[CONTRIBUTION FROM THE DEPARTMENT OF CHEMISTRY, MASSACHUSETTS INSTITUTE OF TECHNOLOGY]

The Cross-linking of Gelatin Using a Water-soluble Carbodiimide

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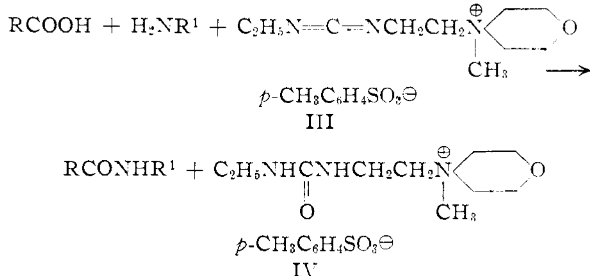
Treatment of an aqueous gelatin solution with a water-soluble carbodiimide (III) resulted in very rapid gelation, shown to be due to amide bond formation.

Recently[2] we reported that water-soluble carbodiimides may be used to form peptide bonds in fully aqueous solution. It is of obvious interest to study the effect of these unusual reagents on proteins.

Gelatin may be regarded as a partially degraded protein, being derived from collagen by acidic or basic hydrolysis. In addition to amino and carboxy terminal groups, the peptides possess side-chains, some of which end in carboxy groups (due principally to glutamic and aspartic acid residues) and others bear free amino functions (accounted for chiefly by lysine).

We have found that treatment of an aqueous solution of gelatin with a water-soluble carbodiimide [1-ethyl-3-(2-morpholinyl-(4)-ethyl)-carbodiimide metho-*p*-toluenesulfonate (III)] results in very rapid (30 seconds) gelation, presumably due to cross-linking by amide bond formation.



Previously, known methods for denaturing, insolubilizing, hardening or tanning protein-type materials with chemical agents, such as aldehydes (formaldehyde or glyoxal) or metallic ions (chrome-alum), are slower acting, leave a residue in the treated material, and operate by complex and poorly understood mechanisms. The carbodiimides II and III are especially useful for amide and peptide bond formation in high yield,[2] by virtue of their accessibility from commercially available intermediates and favorable physical properties.

The normal gelation of gelatin is considered generally to involve hydrogen bond formation through amide groups.[3] It is of interest to demonstrate that the carbodiimide reagent is operating by a different mechanism, namely, formation of new amide bonds. The following experiments convincingly demonstrate this latter mechanism.

Gelatin will not gel in a 2 *M* urea solution at 25° (considered to be due to efficiency of urea in the disruption of hydrogen bonds), but the addition of III causes immediate gelation. Benzoyl gelatin,[4] which contains essentially no free amino groups, gels reversibly as a 5% solution in 10–15 minutes at 25° with or without added III.

Treatment of gelatin with a high ratio (equal weight dry basis) of water-soluble carbodiimide leads to very rapid gelation, but after 10–16 hours storage at 25°, the mixture liquefied. However, if the gelled solution of gelatin and carbodiimide is heated immediately on a steam-cone for 10 minutes, the mixture remains solid at the elevated temperature and on storage for five days at 25°.

At ratios of 10–25% by weight of carbodiimide to a 5% gelatin solution, the rate of gelation is less. The melting point of the treated gelatin is increased to 50–90° and no liquefaction was observed on storage.

In a separate experiment a 5% gelatin solution was freeze-dried immediately after gelation with a nearly equal weight of III. The resulting powder was washed free of water-soluble components and divided into three portions. The first portion (a control) was suspended in water corresponding to a 5% solution and stored three days at 25°, but the mixture did not become homogeneous. A second portion, suspended in water containing the urea IV, likewise did not dissolve on storage. The third portion, treated with an additional quantity of III, did dissolve on storage, thereby establishing excess III as the factor producing liquefaction.

On storing the gelatin–carbodiimide mixture, the rate of disappearance of carboxyl groups (Sörensen titration[5]) paralleled the loss of free amino functions (Van Slyke determination), further evidence that amide bond formation was taking place. Immediately after gelation the carboxy value dropped to 25% of the original gelatin figure and after reliquefaction only 10% of the amino and carboxyl groups remained. Liquefaction is considered to be associated with phase-separation, and the phenomenon is being investigated further.

