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Cleavage of Olefinic Peptides under the Conditions of Oxymercuration

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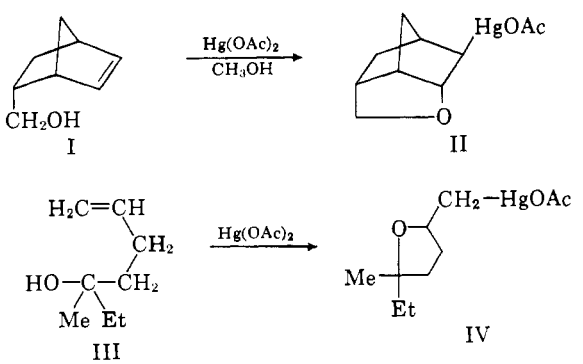
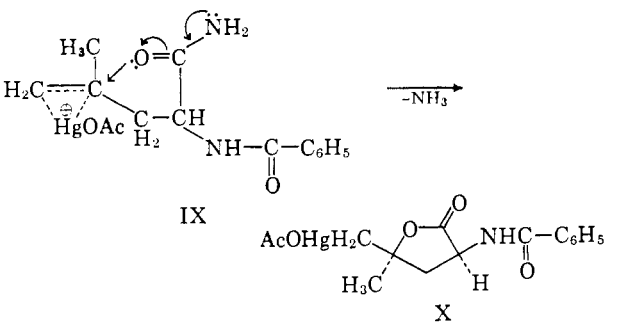
Under the conditions of oxymercuration, i.e., mercuric acetate in ethanolic buffer solution at 75° for 15–100 minutes, the model peptides DL-2-benzamido-4-methyl-4-pentenoic acid amide (V), DL-2-*p*-toluenesulfonamido-4-methyl-4-pentenoylglycine (VI), and DL-2-benzamido-4-pentenoic acid amide (VII) liberated ammonia or glycine in yields up to 30%. DL-3,4-Dehydroprolinamide (VIII) showed cleavage only to the extent of 2–7%. *O*-Methyldihydrorufomycin A (XI), a cyclic heptapeptide containing the unsaturated amino acid Δ^4-norleucine, liberated the adjacent tryptophan unit only to a minor extent, and nonspecific cleavage catalyzed by mercuric ions up to 46% of one peptide bond was observed instead. It is concluded that the extent of peptide cleavage under conditions of oxymercuration depends on the environment and substitution of the double bond.

Nonhydrolytic, nonenzymatic methods for cleavage of peptides involve participation of amide carbonyls in internal nucleophilic displacement reactions (Witkop, 1961). Among the reagents and methods used there have been *N*-bromosuccinimide, *N*-bromoacetamide, *N*-bromocarbamide (Funatsu *et al.*, 1964), anodic oxidation (Iwasaki *et al.*, 1963) and solvolytic cleavage (Iwasaki and Witkop, 1964). We have now investigated the use of mercuric acetate for cleavage of peptide bonds.

Participation of neighboring hydroxyl groups in the oxymercuration of norbornene (I → II) (Henbest and Nicholls, 1959) or of acyclic olefins (III→IV) (Sand and Singer, 1903) suggests an extension of this principle (Traylor and Baker, 1963) to the cleavage of peptides.



Cleavage of Amides and Peptides of 2-Acylamino-(4-methyl)-4-pentenoic Acids.—The model amides and peptides used in this study had been previously synthesized (Izumiya *et al.*, 1962). They were DL-2-benzamido-4-methyl-4-pentenoic acid amide (V), DL-2-*p*-toluenesulfonamido-4-methyl-4-pentenoylglycine (VI), DL-2-benzamido-4-pentenoic acid amide (VII), and DL-3,4-dehydroprolinamide (VIII). Participation of the amide group would best be formulated through a mercurinium (Lucas *et al.*, 1939) intermediate (IX), going to the iminolactone and lactone (X) (Izumiya and Witkop, 1963), analogous to the assumption of reactive



bromonium intermediates for the cleavage of peptide bonds by *N*-bromosuccinimide. The yields achieved in this cleavage depended on the conditions and on the type of peptide. They did not exceed 30%.

Under the conditions of oxymercuration the γ,δ-unsaturation in tetrahydrophenylalanine peptides (Wilchek and Patchornik, 1962) participates and undergoes cleavage in excess of 50% (A. Patchornik, personal communication). Table I summarizes the observations made so far on the influence of double bond substituents on the cleavage yield and the conditions of oxymercuration.

