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Synthetic Peptide Models of Enzyme Active Sites. I. *cyclo*-Glycyl-L-histidyl-L-serylglycyl-L-histidyl-L-seryl

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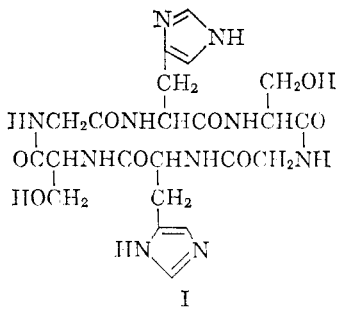
RECEIVED JANUARY 13, 1962

The synthesis of *cyclo*-glycyl-L-histidyl-L-serylglycyl-L-histidyl-L-seryl, a possible model enzyme, has been investigated. Improved methods for the synthesis of the tripeptide, glycyl-L-histidyl-L-serine, have been developed, and the cyclic dimerization of this compound and its derivatives by various reaction sequences, especially through the use of water-soluble carbodiimides, has been studied. A compound has been obtained, the properties of which correspond to those expected of the desired cyclic hexapeptide. This compound shows no significant activity above that expected for a histidine-containing peptide in the catalysis of the hydrolysis of *p*-nitrophenyl acetate under standard conditions. The decomposition of glycyl-L-histidyl-L-serine to L-serine and 3-(4 (or 5)-imidazolylmethyl)-2,5-piperazinedione under unusually mild conditions has been observed.

Various lines of evidence point to the conclusion that the active sites of certain hydrolytic enzymes, notably chymotrypsin and trypsin, partially consist of the functional groups on the side chains of the amino acids L-histidine and L-serine in some type of close steric relationship.[3] The exact nature of this relationship is unknown and, indeed, many of the proposed mechanisms for the catalytic activity of these enzymes require different kinds of relationships. In spite of this and many other uncertainties concerning the nature of these active sites, it seemed worthwhile to construct a relatively simple peptide embodying as many as possible of the known features of the active sites. If such a peptide could be shown to have a significant degree of catalytic activity in model enzyme reactions, it could prove useful as a model in the investigation of many enzymatic phenomena.[4]

The peptide which was chosen for study as a possible "model enzyme" was *cyclo*-glycyl-L-histidyl-L-serylglycyl-L-histidyl-L-seryl (I). The probable conformation of cyclic hexapeptides, based on the assumption of *trans* planar amide bonds



I

and maximum intramolecular hydrogen bonding, has been discussed.[5] Employing these assumptions, models of the cyclic hexapeptide I indicate that the side chains of L-histidine and L-serine are held in positions such that hydrogen bonding between the hydroxyl group and the imidazole group can take place. Thus, it is probable that I is similar to the postulated active sites of certain enzymes in that it consists of a peptide chain in which the side chain functional groups of L-histidine and L-serine are held in a close steric relationship. The present state of knowledge will not permit the assumption of similarities other than these.

The synthesis of I and the determination of possible esterase activity in a model reaction were undertaken. The synthesis of cyclic peptides has been well reviewed.[6,7] In the present case, it was decided that the best route to I was through the cyclic dimerization[8] of the tripeptide glycyl-L-histidyl-L-serine or a derivative. The third amino acid in the sequence, glycine, was chosen to enhance the cyclization by providing a relatively unhindered amine for the formation of the amide link with an activated carboxyl group, and to prevent undesirable steric effects which would result from having three bulky side chains in "axial" positions of the cyclic hexapeptide.[5]

The tripeptide was built up by a stepwise sequence, *i.e.*, the formation of the histidylserine bond first, followed by the formation of the glycylhistidine bond. Carbobenzyloxy-L-histidine azide[9,10] and dicarbobenzyloxy-L-histidine[11,12] with N,N'-dicyclohexylcarbodiimide[13] were used for the formation of the L-histidyl-L-serine bond. The latter method was found to be better with respect to yields and ease of product purification. For the formation of the glycyl-L-histidine bond, carbobenzyloxyglycine azide[14] and carbobenzyloxyglycine *p*-nitrophenylester were employed. The active ester method gave excellent yields of the blocked tripeptide, carbobenzyloxyglycyl-L-histidyl-L-serine methyl ester (V). The free tripeptide, glycyl-L-histidyl-L-serine (VI), was obtained in good

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(2) National Institutes of Health Fellow, 1958–1961.

(3) For recent reviews, see (a) D. E. Koshland, Jr., *Adv. Enzymol.*, **22**, 45 (1960); (b) M. L. Bender, G. R. Schonbaum and G. A. Hamilton, *J. Polymer Sci.*, **49**, 75 (1961).

(4) After the completion of this work, a similar approach to the investigation of enzyme mechanisms was reported by K. D. Kopple and D. E. Nitecki, *J. Am. Chem. Soc.*, **83**, 4103 (1961).

(5) R. Schwyzer and P. Sieber, *Helv. Chim. Acta*, **41**, 2186 (1958).

(6) R. Schwyzer, "Ciba Foundation Symposium, Amino Acids and Peptides with Antimetabolic Activity," 1958, p. 171.

(7) R. Schwyzer, *Rec. Chem. Progr. (Kresge-Hooker Sci. Lib.)*, **20**, 147 (1959).

(8) J. C. Sheehan and W. L. Richardson, *J. Am. Chem. Soc.*, **76**, 6329 (1954); J. C. Sheehan, M. Goodman and W. L. Richardson, *ibid.*, **77**, 6391 (1955).

(9) R. W. Holley and E. Sondheimer, *ibid.*, **76**, 1326 (1954).

(10) R. F. Fischer and R. R. Whetstone, *ibid.*, **76**, 5076 (1954).

