[CONTRIBUTION FROM EMORY UNIVERSITY]

Resistant Peptidases of Swine Kidney

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Peptidases of renal tissue appear to be of two types. One type was found to be readily soluble and was destroyed by digestion with trypsin; a second type was found to be insoluble but released into solution by, though resistant to, trypsin and other proteolytic enzymes. Various forms of a resistant peptidase active in the hydrolysis of cysteinylglycine and leucinamide were isolated from digests of kidney tissue by ion-exchange chromatography; these forms differed in the content of protein. Protein could be removed more or less completely by exhaustive digestion and other procedures; the remaining active fragments appear to contain nucleotides combined with "acid labile" amino acids and peptides.

In previous reports[1] the purification and partial characterization of a renal peptidase was described. This peptidase, active in the hydrolysis of cysteinylglycine, appeared to contain little or no protein but did contain nucleotides. The activity was resistant to enzymatic attack and was not removed from solution by a variety of procedures for the removal of protein. However, a peptidase with similar activities and prepared from kidney tissue by conventional methods has been characterized as a protein.[2]

Experimental

Acetone powders of kidney tissue were prepared from frozen kidneys by two procedures; in the first procedure[2] precautions were taken to avoid drastic conditions. The second procedure was as follows:

Ten pounds frozen kidney tissue was ground in a heavy duty meat grinder and blended with 5 gal. of acetone at room temperature; the material was mixed thoroughly several times during a period of 24 hr. at room temperature. The supernatant acetone was removed by suction and fresh acetone at room temperature was added to restore the original volume and after 24 hr. the acetone was removed by suction and the procedure was repeated once more. Finally, the material was filtered by suction, washed with acetone and ether and dried at room temperature in air. From 10 lb. kidney tissue, about 1400 g. powder was obtained. A third procedure for the preparation of acetone powders involving the boiling of ground kidney tissue in acetone was suitable for the preparation of the resistant peptidases but reduced considerably the yield of labile peptidases.

Labile and Resistant Peptidases.—Soluble peptidases were extracted with water (1 g. of acetone powder to 14 ml. of water). The residue was suspended in NaCl–$NaHCO_3$ (0.1 *M* in each case) in the ratio of 14 ml. for each gram of original powder. The water extract, the suspended residue and a homogenate in water were digested with a mixture of trypsin and chymotrypsin (0.1 mg. per ml. for 24 hr. at 37°). It is apparent (Table I) that the activity extracted with water was destroyed by the digestion whereas the washed residue was without appreciable activity prior to digestion but developed considerable activity during the digestion. The soluble activity in NaCl–$NaHCO_3$ solution (as for the residue) was destroyed more rapidly than in the aqueous solution adjusted to the same *p*H.

The proteolytic enzymes used in these studies were crystalline preparations of Worthington Biochemical Laboratories. Toluene and chloroform were added to all digests as preservatives. The assays were as previously described[3] titrations were made with Meniscomatic burets (American Instrument Co.) with 0.2 *M* alcoholic sodium hydroxide and with stirring with magnetic stirrers.

TABLE I

LABILE AND RESISTANT PEPTIDASES

Preparation	Leucinamidase, units/ml. Control	Leucinamidase, units/ml. Digest	Cysteinylglycinase, units/ml. Control	Cysteinylglycinase, units/ml. Digest
Water extract	0.08	0.00	1.4	0.0
Residue	.06	.40	1.4	15.1
Homogenate	.14	.43	3.5	15.8

In a study of the specificity of destruction of the soluble peptidases by proteolytic enzymes, it was found (Table II) that trypsin was responsible for a rapid destruction of activity and was somewhat more active at *p*H 8 than at 7. However, the activity of the residue was released by trypsin as well as by the other proteolytic enzymes.