Unspecific Cleavage of Rufomycin A by Mercuric Ions.—Naturally occurring olefinic amino acids are comparatively rare. The antibiotic cyclopeptide rufomycin A (Ueyanagi *et al.*, 1963), whose *O*-methyldihydro derivative is pictured in structure XI (Iwasaki and Witkop, 1964), contains 2-amino-4-hexenoic acid whose double bond reacts easily with *N*-bromosuccinimide leading to oxidative cleavage and up to 48% liberation of the

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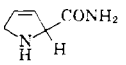
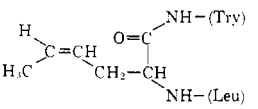
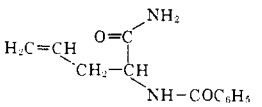
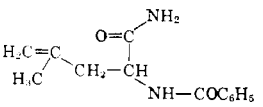
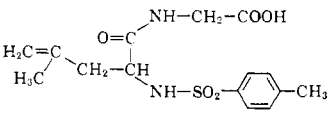
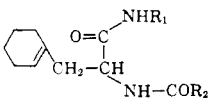
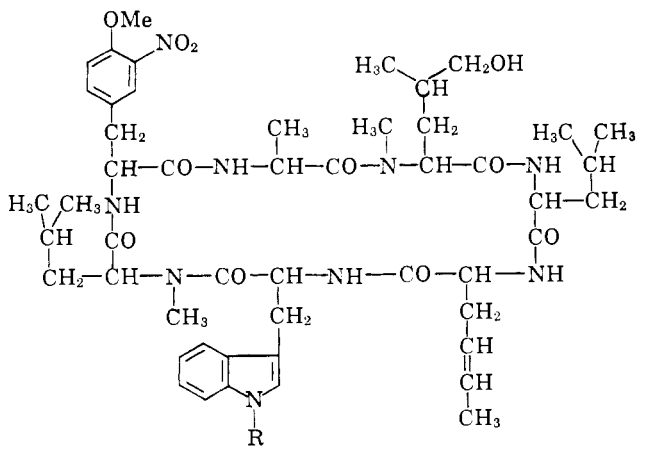
TABLE I

INFLUENCE OF POSITION AND SUBSTITUTION ON THE REACTIVITY OF THE DOUBLE BOND AS REFLECTED BY CLEAVAGE OF UNSATURATED AMINO ACID AMIDES AND PEPTIDES UNDER THE CONDITIONS OF OXYMERCURATION

Peptide	Formula	Cleavage under Conditions of Oxymercuration
3,4-Dehydro-DL-prolinamide (VIII)		2–7%
Δ^4-Norleucine (in rufomycin deriv. XI)		Not ascertained because unspecific cleavage of other peptide bonds supervenes
DL-2-Benzamido-4-pentenoic acid amide (VII)		20–25%
DL-2-Benzamido-4-methyl-4-pentenoic acid amide (V)		25–30%
DL-2-*p*-Toluenesulfonamido-4-methyl-4-pentenoylglycine (VI)		25–30%
Tetrahydrophenylalanyl peptides (Patchornik, 1964)		Up to 55%



XI

adjacent substituted tryptophan residue. However, such a cleavage was not observed under conditions of oxymercuration. Table II summarizes the conditions of the two-step procedure: In the first step oxymercuration in different solvents at room or elevated temperatures was carried out. This was followed by a separate hydrolysis step of the possible intermediate iminolactone.

The peptide bond affected most by these conditions is the one between *N*-methyl-δ-hydroxyleucine and leucine. There is a marked change of the ultraviolet-absorption spectrum during oxymercuration, suggestive of interaction of the mercuric acetate with the tryptophan chromophore (Fig. 1A,B). The spectral changes of indoles and tryptophan derivatives observed on interaction with mercuric acetate are the subject of a separate paper (Ramachandran and Witkop, 1964). There is no cleavage of tryptophyl peptides under the conditions of oxymercuration. Of the maximal release of 46% of new NH_2-terminals, leucine was the major component (Table II). The δ-hydroxyl of *N*-methyl-δ-hydroxyleucine may labilize the adjacent peptide bond, especially under the influence of mercuric ions. It is not surprising that acidic conditions at room temperature cleave this particular peptide bond (Table III). The acid lability of the peptide bond between leucine and 2-amino-4-hexenoic acid is difficult to rationalize. Perhaps it is a result of the overall configuration of the cyclic peptide.

