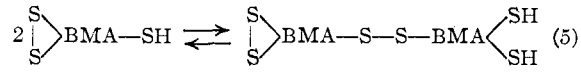
molecule has undergone a significant degree of swelling.

It may be pointed out that there are other reactions which take place in weakly acid solutions which may involve thiol–disulfide interchange. The sulfhydryl enzymes ficin[22] and papain[23] require activation by a reducing agent such as cysteine, and this activation can be produced at *p*H values as low as 4. Gorin, Dougherty and Tobolsky[24] have postulated that in general thiol–disulfide interchange is an intermediate step in the reduction of a disulfide by an excess of mercaptan. If this is true also in the activation of ficin and papain, these reactions serve as examples of exchange reactions proceeding rapidly in weakly acidic solution.

The slow reversal of the aggregation by raising the *p*H or by addition of $HgCl_2$ suggests that a monomer–dimer equilibrium exists, although it is evident that unknown factors exert an apparently erratic influence on either the rate of attainment of equilibrium or the extent of reaction at equilibrium, or in both. However on the assumptions that only the protein components are involved in the equilibrium state, we can try to distinguish between the following two possibilities: (1) BMA can react with itself or with BSA to give aggregates; or (2) BMA can react only with itself. The experimental data indicate that with pure BMA a maximum of 87% of dimer is formed at "equilibrium," while with ordinary BSA (57% BMA and 43% BSA), a maximum of 65% dimer is formed. For case 1, the figure for pure BMA leads to an equilibrium constant for the reaction (eq. 5) of $2K = 1.72 \times 10^5$ liters per mole, where K is the intrinsic constant for the reaction, assumed in what follows to be the same for



(5)

the disulfide group of both BMA and BSA. From this equilibrium constant we can calculate that the expected maximum yield of dimer from ordinary BSA is 70%. For case 2 a similar calculation leads to a maximum yield of only 47%. Thus this interpretation supports the view that the disulfide involved in the exchange can be supplied by either BSA or BMA.

Markus and Karush[25] have shown that a certain one of the 17 disulfide bonds in human serum albumin is particularly susceptible to reduction. Only this bond is reduced by mercaptoethylamine at *p*H 7 in the absence of detergent, whereas all the others undergo reduction when detergent is added. If bovine and human albumins behave similarly in this respect, it is an attractive hypothesis to assume that the thiol–disulfide exchange leading to aggregation at low *p*H involves the one sensitive disulfide bond in BSA.

The question arises as to the sources of the favorable free energy change accompanying the low *p*H aggregation reaction. In the mechanism proposed in equation 2, only an exchange of bonds occurs, and no new bonds are formed. Furthermore, there are factors which are adverse to the dimerization reaction. At low *p*H, BSA carries a large net positive charge, so that there is an electrostatic repulsion between BSA molecules which must be overcome in the formation of a dimer. In addition, one expects a considerable decrease in entropy in a protein dimerization reaction, primarily as the result of loss of translational and rotational entropy. One may speculate that any of a variety of additional factors may be involved, short-range dipole interactions, "hydrophobic" forces, etc., which have been proposed in other protein–protein interactions, but it is further possible in this case that when the intramolecular S–S bond is broken and replaced by an intermolecular bond, conformational changes may occur in the polypeptide chains near the original bond, which contribute to a favorable free energy change for the dimerization reaction.

(22) S. A. Bernhard and H. Gutfreund, *Biochem. J.*, **63**, 61 (1956).
(23) J. R. Kimmel and E. L. Smith, *J. Biol. Chem.*, **207**, 515 (1954).
(24) G. Gorin, G. Dougherty and A. V. Tobolsky, THIS JOURNAL, **71**, 3551 (1949).
(25) G. Markus and F. Karush, *ibid.*, **79**, 134 (1957).

NEW HAVEN, CONN.

[CONTRIBUTION FROM THE DEPARTMENT OF BIOPHYSICS, WEIZMANN INSTITUTE OF SCIENCE]

