[CONTRIBUTION FROM THE DEPARTMENT OF BIOCHEMISTRY, FACULTY OF MEDICINE, LAVAL UNIVERSITY]

New Syntheses of Hydroxyproline[1]

BY ROGER GAUDRY AND CLAUDE GODIN

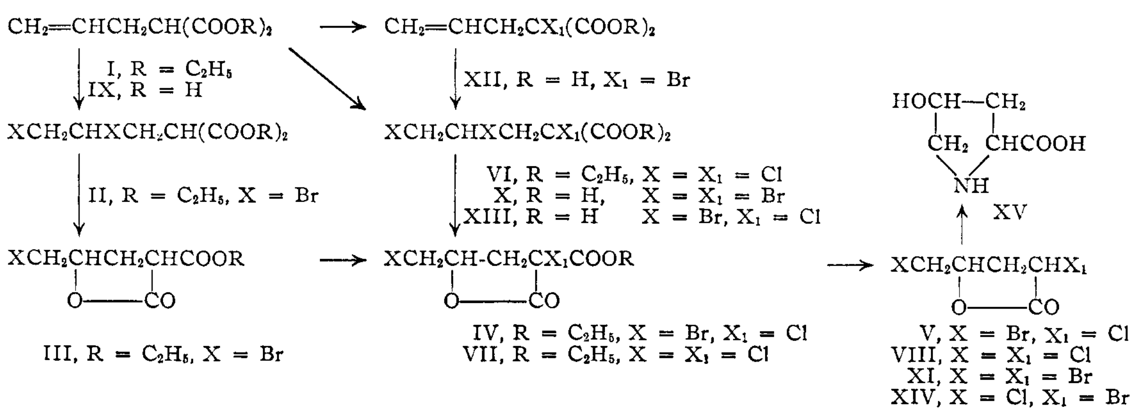
RECEIVED JULY 2, 1953

The four 2,5-dihalogenated-4-valerolactones containing chlorine or bromine have been prepared from the diethyl ester of allylmalonic acid, and their cyclization with ammonia has been investigated. The dichlorolactone, which gave the best yield of hydroxyproline (54%), was obtained in a 90% over-all yield from diethyl allylmalonate. 2-Carbethoxy-5-phthalimido-4-valerolactone has been prepared by condensing 1,2-epoxy-3-phthalimidopropane with diethyl malonate. Chlorination or bromination of this lactone gave the corresponding 2-halogenated lactones which upon acid hydrolysis and treatment with barium hydroxide gave hydroxyproline in a 54% yield from diethyl malonate, by way of the chlorolactone. 2-Cyano-5-phthalimido-4-valerolactone has been prepared by condensing 1,2-epoxy-3-phthalimidopropane with ethyl cyanoacetate. Chlorination or bromination of this lactone gave the corresponding 2-halogenated-2-amidolactones which, upon acid hydrolysis and treatment with hot barium hydroxide, gave hydroxyproline in a 48% yield from ethyl cyanoacetate by way of the chlorolactone. Allohydroxy-DL-proline has been converted to hydroxy-DL-proline by inverting the configuration at C_4. The ethyl ester of hydroxyproline hydrochloride has been isolated.

Hydroxyproline (XV) was first isolated from gelatine by Fischer[2] in 1902 and was first synthesised in 1905 by Leuchs.[3] Other syntheses were later developed by Fischer and Krämer[4] in 1908, by Leuchs and co-workers[5] in 1912, by Hammarsten[6] in 1916, by Traube and co-workers[7] in 1923, by Feofilaktov and Onishchenko[8] in 1938 and by McIlwain and Richardson[9] in 1939. However, none of these is really satisfactory for the preparation of substantial quantities of this amino acid.

Two methods of synthesis were investigated, the first one based on the cyclization with ammonia of a 2,5-dihalogenated-4-valerolactone and the second one based on the cyclization with barium hydroxide of a 2-halogenated-5-amino-4-valerolactone. The halogen atom was chlorine or bromine.

The four 2,5-dihalogenated-4-valerolactones were prepared from the diethyl ester of allylmalonic acid I or free allylmalonic acid IX.



The 2,5-dichloro-4-valerolactone (VIII)[5] was prepared in a 90% over-all yield from the diethyl ester of allylmalonic acid by reaction with sulfuryl chloride followed by acid hydrolysis, decarboxylation and vacuum distillation. This lactone was also prepared in good yield by treatment of allylmalonic acid (IX) with sulfuryl chloride or with water and chlorine. It was also prepared by reaction of the ethyl ester of allylcyanoacetic acid with sulfuryl chloride. Acid hydrolysis and decarboxylation gave the lactone in a 34% yield.

The 2-chloro-5-bromo-4-valerolactone (V) was prepared from allylmalonic diethyl ester in a 59% yield. By reaction with one equivalent of bromine in the cold, followed by vacuum distillation, 2-carbethoxy-5-bromo-4-valerolactone (III) was obtained in a 72% yield. This lactone upon reaction with sulfuryl chloride, acid hydrolysis, decarboxylation and vacuum distillation gave 2-chloro-5-bromo-4-valerolactone (V).