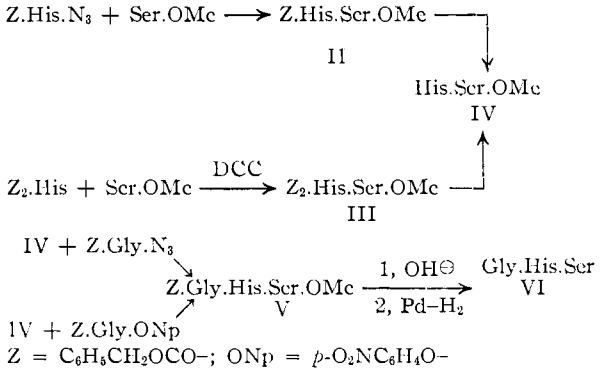
(11) A. Patchornik, A. Berger and E. Katchalski, *ibid.*, **79**, 6416 (1957).

(12) S. Akabori, K. Okawa and F. Sakiyama, *Nature*, **181**, 772 (1958).

(13) J. C. Sheehan and G. P. Hess, *J. Am. Chem. Soc.*, **77**, 1067 (1955).

(14) B. F. Erlanger and E. Brand, *ibid.*, **73**, 3508 (1951).

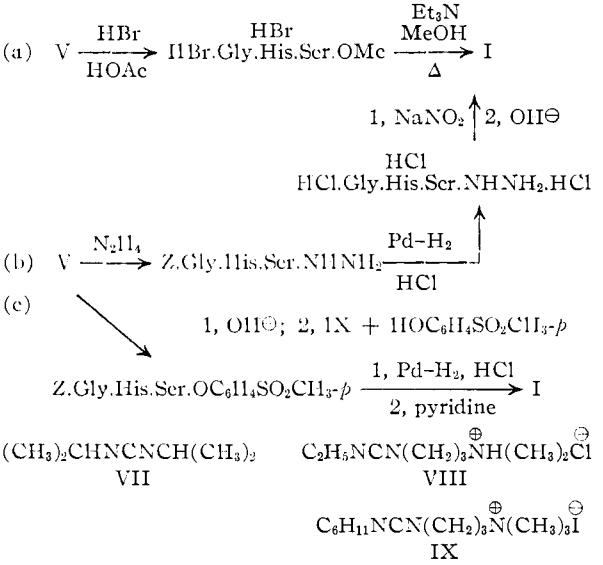
yield from V by ester hydrolysis followed by catalytic hydrogenolysis. These transformations are shown below.



In the investigation of various cyclic dimerization procedures, it was assumed that compound I could be detected by paper chromatography and paper electrophoresis as a spot containing an imidazole group (Pauly reagent) but no amino group (ninhydrin reagent). Since the formation of a cyclic monomer or a cyclic trimer (or polymer) is unlikely, this assumption seemed reasonable. In general, therefore, the results discussed for the following reactions have come from the interpretation of paper chromatographic and paper electrophoretic evidence.

A variety of methods for the cyclic dimerization reaction were tried. Preliminary experiments with glycyl-L-histidyl-L-serine methyl ester (method **a**[15]), glycyl-L-histidyl-L-serine azide (method **b**[8]) and glycyl-L-histidyl-L-serine *p*-methanesulfonylphenyl ester (method **c**[16]) did not appear promising. Method **a** led to results that could best be interpreted in terms of the formation of a series of polymeric peptides. Also, the hydrogen bromide–acetic acid removal of the carbobenzyloxy group was unsatisfactory in this case, giving a mixture of products. Method **b** also led to a complex product mixture probably consisting of polymers and products from the known decomposition of serine azides.[17] Several difficulties were encountered in the use of method **c**. No suitable solvent could be found for the final cyclic dimerization reaction, and the high temperatures required in this method were not compatible with the lability of the histidylserine peptide bond (see below). In none of the above cases was a compound detected, the properties of which corresponded to those expected of I.

Since the unblocked tripeptide VI could be prepared readily, it was decided to investigate the carbodiimide method of peptide bond formation. The only suitable pure solvent which could be found for VI was water. However, through the use of a water-dimethylformamide mixture, it was possible to try N,N′-diisopropylcarbodiimide (VII) in a homogeneous system (method **d**). The resulting product mixture was very complex (probably involving acylurea formation). The use of 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (VIII)[18] (method **e**) led to a mixture that was probably predominantly polymeric, but which also contained two minor components which were Pauly-positive and ninhydrin-negative. The reaction of the tripeptide VI with



1-cyclohexyl-3-(3-dimethylaminopropyl) carbodiimide methiodide (IX)[18] was investigated in some detail with respect to the effects of peptide concentration, carbodiimide concentration, reaction temperature and reaction time. There were three principal peptide products in the reaction mixture, the relative amounts of which depended on the conditions of the reaction.[19] One of these products (product B) satisfied the criteria established for the identification of the cyclic hexapeptide I (Pauly-positive and ninhydrin-negative). The concentration of one of the other components of the reaction mixture (product A) (Pauly- and ninhydrin-positive) was found to be greatest during the early stages of the reaction and to decrease as the reaction proceeded. This observation, combined with the paper chromatographic and paper electrophoretic behavior, suggest that this compound may be the linear hexapeptide, glycyl-L-histidyl-L-serylglycyl-L-histidyl-L-serine. It was found that, by using a 10–15% w./v. solution of the tripeptide VI in water, 3–4 equivalents of the carbodiimide IX and a reaction time of approximately 10 hours at room temperature (25–28°), the only detectable peptide product obtained was product B. Although the purification of this material was complicated by a number of factors, particularly its instability in solution, a fairly pure sample was isolated by a combination of chromatography on a cellulose column, preparative paper chromatography and precipitation from various solvents. This material gave a single, discrete, Pauly-positive, hypochlorite-positive, ninhydrin-negative spot on paper chromatography and paper electrophoresis. The infrared spectrum was typical for a higher pep-

(15) H. Brockmann, H. Tummes and F. A. von Metzsch, *Naturwiss.*, **41**, 37 (1954).

