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SEQUENCES OF AMINO ACID RESIDUES IN BACITRACIN

by

John F. Griffith

A Dissertation Submitted to the
Graduate Faculty in Partial Fulfillment of
The Requirements for the Degree of
DOCTOR OF PHILOSOPHY

Major Subject: Biochemistry

Approved:

Signature was redacted for privacy.

In Charge of Major Work

Signature was redacted for privacy.

Head of Major Department

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Iowa State College

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INTRODUCTION

Recent years have produced many variations and a few improvements in the methods for amino acid residue sequence analysis of peptides. The use of 2,4-dinitrofluorobenzene by Sanger and alkaline phenyl isothiocyanate by Edman are two examples of aminoid blocking reagents which have found wide application. Most of the methods of sequence analysis are of a qualitative nature, used in conjunction with such techniques as paper and column chromatography, paper electrophoresis, and counter current distribution. Because these are qualitative methods, the peptide under consideration must be homogeneous, or at least free of other peptide material, so that other substances present cannot cause misleading results. Unfortunately few, if any, of the naturally occurring peptides are homogeneous, so that often laborious purification procedures must be carried out (e.g., oxytocin). In spite of this, many natural peptides have been purified, and some, such as Gramicidin S, insulin, tyrocidin, oxytocin, and vasopressin, have had their amino acid residue sequences elucidated.

It has become clear that few of the naturally occurring peptides have a simple, straight chain structure. Several have been shown to be of a cyclic nature, especially those

having antibiotic properties; some contain non-peptide moieties. The presence of such cyclic arrangements of residues and other unorthodox linkages, together with the difficulty in separating and purifying similar peptides has suggested that a quantitative approach to structure analysis might permit structure determination of a complex peptide which is the major component of a mixture.

The antibiotic, bacitracin, is such a mixture of peptides. It is a family of at least six similar peptides with a major component, bacitracin A, providing most of the antibiotic activity. The investigation upon which this thesis is based is concerned with determining the sequence of amino acid residues in bacitracin by application of quantitative methods to the crude material.

REVIEW OF THE LITERATURE

The polypeptide antibiotic, bacitracin, which is produced by a strain of *Bacillus licheniformis* (23) was discovered by Johnson and Meleney (19) in June, 1943. It was isolated from the damaged tissue and street dirt from a compound fracture of a seven year old child named Tracey. The name bacitracin was thus derived.

The literature through 1952 relating to the chemistry of bacitracin has been reviewed by Ellinger (11). This review will therefore be limited to the literature dealing with the chemistry of bacitracin from 1953 to date, plus any prior work which it is necessary to include for the purpose of comparison or clarity of description.

Bacitracin has been shown by Newton and Abraham (31), and by Craig *et al.* (9) to consist of a family of similar peptides. These peptides have been separated by both groups of investigators, using counter current distribution, and the main component has been designated bacitracin A. This peptide comprises 44 to 75 per cent of commercial bacitracin (9, 31, 32), the yield varying considerably with the source and the distributing solvents. The minor peptides have been designated with letters B through G. Different investigating groups have endeavored to maintain interconsistency in these

designations by comparing the composition, ultraviolet absorption spectra, relative quantities, and activities of these peptides. Some inconsistencies may still exist, however, since Craig *et al.* (9) report a total of five peptides in commercial bacitracin and Newton and Abraham report ten (32). The reported compositions are summarized in Table 1. Newton and Abraham obtained their separation of approximately 0.7 g. of crude bacitracin hydrochloride using 221 transfers with

Table 1

Composition of commercial bacitracin

Fraction	Percentage of total material	
	Craig *et al.* (9)	Newton & Abraham (29)
A	60	37
A_1	--	7
B	14	12
C	--	5.5
D	5	17.5
E	0-3	17.5
F_1	18	2.5
F_2	18	5.5
F_3	18	11
G	--	2

the solvent system n-amyl alcohol:n-butyl alcohol:0.05 M potassium phosphate buffer, pH 7.0 (4:1:5). The system employed by Craig *et al.* was sec-butyl alcohol: 3 per cent aq. acetic acid, a less selective system than that of Newton and Abraham but one in which bacitracin is more stable. An initial distribution of 527 transfers was followed by a 900 transfer distribution after removal of bacitracins D and E from the apparatus. This is the method by which Craig *et al.* obtain their standard preparation of bacitracin A.

Flodin and Porath (12) have recently resolved bacitracin into two main peaks and two very minor ones by zone electrophoresis on starch. One of the main peaks appeared to be about 20 per cent larger than the other one, and was reported to contain the bulk of the activity. Both of the main peaks were reported to be heterogeneous by other methods of separation.

Bacitracin A

Amino acid composition

Craig *et al.* (6) hydrolyzed their standard preparation of bacitracin A with 6 N hydrochloric acid in an evacuated sealed tube at 110° for 24 hours. The amino acids in the

hydrolysate were determined by the ion exchange technique of Moore and Stein (29). The results are shown in Table 2, compared with a starch column analysis of a bacitracin preparation. Each amino acid was isolated in crystalline form by counter current distribution of an acid hydrolysate of commercial bacitracin since this substance has substantially the same qualitative amino acid composition as bacitracin A (2).

Table 2

Comparison of Amino Acid Compositions of Commercial Bacitracin[a] and Bacitracin A[b]

Amino acid	Bacitracin		Bacitracin A	
	Percentage	Molar proportion[c]	Percentage	Molar proportion[c]
D-Phenylalanine	11	1.0	10.8	1.03
L-Leucine	9	1.1	8.2	1.02
L-Isoleucine	22	2.8	14.6	1.83
L-Cysteine	14	1.9	7.4	1.00
D-Glutamic acid	10	1.1	8.1	0.89
DL-Aspartic acid	17	2.0	14.7	1.77
L-Histidine	10	1.0	7.7	0.80
L-Lysine	9	1.0	8.1	0.89
D-Ornithine	--	1 (?)	6.5	0.80
Ammonia	1.5	1.2	1.3	1.08
Total	103.5		87.4	

[a]Adapted from Barry, Gregory, and Craig (2).

[b]Adapted from Craig, Hausmann, and Weisiger (6).

[c]Based on a hypothetical molecular weight of 1411 for bacitracin A.

Isolated amino acids were identified by paper chromatography, carbon and hydrogen analyses, and optical rotations. The latter data made possible the assignment of the optical configurations shown in Table 2. The racemic aspartic acid indicates a residue each of D- and L-aspartic acid. The data also indicate a single residue of cysteine rather than one of cystine, which was believed present from earlier data (2).

In a recent communication, Craig *et al.* (8) report that bacitracin A apparently contains a third residue of isoleucine, it being of the D-configuration, making a total of two L- and one D-isoleucine residues. This residue, according to Piez (35) appeared in a small chromatographic fraction which Craig *et al.* (6) identified as methionine but failed to explain since bacitracin has been shown definitely to be free of methionine (2).

Stability

Bacitracin A gave no change in distribution pattern after being stored several months at 6°, according to Craig *et al.* (9); this was not the case with a preparation of crude bacitracin (5). Precipitation of the peptide two or three times from methanol solution with acetone did not lower its antibiotic activity (9), though water solubility was reduced

due to removal of solvated acetic acid. The difference in distribution was caused by a gradual conversion of bacitracin A to a peptide with the same ultraviolet absorption spectrum as bacitracin F. The standard preparation of bacitracin A will also undergo this conversion to bacitracin F if permitted to stand several days at room temperature in a phosphate solution of pH 7 or slightly above. Newton and Abraham (33) did not mention this conversion, but did indicate that treatment for two hours at 37° with 0.1 N sodium hydroxide almost completely inactivated the peptide with relatively little change in the ultraviolet absorption spectrum.

Bacitracin A takes up oxygen very slowly at pH 8 (33). This oxygen uptake is greatly accelerated by the presence of cupric ions. Similar observations had been made for bacitracin by Sharp *et al.* (39).

Hydrolysis of bacitracin A with 0.5 N hydrochloric acid for 20 minutes at 100° (33) completely destroys its antibacterial activity.

Titration behavior

Newton and Abraham (33) titrated bacitracin A hydrochloride with 0.1 N sodium hydroxide and obtained three breaks in the curve. One at pH 2.5-5.5 they attributed to the

presence of two carboxyl groups. The portion of the curve from pH 5.5-8.5 was believed due to the imidazole group of histidine, and the portion from pH 8.5-11.5 to either the δ-amino group of ornithine or the ϵ-amino group of lysine. Titration of the product of acid inactivation revealed one additional carboxyl group in the range pH 2.5-5.5, due to the hydrolysis of an amide, and two additional groups in the range pH 5.5-11.5. Of the latter, one could be ammonia from the amide and the other a free thiol group.

Functional groups

Amino groups. Several groups of investigators have studied the products obtained on reacting bacitracin A with fluoro-2,4-dinitrobenzene (FDNB), the reagent of Sanger (37). Craig *et al.* (7) obtained several partial substitution products, which were separated by counter current distribution, hydrolyzed, and their composition determined by two-dimensional paper chromatography. The dinitrophenyl (DNP) amino acids were identified by their yellow color on the paper and by the fact that the amino acids from which they were derived were reduced in intensity. In this manner δ-DNP-ornithine was identified from di-DNP-bacitracin A. The other DNP-amino acid was not named, it merely being stated that another free

amino group was present in bacitracin A. Craig _et al._ (8) later identified it as DNP-isoleucine, and stated that its yield was improved if the bacitracin A was oxidized with performic acid.

Newton and Abraham (32, 33) obtained DNP-leucine or DNP-isoleucine, i-DNP-histidine and δ-DNP-ornithine from DNP-bacitracin A. More recently, Lockhart, Newton, and Abraham (22), have cleared up their uncertainty concerning the leucines, stating that they have definitely isolated DNP-isoleucine and not DNP-leucine.

Porath (36) found DNP-isoleucine from DNP-bacitracin A. None of these investigators have found any ϵ-DNP-lysine and have therefore generally concluded that the ϵ-amino group of the lysine residue is not free, but is probably bound to a carboxyl group of aspartic acid or glutamic acid. Isoleucine is apparently N-terminal, on the basis of the DNP- studies. The δ-amino group of ornithine and the imidazole group of histidine are free.

The application of Edman's method (10) of blocking N-terminal residues with phenylisothiocyanate and removing them as phenylthiohydantoins has not been very successful with bacitracin A. Porath (36) found no N-terminal residue this way, and Lockhart _et al._ (22), who applied the method to commercial bacitracin rather than bacitracin A, recovered

only five per cent of the theoretical amount of isoleucine-PTH.

Ingram (17) methylated commercial bacitracin and obtained δ-N-dimethyl ornithine from the hydrolysate, but could not demonstrate the presence of an α-N-dimethyl amino acid. He therefore suggested that there is no α-amino group in the molecule.

Carboxyl groups. There are no known reports in the literature of positive identification of free carboxyl groups on either bacitracin or bacitracin A. Titration and electrophoresis data (7, 33) indicate that the molecule is neutral, so that there are probably equal numbers of free carboxyl and amino groups, i.e., two of each. One carboxyl group is bound as an amide and another is probably bound to the ϵ-amino group of lysine.

The sulfur. Hydrolysis of bacitracin A yields a single molecule of cysteine (6), but the intact peptide gives none of the characteristic color tests for a thiol group (33). Hydrolysis for 20 minutes at 100° with 0.5 N hydrochloric acid releases a thiol group which gives a strong nitroprusside reaction, and which will reduce potassium ferricyanide at room temperature (32). Bacitracin A will also yield S-DNP-cysteine on treatment with FDNB. Bromine water (33)

and performic acid (8) oxidize the sulfur, so that cysteine is converted to cysteic acid.

Newton and Abraham (33) carried out a hydrogenolysis of bacitracin A with Raney nickel and reported that cysteine was not present in a hydrolysate of the product, but appeared to have been replaced by alanine. Two other new substances were present in the hydrolysate which reacted slower with ninhydrin than the amino acids. One of these substances had an R_f value slightly higher than alanine in butanol: acetic acid, and the other had an R_f value slightly higher than leucine. These were not identified. The desulfurized bacitracin, on treatment with FDNB and subsequent hydrolysis contained DNP-alanine, indicating that the hydrogenolysis produced an N-terminal alanine residue, in place of the nonterminal cysteine.

When the hydrolysate of the hydrogenolysis product was oxidized with periodate and 2,4-dinitrophenylhydrazine added, a 2,4-dinitrophenylhydrazone was obtained. This had a m.p. of 112-114° after recrystallization from ethanol, but was not characterized.

The above findings led Craig <u>et al.</u> (7), and Newton and Abraham (33) to speculate that the sulfur of cysteine is probably involved in a labile heterocyclic ring. Bacitracin A does not give a positive nitroprusside reaction on standing

with nitroprusside and ammonia, while a thioester does. Since bacitracin A was not readily oxidized to a disulfide by iodine (33), a simple thiazolidine with an unacylated nitrogen was excluded by Newton and Abraham. Newton and Abraham suggested that the 2,4-dinitrophenylhydrazone they obtained was derived from an aldehyde which was the oxidation product of an α-amino alcohol. This amino alcohol they believed to be a by-product of the Raney nickel reduction of the heterocyclic ring, the other products being alanine and hydrogen sulfide. The amino alcohol could have arisen from a potential amino aldehyde, since Raney nickel will reduce carbonyl compounds (26). Newton and Abraham therefore suggested that a thiazoline ring is present in bacitracin A as part of the peptide chain:

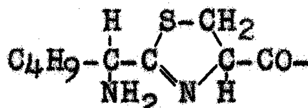
$$
\begin{array}{ccccc}
 & R & S & \text{—} & CH_2 \\
 & | & | & & | \\
-NH- & CH- & C & & CH-CO- \\
 & & & \overset{\diagdown\!\!\!=}{}N\diagup &
\end{array}
$$

Mild treatment of such a ring with acid or oxidation with performic acid or bromine water would produce a new peptide bond and a free thiol or sulfonic acid group, respectively. Newton and Abraham did expect that a thiazoline might be basic enough to be titrated, but stated that there was no room on the titration curve for a basic group in addition

to the δ-amino group of ornithine and the imidazole of histidine.

Porath (36) reported the probable presence of a thiazoline or thiazolidine ring, but gave no evidence for it. He could find no indication of a disulfide bond in bacitracin A by polarography and ruled out any sort of dimerization as a result of ultracentrifugation experiments.

Craig _et al._ (8), in proposing a structure for bacitracin A stated that absorption spectra and other observations (not specified) were consistent with a thiazoline ring system. They proposed that the carbonyl carbon of isoleucine is part of the ring, and pictured the isoleucine in the _D_-configuration, and as a terminal residue:



Ultraviolet absorption spectrum

Craig _et al._ reported a characteristic weak absorption maximum for bacitracin A at 250-255 mμ. On standing several days at 24° in 0.1 M sodium carbonate, the whole spectrum shifted toward higher wavelengths to give a maximum at 290 mμ. This shifted spectrum was similar to that of bacitracin

F, indicating that the latter may be a degradation product of bacitracin A. Bacitracin A, on standing for several days at 24° in 0.1 N hydrochloric acid, gave a complete absence of the maximum at 250-255 mμ.

Molecular weight

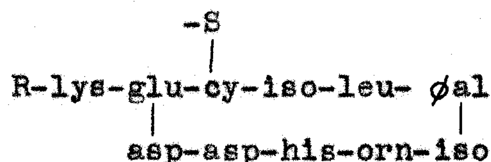
The molecular weight of bacitracin A has been determined by Craig *et al.* (7), using mono- and disubstituted DNP-derivatives. For their calculations, they used as a molecular extinction coefficient for DNP-peptides the value 14,500 ± 10 per cent (3). From the optical density at 350 mμ, they calculated a molecular weight of 1640 for the mono-DNP-derivative or 1470 for the unchanged peptide. The di-DNP-derivative led to a molecular weight of 1310 for the peptide, a value they stated was less accurate than that of 1470. From a sulfur content of 2.2 per cent and the knowledge that only one atom is present, they calculated a molecular weight of 1450.