2-Amino-4-hexenoic acid is remarkable in three respects: (1) in acid hydrolysates of rufomycin or ilamycin (Takita and Naganawa, 1963) it appears not as γ- but as δ-chloro- and δ-hydroxy-2-aminohexanoic acid. Unpublished experiments[1] show that sulfuric acid treatment of *free* 2-amino-4-hexenoic acid leads to 2-amino-4-hydroxyhexanoic acid and its γ-lactone, whereas *peptide-bound,* acylated, or DNP-2-amino-4-hexenoic acid yields 2-amino-5-hydroxyhexanoic acid and, to a minor degree, its δ-lactone. (2) With *N*-bromosuccinimide it yields the expected δ-bromo-γ-lactone. (3) If it reacts with mercuric acetate such interaction may have led to release of NH_2-terminal (substituted) tryptophan whose determination as DNP-tryptophan is difficult because of its instability to hydrolysis.

To summarize, mercuric ions accelerate hydrolysis under the conditions described in Table II. This catalytic effect is not specific; other heavy metal ions such as Co^{2+} (Collman and Buckingham, 1963), Cu^{2+} (Meriwether and Westheimer, 1956), La^{3+}, and Ce^{4+} (Bamann *et al.*, 1961) in a ratio of antibiotic to metal of 50:1 in ethanolic 4.0 M acetate buffer (*p*H 5.5) liberated up to 40% of one peptide bond/mole of compound XI after 48 hours at 75°.

EXPERIMENTAL

Oxymercuration and Cleavage.—From solutions of compounds V, VI, VII, and VIII in ethanol, containing 10 μmoles per ml, 1-μmole aliquots were pipetted into test tubes and 10 μl of a 0.5 M solution of $Hg(OAc)_2$ in 50% acetic acid (5 μmoles) was added to each. The tubes also

[1] J. Ueyanagi, T. Kamiya, M. Fujino, H. Iwasaki, A. Miyake, and S. Tatsuoka, paper presented at the 127th meeting of the Antibiotic Research Association of Japan, Tokyo, November, 1961.

TABLE II

CONDITIONS OF OXYMERCURATION OF *O*-METHYLDIHYDRORUFOMYCIN A (XI) WITH EXCESS MERCURIC ACETATE (RATIO 3:1, W/W) IN A GIVEN SOLVENT AT ROOM TEMPERATURE OR UNDER REFLUX[a]

Sovent	Temperature Reaction Time (hours)	NH_2 Released by TNP Method (%)	NH_2-Terminals Determined by DNP Method
Dimethoxyethane	20° 0.5–72	No change	
Dimethoxyethane	Reflux 6	4.5	Leu
Methanol	20° 12	9.6	Leu
Methanol	Reflux 6	11.7	Leu,Ala,Me-NO_2-Tyr,(Try?)
Methanol	Reflux 12	36	Leu,Ala,Me-NO_2-Tyr,(Try?)
Ethanol	20° 12	14.3	Leu
Ethanol	Reflux 12	42–46	Leu,Ala,Me-NO_2-Tyr,(Try?)
Acetic acid	20° 2–24	9.6–16.7	Leu,Ala
Acetic acid	Reflux 6	23.4	Leu,Ala
Isopropanol	Reflux 12	18.7	Leu,Ala,Me-NO_2-Tyr,(Try?)
1-Propanol	Reflux 12	8.3	Leu,Ala,Me-NO_2-Tyr,(Try?)
1-Propanol	Reflux 24	15.7	Leu,Ala,Me-NO_2-Tyr,(Try?)

[a] After removal of solvent a separate hydrolysis step consisted in letting the sample stand in a mixture of glacial acetic acid–1.0 N hydrochloric acid (4:1) for 17 hours at room temperature.

