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

The Isolation and Determination of Structure of Peptides with Strepogenin Activity. I. Serylhistidylleucylvalylglutamic Acid and Serylhistidylleucylvalylglutamylalanylleucine from Insulin

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A peptide possessing strepogenin activity has been isolated in weighable amounts from acid digests of crystalline beef insulin and demonstrated to be relatively pure. The structure of this peptide was established as serylhistidylleucylvalylglutamic acid. The heptapeptide serylhistidylleucylvalylglutamylalanylleucine was also obtained but in a less pure condition. The strepogenin activity of these peptides was determined and discussed in terms of the specific nutritional requirements of the assay organism *Lactobacillus casei*.

Strepogenin activity can be obtained from partial hydrolysates of certain crystalline proteins.[2,3] The isolation in pure condition of the one or more compounds responsible for this potency and the establishment of the structure of such material has been the aim of this[4-7] and other[8-12] laboratories for many years. Because of its chemical properties and because some pure synthetic peptides have activity,[6,13] the active component of protein hydrolysates has been thought to be peptidic in nature. The present paper is a report of the isolation and determination of structure of the first two such peptides to be obtained in relatively pure form from a protein digest. The source of the peptides was a partial acid hydrolysate of crystalline beef insulin. The active peptides were serylhistidylleucylvalylglutamic acid and serylhistidylleucylvalylglutamylalanylleucine.

Experimental

The Assay.—The *Lactobacillus casei* method of Sprince and Woolley[6,14] was employed. Wilson's liver fraction L was assigned a value of one unit per mg. and served as the strepogenin standard. It was important to adjust all samples to *p*H 7 before assay. Acid itself stimulated growth.

Source of Material.—Crystalline beef insulin (Lilly)[15] (1 g.) plus 25 ml. of concd. HCl were mixed in a tightly stoppered flask and immediately placed at 37° and held for 20 hr. The acid was removed as completely as possible by distillation under reduced pressure in a 37° water-bath. The residue was dried *in vacuo* over KOH for 18 hr., dissolved in 100 ml. of water, adjusted to *p*H 7 with NH_4OH, and freed of a small precipitate by filtration. This preparation had a total of 8×10^4 units of activity.

It was found very important to follow precisely the directions given for hydrolysis and for fractionation. Seemingly small variations in technique frequently led to greatly diminished activity; however, the procedures described gave reproducible results.

Fractionation of Peptides.—The fractionation was done on the ion-exchange resins Dowex-2 and Dowex-50 (200–400 mesh) which had been prepared according to the directions of Hirs, Moore and Stein.[16] The separations were followed by strepogenin assay of the eluate fractions. Because of the very large number of closely related peptides produced by the non-specific acid cleavage of the protein, it was not possible to isolate pure material from a single chromatographic column. Consequently, repeated fractionation on a variety of columns was required.

Isolation of the Pentapeptide. Step 1.—A column of Dowex 2-x4 resin (formate form) 2×49 cm. was prepared and equilibrated with a buffer made by adjusting 0.08 *M* formic acid to *p*H 5.5 with ammonium hydroxide. The hydrolysate described above was applied to the column and washed on with 10 ml. of buffer three times, after which a reservoir containing the buffer was attached to the column. The flow rate was adjusted to 30 ml. per hr. and fractions were collected every 30 min. on a Technicon Time-Flow machine. These fractions were diluted appropriately, neutralized and assayed. A rather broad band of active material was obtained between tubes 13 and 93 which contained 2.6×10^4 units. These fractions were combined and evaporated to dryness at 30°. The ammonium formate was removed by sublimation *in vacuo* at 37°.

Step 2.—A 2×36 cm. Dowex 50-x4 (ammonium form) column was equilibrated with 0.08 *M* ammonium formate *p*H 4.2. The crude sample from step 1 was dissolved in 10 ml. of water and adjusted to *p*H 4.0 with formic acid. This was applied to the column and elution was begun with the *p*H 4.2 ammonium formate buffer. The first 760 ml. of the effluent was discarded and the next 790 ml. collected, evaporated, and the buffer removed by sublimation (8×10^3 strepogenin units).