Porath (36) determined the molecular weight of bacitracin purified by charcoal chromatography. By ultracentrifugation, he obtained a molecular weight of 1390. Porath's bacitracin A preparation appeared to be identical with a sample supplied to him by Craig. Small variations could

be accounted for by the presence of associated solvent molecules, which might differ, depending upon the method of purification.

Amino acid residue sequences

Porath (36) partially hydrolyzed bacitracin A with 6 N hydrochloric acid at 37° for 72 hours. Zone electrophoresis and charcoal and paper chromatography were used to fractionate the resulting peptide mixture. He experienced difficulty in isolating peptides containing cystine, glutamic acid, or aspartic acid, since they were frequently rendered insoluble on evaporation of the solvent. One fragment contained only lysine and a ninhydrin-negative compound which could not be identified. Porath proposed the following sequence, pointing out that the molecule is highly asymmetrical. He indicated

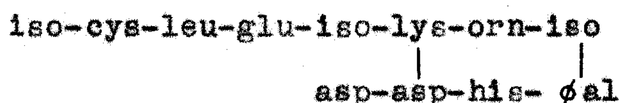


that the lysine, cysteine, and glutamic residues are probably part of a labile heterocyclic ring system. Porath believes that the heterocyclic ring together with the D-amino

acid residues is primarily responsible for the antibiotic activity, though the asymmetrical shape of the molecule may contribute. No experimental evidence was presented for the structure, and Porath felt that he had not definitely established the amino acid residue sequence.

Lockhart _et al._ (22) used 11 N hydrochloric acid at 37° to hydrolyze partially bacitracin A or its performic acid oxidation product for varying periods from one to six days. Paper electrophoresis and paper chromatography were used to separate the peptides, and their N-termini were determined by Sanger's (37) method. Six days of hydrolysis using the above conditions released significant amounts of free histidine, ornithine, aspartic acid and glutamic acid. Dipeptides identified were leucylglutamic acid, β-sulfoalanylleucine, and isoleucylcysteic acid. A tripeptide of leucine, glutamic acid, and N-cysteic acid, and a tetrapeptide of N-isoleucine, cysteic acid, leucine, and glutamic acid were also identified. These results led them to propose the partial sequence -iso-cys-leu-glu-. From unpublished data, they suggested that the isoleucine end extended through phenylalanine, isoleucine, and ornithine, with ornithine N-terminal. They also believed that an aspartic acid residue was not far removed from that of glutamic acid.

Craig *et al.* (8) have proposed in a communication a nearly complete structure for bacitracin, except for a "complication of cross linkages", one of which may involve the amino group of the isoleucine residue next to cysteine. They also used hydrochloric acid partial hydrolysis and treated the products with FDNB, the DNP-peptides being purified by counter current distribution, two dimensional paper chromatography, and paper electrophoresis. These DNP-peptides were then completely hydrolyzed and the DNP-amino acids extracted. These, and the remaining amino acids in the hydrolysates were separately characterized by the same methods used to purify the DNP-peptides. The data obtained led to the conclusion that there are three, and not two, residues of isoleucine. By characterizing over 30 peptide fragments, Craig *et al.* proposed the following structure, with isoleucine N-terminal and aspartic acid C-terminal. No attempt was



made to locate the amide group, but a thiazoline ring which was included involved isoleucine and cysteine (p. 14). It is interesting to note that this structure, including the amide group and a molecule of acetic acid present in the

standard preparation of Craig *et al.* (8, 9), has a calculated molecular weight of 1451.

Other Bacitracin Peptides

Relatively little has been published about the other peptides of bacitracin. There are perhaps several reasons for this, the most important being the difficulty of obtaining any quantity of them in pure form, though this has undoubtedly been done in the process of purifying bacitracin A. Another probable reason is their low activity and therefore relative unimportance. The antibacterial activities of the various bacitracins have been reported by Newton and Abraham (32), in units per mg.: A, 36.0; B, 13.5; C, 18.0; D, 0.5; E, 0.3; F_1, 2.0; F_2 1.0; F_3, 0.5; G, 5.0. The unit of activity is that defined by Arriagada, *et al.* (1).

Amino acid composition

No quantitative estimations of the amino acid compositions of the peptides other than bacitracin A have been published. Crude bacitracin contains a substance which on acid hydrolysis behaves very much like valine, both microbiologically with *L. mesenteroides* (32) and on chromatography (5),

but when isolated as a crystalline material is not valine. It has not been characterized as yet, so will be referred to as "valine". This "valine" is apparently associated with bacitracin B (5, 9) as an N-terminus, because DNP-"valine" has been obtained from both crude DNP-bacitracin (22) and DNP-bacitracin B (32). DNP-isoleucine has also been isolated from a hydrolysate of DNP-bacitracin B. Bacitracins D and E have also been reported to contain "valine".

Bacitracins C and G yield on hydrolysis a substance which behaves like glycine. The amino group of this glycine is free, since it forms a DNP-derivative. Newton and Abraham have considered the fact that this glycine might come from the degradation of purines during hydrolysis.

All of the bacitracins contain cysteine, ornithine, lysine, histidine, aspartic acid, glutamic acid, phenylalanine, leucine and/or isoleucine residues. Bacitracins D and E contain little if any amide nitrogen.

Ultraviolet absorption spectra

Craig *et al.* (9), and Newton and Abraham (32) have studied the ultraviolet absorption spectra of the various bacitracin peptides. Bacitracins B, D, and E, like A, show broad, weak maxima at 250-255 mμ. Bacitracins C and G both

have a strong, sharp maximum at 258 mμ with a shoulder at 268 mμ. Bacitracin F has a shoulder at 250-255 mμ and a broad maximum at 288-290 mμ.

EXPERIMENTAL

Materials

Bacitracin

Crude bacitracin was supplied by Commercial Solvents Corporation, Terre Haute, Indiana in two lots. Lot B510221-A had a labeled potency of 55 units per mg. and lot C-373 was labeled 63 units per mg. In the text these will be referred to as lot B and lot C, respectively. Lot B was a pale yellow powder and lot C was white.

Bacitracin A was supplied by Dr. L. C. Craig of the Rockefeller Institute for Medical Research, New York, N. Y., and was assumed to be some of his standard preparation (9) containing acetic acid.

Chemicals and reagents

Chemicals and reagents were of reagent or c.p. grade except where purity was not important, where technical grade was used. Ninhydrin used for quantitative determinations was obtained from Dougherty Chemical Company.

Bacterial cultures

The following bacterial cultures which were used in routine microbiological assays were regularly maintained: *Lactobacillus arabinosus* 17-5, ATCC 8014; *Leuconostoc mesenteroides* P-60, ATCC 8042; *Leuconostoc citrovorum*, ATCC 8081; *Lactobacillus brevis*, ATCC 8287; and *Streptococcus faecalis*, ATCC 8042.

Methods

Microbiological assays

The microbiological assays were carried out by routine procedure. The media used are those referred to as q, r, s, u and x by Fox *et al.* (14), with the amino acid to be assayed omitted. Table 3 shows the medium used with each culture.

Standard curves covered the range 0-50 µg. except for histidine, which was run between 0-20 µg. Final pH of media before inoculation was 6.8 - 7.0 except for medium u, which contained arabinose. In this case the final pH was 6.5 in order to reduce darkening while in the autoclave. Standards were usually set up in duplicate, sufficient water being added to give a volume of 2.50 ml.

Table 3

Microorganisms and Media for Microbiological Assays

Amino acid	Microorganism	Medium[a]
Aspartic acid	*L. brevis, L. mesenteroides* P-60	q
Cystine	*L. mesenteroides*	q
Glutamic acid	*L. citrovorum*	x
Histidine	*L. mesenteroides* P-60	q, r
Isoleucine	*L. arabinosus* 17-5	q
Leucine	*L. arabinosus* 17-5	q
Lysine	*L. mesenteroides* P-60	s
Phenylalanine	*L. brevis*	u
Valine	*S. faecalis*	q

[a]Letters refer to media used by Fox *et al.* (14).

Samples were hydrolyzed 18 to 20 hours at 120° with 6 N hydrochloric acid. In some cases, as indicated in the text, constant boiling hydrochloric acid (glass distilled) was used, inasmuch as this seemed to reduce the formation of humin during hydrolysis (18). The hydrolyzed samples were evaporated to dryness *in vacuo*, the residues taken up in distilled water and again evaporated to dryness. The residues were

then redissolved in water, adjusted to pH 6.8 with 0.2 N sodium hydroxide, and diluted to a known volume. In sequence study II, the complete hydrolysis was carried out by refluxing with a 1:1 mixture of concentrated hydrochloric acid and 98 per cent formic acid for 18 hours. According to Miller and du Vigneaud (24) and Hess and Sullivan (15), the destruction of cystine is reduced under these conditions.

Samples were usually set up in duplicate at three dilutions, with an attempt made to keep the amino acid assayed between 10-30 µg. Sufficient water was added to give a volume of 2.50 ml. To standards and samples was added 2.50 ml. of the appropriate medium to give a total volume of 5.00 ml. in each assay tube.

Tubes were autoclaved 15 minutes at 120°, allowed to cool, and inoculated with a fresh 18 hour culture of the appropriate organism, washed and suspended in isotonic saline. Incubations were for 72 hours at 37°. The assays were titrated with 0.05 N sodium hydroxide with the use of brom thymol blue indicator.

The average titration values for the standard at each level were plotted, obviously aberrant values being discarded, and the sample values were read from this standard curve. Sample values falling near either extreme of a curve or on atypical portions were not used, otherwise titration values

were averaged for a given dilution and this value read from the curve. Values for a given sample from the curve were calculated as ug. per ml. and averaged to give the final assay value.

If samples did not give the same growth response curve as the standards, the assay was repeated, quantity of sample permitting.

Chromatographic identifications on paper

Qualitative identification of amino acids was made by one dimensional chromatography on Whatman no. 1 paper either by ascending or descending technique. Solvents used were butanol:acetic acid:water, 4:1:5 (34); and phenol:pH 8.3 borate buffer, mobile phase:100 ml. of liquefied phenol to 15 ml. of buffer, and equilibrating phase:15 ml. of mobile phase in 85 ml. of buffer (21). Papers used with the phenol:pH 8.3 buffer system were first sprayed with the buffer and allowed to dry.

Ascending technique. Sheets of paper 8½ by 11 inches were used. Spots were applied along a line ½ inch from the long edge of the paper and dried. The paper was rolled into a cylinder with the short edges not quite meeting and stapled in three places. The paper cylinder was placed in a ½ inch

layer of solvent in the bottom of a 1 gallon wide mouth, screw cap jar and the solvent allowed to ascend to the upper edge of the cylinder.

Descending technique. Strips of paper 22½ inches long and 1 to 6 inches wide were used. Solutions were applied on spots along a line 3 inches from one end of the strip and chromatographed by descent in a Research Equipment Corporation, Model MC500, glass chromatographic assembly until the solvent was within 4 inches of the bottom of the strip.

Location of amino acids. The dried papers were sprayed with a ninhydrin reagent prepared by mixing 50 ml. of 0.2 per cent ninhydrin in water-saturated butanol, 15 ml. of glacial acetic acid, and 2 ml. of 2,4,6-collidine (21). This reagent produces a variety of colors with amino acids, from light blue through purple to red and brown. The wet paper was placed in a 105° oven for 2 minutes to develop the colors.

Quantitative chromatography on Dowex-50

Peptides to be assayed were hydrolyzed 24 hours at 120° with constant boiling hydrochloric acid. The hydrolysates were evaporated to dryness *in vacuo* and the residues dissolved in 1.00 ml. of water. A 0.50 ml. volume of each solution was diluted with an equal volume of pH 3.42 0.1 M citrate buffer,

and was placed on a Dowex-50 column with a dropper pipet, followed by three 0.3 ml. buffer washings. The hydrolysates were chromatographed on the Dowex-50 columns by the method of Moore and Stein (29). A 100 cm. temperature-controlled column was used for the separation of the neutral and acidic amino acids and a 15 cm. column was used for the basic amino acids. An effort was made to follow the procedure and recommendations of Moore and Stein as closely as possible. The 100 cm. column was not operated above 50° inasmuch as there was no tyrosine present in any of the samples analyzed. Inasmuch as there was no methionine present in the samples, no thiodiglycol was added to the buffers, otherwise buffers of pH 3.42, 4.25, 5.0, and 6.8 were prepared according to Moore and Stein.

Effluent fractions of 1.0 ml. were collected in 18 by 150 mm. test tubes, mounted on a Research Equipment and Service Company, Model 230, automatic fraction collector equipped with a photoelectric drop counter. The pH of the fractions was adjusted to 5 by adding a previously determined number of drops to 0.5 N hydrochloric acid or N sodium hydroxide to each tube. Aliquots of 0.50 ml. of each fraction were pipetted into photometer tubes for amino acid analysis by the quantitative ninhydrin method of Troll and Cannan (41).

The photometer tubes consisted of optically matched 18 by 50 mm. Kimble soft glass culture tubes (28).

The reagents were prepared according to Troll and Cannan and were added to the tubes with a 2 ml. Alfred Bicknell Associates automatic pipet in the case of the KCN-pyridine and 80 per cent phenol. The ninhydrin solution was added with a 0.2 ml. pipet of the same type. The tubes were stoppered after addition of the ninhydrin and the reaction was allowed to proceed 5 minutes at 95-100°. After cooling, 7.5 ml. of 60 per cent ethanol was added to each tube with a Fisher Volustat. Absorbancy readings were made against reagent blanks (fractions from the column containing no amino acid) to the nearest 0.01 unit at 570 mμ on a Coleman Junior spectrophotometer. If the absorbancy was greater than 1.0 the samples were diluted with sufficient 60 per cent ethanol, in 5.0 ml. increments, to bring the reading below 1.0.

The absorbancy readings were converted directly to micromoles of leucine from a standard curve and these values multiplied by the proper color yield factor for the amino acid being determined. In cases where two or more amino acids with different color yield factors were eluted to give superimposed peaks, the identities of the amino acids present were established by paper chromatography of the fractions after concentration. In such cases, the quantities of amino acids

present could be expressed only as equivalent micromoles of leucine. Curves of micromoles amino acid vs. ml. eluted were plotted and the total quantity of a given amino acid obtained by integrating the area under the proper peak on the curve.

The color yield for each amino acid to be determined was obtained by running the ninhydrin reaction in duplicate on various concentrations of buffered solutions of a given amino acid. Aqueous solutions of 0.10, 0.20, and 0.30 micromoles were pipetted into photometer tubes and evaporated to dryness _in vacuo_. Each residue was redissolved in 0.50 ml. of the buffer solution, in which it would be found on elution from the resin column, i.e., pH 3.42 or pH 4.25 citrate buffer, or pH 6.8 phosphate buffer. In one determination, the pH of the resulting solution was adjusted to 5 by addition of a calculated number of drops of N sodium hydroxide or 0.5 N hydrochloric acid. In another determination, the pH of each solution was left unchanged.

Study of carboxoid blocking conditions

A 16.1 mg. sample of DL-leucyl-DL-valine was dissolved in 30.0 ml. water and 23.7 mg. of benzoyl-DL-leucyl-DL-valine was dissolved in 5.0 ml. of N ammonium hydroxide plus 25.0 ml. water. Seven 4.0 ml. aliquots were taken from each

solution and placed in separate weighing bottles, where they were evaporated to dryness *in vacuo*. To six of the identical samples of each peptide were added 2, 10, or 50 molar excesses of ammonium thiocyanate and phenyl isothiocyanate, respectively, in 10 per cent acetic acid in acetic anhydride. The solutions were heated 30 minutes on a boiling water bath, then evaporated *in vacuo*. The dry residues were left in contact with the air for three days at this stage (16).

