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Partial Hydrolysis Studies with Bacitracin A

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A study of the partial hydrolysis by hydrochloric acid of bacitracin A has been made Numerous peptides and DNP derivatives have been isolated and characterized by a combination of countercurrent distribution, paper chromatography, zone electrophoresis and ultimate analysis The data obtained can be completely rationalized, except for one peptide sequence, on the basis of a formula containing two rings. The one sequence may indicate a third ring

In a previous publication[1] dealing with the nature of the antibiotic polypeptide, bacitracin, the isolation of a single polypeptide has been described. The qualitative and quantitative amino acid compositions[2] of this peptide, bacitracin A, were determined and the molecular weight was established by the method of partial substitution.[3] These results were in essential agreement with the results on bacitracin A from other laboratories.[4,5]

A reliable foundation for partial hydrolysis studies with bacitracin A has thus been made. Such studies, aside from the problem of the structure of this interesting antibiotic, promised to be of considerable interest because of the unique linkages thought to surround the cysteine and lysine residues. Since all functional groups of both these residues were found to be covered[3] either rings or cross linkages were strongly indicated.

Bacitracin A is gradually transformed at *p*H 7 or higher to an F type[3,4] of bacitracin. The transformation involves loss of ammonia and some, as yet obscure rearrangement connected with the cysteine residue. It would appear to be of great interest to learn which of the other residues are also involved in the transformation. This question will be taken up in a forthcoming paper.

An interesting account of rearrangements with cysteamine in which there is a transfer of acyl groups or amino acid residues from sulfur to nitrogen has recently been described by Wieland and co-workers.[6] The further possibility of histidine being involved in similar rearrangements is mentioned. In general these rearrangements take place at neutral or alkaline *p*H but would be much less likely to occur in acid solution. They may well be of fundamental importance to protein chemistry and could explain in part some of the unique transformations noted with bacitracin A. Before this possibility can be explored in the most intelligent way some idea of the possible amino acid sequences involved must be forthcoming.

With peptides containing hydroxyamino acids, rearrangements[7] of peptide linkages from N to O are thought to occur in acid solution. Such linkages are thought to present a weak point in the peptide chain where it can be split preferentially. A similar behavior might be expected with a cysteine containing peptide in which the oxygen at the susceptible point has been replaced by sulfur. On the other hand, a somewhat different state of affairs might be presented when these linkages are in a ring rather than in a chain.

In either case it is not certain that the amino acid residue which could form the thioester linkage would rearrange again only to the nitrogen of the cysteine when a more alkaline *p*H is restored. If other nitrogens in other amino acid residues should be involved a very complicated and confusing state of affairs could result.

If the sulfur of bacitracin A could be oxidized with performic acid as Sanger[8] has done with insulin, part of the uncertainty of secondary transformation might be removed. In experiments along this line to be reported later the sulfur of bacitracin A in fact was found to be oxidized since on subsequent complete hydrolysis cysteic acid appeared. However, with the particular conditions used the sulfur did not prove to be the only point in the molecule susceptible to oxidation.

Although oxidation of the sulfur prior to partial hydrolysis could well give a result with less ambiguity or uncertainty for the indication of a possible structure it seemed also of considerable interest to make a study of direct hydrolysis. The plan of attack in this case involved acid hydrolysis with hydrochloric acid, evaporation of the excess acid and countercurrent distribution of the acid residue in a system containing hydrochloric acid. Dilute acid conditions were maintained throughout subsequent distributons. At only one point in the whole fractionation procedure was there any departure from acid conditions. When conversion to dinitrophenyl derivatives (DNP) was required the *p*H was raised in order to cause the peptides to react. However, this was done at room temperature always in the presence of excess fluorodinitrobenzene by addition of sufficient triethylamine to reach a *p*H of 8. At the end of a two-hour reaction time the *p*H was reduced again.

Methods

Fractionation of the hydrolysis products was primarily accomplished by countercurrent distribution but each step was carefully studied and controlled by paper chromatography and paper electrophoresis both before and after complete hydrolysis.