Poly-hydroxy-L-proline

BY JOSEPH KURTZ, GERALD D. FASMAN,[1] ARIEH BERGER AND EPHRAIM KATCHALSKI

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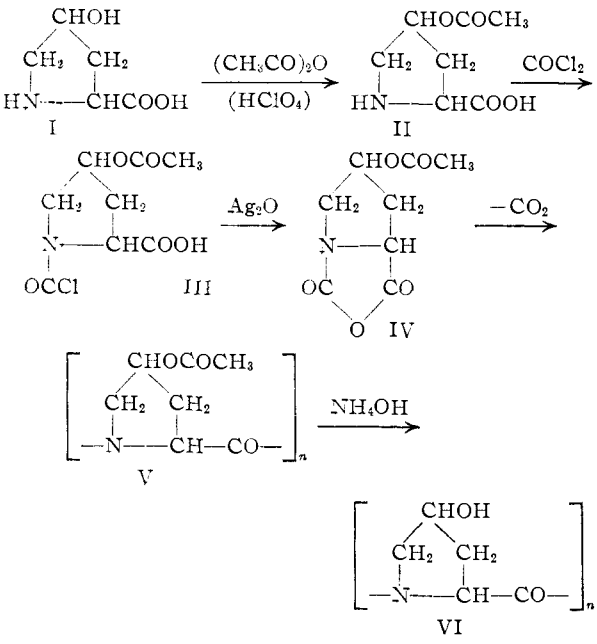
O-Acetyl-hydroxy-L-proline (II) yielded on treatment with phosgene the intermediate N-carbonyl chloride III which was cyclized by means of silver oxide to O-acetyl-N-carboxyhydroxy-L-proline anhydride (IV). Poly-O-acetylhydroxy-L-proline (V) was derived from IV on polymerization in pyridine. Deacetylation of V with aqueous ammonia gave rise to polyhydroxy-L-proline (VI). Osmotic and sedimentation measurements in aqueous solution gave average molecular weights of 10,600 and 10,700, respectively, for the sample of VI synthesized. O-*p*-Tolylsulfonylhydroxy-L-proline (X) was synthesized and converted into the corresponding N-carboxyanhydride XI, by means of phosgene and silver oxide. Poly-O-*p*-tolylsulfonylhydroxy-L-proline (XII) was obtained by polymerization of XI in pyridine. V showed mutarotation in formic acid. The specific optical rotation changed within several hours from +25° to −175°. The form with $[\alpha]^{25}D$ −175° could be reversed to the form with $[\alpha]^{25}D$ +25° by means of dimethylformamide. Mutarotation in acetic acid was observed also with XII. In this case reversal could be effected by means of pyridine.

In a previous publication[2] the synthesis of poly-L-proline was reported. This compound proved to be a useful high molecular weight model in the study of collagen and gelatin.[3] As collagen also

(1) Children's Cancer Research Foundation, 35 Binney Street, Boston, Mass. Weizmann Fellow, 1954–1955.
(2) A. Berger, J. Kurtz and E. Katchalski, THIS JOURNAL, **76**, 5552 (1954).
(3) P. M. Cowan and S. McGavin, *Nature*, **176**, 501 (1955); P. M. Cowan, S. McGavin and A. C. T. North, *ibid.*, **176**, 1062 (1955).

contains a considerable amount of hydroxy-L-proline it was desirable to synthesize polyhydroxy-L-proline and to investigate its chemical and physical properties.

The synthesis of polyhydroxy-L-proline is



O-Acetylhydroxy-L-proline (II) was obtained by the acetylation of hydroxy-L-proline (I) according to Sakami and Toennies.[4] The intermediate N-carbonyl chloride III, which was obtained by passing phosgene through a suspension of II in dioxane, did not cyclize to the desired N-carboxyanhydride IV on heating. Cyclization was effected by treatment of III with silver oxide in acetone solution. The crystalline O-acetyl-N-carboxyhydroxy-L-proline anhydride thus obtained yielded poly-O-acetylhydroxy-L-proline (V) on polymerization in pyridine. Polyhydroxy-L-proline (VI) was derived from V by deacetylation with ammonium hydroxide. The constitution of VI was ascertained by the quantitative yield of hydroxy-L-proline on acid hydrolysis.

Osmotic measurements in aqueous solution (0.1 N sodium acetate, pH 7.3) for the polyhydroxy-L-proline prepared gave an average molecular weight of 10,600 corresponding to an average degree of polymerization n = 94. Sedimentation, diffusion and partial specific volume measurements gave in the same solvent (ρ = 1.00451 g./cm.^{3}) S_{20} = 1.07 Svedberg units, D_{20} = 7.72 × 10^{-7} cm.^{2}/sec. and V = 0.683 cm.^{3}. From these data an average molecular weight of 10,700 (n = 95) was calculated.

An average molecular weight of 5750 (n = 51) was calculated from the values obtained by potentiometric end group titration in aqueous solution. Carboxyl end groups (pK 3.75) were found to be in slight excess over the imino end groups (pK 7.90). The discrepancy in the values for the molecular weight determined by physical and by chemical methods may be due to dimerization in aqueous solution or alternatively due to some strongly absorbed low molecular weight molecules.

Acetylation of the polyhydroxy-L-proline, n = 94, by means of acetic anhydride in dichloroacetic acid, in the presence of perchloric acid, yielded poly-O-acetylhydroxy-L-proline quantitatively. The infrared absorption spectrum of the product showed the same absorption bands as that of V. The specific viscosity of the reacetylated sample, η_{sp} = 0.16 (c 0.5%, in glacial acetic acid), was practically identical with that of the poly-O-acetylhydroxy-L-proline sample, V. This indicates that no appreciable degradation occurred during the deacetylation of V with concentrated aqueous ammonia.