TABLE II

PROTEOLYTIC DESTRUCTION OF SOLUBLE PEPTIDASES

Treatment, 1 mg./ml. 24 hr.	Leucinamidase activity, unit/ml. Soluble *p*H 7	Soluble *p*H 8	Residue *p*H 8
Refrigerated control	0.32	0.26	0.31
37° control	.32	.29	0.48
Protease	.10	.00	1.10
Chymotrypsin	.26	.22	1.32
Trypsin	.15	.00	1.03
Papain	.29	.29	0.94

A variety of enzymes were tested for the ability to release the peptidases resistant to digestion from a washed residue of the kidney powder; a compilation of such studies is given in Table III. It is apparent that trypsin, chymotrypsin and papain were essentially equal in their ability to release the peptidases from the washed residue. Inasmuch as the *p*H was near 8, it is not surprising that pepsin was inactive; digestion at this *p*H with pepsin had no effect on the released peptidases and, below *p*H 6, activity disappeared in the presence or in the absence of proteolytic enzymes. The effect of time of digestion in the release of peptidases is illustrated in Table IV. Two levels of chymotrypsin (0.1 and 1.0 mg. per ml.) were used in the digests; it was observed that the release of peptidase activity was increased by an increase of the level of chymotrypsin. The amount of peptidase activity released by the higher level of chymotrypsin seemed to be greater but the addition of a second 0.1 mg. of chymotrypsin served to increase the level of peptidase activity to that achieved with 1.0 mg.

In view of the possibility that materials in the crude digest might protect the peptidase activities from proteolysis, the material purified to a high level of activity by ion-exchange chromatography as described below was checked for stability in the presence of proteolytic enzymes; the results are given in Table V. As a further check on the stability of the materials to proteolysis, the purified material was tested in the presence of urea and EDTA; the reasoning was that the presence of a denaturing agent such as urea or that the removal of metals by EDTA might make the material subject to proteolysis. As may be seen, the material could be assayed at 0 time with no apparent loss of activity with either reagent but with incubation at 37° activity was lost especially with the higher concentrations of both reagents. An intermediate concentration of urea that destroyed half or less of the activity was selected and the proteolytic enzymes, 5 mg. per ml. of peptidase solution, were permitted to act for a period of 3 hr.; it is evident that

(1) F. Binkley, THIS JOURNAL, **81**, 1237 (1959), and F. Binkley, C. K. Olson and C. Torres, Abstracts, 128th Meeting, American Chemical Society, Minneapolis, 1955. These studies were supported by grants from the Rockefeller Foundation and from the U. S. Public Health Service.

(2) D. H. Spackman, E. L. Smith and D. M. Brown, *J. Biol. Chem.*, **212**, 255 (1955), E. L. Smith and D. H. Spackman, *ibid.*, **212**, 271 (1955).

(3) F. Binkley, V. Alexander, F. E. Bell and C. Lea, *J. Biol. Chem.*, **226**, 559 (1957),

TABLE III

ENZYMATIC RELEASE OF RESISTANT PEPTIDASES

Enzyme in digest, 0.1 mg./ml. 24 hr.	Leucinamidase, units/ml.	Cysteinyl-glycinase, units/ml.
None	0.06	1.7
Trypsin	.60	15.7
Chymotrypsin	.55	14.3
Pepsin	.08	1.7
Papain	.60	16.4
Hyaluronidase	.07	2.0
Ribonuclease	.07	2.6
Deoxyribonuclease	.07	2.0
Carboxypeptidase	.05	2.7

TABLE IV

TIME FACTORS IN PEPTIDASE RELEASE

Chymotrypsin, mg./ml.	1 hr., units/ml.	3 hr., units/ml.	24 hr., units/ml.
None	0.00	0.00	0.06
0.1	.17	.27	.35
0.1 then 0.1 at 5 hr.	..	..	.45
1.0	.29	.47	.45

the proteolytic enzymes were without effect in the presence or absence of denaturing or metal chelating agents.