Following partial hydrolysis of bacitracin A an initial countercurrent distribution, Fig. 1, served to give a group separation. Cuts were taken so as to include those peptides which appeared to be present in largest amount but yet avoid as far as possible overlapping of families. Unquestionably a certain number of significant peptides were

(1) L. C. Craig, J. R. Weisiger, W. Hausmann and E. J. Harfenist, *J. Biol. Chem.*, **199**, 259 (1952).

(2) L. C. Craig, W. Hausmann and J. R. Weisiger, *ibid.*, **199**, 865 (1952).

(3) L. C. Craig, W. Hausmann and J. R. Weisiger, *ibid.*, **200**, 765 (1953).

(4) G. G. F. Newton and E. P. Abraham, *Biochem. J.*, **53**, 604 (1953).

(5) J. Porath, *Acta Chem. Scand.*, **6**, 1237 (1952).

(6) Von Th. Wieland, E. Bokelmann, L. Bauer, H. U. Lang and H. Lau, *Ann. Chem.*, **584**, 129 (1953).

(7) D. F. Elliot, "The Chemical Structure of Proteins," Ciba Foundation Symposium, J. & A. Churchill Ltd., London, 1953, p. 129.

(8) F. Sanger, *Biochem. J.*, **44**, 126 (1944).

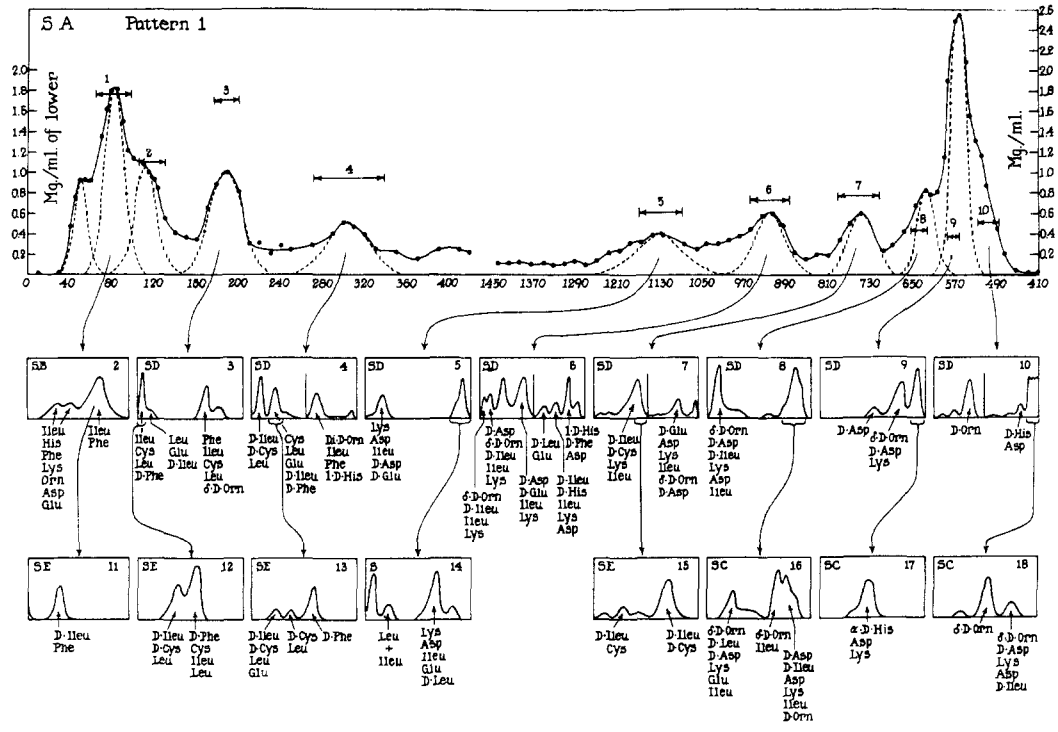


Fig. 1.—Distribution patterns of partial hydrolysates. Number of transfers in patterns: 1, 1040; 2, 536; 3, 202; 4, 530; 5, 219; 6, 660; 7, 920; 8, 195; 9, 223; 10, 439; 11, 51; 12, 600; 13, 411; 14, 196; 15, 205; 16, 219; 17, 64; 18, 216.

discarded by this procedure but definite simplification resulted.