The 2,5-dibromo-4-valerolactone (XI) was prepared in an 85% yield by treating allylmalonic acid (IX) in chloroform with bromine. Vacuum distillation of the halogenated product gave the pure lactone. The crystalline 2-carboxy-2,4,5-tribromovaleric acid (X) can be isolated in a small yield from the reaction mixture of allylmalonic acid and bromine.

The 2-bromo-5-chloro-4-valerolactone (XIV),[3] was prepared by treating allylmalonic acid (IX) in chloroform with N-bromosuccinimide to give 2-carboxy-2-bromo-4,5-pentenoic acid (XII) which was allowed to react with one equivalent of sulfuryl chloride to give the crude 2-carboxy-2-bromo-4,5-dichlorovaleric acid (XIII). Decarboxylation of this product gave 2-bromo-5-chloro-4-valerolactone (XIV) in a 60% yield.

(1) Paper presented before the Division of Organic Chemistry at the 123rd Meeting of the American Society, Los Angeles, Calif., March 1953.

(2) E. Fischer, *Ber.*, **35**, 2660 (1902).
(3) H. Leuchs, *ibid.*, **38**, 1937 (1905).
(4) E. Fischer and A. Krämer, *ibid.*, **41**, 2728 (1908).
(5) H. Leuchs, M. Giua and J. F. Brewster, *ibid.*, **45**, 1960 (1912).
(6) E. Hammarsten, *Compt. rend. trav. lab. Carlsberg*, **11**, 223 (1916).
(7) W. Traube, R. Johow and W. Tepohl, *Ber.*, **56**, 1861 (1923).
(8) V. V. Feofilaktov and A. S. Onishchenko, *Compt. rend. acad. sci. U.R.S.S.*, **20**, 133 (1938).
(9) H. McIlwain and G. M. Richardson, *Biochem. J.*, **33**, 44 (1939).

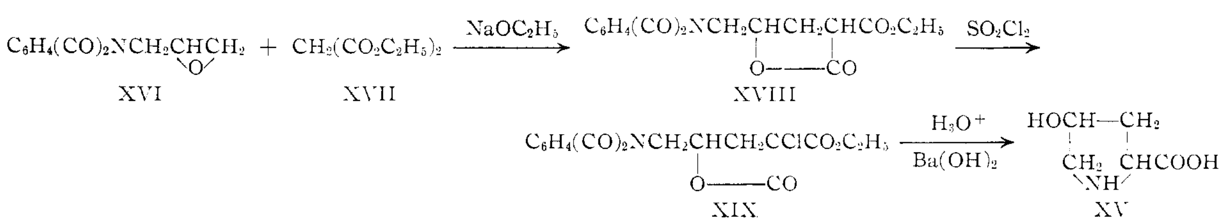
The four 2,5-dihalogenated-4-valerolactones were cyclized with an excess of ammonia into hydroxyproline, under the same experimental conditions. The yields of hydroxyproline copper salts are given in Table I.

TABLE I

2,5-Dihalogenated-4-valerolactones	Yields of hydroxyproline, %
2,5-Dichloro-	54.4
2-Bromo-5-chloro-	34.6
2-Chloro-5-bromo-	30.9
2,5-Dibromo-	16.1

In all cases approximately equal amounts of DL- and allo DL-forms of hydroxyproline were obtained and isolated as copper salts. Whereas the DL-form is easily separated because of the insolubility in water of its copper salt, the allo DL-form is difficult to obtain pure because its copper salt is very soluble.

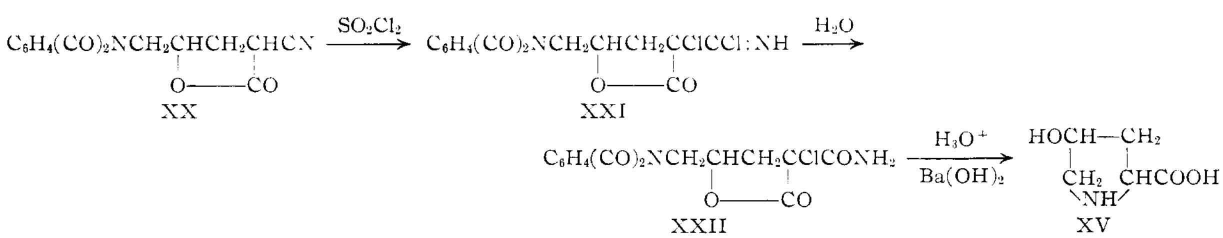
To overcome this difficulty, a method of synthesis was investigated, which was found to give the allo DL-form in a high state of purity. 1,2-Epoxy-3-phthalimidopropane (XVI), easily prepared from epichlorohydrin and potassium phthalimide, was condensed with diethyl malonate (XVII) to give a 74% yield of 2-carbethoxy-5-phthalimido-4-valerolactone (XVIII). This lactone was allowed to react with bromine to give 2-carbethoxy-2-bromo-5-phthalimido-4-valerolactone.[10] Similarly, 2-carbethoxy-2-chloro-5-phthalimido-4-valerolactone (XIX) was prepared by reaction with sulfuryl chloride. The chloro- and the bromolactones were not readily obtained in a crystalline form. They were hydrolyzed in acid media and cyclized with an excess of hot barium hydroxide. A 73% yield of hydroxyproline copper salt was obtained through the chloro compound and a 62.5% yield through the bromo compound. In this synthesis the DL- and allo DL-forms were also obtained in equal quantities and both forms of the copper salt were obtained pure without difficulty. An acid synthetic resin, Dowex-50, was used to separate the amino acid from the inorganic salts.