Step 3.—A 1×120 cm. Dowex 50-x4 (sodium form) column was equilibrated with *p*H 4.8 sodium acetate, 0.1 *M* with respect to sodium. The sample from step 2 was dissolved in 10 ml. of water, acidified to *p*H 4 with acetic acid, and adsorbed to the column. Elution was performed with a gradient of *p*H.[17] The reservoir contained 0.1 *M* sodium acetate *p*H 6.0, and the mixing bowl was started with 0.1 *M* sodium acetate *p*H 4.8. The volume of the mixing bowl was 250 ml. The flow rate was 4 ml. per hr. Each 30-min. fraction was assayed in order to allow sharp demarcation of the pentapeptide from other active peptides which emerged earlier from the column. The peak of activity between 977 and 1037 ml. (*i.e.*, the slowest peak) contained 1.5×10^3 units and was principally the pentapeptide.

Step 4.—A 1×49 cm. Dowex 2-x4 (formate form) column was equilibrated with 0.1 *M* ammonium formate *p*H 6.1. The pentapeptide-containing fraction from step 3 (60 ml.) was adjusted to *p*H 7.0 with NH_4OH and adsorbed on the column. After washing, elution was begun with 0.08 *M* ammonium formate *p*H 5.7. The sharp peak of activity which emerged in tubes 80 to 93 contained 1.0×10^3 units. This fraction was evaporated and freed of buffer by sublima-

(1) With the technical assistance of V. Armbrust.

(2) D. W. Woolley and H. Sprince, *Federation Proc.*, **4**, 164 (1945).

(3) H. Sprince and D. W. Woolley, THIS JOURNAL, **67**, 1734 (1945).

(4) D. W. Woolley, *J. Exp. Med.*, **73**, 487 (1941).

(5) D. W. Woolley, *J. Biol. Chem.*, **171**, 443 (1947).

(6) D. W. Woolley and R. B. Merrifield, THIS JOURNAL, **76**, 316 (1954).

(7) R. B. Merrifield and D. W. Woolley, *Arch. Biochem. Biophys.*, **56**, 265 (1955).

(8) M. L. Scott, L. C. Norris and G. F. Heuser, *J. Biol. Chem.*, **166**, 481 (1946).

(9) C.-H. de Verdier and G. Ågren, *Acta Chem. Scand.*, **2**, 783 (1948).

(10) L. D. Wright, J. S. Fruton, K. A. Valentik and H. R. Skeggs, *Proc. Soc. Exp. Biol. Med.*, **74**, 687 (1950).

(11) M. S. Dunn and L. E. McClure, *J. Biol. Chem.*, **184**, 223 (1950).

(12) E. Kodicek and S. R. Mistry, *Arch. Biochem. Biophys.*, **44**, 30 (1953).

(13) D. W. Woolley, R. B. Merrifield, C. Ressler and V. du Vigneaud, *Proc. Soc. Exp. Biol. Med.*, **89**, 669 (1955).

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

(15) Kindly supplied by Eli Lilly and Co.

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

(17) H. Busch, R. B. Hurlbert and V. R. Potter, *ibid.*, **196**, 717 (1952).

tion. The remaining white solid weighed 12 mg. It was the pentapeptide serylhistidylleucylvalylglutamic acid.

Isolation of the Heptapeptide.—Although the heptapeptide and pentapeptide were rather well separated from most of the peptides of the insulin hydrolysate by the columns described in steps 1 and 2 above, they were not well separated from each other. However, this separation was achieved with the gradient elution system described in step 3. The heptapeptide emerged as the first sharp activity peak between 755 and 811 ml. (1.4×10^3 units). This was further purified and freed of buffer in a manner similar to that described in step 4 above. The white solid which remained after sublimation weighed 9 mg. and had an activity of 98 units per mg. It was the heptapeptide serylhistidylleucylvalylglutamylalanylleucine.

Fractionation of Other Active Peptides.—In addition to the penta- and heptapeptides several other active peptide fractions have been obtained from the experiments, but the purity and structures of these substances have not been determined. After elution with 0.08 *M* ammonium formate *p*H 5.5, the Dowex 2 column of step 1 above was eluted successively with 2000 ml. of 0.08 *M* ammonium formate *p*H 4.3, and 1500 ml. of 0.08 *M* ammonium formate *p*H 3.2, and finally with 200 ml. of 0.5 *M* formic acid. These fractions contained 8.5×10^3, 4.0×10^3 and 5.0×10^3 units, respectively. Each of these fractions was separated into several different but active components by further chromatography. The total recovery of activity from the original Dowex 2 column was 54 per cent.