(16) R. Schwyzer and P. Sieber, *Helv. Chim. Acta*, **41**, 2199 (1958).

(17) J. S. Fruton, *J. Biol. Chem.*, **146**, 463 (1942).

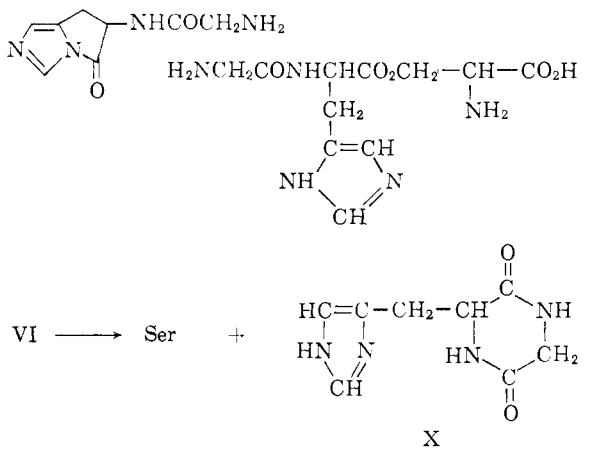
(18) J. C. Sheehan, P. A. Cruickshank and G. L. Boshart, *J. Org. Chem.*, **26**, 2525 (1961).

(19) These products had the folowing Rf values on paper chromatography with Whatman No. 1 paper and 1-butanol-pyridine-acetic acid-water (4:4:1:5): VI, 0.32; A, 0.44; B, 0.55; C, 0.62.

tide. Hydrolysis led to an approximately equimolar mixture of glycine, histidine and serine. The molecular weight, determined cryoscopically in dimethyl sulfoxide by a modification of the method of Schwyzer and Sieber,[20] was found to be 588 ± 10% (calculated molecular weight 562). There seems to be little doubt that this is the desired cyclic hexapeptide.

It is interesting to note the degree to which the composition of the product mixture depends on the nature of the carbodiimide which is employed. Also of interest is the observation that high dilution conditions are not necessary to prevent the formation of high polymers and, in fact, the use of high concentrations gave considerably simpler product mixtures.

During these investigations, it was observed that, on storage in water at room temperature, the tripeptide VI decomposed to form two new compounds, which have been identified as L-serine and the diketopiperazine of glycine and L-histidine (X). The cleavage of a peptide bond under such mild conditions was unexpected, and the reaction is undergoing further investigation.[21]



This reaction is of interest in that it can be interpreted to support the view that the presence of a serine hydroxyl and a histidine imidazole in proper steric relationship to an amide group can catalyze the decomposition of that amide bond. It is possible that a similar kind of decomposition occurs with glycyl-L-histidine amide.[22]

The results of the determination of the catalytic activity of the cyclic hexapeptide I and the tripeptide VI in the hydrolysis of *p*-nitrophenyl acetate under the conditions of Katchalski, *et al.*,[23] are summarized in Table I. It can be seen that I does not exhibit catalytic activity above that expected for a compound containing an imidazole group (note that the value for K_2 for VI should not be compared to that of I since, under these conditions, the free amine of VI is probably acetylated[24]). Evidently, the relationship between the side chain functional groups of histidine and serine which exists in compound I is not a sufficient condition for the catalysis of the hydrolysis of *p*-nitrophenyl acetate under the conditions employed.

TABLE I

HYDROLYSIS OF *p*-NITROPHENYL ACETATE IN 0.2 *M* PHOSPHATE BUFFER, *p*H 7.76 [*p*-NITROPHENYL ACETATE] = 2.82×10^{-5} *M*, 28.0 ± 0.5°

Catalyst	Concn. of catalyst, mole l.$^{-1}$ × 10^4	k_1, min.$^{-1}$ × 10^2	c, mole l.$^{-1}$ × 10^4	k_2, mole^{-1} min.$^{-1}$ l.$^{-1}$
None		4.74		
Histidine·HCl	5.55	8.01	5.55	5.86
I	2.16	7.96	4.32	7.41
VI	4.57	11.45	4.57	14.6
His·HCl[23]				5.7
Copoly His, Ser[23]				9.7
Chymotrypsin[23]				10^4

Experimental

Melting points were taken on a calibrated micro-hot-stage. Microanalyses were by Dr. S. M. Nagy and associates at M.I.T. Infrared spectra were obtained on Perkin–Elmer spectrophotometers, model 21 and Infracord 137. Spectra were taken of all isolated compounds and were in agreement with the assigned structures. Unless otherwise specified, paper chromatography was carried out on Whatman No. 1 chromatography paper by the ascending technique. The developing solvents most commonly used were 1-butanol–pyridine–acetic acid–water (4:4:1:5) and the 1-butanol phase of 1-butanol–acetic acid–water (4:1:5), hereafter BPAW and BAW, respectively. Spots were detected by spraying with ninhdrin, Pauly and *t*-butyl hypochlorite reagents. Electrophoresis was carried out on a modified Durrum apparatus at 2.5 kilovolts, 20–40 milliamps., 13–25°, 1–4 hours, in *p*H 1.81 buffer on Whatman No. 3MM paper.

In the following experiments, ion exchange resin was frequently used to remove inorganic salts from amino acid derivatives. This process, hereafter referred to as "desalting on ion exchange resin," consisted of dissolving the mixture in a small amount of water, placing the solution on a column of Dowex 50W-X8, 200–400 mesh, hydrogen form, of an appropriate size, and eluting with water until the eluate was anion free. The material remaining on the column was then eluted with an appropriate amount of 3% aqueous ammonia, excess ammonia was removed under reduced pressure, and the solution was lyophilized.