TABLE III

LABILITY OF PEPTIDE BONDS IN *O*-METHYLDIHYDRORUFOMYCIN A TO ACID OF VARYING STRENGTH AT ROOM TEMPERATURE FOR 24 HOURS

Acid	Strength (N)	Liberation of One New NH_2-Terminal Residue/Mole (%)	Composition of Total DNP-NH_2-Terminals (%)		
			Ala	Leu	δ-OH-α-aminohexanoic Acid (from Δ^4-norleucine)
Hydrofluoric	6.0	9			
Hydrofluoric	14.0	22	17	35	48
Hydrochloric	6.0	40	3	14	83
Hydrobromic	6.0	54	1	17	82
Hydrobromic	4.5	41			

contained 0.5 ml of a 4.0 M acetate buffer of *p*H 5.5. One set of tubes was heated at 75 ± 2° for 15 minutes, and another set of tubes, containing the same amounts of samples and reagent, was kept at room temperature. A set of blanks of the samples with no reagent added was also prepared. Into the tubes containing $Hg(OAc)_2$ was passed a stream of H_2S to precipitate mercuric ions as the sulfide. In some experiments thiolacetic acid was used to remove mercuric ion. However, the presence of excess thiol caused the formation of red colors with ninhydrin, which faded at a rapid rate.

Excess H_2S was displaced by a stream of N_2. The amount of free amino groups present in solution was measured by the ninhydrin reaction (Rosen, 1957). Correction was made for the values from blanks. Standards were run, with and without addition of mercuric acetate. The presence of HgS during the development of color caused a slightly lower color yield, which however was reproducible. Before measurement of color all tubes were centrifuged at low speed to pack the HgS at the bottom of the tubes. Samples treated with $Hg(OAc)_2$ at room temperature showed no increase in amino-group content. Samples subjected to heating at 75° showed the presence of amino groups corresponding to cleavage yields of 30.0, 22.6, 15.7, and 2.8%, respectively, for compounds V, VI, VII, and VIII.

Influence of reaction conditions: compound V (0.5 μmole) in the presence of 10 μmoles of reagent and 0.5 ml ethanol was heated at 75°. The cleavage yield was examined at various time intervals:

Time (min)	20	40	80	100
Cleavage yield (%)	4.2	8.3	21.5	24.0

A medium of pure ethanol gave no better cleavage yields than alcoholic acetate buffer of *p*H 5.5.

With compound VI, replacement of ethanol by methanol did not give cleavage yields in excess of 5–10%. Replacement of the alcohols by 0.1 ml 50% acetic acid gave a maximum cleavage yield of only 15–18%.

In another series of experiments 2-μmole amounts of samples V, VI, and VII were refluxed for 16 hours in the presence of 10 μmoles of $Hg(OAc)_2$ and 20 μmoles of acetic acid. Cleavage yields were 26.5, 8.3, and 22.6%, respectively. The low yield of 8.3% glycine from compound VI is probably owing to the formation of diketopiperazine from glycine as a result of prolonged refluxing. Assay by the DNP method gave much higher yields (*vide infra*).

With dehydroprolinamide under any conditions cleavage yields did not exceed 7%. Even this limited cleavage is unexpected because there is none with *N*-bromosuccinimide. This is the first example of reactivity of the double bond in 3,4-dehydroproline toward an electrophilic reagent.

Identification and Isolation of Glycine from the Cleavage of compound VI.—One μmole of compound VI in 0.1 ml ethanol was mixed with 10 μmoles of $Hg(OAc)_2$ in 0.5 M acetic acid and 0.3 ml of *p*H 5.5 buffer. The mixture was heated for 15 minutes in a stoppered tube at 100°, and then cooled. The reaction mixture was passed through a 1.5-ml column of Dowex-50 × 8 in the H^+ form, and the column was washed with water. The column was now eluted with 1.0 M ammonia and the eluate was taken to dryness and dissolved in a small volume of water. An aliquot was chromatographed (ascending) on paper (*n*-BuOH-AcOH-pyridine-water, 30:6:20:24), as was a sample of authentic glycine. Both the amino acid released by oxymercuration as well as glycine had identical R_F values.