After the preliminary separation into groups each group was converted to a mixture of DNP[9] (dinitrophenyl) derivatives and again distributed in a suitable system. The patterns from these distributions were determined by ultraviolet absorption at a wave length of 350 mμ. A complete absorption spectrum curve was taken wherever there was reason to suspect deviation from the simple DNP amino acid type. Weight determinations were made on the solutions from certain tubes in each band. This permitted an estimate of molecular weight[10] after the number and type of different dinitrophenyl groups present had been shown by hydrolysis and subsequent fractionation. The weight extinction ratio throughout a band served as a further indication of homogeneity.

The conditions used for conversion of the peptides to DNP derivatives were the following. A residue of the peptide hydrochloride was dissolved in 5 ml. of 66% aqueous ethanol and a large excess of fluorodinitrobenzene added. After shaking the mixture, sufficient triethylamine was added to bring the *p*H to 8. This *p*H was maintained while the reaction proceeded in the absence of light for 2 hours at room temperature. The ethanol was quickly evaporated under reduced pressure in the rotary evaporator[11] at 25° and water added. The excess FDNB was extracted with ether containing 1% triethylamine. The aqueous layer was again evaporated and the residue was immediately placed in the acid system for distribution.

The amino acids present in a given preparation or pure peptide fraction obtained from the distribution were usually deduced from hydrolysis and two-dimensional ascending paper chromatography in the solvent systems found to be the most suitable for separating a mixture of all the amino acids. These systems were 2-butanol, 3% aqueous ammonia and 2-butanol, formic acid. If the strong uniform spots from amino acids obviously present in the peptide were accompanied also by weak ones the preparation was regarded as impure and subjected to further fractionation.

Paper electrophoresis was carried out essentially according to the technique described by Kunkel.[12] The buffer most used contained 0.8% pyridine and 0.2% acetic acid. The *p*H approximated 5.6. For a more alkaline buffer to detect the loss of ionization of weak amino or imidazole groups a veronal buffer 0.05 molar at *p*H 8 was employed.

In order to decide which amino acid of a DNP-peptide had the DNP group attached a 24-hr. hydrolysis at 108° in 6 *N* hydrochloric acid was used. This resulted in considerable destruction of the DNP derivatives of many of the amino acids but did not have the disadvantage of unknown spots due to residual peptides. The hydrolysate from 1–3 mg. of the peptide was evaporated to dryness and taken up in a small volume of water. The solution or suspension was extracted with ethyl ether. The ether extract was evaporated to dryness and weighed. The residue was then studied by countercurrent distribution in a system made from chloroform, glacial acetic acid and 0.1 *N* aqueous HCl (2:2:1). The solute in a single band from this system was then partitioned in a system differing by the substitution of benzene for chloroform or in a system made by equilibrating a solution containing 2 *M* K_2HPO_4, 2 *M* NaH_2PO_4 and water (volume proportions 9:1:5, respectively) with ethyl acetate. The partition ratios of the DNP amino acids from bacitracin A and some of their probable transformation products are given in Table I.

Identification of the DNP amino acids by C.C.D. (countercurrent distribution) may be somewhat slower than by one of the chromatographic methods but it has the advantage of being a quantitative method as well as offering versatility. Although analysis of the distribution was made by optical density at 350 mμ any deviation in spectrum could be investigated quickly at other wave lengths. One-half mg. of a DNP-amino acid proved ample for the countercurrent distribution and spectral study.

It can be seen readily from Table I that all the DNP amino acids which are present in bacitracin A can be distinguished easily from each other except leucine and isoleucine. A good system for differentiating this pair has not been found. Nor has it been possible to find systems for differentiating them by paper chromatography.

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

(10) A. R. Battersby and L. C. Craig, THIS JOURNAL, **74**, 4023 (1952).

(11) L. C. Craig, J. D. Gregory and W. Hausmann, *Anal. Chem.*, **22**, 1462 (1950).