Dicarbobenzyloxy-L-histidyl-L-serine Methyl Ester (III).—To a stirred suspension of 1.47 g. (0.00945 mole) of L-serine methyl ester hydrochloride[25] in 35 ml. of methylene chloride was added 1.33 ml. of triethylamine. When only a faint cloudiness remained, the solution was cooled to 0° and, with continued stirring, 1.95 g. (0.00945 mole) of N,N'-dicyclohexylcarbodiimide was added, followed by a solution of 4.09 g. (0.00899 mole) of dicarbobenzyloxy-L-histidine methanolate[12] in 35 ml. of methylene chloride. Dicyclohexylurea began to precipitate after 3 minutes. The mixture was stirred at 0° for 2 hours, then at room temperature overnight. The dicyclohexylurea was removed by filtration (1.57 g. (74%)). The filtrate was washed successively with 50-ml. portions of 0.2 *N* hydrochloric acid, 1% sodium bicarbonate solution and water, dried, then concentrated to a solid under reduced pressure. The solid residue was dissolved in 50 ml. of hot methylene chloride, filtered, then crystallized by cooling and slowly adding 100 ml. of petroleum ether. The yield was 3.78 g. (76%), m.p. 149–150° (dec. $[\alpha]^{27}D + 12°$ (*c* 1 in methanol). Recrystallization from ethanol afforded colorless needles, 3.14 g., m.p. 152–153° dec.

Anal. Calcd. for $C_{26}H_{28}N_4O_8$: C, 59.53; H, 5.38; N, 10.68. Found: C, 59.62; H, 5.71; N, 10.84.

(20) R. Schwyzer and P. Sieber, *Helv. Chim. Acta*, **41**, 2190 (1958).

(21) Two possible intermediates for this decomposition are shown below, but it seems unlikely that either could arise under these conditions.

(22) D. G. Smyth, A. Nagamatsu and J. S. Fruton, *J. Am. Chem. Soc.*, **82**, 4600 (1960).

(23) E. Katchalski, G. D. Fasman, E. Simons, E. R. Blout, F. R. N. Gurd and W. L. Koltun, *Arch. Biochem. Biophys.*, **88**, 361 (1960).

(24) W. L. Koltun and F. R. N. Gurd, *J. Am. Chem. Soc.*, **81**, 301 (1959).

(25) Prepared by the method of R. L. M. Synge, *Biochem. J.*, **42**, 99 (1948).

L-Histidyl-L-serine Methyl Ester Dihydrochloride (IV).—To a solution of 0.525 g. (0.001 mole) of III in 25 ml. of methanol were added 2 ml. of *N* hydrochloric acid and 0.1 g. of 10% palladium-on-charcoal. The hydrogenolysis was carried out at room temperature and pressure, and was complete in 1 hour. The mixture was filtered through Celite then concentrated under reduced pressure to 0.322 g. of a pale yellow oil. All attempts to crystallize this oil failed, and it was used without further purification.

Carbobenzyloxyglycyl-L-histidyl-L-serine Methyl Ester (V). (a). Via Carbobenzyloxyglycine-*p*-Nitrophenyl Ester.—To a stirred solution of 2.93 g. (0.00889 mole) of IV in 60 ml. of dimethylformamide was added 2.49 ml. of triethylamine, followed by a solution of 2.93 g. (0.00889 mole) of carbobenzyloxyglycine *p*-nitrophenyl ester in 15 ml. of dimethylformamide. The solution turned yellow-orange immediately. The solution was stirred at room temperature for 12 hours and concentrated under reduced pressure at 35° to an orange-brown oil, which was dissolved in 20 ml. of methanol, diluted with a solution of 2.46 g. of sodium bicarbonate in 30 ml. of water, concentrated to a volume of 30 ml., then cooled at 0° overnight. The crystals were collected by filtration and washed successively with 40 ml. of 5% sodium bicarbonate solution and 100 ml. of water. Treatment of the crystals in warm methanol with activated charcoal, followed by two recrystallizations from methanol, yielded 3.35 g. (84%) of colorless prisms, m.p. 168–169° dec. (lit.[10] m.p. 163–165°), $[\alpha]^{26}D$ + 1.8° (*c* 2.7 in dimethylformamide).

Anal. Calcd. for $C_{20}H_{25}N_5O_7$: C, 53.68; H, 5.63; N, 15.65. Found: C, 53.92; H, 5.72; N, 15.83.

(b) Via Carbobenzyloxyglycine Azide.—To a cooled (−5°) solution of 0.219 g. (0.983 mmole) of carbobenzyloxyglycine hydrazide[8] in 1.2 ml. of acetic acid, 2.5 ml. of *N* hydrochloric acid and 3 ml. of water was added a cold solution of 0.09 g. of sodium nitrite in 1 ml. of water. After standing for 2 minutes at −5°, the colorless precipitate was dissolved in 6 ml. of ether, the layers were separated, and the ether layer was washed successively with cold 5-ml. portions of water, 3% sodium bicarbonate solution, and water. After drying over anhydrous magnesium sulfate at −5° for 10 minutes, the solution was added to a cooled (0°) solution of 0.288 g. (0.983 mmole) of L-histidyl-L-serine methyl ester dihydrochloride and 0.137 ml. of triethylamine in 10 ml. of dimethylformamide. The resulting solution was stirred at 0° for 3 hours, then at room temperature overnight. The pale yellow solution was filtered to remove a small amount of insoluble material, then concentrated at room temperature under reduced pressure to a yellow oil. The addition of 0.2 g. of sodium bicarbonate to a solution of the oil in 10 ml. of water followed by cooling yielded a solid, 0.144 g., m.p. 138–150° dec. Three recrystallizations from methanol yielded 0.043 g. (9.8%) of prisms, m.p. 166–167° dec., $[\alpha]^{26}D$ +0.4° (*c* 0.74 in dimethylformamide).

