruminal degradation of casein is only about one-eighth of the corresponding value for DMU–casein, while the degradation rate constant, k_d, of casein in the ruminal fluid is about 8 times the value of DMU–casein. The lower K_m value indicates a lower affinity by ruminal digestive enzymes for DMU–casein than for casein (Cornish-Bowden, 1979). Estimated ruminal escape (Table VII) was improved by a factor of 5.2 (from 7.5 to 39.0%). Note that the relative magnitude of protection is the important criterion here.

Table VIII also shows that the degradation of DMU–casein compared to that of the untreated control decreases with increasing amounts of DMU used to modify the casein. Ruminal protection achieved with the highest ratio of DMU to casein (Table VIII) was comparable to that obtained previously with formaldehyde treatment (Friedman and Broderick, 1977). Treating wheat gluten and soy protein (Promine-D) with DMU also increases their resistance to ruminal degradation but less than with casein. The results corroborate those of Mahadevan et al. (1980), who found that cross-linking proteins through oxidation of sulhydryl groups to disulfide links increased their resistance to ruminal degradation.

The PER of DMU casein is only slightly less than that of untreated casein (Table VII). This result indicates that proteins protected by DMU treatment are still digestible in the small intestine and that their amino acids were still utilized by the animal.

Since casein, when fed to ruminants, escapes the rumen to the extent of 10% or less, while about 25–30% of feed protein supplements, e.g., soybean meal, escape, improvement in ruminal escape of soybean meal protein to about 75–80% (an effect of smaller magnitude than obtained here with casein) without reducing protein quality would importantly increase protein utilization by ruminants.

Dimethylolurea appears especially promising as a protective reagent because (a) the PER of DMU casein was only slightly less than that of casein (Table VIII), (b) DMU appears to modify only tyrosine residues (a "semidispensable" amino acid since it can spare phenylalanine), (c) additional studies showing that DMU also reacts with proteins in the solid state suggest that it may be useful for large-scale, economical modification of feed proteins, and (d) chemical modification with DMU introduces two nitrogen atoms into the protein per mole of DMU; some of this nonprotein nitrogen may be transformed to ammonia and then used for microbial biosynthesis of amino acids. Ammonia release from DMU-treated proteins was proportionately higher than amino acid release, suggesting hydrolysis of some reacted DMU without loss of protein protection. It remains to be shown whether DMU-cross-linked tyrosine residues, which can be viewed as benzyl ureas, are hydrolytically cleaved by

ureases or other enzymes [cf. Amos et al. (1980)]. Finally, our observations that DMU is a selective reagent for tyrosine residues in proteins suggests that it may have special value in studying the role of tyrosine in structural proteins and enzymes.

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Physicochemical Aspects of Sweetness in Cocoa Drinks

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The role of lecithin and glycerol monosterate in modifying the sweetness of cocoa drinks was investigated. While both surfactants enhanced the sweetness of the drinks and both reduced their surface tension, only glycerol monostearate increased their viscosity. Statistical analysis of panellists' responses showed that a significant inverse relationship exists between surface tension and sweetness response, the latter being measured in terms of both intensity and persistence. These results help to resolve conflicting reports of the effects of hydrocolloids on basic taste. They are discussed in terms of possible mechanisms of taste chemoreception.

Many surfactants or emulsifying agents find frequent use in food systems, being generally chemically similar to stabilizers, solubilizers, and wetting agents (Nash and Brickman, 1972). However, most studies of the gustatory effects of hydrocolloids in foods have been concerned mainly with the investigation of viscosity [e.g., Moskowitz and Arabie (1970), Vaisey et al. (1969), and Pangborn et al. (1978)], and relatively little work has shown quantitative relationships between taste intensity and other physical properties of the stimulant. In their attempts to elucidate relationships between relative sweetness of molecules and various physicochemical properties, Ferguson and Lawrence (1958) suggested that surface tensions of solutions of sapid molecules could affect their penetration into taste bud pores or alter the permeability characteristics of taste cells and thereby affect taste response. More recently, DeSimone (1980) has suggested that a lowering of surface tension may cause taste intensity to be lowered. Harkins (1954) has stated that the importance of surface effects is best illustrated by highly disperse or colloidal systems, since colloidal particles generally exhibit a relatively high activity of so-called surface forces. It is not known whether DeSimone's conclusions are applicable to such colloidal systems.

Thermodynamically, a physicochemical system tends to assume the condition in which its free energy is lowest. Since surface molecules attract each other, forming a film of greater or less strength, there is a resulting surface tension which may in turn affect molecular volume. Thus, modification of the effects of stimulus molecules in the dynamics of taste chemoreception by modifying surface tension should be very significant. West (1963) noted that the degree of molecular association and the strength of intermolecular forces of attraction in colloidal solutions are governed by surface tension, and since according to Shallenberger's AH-B concept (Shallenberger and Acree, 1967) these considerations underlie the stereochemistry of taste, it is conceivable that surface tension effects may be more important than viscosity effects in gustation.

Although sucrose like all sugars is surface inactive (Browne and Zerban, 1941), it may form complexes with surfactants (Birch and Ogunmoyela, 1980a) which enhance sweetness response. In the work reported in this paper, the viscosities and surface tension values of solutions of sucrose-sweetened cocoa drinks to which increasing con-

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