(12) H. G. Kunkel, "Zone Electrophoresis in Methods of Biochemical Analysis," Vol. I, Interscience Publishers, New York, N. Y., 1953, p. 141.

TABLE I

PARTITION RATIOS OF THE AMINO ACIDS AND REACTION PRODUCTS FROM BACITRACIN A

DNP-amino acid	$CHCl_3$, HAc, HCl system	Benzene, HAc, HCl system	Phosphate system
Leucine	0.18	2.5	4.4
Isoleucine	.18	2.2	4.4
Phenylalanine	.19	2.0	10.6
Lysine (di-DNP)	.16	1.1	40
Ornithine (di-DNP)	.42	0.40	8.8
Cystine (di-DNP)	.94	...	...
Cysteine (di-DNP)	.62	.40	...
Histidine (di-DNP)	6.9	...	...
Glutamic acid	1.9	.072	0
Aspartic acid	3.8	.034	0
Dinitroaniline	0.71	.38	...
Dinitrophenol	0.29	9	0.87

Information concerning the identity of several of the other DNP amino acids, however, was derived by paper chromatography. The positions and behavior of δ-DNP-ornithine, α-mono-DNP-histidine and imidazole-mono-DNP-histidine on the two-dimensional paper chromatograms prepared with the systems used for study of the free amino acids are very characteristic. DNP-aspartic acid and DNP-glutamic acid give a spot in the same place but are distinguishable from all the other DNP amino acids.

Di-DNP-histidine can be recognized easily since it apparently undergoes extensive transformation into a very slightly soluble product during hydrolysis. However, a suspension of the hydrolysate in 50% aqueous ethanol placed on a paper chromatogram and studied by two-dimensional chromatography in the systems above gives a series of characteristic spots.

When a peptide containing di-DNP-histidine and also another DNP-amino acid is hydrolyzed the degradation products of the histidine will be extracted in part by ether and may interfere with identification of the other amino acid by C.C.D. For this difficulty a higher number of transfers was required. The degradation products from histidine showed a different type of absorption spectrum in the ultraviolet region and could be detected easily.

Paper electrophoresis was of considerable help in studying hydrolysates of DNP peptides. Thus δ-DNP-ornithine gave a neutral yellow spot which turned blue on spraying with the ninhydrin reagent. Dinitrophenol could be recognized because it travels somewhat faster to the anode than do DNP-leucine or DNP-isoleucine and the yellow spot almost disappeared when a drop of mineral acid was added. Dinitroaniline gave an insoluble spot at the origin. DNP-aspartic and DNP-glutamic acids gave yellow spots migrating rapidly toward the anode. They could be separated by a prolonged run.

The behavior of S-DNP-cysteine in paper chromatography was especially interesting. In the pyridine acetate buffer at *p*H 5.6 it gave a neutral yellow spot which stained blue with ninhydrin. But at a more alkaline *p*H, *e.g.*, at *p*H 8, a continuous yellow streak from the origin toward the anode was formed. This did not turn blue on spraying with ninhydrin. Obviously in the more alkaline buffer a rearrangement of the DNP group from the S to the N takes place, probably with the formation of di-N-DNP-cystine.

The S-DNP-cysteine used in this study was made by treating reduced glutathione with the DNP-reagent and subsequent acid hydrolysis. It was crystallized from ethanol–pyridine.

The residue from a particular C.C.D. band of the DNP derivatives of the peptides could be dissolved in a small volume of glacial acetic acid and lyophilized. This usually gave a fluffy yellow residue which often showed signs of being crystalline. Irrespective of crystallinity it was often in a suitable form for C, H, N, S or Cl analysis. Such analytical data were found to be very characteristic and of great value in identification.

The fractionation approach required extensive evaporation of many different dilute solutions under mild conditions. For this purpose only the rotary evaporator[11] previously described was used. An appropriate reduced pressure was employed so that the temperature of the water-bath which heated the distillation flask was never above 25°.

Experimental

A 4-g. sample of the standard preparation of bacitracin A[1] was hydrolyzed for 3 hr. in 600 ml. of 12 *N* hydrochloric acid at a temperature of 80°. During the hydrolysis a slow stream of nitrogen was passed through the flask. Considerable gaseous HCl passed out through the reflux condenser.