Purification of Enzyme.—The powder prepared from 10 lb. of kidney tissue (or the kidney tissue pulled dry on the filter) was blended with 10 l. of a solution of 0.1 *M* NaCl and 0.1 *M* $NaHCO_3$, chloroform and toluene were added as preservatives, and the mixture was shaken thoroughly. On each of three consecutive days, 3 g. of Panprotease (Worthington) was added, digestion was permitted to proceed for about one week, and the digest was filtered by gravity. The filtrate was stored at room temperature until used. To each liter of filtrate was added 600 ml. of ethanol; after several hours at 0°, the precipitate was removed and discarded. An additional 600 ml. of ethanol was added and after a few hours at 0° the precipitate was collected by centrifugation and dissolved in about one tenth of the original volume 0.1 *M* Tris buffer, *p*H 8.0. The material was warmed to 37° and for each liter of solution 1 g. of Panprotease was added and digestion was permitted to continue overnight. For each 100 ml. solution 10 g. of kaolin was added, the mixture was shaken for 30 min. and centrifuged. The supernatant solution was treated exhaustively with chloroform and octanol.[4]

TABLE V

RESISTANCE OF PURIFIED PEPTIDASE TO PROTEOLYSIS

Treatment	Units/ml. 0 time	Units/ml. 30 min.	Units/ml. 3 hr.
Control	105	101	95
Trypsin	105	105	101
Chymotrypsin	105	105	105
Papain	105	101	98
2 *M* urea	129	..	80
4 *M* urea	115	..	71
6 *M* urea	110	..	9
4 *M* urea, trypsin	..	..	85
4 *M* urea, chymotrypsin	..	..	81
4 *M* urea, papain	..	..	88
0.1 *M* EDTA	104	3	..
.01 *M* EDTA	105	62	40
.001 *M* EDTA	108	75	..
.01 *M* EDTA, trypsin	..	..	39
.01 *M* EDTA, chymotrypsin	..	..	40
.01 *M* EDTA, papain	..	..	40

The mixture of chloroform and octanol was washed with bicarbonate before use. The solution was mixed with an equal volume of chloroform and octanol (95 volumes of chloroform and 5 volumes of octanol) and was shaken in a glass stoppered bottle as vigorously as possible at room temperature with a mechanical shaker for 30 min.; the mixture was then centrifuged at the highest permissable speed in a Size 2 International centrifuge for 30 min. The supernatant material was removed by suction from the emulsion and the procedure was repeated until no more emulsion was formed in subsequent treatments; six to eight treatments were usually required. The solution was then subjected to treatment with magnesium acetate and ammonium hydroxide much as previously described.[3] The solution (1 liter or more) was cooled to near 0° and sufficient magnesium acetate to render the solution 0.1 *M* was added and the mixture was stirred until the magnesium acetate was dissolved. Ammonium hydroxide was added dropwise and with vigorous stirring until *p*H 9.6 (*p*H meter) was reached and the solution was allowed to stand at 0° for 30 min. The mixture was then centrifuged in the PR-2 centrifuge for 30 min. at 3000 r.p.m. at 0°. The precipitate was discarded and cold ethanol, 20 ml. per 100 ml. of solution, was added to the supernatant; the precipitate was removed by centrifugation and dissolved in water and the *p*H was corrected to 8 with dilute acetic acid. An additional 60 ml. of ethanol per 100 ml. of solution (a total of 80 ml. per 100 ml. of solution from the removal of the precipitate formed upon the addition of magnesium acetate and ammonium hydroxide) was added and the precipitate was collected by centrifugation. With the somewhat higher *p*H (9.6 as contrasted with 9.3 in the earlier procedure) the bulk of the peptidase activity was found in the second alcohol fraction rather than in both fractions as previously reported.[3] The second alcohol fraction was dissolved in cold water and the *p*H was corrected to 8 by the addition of dilute acetic acid.