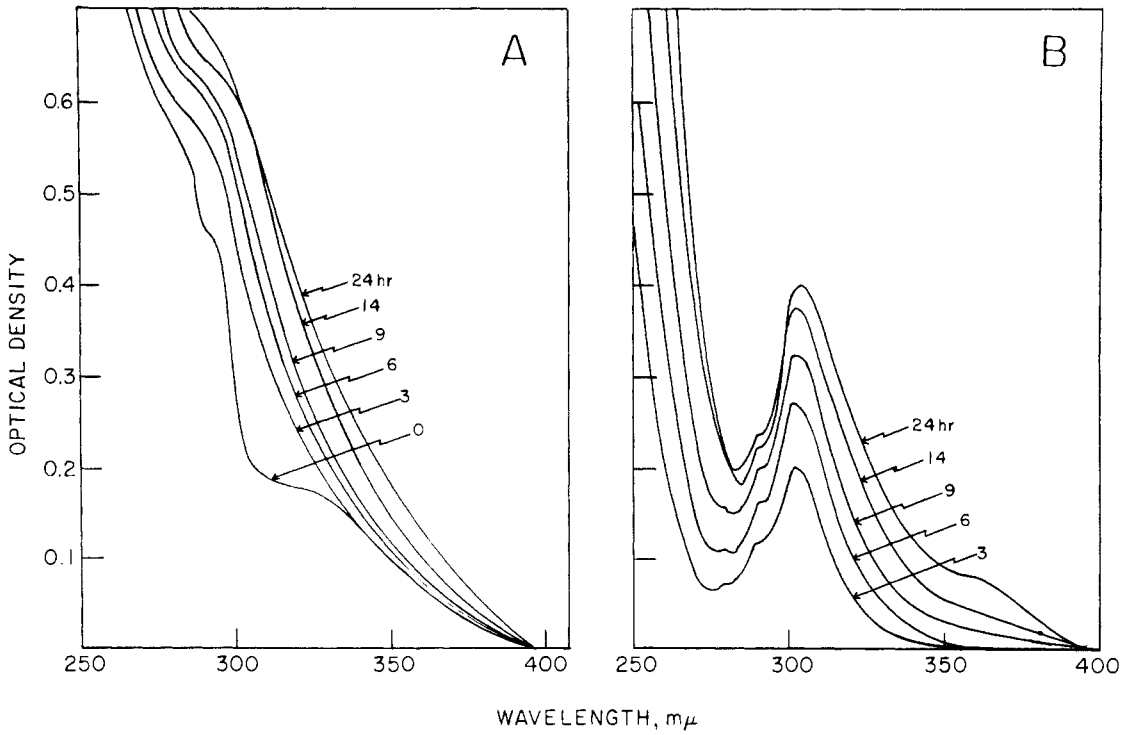


FIG. 1.—Changes in ultraviolet-absorption and difference spectra of *O*-methyldihydrorufomycin A in the course of oxymercuration. (A) Ultraviolet-absorption spectrum; (B) difference spectrum.

For the isolation of liberated glycine, samples containing 500 μmoles of compound VI were treated as follows: (a) Two ml of 0.5 M $Hg(OAc)_2$ in 50% acetic acid (equivalent to 2 moles of reagent per mole of sample) and 5 ml ethanol were added. After 15 hours at room temperature the sample was heated in a water bath for 30 minutes. (b) The sample was treated as in (a) but a 4-fold excess of reagent was used. (c) To the sample a weighed 4-fold excess of solid $Hg(OAc)_2$ and 5 ml of ethanol were added and the mixture was refluxed for 30 minutes. The reaction mixtures were treated with H_2S to precipitate mercuric ion and the filtrate was concentrated to 2 ml. A 2-fold excess of bicarbonate and a 10-fold excess of 1-fluoro-2,4-dinitrobenzene and 4 ml of ethanol were added and the mixtures were shaken for 3 hours. Ethanol was removed by evaporation and the aqueous phase was extracted repeatedly with ether to remove excess reagent. After removal of ether by aeration, the aqueous phase was adjusted to *p*H 1–2 with hydrochloric acid. After cooling, the DNP-glycine that separated was collected. Based on the weight of DNP-glycine (mp 205–206°), the cleavage yields were 8.3, 18, and 28% for reaction conditions a, b, and c, respectively.