The number of active substances which had to be separated is illustrated in Fig. 1. This shows the elution pattern observed merely in step 2 of the main fractionation. In this particular experiment a 1 × 125 cm. Dowex 50-x4 column was employed. Elution was begun with 0.08 *M* ammonium formate *p*H 4.3 at a rate of 7 ml. per hr. After 280 30-min. fractions, the buffer was changed to 0.1 *M* *p*H 5.5. The hepta- and pentapeptides were contained in the fractions with peaks at 1.6 and 1.71., respectively.

Properties and Purity of the Peptides.—The purity of the pentapeptide was demonstrated by (a) ion-exchange column chromatography, (b) countercurrent distribution, (c) zone electrophoresis on paper, (d) paper chromatography, and (e) qualitative and quantitative amino acid analyses of hydrolysates.

Ion-exchange Chromatography.—Three mg. of the pentapeptide was chromatographed exactly as described in step 3 above. The eluate fractions were analyzed both by strepogenin assay and by the colorimetric ninhydrin method of Moore and Stein.[18] In absolute figures tube 322 contained 6.5 strepogenin units per ml. and 1.14×10^{-5} leucine equivalents per ml. The data of Fig. 2 show first, that only one peak was obtained from the pentapeptide sample and second, that this peak was homogeneous by this test because of the constant ratio of activity to ninhydrin color over the entire peak.

Countercurrent Distribution.—Three mg. of the pentapeptide was distributed for 100 transfers in a countercurrent machine[19] between 0.1 *N* HCl (10 ml.) and *sec*-butyl alcohol (15 ml.). It moved as a single symmetrical activity peak with a maximum in tube 20, and closely approximated the theoretical curve for $K = 0.167$ (Fig. 3). From tubes 12 to 28, 72% of the initial activity was recovered. In the system 0.1% acetic acid–*sec*-butyl alcohol $K = 0.062$.

Zone Electrophoresis on Paper.—Paper electrophoresis[20] of the pentapeptide in 0.1 *M* ammonium formate *p*H 4.0 (10 v./cm., 17 hr., 4°) showed a single band of activity[21] at a distance of 25.4 cm. toward the cathode from the starting line. This corresponded with the only visible ninhydrin-positive zone. Other buffers at different *p*H values gave similar correspondence between activity and ninhydrin color. Electrophoresis on a pad of 10 to 20 sheets of Whatman #1 paper has been used to purify other preparations, but offered no special advantage over the ion-exchange columns. The isoelectric point of the pentapeptide was determined to be *p*H 5.1 by paper electrophoresis using sodium cacodylate of 0.1 ionic strength at various *p*H values. The isoelectric point of the tetrapeptide histidylleucylvalylglutamic acid, to be described later, was also 5.1; however, in agreement with its lower molecular weight, this peptide had a greater mobility than the pentapeptide at *p*H 5.5 to 7.5.

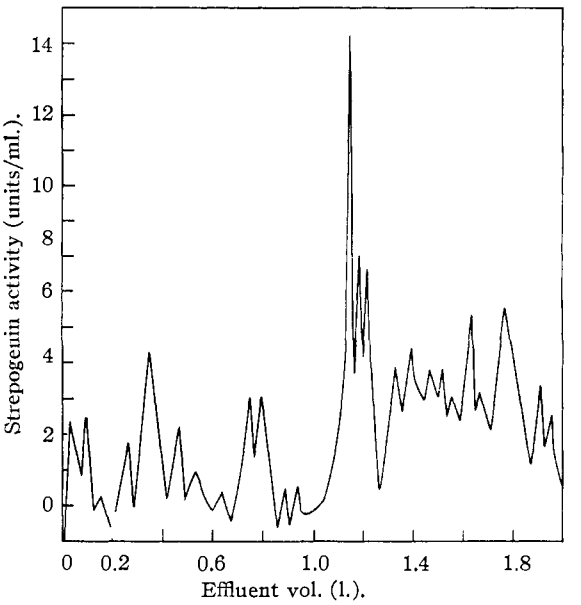

Fig. 1.—Fractionation of strepogenin activity by chromatography on an ion-exchange column; column, 1 × 125 cm. Dowex 50-x4; sample, insulin digest (8.2×10^3 strepogenin units) previously fractionated as in step 1 of text.

