[CONTRIBUTION FROM THE LABORATORIES OF THE ROCKEFELLER INSTITUTE FOR MEDICAL RESEARCH]

The Isolation and Determination of Structure of Peptides with Strepogenin Activity. II. The Disulfide of Leucylvalylcysteinylglycylglutamylarginine from Insulin[1]

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The disulfide of leucylvalylcysteinylglycylglutamylarginine was isolated in analytically-pure condition by the use of ion-exchange resins from an enzymic digest of insulin and was shown to have 200 units of strepogenin activity per mg. The peptide was pure by all criteria applied, and the structure was determined by amino acid analysis, oxidation, partial hydrolysis and identification of the degradation products.

Peptides with strepogenin activity[2] have been isolated previously from partial acid-hydrolyzates of insulin.[3] Since an enzymic digest would approximate more closely the peptides available to animals from the alimentary digestion of proteins, it was decided to isolate a peptide with strepogenin activity from an enzymic hydrolyzate. The manner in which this was accomplished will be described in this paper.

Experimental

Biological Assay.—*Lactobacillus casei* was used as described previously.[2,4] Wilson's liver fraction L was assigned the value of one unit per mg. and served as the standard. One unit was sufficient to give approximately half-maximal growth in ten ml. of basal medium, incubated for 20 hr. at 37°.

Enzymic Digestion.—Crystalline beef insulin (Lilly 535664)[5] (500 mg.) was dissolved in 100 ml. of H_2O containing 1 ml. of 88% formic acid. Crystalline pepsin (Worthington Biochemical Corp., Freehold, N.J.) (5 mg.) in 1 ml. of 0.1 *M* formic acid was added and the mixture incubated under toluene at 37° for 24 hr. The *p*H was then adjusted to 7.9 with 1 *M* NaOH and 5 mg. each of crystalline chymotrypsin and crystalline trypsin (both from Worthington) added. The *p*H was maintained by periodic addition of NH_4OH during incubation at 37° for 24 hr. The mixture was then filtered and adjusted to *p*H 3.9 with HCl. Such a preparation had a total of 22000 units of strepogenin activity.

Fractionation of the Peptides.—Fractionation was accomplished on the ion-exchange resins Dowex-2, Dowex-50 and IRC-50. The resins were prepared and poured into columns by the methods of Moore and Stein.[6,7] All buffers were saturated with toluene. The separation was followed by strepogenin assay of the eluted fractions.

Preliminary Experiments.—A column of Dowex-50 x 4 (200–400 mesh) (Na-form), 0.9 × 122 cm. was prepared and equilibrated with *p*H 4.0 sodium acetate, 0.1 *M* in Na^+. The insulin digest (*p*H 3.9) was passed through the column, followed by 200 ml. of *p*H 4.0, 0.1 *M* sodium acetate buffer. Up to this point, no strepogenin activity had passed through the column. The column was developed by the use of 0.1 *M* sodium acetate, the *p*H of which was made to rise gradually from 4 to 6 by means of a 250 ml. mixing bowl.[8] Half-hour fractions (3.8 ml.) were collected on a Technicon Time-Flow machine. At tube 430, 0.1 *M* sodium acetate, and at tube 493 0.1 *M* sodium bicarbonate replaced the *p*H 6 buffer in the reservoir. The elution pattern is shown in Fig. 1. The main peak emerging at *p*H 5.6 was then adjusted to *p*H 5.2 and poured on to a Dowex-50 column equilibrated and operated at a constant *p*H of 5.6 (0.1 *M* in Na^+). After about 100 ml. of buffer had passed through, a major and a minor peak of activity had emerged. The major peak was freed from salt on a Dowex-2 column in the general manner described in a subsequent section.

Material so obtained was never pure as judged by quantitative amino acid analysis. The major amino acid constituents were leucine, valine, cystine, glycine, glutamic acid and arginine. In subsequent experiments which led to the isolation of the pure compound, both strepogenin assay and Sakaguchi[9] analysis for arginine were used to follow the purification.