All the samples, including the untreated aliquot of each peptide, were hydrolyzed 10 hours with 6 N hydrochloric acid at 120°. The hydrolysates were evaporated *in vacuo*, then taken up in a few ml. of water and adjusted to pH 6.8 ± 0.2 with 0.2 N sodium hydroxide. The samples were again evaporated *in vacuo* and the dry residue allowed to stand one hour with 1 ml. of ethyl acetate. The ethyl acetate was decanted and the residue washed with 2-3 ml. of dry ether. The dry sample was dissolved in 40.0 ml. water and microbiological assays run for leucine and valine.

Sequence study I

A 2.00 mg. sample of bacitracin, lot B, was hydrolyzed at 120° for 14 hours with 5.0 ml. of 6 N hydrochloric acid.

The hydrolysate was evaporated to dryness *in vacuo*, taken up in 5 ml. of water, and again evaporated.

A 10.0 mg. sample of bacitracin, lot B, was dissolved in water to give a 10.0 ml. solution, and four 2.0 ml. aliquots were pipetted into numbered 10 ml. beakers. Two ml. of pyridine containing 40 mg. of phenyl isothiocyanate was pipetted into each beaker and a drop of brom thymol blue added. The pH was adjusted to 8-9 by dropwise addition of 0.2 N sodium hydroxide until the indicator was blue-green, and the samples were allowed to react at 37° for eight hours with occasional readjustment of pH with sodium hydroxide. The samples were then evaporated *in vacuo* over sulfuric acid.

Two ml. of dry dioxane saturated with dry hydrogen chloride (13) was added to beakers AT_2, AT_3, and AT_4, and allowed to stand at room temperature for 6 hours. The dioxane-HCl was evaporated off *in vacuo* over sodium hydroxide.

In this manner, the samples were subjected to a total of 1, 2, 3, and 4 phenyl isothiocyanate treatments and 1, 2, and 3 dioxane-HCl treatments respectively. Finally the dry residues of the treated samples were hydrolyzed in 4.0 ml. of 6 N hydrochloric acid at 120° for 18 hours. The hydrolysates were evaporated to dryness twice *in vacuo* after being dissolved in water. They were finally redissolved

in water, their pH adjusted to 6.8, and diluted to 50.0 ml. for assay.

Sequence study II

A 33.0 mg. sample of bacitracin, lot B, was dissolved in water plus 10-12 drops 0.1 N hydrochloric acid and made up to 25.0 ml. Six 4.0 ml. aliquots were pipetted into weighing bottles labeled AT_0 through AT_6. Aminoid blocking reactions were carried out by adding 4.0 ml. of pyridine containing 22 mg. of phenyl isothiocyanate, 1 drop of brom thymol blue, and sufficient 0.2 N sodium hydroxide to raise the pH to 8-9. The blocking reaction was allowed to proceed at 37° for 4 hours with occasional readjustment of the pH. At the end of this time the samples were evaporated to dryness *in vacuo* and 5.0 ml. of dry dioxane-HCl added. This was allowed to react 6 hours at room temperature, then evaporated off *in vacuo*. This constituted a complete aminoid blocking and cleavage reaction. Each sample was subjected to a different number of such blocking reactions from none to six, each followed by a cleavage reaction, except after the final blocking reaction.

The treated samples and the control, AT_0, were dissolved in a mixture of 2.9 ml. of conc. hydrochloric acid and 3.1 ml.

of 98 per cent formic acid (15, 24) and transferred to 5 in. Pyrex test tubes with ground glass joints. These were fitted with reflux condensers, and the samples were hydrolyzed in an oil bath at 110-120° for 18 hours. The solutions were then evaporated *in vacuo* over sodium hydroxide and sulfuric acid. The residues were taken up with water and again evaporated. They were dissolved again in water and their pH adjusted to 6.9. The solutions were slightly cloudy at this point, so they were filtered into 50 ml. volumetric flasks and made up to volume for assay.

Rate study I. Partial hydrolysis with hydrochloric acid-acetic acid

Partial hydrolysis. Four 15.0 mg. samples of bacitracin, lot B, were weighed into weighing bottles and dissolved in 2.0 ml. of 1:1 10 N hydrochloric acid-glacial acetic acid. The bottles were stoppered and the samples were allowed to hydrolyze 1, 2, 4, and 8 days, respectively at 37°. Hydrolysis was stopped by evaporating the samples to dryness *in vacuo* over sodium hydroxide at room temperature. The dry residues were redissolved in 2 ml. of water and again evaporated to dryness. Each sample was then dissolved in water and diluted to 25.0 ml. Four 6.0 ml. aliquots were taken

from each sample solution and placed in separate weighing bottles labeled T_0, CT_1, AT_1, or AT_2 (see Table 4), together with a number corresponding to the number of days the sample was partially hydrolyzed. These solutions were evaporated *in vacuo*.

Table 4

Key to Symbols Used

Symbol	Definition
DNP	2,4-dinitrophenyl
PTC	phenylthiocarbamyl
PH	3-phenylhydantoin
PTH	3-phenyl-2-thiohydantoin
T_0 or AT_0	peptide hydrolysate having had no previous blocking treatment
CT_1	one carboxoid blocking treatment prior to complete hydrolysis
AT_1	one aminoid blocking treatment prior to complete hydrolysis
AT_n	n successive aminoid blocking treatments prior to complete hydrolysis
XAT_n	alkaline hydrolysate of extract of phenylthiohydantoins in hydrolysate of sample given n aminoid blocking treatments
R	recovery sample

<u>Blocking treatments</u>. Samples labeled AT_1 or AT_2 were dissolved in 3.0 ml. of water and 3.0 ml. of pyridine containing 20 mg. of phenyl isothiocyanate. Two drops of brom thymol blue were added to each, and sufficient 0.2 N sodium hydroxide to give a blue-green color with the indicator (pH 8-9). The samples were kept at 37° for 8½ hours with occasional readjustment of pH. The samples were then evaporated *in vacuo* over sodium hydroxide.

Samples labeled AT_2 were dissolved in 5.0 ml. of dry dioxane-HCl and allowed to stand at room temperature for 16 hours. They were then evaporated to dryness *in vacuo* over sodium hydroxide and sulfuric acid. The residues were treated with alkaline phenyl isothiocyanate exactly as before, then evaporated *in vacuo*.

Samples labeled CT_1 were dissolved in 4.0 ml. of 10 per cent acetic acid in acetic anhydride. Four mg. of ammonium thiocyanate was added to samples CT_1-1 and CT_1-2; 10 mg. of ammonium thiocyanate was added to CT_1-4 and CT_1-8. The solutions were heated 30 minutes on a boiling water bath, then evaporated *in vacuo*. The dry residues were left open to the air for about four days.

Samples labeled T_0 were used as controls, and were therefore subjected to no blocking treatment.

Complete hydrolysis. All of the samples were hydrolyzed with 5.0 ml. of 3 N hydrochloric acid at 120° for 16 hours. The hydrolysates were evaporated _in vacuo_, redissolved in 3-4 ml. of water and their pH adjusted to 6.8 with 0.2 N sodium hydroxide. The neutral solutions were diluted to 25.0 ml. with water and filtered prior to microbiological assay.

Rate study II. Partial hydrolysis with hydrochloric acid-acetic acid

Seven 75.0 mg. samples of bacitracin, lot B, were weighed into weighing bottles and dissolved in 10 ml. of a 1:1 mixture of 10 N hydrochloric acid-glacial acetic acid. The solutions were placed in a 37° oven and one removed and evaporated _in vacuo_ at 1, 2, 4, 8, 16, 32, and 64 days. The residues were taken up in 2 ml. of water and again evaporated to dryness _in vacuo_.

Each residue was redissolved in 0.5 ml. of water and 2 λ of each solution was chromatographed, by descending technique, with butanol:acetic acid:water. A sample of unhydrolyzed bacitracin, lot B, was also chromatographed.

Terminal residue study with recovery samples

Five 12.0 mg. samples of bacitracin, lot B, were weighed into weighing bottles and labeled T_0, CT_1, AT_1, RT_0, and RAT_1. To RT_0 and RAT_1 were added 2.00 mg. of L-isoleucine and 1.00 mg. of L-leucine.

Blocking treatments. Samples AT_1 and RAT_1 were dissolved in 5.0 ml. of water plus 5.0 ml. of pyridine containing 23 mg. of phenyl isothiocyanate. Two drops of brom thymol blue were added to each and sufficient 0.2 N sodium hydroxide to raise the pH to 8-9. The reaction was allowed to proceed 6 hours at 37° with occasional readjustment of pH, then the solutions were evaporated in vacuo over sulfuric acid.

Sample CT_1 was dissolved in a mixture of 9.0 ml. of acetic anhydride and 1.0 ml. of acetic acid containing 23 mg. of phenyl isothiocyanate. The solution was heated 30 minutes on a boiling water bath, then evaporated in vacuo over sodium hydroxide.

Samples T_0 and RT_0 were left untreated as controls.

Complete hydrolysis. All the samples were hydrolyzed 20 hours with 10.0 ml. of constant boiling hydrochloric acid at 120°. The hydrolysates were evaporated to dryness in vacuo, the residues dissolved in 10 ml. of water, and their pH adjusted to 6.6-7.0 with 0.2 N sodium hydroxide. The

solutions were filtered into 100 ml. volumetric flasks and diluted to volume with water prior to microbiological assay.

Preparation of desulfurized bacitracin

Preparation of Raney nickel catalyst. Thirty-eight grams of nickel-aluminum alloy was dissolved in sodium hydroxide, following the procedure of Monzingo (25). After all the alloy had been added at 5-10°, the suspension was allowed to stand overnight at room temperature. From this point, the procedure of Monzingo et al. (27), was followed. The suspension was heated on a water bath at 50° for one hour with occasional stirring. The liquid was then decanted and the Raney nickel resuspended in 200 ml. of water (redistilled water was used throughout), allowed to settle, and the liquid again decanted. The nickel was transferred by means of water washings to a 250 ml. glass stoppered mixing cylinder, the water decanted, and a solution of 5 g. of sodium hydroxide in 50 ml. of water added. This mixture was shaken and the alkali decanted. The nickel was then washed 15 times with 200 ml. portions of water, the suspension then being neutral to litmus. The nickel was washed 11 more times with water, 3 times with 95 per cent ethanol, and 3 times with 50 ml.

volumes of absolute ethanol. The active Raney nickel was stored under absolute ethanol.

Hydrogenolysis of bacitracin. A 0.5 g. sample of bacitracin, lot B, was dissolved in a mixture of 25 ml. of ethanol and 10 ml. of water. A suspension of about 5 g. of Raney nickel in 25 ml. of ethanol was added, and the mixture refluxed on a water bath for one hour. The catalyst was filtered off and washed several times with aq. ethanol (5:1), the washings being added to the filtrate. The solution was evaporated to about 5 ml. over calcium chloride *in vacuo*. This was diluted to about 150 ml. with water and the pH adjusted to 5.0 with 0.1 N hydrochloric acid. The solution was extracted three times with one per cent (w/v) 8-hydroxyquinoline in chloroform, using one 50 ml. portion and two 25 ml. portions. This was followed by four 50 ml. extractions with chloroform. It was necessary to add 10 ml. of ethanol with the first chloroform washing to prevent the formation of an emulsion. The aq. solution was evaporated *in vacuo* to about 100 ml. over calcium chloride, Anhydrone, and sodium hydroxide. A few ml. of ethanol was added to the solution, which was warmed slightly to dissolve suspended material. The product was then lyophilized, yielding 284 mg. of a white powder.

Qualitative analysis of product. The product was tested by sodium fusion for the presence of sulfur and halogen, as was bacitracin. Neither bacitracin nor the hydrogenolysis product contained halide, but the latter still contained a small amount of sulfur when compared with bacitracin. Neither bacitracin nor the hydrogenolysate gave any color with sodium nitroprusside in the presence of cyanide and ammonia; cysteine gave a strong magenta color under these conditions. Hydrolysis of both bacitracin and the hydrogenolysate in 0.1 N hydrochloric acid for 20 min. at 100° resulted in bacitracin giving a fairly strong nitroprusside test, while the hydrogenolysate gave a very weak one.

Sequence study of desulfurized bacitracin

Ninety mg. of the hydrogenolysis product of bacitracin dissolved in 50.0 ml. of water was divided into seven 7.0 ml. aliquots in weighing bottles. The bottles were labeled T_0, CT_1, AT_1, AT_2, AT_3, AT_4, and AT_5; and the aliquots were evaporated *in vacuo*.

Blocking treatments. The AT samples were dissolved in 5.0 ml. of water plus 5.0 ml. of pyridine containing 44 mg. of phenyl isothiocyanate. Two drops of brom thymol blue were added, and the pH adjusted to 8-9 by dropwise addition of

triethylamine (18). The blocking reaction was allowed to proceed at 37° for 12 hours with occasional readjustment of pH by addition of triethylamine, then the solutions were evaporated _in vacuo_ over sodium hydroxide and sulfuric acid. The dry residues were washed with 5 ml. of benzene to remove excess reagents.

Samples AT_2-AT_5 were taken up in 5.0 ml. of dry dioxane-HCl and allowed to stand three hours at room temperature, then they were evaporated _in vacuo_. Alternate phenyl isothiocyanate and dioxane-HCl treatments were carried out on these samples until each had received a number of blocking treatments equal to the sample subscript number. No dioxane-HCl treatment was carried out on a sample subsequent to its final blocking treatment.

Sample CT_1 was dissolved in 10.0 ml. of 10 per cent acetic acid in acetic anhydride containing 12 mg. of ammonium thiocyanate. The solution was heated on a boiling water bath for 30 minutes, then evaporated _in vacuo_. The dry residue was left open to the air about four days.

Sample T_0 was left untreated to serve as a control.

Complete hydrolysis. All the samples were hydrolyzed 24 hours in 10.0 ml. of constant boiling HCl at 120°. The hydrolysates were evaporated to about one half ml. on a steam bath, then to dryness _in vacuo_.

Extraction of thiohydantoins and phenylthiohydantoins. Samples CT_1 and AT_1-AT_4 were extracted six times with 5 ml. volumes of ethyl acetate, by warming over steam, allowing the ethyl acetate to cool, and then decanting it into a weighing bottle marked with the sample designation preceded by an X. Sample AT_5 was dissolved in 5 ml. of water and extracted by shaking with three 5 ml. volumes of chloroform, followed by three 5 ml. volumes of ethyl acetate. All the extracts were evaporated to dryness on a water bath.

Alkaline hydrolysis of extracts. The dry extracts were taken up, by warming, in small volumes of ethyl acetate and transferred to 12x75 mm. Pyrex test tubes. The extracts were evaporated to dryness in the test tubes in a water bath, and finally dried *in vacuo*. Two ml. of 0.25 N barium hydroxide was added to each tube, which was then sealed off. The contents of the tubes were hydrolyzed at 140° for 48 hours, the tube cooled, and broken open. The barium was precipitated by the addition of 0.5 ml. of N sulfuric acid. The solutions were then transferred to small weighing bottles and evaporated to dryness *in vacuo*. The residues were taken up in 0.5 ml. of water and 5 λ samples were chromatographed on paper, using butanol:acetic acid, and phenol:pH 8.3 borate buffer. The dry, extracted hydrolysates of the original samples were

also dissolved in 0.5 ml. of water and chromatographed using the same solvent systems.

Performic acid oxidation of bacitracin A

Performic acid was prepared by mixing 1 ml. of 30 per cent hydrogen peroxide with 9 ml. of 88 per cent formic acid, according to Toennies and Homiller (42). The solution was allowed to stand 30 minutes at room temperature before use (30). A 113.0 mg. sample of bacitracin A was dissolved in 5 ml. of the performic acid and the reaction was allowed to proceed 30 minutes at 24° (38). The oxidation was stopped by the addition of 5 ml. of water, followed by evaporation of the solution *in vacuo* over sodium hydroxide.