Studies on the Role of Ions in the Cleavage of O-Methyldihydrorufomycin A (XI). Control Experiment with Lithium Acetate in Ethanol.—A solution of 6 mg of compound XI and 6 mg of lithium acetate in 3 ml of ethanol was refluxed for 12 hours. An aliquot of 0.5 ml of the mixture was taken out before and after reaction and diluted with ethanol to 10 ml. No difference was found between the ultraviolet spectra of these two solutions. Two ml of the reaction mixture was used for quantitative analysis of released terminal amino groups by the trinitrophenylation (TNP) method. An aliquot of 1 ml of the reaction mixture was taken into each of two 10-ml flasks and evaporated *in vacuo*. Each residue was dissolved in 1 ml of methoxyethanol. To one of them, 1 ml of 0.1% trinitrobenzenesulfonic acid in 0.1 M potassium phosphate buffer (*p*H 7.66) was added, and to the other blank sample, 1 ml of 0.1 M phosphate buffer without trinitrobenzenesulfonic acid. Two other blank samples were made up as follows: (1) 1 ml of methoxyethanol was mixed with 1 ml of 0.1% trinitrobenzenesulfonic acid in 0.1 M phosphate buffer; (2) to 1 ml of methoxyethanol was added 1 ml of 0.1 M phosphate buffer.

All four solutions were allowed to stand for 3 hours at 40° under exclusion of light. To each sample was added 1 ml of 1.0 N hydrochloric acid in methoxyethanol. The ultraviolet spectra of these solutions were recorded in four individual cells. The cells containing the sample solution and the blank No. 2 solution were put in the sample chamber of the ultraviolet machine, while the cells of the sample solution without trinitrobenzenesulfonic acid and the blank No. 1 solution were in the reference chamber. From the optical density at λ_{max} 330–350 mμ, the amount of free NH_2-terminal group was calculated under the assumption that OD of 1 μmole NH_2/1 ml is 11 (Satake *et al.*, 1960).

The amount of liberated terminal amino groups was only 5.6%. The rest of the reaction mixture (*ca.* 1 ml) was evaporated *in vacuo* and the residue was dinitrophenylated with the BFDN methoxyethane solution and 2% triethylamine. No DNP-amino acids were found by thin-layer chromatography in the system chloroform–methanol–acetic acid 95:5:1.

Cleavage of O-Methyldihydrorufomycin A (XI) under the Conditions of Oxymercuration.—A solution of 90 mg of compound XI and 300 mg of mercuric acetate in 45 ml of ethanol was refluxed for 24 hours. After 3, 6, 9, 14, and 24 hours, 8 ml of the reaction mixture was withdrawn for analysis. Changes in the ultraviolet-absorption spectrum were observed with a 250-μl aliquot of each reaction mixture in 5 ml of ethanol (Fig. 1A,B).

Each aliquot was evaporated *in vacuo* and to the residue was added 4 ml of a mixture of acetic and 1.0 N hydrochloric acids (4:1). The mixture was allowed to stand at room temperature for 17 hours and evaporated *in vacuo*. To remove hydrochloric acid and acetic acid the addition and evaporation of ethanol to the residue

was repeated several times. Each residue was dissolved in 8 ml of ethanol, from which 2 ml was used for quantitative analysis of released terminal amino groups as described (see Fig. 2). The residual 6 ml of the reaction mixture was evaporated *in vacuo* and the residue was dinitrophenylated. After hydrolysis with hydrochloric-acetic acid (1:1) for 12 hours at 105°, DNP-amino acids were extracted with ether from the acidic aqueous solution and submitted to thin-layer chromatography (Table II).

Quantitative Analysis of Individual N-*Terminal Amino Acids Released under the Conditions of Oxymercuration.*—A. Assay by TNP method and gas chromatography.—A solution of 102 mg of compound XI and 340 mg of mercuric acetate in 51 ml of ethanol was refluxed for 10 hours. The reaction mixture was evaporated *in vacuo* and the residue was hydrolyzed with 102 ml of a mixture of acetic–1.0 N hydrochloric acid (4:1) at room temperature for 17 hours. An aliquot of 2 ml of the reaction mixture was used for the assay of released NH_2-terminals by the TNP method and gave a value of 22%. The remainder of the reaction mixture was divided into two equal volumes (50 ml each), to one of which was added 5 μmoles of leucine to obtain a recovery value. Both samples were evaporated *in vacuo* and the residues were dinitrophenylated with 1 ml of BFDN in dimethoxyethane solution and 3 ml of 2% triethylamine for 8 hours. The solvent was evaporated *in vacuo* and each residue was hydrolyzed with 5 ml of acetic-hydrochloric acid (1:1) at 105° for 12 hours in a sealed tube. Five μmoles of DNP-valine were added to both hydrolysates as internal standard. The DNP-amino acids were extracted with ether and methylated with excess diazomethane. Thin-layer chromatograms of DNP-amino acids, in the system chloroform–methanol–acetic acid, 95:5:1, and of their methyl esters in the system toluene–pyridine–acetic acid, 80:10:1, showed the presence of DNP-leucine, -alanine, *O*-methylnitrotyrosine, and a spot which at first looked like tryptophan.