Isolation of Pure Peptide. Column 1.—A column of IRC-50 (Amberlite XE 64) resin (50–500 mesh) (Na-form), 0.9 × 90 cm., was prepared and equilibrated with *p*H 4.0 sodium acetate buffer, 0.1 *M* in Na^+. The enzymic digest of 500 mg. of insulin (*p*H 3.9) was passed through the column at a flow rate of about 8 ml. per hr. When all of the sample had passed into the column, elution was begun with *p*H 4.0 sodium acetate buffer, and half-hour fractions were collected. At tube 131, *p*H 5.0 sodium acetate, 0.1 *M* in Na^+ replaced the *p*H 4.0 buffer. The *p*H of the buffer was changed to 6.0 at tube 270, to 6.9 at 614, to 7.9 at 697, and at tube 712, 0.1 *M* NaOH replaced the buffers. In all these changes, [Na^+] was maintained constant at 0.1 *M*. The elution pattern is shown in Fig. 2.

The biologically inactive arginine-containing fraction in tubes 40–90 was desalted on Dowex-2 as described below and purified by electrophoresis on filter paper[10] at *p*H 5.0 (pyridine acetate buffer). The material that did not move at this *p*H but gave a positive ninhydrin and Sakaguchi reaction was eluted and refluxed with 6 *N* HCl overnight. Two-dimensional chromatography of the hydrolyzate in *sec*-butyl alcohol–ammonia–water followed by *sec*-butyl alcohol–formic acid–water[11] revealed the presence of leucine, valine, glycine, glutamic acid, arginine, phenylalanine, tyrosine and threonine. The biological inactivity of this material showed clearly that the mere presence of an arginine-containing peptide was not sufficient for activity.

Column 2.—A column of Dowex-50 x 4 resin (200–400 mesh) (Na-form), 0.9 × 65 cm., was prepared and equilibrated with *p*H 4.0 sodium acetate buffer, 0.1 *M* in Na^+. The contents of tubes 140 to 210 from column 1 were adjusted to *p*H 3.9 and passed through the column. Development was achieved by the use of the same *p*H-gradient as described in the Preliminary Experiments (250-ml. mixing bowl filled with *p*H 4.0 buffer, reservoir *p*H 6.0 buffer). Half-hour fractions (2.7 ml.) were collected. At tube 266, 0.1 *M* sodium acetate (unbuffered) and at tube 360, 0.1 *M* sodium bicarbonate replaced the solvent in the reservoir. The elution pattern is shown in Fig. 3.

Column 3.—A column of Dowex-2 x 4 (200–400 mesh) (acetate-form), 0.9 × 50 cm., was prepared and equilibrated with *p*H 8.9 ammonium acetate, 0.1 *M* in acetate. The sample to be desalted (*e.g.*, tubes 174 to 194 from column 2) was adjusted to *p*H 9.2 with ammonia, poured through the column, and followed by about 200 ml. of *p*H 8.9 ammonium acetate. The column was developed with *p*H 6.0 ammonium acetate, 0.1 *M* in acetate, and half-hour fractions (5.8 ml.) collected. Since NH_4^+ interfered with the Sakaguchi reaction, an aliquot (0.2 ml.) of each eluted fraction was pipetted into a test-tube, 0.2 ml. of 10% NaOH added and

(1) This investigation was supported in part by a grant from the U. S. Public Health Service.

(2) H. Sprince and D. W. Woolley, *J. Exp. Med.*, **80**, 213 (1944).

(3) R. B. Merrifield and D. W. Woolley, THIS JOURNAL, **78**, 358 (1956).

(4) D. W. Woolley, *J. Biol. Chem.*, **172**, 71 (1948).

(5) Kindly supplied by Dr. Otto Behrens of Eli Lilly & Co., Indianapolis, Ind.

(6) C. H. W. Hirs, S. Moore and W. H. Stein, *J. Biol. Chem.*, **195**, 669 (1952).

(7) C. H. W. Hirs, S. Moore and W. H. Stein, *ibid.*, **200**, 493 (1953).

(8) S. Moore and W. H. Stein, *ibid.*, **211**, 893 (1954).

(9) C. J. Weber, *ibid.*, **86**, 217 (1930).