Polyhydroxy-L-proline is very water soluble, but, unlike poly-L-proline, is not precipitated from its aqueous solution by trichloroacetic acid. It is insoluble in glacial acetic acid, cold formic acid and dimethylformamide. It can be precipitated from aqueous solution by dimethylformamide. The solubility behavior of polyhydroxy-L-proline appears to be determined by the presence of free hydrophilic hydroxyl groups and the absence of amide hydrogens.

The infrared absorption spectrum of poly-O-acetyl-hydroxy-L-proline in perfluorocarbon mulls showed absorption bands at 6.94 μ (1440 cm.$^{-1}$), 6.06 μ (1650 cm.$^{-1}$), 5.75 μ (1740 cm.$^{-1}$), 3.40 μ (2940 cm.$^{-1}$) and 2.86 μ (3500 cm.$^{-1}$). The band at 6.94 μ is due to CH deformation, the one at 6.06 μ due to the amide carbonyl and that at 5.75 μ due to the ester carbonyl stretching frequencies. The band at 3.40 μ is the CH stretching frequency, and the one at 2.86 μ (3500 cm.$^{-1}$) corresponds probably to the analogous band at 3495 cm.$^{-1}$ observed in poly-L-proline[2] and is most likely due to the OH band of adsorbed water.[5] In polyhydroxy-L-proline as compared with poly-O-acetyl-hydroxy-L-proline, the CH band at 6.93 μ remained unaltered, while the amide carbonyl showed a slight shift to lower frequency (6.17 μ, 1620 cm.$^{-1}$), probably because of hydrogen bonding with hydroxyl groups, and the ester CO-band disappeared entirely. A very strong band at 2.94 μ (3400 cm.$^{-1}$) due to the free hydroxyl groups appeared.

Poly-O-tolylsulfonylhydroxy-L-proline (XII) was synthesized as follows: N-carbobenzoxy-O-p-tolylsulfonylhydroxy-L-proline methyl ester (VII)[6] gave on alkaline hydrolysis N-carbobenzoxy-O-p-tolylsulfonylhydroxy-L-proline (VIII) which yielded on decarbobenzoxylation with hydrogen bromide in glacial acetic acid[7] O-p-tolylsulfonylhydroxy-L-proline (X). Alternatively X could be prepared by decarbobenzoxylation of VII, to yield O-p-tolylsulfonylhydroxy-L-proline methyl ester hydrobromide (IX), followed by alkaline hydrolysis. X was allowed to react with phosgene to yield the intermediate carbonyl chloride which, analogously to III, was cyclized with silver oxide to the O-p-tolylsulfonyl-N-carboxyhydroxy-L-proline anhydride (XI). Polymerization of XI in pyridine yielded poly-O-p-tolylsulfonylhydroxy-L-proline (XII). The p-tolylsulfonyl group of XII could not be removed by

(4) W. Sakami and G. Toennies, *J. Biol. Chem.*, **144**, 203 (1942).

(5) E. R. Blout and G. D. Fasman, "Glue and Gelatin Conference," Cambridge, England. 1957, in press.

(6) A. A. Patchett and B. Witkop, THIS JOURNAL, **79**, 185 (1957).

(7) D. Ben-Ishai and A. Berger, *J. Org. Chem.*, **17**, 1564 (1952).

phosphonium iodide in glacial acetic acid,[8] by 0.1 *N* sodium hydroxide or by boiling with benzylamine. Treatment of XII with metallic sodium in liquid ammonia[9] also failed to yield polyhydroxy-L-proline.

The method of cyclization with silver oxide described above for the preparation of IV and XI, was found useful also in the preparation of N-carboxy-L-proline anhydride from L-proline. This procedure, which is described in detail below, is much simpler and gives considerably higher yields than that described previously[2] where the anhydride was prepared from N-carbobenzoxy-L-proline.

As with poly-L-proline[10] mutarotation was observed with poly-O-acetylhydroxy-L-proline (V) and with poly-O-*p*-tolylsulfonylhydroxy-L-proline (XII). Poly-O-acetylhydroxy-L-proline (V), precipitated from the pyridine polymerization mixture by ether, showed a specific optical rotation of $[\alpha]^{25}D$ +25° (*c* 0.5), immediately after dissolution in 90% formic acid. The optical rotation of the solution decreased with time to a final value of $[\alpha]^{25}D$ −175° after six hours at room temperature (Fig. 1, curve A). Precipitation of the polymer from the highly levorotatory solution with ether yielded a preparation with $[\alpha]^{25}D$ −175°, immediately after dissolution in 90% formic acid.