Carbobenzyloxyglycyl-L-histidyl-L-serine.—To a solution of 0.005 g. (0.011 mmole) of carbobenzyloxyglycyl-L-histidyl-L-serine methyl ester (from method **a**) in 0.43 ml. of methanol was added 0.43 ml. of 0.525 *N* sodium hydroxide. By paper chromatography of aliquots with butanone–pyridine–water (4:1:1.6), the hydrolysis was found to be complete in 30 minutes, and to give only one detectable product (R_f 0.64) (ester, R_f 0.94). For large-scale runs, proportional quantities of reagents were used. After a reaction time of 1 hour, two equivalents of *N* hydrochloric acid was added, and removal of the methanol under reduced pressure followed by lyophilization yielded a colorless solid. All attempts to crystallize this compound as the sodium salt, lithium salt, free acid or hydrochloride failed, and it was used for the next reaction without further purification.

Glycyl-L-histidyl-L-serine (VI).—To a solution of carbobenzyloxyglycyl-L-histidyl-L-serine (obtained from the hydrolysis of 1 g. (0.00223 mole) of carbobenzyloxyglycyl-L-histidyl-L-serine methyl ester) in 25 ml. of water and 20 ml. of methanol were added 5.6 ml. of *N* hydrochloric acid and 0.16 g. of 10% palladium-on-charcoal. Hydrogenolysis was carried out at room temperature and pressure for 1.75 hours (carbon dioxide evolution ceased after 1 hour). After removal of the catalyst by filtration through Celite and removal of the methanol by concentration under reduced pressure, the colorless solution was desalted on ion exchange resin. Two recrystallizations of the residue from water–ethanol yielded 0.574 g. (86%) of hygroscopic needles, m.p. 202–203° dec., $[\alpha]^{24}D$ +8.9°.

Anal. Calcd. for $C_{11}H_{17}N_5O_5$: C, 44.14; H, 5.73; N, 23.40. Found: C, 44.14; H, 5.91; N, 23.52.

Carbobenzyloxyglycyl-L-histidyl-L-serine Hydrazide.—A solution of 0.224 g. (0.5 mmole) of V and 0.125 ml. (2.5 mmoles) of hydrazine hydrate in 10 ml. of methanol was refluxed under nitrogen for 17 hours. During reflux, the product crystallized as fine needles. After cooling to room temperature, the crystals were collected by filtration, giving 0.185 g. (83%) of the hydrazide, m.p. 204–206° dec. Washing the crystals with 4 ml. of hot methanol yielded 0.161 g. (72%) of colorless needles, m.p. 208.5–209.5° dec.

Anal. Calcd. for $C_{19}H_{25}N_7O_6$: C, 51.00; H, 5.64; N, 21.92. Found: C, 51.07; H, 5.66; N, 21.84.

***cyclo*-Glycyl-L-histidyl-L-serylglycyl-L-histidyl-L-seryl (I). Preliminary Experiments. (a) Via Glycyl-L-histidyl-L-serine Methyl Ester.**—A solution of 0.006 g. (0.013 mmole) of carbobenzyloxyglycyl-L-histidyl-L-serine methyl ester in 1.5 ml. of 2 *N* hydrogen bromide in acetic acid was allowed to stand at room temperature for 45 minutes. The solution was concentrated under reduced pressure to a yellow oil, which was twice flushed with ethanol. A solution of the oil in 6 ml. of ethanol containing 1 drop of triethylamine was then refluxed under nitrogen for 24 hours. The reaction was followed by the removal of aliquots at intervals during the reflux. The aliquots were chromatographed on paper with BAW and the spots detected with Pauly and ninhydrin reagents. A Pauly-positive, ninhydrin-positive streak of R_f 0.00–0.17 became more intense as the reaction progressed. No ninhydrin-negative spot was detected.

(b) Via Glycyl-L-histidyl-L-serine Azide.—A solution of 0.309 g. (0.691 mmole) of carbobenzyloxyglycyl-L-histidyl-L-serine hydrazide in 15 ml. of methanol, 5 ml. of water and 2.14 ml. of *N* hydrochloric acid containing 0.05 g. of 10% palladium-on-charcoal was hydrogenated at room temperature and pressure for 1.25 hours. After removal of the catalyst by filtration through Celite and removal of the methanol by concentration under reduced pressure, the solution was lyophilized, giving a yellow solid. To a cooled (0°) solution of the solid in 5 ml. of water was added a cold solution of 0.0476 g. (0.691 mmole) of sodium nitrite in 2 ml. of water. After standing for 5 minutes at 0° with occasional swirling, the solution was diluted to a volume of 30 ml. with cold water, the *p*H was adjusted to 8 with 2 *N* sodium hydroxide, and the resulting solution was stored at 0° for 68 hours. The solution was then concentrated to a volume of 7 ml., cooled, and a small amount of insoluble material was removed by filtration. The filtrate was then desalted on ion exchange resin. The residue, 0.146 g., consisted of a complex mixture of Pauly-positive, ninhydrin-positive products (at least 6 spots of R_f 0.00–0.10 in BAW and, in BPAW, R_f 0.10–0.31, 0.36). An attempted fractionation of this material on a 2.7 × 53 cm. column of Dowex 50W-X2, 200–400 mesh, with *p*H 5.60 pyridine–formic acid buffer (flow rate 0.1–0.3 ml./minute, 400 fractions of 0.5–2 ml. collected) gave no separation of the components of the mixture. No Pauly-positive, ninhydrin-negative component was detected at any stage of the reaction.