Experimental[6]

1-Ethyl-3-(2-morpholinyl-(4)-ethyl)-thiourea (I).—To a solution of 10 g. (0.077 mole) of N-(2-aminoethyl)-morpholine in 100 ml. of ether was added slowly 6.7 g. (0.077 mole) of ethyl isothiocyanate. After ebullition had ceased, the reaction solution was stored overnight at 0°. The crystalline product I amounted to 14.0 g. (84%), m.p. 94–96°.

Anal. Calcd. for $C_9H_{19}N_3OS$: C, 49.75; H, 8.81; N, 19.34. Found: C, 49.80; H, 8.83; N, 19.35.

1-Ethyl-3-(2-morpholinyl-(4)-ethyl)-carbodiimide (II).—A solution of 5.0 g. (0.023 mole) of 1-ethyl-3-(2-morpholinyl-(4)-ethyl)-thiourea in 100 ml. of dry acetone was stirred at room temperature with 7.0 g. of mercuric oxide (Merck Yellow) for 5 hours. The precipitated mercuric sulfide was

(1) Aided by a Fellowship from the National Institutes of Health.

(2) J. C. Sheehan and J. J. Hlavka, *J. Org. Chem.*, **21**, 439 (1956).

(3) C. Robinson and M. Bott, *Nature*, **168**, 325 (1951).

(4) A sample of benzoyl gelatin, prepared by Schotten–Baumann benzoylation of gelatin, was furnished by Dr. E. R. Blout, Polaroid Corporation, Cambridge, Mass.

(5) G. Wilson, "Laboratory Manual of Physiological Chemistry," The Williams and Wilkins Co., Baltimore, Md., 1952, p. 72.

(6) All melting points are corrected. We wish to thank Dr. S. M. Nagy and associates for the microanalytical data.

removed by filtration and the filtrate was again stirred 12 hours with 7.0 g. of mercuric oxide. After filtration the acetone was distilled under reduced pressure and the residue was purified by distillation at 80° (0.05 mm.), yield 3.5 g. (76%).

Anal. Calcd. for $C_9H_{17}N_3O$: C, 58.98; H, 9.35; N, 22.93. Found: C, 59.11; H, 9.34; N, 23.10.

1-Ethyl-3-(2-morpholinyl-(4)-ethyl)-carbodiimide Metho-*p*-toluenesulfonate (III).—A mixture of 2.0 g. (0.011 mole) of methyl *p*-toluenesulfonate and 2.0 g. (0.11 mole) of carbodiimide II was heated on a steam-bath for 10 minutes. On trituration with benzene the mixture crystallized; 3.6 g. (90%), m.p. 86–88°. Recrystallization from an acetone–benzene mixture raised the m.p. to 91–93°.

Anal. Calcd. for $C_{17}H_{27}N_3O_4S$: C, 55.27; H, 7.37; N, 11.38. Found: C, 55.41; H, 7.50; N, 11.50.

Reaction of Gelatin[7] with an Excess of 1-Ethyl-3-(2-morpholinyl-(4)-ethyl)-carbodiimide Metho-*p*-toluenesulfonate (III). Experiment 1.—To a solution of 2.0 g. of gelatin[7] in 25.0 ml. of water was added 1.5 g. of III. The solution gelled almost immediately (30 seconds), but liquefied on storage at room temperature for 16 hours. The resulting solution was dialyzed (using $^{8}/_{32}''$ cellulose casing) for 4 days. The solution was freeze-dried to a white powder; weight 2.0 g. A Sorensen titration[5] on 0.4 g. required 0.25 ml. of 0.103 *N* sodium hydroxide. An untreated gelatin sample (0.4 g.) required 2.6 ml. A Van Slyke analysis yielded 0.19% nitrogen, while untreated gelatin yielded 0.64% nitrogen.

Experiment 2.—A solution of gelatin in 50.0 ml. of water was gelled with 2.25 g. of carbodiimide III. The gel was immediately freeze-dried, followed by trituration with 500 ml. of water. The resulting material after drying weighed 3.0 g. The wash water on evaporation yielded 2.3 g. of urea IV.

A 0.5-g. sample of the above-treated gelatin was dissolved in 20 ml. of a 50% calcium chloride solution by heating on a steam-bath for 1 hour. A Sorensen titration[5] required 0.65 ml. of 0.103 *N* sodium hydroxide.