The solution was dialyzed against 0.1 *M* Tris buffer, *p*H 7.5, containing 0.001 *M* magnesium acetate and 0.001 *M* zinc acetate at 0°; dialysis overnight was possible under these conditions. The dialyzed material was treated batchwise with Amberlite IR-45 (as the free base) until the resin began to absorb the activity; 25 g. of wet resin was added to 100 ml. of the solution, assays were made and additional batches were added until the activity started to decrease. In a typical experiment 250 ml. of a solution of peptidases was treated with 60-g. batches of Amberlite IR-45. The resin was added and the mixture was shaken gently for 30 min. at room temperature and was filtered. The resin was washed with water to bring the total volume of filtrate to 250 ml. and the filtrates were tested for activity in the hydrolysis of cysteinylglycine, absorption in the ultraviolet and content of protein.[5] In this particular case, treatment was stopped after the second treatment, but in other cases it has been possible to extend the procedure without loss of activity to a fourth treatment with further reduction of ultraviolet absorption and apparent protein content.

TABLE VI

TREATMENT WITH AMBERLITE-45

Solution	Activity, units/ml.	E_{2600}	"Lowry protein,[5]" mg./ml.
Original	810	25	6.50
First treatment	800	16	1.35
Second treatment	670	14	0.95

Ion-exchange Purification.—Ecteola-cellulose[6] in a large column 6.5 by 60 cm. was equilibrated with Tris buffer 0.01 *M*, *p*H 8, containing 0.0001 *M* zinc and magnesium acetates. About 300,000 units cysteinylglycinase (250–300 ml. of the above solution and the yield from about 30 l. of digest filtrate) were added to the column and the column was washed with 6 l. of water followed by 2 l. of the *p*H 8 Tris buffer. Gradient elution was undertaken with solutions of sodium chloride made up in Tris buffer containing zinc and magnesium ions as above but at *p*H 7. Two l. of the *p*H 7 Tris buffer were placed in a lower mixing chamber and 4 l. of 0.2 *M* NaCl made up in the *p*H 7 buffer were placed in an upper chamber feeding into the lower mixing chamber. When 4 l. of eluate had been collected (25-ml. samples), 0.5 *M* NaCl made up in the *p*H 7 buffer was

(4) M. G. Sevag, D. B. Lackman and J. Smolens, *J. Biol. Chem.*, **124**, 425 (1938).

(5) D. H. Lowry, N. S. Rosebrough, A. L. Farr and R. S. Randall, *ibid.*, **143**, 265 (1951).

(6) E. A. Peterson and H. A. Sober, THIS JOURNAL, **78**, 751 (1956).

placed in the upper chamber and elution was continued as long as material absorbing in the ultraviolet was found in the eluate. The bulk of the material absorbing in the ultraviolet was eluted shortly after the addition of the 0.5 *M* NaCl; some activity was eluted with this material but in a manner as to suggest gross contamination with unrelated nucleotides. Although it was possible to operate the column at room temperature by adding antibiotics, the dilute solutions were unstable, and it was found desirable to operate the column at 4°; activity in the dilute solutions could be preserved by the addition of protein, the addition of 1 mg. of crystalline trypsin or chymotrypsin per tube was sufficient to preserve activity in the tubes used for assay. The results are illustrated in Fig. 1.

Two criteria of homogeneity have been applied to fractions III and IV from several columns. Paper electrophoresis was applied in the range of *p*H from 7 to 9 with 0.02 *M* Tris–sulfate buffers. The papers after 4 to 18 hr. electrophoresis were cut into small strips and the strips were eluted with 0.1 *M* Tris buffer, *p*H 8; the absorption in the ultraviolet and activity were determined. It should have been possible to detect a 10% dissociation of the two properties, but, invariably, absorption and activity were in the same position on the paper. Rechromatography on small columns of Ecteola-cellulose, 1 by 20 cm., with gradients varied from 0.3 to 0.6 *M* and from *p*H 8.5 to 6.6 did not reveal any dissociation of absorption in the ultraviolet from activity. Thus, it would appear that fractions III and IV were relatively homogeneous preparations of a resistant peptidase.