The synthesis of hydroxyproline from 2-cyano-5-phthalimido-4-valerolactone (XX) was also investigated. This lactone was obtained in a 65% yield by condensing 1,2-epoxy-3-phthalimidopropane with ethyl cyanoacetate. By reaction with sulfuryl chloride or bromine, a chloro- or a bromolactone was obtained. Acid hydrolysis and cyclization with barium hydroxide gave hydroxyproline in a 73% yield through the chlorolactone and a 62.5% yield through the bromolactone. These lactones were identified as 2-amido-2-chloro-5-phthalimido-4-valerolactone (XXII) and 2-amido-2-bromo-5-phthalimido-4-valerolactone (XXII, Cl = Br). When 2-cyano-5-phthalimido-4-valerolactone (XX) in dry chloroform or glacial acetic acid was treated with sulfuryl chloride or bromine, hydrogen chloride or hydrogen bromine apparently reacted with the nitrile group to give the imino chloride (XXI) or imino bromide (XXI, Cl = Br), as in the first step of Stephen's reaction. In presence of water the imino chloride or imino bromide was hydrolyzed to the amide.

In order to prove definitely the structure of these amides, they were synthesized from 2-amido-5-phthalimido-4-valerolactone, prepared by two different ways: firstly, in a 94% yield from 2-cyano-5-phthalimido-4-valerolactone (XX) by treatment with concentrated sulfuric acid; secondly, in a 10% yield from 2-carbethoxy-5-phthalimido-4-valerolactone (XVIII) by reaction with concentrated aqueous ammonia. 2-Amido-5-phthalimido-4-valerolactone (XX, CN = $CONH_2$) by reaction with sulfuryl chloride or bromine gave 2-amido-2-chloro-5-phthalimido-4-valerolactone (XXII) or 2-amido-2-bromo-5-phthalimido-4-valerolactone (XXII, Cl = Br).

Hydroxy-DL-proline and allohydroxy-DL-proline were recovered from their copper salts by means of hydrogen sulfide, and were recrystallized from 80% ethanol. Allohydroxy-DL-proline was converted to hydroxy-DL-proline in a 20% yield, using the procedure devised by Neuberger,[11] who inverted hydroxy-L-proline to allohydroxy-L-proline and applied by Greenstein and Robinson[12] to the inversion of allohydroxy-D-proline to hydroxy-D-proline.



The hydroxyproline ethyl ester hydrochloride was also isolated. It is formed when dry hydroxy-

(10) A. N. Dey, *J. Chem. Soc.*, 1166 (1937).

(11) A. Neuberger, *J. Chem. Soc.*, 429 (1945).

(12) J. P. Greenstein and D. S. Robinson, *J. Biol. Chem.*, **195**, 383 (1952).

proline hydrochloride is dissolved in absolute ethanol or when dry hydroxyproline is treated with absolute ethanol saturated with dry hydrogen chloride. The DL-form is obtained readily and melts at 142°.[13] The allo DL-form crystallized very slowly and melts at 133–136°.

Hydroxyproline has a sharp melting point, easily reproduced and giving a good indication of purity. The best method for synthesizing large quantities of hydroxyproline is that using the chlorination of 2-carbethoxy-5-phthalimido-4-valerolactone. The over-all yield is 54% based on 1,2-epoxy-3-phthalimidopropane and only one intermediate compound, 2-carbethoxy-5-phthalimido-4-valerolactone, has to be isolated.

Experimental

2-Carbethoxy-5-bromo-4-valerolactone (III).—To allylmalonic diethyl ester[14] (I) (40 g., 0.2 mole) cooled at −5°, bromine (32 g., 0.2 mole) was added dropwise with stirring. The mixture was stirred for one hour in the cold and then fractionated *in vacuo* to give the lactone; yield 36 g. (71.7%) b.p. 153–154° (3 mm.). *Anal.* Calcd. for $C_8H_{11}O_4Br$: Br, 31.87. Found: Br, 31.96.

2-Chloro-5-bromo-4-valerolactone (V).—To allylmalonic diethyl ester[14] (I) (40 g., 0.2 mole) cooled at −5°, bromine (32 g., 0.2 mole) was added dropwise with stirring. The mixture was stirred for one hour in the cold and air was blown through the solution to remove bromine in excess. Sulfuryl chloride (27 g., 0.2 mole) was added very slowly to the solution and the mixture was heated under reflux for two hours. The excess of sulfuryl chloride was removed under reduced pressure. The residue was dissolved in 200 ml. of glacial acetic acid, 100 ml. of concentrated hydrochloric acid was added, and the mixture was heated under reflux for two hours. The solution was then evaporated under reduced pressure and the residue fractionated *in vacuo;* yield 25 g. (58.7%), b.p. 136–138° (3 mm.). *Anal.* Calcd. for $C_5H_6O_2ClBr$: Cl + Br, 54.0. Found: Cl + Br, 52.48.