The mixture of DNP-amino acid methyl esters was submitted to gas chromatography and the amount of leucine and alanine were calculated (Ishii and Witkop, 1963) as 8.3 and 4.2%, respectively.

B. Paper chromatography of DNP-amino acids.—A solution of 24 mg of compound XI and 80 mg of mercuric acetate in 12 ml of ethanol was refluxed for 10 hours. The reaction product was hydrolyzed with 24 ml of acetic acid and hydrochloric acid 1.0 N (4:1) for 17 hours at room temperature. The solvent was removed *in vacuo*, the residue was diluted in 4 ml of 50% ethanol, and mercuric ion was removed by hydrogen sulfide. Nitrogen was passed in to remove excess hydrogen sulfide. The reaction mixture was filtered and the precipitate was washed with 50% ethanol containing 20% acetic acid. The solvent was removed by evaporation *in vacuo* and the residue was dinitrophenylated with 25 ml of BFDN in 1 ml of ethanol and 20 mg of bicarbonate in 0.5 ml of water for 3 hours, and hydrolyzed with 1 ml of hydrochloric-acetic acid (1:1) at 105° for 20 hours. The DNP-amino acids were extracted with ether and their amounts were assayed by paper chromatography in *t*-amyl alcohol–*p*H 6 phthalate buffer and in Biserte's two-dimensional system. The cleavage yields were: DNP-nitrotyrosine, 8.4 ± 1.2%; DNP-leucine, 37.4 ± 2.2%; DNP-alanine, 3.6%; DNP-2-amino-5-hydroxyhexanoic acid 33.7 ± 3.1%. The latter DNP-amino acid has an R_F slightly lower than that of leucine in the pyridine-toluene-chloroethanol-ammonia solvent system, and an R_F slightly lower than that of dinitrophenol in the *p*H 6 phosphate buffer (1.5 M) solvent system. On hydrolysis of the DNP-amino acid, extracted from paper chromatograms with 28% ammonia, the open acid corresponding presumably to 2-amino-5-hydroxyhexanoic acid is formed and has an R_F of 0.32 (butanol–pyridine–water–acetic acid), while 2-amino-4-hexanoic acid has an R_F of 0.55. However, the latter on acid hydrolysis decomposes completely to yield 2-amino-4-hydroxyhexanoic acid and its γ-lactone which in the above solvent system has the R_F values of 0.33 and 0.67, respectively.

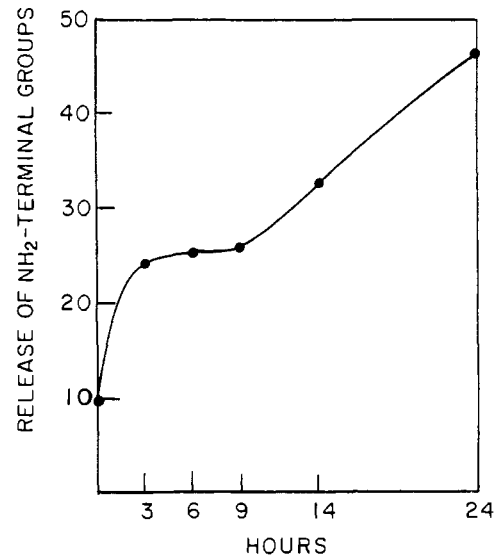


Fig. 2.—Release of new NH_2-terminal groups under the conditions of oxymercuration of *O*-methyldihydrorufomycin A.

The Catalytic Influence of Mercuric and Other Metal Ions on the Hydrolytic Cleavage of Dihydrorufomycin A.—Samples (2 μmoles) of dihydrorufomycin A were heated at 75° for 12 hours and 24 hours in the presence of 20 μmoles of mercuric acetate, cobaltous acetate, or cupric acetate, and 1 ml of ethanol. After the reaction the metal was precipitated as the sulfide, and aliquots of the supernatants were removed for analysis of free amino groups. After 12 hours with all three metals, there was hydrolysis to the extent of 14–16% of one peptide bond per mole and after 24 hours to the extent of about 40% of one peptide bond.