Paper Chromatography.—The R_F values of the penta- and heptapeptides on ascending paper chromatograms were found to be 0.37 and 0.52 in propanol–water (2:1), and 0.22 and 0.39 in *sec*-butyl alcohol–formic acid.[22] By descending chromatography in *m*-cresol–0.03% NH_3,[23] the R_F values were 0.33 and 0.56 for the penta- and heptapeptides, respectively. The strepogenin activity corresponded to the ninhydrin area in each solvent and was not found in other parts of the chromatograms.[21]

Quantitative Amino Acid Analyses.—One mg. of the pentapeptide was refluxed in 20 ml. of redistilled HCl for 20 hr., the acid was removed under reduced pressure, and the hydrolysate analyzed quantitatively by the ion-exchange method of Moore and Stein.[24] The data of Table I show that there were equimolar ratios of the five amino acids, serine, glutamic acid, valine, leucine and histidine. With the exception of 0.06 equivalent of aspartic acid and

TABLE I

QUANTITATIVE AMINO ACID ANALYSES

	Molar ratios[a]	
	Pentapeptide[b]	Heptapeptide[c]
Serine	1.00	1.0
Glutamic acid	1.00	1.2
Alanine	0.00	0.9
Valine	1.09	1.3
Leucine	1.03	1.8
Histidine	1.03	1.2

[a] Figures are corrected for losses of glutamic acid and histidine[24] and serine: E. J. Harfenist, THIS JOURNAL, **75**, 5528 (1953). [b] The pentapeptide also contained 0.06 mole of aspartic acid and phenylalanine, but no detectable amounts of other amino acids. [c] The heptapeptide also contained 0.04 mole aspartic acid, 0.06 mole phenylalanine and 0.1 mole glycine.

(18) S. Moore and W. H. Stein, *J. Biol. Chem.*, **211**, 907 (1954).

(19) L. C. Craig, J. D. Gregory and G. T. Barry, *Cold Spring Harbor Symposia on Quant. Biol.*, **14**, 24 (1949).

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

(21) It was found essential to elute the paper before the solvent was allowed to evaporate. If the sheets became dry, only a small percentage of the total activity could be eluted from them.

(22) W. Hausmann, THIS JOURNAL, **74**, 3181 (1952).

(23) F. Sanger and H. Tuppy, *Biochem. J.*, **49**, 463 (1951).