The white residue was dissolved in 2 ml. of methanol and 20 ml. of acetone added, precipitating the oxidation product. The solvents were evaporated off *in vacuo* in order to remove any remaining formic acid. The dry residue was dissolved in 2 ml. of warm methanol and the solution was transferred to a tared centrifuge tube with three 2 ml. methanol washings. The oxidation product was again precipitated by the addition of 20 ml. of acetone, and was centrifuged out. The slightly opalescent supernatant was decanted into another tared centrifuge tube, while the centrifugate was washed three

times with 15 ml. volumes of acetone by centrifugation. It was finally dried *in vacuo* and weighed; yield 74 mg. This was designated fraction A.

An equal volume of acetone was added to the original supernatant, producing more white precipitate, which was centrifuged out, washed, dried, and weighed as with fraction A. This material was designated fraction B; yield 22 mg.

The supernatant from fraction B produced more precipitate on standing so the solvent was evaporated off and the yellow residue, designated fraction C, was dried *in vacuo* and weighed; yield 22 mg. The combined yield of the three fractions was 118 mg. (100 per cent of theoretical).

Sequence study of oxidized bacitracin A

Two 10.0 mg. samples of performic-oxidized bacitracin A, fraction A, were dissolved in 2.0 ml. of pyridine and 3.0 ml. of water. The samples were labeled AT_0 and AT_1. To AT_1 was added 1.0 ml. of pyridine containing 22 mg. of phenyl isothiocyanate, and 1 drop of brom thymol blue plus 3 drops of triethylamine. The solution was kept at 37° for two hours, then, together with sample AT_0, evaporated to dryness *in vacuo* over sulfuric acid. The residues were dissolved in 5.0 ml. of constant boiling hydrochloric acid and hydrolyzed for 24 hours at 120°. The hydrolysates were

evaporated *in vacuo* over sodium hydroxide and sulfuric acid.

The residues were dissolved in 1.00 ml. of water and 0.50 ml. portions of each were mixed with equal volumes of pH 3.42 0.1 M citrate buffer. These solutions were analyzed for their amino acid content by quantitative chromatography on Dowex-50 columns, following the procedure of Moore and Stein (29). The effluent fractions were assayed by the ninhydrin procedure of Troll and Cannan (41).

RESULTS

Ninhydrin Color Yields

The quantitative ninhydrin reaction of Troll and Cannan (41) is reported to give equal color yields on a molar basis in water solutions of all the common amino acids except proline and hydroxyproline. This is not the case when the amino acids are in buffered solutions of the compositions obtained from Moore-Stein ion exchange columns. The color yields for some amino acids under such conditions are shown in Table 5.

Carboxoid Blocking Reaction Conditions

Table 6 shows the recoveries of leucine and valine with different carboxoid blocking conditions. For each peptide, the results are expressed as the percentage of amino acid found compared to the untreated control sample. Each value, including its standard deviation, was calculated from the mean of six titration values for each sample.

Table 5

Color Yields from Amino Acids on a Molar Basis Relative to Leucine

Amino acid	pH of buffer			
	3.42	4.25	6.8	5.0
Alanine	.79			---
Aspartic acid	.46			.23
Cysteic acid	---			.25
Cysteine	---			.30
Half-cystine				.24
Glutamic acid	.60			.30
Histidine			.80	.71
Isoleucine		1.02		1.00
Leucine		1.00		1.00
Lysine			.21	.33
Ornithine			---	.35
Phenylalanine		---		.90
Valine		.98		---

Table 6

Percentage Recovery[a] of Amino Acids in Carboxoid Treated Peptides[b]

Peptide	Molar excess of blocking reagent					
	Ammonium thiocyanate			Phenyl isothiocyanate		
	2	10	50	2	10	50
Leucylvaline:						
leucine	79±6	72±6	101±8	70±6	95±11	96±12
valine	30±2	19±1	36±1	56±2	59±2	50±3
Benzoylleucylvaline:						
leucine	89±6	102±6	88±5	65±3	83±6	104±5
valine	33±2	29±1	28±2	45±5	48±3	51±3

[a]Including standard deviations.

[b]Untreated samples equal 100 per cent.

Bacitracin Sequence Studies

Tables 7 and 8 show the results of two series of sequential residue analyses of commercial bacitracin. The assay organisms employed utilized only the L- forms of the amino acids in the hydrolysates. No organisms were available for the assay of ornithine.

Table 7

Sequential Residue Analysis of Commercial Bacitracin
Study I

Amino acid	Percentage amino acid found				
	AT_0	AT_1	AT_2	AT_3	AT_4
Cystine	3.5	1.6	1.3	1.3	1.6
Histidine	8.4	8.8	8.2	8.4	7.9
Isoleucine	36.	19.	17.	21.	20.
Leucine	9.5	8.9	9.0	8.9	8.9
Lysine	8.8	9.2	10.7	11.8	11.8

Rate Studies

Table 9 gives the microbiological assay results of partial hydrolysates of commercial bacitracin which have been subjected to various blocking treatments. The data are presented graphically in Figures 1-5. The zero time value is taken from the mean T_0 value for each amino acid. Figure 6 is a chromatogram showing the progressive liberation of amino acids during the partial hydrolysis of commercial bacitracin.

Table 8

Sequential Residue Analyses of Commercial Bacitracin
Study II

Amino acid	Percentage amino acid found						
	AT_0	AT_1	AT_2	AT_3	AT_4	AT_5	AT_6
Aspartic acid[a]	5.3	6.0	5.6	4.8	4.9	5.2	5.0
Cystine	6.7	---	4.7	1.9	0.8	0.7	0.7
Glutamic acid	2.2	1.8	1.6	1.1	1.2	0.8	0.6
Histidine	8.1	9.3	9.3	7.8	7.7	6.8	7.1
Isoleucine	33.	29.	29.	31.	29.	32.	---
Leucine	6.2	7.1	7.2	5.9	5.7	5.2	5.5
Lysine	2.2	2.8	2.8	2.4	2.6	2.8	2.6
Ornithine	---	---	---	---	---	---	---
Phenylalanine	0.4	0.4	0.4	0.3	0.5	0.4	0.4

[a] *L. brevis*.

Table 9

Rate Study I. Partial Hydrolysis with HCl:Acetic Acid

(Values are number of residues, based on a molecular weight of 1470 for bacitracin)

Amino acid	Days of partial hydrolysis	Blocking treatment			
		T_0	CT_1	AT_1	AT_2
Aspartic acid[a]	1	.96	1.22[b]	1.01[b]	1.08[b]
	2	1.01	1.44	1.00[b]	1.02
	4	1.05[b]	1.00[b]	.90[b]	.63[b]
	8	1.00[b]	0.88	1.05[b]	.40[b]
Histidine[c]	1	.66	.41	---	.65
	2	.66	.39	---	.63
	4	---	---	.60	.55
	8	.59	.28	.42	.34
Lysine	1	.71	.62	.72	.67
	2	.70	.68	.74	.62
	4	.71	.65	.70	.60
	8	.69	.58	.72	.52
Isoleucine	1	1.98	2.04	1.69	1.59
	2	2.04	2.11	1.67	1.37
	4	2.00	2.02	1.52	1.04
	8	1.99	1.98	1.30	.76
Leucine	1	.86	.83	.80	.72
	2	.91	.77	.80	.63
	4	.87	.74	.75	.59
	8	.90	.63	.72	.56

[a]*L. brevis*.

[b]From single titration value.

[c]All data from single titration values.