2-Carbethoxy-2,5-dichloro-4-valerolactone (VII).—Sulfuryl chloride (580 g., 4.3 moles) was added dropwise to allylmalonic diethyl ester[14] (I) (400 g., 2 moles) in the cold. The mixture was then heated under reflux for 20 minutes and the sulfuryl chloride in excess was removed under reduced pressure. The residue was fractionated to give the lactone; yield 420 g. (87%), b.p. 148–150° (5 mm.); lit. 178–179° (5 mm.).[5] *Anal.* Calcd. for $C_8H_{10}O_4Cl_2$: Cl, 29.45. Found: Cl, 29.40. The solid isomer crystallized out. It was filtered and recrystallized from ethanol; yield 184.6 g. (38%), m.p. 56–57°; lit. m.p. 55°.[5] *Anal.* Calcd. for $C_8H_{10}O_4Cl_2$: Cl, 29.45. Found: Cl, 29.72.

2,5-Dichloro-4-valerolactone (VIII).—Sulfuryl chloride (580 g., 4.3 moles) was added dropwise to cooled allylmalonic diethyl ester (I) (400 g., 2 moles). The mixture was stirred for one hour at room temperature and refluxed for 20 minutes. The excess of sulfuryl chloride was then evaporated under reduced pressure, the crude 2-carbethoxy-2,4,5 trichlorovaleric ethyl ester was dissolved in 400 ml. of glacial acetic acid, 300 ml. of hydrochloric acid was added and the mixture was heated under reflux for 3 hours. The solution was then evaporated under reduced pressure and fractionated to give the dichlorolactone; yield 306 g. (90.5%), b.p. 137–140° (5 mm.); lit. 159–161° (13 mm.).[5] *Anal.* Calcd. for $C_5H_6O_2Cl_2$: Cl, 42.0. Found: Cl, 42.6.

2-Carboxy-2,4,5-tribromovaleric Acid (X).—To allylmalonic acid[15] (IX) (7.2 g., 0.05 mole) in chloroform (50 ml.) bromine (24.0 g., 0.15 mole) was added dropwise at room temperature with stirring. The mixture was then stirred for 2 hours. The solvent was removed *in vacuo*. The solid residue was recrystallized from chloroform; yield 5.2 g. (27.2%), m.p. 123–124°. *Anal.* Calcd. for $C_6H_7O_4Br_3$: Br, 62.66. Found: Br, 62.29.

2,5-Dibromo-4-valerolactone (XI).—Allylmalonic acid[15] (IX) (28.8 g., 0.2 mole) was dissolved in chloroform (100 ml.). Bromine (80.0 g., 0.5 mole) was added dropwise with stirring at room temperature. The mixture was stirred for 2 hours at that temperature. Upon fractional distillation, lactonization and decarboxylation occurred and the lactone was obtained; yield 44.2 g. (85.8%), b.p. 159–161° (3 mm.). *Anal.* Calcd. for $C_5H_6O_2Br_2$: Br, 62.01. Found: Br, 62.70.

2-Bromo-5-chloro-4-valerolactone (XIV).—Allylmalonic[15] acid (IX) (36.0 g., 0.25 mole) dissolved in 200 ml. of chloroform and N-bromosuccinimide (44.5 g., 0.25 mole) was slowly added. The temperature was kept below 25°. The solution was evaporated to dryness, the residue was dissolved in water and the solution was extracted with ether to give the crude 2-carboxy-2-bromo-4,5-pentenoic acid. The acid was treated with sulfuryl chloride (40.5 g., 0.3 mole) with stirring for 1 hour, the sulfuryl chloride in excess was removed under reduced pressure, the residue was dissolved in water and the solution was extracted with ether. Fractional distillation gave the desired lactone; yield 36.8 g. (69%), b.p. 136–140° (3 mm.); lit. b.p. 156–164° (11 mm.).[3] *Anal.* Calcd. for $C_5H_6O_2ClBr$: Cl + Br, 54.0. Found: Cl + Br, 52.1.

Hydroxyproline Copper Salts. (Cyclization of a 2,5-Dihalogenated-4-valerolactone with Ammonia).—2,5-Dichloro-4-valerolactone (VIII) (169 g., 1 mole) was dissolved in 750 ml. of concentrated ammonium hydroxide. The solution was heated for 24 hours at 100° in an autoclave. The excess of ammonium hydroxide was then evaporated under reduced pressure, 180 ml. of concentrated hydrochloric acid was added and the solution was evaporated to dryness. To the dry residue was added alcohol and the ammonium salts were filtered off. The alcoholic solution was evaporated to dryness, and the crude hydroxyproline hydrochloride was dissolved in water. The hot solution was stirred for one hour with 125 g. of yellow lead oxide. The solution was then cooled and filtered cold. The last traces of chloride were removed with a few grams of silver carbonate and the solution was filtered. The solution was then saturated with hydrogen sulfide and filtered. The solution was evaporated to dryness. The residue was dissolved in water, decolorized with norit and diluted to 1 liter. The solution was heated under reflux for one hour with an excess of copper carbonate (30 g.) and filtered hot. Upon cooling the blue hydrated hydroxy-DL-proline copper salt crystallized out; yield 54.8 g. (27.7%). *Anal.* Calcd. for $C_{10}H_{16}O_6N_2Cu\cdot 2H_2O$: N, 7.07. Found: N, 7.10. The filtrate was evaporated under reduced pressure to a very small volume and the violet anhydrous allohydroxy-DL-proline copper salt was filtered off; yield 43.0 g. (26.6%). It was recrystallized from a small volume of water. *Anal.* Calcd. for $C_{10}H_{16}O_6N_2Cu$: N, 8.65. Found: N, 8.64.