(10) H. G. Kunkel and A. Tiselius, *J. Gen. Physiol.*, **35**, 89 (1951).

(11) W. Hansmann, THIS JOURNAL, **74**, 3181 (1952).

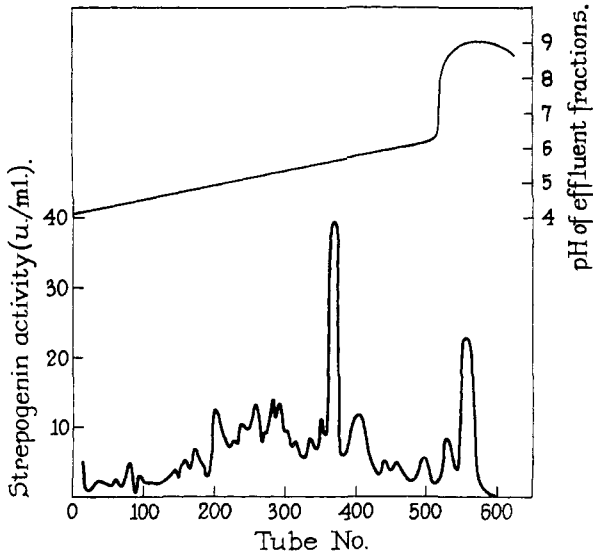

Fig. 1.—Fractionation of strepogenin activity from an enzymic insulin digest by chromatography on Dowex-50 x 4. Tube volume = 3.8 ml.

the contents of the tube evaporated to dryness *in vacuo*. Then, 0.6 ml. of water was added, followed by 0.1 ml. of the α-naphthol reagent as described elsewhere.[9] Tubes 12 to 15 contained the bulk of the active material which was freed from ammonium acetate by sublimation in high vacuum at 40°. Strepogenin assay and color intensity from the Sakaguchi reaction on an aliquot of the purified peptide so obtained indicated a biological activity of 130 units per micro-mole of arginine. Similar analyses before passage through Dowex-2 gave the same value. The Dowex-2 column therefore served primarily for desalting rather than for separation from other peptides.

Quantitative Amino Acid Analysis.—One and two tenths mg. of peptide prior to desalting on Dowex-2 was refluxed with 30 ml. of constant boiling HCl for 16 hr. The acid was then removed under reduced pressure, and the hydrolyzate analyzed by the ion-exchange method of Moore and Stein.[12] As shown in Table I, essentially equimolar ratios

TABLE I

QUANTITATIVE AMINO ACID ANALYSIS

Amino acid	Micro-moles	Ratio (leucine = 1)
Glutamic acid	1.78	1.03
Glycine	1.77	1.03
Half-cystine	1.80	1.04
Valine	1.71	0.99
Leucine	1.72	1
Arginine	1.75	1.01
Aspartic acid	0.074	0.04
Phenylalanine	0.037	0.02

of six amino acids were found. Since arginine is not recovered quantitatively by the Dowex-50 column, a separate aliquot of the sample was hydrolyzed and the arginine determined with the Sakaguchi reagents.

Evidence of Purity.—The purity of the peptide was demonstrated by a number of methods. As shown in Table I, six amino acids in virtually equimolar amounts were present, and only relatively trace amounts of phenylalanine and aspartic acid were detected. The latter two amino acids could not be detected when a similar amount of hydrolyzed peptide was chromatographed on paper. The fact that the effluent curves for strepogenin activity and arginine-containing material practically coincided (see Fig. 3, tubes 174 to 194) was additional evidence for a high degree of purity of the isolated material. The peptide was also shown to be electrophoretically[10] homogeneous between *p*H 1 and 8 (paper electrophoresis). With the satisfaction of these

(12) S. Moore and W. H. Stein, *J. Biol. Chem.*, **192**, 663 (1951).