Figure 1

L-Aspartic Acid in Treated Partial Hydrolysates

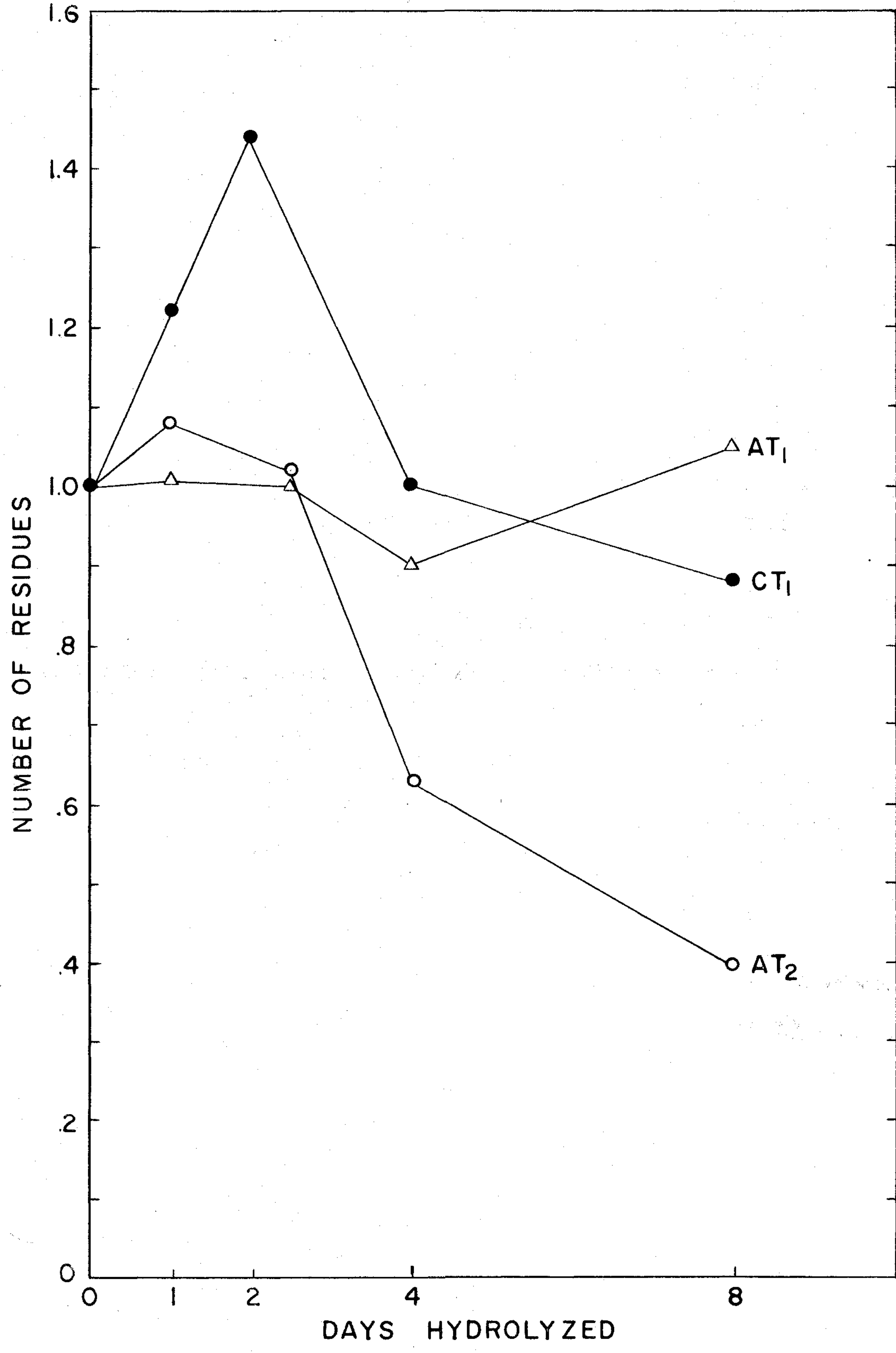

NUMBER OF RESIDUES
1.6
1.4
1.2
1.0
.8
.6
.4
.2
0
0
1
2
4
8
DAYS HYDROLYZED
AT1
CT1
AT2

Figure 2

Histidine in Treated Partial Hydrolysates

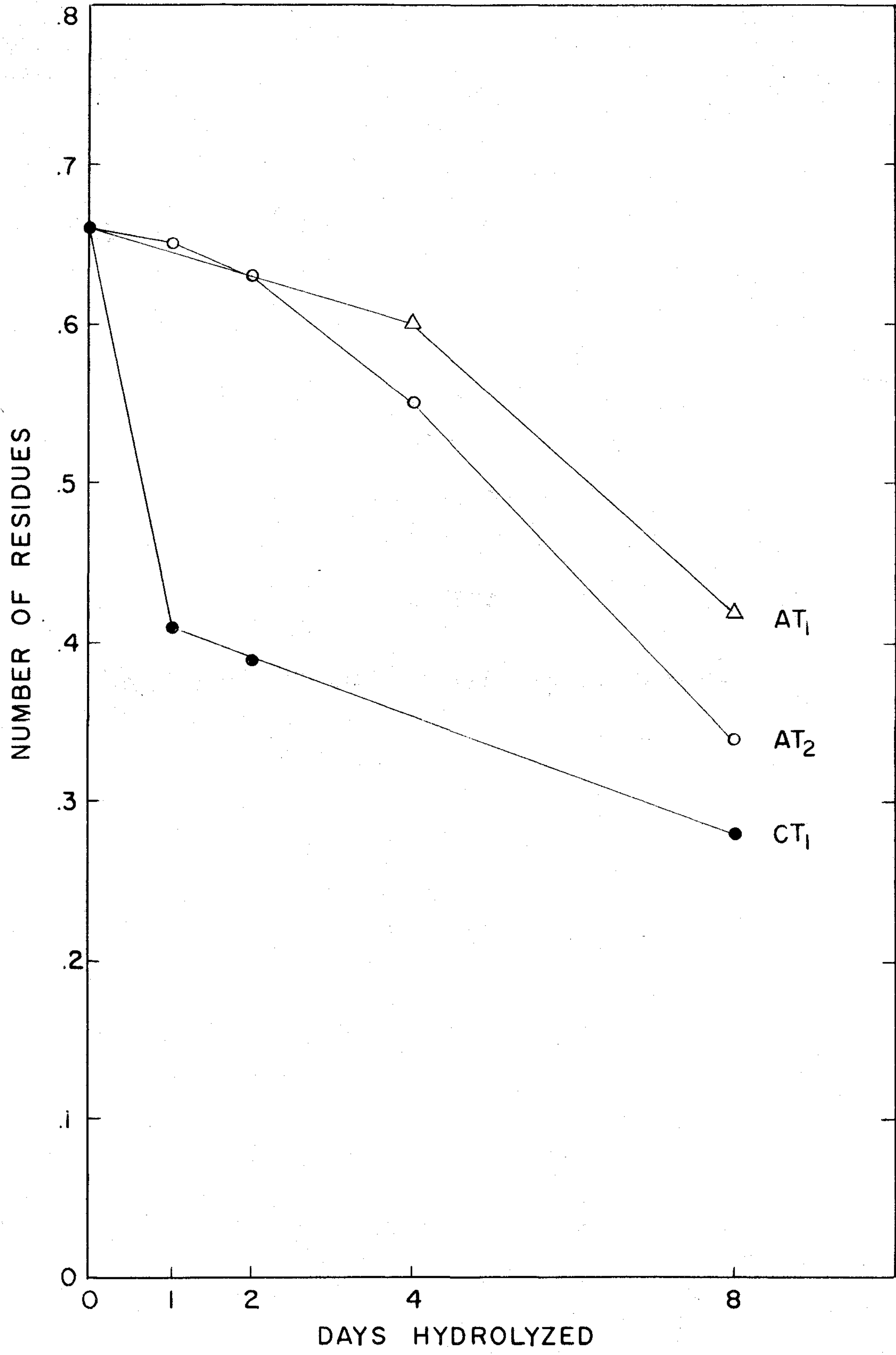

NUMBER OF RESIDUES
.8
.7
.6
.5
.4
.3
.2
.1
0
0
1
2
4
8
DAYS HYDROLYZED
AT1
AT2
CT1

Figure 3

Lysine in Treated Partial Hydrolysates

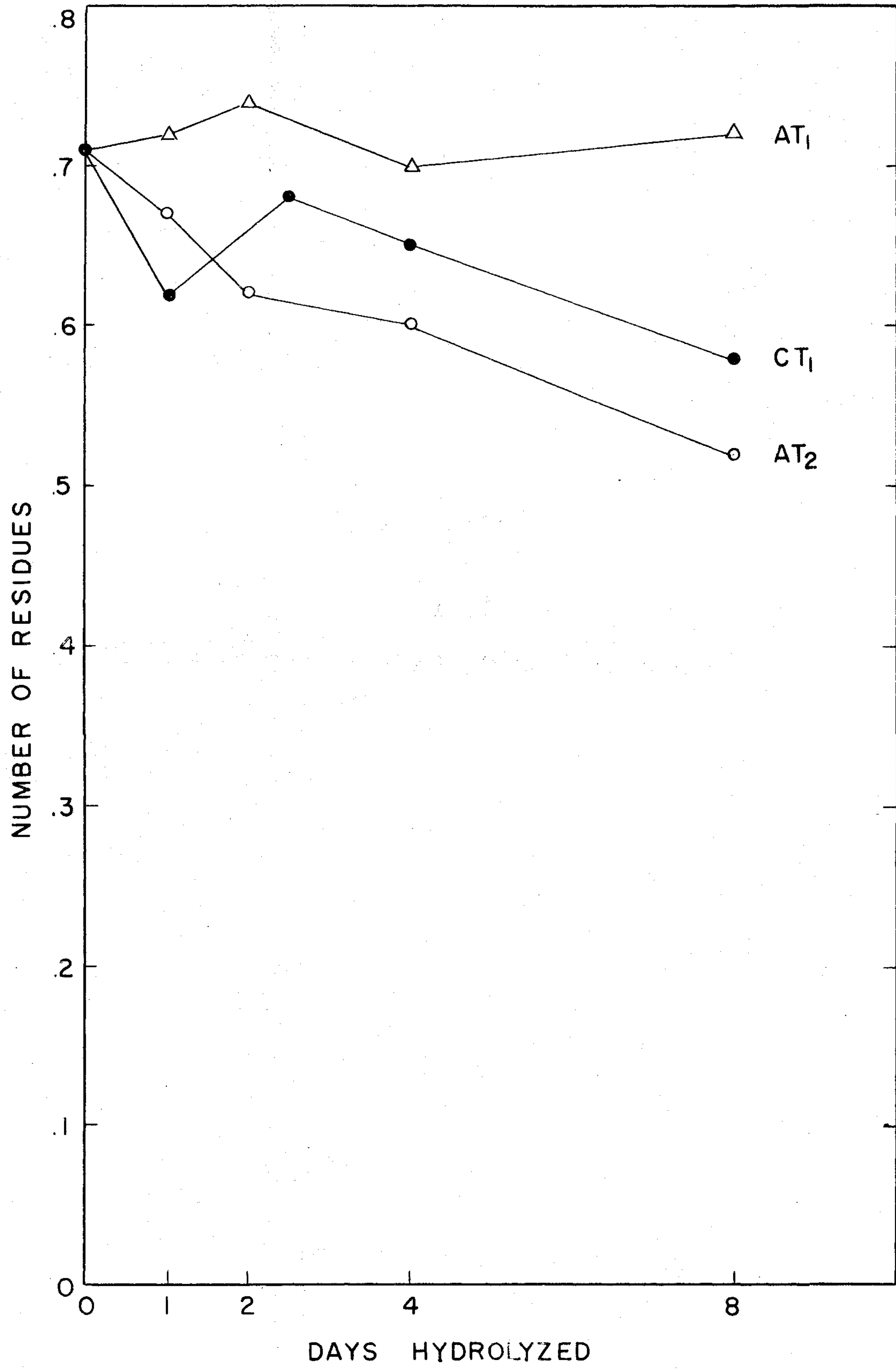
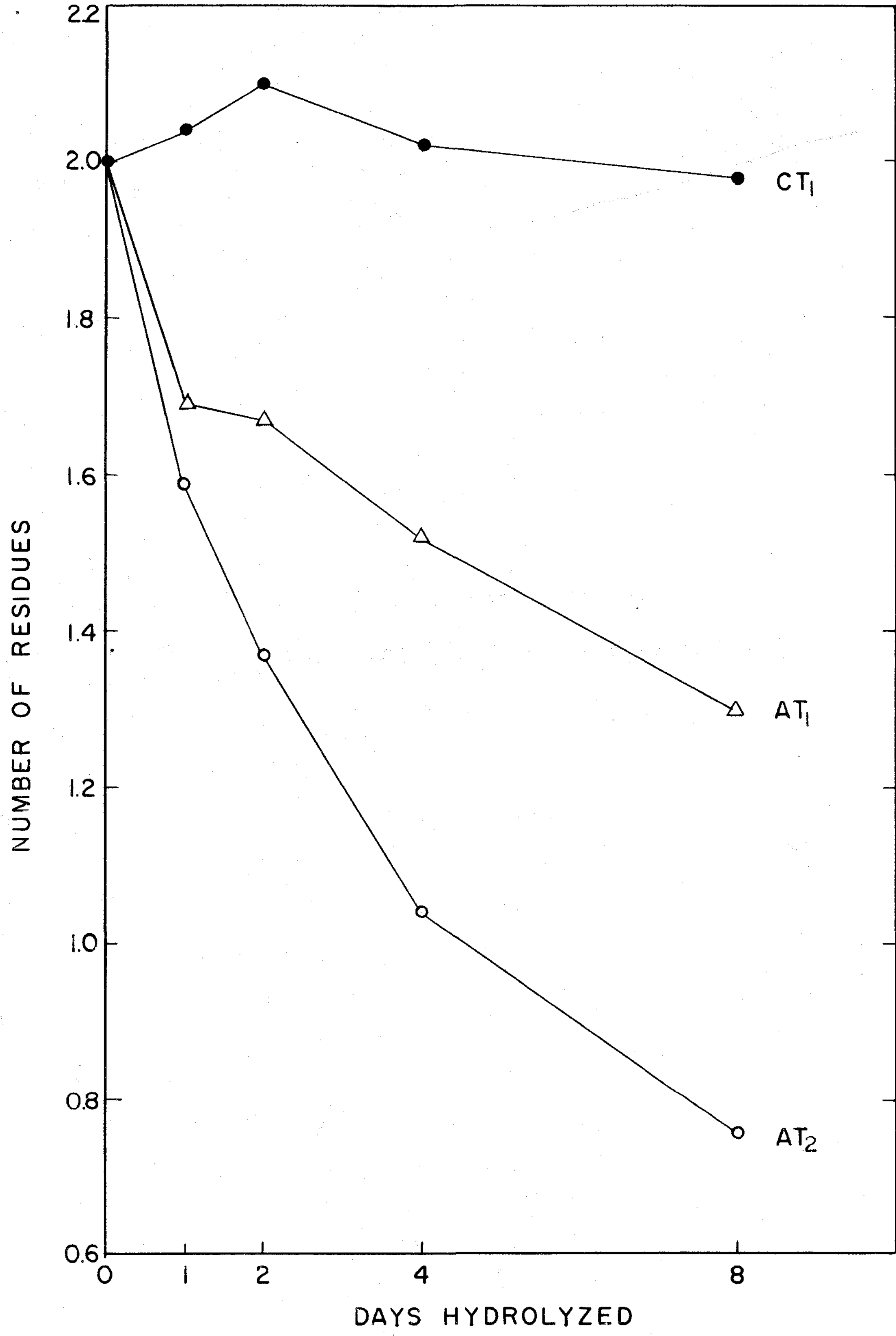

.8
.7
.6
.5
.4
.3
.2
.1
0
NUMBER OF RESIDUES
0
1
2
4
8
DAYS HYDROLYZED
AT1
CT1
AT2

Figure 4

L-Isoleucine in Treated Partial Hydrolysates



NUMBER OF RESIDUES
2.2
2.0
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0
1
2
4
8
DAYS HYDROLYZED
CT1
AT1
AT2

Figure 5

Leucine in Treated Partial Hydrolysates

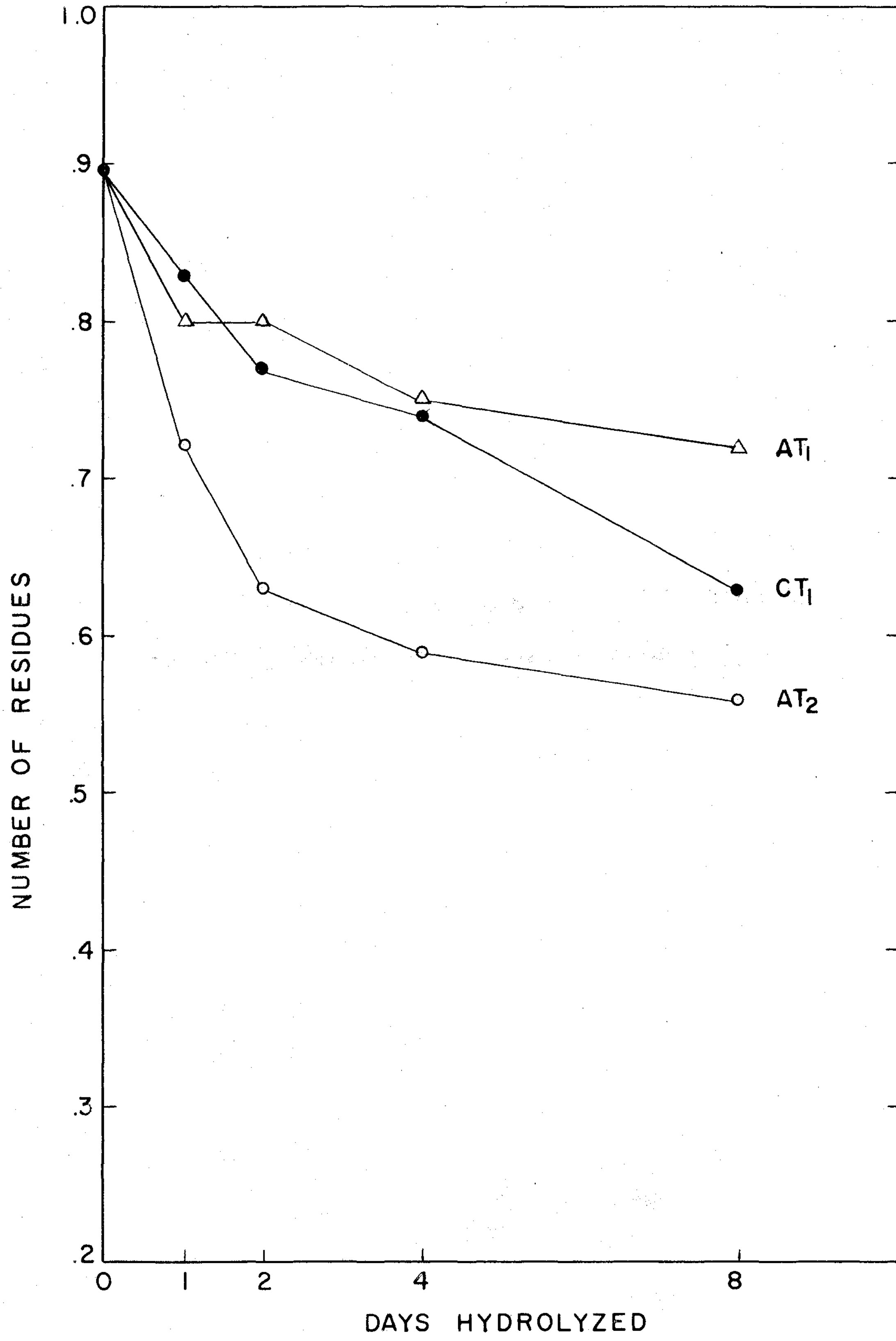

1.0
.9
.8
.7
.6
.5
.4
.3
.2
NUMBER OF RESIDUES
0
1
2
4
8
DAYS HYDROLYZED
AT1
CT1
AT2

Figure 6

Chromatogram of Bacitracin and Partial
Hydrolysates of Bacitracin

(Solvent System: Butanol:Acetic Acid:Water)

data given are for DL-aspartic acid. Samples RT_0 and RAT_1 were the same as T_0 and AT_1 respectively, except that they contained 1.00 mg. of L-leucine and 2.00 mg. of L-isoleucine.

The recoveries of added isoleucine and leucine were calculated from the differences between the actual amounts of these amino acids found in the recovery and non-recovery samples. The results (percentage recovered):

	RT_0	RAT_1
isoleucine	87	4.6
leucine	106	11.5

Desulfurized bacitracin

Figures 7 and 8 show the chromatograms of the hydrolyzed treated peptides and the hydrolyzed thiohydantoin and phenylthiohydantoin extracts from the sequential blocking treatments of desulfurized bacitracin.

Oxidized bacitracin A

Figure 9 shows the effluent pattern obtained from the Dowex-50 columns for a hydrolyzed 5.0 mg. sample of performic oxidized bacitracin A. The number of residues shown in

Terminal Residue Studies

Bacitracin

Table 10 shows the microbiological assay results obtained from the residue study of commercial bacitracin. Only the amino acids known to be present in the L-configuration were assayed. Aspartic acid was assayed with *L. mesenteroides* which will utilize both the D- and L- forms, therefore the

Table 10

Bacitracin Terminal Residue Study

Amino acid	Number of residues				
	T_0	RT_0	AT_1	RAT_1	CT_1
Aspartic acid[a]	1.88	1.89	2.02	1.97	1.79
Histidine (1st detm.)	.72	----	.75	----	.63
Histidine (2nd detm.)	.79	.81	.78	.83	.70
Isoleucine	1.92	1.66[b]	1.63	1.72[b]	1.55
Leucine	0.89	0.96[c]	0.94	1.06[c]	0.88
Valine	.14	.14	.11	.11	.13

[a] *L. mesenteroides*.

[b] Calculated after correcting for added isoleucine.

[c] Calculated after correcting for added leucine.