(c) Via Glycyl-L-histidyl-L-serine *p*-Methanesulfonylphenyl Ester.—To a stirred, cooled (0°) solution of 0.0965 g. (0.223 mmole) of carbobenzyloxyglycyl-L-histidyl-L-serine and 0.057 g. (0.335 mmole) of *p*-methanesulfonylphenol[26,27] in 3 ml. of dimethylformamide was added a solution of 0.118 g. (0.335 mmole) of 1-cyclohexyl-3-(3-dimethylaminopropyl)-carbodiimide methiodide (IX)[18] in 2 ml. of dimethylformamide. The solution was stirred at 0° for 3 hours, then at room temperature for 2 hours. The pale yellow solution was concentrated at room temperature under reduced pressure to a yellow oil. The addition of 8 ml. of water to a solution of the oil in 2 ml. of ethanol followed by cooling caused the product to separate as an oil. The supernatant was decanted, and the oil was washed with two 2-ml. portions of water, then dried, giving 0.036 g. (27%) of a yellow oil, R_f 0.92 in BAW.

To a solution of the ester in 4 ml. of methanol containing 0.56 ml. of *N* hydrochloric acid was added 0.05 g. of pal-

(26) F. G. Bordwell and G. D. Cooper, *J. Am. Chem. Soc.*, **74**, 1058 (1952).

(27) F. G. Bordwell and P. J. Bouton, *ibid.*, **79**, 717 (1957).

ladium-on-charcoal, and the stirred mixture was hydrogenated at room temperature and pressure for 4 hours. After filtration and the removal of the methanol under reduced pressure, the pale yellow solution was lyophilized, giving a yellow oil, R_f 0.04 in BAW with a small amount of Pauly-pink contaminant at R_f 0.31.

A stirred suspension of 0.005 g. of the amino ester in 2.5 ml. of pyridine and 2.5 ml. of dimethylformamide was stored at room temperature for 42 hours, then at 90° for 24 hours. At intervals during this time, aliquots were removed, concentrated to dryness under reduced pressure, then chromatographed on paper with BPAW and BAW. Almost no reaction took place at room temperature. At 90°, a complex mixture of products was obtained, resulting in Pauly-positive, ninhydrin-positive streaks of R_f 0.00–0.49 and 0.60–0.84 in BPAW and of R_f 0.00–0.22 in BAW. A small Pauly-positive, ninhydrin-negative spot was detected with BAW, R_f 0.37.

(d) **Via Glycyl-L-histidyl-L-serine (VI) and N,N'-Diisopropylcarbodiimide.**—To a solution of 0.003 g. (0.01 mmole) of glycyl-L-histidyl-L-serine (VI) in 0.2 ml. of water and 0.3 ml. of dimethylformamide was added 0.01 ml. (0.063 mmole) of N,N'-diisopropylcarbodiimide (VII), and the resulting solution was stored at room temperature for 62 hours. During this time, aliquots were removed, quenched with dilute hydrochloric acid, concentrated to dryness, then chromatographed on paper with BPAW. A complex mixture of products was obtained, giving a Pauly-positive, ninhydrin-positive streak of R_f 0.14–0.91.

The use of diethyl phosphite in place of dimethylformamide gave a similar product mixture.

(e) **Via Glycyl-L-histidyl-L-serine (VI) and 1-Ethyl-3-(3-dimethylaminopropyl)-carbodiimide Hydrochloride (VIII)**—A series of experiments designed to test the effects of peptide concentration, carbodiimide concentration and reaction time were carried out. In each case, 1–3 mg. (0.003–0.01 mmole) of VI and the appropriate amount of carbodiimide were dissolved in sufficient water to bring the solution to the desired concentration, and stored at room temperature. Aliquots were removed at intervals during the reaction, quenched with dilute hydrochloric acid, concentrated under reduced pressure if necessary, then chromatographed on paper with BPAW. The following sets of conditions were investigated: weight % of VI, equivalents of VIII: 0.05, 2.7; 0.05, 13; 0.3, 3.5; 0.3, 15; 1.0, 2.7; 5.0, 3.0; 10, 3.4.

The reaction was found to proceed faster with higher conconcentrations of starting peptide with more equivalents of carbodiimide as expected, but the product mixture was found to be *independent* of these factors. In all cases, a Pauly-positive, ninhydrin positive streak of R_f 0.00–0.40 (VI, R_f 0.26) was obtained, with small Pauly-positive, ninhydrin-negative spots at 0.41 and 0.48.

(f) **Via Glycyl-L-histidyl-L-serine (VI) and 1-Cyclohexyl-3-(3-dimethylaminopropyl)-carbodiimide Methiodide (IX).**—A series of experiments designed to test the effects of tripeptide concentration, carbodiimide concentration, reaction temperature and reaction time were carried out. In each case, 1–3 mg. (0.003–0.01 mmole) of VI and the appropriate amount of carbodiimide were dissolved in sufficient water to bring the solution to the desired concentration, and the solution was stored at the desired temperature. Aliquots were removed from the reaction mixture at intervals during the reaction, quenched with dilute hydrochloric acid, lyophilized or concentrated under reduced pressure if necessary, then chromatographed on paper with BPAW. The following sets of conditions were investigated at room temperature: weight % of VI, equivalents of IX: 0.05, 1.2; 0.1, 0; 0.1, 1.2; 0.1, 1.9; 0.1, 7.0; 0.1, 15; 0.1, 29; 0.5, 1.2; 1.0, 3.0; 2.0, 3.0; 5.0, 3.0; 10.0, 3.0. At 40°: 0.1, 0; 0.1, 3.5; 0.1, 15. The results have been discussed above.

cyclo-**Glycyl-L-histidyl-L-serylglycyl-L-histidyl-L-seryl (I).**—A solution of 0.199 g. (0.665 mmole) of VI and 0.952 g. (2.71 mmoles) of IX in 3.0 ml. of water was stored at room temperature for 26 hours.