Glycylglycinase and alkaline phosphatase activity, readily demonstrated in the crude extracts, could not be demonstrated in fraction III or IV. Leucinamidase activity was present; about 2500 units were found in fraction III. Although the eluates contained Tris buffer and a direct determination of nitrogen was not possible, the total nitrogen could be estimated from the content of amino acids and nucleotides; calculations with fraction III indicated about 2 mg. of total nitrogen. The specific activity in the hydrolysis of cysteinylglycine was 30,000 units; the specific activity in the hydrolysis of leucinamide was 1200. Semenza[7] has reported the purification of a cysteinylglycinase of the labile type and in the report has indicated the need of clarification of the expression of specific activity. Semenza assayed his preparations in a total volume of 1 ml. with correction for dilution and expressed unitages in terms of 10% hydrolysis; our assays are in a total volume of 10 ml. with the same concentration of substrate and our unitages are expressed in terms of 50% hydrolysis. Our unitages are not corrected for the dilution (1 to 10) of the enzyme in the assay sample. Semenza used a digestion period of 10 min.; we use a period of 15 min. Thus, our unit is equal to about 33 Semenza units. The proteolytic coefficients quoted by Semenza must be grossly in error and are not substantiated by his reported activities; it would appear that he has used natural logarithms to calculate the coefficient whereas the coefficient is defined in terms of decimal logarithms.

Composition of Fraction IV.—Fraction IV, concentrated by lyophilization, was dissolved in a total volume of 50 ml. and analyzed for content of guanine (absorption in the ultraviolet at 2600 with guanylic acid as the standard), total phosphate and phosphate released by hydrolysis in 1 *N* HCl for 15 min. at 100° by the method of Allen,[8] total pentose by the orcinol method as modified by Ceriotti[9] and amino acids by a ninhydrin procedure as described by Moore and Stein[10] with glycine as the standard. The results are given in Table VII; amino acids were determined in hydrolysates prepared with 8 *N* HCl for 24 hr. at 105° (designated as total amino acids) and with 1 *N* HCl for 1 hr. at 100° (designated as acid labile amino acids). The identification of guanine as the predominant purine or pyrimidine has rested upon the absorption in the ultraviolet and upon chromatographic techniques. The ratios of absorption at 2500 Å./2600 Å. and 2800 Å./2600 Å. were 0.9 and 0.60, respectively; a sample of guanylic acid at *p*H 7 and run at the same time was found to have identical ratios. A hydrolysate of the material prepared with barium hydroxide at room temperature was found to yield guanine nucleotides;

(7) G. Semenza, *Biochem. et Biophys. Acta*, **24**, 401 (1957).
(8) R. J. L. Allen, *Biochem. J.*, **34**, 858 (1940).
(9) G. Ceriotti, *J. Biol. Chem.*, **214**, 59 (1955).
(10) S. Moore and W. H. Stein, *ibid.*, **211**, 907 (1954).

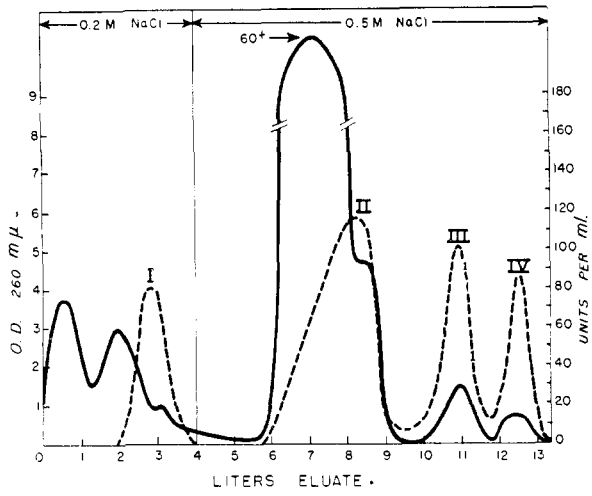

Fig. 1.—Purification with Ecteola-cellulose: solid line, optical density at 260 mμ; broken line, activity in the hydrolysis of cysteinylglycine.