2-Cyano-5-phthalimido-4-valerolactone (XX).—Ethyl cyanoacetate (23.7 g., 0.21 mole) was added to a solution of sodium (4.6 g., 0.2 mole) in absolute ethanol (200 ml.) cooled at 10°. The solution was stirred for 15 minutes and 1,2-epoxy-3-phthalimidopropane[16] (XVI) (40.6 g., 0.2 mole) was added with stirring over a period of one hour. The mixture was then heated very slowly up to 40–45° and stirred at that temperature for 18 hours. The mixture was then poured on ice, acidified, diluted to one liter and stored in the refrigerator for 24 hours. A yellow-brown product was then filtered, washed with water and dried to give 44.2 g. of the crude lactone. Upon recrystallization from 100 ml. of glacial acetic acid the pure lactone was obtained; yield 35.0 g. (65%), m.p. 187°. After many recrystallizations from acetic acid the m.p. was 189–190°. *Anal.* Calcd. for $C_{14}H_{10}O_4N_2$: N, 10.37. Found: N, 10.36.

Hydroxyproline Copper Salts (from 2-Carbethoxy- or 2-Cyano-5-phthalimido-4-valerolactone).—2-Carbethoxy-5-phthalimido-4-valerolactone[10] (XVIII) (9.5 g., 0.03 mole) was dissolved in glacial acetic acid (100 ml.). Sulfuryl chloride (4.3 g., 0.032 mole) was added dropwise with stirring. The mixture was then heated for 2 hours at 70°. The solution was evaporated to dryness *in vacuo*. The residue was washed several times with water, glacial acetic acid (100 ml.) and concentrated hydrochloric acid (100 ml.) were added and the mixture was heated under reflux for 3 hours. The mixture was evaporated to dryness under reduced pressure, the residue dissolved in water and phthalic acid filtered off (calcd. 4.9 g., found 4.9 g.). The filtrate

(13) All melting points are uncorrected.

(14) J. F. Bihkman, *Chem. Centr.*, **78, II**, 1210 (1907).

(15) R. Marbury, *Ann.*, 119 (1897).

(16) M. Weizmann and S. Malkowa, *Bull. soc. chim. France*, **47**, 356 (1930).

was neutralized with barium hydroxide and an excess of barium hydroxide (12.6 g., 0.04 mole) was added. The mixture was heated under reflux for 6 hours, and filtered cold. The filtrate, neutralized with hydrochloric acid, was poured through a glass column containing Dowex-50 cation-exchange resin in the acid form. The amino acid and the barium ion remained on the resin, while the anions were removed by rinsing with water. The amino acid was eluted from the resin with 200 ml. of 2 *N* ammonium hydroxide. The eluate was evaporated to dryness *in vacuo*. The residue was dissolved in water, decolorized with norit and heated under reflux for 1 hour with an excess of copper carbonate (10 g.). The mixture was filtered hot. Upon cooling the hydroxy-DL-proline copper salt crystallized out. It was filtered off, washed with water and dried at 110°; yield 1.75 g. (36.4%). The filtrate was evaporated to dryness *in vacuo*. The residue was dissolved in hot water (10 ml.) and ethanol was added (400 ml.). The allohydroxy-DL-proline copper salt crystallized out. It was filtered off, washed with alcohol and dried at 110°; yield 1.75 (36.4%).

2-Carbethoxy-2-chloro-5-phthalimido-4-valerolactone (XIX).—2-Carbethoxy-5-phthalimido-4-valerolactone[10] (XVIII) (6.3 g., 0.02 mole) was dissolved in glacial acetic acid (100 ml.). Sulfuryl chloride (3.0 g., 0.022 mole) was added dropwise with stirring. The mixture was heated for 2 hours at 70° and evaporated to dryness *in vacuo*. The residue was washed several times with water. The white sticky solid obtained was dissolved in a hot mixture of acetone and ethanol (1:1). To the cooled solution, water was added until it became cloudy and the solution was stored in the refrigerator for several days. A white solid separated out. It was filtered and air dried, m.p. 55°. *Anal.* Calcd. for $C_{16}H_{14}O_6NCl$: N, 3.98; Cl, 10.09. Found: N, 4.06; Cl, 9.94.

2-Amido-5-phthalimido-4-valerolactone (XX, CN = CONH$_2$).—2-Cyano-5-phthalimido-4-valerolactone (XX) (9.0 g., 0.033 mole) was dissolved in concentrated sulfuric acid (25 ml.) cooled at 0°. The solution was kept for 24 hours at room temperature. The mixture was poured on ice. A white solid was formed. It was filtered off, washed with water and recrystallized from boiling ethanol; yield 8.9 g. (93.6%), m.p. 217–218°. *Anal.* Calcd. for $C_{14}H_{12}O_5N_2$: N, 9.72. Found: N, 9.76.