Partial removal of the urea was accomplished as follows: a mixture of 1200 ml. of 1-butanol, 750 ml. of water and 40 ml. of acetic acid was equilibrated and the layers were separated. A 2.2 × 31-cm. column of cellulose powder (Whatman, standard grade) was washed with the entire aqueous layer, then with 300 ml. of the 1-butanol layer. The reaction mixture was diluted with 5 ml. of the 1-butanol phase, placed on the column, then eluted with 250 ml. of the 1-butanol phase at a rate of 2 ml. per minute. During the elution, a pale yellow band came off. The column was then washed with 150 ml. of water and the eluate lyophilized, giving 0.382 g. of a pale yellow solid. Paper chromatography with BPAW showed the 1-butanol eluate to contain mostly urea, R_f 0.74, with a small amount of product, R_f 0.45, and the aqueous eluate to contain about equal parts of urea and peptide product.

The solid obtained from the aqueous eluate was further purified by preparative scale paper chromatography as follows: sheets of Whatman No. 3MM chromatography paper were washed by allowing them to stand for 12 hours in each of 15 l. of 0.1 *N* hydrochloric acid, 15 l. of 0.1 *N* sodium hydroxide and three 15-l. portions of water, then allowed to dry. The solid obtained above was dissolved in 1 ml. of water, placed equally on 7 sheets of washed paper in 0.5 × 46.5-cm. streaks, then the chromatograms were developed by the descending technique with BPAW. After the chromatograms were dried, strips were cut from the edges, and the locations of the bands were detected with hypochlorite spray (the streak due to the urea turned yellow when the chromatograms were dried, providing a convenient reference line for variations in R_f across the width of the paper). A 4-cm. strip including the region of the product was cut from each chromatogram, and eluted with water. Lyophilization of the eluates gave 0.237 g. of an oily solid. Paper chromatography in BPAW showed the major component to be the desired Pauly-positive, ninhydrin-negative material at R_f 0.46, with traces of other materials at R_f 0.29, 0.34, 0.40, 0.56 and 0.82.

A solution of the oily solid in 0.5 ml. of ethanol was filtered, diluted with 6 ml. of 2-propanol, cooled and filtered again. The filtrate was concentrated under reduced pressure to 0.216 g. of a pale yellow oil. The oil was then twice dissolved in 1 ml. of ethanol and precipitated by the addition of 6 ml. of acetone and cooling, giving 0.123 g. of an amorphous solid. Paper chromatography with BPAW showed the major component to be a Pauly-positive, ninhydrin-negative spot at R_f 0.48, with a trace contaminant at R_f 0.32. The solid was treated with 0.1 g. of activated charcoal in methanol solution at room temperature, then was thrice precipitated from 1 ml. of 2-propanol with 6 ml. of acetone and cooling, giving 0.071 g. of a colorless solid, m.p. 173–180° dec.

This material gave a single, discrete, Pauly-positive, ninhydrin-negative spot on paper chromatography with BPAW and on electrophoresis. The electrophoretic mobility of this material relative to that of the starting tripeptide VI (*e.g.*, 25.5 cm. and 37.0 cm., respectively) is the expected mobility for a compound of the desired type. Hydrolysis of a small sample with 20% hydrochloric acid at 110° for 24 hours showed it to contain approximately equimolar quantities of glycine, histidine and serine. The infrared spectrum was typical for higher peptides, showing a broad band at 1675–1550 cm.$^{-1}$ (KBr). A cryoscopic molecular weight determination in dimethylsulfoxide gave a value of 588 (calculated molecular weight 562).

3-(4(or 5)-Imidazolylmethyl)-2,5-piperazinedione (X).—To a stirred, cooled (0°) suspension of 0.602 g. (2.49 mmoles) of L-histidine methyl ester dihydrochloride[25] in 20 ml. of methylene chloride was added 0.75 ml. of triethylamine, followed by 0.910 g. (2.76 mmoles) of carbobenzyloxyglycine *p*-nitrophenyl ester. The solution was stirred at 0° for 1 hour, then at room temperature overnight. The yellow solution was concentrated under reduced pressure to a yellow oil, which was dissolved in 30 ml. of 0.5 *N* hydrochloric acid and extracted with four 10-ml. portions of ether. To the aqueous layer was then added 0.1 g. of 30% palladium-on-charcoal, and hydrogenolysis was carried out at room temperature and pressure for 2.0 hours. After filtration through Celite, the solution was lyophilized, giving a colorless solid. A solution of the solid in 60 ml. of methanol containing 3 ml. of triethylamine was then refluxed for 48 hours. Concentration of the solution under reduced pressure gave a mixture of needles and prisms. These crystals were desalted on ion exchange resin. Three recrystallizations of the residue from water–acetone gave 0.141 g. (29%) of colorless prisms, m.p. 242–243° dec., $[\alpha]^{28}D$ + 52° (*c* 1.36 in water).

Anal. Calcd. for $C_8H_{10}N_4O_2 \cdot 1/2H_2O$: C, 47.38; H, 5.44; N, 27.63. Found: C, 47.60; H, 5.43; N, 27.93.

Decomposition of Glycyl-L-histidyl-L-serine (VI) in Water.—A solution of 1 mg. of glycyl-L-histidyl-L-serine (VI) in 0.1 ml. of water was heated on a steam-bath for 3 hours, giving a mixture of unchanged VI and two decomposition products, one Pauly-negative and ninhydrin-positive, the other Pauly-positive and ninhydrin-negative. The former compound was identical in behavior with an authentic sample of L-serine on paper chromatography in BPAW, BAW and 1-butanol-butanone–water–diethylamine (10:10:5:1), and on electrophoresis. The latter compound was identical in behavior to X on paper chromatography in BPAW and BAW, and on electrophoresis.