in the formate techniques of chromatography,[11] guanosine monophosphate and diphosphate were identified. Similar results were obtained with fraction III.[1] By a change of *p*H of the buffer in the gradient as described above from *p*H 7 to 7.5, it was possible to elute the unrelated nucleotides before fraction I and to obtain this material in a homogeneous manner. The material was lyophilized and submitted to analysis as for fraction IV (Table VIII). The content of guanine is not given since the absorption in the ultraviolet revealed a maximum at 2800 with no indication of purine absorption; the ratio of absorption 2800 Å./2600 Å. was 1.6. On the basis of total nitrogen, fraction I was found to have a specific activity of 2000 units per mg. N in the hydrolysis of cysteinylglycine and a proteolytic coefficient of 120 in the hydrolysis of leucinamide.

TABLE VII

COMPOSITION OF FRACTION IV

Component	Mmoles per l.
Guanine	1.20
Total phosphate	2.44
Labile phosphate	1.19
Pentose	1.27
Amino acids, total	5.00
Amino acids, labile	2.06

TABLE VIII

COMPOSITION OF FRACTION I

Component	Mmoles per l.
Pentose	1.1
Total phosphate	1.9
Labile phosphate	1.0
Amino acids, total	26.6

From time to time, concentrates were found to have a high protein content and to behave in an unusual manner in chromatography. From such a concentrate, fraction I was isolated, subjected to redigestion with chymotrypsin and trypsin at 40° for 6 hr. and rechromatographed on Ecteola-cellulose; two fractions were obtained in the gradient up to 0.2 *M* NaCl (Table IX). The amino acids are expressed as moles per mole of total phosphate. There was no loss of activity during the digestion and the three fractions were equally active when the activity was expressed on the basis of phosphate content. It would appear that digestion with trypsin and chymotrypsin had removed much protein and, particularly, protein with amino acids absorbing in the ultraviolet.

(11) R. B. Hurlbert, H. Schmitz, A. F. Brumm and V. R. Potter, *ibid.*, **209**, 23 (1954).

TABLE IX

EFFECT OF REDIGESTION WITH CHYMOTRYPSIN AND TRYPSIN

Fraction	Total amino acids	Labile amino acids	Ratio 2600/2800
Before digestion	125	18	0.88
Tubes 75–99			
After digestion	6.7	3.8	0.91
Tubes 70–120			
After digestion	1.6	1.6	1.57
Tubes 180–190			

Evidence of Polynucleotide Nature.—Although the peptidases were not inactivated by treatment with ribonuclease and no action of ribonuclease was detected by changes in optical properties or by release of titrable acidic groups, the material when treated with alkali had a marked "hyperchromic effect." A solution with an absorption of 0.33 at 2600 in 0.1 *N* NaOH when freshly prepared was found to have an absorption of 0.43 after 20 hr. at 37°. It is of interest that Magasanik and Chargaff[12] found a marked hyperchromic effect with polynucleotide "cores" rich in guanylic acid.

Amino Acid Composition.—Fraction IV was hydrolyzed with 6 *N* HCl for 24 hr. at 100° and the HCl was removed by repeated evaporation. A solution of the hydrolysate in water was subjected to two dimensional descending chromatography; the first dimension was with 1-butanol–water–acetic acid in the proportions of 4–5–1; the second dimension was with *n*-propanol–water in the proportions of 7–3. The amino acids were detected with a ninhydrin stain. Aspartic acid, glutamic acid, serine, isoleucine and leucine were identified; another component found somewhere near histidine was not identified. An aliquot of the hydrolysate containing 2 mg. of "leucine equivalents" was placed upon a column of Dowex 50 × 4 and the amino acids were eluted according to the procedures of Moore and Stein[13] with analysis of the eluates in terms of leucine equivalents by a photometric ninhydrin procedure.[10] The results are given in Table X; the amino acids found by paper chromatography were detected in the ion-exchange methods and, in addition, an unknown component was found. However, no basic amino acids were detected and the material behaving somewhat like histidine in paper chromatography must not be histidine. The results are not corrected for color yield or for destruction of amino acids during hydrolysis. Traces of glycine and alanine are found in the hydrolysates but it would appear these arise from the breakdown of the purines.