Boiling of V ($[\alpha]^{25}D$ −175°) in dimethylformamide for several seconds, cooling and precipitation by ether yielded V ($[\alpha]^{25}D$ +25°), showing the original mutarotation in formic acid (Fig. 1, curve B).

A material with $[\alpha]^{25}D$ +10° was obtained when a concentrated solution of V($[\alpha]^{25}D$ −175°) in formic acid was diluted with pyridine (40 volumes), left for 24 hours at room temperature and precipitated with ether. The rate of mutarotation of this sample in 90% formic acid was faster than that of the polymer originally isolated.

The mutarotation in glacial acetic acid of poly-O-tolylsulfonylhydroxy-L-proline (XII) precipitated from pyridine by ether, $[\alpha]^{25}D$ 0°, immediately after dissolution in glacial acetic acid, is given in Fig. 1, curve C. The polymer precipitated by ether from its solution in acetic acid ($[\alpha]^{25}D$ −120°) could be transformed to XII ($[\alpha]^{25}D$ 0°) by dissolution in pyridine and precipitation with ether after 12 hours.

The specific rotation of polyhydroxy-L-proline (VI), $[\alpha]^{25}D$ −400° (*c* 0.5, in water), obtained from V ($[\alpha]^{25}D$ +25°) by treatment with aqueous ammonia, did not change within two weeks at room temperature. The treatment with aqueous ammonia seems therefore to produce the form of VI with the highest levorotation in water.

Experimental

All melting points are uncorrected.

O-Acetyl-N-carboxyhydroxy-L-proline Anhydride (IV).—Dry phosgene was passed at room temperature through a suspension of O-acetylhydroxy-L-proline[4] (4.0 g.) in anhydrous dioxane (80 ml.) until a clear solution was obtained (about 30 minutes). Excess phosgene was removed by a stream of dry carbon dioxide and the solvent was distilled off *in vacuo* at 45°. The N-carbonyl chloride III, left as an oily residue, was dissolved in dry acetone (150 ml.) and the resulting solution was stirred mechanically with silver oxide (4.0 g.)[11] until free of chloride (3 to 6 hours at room temperature). The solution was decanted from the precipitate,[12] evaporated to dryness *in vacuo*, and the crystalline residue was dissolved in ethyl acetate (5.0 ml.). The anhydride which crystallized out on the addition of petroleum ether (100 ml.) was collected, washed with petroleum ether and dried *in vacuo;* yield 4.0 g. (87%), m.p. 120° dec., $[\alpha]^{25}D$ −75° (*c* 1.0, in ethyl acetate).

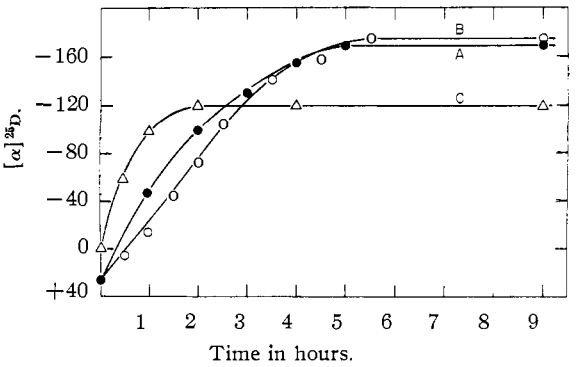


Fig. 1.—Mutarotation of poly-O-acetyl-hydroxy-L-proline (V) (curves A and B, explanation see text) in 90% formic acid and of poly-O-*p*-tolylsulfonyl-hydroxy-L-proline (XII) in glacial acetic acid (curve C).

Anal. Calcd. for $C_8H_9O_5N$: C, 48.2; H, 4.6; N, 7.0; CO_2, 22.1; neut. equiv., 199. Found: C, 48.4; H, 4.6; N, 6.9; CO_2, 21.2 (loss of weight on heating to 120° for 30 minutes); neut. equiv., 204, determined by titration in methanol with 0.1 *N* sodium methoxide using thymol blue as indicator.

Poly-O-acetylhydroxy-L-proline (V).—O-Acetyl-N-carboxyhydroxy-L-proline anhydride (1.0 g.) was dissolved in dry pyridine (10 ml.) whereupon carbon dioxide evolution began. The solution was stirred at room temperature for two days and the polymer formed was precipitated with ether (50 ml.). The precipitate was collected, washed with ether and dried; yield 0.7 g. (90%); $[\alpha]^{25}D$ +25° (*c* 1.0 in 90% formic acid) immediately after dissolution, changing to the final value of $[\alpha]^{25}D$ −175° (*c* 1.0, in 90% formic acid) within 6 hours.

Anal. Calcd. for $(C_7H_9O_3N)_n$: C, 54.2; H, 5.9; N, 9.0; CH_3CO, 27.7. Found: C, 53.0 (53.8 by wet combustion[13]); H, 6.0; N, 8.9; CH_3CO, 26.7.