Catalysis of the Hydrolysis of *p*-Nitrophenyl Acetate by L-Histidine, Glycyl-L-histidyl-L-serine (VI) and cyclo-Glycyl-L-histidyl-L-serylglycyl-L-histidyl-L-seryl (I).—The conditions of Katchalski, *et al.*,[23] were employed. At zero time, 0.500 ml. of a solution of 0.001587 g. of *p*-nitrophenyl acetate[28] in 10.0 ml. of purified, peroxide-free dioxane was mixed thoroughly with 15.0 ml. of 0.20 *M* phosphate buffer, *p*H 7.76, at 28.0 ± 0.5°, containing the desired histidine-containing compound. The changes in optical density at 400 mμ were followed on a Coleman Junior spectrophotometer. Readings were taken at 1–5 minute intervals for the first 50% of the reaction, then an infinite time reading was taken after 10 half-lives. The first-order rate constant, k_1, was calculated from eq. 1. It was found that k_1, in all

$$k_1 = \frac{1}{t} \ln \frac{OD_t}{OD - OD_t} \qquad (1)$$

cases (including the absence of a histidine-containing compound), dropped rapidly during the first 15% of reaction, then became constant within experimental error (probably a very gradual decrease in k_1). The values reported for k_1 were calculated from the data obtained between 20% and 40% reaction. The second-order rate constant, k_2, was calculated from eq. 2, where c = molarity of histidine residues and k_w = the first-order rate constant in the absence of a

$$k_2 = (k_1 - k_w)/c \qquad (2)$$

histidine-containing compound. The results are summarized in Table I, together with some of the data from Katchalski, *et al.*,[23] for comparison.

(28) F. Chattaway, *J. Chem. Soc.*, **134**, 2495 (1931).

[CONTRIBUTION FROM THE DEPARTMENT OF BIOCHEMISTRY, CORNELL UNIVERSITY MEDICAL COLLEGE, NEW YORK, NEW YORK]

Synthesis of D-Leucine-oxytocin, a Biologically Active Diastereoisomer of Oxytocin, and Demonstration of its Separability from Oxytocin upon Countercurrent Distribution[1]

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RECEIVED FEBRUARY 22, 1962

The synthesis of the D-leucine isomer of oxytocin is described. The required nonapeptide intermediate, S-benzyl-N-carbobenzoxy - L - cysteinyl - L - tyrosyl - L - isoleucyl - L - glutaminyl - L - asparaginyl - S - benzyl - L - cysteinyl - L - prolyl-D-leucylglycinamide, was obtained by coupling S-benzyl-N-carbobenzoxy-L-cysteinyl-L-tyrosyl-L-isoleucyl-L-glutaminyl-L-asparagine with S-benzyl-L-cysteinyl-L-prolyl-D-leucylglycinamide by means of dicyclohexylcarbodiimide. The protected nonapeptide so formed was reduced with sodium in liquid ammonia and subsequently oxidized to the octapeptide amide, D-leucine-oxytocin, which was purified by countercurrent distribution. The D-leucine-oxytocin was found to possess oxytocic and avian depressor activities of approximately 20 units per mg. and a milk-ejecting activity of at least 50 units per mg. The separability of this diastereoisomer from oxytocin upon countercurrent distribution was demonstrated by subjection of a mixture of the D-leucine-oxytocin and tritium-labeled oxytocin to this procedure.

A recent communication from this Laboratory[2] described the synthesis of tritium-labeled oxytocin from L-leucine labeled with a crude preparation of leucine of high specific activity prepared by the catalytic reduction of dehydro-L-leucine with tritium gas. It was hoped that radioactivity not due to L-leucine would be eliminated during the crystallizations of the intermediates in some of the steps of the synthesis as well as during the purification of the hormone by countercurrent distribution. In fact, the countercurrent distribution of the final radioactive hormone preparation gave no evidence of the presence of impurities. However, there was a possibility that the tritium-labeled leucine used in the radioactive synthesis may have contained some racemic leucine. If this were true, it is conceivable that any resulting diastereoisomer may not have been eliminated completely in the course of the purification of the crystalline protected peptide intermediates, carbobenzoxy-L-prolyl-L-leucine, carbobenzoxy-L-prolyl-L-leucylglycinamide and S-benzyl-N-carbobenzoxy-L-cysteinyl - L - prolyl - L - leucylglycinamide. Furthermore, any radioactive D-leucine diastereoisomer which might have been carried through the protected nonapeptide stage would then be present in the radioactive oxytocin as radioactive D-leucine-oxytocin and might not have been separated from the oxytocin during countercurrent distribution.

It therefore seemed desirable to synthesize the non-radioactive D-leucine isomer of oxytocin and study its separability from oxytocin upon countercurrent distribution under the conditions previously employed for the purification of the radioactive hormone. If the two diastereoisomers were separable under these conditions, it would appear unlikely that the final radioactive oxytocin preparation had been contaminated with the D-leucine isomer.

The D-leucine isomer of oxytocin was also of interest in its own right, since a study of its properties would make a basic contribution to the general question of the relationship of diastereoisomerism to the biological activity and other properties of the hormone. Investigations along these lines were initiated a few years ago in this Laboratory and are being continued at the present time.

For the synthesis of the D-leucine-oxytocin the same route was employed as for the synthesis of the tritium-labeled oxytocin.[2] Thus, the C-termi-

(1) This work was supported in part by a grant from the National Heart Institute, U. S. Public Health Service, Grant No. H-1675.

(2) V. du Vigneaud, C. H. Schneider, J. E. Stouffer, V. V. S. Murti, J. P. Aroskar and G. Winestock, *J. Am. Chem. Soc.*, **84**, 409 (1962).