TABLE X

AMINO ACID CONTENT OF FRACTION IV

Amino acid	Effluent, ml.	Leucine equiv. (mg.)
Unknown	84–94	0.28
Aspartic acid	158–180	.35
Glutamic acid	252–276	.25
Serine	182–200	.27
Isoleucine	438–450	.33
Leucine	450–462	.31
		1.89

(12) B. Magasanik and E. Chargaff, *Biochem. et Biophys. Acta*, **7**, 396 (1951).

(13) S. Moore and W. H. Stein, *J. Biol. Chem.*, **211**, 893 (1954).

ATLANTA 22, GEORGIA

[CONTRIBUTION FROM PASADENA FOUNDATION FOR MEDICAL RESEARCH]

Peptide Studies. III. An Antibacterial Tripeptide of L- and D-Valine[1]

BY S. SHANKMAN, S. HIGA AND V. GOLD

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The relationship between antibacterial activity and configuration of five tripeptides of L- and D-valine has been investigated, employing *Streptobacterium plantarum, Leuconostoc citrovorum* parent and mutant, *Lactobacillus casei* and *Lactobacillus fermenti*. The tripeptide L-valyl-L-valyl-D-valine is shown to be antibacterial at 0.05 mg. per ml. for three cultures. The other peptides or derivatives were not antibacterial at 20 times this concentration.

Previous work on peptides with antibacterial activity has shown the presence of D-amino acids in such peptides[2] or has been concerned with large polymers of a single amino acid.[3] Work in this Laboratory has been directed to the systematic investigation of optical configuration and biologic activity of peptides.

The eight pure tripeptides of valine have been synthesized previously[4] and their utilization by lactic acid bacteria has been studied.[5] The present report is a study of five of these peptides and some lower molecular weight derivatives for the possible inhibition of bacterial growth. The compound L-valyl-L-valyl-D-valine has been found to be antibacterial. None of the other compounds tested had significant antibacterial properties.

(1) Supported in part by grant CY-3609 from the National Institutes of Health, National Cancer Institute.

(2) T. S. Work in Biochemical Society Symposia No. 1, Cambridge Univ. Press, 1948, p. 61.

(3) D. W. Watson and W. L. Bloom, *Proc. Soc. Exp. Biol. & Med.* **39**, 27 (1952).

(4) S. Shankman and Y. Schvo, THIS JOURNAL, **80**, 1164 (1958).

(5) S. Shankman, S. Higa, H. A. Florsheim, Y. Schvo and V. Gold, *Arch. Biochem. & Biophys.*, in press.

Valine was added to the medium employed for utilization studies.[5] The test period was 65–72 hr. unless otherwise noted. Other conditions have been described previously.[5] Compounds tested and antibacterial findings are given in Table I.

The data presented in Table I indicate that of the peptides tested, L-valyl-L-valyl-D-valine was much more effective as an antibacterial agent than any other compound. That such activity was due to the structure of inhibitor, and not to concentration effects, is demonstrated by the lack of toxicity of glycine or mixed amino acids. The slight antibacterial activity of phthalyl-D-valine and phthalyl-L-valine for *S. plantarum* has been observed previously.[6]

Neither L-valyl-L-valyl-D-valine nor L-valyl-L-valyl-L-valine inhibited *L. fermenti* under these test conditions. For other compounds listed, inhibitions for *L. fermenti* were approximately those of *S. plantarum*. The LLD tripeptide inhibited *L. casei* at the start of this work with a half-maximum concentration for inhibition of 0.25 mg./ml. Later studies

(6) F. N. Minard and S. W. Fox, THIS JOURNAL, **71**, 1160 (1949).