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

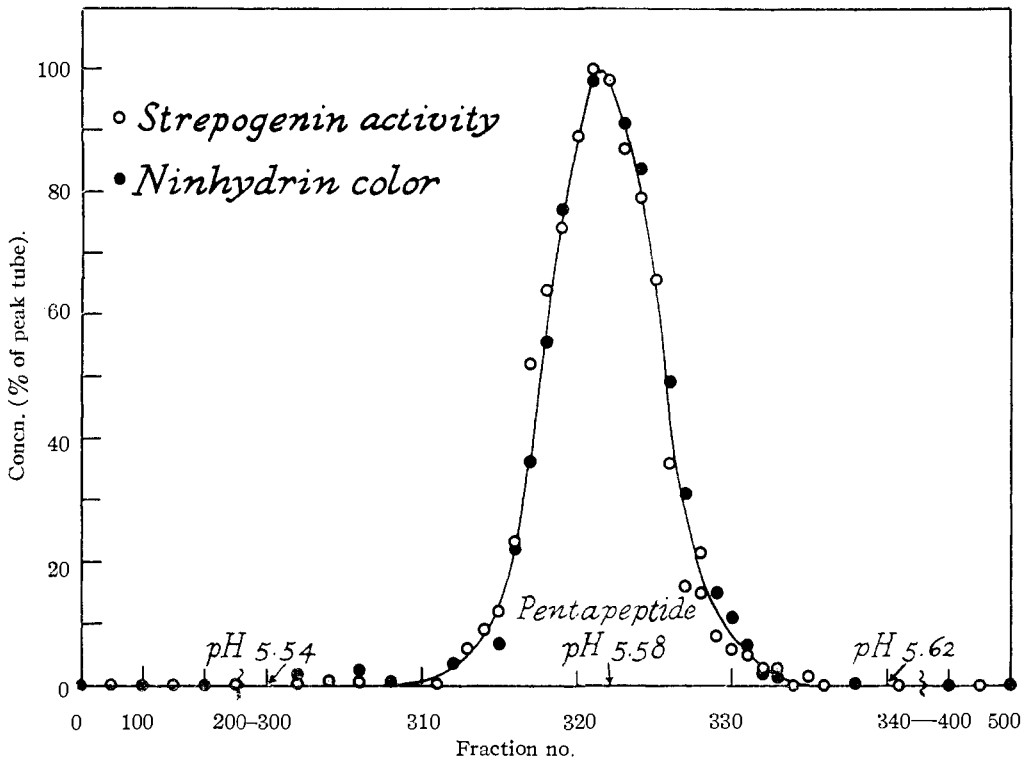

Fig. 2.—Ion-exchange chromatogram of the pentapeptide; column, 1 × 120 cm. Dowex 50-x4; sample, 3.0 mg. of pentapeptide. Elution: 0.1 *M* sodium acetate with *p*H gradient of 4.8 to 6.0 as described in step 3 of text.

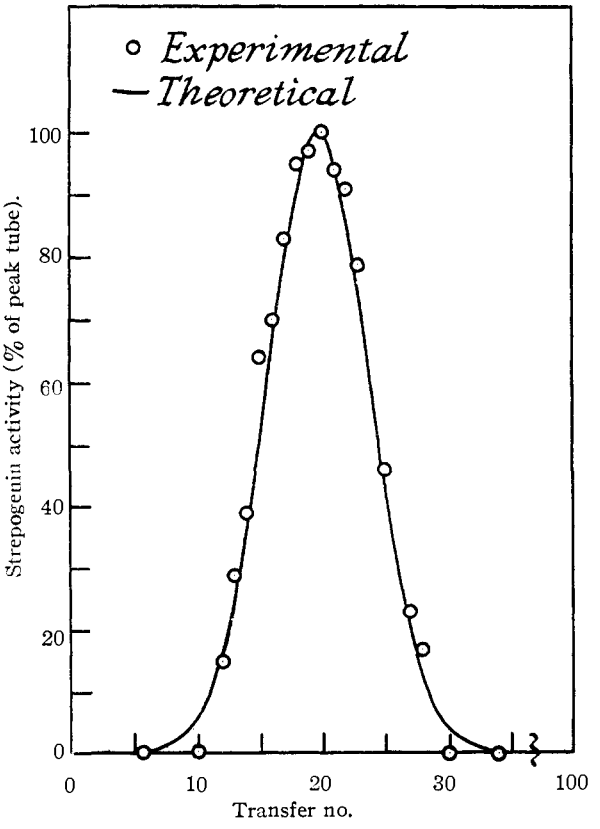

Fig. 3.—Countercurrent distribution of the pentapeptide. System: 0.1 *N* HCl-*sec*-butyl alcohol; 10 ml. of lower phase, 15 ml. upper phase; total transfers 100; sample, 3.0 mg. of pentapeptide.

phenylalanine no traces of other amino acids were detected. Two-dimensional paper chromatograms of the hydrolysates in the system *sec*-butyl alcohol–ammonia, and *sec*-butyl alcohol–formic acid[22] also showed these five amino acids and revealed no other ninhydrin-positive material. Such a chromatogram will completely separate all of the amino acids of insulin.

Similar paper chromatograms of the heptapeptide showed only serine, glutamic acid, valine, leucine, histidine and alanine, but the ion-exchange column analysis detected small amounts of aspartic acid, phenylalanine and glycine. In addition, the constituent amino acids were only approximately equimolar in amount in the heptapeptide. By this criterion, it was therefore considerably less pure than the pentapeptide.

Structure of the Peptides.—The structure of the pentapeptide was established from (a) the quantitative amino acid analysis, (b) amino-end group determinations by the dinitrophenyl, and thiohydantoin methods, (c) carboxyl end-group determination by the carboxypeptidase method, and (d) the separation and identification of smaller fragments resulting from degradation procedures.