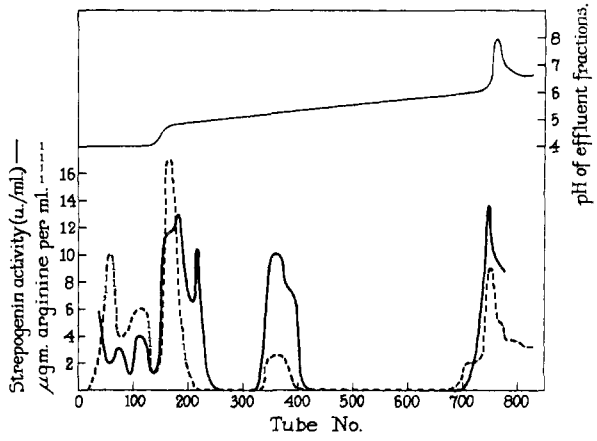

Fig. 2.—Fractionation of strepogenin activity from an enzymic insulin digest by chromatography on IRC-50. Tube volume = 4.0 ml.

criteria of purity, a study of the amino acid sequence of the peptide was undertaken.

Structure of the Peptide.—Since the peptide contained cystine (or cysteine), the possibility of disulfide interchange[13] could not be overlooked. Therefore, before attempts were made to determine the amino acid sequence by a combination of dinitrophenylation and partial hydrolysis, it was thought best to oxidize the cystine to cysteic acid. Because this was done, it was necessary to keep in mind the failure of DNP-peptides containing cysteic acid to extract into organic solvents.

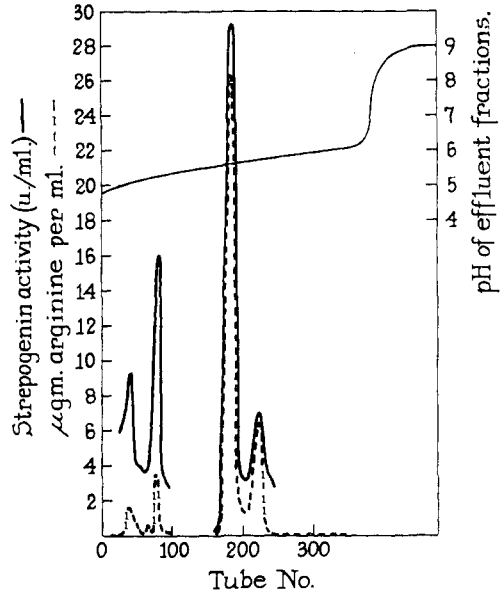

Fig. 3.—Fractionation of strepogenin activity from tubes 140–210 of Fig. 2 by chromatography on Dowex-50 x 4. Tube volume = 2.7 ml.

Oxidation of the Peptide.—The desalted peptide (0.7 mg.) was oxidized at −10° with performic acid as described by Hirs.[14] The product was dissolved in a little water and chromatographed in phenol–water. No free cysteic acid (R_f = 0.10) could be detected; all material remained at the origin. Consequently, a cysteinyl residue bound only by disulfide linkage to the rest of the chain could not have been present.

The oxidized peptide was purified by paper electrophoresis[10] in *p*H 5.0 pyridine acetate buffer (7 v., per cm., 3 hr.,

(13) A. P. Ryle and F. Sanger, *Biochem. J.*, **60**, 535 (1955).

(14) C. H. W. Hirs, *J. Biol. Chem.*, **219**, 611 (1956).

room temp.). Two spots giving both a positive ninhydrin and Sakaguchi reaction were found 1.5 cm. from the origin toward the cathode (electrophoretically neutral) (spot A) and 2 cm. toward the anode (spot B). Cysteic acid moved 10.5 cm. toward the anode under these conditions. Spot A was assumed to represent unoxidized peptide. Spot B represented 80% of the material applied to the paper (based on Sakaguchi color intensity) and was assumed to be the desired oxidized peptide.