When 0.1 g. of the gelatin prepared above was suspended in 2.0 ml. of water containing 75 mg. of urea IV, no liquefaction occurred even after 9 days. If, however, carbodiimide was substituted for IV, liquefaction occurred.

When conductometric amino titrations[8] were carried out on the gelatins prepared in both runs 1 and 2, a decrease in amino content as compared to untreated gelatin was observed.

Experiment 3.—A solution of 0.2 g. of gelatin, 0.45 g. of urea and 0.15 g. of carbodiimide III in 4.0 ml. of water gelled after 20 seconds. On storage overnight the reaction mixture liquefied.

Reaction of Carbodiimide III and Benzoyl Gelatin.[4]—To a solution of 0.1 g. of benzoyl gelatin[4] in 2 ml. of water was added 0.1 g. of III. On stirring no sudden gelation occurred as with the control gelatin.

(7) The gelatin was obtained from the Atlantic Gelatin Co., Division of General Foods, Lot #6200-X.

(8) S. Ellis and J. Parkhurst, *Biochem. J.*, **52**, 350 (1952).

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The Biogenesis of Nicotine. III. Further Observations on the Incorporation of Ornithine into the Pyrrolidine Ring[1]

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Radioactive nicotine was isolated from two groups of *Nicotiana tabacum* plants, which were harvested one and three weeks after the administration of equal counts of ornithine-2-C^{14}. The nicotine from the two experiments had almost the same specific activity, indicating that little or no metabolic breakdown of nicotine had occurred during the last two weeks of the second experiment. The nicotine from both experiments was degraded unambiguously and all the activity in the alkaloid was shown to be equally divided between the two α-carbons of the pyrrolidine ring.

Introduction

It has been shown that radioactive nicotine is obtained when ornithine-2-C^{14} is fed intact *N. tabacum*[1a] or *N. rustica*[2] plants, or to sterile roots[3] of the former species. Degradation of the radioactive nicotine[1a,2] indicated that half the activity was located on C-2 of the nicotine molecule I. This result could be explained by postulating that ornithine was metabolized to a symmetrical compound before incorporation into nicotine. In the biogenetic scheme suggested by the author,[1b] the mesomeric anion of Δ^1-pyrroline functioned as the symmetrical intermediate. Putrescine (1,4-diaminobutane) is another plausible intermediate, which would result from ornithine on decarboxylation. However the feeding of radioactive putrescine to *Datura stramonium* plants did not yield radioactive hyoscyamine,[4] whereas radioactive ornithine was incorporated into the pyrrolidine ring of the hyoscyamine.[5] Furthermore in other biological systems the pyrrolidine ring has been shown to arise from ornithine *via* glutamic-γ-aldehyde, putrescine not being an intermediate.[6]

It was considered of interest to investigate changes in the activity of the nicotine after feeding ornithine-2-C^{14} for varying lengths of time. In the present work radioactive ornithine was administered to two groups of *N. tabacum* plants growing in inorganic nutrient solution. The first group was harvested after 7 days and the second after 21 days. The degradative steps which were used to locate the positions of activity in the radioactive nicotine are shown in Fig. 1. Treatment of nicotine with hydrogen iodide yielded methyl iodide which was absorbed in triethylamine affording methyltriethylammonium iodide (II). Oxidation of nicotine with concentrated nitric acid yielded

(1) Parts I and II of this series are considered to be: (a) E. Leete, *Chemistry & Industry*, 537 (1955), and (b) E. Leete, THIS JOURNAL, **78**, 3520 (1956), respectively. This work has been supported in part by a grant from the Research Corporation, New York.

(2) L. J. Dewey, R. U. Byerrum and C. D. Ball, *Biochim. Biophys. Acta*, **18**, 141 (1955).

(3) R. F. Dawson, private communication.

(4) D. G. M. Diaper, S. Kirkwood and L. Marion, *Can. J. Chem.*, **29**, 964 (1951).

(5) (a) E. Leete, L. Marion and I. D. Spenser, *ibid.*, **32**, 1116 (1954); (b) *Nature*, **174**, 650 (1954).

(6) M. R. Stetten in "Amino Acid Metabolism," edited by W. D. McElroy and H. B. Glass, John Hopkins Press, Baltimore, Md., 1955, pp. 277–290.