Dinitrophenyl End-group Analysis.—The dinitrophenyl (DNP) derivative was prepared from 4 mg. of the pentapeptide according to Sanger's DNP method[25] and extracted from acid solution into ethyl acetate. Material so obtained was divided into 2 parts, each of which was hydrolyzed in a sealed tube with 0.3 cc. of acetic acid and 0.3 cc. of HCl at 105°. One portion was hydrolyzed for 2 hr. and the other for 28 hr. The hydrolysates were evaporated to dryness, dissolved in water and extracted with ether. The ether phase from the 2-hr. hydrolysate was shown by paper chromatography to contain only DNP serine (R_F 0.14 in benzyl alcohol–phthalate *p*H 6.[26] After 28 hr. hydrolysis, there was considerable loss of DNP serine. The aqueous phases of the hydrolysates were still yellow and were shown to contain histidine with a DNP-group on the imidazole ring. Thus the aqueous phase of the 28-hr. hydrolysate showed a yellow spot of R_F 0.24 in *sec*-butyl alcohol–formic acid,[22] corresponding to that of *im*-DNP-histidine which had been prepared from carnosine. After spraying with nin-

(25) F. Sanger, *Biochem. J.*, **39**, 507 (1945).

(26) S. Blackburn and A. G. Lowther, *ibid.*, **48**, 126 (1951).

hydrin this spot turned purple indicating an unsubstituted α-amino group. In addition to the *im*-DNP-histidine, glutamic acid, leucine and valine appeared and no other ninhydrin-positive spots were visible.

Thiohydantoin End-group Analysis.—The pentapeptide (3.9 mg.) was converted to the phenylthiocarbamyl derivative by the method of Landmann, *et al.*[27] The dry product was washed well with benzene, and dissolved in 5 ml. of anhydrous dioxane which had been saturated with dry hydrogen chloride. After 2 hr. at 25°, the solvent was removed and the cleaved phenylthiohydantoin was extracted into ether and chromatographed in the xylene–acetic acid–phthalate system.[27] When the paper was sprayed with Grote's reagent,[28] it showed a red spot at R_F 0.83. The phenylthiohydantoin prepared from serine gave a spot of R_F 0.82. The residue remaining after ether extraction, containing the tetrapeptide histidylleucylvalylglutamic acid, was dissolved in water and a portion hydrolyzed. Paper chromatograms revealed approximately equal amounts of histidine, leucine, valine and glutamic acid, and only a trace (0.05 equiv.) of serine. On the basis of the ninhydrin values the yield of tetrapeptide was nearly quantitative. A single attempt to extend the method to the next N-terminal residue (histidine) was unsuccessful. The phenylthiocarbamyl derivative was prepared and treated with dioxane–HCl as before. An ether extract of the neutralized residue contained no histidine hydantoin (or other hydantoins) as judged from paper chromatograms or from the absorption spectrum. In addition, when the aqueous phase was chromatographed it showed no ninhydrin-positive material. However, after hydrolysis in HCl–formic acid paper chromatograms revealed histidine, leucine, valine and glutamic acid with a recovery of about 70%. From these results it was concluded that the phenylthiocarbamyl peptide had formed, but that dioxane–HCl had not effected the desired ring closure.

Partial Hydrolysis of the Tetrapeptide.—The isolated tetrapeptide resulting from the thiohydantoin cleavage of the pentapeptide was hydrolyzed in concd. HCl for 70 hr. at 37° and the resulting smaller peptides separated by paper electrophoresis. A small indicator strip of the paper was sprayed with ninhydrin to locate the amino acids and peptides as shown in Table II. With this as a guide, the remainder of the paper was cut into appropriate bands, eluted with water, and the resulting peptides hydrolyzed and run on paper chromatograms. From these data the structures in Table II were assigned. The relative electrophoretic positions were consistent with these compositions. They were also in keeping with the mobilities of the pentapeptide and serylhistidine (Table II, footnote).

TABLE II

ELECTROPHORETIC SEPARATION[a] OF A PARTIAL HYDROLYSATE OF THE TETRAPEPTIDE HISTIDYLLEUCYLVALYLGLUTAMIC ACID

Spot No.	Distance migrated[b] (mm.)	Amino acid composition	Probable structure
1	−217	his	his
2	−166	his, leu	his-leu
3	−128	his, leu, val	his-leu-val
4	− 75	his, leu, val, glu	his-leu-val-glu
5	− 28	leu, val	(leu-val, leu, val)[c]
6	+ 4	leu, val, glu	leu-val-glu
7	+ 34	val, glu	val-glu
8	+ 73	glu	(glu-x)[d]
9	+113	glu	glu

[a] Run in 0.1 *M* pyridine acetate, *p*H 5.0 for 206 min.; 600 v., 4°. Ser-his and ser-his-leu-val-glu moved −173 and −52 mm., respectively. [b] (+) indicates movement toward anode, (−) toward cathode. [c] This mixture was not separated. [d] Present in trace amounts. Only glutamic acid could be identified.