Days hydrolyzed

0 1 2

Days hydrolyzed

Figure 7

Chromatogram of Samples from Sequence Study of Desulfurized Bacitracin

(Solvent System: Butanol:Acetic Acid:Water)

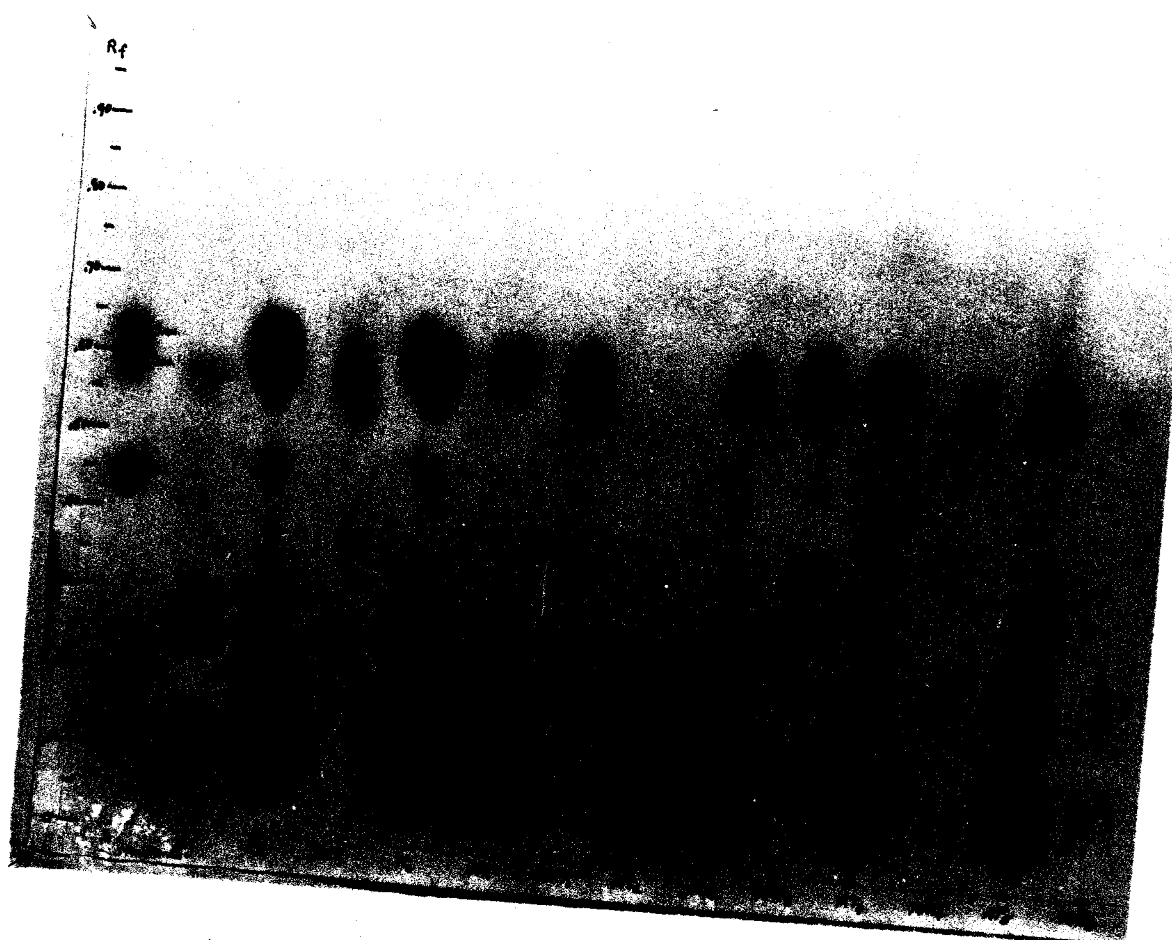

Rf

Figure 8

Chromatogram of Samples from Sequence Study
of Desulfurized Bacitracin

(Solvent System: Phenol:Borate Buffer, pH 8.3)

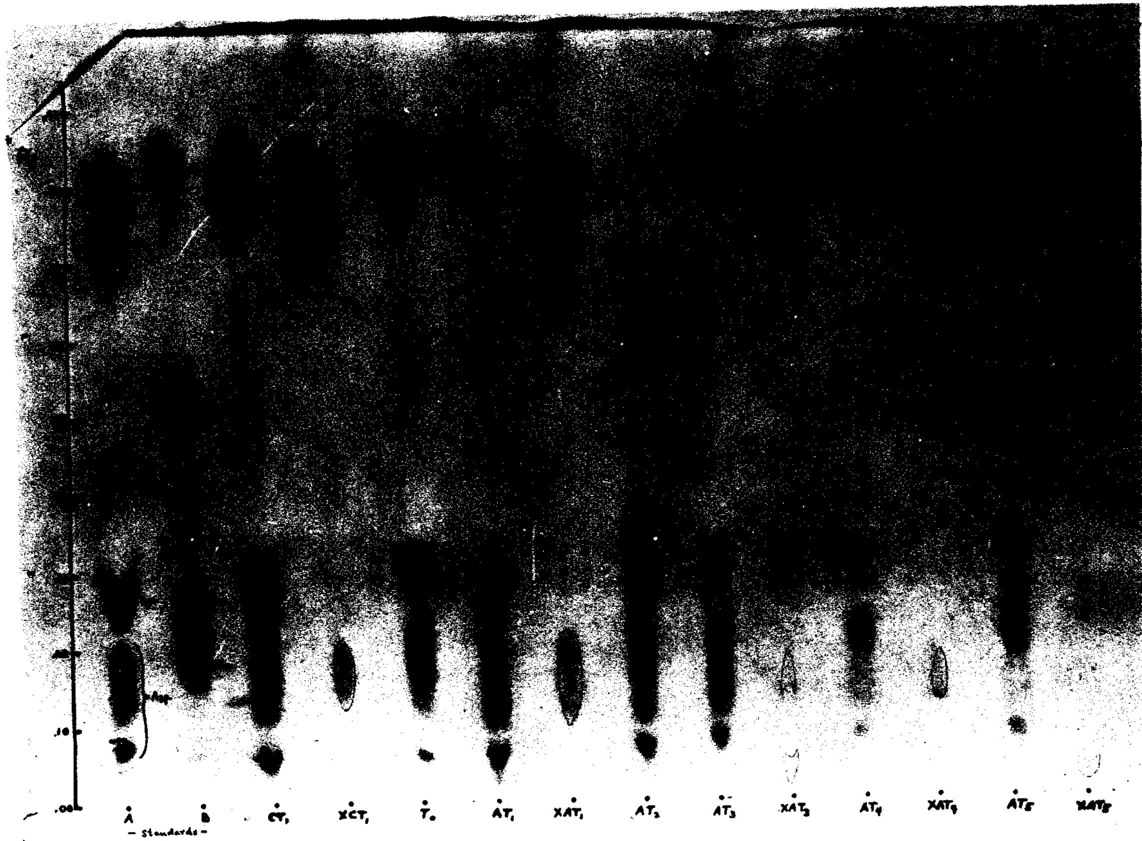
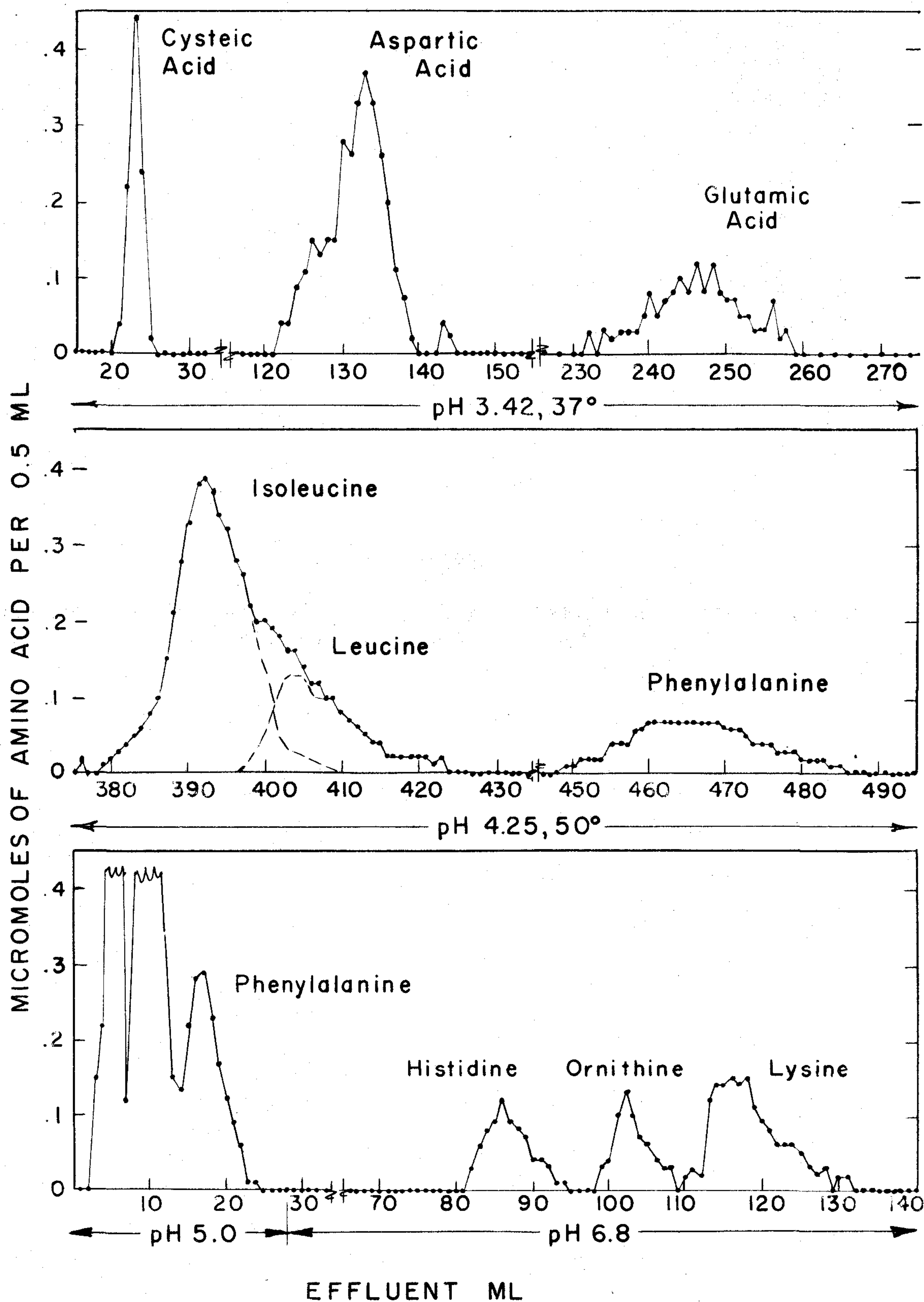

.10
.00
A
B
— Standards —
CT_1
XCT_1
T_0
AT_1
XAT_1
AT_2
AT_3
XAT_3
AT_4
XAT_4
AT_5
XAT_5

Figure 9

Effluent Patterns from Ion Exchange Columns of Hydrolysate of Oxidized Bacitracin A



Cysteic Acid
Aspartic Acid
Glutamic Acid
pH 3.42, 37°
Isoleucine
Leucine
Phenylalanine
pH 4.25, 50°
Phenylalanine
Histidine
Ornithine
Lysine
pH 5.0
pH 6.8
MICROMOLES OF AMINO ACID PER 0.5 ML
EFFLUENT ML

Table 11 were calculated by integrating the points in micromoles per 0.5 ml. under each peak of the effluent patterns (the effluent pattern for AT_1 is not shown), multiplying each sum by 2 to give the number of micromoles of amino acid per

Table 11

Amino Acid Composition of Oxidized Bacitracin A

Amino acid	Number of residues[a]	
	AT_0	AT_1
Cysteic acid	0.58	0.62
Aspartic acid	1.91	2.24
Glutamic acid	0.91	1.06
Isoleucine + leucine	3.70	3.48
Phenylalanine	0.96	1.04
Histidine	0.45	0.49
Ornithine	0.38	0.43
Lysine	0.94	0.99

[a]Calculated from a molecular weight of 1520 based on a molecular weight of 1450 for bacitracin A.

5.0 mg. sample, and finally dividing the number of micro-moles by 3.3, the calculated number of micromoles of oxidized bacitracin A per 5.0 mg.

DISCUSSION

Carboxoid Blocking Reaction Conditions

This experiment was carried out in order to supplement the results of Hurst (16) with regard to the microbiological recovery of the N-terminal residue of a peptide following a reaction which is intended to block only the C-terminal residue. The data of Table 6 indicate that either blocking reagent gave about the same recovery of leucine, except perhaps at the two mole excess level where the benzoylleucylvaline treated with phenyl isothiocyanate gave considerably less leucine than the one treated with ammonium thiocyanate.

The valine data indicate that phenyl isothiocyanate is a poor C-terminal blocking reagent, having removed only about half of the valine as the phenylthiohydantoin. On the other hand, ammonium thiocyanate consistently removed better than 70 per cent of the valine as the thiohydantoin. The level of excess blocking reagent did not appear to be significant, though in general the best results were obtained with a 50 mole excess of reagent for both leucine and valine.

Sequence Studies on Bacitracin

These two experiments were carried out before any specific information had appeared in the literature concerning the optical configurations of the known amino acid residues. The data of Table 8 confirm the findings of Craig, Hausman, and Weisiger (6) that glutamic acid, phenylalanine, and one residue of aspartic acid are of the D-configuration. The low assay results for these amino acids can be explained by the inability of the assay organisms to utilize the D-forms. The low values for lysine are inconsistent with the data of Table 7.

An assumption that bacitracin was composed of straight chain peptides is not supported by the data of Tables 7 and 8, inasmuch as no consistent or well-defined decrements can be seen in the assays with successive aminoid blocking treatments. A relative decrease of 50 per cent for isoleucine in Table 7 after one treatment is not found in Table 8; the same is true for cystine in Table 7, where all the values are so low as to be within experimental error. The decrement in cystine in Table 8 from 4.7 to 1.9 per cent after three treatments may be significant, and would therefore indicate that cysteine is the third residue from the N-terminus. The decrease from 6.7 to 4.7 per cent in two treatments seems to

point to partial blocking of cysteine which is nearly complete after the third treatment. The *D*-isoleucyl-cysteinyl structure involving a thiazoline ring which has been proposed by Craig, Hausmann, and Weisiger (8) might undergo gradual degradation, with concomitant blocking of the resulting cysteine residue, as a result of several blocking treatments. No drop in isoleucine could be expected from microbiological assay results if the N-terminus is a *D*-isoleucine residue. The cystine found in the untreated sample (Table 8) compares favorably with the value of 7.4 per cent reported by Craig *et al.* (6) from chromatographic analysis.

In general, there is no definite indication from these studies of an N-terminus in bacitracin, at least in the major peptide. If an N-terminus exists, it does not appear to be susceptible to quantitative blocking with phenyl isothiocyanate.

Rate Studies

Inspection of the chromatograms of partial hydrolysates of bacitracin in Figure 6 shows that both aspartic acid and histidine are rapidly liberated under the conditions employed. Glutamic acid appears to be liberated almost as rapidly, followed by ornithine and/or lysine, and cysteine. The

leucines seem to be liberated at the slowest rate. At the end of 64 days there is still some material giving R_f values higher than leucine (.62) and which may therefore be leucine or isoleucine peptides.

The data of Table 9 also give definite indications that certain peptide bonds were preferentially hydrolyzed to varying degrees while others were not at all during the period employed. These observations are similar to those of Consden, et al. (4) with Gramicidin S, under the same hydrolytic conditions.

Figure 1 indicates the degree of hydrolysis of peptide bonds involving L-aspartic acid or a bond one residue removed from the amino group of L-aspartic acid. The data does not apply to D-aspartic acid, since the assay organism used, L. brevis, cannot utilize this isomer at the assay levels. The curves indicate that the L-aspartic acid amino group is not liberated, but that the bond adjacent to it is hydrolyzed. These facts are more clearly illustrated in Figure 10 which shows the percentage of a given bond opened, based on the control samples, as a function of time. These curves, as are those of Figures 11-14, are plotted from data calculated from Table 9. No attempt was made to calculate the rate of liberation of the carboxyl group of aspartic acid, since the thiocyanate blocking reaction gives only a small percentage