Dinitrophenylation and Partial Hydrolysis of Oxidized Peptide.—The oxidized peptide was treated with 2,4-dinitrofluorobenzene (DNFB) and triethylamine for 4 hr. as described by Sanger.[15] After extraction with ether and benzene, the water layer was evaporated to dryness and dried *in vacuo* over P_2O_5. The peptide then was hydrolyzed partially with 5 ml. of concentrated HCl at 37° for 48 hr. The hydrolyzate was evaporated and dried over P_2O_5 and NaOH. The residue was dissolved in 3 ml. of water and extracted three times with ethyl acetate. Chromatography in *t*-amyl alcohol–*p*H 6 phthalate buffer[16] and *n*-butanol–ammonia revealed the presence of DNP-leucine (R_f = 0.9) and some slowly moving yellow material. The aqueous and organic layers were then analyzed by paper electrophoresis as shown in Table II. The spots were eluted with 6 *N* HCl and refluxed overnight. The hydrolyzates were concentrated to dryness, dissolved in water and extracted with ethyl acetate. The organic layer was chromatographed in buffered *t*-amyl alcohol,[16] the aqueous layer in phenol–water and *sec*-butyl alcohol–ammonia.[11] In each case, the only DNP-amino acid to appear in the organic extract was DNP-leucine. From the results of these chromatograms the amino acid sequences shown in Table II were assigned. The composition of the peptides was in keeping with their relative mobilities in electrophoresis. The fact that the only DNP-amino acid to be isolated was DNP-leucine, located leucine in the amino-terminal position. From the quantitative analysis of a hydrolyzate of the peptide, it was evident that the amino acids were present in 1:1 ratios. Because the original peptide gave no positive test for SH with nitroprusside prior to reduction, the isolated material must have been a disulfide. These data, in addition to the amino acid sequences of peptides isolated from a partial hydrolyzate of the DNP-oxidized peptide, permit only one structure to be assigned that is consistent with all data. That structure is the disulfide of leucylvalylcysteinylglycylglutamylarginine. Evidently spot 7 in Table II represented unhydrolyzed peptide.

TABLE II

ELECTROPHORETIC SEPARATION[a] OF A PARTIAL HYDROLYZATE OF THE OXIDIZED DNP-PEPTIDE

Spot no.	Distance[b] migrated (cm.)	Color[c]	Amino acid composition and sequence
		Ethyl acetate layer	
1	+ 8.7	Yellow	DNP-leu
2	+ 6.0	Yellow	DNP-leu-val
3	+11.0	Yellow	2,4-Dinitrophenol
		Water layer	
4	+15.5	Yellow	DNP-leu-val-cya[d]
5	+13.0	Yellow	DNP-leu-val-cya-gly
6	+ 4.5	Colorless	val-cya-gly-glu
7	+10.0	Yellow	DNP-leu-val-cya-gly-glu-arg
8	− 3.8	Colorless	gly-glu-arg
9	− 2.0	Colorless	val gly[e]
10	−16.0	Colorless	arg
		Standards	
Cya	+14.5		
DNP-leu	+ 8.0		
Gly	− 2.0		
Arg	−16.0		
2,4-Di-nitro-phenol	+11.0		

[a] Run in 0.1 *M* pyridine acetate, *p*H 5.0, for 120 min. at 13 volts per cm., room temp. [b] + indicates movement toward the anode, − toward the cathode. [c] "Yellow" indicates a DNP-derivative. "Colorless" indicates peptides visualized with ninhydrin. [d] "Cya" denotes cysteic acid. [e] Since this was a neutral spot, glycine and valine were probably present as the free amino acids in the partial hydrolyzate.

Dinitrophenylation of the Peptide.—The unoxidized peptide was treated with DNFB and triethylamine as above,[15] extracted with ether and then with ethyl acetate. The organic layer was colorless; the presence of arginine kept the peptide in the aqueous phase. The DNP-peptide was refluxed overnight with 6 *N* HCl, the acid evaporated and the residue extracted with ethyl acetate. Chromatography in buffered *t*-amyl alcohol[16] revealed the presence of DNP-leucine as the only DNP-amino acid. This result again excluded the possibility that a cysteine residue was attached to the peptide by disulfide linkage. Had this been the case, some mono-DNPcystine would have been formed. When synthetic mono-DNPcystine[17] was refluxed with 6 *N* HCl, some bis-DNPcystine and free cystine were formed.[18]

Additional evidence favoring the symmetrical disulfide structure was the following: Treatment of the unsubstituted peptide with sodium in liquid ammonia[18] resulted in no appreciable loss in growth-factor activity (90% of activity was recovered). If a cysteinyl residue attached by disulfide linkage to the peptide chain had been present and essential for the biological activity, one would have expected a significant loss of activity. These results corroborated the results from the quantitative amino acid analysis, which indicated a 1:1 ratio between half-cystine (cysteine) and any of the other amino acids.