The polymer had a specific viscosity of $\eta_{sp} = 0.14$ (*c* 0.5 g. in 100 ml. of glacial acetic acid) at 21°, as determined in an Ostwald viscometer.

Poly-O-acetylhydroxy-L-proline is soluble in glacial acetic acid, formic acid and dimethylformamide. It is insoluble in water and ethanol.

Polyhydroxy-L-proline (VI).—Poly-O-acetylhydroxy-L-proline (V) (0.6 g.) was suspended in 29% aqueous ammonia (9 ml.), and the solution which became clear after 4 hours was left overnight at room temperature and evaporated *in vacuo* to dryness. The solid residue was extracted with ethanol in a Soxhlet apparatus and then heated to 75° at 1 mm. for four hours in order to remove the last traces of acetamide and ammonium acetate. The preparation thus obtained (0.4 g., 92% of the theoretical) gave a negative Nessler test.

Anal. Calcd. for $(C_5H_7O_2N)_n$: C, 53.1; H, 6.2; N, 12.4. Found: C, 51.6 (52.0 by wet combustion[13]); H, 6.3; N, 12.3; $[\alpha]^{25}D$ −400° (*c* 1.0, in water).

Polyhydroxy-L-proline is soluble in water, hot formic acid

(8) E. Fischer, *Ber.*, **48**, 93 (1915); R. Schoenheimer, *Z. physiol. Chem.*, **154**, 203 (1926).

(9) V. du Vigneaud and O. K. Behrens, *J. Biol. Chem.*, **117**, 27 (1937).

(10) J. Kurtz, A. Berger and E. Katchalski, *Nature*, **178**, 1066 (1956).

(11) B. D. H. Laboratory reagent grade silver oxide was used.

(12) The precipitate was washed with acetone, with dilute nitric acid until the washings were silver free, with water and alcohol and dried to constant weight; yield of silver chloride 3.12 g., 94% of the theoretical, assuming a quantitative yield of O-acetyl-N-chlorocarbonylhydroxyproline.

(13) D. D. Van Slyke and J. Folch, *J. Biol. Chem.*, **136**, 509 (1940).

and dichloroacetic acid. It is insoluble in glacial acetic acid, alcohol and dimethylformamide. Unlike poly-L-proline, polyhydroxy-L-proline is not precipitated from its aqueous solution by trichloroacetic acid.

Preliminary experiments showed that in aqueous solution the polyhydroxy-L-proline obtained does not pass to a measurable extent through a dialysis membrane (Commercial cellophane dialysis tubing). For potentiometric titrations the polymer was dissolved in distilled water, dialyzed against distilled water for three days and then lyophilized. For osmotic, sedimentation and diffusion measurements, the polymer was dissolved in 0.1 *M* sodium acetate (*p*H 7.3) and dialyzed against this salt solution for three days.

The potentiometric titration of the end groups of polyhydroxy-L-proline was performed using a Radiometer (Copenhagen) Titrator in connection with an Agla Micrometer Syringe. Readings were taken at 0.05 *p*H interval. A solution of 35.5 mg. of polyhydroxy-L-proline in 2 ml. of water had a *p*H of 4.6. The polymer was titrated with 0.1 *N* KOH to *p*H 11.2 and with 0.1 *N* HCl to *p*H 2.6. Water blanks at the appropriate concentrations were run and the values subtracted from the titration data. The amount of imino end groups (*pK* 7.90) and carboxyl end groups (*pK* 3.75) found corresponded to equivalent weights of 7900 and 4500, respectively. From these data an average molecular weight of 5750 was calculated[14] assuming "water initiation."

The osmotic measurements were carried out with a modified Zimm osmometer.[15] Solutions of polymer (4.18, 3.10 and 1.04 mg. per ml.) in 0.1 *M* sodium acetate (*p*H 7.3) gave at 30° osmotic pressures of 110, 84 and 30 mm. of decalin, respectively, against the dialysate.

The sedimentation measurements were carried out in a Spinco Ultracentrifuge at 220,000 *g* using a synthetic boundary cell. A sedimentation constant of $S_{20} = 1.07$ Svedberg units was obtained for a solution of 5 mg. of polymer per ml. of 0.1 *M* sodium acetate.

The diffusion measurements were carried out in a Claesson cell[16] at 20° at a concentration of 10 mg. of polymer per ml. of 0.1 *M* sodium acetate; $D_{20} = 7.72 \times 10^{-7}$ cm.²/sec.

The partial specific volume was determined pycnometrically[17] in water at 20°, in the concentration range of 1 to 10 mg. per ml. A value of $V = 0.683$ cm.³ was obtained.