Figure 10

Rate of Fission of Linkages Associated with L-Aspartic Acid

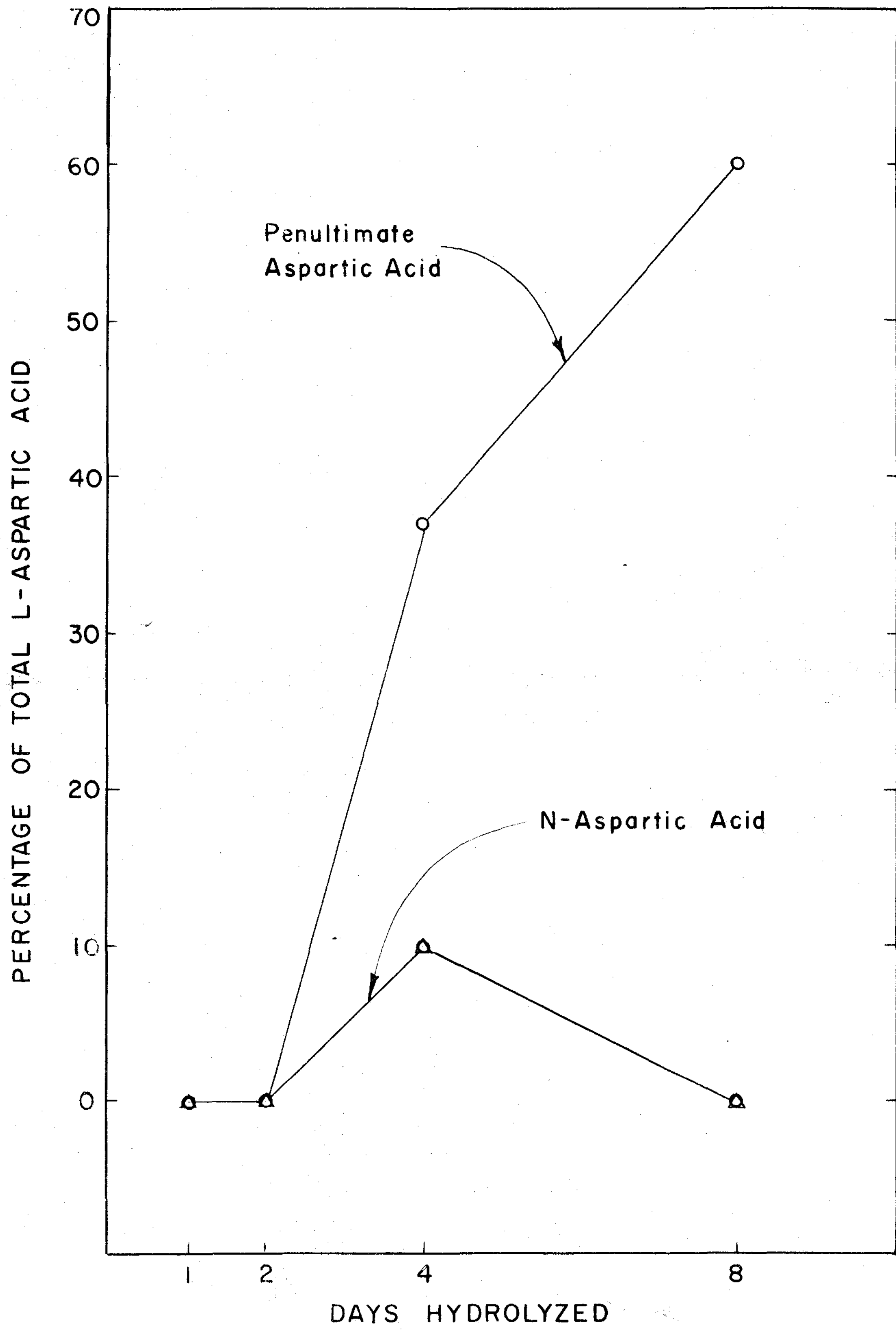
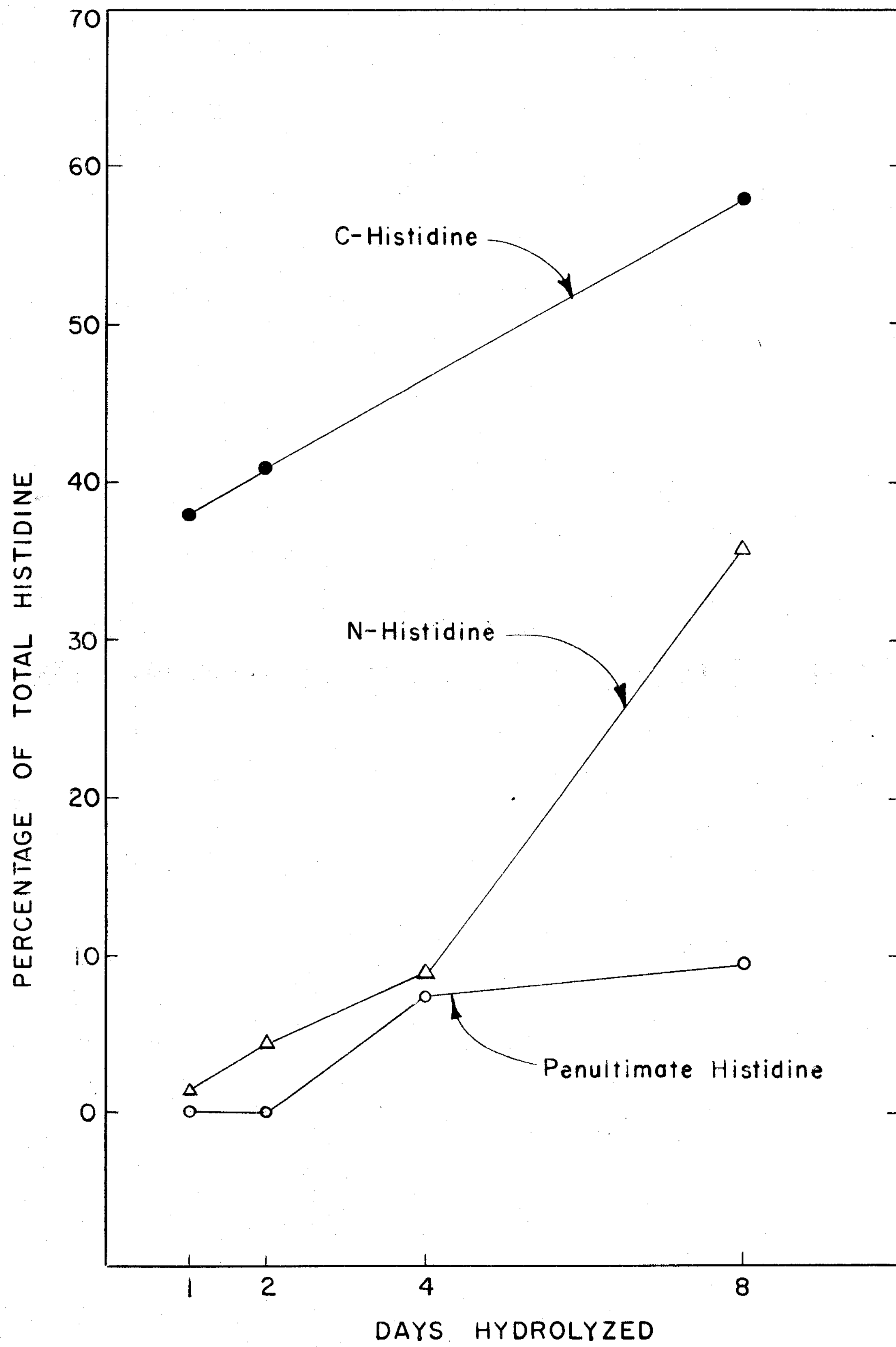

Penultimate
Aspartic Acid
N-Aspartic Acid
PERCENTAGE OF TOTAL L-ASPARTIC ACID
DAYS HYDROLYZED
70
60
50
40
30
20
10
0
1
2
4
8

Figure 11

Rate of Fission of Linkages Associated with Histidine



70
60
50
40
30
20
10
0
PERCENTAGE OF TOTAL HISTIDINE
C-Histidine
N-Histidine
Penultimate Histidine
1
2
4
8
DAYS HYDROLYZED

Figure 12

Rate of Fission of Linkages Associated with Lysine

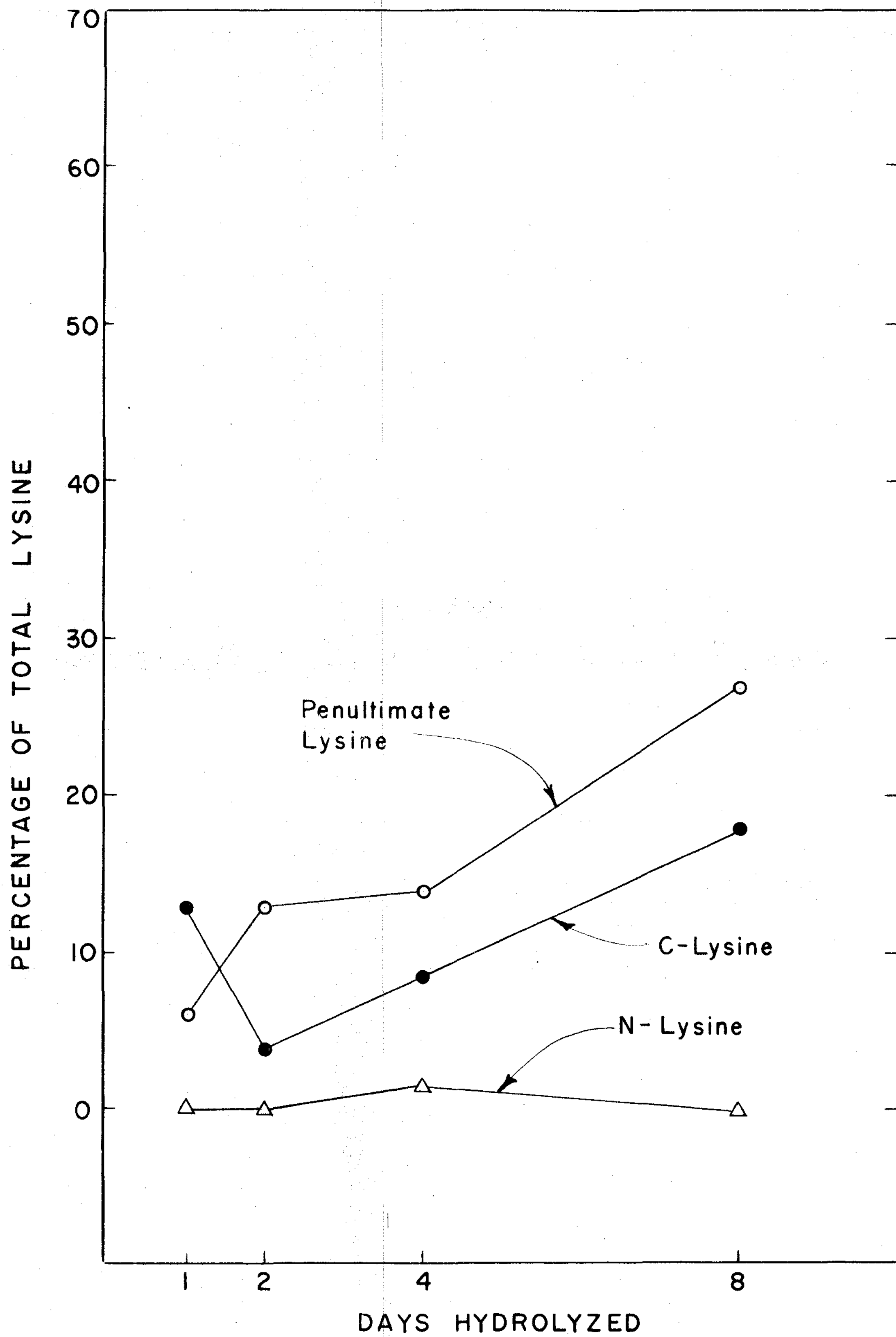

70
60
50
40
30
20
10
0
PERCENTAGE OF TOTAL LYSINE
Penultimate
Lysine
C-Lysine
N-Lysine
1
2
4
8
DAYS HYDROLYZED

Figure 13

Rate of Fission of Linkages Associated with L-Isoleucine

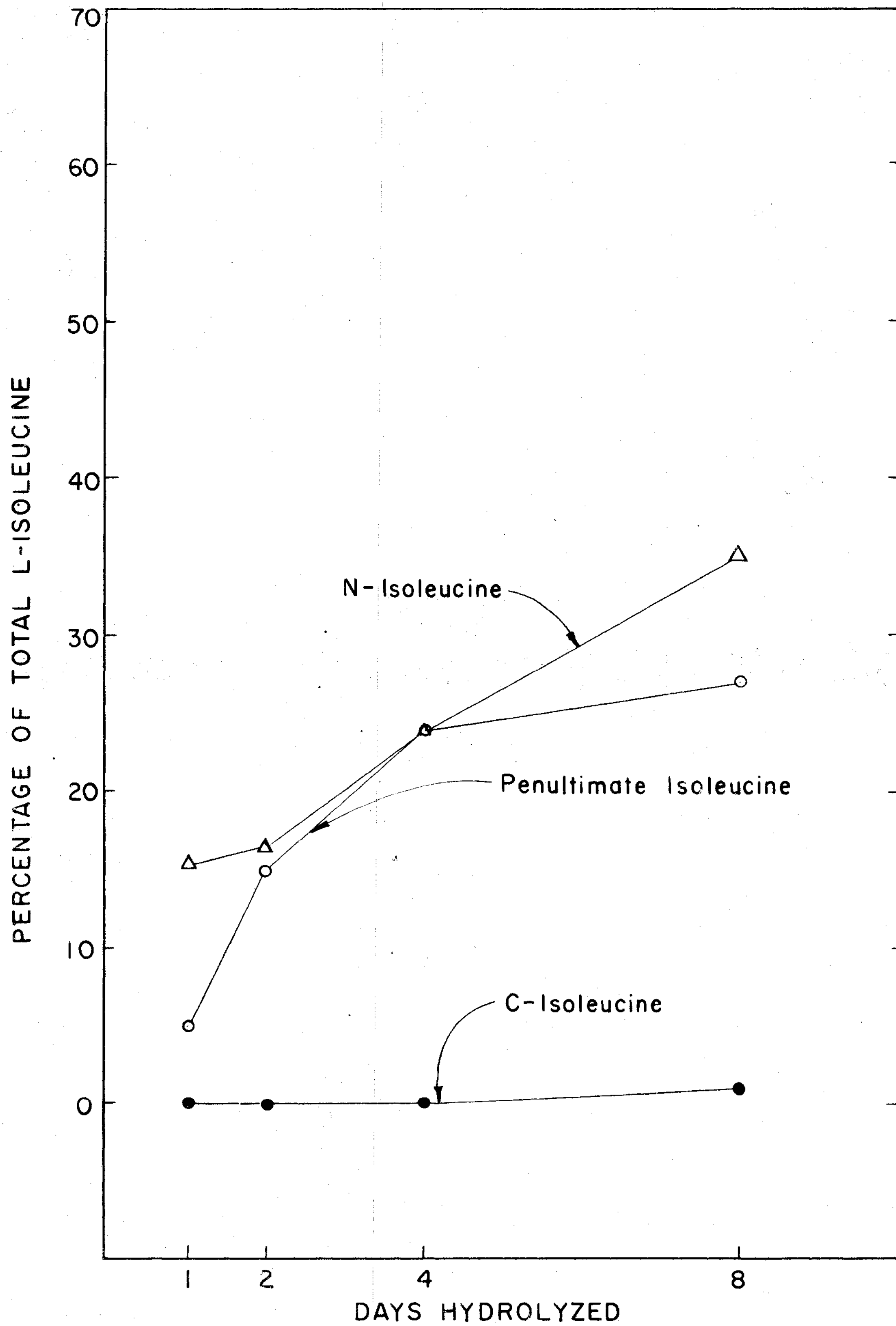
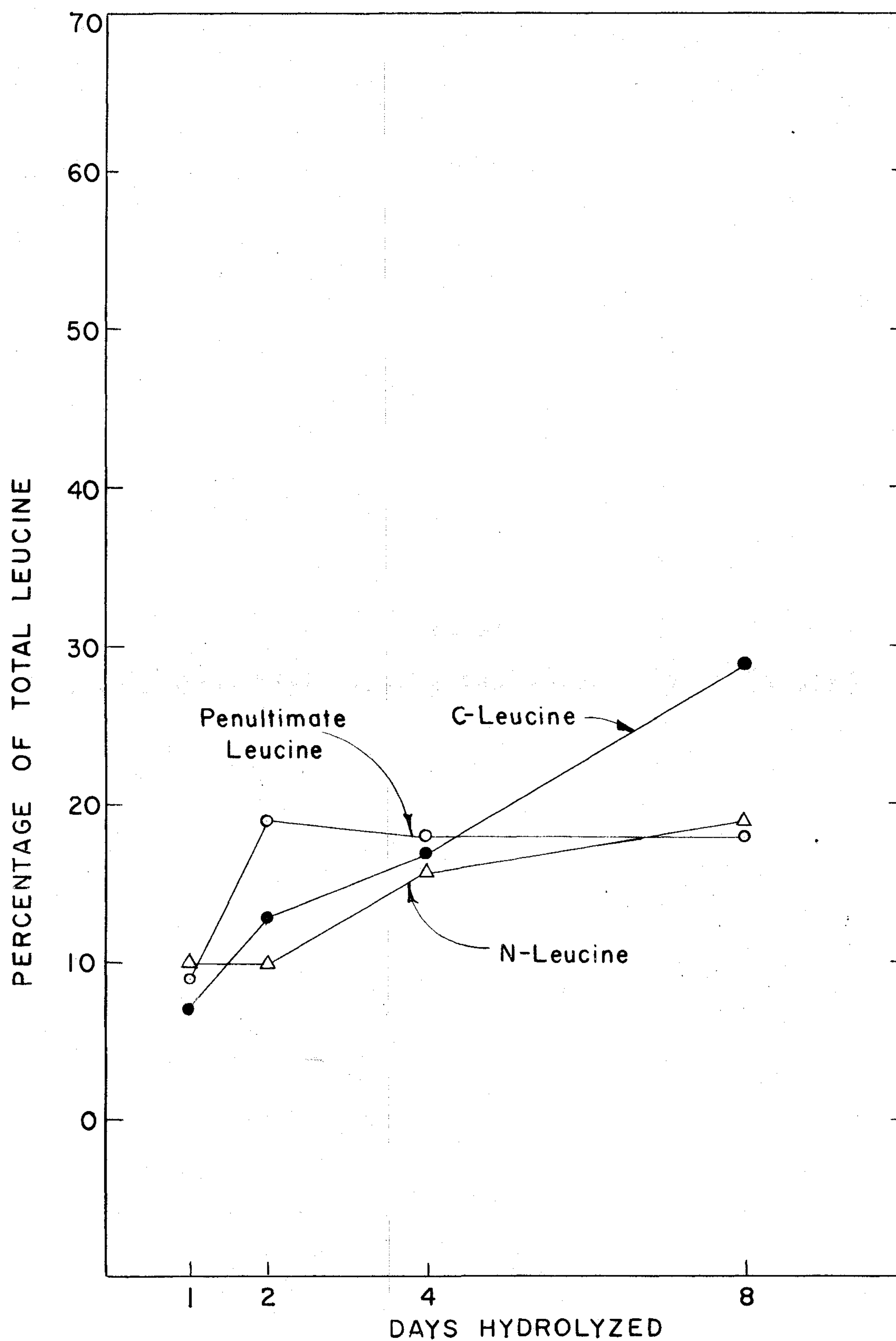