Biological Activity of the Peptide.—Table III shows that the strepogenin activity of various preparations of the peptide was approximately 200 units per mg. Sufficient material for an assay based on dry weight of pure peptide was never available. The specific activity was calculated on the basis of the color intensity obtained with Sakaguchi's reagents[9] or with ninhydrin after hydrolysis. Because the Sakaguchi color intensity was the same before and after hydrolysis, it was unnecessary to hydrolyze the peptide before analysis for arginine. In order to check the accuracy of the assays, synthetic serylhistidylleucylvalylglutamic acid,[19] previously reported to possess 80 units per mg., was assayed alongside the arginine peptide with results shown in Table III. As with most pure peptides,[20] the dose-response curve flattened out sooner than with liver standard. The activity values were the averages calculated from turbidity measurements at levels up to about 0.7 μg. per ml. of sample and were reproducible within 10% from one experiment to another.

TABLE III

BIOLOGICAL ACTIVITY OF THE PEPTIDE AT VARIOUS STAGES OF PURIFICATION

Sample	Units/micro-mole arginine	Units per mg.
Column 2, tubes 173–195	130	190
Column 3, tubes 12–15. First prepn.	135	200
Column 3, tubes 12–15. Second prepn.	132	195
Ser-his-leu-val-glu		80

Does the Peptide Contain Glutamine or Glutamic Acid?—Since ammonia was not rigorously excluded during the isolation and hydrolysis of the peptide, an analysis for ammonia in the hydrolyzate could not give an indication of the presence of an amide in the peptide. More definitive evidence was therefore sought. The peptide was refluxed with 0.1 *M* HCl for ten minutes. Under such conditions, a glutamine containing peptide (*viz.*, L-glutaminyl-L-asparagine)[21] was converted from an electrophoretically neutral,

(15) F. Sanger and E. O. P. Thompson, *Biochem. J.*, **53**, 353 (1953).
(16) S. Blackburn and A. G. Lowther, *ibid.*, **48**, 126 (1951).
(17) F. R. Bettelheim, *J. Biol. Chem.*, **212**, 235 (1955).
(18) V. du Vigneaud, C. Ressler, J. M. Swan, C. W. Roberts and P. G. Katsoyannis, THIS JOURNAL, **76**, 3115 (1956).
(19) R. B. Merrifield and D. W. Woolley, *ibid.*, **78**, 4646 (1956).
(20) D. W. Woolley, R. B. Merrifield, C. Ressler and V. du Vigneaud, *Proc. Soc. Expl. Biol. Med.*, **89**, 669 (1955).
(21) Kindly supplied by Dr. V. du Vigneaud.

to an acidic compound. This treatment did not alter the biological activity of the isolated peptide significantly (195 units per mg. before hydrolysis and 170 units per mg. after) or change the electrophoretic behavior on paper between *p*H 1 and 8. The fact that the original peptide contained glutamic acid and not glutamine was also implied by the fact that it was electrophoretically neutral at *p*H 5.0. The corresponding glutamine peptide should have been basic. A more positive way of showing that the peptide contained glutamic acid in preference to glutamine would have been by hydrolysis with carboxypeptidase, followed by chromatographic identification of glutamic acid or glutamine. However, carboxypeptidase did not attack this peptide.

Discussion

Since this peptide survived the action of pepsin, trypsin, chymotrypsin, carboxypeptidase and dilute acid, it should be a suitable material with which to investigate the strepogenin requirement of growing animals.[22,23] Sufficient quantities for such an experiment could be obtained only through chemical synthesis.

As was the case with the acid digest of insulin,[3] many peptides with strepogenin activity were present in enzymic hydrolyzates (see Fig. 1). That more than one arginine-containing peptide with biological activity was present was shown in Figs. 2 and 3. Because these materials appeared to be present in even smaller amounts than the peptide isolated, it seemed impractical to attempt to isolate them. The peptide isolated in pure form accounted for 4.2% of the strepogenin activity of the enzymic hydrolyzate of insulin. Undoubtedly, only a fraction of the peptide found in the hydrolyzate was isolated in pure condition.