Hydrolysis with Carboxypeptidase.—One mg. of crystalline carboxypeptidase (Worthington) was dissolved in 0.25 ml. of 10% LiCl and 0.25 ml. of *p*H 8 sodium phosphate added. One mg. of the pentapeptide was dissolved in 0.1 ml. of water and mixed with 0.05 ml. of the carboxypeptidase solution. After 24 hr. a 0.1-ml. aliquot was fractionated by paper electrophoresis using the conditions described in Table II. Half of the paper was then developed with ninhydrin. In addition to glutamic acid, and leucine and valine, a yellow spot which turned purple on standing, appeared at −173 mm. The corresponding area from the other half was eluted with 5 ml. of water, filtered and hydrolyzed for 20 hr., at 105° in 6 *N* HCl. Chromatograms showed only serine and histidine. Because serine was N-terminal in the pentapeptide, this must have been serylhistidine. A decision between glutamic acid and glutamine could be made from this same carboxypeptidase experiment from which glutamic acid, not the amide, was derived. Pure glutamine was not hydrolyzed under these conditions. The electrophoretic mobility of the pentapeptide also indicated that both of the carboxyl groups of the glutamic acid residue were free.

The Structure of the Heptapeptide.—The structure of the heptapeptide was assigned from amino acid analyses, from DNP and carboxypeptidase end-group analyses, and by analogy with the pentapeptide. Quantitative ion-exchange chromatography (Table I) showed approximately one mole each of serine, histidine, valine, glutamic acid and alanine, and 1.8 moles of leucine, indicating a heptapeptide. In addition, there was a small amount of aspartic acid, glycine and phenylalanine. The deviations of the ratios from whole numbers may therefore be attributed to contamination of the heptapeptide with small amounts of other peptides. The low leucine might be due in part to the presence of a few per cent. of the corresponding hexapeptide which terminates in alanine, although a chromatographic peak attributable to this compound was not observed. The presence of substantial amounts of this peptide should also have been revealed by liberation of alanine concomitantly with leucine in the carboxypeptidase experiment described below. The DNP method previously described for the pentapeptide showed N-terminal serine. From these data the heptapeptide was assumed to have the first five residues in common with the pentapeptide, leaving only the alanine and second mole of leucine to be located.

When 0.2 mg. of the heptapeptide was incubated at 37° with 0.03 mg. of carboxypeptidase in 0.12 ml. of 10% LiCl for 1 hr., the only free amino acid which could be detected was leucine. After 4 hr., a faint spot of alanine appeared on the chromatogram in addition to the leucine. With larger amounts of enzyme, glutamic acid and valine were also liberated. This experiment indicated a C-terminal leucine residue which was preceded by alanine. It also showed that this peptide contained glutamic acid rather than glutamine. Thus it was concluded that the sequence of the heptapeptide was serylhistidylleucylvalylglutamylalanylleucine.

Biological Activity of the Peptides.—The results of microbiological assays of several preparations of the peptides are summarized in Table III. The strepogenin activity of the pentapeptide was approximately 80 units per mg. and did not vary significantly for samples obtained from ion-exchange columns, countercurrent distribution or paper electrophoresis. Since sufficient material was not always available for accurate dry weight measurement, the specific activity of some samples was calculated from colorimetric ninhydrin data obtained on acid hydrolysates. These

TABLE III

STREPOGENIN ACTIVITY OF VARIOUS PREPARATIONS OF THE PEPTIDES

Peptide	Additional purification[a]	Activity[b] (units/mg.)
Ser-his-leu-val-glu	None	86[c]
	None	78[d]
	Countercurrent distribution	85[d]
	Paper electrophoresis	80[d]
Ser-his-leu-val-glu-ala-leu	None	98[c]

[a] Additional to the column chromatography described in the text. [b] By the *L. casei* method. [c] Based on dry weight. [d] Based on ninhydrin analysis of an acid hydrolysate.

(27) W. A. Landmann, M. P. Drake and J. Dillaha, THIS JOURNAL, **75**, 3638 (1953).