2-Halogenated-2-amido-5-phthalimido-4-valerolactone (XXII). (a) **From 2-Cyano-5-phthalimido-4-valerolactone (XX).**—2-Cyano-5-phthalimido-4-valerolactone (5.4 g., 0.02 mole) was dissolved in glacial acetic acid (100 ml.). Sulfuryl chloride (4.0 g., 0.03 mole) was added and the mixture heated for 5 hours at 80°. The mixture was evaporated to dryness *in vacuo*. The residue was washed several times with water and filtered off. The white solid obtained was recrystallized from boiling ethanol; yield 5.9 g. (92.1%), m.p. 208°. *Anal.* Calcd. for $C_{14}H_{11}O_5N_2Cl$: N, 8.68; Cl, 11.00. Found: N, 8.57; Cl, 10.61.

When bromine was used instead of sulfuryl chloride, the yield was 95.9%, m.p. 201–202°. *Anal.* Calcd. for $C_{14}H_{11}O_5N_2Br$: N, 7.62; Br, 21.79. Found: N, 7.64; Br, 20.42.

(b) **From 2-Amido-5-phthalimido-4-valerolactone (XX, CN = CONH$_2$).**—2-Amido-5-phthalimido-4-valerolactone (3.6 g., 0.012 mole) was dissolved in chloroform (100 ml.). Sulfuryl chloride (2.7 g., 0.02 mole) was added and the mixture was kept at room temperature for 24 hours. The mixture was evaporated to dryness *in vacuo* and the residue washed with water several times. The white solid obtained was filtered off and recrystallized from boiling ethanol; yield 3.5 g. (92.1%), m.p. 208°. *Anal.* Calcd. for $C_{14}H_{11}O_5N_2Cl$: N, 8.68; Cl, 11.00. Found: N, 8.68; Cl, 10.67.

When bromine was used instead of sulfuryl chloride, the yield was 98.1%, m.p. 202°. *Anal.* Calcd. for $C_{14}H_{11}O_5N_2Br$: N, 7.62; Br, 21.79. Found: N, 7.64; Br, 21.87.

Hydroxy-DL-proline (XV).—Anhydrous hydroxy-DL-proline copper salt (6.4 g., 0.02 mole) in water (200 ml.) was acidified with glacial acetic acid (5 ml.). The mixture was saturated with hydrogen sulfide. Copper sulfide was filtered off and the solution evaporated to dryness *in vacuo*. The residue was dissolved in hot water (10 ml.) filtered hot and ethanol (100 ml.) added. Upon cooling, a white solid separated out. It was recrystallized by dissolving in a small volume of hot water and adding 4 volumes of ethanol; yield 4.9 g. (94%), m.p. 247° (open capillary); lit. 255°.[3] *Anal.* Calcd. for $C_5H_9O_3N$: N, 10.68. Found: N, 10.69.

Allohydroxy-DL-proline (XV).—The same experimental procedure was used; yield 4.4 g. (84.6%), m.p. 238° (open capillary); lit. 245°.[3] *Anal.* Calcd. for $C_5H_9O_3N$: N, 10.68. Found: N, 10.71. Mixed m.p. of DL- and allo-DL-forms was 230°.

Hydroxyproline Ethyl Ester Hydrochlorides.—They were obtained by treating dry hydroxyproline hydrochloride with absolute ethanol.

(a) Hydroxy-DL-proline ethyl ester hydrochloride readily separated out; m.p. 142°. *Anal.* Calcd. for $C_7H_{14}O_3NCl$: N, 7.16; Cl, 18.15. Found: N, 7.06; Cl, 18.50.

(b) Allohydroxy-DL-proline ethyl ester hydrochloride very slowly separated out; m.p. 133–136°. *Anal.* Calcd. for $C_7H_{14}O_3NCl$: N, 7.16. Found: N, 7.56.

N-Acetyl-allohydroxy-DL-proline.—Allohydroxy-DL-proline (13.1 g., 0.1 mole) was dissolved in glacial acetic acid (100 ml.). The solution was heated at its boiling point. Acetic anhydride (10.2 g., 0.1 mole) was added dropwise with good stirring. The solution was allowed to cool and then evaporated to dryness *in vacuo* on a water-bath at 35°. The oily residue was cooled and a solid separated out. Acetone (20 ml.) was added, the solid was filtered off and recrystallized from a mixture of ethanol and ether (1:4); yield 16.1 g. (93%), m.p. 143–144°; lit. 145.5° cor. for N-acetyl-allohydroxy-D-proline,[12] and 144–145° for N-acetyl-allohydroxy-L-proline.[11]

N-Acetyl-allohydroxy-DL-proline Methyl Ester.—N-Acetyl-allohydroxy-DL-proline (5.2 g., 0.03 mole) in dry dioxane (100 ml.) was cooled at −10°. The mixture was treated with small quantities of diazomethane in ether until the yellow color of diazomethane remained. The excess of diazomethane was destroyed with a few drops of glacial acetic acid and the solution was evaporated to dryness *in vacuo* at room temperature. The solid residue was dissolved in ethanol (10 ml.) and ether (50 ml.) was added. Upon cooling a solid separated out; yield 5.3 g. (95%), m.p. 79–80°; lit. 78° for N-acetyl-hydroxy-L-proline methyl ester.[11]