Sanger and Tuppy,[24] using a peptic digest of the oxidized B-chain of insulin, identified the peptide leucylvalylcysteylglycylglutamylarginylglycylphenylalanine[25] (Bp4) as a spot on paper chromatograms. Later, from unoxidized insulin, Sanger, *et al.*,[26] obtained the unsymmetrical disulfide involving leucylvalylcysteinylglycylglutamylarginylglycylphenylalanylphenylalanine and cysteinylaspartic acid. The structure of this disulfide was deduced from a study of the products of the oxidation with performic acid. This sequence was confirmed by Haugaard and Haugaard[27] who obtained a fraction, the amino acid composition of which corresponded to the unsymmetrical disulfide involving leucylvalylcysteinylglycylglutamic acid and cysteinylaspartic acid by chromatographing subtilisin-digested insulin on Dowex-50 x 4. In neither case was the proposed structure based on an isolated peptide of proved purity. The present work, which deals with analytically-pure material, confirms the amino acid sequence from leucine to arginine.

All the evidence indicated that material isolated in the present work was the symmetrical disulfide. This disulfide is not a part of the structure of insulin as proposed by Sanger and co-workers.[26] However, one can picture the formation of this symmetrical disulfide from disulfide interchange[13] of the unsymmetrical peptides deduced by Sanger. If the Sanger structure for insulin is correct, one must assume that this disulfide interchange occurred and was responsible for the formation of the peptide herein described.

Although it is commonly held that protein digests prepared by the combined action of several enzymes are too complex to permit isolation of pure peptides by the use of existing methods, the present work shows that this is not necessarily so, when several columns of different ion-exchange resins are used sequentially.

(22) M. Womack and W. C. Rose, *J. Biol. Chem.*, **162**, 735 (1946).
(23) D. W. Woolley, *ibid.*, **159**, 753 (1945).
(24) F. Sanger and H. Tuppy, *Biochem. J.*, **49**, 481 (1951).
(25) "Cysteyl" indicates a cysteic acid residue.
(26) A. P. Ryle, F. Sanger, L. F. Smith and R. Kitai, *Biochem. J.*, **60**, 541 (1955).
(27) E. S. Haugaard and N. Haugaard, *Compt. rend. lab. Carlsberg*, **29**, No. 21, 350 (1955).

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Solubility and Mechanism of Dye-uptake in Protein–Dye Salts[1,2]

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The precipitation of the basic protein lysozyme by the acidic dye, Orange II, has been studied systematically. From a titration of dye into the protein solution and analyses for dye in filtrate samples at frequent intervals, a plot is constructed of dye uptake as a function of free dye concentration. This graph summarizes quantitatively the solubility and stoichiometry of the precipitate. A reversible equilibrium exists between the solution and the amorphous precipitate. The precipitate continues to bind dye after lysozyme is completely precipitated from solution, up to an extrapolated maximum value which at *p*H's less than 4 is equal to the number of positively charged groups in lysozyme. The mutual precipitation of lysozyme and Orange II shows quantitative agreement with a solubility-product model, while the further binding of dye by the precipitate can be described as a simple association where all the dye-binding sites have the same binding energy. The lysozyme–Orange II system was studied with temperature, *p*H, ionic strength and initial protein concentration as experimental variables.

The ability of ionic dyes to precipitate proteins from solution has long been known. Although such diverse substances as colloidal metal oxides, tannic acid, polyphosphates, ionic detergents and trichloroacetic acid are also effective protein precipi-

(1) Part of a thesis presented to the Graduate School of the University of Wisconsin by D. B. Wetlaufer in partial fulfillment of the requirements for the Ph.D. degree, 1954.
(2) Published with the approval of the Director of the Wisconsin Agricultural Experiment Station. A preliminary report of this work was presented at the 125th meeting of the American Chemical Society, Kansas City, Mo., March 25, 1954.
(3) Predoctoral Fellow of the National Science Foundation, 1952-1954.