Hydrolysis of Polyhydroxy-L-proline.—Polyhydroxy-L-proline (VI) (21.0 mg.) was hydrolyzed in 6 *N* hydrochloric acid (1 ml.) at 110° for 24 hours. The amount of hydroxy-L-proline (23.5 mg.) determined[18] in the clear hydrolysate, corresponded to 97% of the theoretical. Paper chromatographic analysis, using butanol–acetic acid–water (4:1:5) as developer, gave with ninhydrin one yellow spot with an R_f of 0.12, identical with that of an authentic sample of hydroxy-L-proline.

The hydroxy-L-proline formed on the hydrolysis of VI gave an optical rotation of $[\alpha]^{25}D$ −43.9° (*c* 0.157, in 6 *N* hydrochloric acid). An authentic sample of hydroxy-L-proline gave under the same conditions $[\alpha]^{25}D$ −42.2°.

Acetylation of Polyhydroxy-L-proline.—To a solution of polyhydroxy-L-proline (100 mg.) in dichloroacetic acid (2 ml.), acetic anhydride (1 ml.) and 70% perchloric acid (0.1 ml.) were added. After two hours at room temperature the acetylated polymer was precipitated with ether (10 ml.), collected, washed with ether and dried *in vacuo;* yield almost quantitative.

Anal. Calcd. for poly-O-acetylhydroxyproline (V): CH_3CO, 27.7. Found: CH_3CO, 26.2.

The reacetylated polyhydroxyproline had a specific viscosity of $\eta_{sp} = 0.16$ (*c* 0.5 g. in 100 ml. of glacial acetic acid) at 21.0° and an optical rotation of $[\alpha]^{25}D$ −170° (*c* 0.43, in 90% formic acid). The solubility characteristics of this product were found to be identical with those of V.

N-Carbobenzoxy-O-*p*-tolylsulfonylhydroxy-L-proline (VIII).—N-Carbobenzoxy-O-*p*-tolylsulfonylhydroxy-L-proline methyl ester (VII)[6] (8.0 g.) was dissolved in methanol (90 ml.), 1 *N* sodium hydroxide (26 ml.) was added and the mixture kept at 0° overnight. After the addition of 1 *N* hydrochloric acid (26 ml.) the methanol was removed *in vacuo* and the mixture was extracted with ether (3 × 100 ml.). The dried ethereal solution was evaporated to dryness; yield 7.6 g. (98%).

Anal. Calcd. for $C_{20}H_{21}O_7NS$: neut. equiv., 420. Found: neut. equiv., 426, determined by titration in dioxane with 0.1 *N* sodium methoxide using thymol blue as indicator.

The benzylamine salt of VIII was obtained by adding an ethereal solution of benzylamine to an ethereal solution of VIII. Crystallization occurred on standing; m.p. 120–122°.

Anal. Calcd. for $C_{27}H_{30}O_7N_2S$: C, 61.5; H, 5.7; N, 5.3; neut. equiv., 527. Found: C, 61.6; H, 5.4; N, 5.7; neut. equiv., 521 determined by titration in dioxane with 0.1 *N* sodium methoxide using thymol blue as indicator.

O-*p*-Tolylsulfonylhydroxy-L-proline Methyl Ester Hydrobromide (IX).—N-Carbobenzoxy-O-*p*-tolylsulfonylhydroxy-L-proline methyl ester (VII) (2.0 g.) was dissolved in 33% hydrobromic acid in glacial acetic acid[7] (3 ml.) and allowed to stand at room temperature for two hours. On the addition of anhydrous ether (50 ml.) an oil separated out which crystallized on scratching. The supernatant was decanted and the precipitate was washed with dry ether. It was dissolved in anhydrous methanol (10 ml.) and ether was added to opalescence. The crystals, formed on cooling overnight in the refrigerator, were collected and dried *in vacuo* over phosphorus pentoxide; yield 1.7 g. (98%), m.p. 131–132°.

Anal. Calcd. for $C_{13}H_{15}O_5NSBr$: C, 41.1; H, 4.8; N, 3.7. Found: C, 41.2; H, 4.7; N, 3.1.

O-*p*-Tolylsulfonylhydroxy-L-proline (X). a. **From VIII.**—N-Carbobenzoxy-O-*p*-tolylsulfonylhydroxy-L-proline (VIII) (2.0 g.) was dissolved in 33% HBr in glacial acetic acid[7] (3.0 ml.). After half an hour at room temperature dry ether (30 ml.) was added and the oil which separated out was caused to crystallize by scratching. The supernatant was decanted, the precipitate washed twice with dry ether and dissolved in absolute ethanol (3.0 ml.). On the addition of a slight excess of pyridine a precipitate was formed which was twice washed with absolute ethanol and recrystallized from hot water; yield 0.53 g. (39%), m.p. 162–165°. The material was dried for four hours at 100° before analysis.