(28) I. W. Grote, *J. Biol. Chem.*, **93**, 25 (1931).

values agreed within experimental error with those obtained from direct weight data. The heptapeptide had a somewhat greater activity (about 100 units per mg.). One must remember, however, that the heptapeptide was not as pure as the pentapeptide.

The strepogenin assays were subject to several errors, and for this reason the values for potency were only approximate. With the isolated peptides the dose–response curves flattened out sooner than with the standard. Thus, values for different levels of either the penta- or heptapeptides agreed well up to about 0.7 μg. per ml., but above this increasing levels gave decreasing values for potency. The shapes of the dose–response curves were similar to that previously reported for a synthetic peptide (see ref. 13, Fig. 1, peptide 2). The activity values were the averages calculated from turbidity measurements at several low levels of sample. They were reproducible to about 15% from one experiment to another. When it was important to compare the potency of two samples (as for Table III), they were assayed in the same experiment to minimize errors between assays.

Reliable data on the potency of the small peptides derived from the pentapeptide by partial acid or enzymatic hydrolysis are not available, but preliminary experiments indicated that none of these had activity greater than 9 units per mg. The simple histidine peptide carnosine was also inactive in this test.

Discussion

When a biologically active substance is obtained for the first time from a natural product, it is difficult to be sure that the activity belongs to the compound isolated. The possibility still remains of minute quantities of impurities of very high potency. For this reason it is important to employ a variety of purification procedures. Since the specific activity of the pentapeptide-containing fraction remained constant after fractionation by ion-exchange chromatography, paper electrophoresis and countercurrent distribution, the activity can reasonably be attributed to this peptide. Furthermore, observations on synthetic oxytocin have already proved that a single peptide can possess high strepogenin activity.[6]

It should be pointed out, however, that the isolated penta- and heptapeptides represented only a small fraction (2.3%) of the strepogenin activity which was present in the insulin hydrolysate. This low value was not due primarily to losses during isolation. Since the sequence serylhistidylleucylvalylglutamic acid represents only 10% of the weight of insulin, according to Sanger's structure,[28,29] only 11 to 15% of the total activity could have been accounted for by these peptides even if they were to have been formed quantitatively during hydrolysis. Other more active components, presumably peptides, must be present in the hydrolysate. Several have already been isolated[6] from certain of the fractions described in this paper, but their structures are not yet known.

Although serylhistidylleucylvalylglutamic acid was obtained from paper chromatograms of digests of the phenylalanine chain of oxidized-insulin by Sanger,[23] the present structure proof is important because, except for the N-terminal residue, this sequence was deduced by him entirely from the qualitative composition of the several small peptides derived from the original hydrolysate rather than from data on the purified isolated peptide itself. Sanger also deduced the sequence serylhistidylleucylvalylglutamylalanylleucyl, but did not actually detect a heptapeptide of this composition.

The data presented here throw some light on the peptide needs of *Lactobacillus casei*, but the exact requirement which the penta- and heptapeptides satisfy is not entirely clear. Chain length may be of some importance, since, on a molar basis, the heptapeptide appeared to be about 50% more active than the pentapeptide,[30] and all smaller peptides which have been tested have had much lower activities (see also 13). Loss of serine as in histidylleucylvalylglutamic acid, or in any of the smaller peptides derived therefrom, reduced the activity at least ten fold. However, the presence of serine in other peptides such as serylhistidine, glycylserine, or serylglycylglutamic acid[31] did not produce compounds of high activity. The rather non-specific requirements for small peptides of serine and histidine which have been claimed[32] for *Lactobacillus delbrueckii*[33,34] therefore do not seem to exist in *L. casei* under the conditions of the strepogenin assay. Whether the penta- and heptapeptides have growth-promoting activity for other strepogenin-requiring microörganisms has not been determined.

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(29) F. Sanger, L. F. Smith and R. Kitai, *Biochem. J.*, **58**, vi (1954).

(30) Nevertheless, size alone was not enough because other peptides of this general size have proved to be inactive.[5,6]

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(32) In unpublished work by Woolley and Woolley it has not been possible to confirm the claim of Prescott, *et al.*,[34] that carnosine was more active in promoting the growth of *L. delbruckii* than was histidine. Carnosine proved to be less active than histidine with their organism under the conditions they described.

(33) V. J. Peters, J. M. Prescott and E. E. Snell, *ibid.*, **202**, 521 (1953).

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