N-Acetyl-O-toluenesulfonylallohydroxy-DL-proline Methyl Ester.—N-Acetyl-allohydroxy-DL-proline methyl ester (6.2 g., 0.033 mole) was dissolved in dry pyridine (15 ml.). The solution was cooled at 0° and *p*-toluenesulfonyl chloride (6.7 g., 0.035 mole) was added. The mixture was kept at 0° for 18 hours, poured on ice and *N* hydrochloric acid (145 ml.) was added. The solution was stored in the refrigerator for 24 hours. The white solid obtained was filtered off and washed with ether; yield 8.3 g. (74.1%), m.p. 119–120°; lit. 143.5° cor. for N-acetyl-O-toluenesulfonylallohydroxy-D-proline methyl ester,[12] and 60° for N-acetyl-O-toluenesulfonylhydroxy-L-proline methyl ester.[11] *Anal.* Calcd. for $C_{15}H_{19}O_6NS$: N, 4.10. Found: N, 4.26.

N-Acetyl-O-toluenesulfonylallohydroxy-DL-proline.—N-Acetyl-O-toluenesulfonylallohydroxy-DL-proline methyl ester (8.0 g., 0.024 mole) was dissolved in methanol (150 ml.). The solution was cooled at 0°, *N* sodium hydroxide (24 ml.) was added and the mixture was kept at 0° for 18 hours. Normal hydrochloric acid (24 ml.) was added and the mixture was evaporated to dryness *in vacuo*. A solid separated out. It was filtered and washed with water; yield 6.8 g. (90%), m.p. 85–86°; lit. 143.5° cor. for N-acetyl-O-toluenesulfonylallohydroxy-D-proline[12] and 181–182° for N-acetyl-O-toluenesulfonylhydroxy-L-proline.[11] *Anal.* Calcd. for $C_{14}H_{17}O_6NS$: N, 4.28. Found: N, 4.40.

Inversion. Hydroxy-DL-proline Copper Salt.—N-Acetyl-O-toluenesulfonylallohydroxy-DL-proline (6.5 g., 0.02 mole) was dissolved in 0.5 *N* sodium hydroxide (80 ml., 2 equivalents). The solution was heated under reflux for 20 minutes, cooled and neutralized with 0.5 *N* hydrochloric acid (80 ml.). The solution was evaporated to dryness *in vacuo*. The oily residue, N-acetyl-hydroxy-DL-proline, was dissolved in 2 *N* hydrochloric acid (200 ml.). The solution was heated under reflux for 2 hours and evaporated to dryness *in vacuo*. The residue was dissolved in water and decolorized with norit. The solution containing the hydroxy-DL-proline hydrochloride was poured through a glass column containing deacidite "Permutit" anion-exchange resin in the basic form. The hydrochloric acid remained on the resin. The free amino acid in aqueous solution was heated under reflux with an excess of copper carbonate (10 g.) for 1 hour. The solution was then filtered hot. Upon cooling, the hydroxy-DL-proline blue copper salt crystallized out. It is filtered off and dried at 110°; yield 1.1 g. (34.3%). *Anal.* Calcd. for $C_{10}H_{16}O_6N_2Cu$: N, 8.65. Found: N, 8.62.

Acknowledgments.—The authors are indebted to the National Cancer Institute of Canada for financial assistance, and to the National Research Council of Canada for Research Fellowships awarded to one of them (C. G.).

QUEBEC, P. Q., CANADA

[CONTRIBUTION FROM THE DEPARTMENT OF CHEMISTRY, COLUMBIA UNIVERSITY]

Amino Acid Composition of Crystalline Pancreatic Amylase from Swine[1]

BY M. L. CALDWELL, EMMA S. DICKEY, VIRGINIA M. HANRAHAN, H. C. KUNG, JO-FEN T. KUNG AND MARY MISKO[2]

RECEIVED JUNE 26, 1953

The amino acids present in three times crystallized electrophoretically and enzymically homogeneous pancreatic amylase from swine have been determined quantitatively. The results are presented and discussed briefly.

Introduction

Pancreatic amylase is a protein that, as far as is known, contains no non-protein prosthetic groups. It appears to exert its distinctive action, at least in part, by virtue of the active groupings of some of its amino acids[3,4] and probably also because of the arrangement of the amino acids in the protein molecule.[3] Free primary amino groups, presumably from lysine, are essential to the action of pancreatic amylase.[3,4] There is also some indication that lysine may be lost early in the hydrolysis of this protein with an accompanying loss of amylase activity.[3,5]

It is evident that quantitative information about the amino acid make-up of the amylase is essential to an understanding of its action. This report gives results of analyses to determine the amino acid content of crystalline electrophoretically and enzymatically homogeneous, highly active swine pancreatic amylase.