70
60
50
40
30
20
10
0
PERCENTAGE OF TOTAL L-ISOLEUCINE
N-Isoleucine
Penultimate Isoleucine
C-Isoleucine
1
2
4
8
DAYS HYDROLYZED

Figure 14

Rate of Fission of Linkages Associated with Leucine


70
60
50
40
30
20
10
0
PERCENTAGE OF TOTAL LEUCINE
Penultimate
Leucine
C-Leucine
N-Leucine
1
2
4
8
DAYS HYDROLYZED

of theoretical thiohydantoin with aspartic and glutamic acids (40).

Figure 2 is a graphical representation of the histidine data from Table 9. Both the carboxyl and amino group were rapidly opened under the experimental conditions. But, as can be seen more readily in Figure 11, the bond adjacent to the histidine amino group did not open as rapidly. The indication of rapid hydrolysis of both peptide bonds involving histidine is substantiated by the fact that a well defined histidine spot was found on the paper chromatogram of the eight day hydrolysate (Figure 6).

The lysine α-amino group was not observed to undergo liberation as can be seen from Figures 3 and 12, nor did the carboxyl group to any great extent. The peptide bond adjacent to the lysine α-amino group was opened to a greater extent.

Neither of the L-isoleucine carboxyl groups, as seen in Figures 4 and 13 were liberated at all in eight days. Any D-isoleucine present (8) could not be detected by microbiological assay. Because two residues of L-isoleucine have been found, it is not possible to distinguish between them from the rate data alone. There is no reason to expect that peptide bonds involving their amino groups should hydrolyze at the same rate, since the other residue bound to isoleucine would also be a determining factor. The bonds adjacent to

the isoleucine amino groups were collectively hydrolyzed to about the same extent as the amino groups themselves were liberated.

As shown in Figures 5 and 14, the bond adjacent to the leucine amino group undergoes a rapid initial hydrolysis, but then hydrolysis stops at two days with less than 20 per cent of the bond hydrolyzed. This seems to indicate that about 82-83 per cent of this bond is stable toward the hydrolytic conditions employed. It is interesting to note that the bond adjacent to the leucine amino group is not a conventional peptide bond in the bacitracin A sequences proposed by Craig _et al._ (8) and Lockhart _et al._ (22) but is part of the proposed thiazoline ring (33) involving cysteine. Whether or not such a ring system is stable toward hydrochloric acid-acetic acid hydrolysis at 37° is not known, but if the proposed structure of bacitracin A is correct (8), the initial 17-18 per cent hydrolysis of the bond in question in commercial bacitracin could have taken place in minor bacitracin peptides with different residue sequences, and/or without the thiazoline ring system.

When the rate curves of Figures 10-14 are compared it can be seen that several of them coincide rather closely, while others are similar either in general level of hydrolysis or in their slopes, even though displaced from one

another. The C-leucine curve very closely coincides with that of penultimate lysine. Likewise, except for the initial points, C-lysine almost exactly coincides with penultimate histidine. Both showing no hydrolysis, C-isoleucine is similar to N-lysine. Although they have quite different slopes, both C-histidine and penultimate aspartic acid show much greater extents of hydrolysis than any of the other curves, and may therefore be related.

Table 12 indicates some of the two-residue sequences, with or without an intervening residue designated by x, which are indicated on comparison of all the rate curves of Figures 10-14. Some sequences for which there is strong negative evidence are also listed.

All possible combinations of the sequences shown in Table 12 result in the four longer partial sequences:

-leu-iso-lys-x-his-x-asp- (1)

-leu-iso-lys-x-iso---his-x-asp- (2)

-leu-x-iso-lys-x-his-x-asp- (3)

-leu-x-iso-lys-x-iso---his-x-asp- . (4)

Table 12

Partial Sequences from Rate Study I

Possible	Improbable
his-x-asp[a,b]	iso-x-his[c]
lys-x-his	iso-x-iso
lys-x-iso[a]	iso-leu[d]
leu-x-lys	iso-iso
leu-x-iso[a,b]	
leu-iso (?)	
iso-lys[a]	

[a]Agrees with Craig *et al.* (8).

[b]Agrees with Porath (36).

[c]Does not agree with Craig *et al.* (8)

[d]Does not agree with Porath (36).

Of these, none agrees with the structure of Porath (36) for bacitracin A, but no. 4 agrees with the sequence of Craig *et al.* (8), part of which is:

-leu-glu-iso-lys-orn-iso-Øal-his-asp-asp.

Terminal Residue Studies

Bacitracin

The initial purpose of this experiment was to absolve some uncertainty concerning the utilization of the phenylthiohydantoins of isoleucine and leucine by the assay organism, *L. arabinosus*. Known amounts of isoleucine and leucine, roughly equivalent to those amino acids bound in bacitracin, were added to two samples. Two other identical bacitracin samples were not recovery samples, i.e., had no added leucine or isoleucine. In the case of isoleucine, 87 per cent was recovered on assay of the untreated samples, but only 4.6 per cent following an alkaline phenyl isothiocyanate treatment. One hundred per cent of the leucine was recovered prior to blocking, and 11.5 per cent afterwards. It therefore can be said that isoleucine-PTH is not utilized for growth in place of isoleucine by *L. arabinosus*, nor does leucine-PTH seem to replace leucine to a significant extent.

The experiment failed to show any N-aspartic acid of either configuration. Two residues of aspartic acid were indicated, for *L. mesenteroides* responds to either isomer with nearly the same growth at assay levels. The decrease from an average of 1.94 residue for the untreated and aminoid

treated samples to 1.79 residue for the carboxoid treated sample represents a drop of 8 per cent in the total aspartic acid, but inasmuch as only one residue can be C-terminal in a given molecule this is equivalent to a drop of 16 per cent of a possible C-terminal aspartic acid residue. Swan (40) has reported that aspartic acid or glutamic acid is converted into a mixture of two anhydrides when heated with acetic anhydride in the presence or absence of ammonium thiocyanate, and that on hydrolysis, only one of these anhydrides can undergo conversion to the thiohydantoin. He reports that in the case of N-acetyl glutamic acid the yield of this thiohydantoin never exceeds 10 per cent, with correspondingly low yields of the aspartic acid thiohydantoin. Thus a drop of 16 per cent in the recovery of one aspartic acid residue, though indicating only a 16 per cent conversion to the phenylthiohydantoin could be indicative of C-aspartic acid in the main portion of bacitracin. (This finding is especially significant when it is considered that the phenyl isothiocyanate used in this experiment is only half as effective as ammonium thiocyanate as a carboxoid blocking reagent (Table 6) under ordinary conditions, i.e., the terminal residue not a dicarboxylic acid.) The findings of Craig _et al._ (8) confirm this.

It is possible to speculate on the optical configuration of this terminal aspartic residue by considering the evidence for a -histidyl-x-aspartyl- sequence (Table 12) from the L. brevis assay. Since this organism cannot utilize D-aspartic acid at assay levels, the sequence may well be histidyl-x-L-aspartyl. The structure of Craig et al. would then contain -histidyl-D-aspartyl-L-aspartic acid. However, the two columns labeled T_0 and CT_1 for aspartic acid in Table 9 give no indication of any carboxoid L-aspartic acid.

The valine assay in Table 10 was made to check the finding of Newton and Abraham (32) that 4200 µg. of crude bacitracin gave the same growth response with L. mesenteroides as 117 µg. of DL-valine. This is equivalent to 0.17 residue of L-valine per molecule of bacitracin, based on a molecular weight of 1450. This value agrees well with the 0.14 residue from the S. faecalis assay in Table 10. At the low level of the assay it is not possible to determine whether or not any of the valine is N-terminal, as reported by Newton and Abraham.

Desulfurized bacitracin

Examination of T_0 in Figures 7 and 8 reveals that very little cysteine was reduced to alanine by hydrogenolysis of

bacitracin. Faint alanine spots (R_f .30) appear in XAT_1 and XAT_3 in Figure 7, but no alanine is evident in any of the hydrolysates in Figure 8. Apparently the small amount of alanine formed from cysteine is N-terminal, having been removed by one aminoid blocking treatment.

A small quantity of valine appears in T_0 and CT_1 of Figure 7, and it appears to be N-terminal, inasmuch as none appears in AT_1 through AT_5. Valine does not appear in any of the PTH hydrolysates, probably because the small amount of valine-PTH formed is degraded by the alkaline hydrolysis to give products other than valine.

Most significant is the removal of a fairly large quantity of leucine and/or isoleucine by both aminoid and carboxoid blocking treatments. Inasmuch as there is no C-L-isoleucine or C-leucine in bacitracin itself (Table 10), the hydrogenolysis must have opened at least one C-isoleucine or C-leucine bond to give free amino acid or a peptide chain with one of these amino acids as the C-terminus. If such is the case, a corresponding N-terminus should appear, but there is no indication of such a residue unless it is also leucine or isoleucine.

A distinct spot appears in all the extract hydrolysates at R_f .24 in Figure 7; this is between aspartic acid (.18) and glutamic acid (.26). Similar spots at R_f .12-.21 in

Figure 8 may be from the same substance. If so, the substance may be glutamic acid, in which case it appears to be both N-terminal and C-terminal. Another unidentified substance appears at R_f .47-.48 in the extract hydrolysates in Figure 7. This may be the degradation product of a known amino acid-PTH, but there is no direct evidence for this.

It must be concluded that hydrogenolysis does more than simply reduce a cysteine residue to N-alanine, and that no sequences can be assigned to the resulting mixture of peptides.

Oxidized bacitracin A

The sample of bacitracin A was oxidized with performic acid in order to convert the cysteine residue to a more stable residue of cysteic acid. Such treatment was expected to destroy any heterocyclic ring system involving the cysteine and, if the proposed structure of Craig *et al.* (8) is correct, to render any N-terminal residue more easily attacked by phenyl isothiocyanate.

The effluent patterns from the Dowex-50 columns shown in Figure 9 indicate that no cysteine or cystine remained in the bacitracin A after performic acid oxidation, but that cysteic acid was present. However, the quantity of cysteic acid found was not equivalent to one residue of cysteine per

molecule of molecular weight 1450, as can be seen in Table 11, but was only about 0.6 residue, leaving 40 per cent of the original cysteine unaccounted for. If the oxidation of bacitracin A were incomplete, the low cysteic acid value could be explained, but if such were the case, some cystine should appear in the effluent fractions. No other material was detected in the effluent fractions which could arise from the degradation of cysteine. The consistently low values for histidine and ornithine also cannot be explained.

The ion exchange resin did not completely resolve isoleucine and leucine, as can be seen from Figure 9, so that the leucine-isoleucine values are given collectively in Table 11. From these values, and noting the asymmetry of the leucine-isoleucine peak, it must be concluded that bacitracin A contains one residue of leucine and three residues of isoleucine. One treatment of oxidized bacitracin A with phenyl isothiocyanate reduced the leucine-isoleucine aggregate by only 0.24 residue, all of which can be attributed to isoleucine inasmuch as the leucine portion of the AT_1 effluent curve (not shown) remained unchanged, while the isoleucine portion had a lower peak. All of the amino acid values for AT_1 are higher than those for AT_0 except isoleucine-leucine. If the phenylalanine values for both AT_0 and AT_1 are corrected to exactly 1.00 residue, the isoleucine-

leucine values become 3.90 and 3.36 respectively, making a drop of 0:54 residue on one aminoid treatment. If this is entirely associated with isoleucine, an N-isoleucine residue is still not clearly indicated, though it is certainly favored over any other N-terminus. The half residue of N-isoleucine may have been removed for the same reason that only about one half a residue of cysteic acid is obtained on performic acid oxidation. This could be that part of the isoleucine and cysteine are degraded by oxidation and/or hydrolysis to give one or more ninhydrin-negative fragments, which would not be detected in the effluent fractions.

SUMMARY AND CONCLUSIONS

1. Both ammonium thiocyanate and phenyl isothiocyanate partially removed the aminoid residue of leucylvaline and benzoylleucylvaline under carboxoid blocking conditions. The benzoyl group only partly protected the aminoid residue.

2. Edman degradation of bacitracin indicates that the peptide does not have a free N-terminus.

3. Partial hydrolysis of bacitracin results in rapid liberation of histidine, with very little liberation of isoleucine, leucine, or lysine in the same period. The rates of liberation of certain functional groups can be matched to give four possible partial sequences for the major peptide of bacitracin. These sequences involve most of the L-amino acid residues of bacitracin. One of these partial sequances agrees with the structure of bacitracin A proposed by Craig, Hausmann, and Weisiger.

4. Raney nickel hydrogenolysis of bacitracin results in only partial reduction of the cysteine residue to alanine, and may produce other changes in the peptide. A carboxyl group of leucine or isoleucine appears to be liberated during the reduction, as is a material with the chromatographic behavior of glutamic acid.

5. Performic acid oxidation of bacitracin A converts approximately 60 per cent of the cysteine to cysteic acid, the rest being unaccounted for.

6. One phenyl isothiocyanate treatment of oxidized bacitracin A removed less than one half of an isoleucine residue. Therefore, the evidence for an N-isoleucine residue in bacitracin A is in doubt, though any other N-terminus is ruled out.

7. Ammonium thiocyanate is a superior carboxoid blocking reagent to phenyl isothiocyanate, though neither would quantitatively remove the carboxoid residue from leucylvaline or benzoylleucylvaline.

8. Different levels of blocking reagent have no great effect on the degree of carboxoid blocking. In general, higher levels give slightly higher recoveries of the aminoid residue.

9. It is possible to distinguish between residues of D- and L- aspartic acid in peptides microbiologically by the selective application of L-utilizing and DL-utilizing assay organisms.

10. The aminoid blocking reaction with phenyl isothiocyanate almost quantitatively removes leucine and isoleucine added to peptide samples, as indicated by microbiological assay with *L. arabinosus*.

11. The performic acid oxidation product of bacitracin A contains three residues of isoleucine, one of which is of the *D*-configuration.

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