Anal. Calcd. for $C_{12}H_{15}O_5NS$: C, 50.5; H, 5.3; N, 4.9; neut. equiv., 285. Found: C, 50.6; H, 5.4; N, 4.9; neut. equiv., 276, determined by titration with 0.1 *N* perchloric acid in glacial acetic acid using thymol blue as indicator.

X is soluble in hot water and hot dimethylformamide. It is insoluble in methanol, ethanol and dioxane.

b. **From IX.**—O-*p*-Tolylsulfonylhydroxy-L-proline methyl ester hydrobromide (IX) (1.0 g.) was dissolved in water (10 ml.) and the solution cooled to 0°; 1.0 *N* sodium hydroxide (5.5 ml.) was added causing a precipitate to separate which was redissolved by the addition of methanol (10 ml.). The reaction mixture was left at 0° overnight and then brought to *p*H 7.0 by the addition of 1.0 *N* hydrochloric acid. The crystals formed were collected, washed with cold water and dried in a desiccator; yield 0.54 g. (72%), m.p. 163–165°.

O-*p*-Tolylsulfonyl-N-carboxyhydroxy-L-proline Anhydride (XI).—A stream of phosgene was passed through a suspension of O-*p*-tolylsulfonylhydroxy-L-proline (X) (2.0 g.) in anhydrous dioxane (40 ml.) at room temperature until a clear solution was obtained (30 minutes). Excess of phosgene was removed by means of a stream of dry carbon dioxide and the solvent was evaporated *in vacuo* at 40–45°. The oily residue was washed twice by trituration with petroleum ether, dissolved in dry acetone (75 ml.) and the resulting solution was mechanically stirred with silver oxide[11] (2.0 g.) until free of chloride (3 to 6 hours at room temperature). The solution was decanted from the precipitate, evaporated to dryness *in vacuo* and the crystalline residue was dissolved in dry ethyl acetate (2.0 ml.) at 50°. The crystals which separated out on the addition of petroleum ether (50 ml.) were collected, washed with petroleum ether and dried *in vacuo;* yield 2.0 g. (91%), m.p. 115° dec.

Anal. Calcd. for $C_{13}H_{13}O_6NS$: C, 50.1; H, 4.2; N, 4.5; S, 10.3; mol. wt., 311. Found: C, 50.0; H, 4.4; N, 4.3; S, 10.4; mol. wt., 320, determined by titration with 0.1 *N*

(14) M. Sela and A. Berger, THIS JOURNAL, **77**, 1893 (1955).

(15) E. Shimoni and A. Berger, to be published.

(16) S. Claesson, *Nature*, **158**, 834 (1946).

(17) T. Svedberg and K. O. Pedersen, "The Ultracentrifuge," Clarendon Press, Oxford, 1940, p. 57.

(18) R. E. Neuman and M. A. Logan, *J. Biol. Chem.*, **184**, 299 (1950).

sodium methoxide in methanol using thymol blue as indicator.[19]

Poly-O-*p*-tolylsulfonylhydroxy-L-proline (XII).—O-*p*-tolylsulfonyl-N-carboxyhydroxy-L-proline anhydride (XI) (2.0 g.) was dissolved in dry pyridine (20 ml.) and left to polymerize for two days at room temperature. The polymer formed was precipitated with ether (100 ml.), collected, washed with ether and dried *in vacuo;* yield 1.0 g. (58%). An average degree of polymerization $n = 30$, was calculated from end group analysis[14]; $[\alpha]^{25}D$ 0.0° (*c* 0.5, in glacial acetic acid) immediately after dissolution, changing to the final value of $[\alpha]^{25}D$ −120° (*c* 0.5, in glacial acetic acid) within three hours.

Anal. Calcd. for $(C_{12}H_{13}O_4NS)_n$: C, 53.9; H, 4.9; N, 5.2; S, 12.0. Found: C, 54.1; H, 4.5; N, 5.2; S, 11.9.

Poly-O-*p*-tolylsulfonylhydroxy-L-proline, $n = 30$, is soluble in glacial acetic acid, dimethylformamide and pyridine. It is insoluble in water and ethanol.

N-Carboxy-L-proline Anhydride.—Dry phosgene was passed at room temperature through a suspension of L-proline (3.0 g.) in dry dioxane (60 ml.) until a clear solution was obtained (one hour). Excess of phosgene was removed with dry carbon dioxide and the solution was concentrated *in vacuo* at 40°. The oily residue was dissolved in dry acetone (150 ml.) and the resulting solution was mechanically stirred with silver oxide[11] (3.0 g.) until free of chloride (6 hours at room temperature). The solution was decanted from the precipitate, evaporated to dryness *in vacuo* and the oily residue washed twice with petroleum ether. The oily material was dissolved in ethyl acetate (5 ml.), and petroleum ether (75 ml.) was added. The oil which separated out crystallized in long colorless needles in the refrigerator; yield 2.5 g. (68%), m.p. 45° dec. The m.p. did not change on recrystallization from ethyl acetate–petroleum ether. Molecular weight 143 (calcd. 141), determined by titration with 0.1 *N* sodium methoxide in methanol using thymol blue as indicator.[19]

(19) A. Berger, M. Sela and E. Katchalski, *Anal. Chem.*, **25**, 1554 (1953).