Experimental

Crystalline Amylase.—Several batches of three times crystallized pancreatic amylase were prepared from swine pancreatin as described previously.[6] They all gave the same high amylase activity of 16,000.[6-8] After activity and other measurements had been made, the crystalline amylase was lyophilized[9] and the dry composite sample used for most of the analyses. Holding the lyophilized protein in a vacuum oven at 100° for 24 hours[10] caused a loss of weight of 1.8%. No further loss of weight occurred in 24 hours at 110° in the vacuum oven. Previous work had shown[6] the three times crystallized pancreatic amylase to be homogeneous by electrophoresis and by sedimentation measurements. In addition, evidence that the crystalline protein is enzymically homogeneous had been obtained by selective inactivation studies[6] and by comparisons of the solubility of the protein and of the active amylase.[6]

Hydrolyses.—For most of the work, and unless otherwise stated, the protein was hydrolyzed according to the method suggested by Block[11] and by Rees[12] by refluxing the protein at 120° and at ordinary pressure for 20 hours with 6 N hydrochloric acid. The hydrochloric acid was removed by repeated distillation under reduced pressure and the residue dissolved in 10% isopropyl alcohol. The completeness of the hydrolysis was confirmed by measurements of amino nitrogen using the micromanometric Van Slyke apparatus.[13-15] Other hydrolysis procedures[13-16] gave the same results as judged by distribution of nitrogen measurements.[13-15]

Methods of Analysis for Individual Amino Acids.—Although the yields of crystalline pancreatic amylase are considered good,[6] nevertheless, the amounts of the crystalline protein available for the analyses were limited. Therefore, the selection of methods for the analyses also was limited. Chromatographic techniques were chosen for most of the work because they promised reliable information with relatively small expenditure of protein. Several colorimetric methods also were employed and in addition the amylase was analyzed for its distribution of nitrogen.[13-15]

Many chromatographic procedures were investigated. Those finally adopted were patterned, in general, upon methods described by Block[11] and by Block and Bolling[17a] but with variations and adaptations proposed by other workers[18,19] and resulting from experience gained during the course of this investigation.[20] Before being adopted for use with the crystalline amylase, the methods of analysis were applied to crystalline egg albumin.[21] The methods finally selected gave values for the amino acid content of crystalline egg albumin[20] that, except for alanine, agreed well with values considered satisfactory for this protein as reported by other workers. However, the values for alanine were consistently higher than those reported by other workers for this protein.

In addition to the usual control of each chromatographic analysis by several concentrations of the pure amino acid[22]

(1) This investigation was supported in part by research grants from the Williams Waterman Fund; in part by a research grant from the National Institutes of Health, Public Health Service; in part by a research grant fron the Nutrition Foundation.

(2) The authors wish to thank Dr. R. J. Block for many helpful suggestions.

(3) J. E. Little and M. L. Caldwell, *J. Biol. Chem.*, **142**, 585 (1942); **147**, 229 (1943).

(4) M. L. Caldwell, C. E. Weill and R. S. Weil, THIS JOURNAL, **67**, 1079 (1945).

(5) H. C. Sherman and M. L. Caldwell, *ibid.*, **44**, 2926 (1922).

(6) M. L. Caldwell, Mildred Adams, Jo-fen Tung Kung and Gloria C. Toralballa, *ibid.*, **74**, 4033 (1952).

(7) H. C. Sherman, M. L. Caldwell and M. Adams, *ibid.*, **50**, 2529, 2535, 2538 (1928).

(8) M. L. Caldwell, S. E. Doebbling and S. H. Manian, *Ind. Eng. Chem., Anal. Ed.*, **8**, 181 (1936).

(9) Apparatus purchased from F. J. Stokes Machine Company, Philadelphia 20, Pa.

(10) J. G. Pierce and V. du Vigneaud, *J. Biol. Chem.*, **186**, 77 (1950).

(11) R. J. Block, *Anal. Chem.*, **22**, 1327 (1950).

(12) M. W. Rees, *Biochem. J.*, **40**, 632 (1946).

(13) (a) D. D. Van Slyke, *J. Biol. Chem.*, **83**, 425 (1929); (b) **9**, 185 (1911); (c) **10**, 15 (1911); (d) **12**, 275 (1912); (e) **12**, 295 (1912); (f) **23**, 407 (1915).

(14) D. G. Doherty and C. L. Ogg, *Ind. Eng. Chem., Anal. Ed.*, **15**, 751 (1943).

(15) J. P. Peters and D. D. Van Slyke, "Quantitative Clinical Chemistry," Vol. II, The Williams and Wilkins Company, Baltimore, Md., 1932, p. 385.

(16) V. Henriques and J. K. Gjaldbäk, *Z. physiol. Chem.*, **67**, 8 (1910).

(17) (a) R. J. Block and D. Bolling, "The Amino Acid Composition of Proteins and Foods," Charles C Thomas Company, Springfield, Ill., 1951, pp. 409–449; (b) p. 136.

(18) E. F. McFarren, *Anal. Chem.*, **23**, 168 (1951).

(19) E. F. McFarren and J. A. Mills, *ibid.*, **24**, 650 (1952).

(20) Unpublished.

(21) Purchased from Armour and Company, Chicago, Ill.

(22) All amino acids used as controls were found to be chromatographically "pure." Each gave only one spot in the chromatographic procedure. Each also gave R_f values with numerous solvents that