In another set of experiments, at the same temperature, in a solvent of ethanol–4.0 M sodium acetate buffer (*p*H 5.5) (1:1) and a ratio of antibiotic to metal of 1:50, neither Co^{2+}, Hg^{2+}, Cu^{2+}, La^{3+}, nor Ce^{4} ions caused significant hydrolysis (less than 10% of one bond) at 10 hours. At 48 hours the maximum hydrolysis amounted to about 40% of one peptide bond.

Effect of Acids on O-Methyldihydrorufomycin (XI).—Samples of 7.2 μmoles of compound XI were mixed with 1 ml each of hydrogen fluoride, hydrochloric acid, and hydrogen bromide of varying concentration. After 24 hours storage at room temperature the acid was evaporated and the dry residue was diluted in 1 ml of ethanol from which aliquots were withdrawn for the analysis of free NH_2 groups. The results are shown in Table III.

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Physical Parameters of κ-Casein from Cow's Milk*

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The association of κ-casein molecules into aggregates in inorganic salt solutions near neutrality precludes a study of the basic unit with these conditions. Previous studies on κ-casein were performed in alkaline phosphate buffers (≈*p*H 12) to avoid molecular aggregation. However, the properties of κ-casein treated in this manner indicate a destruction of the indigenous disulfide bonds. Consequently, the physical parameters of the protein were determined in neutral or acid solvents containing agents capable of dissociating secondary bonds. The weight-average molecular weight of κ-casein, determined by the short-column sedimentation-equilibrium procedure, was approximately 125,000 in 67% acetic acid–0.15 M NaCl and 5.0 M guanidine HCl solutions. A value of approximately 56,000 for the small component was determined from Trautman plots of approach-to-equilibrium data for the polydispersed system in 7.0 M urea, 33% acetic acid–0.15 M NaCl, and, at low protein concentrations, in 5.0 M guanidine HCl. A molecular weight of approximately 28,000 was obtained for a disulfide-reduced sample of κ-casein in both 5.0 M guanidine HCl and 67% acetic acid–0.15 M NaCl solutions. The reduced specimen was monodispersed in these solvents. The molecular weight of κ-casein in alkaline phosphate buffer (*p*H 12.0) was approximately 28,000; but chemical analyses indicated a loss of disulfide bonds. The results of these studies suggest that κ-casein is composed of two subunits of approximately 28,000 mw joined by disulfide bonds or by secondary bonding which is dependent on specific tertiary configuration produced by intramolecular disulfide bonds. Values for the minimum molecular weight of κ-casein calculated from phosphorus, sulfur, and sialic acid analyses support this conclusion.

κ-Casein is believed to function as the "protective colloid" for micellar casein. The interaction of this protein with other casein components in the presence of calcium ions at concentrations where certain of the components are individually insoluble yields a stable micelle (Waugh and Von Hippel, 1956; Waugh, 1958, 1961). The alteration of κ-casein by the enzyme rennin is sufficient to cause coagulation of the micellar proteins (Waugh, 1958; Wake, 1959; Garnier, 1959). Reflecting its capability for strong interactions, κ-casein forms relatively uniform aggregates of high molecular weight in inorganic salt solutions below *p*H 11 (Waugh and Von Hippel, 1956). These aggregates must be dissociated to characterize more precisely the physical parameters of this protein. However dissociation can be accomplished only by using strong agents, e.g., concd acetic acid, guanidine HCl, or urea, which make the determination of thermodynamic or hydrodynamic properties less accurate because of the preferential interactions and the nonideal behavior of the protein. Recent studies of various proteins in dissociating agents similar to those employed here showed that these errors were not greatly significant (Harrap and Woods, 1961; Kielley and Harrington, 1960; Trautman and Crampton, 1959; Yphantis and Waugh, 1957; Criddle *et al.*, 1962).

In this paper we report: (a) some of the physical properties of κ-casein in concentrated acetic acid, guanidine HCl, and urea, (b) a partial compositional analysis of the protein, and (c) data to support the conjecture that the basic unit of κ-casein is composed of two subunits joined by disulfide bonds. Selected portions of this study were reported previously without detail by Swaisgood and Brunner (1963).

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