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REHOVOTH, ISRAEL

[CONTRIBUTION FROM THE BIOPHYSICS RESEARCH LABORATORY OF THE DEPARTMENT OF MEDICINE, HARVARD MEDICAL SCHOOL AND THE PETER BENT BRIGHAM HOSPITAL]

Spectrophotometric Evidence for Enzyme Inhibitor Complexation[1,2]

BY BERT L. VALLEE, THOMAS L. COOMBS AND ROBERT J. P. WILLIAMS[3]

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A mixture of ionic zinc and 1,10-phenanthroline demonstrates absorption maxima at 3125, 3275 and 3425 Å. in a special 5 cm. absorption cell; these maxima have not been previously described. Addition of 1,10-phenanthroline or of 8-hydroxyquinoline-5-sulfonic acid to the zinc metalloenzymes, carboxypeptidase, yeast alcohol dehydrogenase or liver alcohol dehydrogenase, or to the zinc protein complex, insulin, produces ultraviolet absorption spectra, which are entirely analogous to those observed with the ionic zinc–1,10-phenanthroline or 8-hydroxyquinoline-5-sulfonic acid system. These spectra therefore demonstrate the existence of protein–zinc–chelate mixed complexes. Spectral evidence indicates these complexes to be completely dissociable upon dialysis. 1,10-Phenanthroline and 8-hydroxyquinoline-5-sulfonic acid, previously shown to inhibit carboxypeptidase, yeast ADH and liver ADH, had been postulated to exert these inhibitory effects through interaction with the zinc atoms of the enzymes. The spectral data provide direct evidence for this proposed chemical interaction resulting in the formation of enzymatically inactive enzyme–zinc–chelator complexes. These findings offer additional evidence that zinc is an active site for these metalloenzymes and substantiate the previously proposed mechanisms for inhibition by metal chelators.

Introduction

While until recently carbonic anhydrase was the only enzyme known to contain zinc as an integral and functional part of the molecule,[4a] detection of functional zinc atoms in a number of other enzymes[4b] has greatly extended the hitherto unsuspected biochemical role of this element.

The firm binding of zinc to the apoenzyme and its stoichiometry in enzyme–coenzyme complexes have been discussed. Metal binding agents have been employed to inhibit the catalytic activity of such systems.[4b] These inhibition data *imply* that loss in activity occurs through complexing of the agent with the zinc of the metalloenzyme, but no *direct* evidence for the mechanism of this action is at hand.

Zinc ions in aqueous solution form complexes with either 1,10-phenanthroline (OP) or 8-hydroxyquinoline-5-sulfonic acid (80HQ5SA) which exhibit characteristic absorption spectra. The present data demonstrate similar absorption spectra for zinc containing proteins with these agents, constituting direct evidence for the postulated mechanism of inhibition.

Materials and Methods

Standard zinc solutions were prepared from weighed amounts of spectrographically pure zinc metal (Johnson Matthey Co.) dissolved in dilute metal-free hydrochloric acid.

Liver Alcohol Dehydrogenase (LADH) and Yeast Alcohol Dehydrogenase (YADH).—Commercial crystalline preparations were obtained from the Worthington Biochemical Corporation and used without further purification; the LADH had a zinc-to-protein ratio of 1650 μg. Zn/g. (1.9 g. atoms Zn/mole protein); the YADH had a zinc-to-protein ratio of 1760 μg. Zn/g. (4.1 g. atoms Zn/mole protein) and insignificant amounts of all other metals. Both enzymes, when ultracentrifuged in a Spinco Model E Ultracentrifuge, were more than 90% monodisperse and had turnover numbers of 8.2 and 240 moles DPNH per sec. per mole enzyme, respectively.

Carboxypeptidase (Cp).—A Worthington Biochemical Corporation three times crystallized preparation was twice recrystallized further by the method of Neurath, *et al.*[5] The

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(2) Presented in part before a meeting of the Federated Societies of Experimental Biology, March, 1957.

(3) Fellow of Wadham College, Oxford, England.

(4) (a) D. Keilin and T. Mann, *Biochem. J.*, **34**, 1163 (1940); (b) B. L. Vallee, *Advances in Protein Chem.*, **10**, 317 (1955).

(5) H. Neurath, E. Elkins and S. Kaufman, *J. Biol. Chem.*, **170**, 221 (1947).