At the end of the heating period the solution was rapidly evaporated to dryness in a rotary evaporator with a bath temperature never above 25°. A pressure of about 5 mm. (oil pump protected with a trap containing solid NaOH) was maintained during the evaporation. The rotating condenser dipped into an acetone–Dry Ice mixture. No attempt was made to remove the last traces of volatile acid since the slightly brown colored residue was placed directly into a system containing HCl for fractionation by countercurrent distribution.

The system used for the first preliminary distribution was made by equilibrating freshly distilled 90% aqueous phenol with an equal volume of 0.1 *N* hydrochloric acid. The phase volumes in the distribution train were 6 ml. of the upper phase and 10 ml. of the lower phase.

The distribution apparatus was of the fully automatic type previously described.[13] It contained a total of 420 tubes. The sample was placed in a bank of 15 tubes at the beginning.

After completing 1030 transfers the filling device was disconnected and no further upper phase added. However, the apparatus was permitted to operate until all the upper phases had migrated into the fraction collector. The last phase left the train at 1450 transfers. A weight pattern is shown in the upper pattern of Fig. 1. The curve on the left refers to the phenol phases remaining in the machine stripped of upper phases, weight plotted against tube number. The curve on the right refers to the aqueous phases in the effluent, weight plotted against transfer number. Both curves form the "diamond" pattern discussed by Bush and Densen.[14]

Nine cuts out of the ten as shown on the pattern were further studied more or less intensively by paper chromatography and paper electrophoresis, then samples from each were hydrolyzed with 6 *N* HCl for 24 hr. at 108° and again studied with paper chromatography and paper electrophoresis. This study confirmed the indication derived from comparison of experimental with theoretical curves in pattern 1 that all cuts were still complex mixtures. Each cut, however, represented an entirely different family of peptides as the studies reported below will show. The solutes in the various cuts were recovered by extraction of the phenol with chloroform and evaporation of the aqueous phase. A small volume of water was added to the cuts from the left-hand side of pattern 1 of Fig. 1. With cuts 1 and 2 it was necessary to carry out a three-stage diamond pattern to remove all the peptide material from the phenol.

Further fractionation of each cut was carried out except with cut 1 by converting the entire fraction to dinitrophenyl (DNP) derivatives and distribution of these in various systems. The systems used throughout are listed in Table II and are designated in Fig. 1 by the abbreviation given in Table II. Pattern 1 is a true reproduction of the experimental pattern but the patterns below it are schematic reproductions of the actual pattern, simplified in the interest of sav-

TABLE II

SYSTEMS USED IN THE FRACTIONATION SHOWN IN FIG. 1

System	Solvent combination	Vol. proportions
SA	0.1 *N* aqueous HCl, 90% aqueous phenol	1:1
SB	2-Butanol, 1% aq. trichloroacetic acid	1:1
SC	2-Butanol, 0.1 *N* aq. HCl	1:1
SD	Glacial acetic acid, chloroform 0.1 *N* aq. HCl	2:2:1
SE	Glacial acetic acid, benzene 0.1 *N* aq. HCl	2:2:1

(13) L. C. Craig, W. Hausmann, E. H. Ahrens, Jr., and E. J. Harfenist, *Anal. Chem.*, **23**, 1236 (1951).

(14) M. T. Bush and P. Densen, *ibid.*, **20**, 121 (1948).

TABLE III

RESULTS FROM HYDROLYSIS OF PEPTIDES AND STUDY BY P.C., P.E., C.C.D. AND ABSORPTION

Peptide fraction	Phe	Ileu	Leu	Cys	Asp	Glu	His	Lys	Orn	D at 350 Mμ mg./cm.
2_1	+	+			+	+	+	+	+	..
2_2	+	+			+	+	+	+	+	..
2_3	+	+								..
3_1	+(DNP)	+	+							34.6
3_2		+(DNP)	+			+				..
3_3	+	+	+						+(δ-DNP)	25.5
4_1		+(DNP)	+	+(DNP)						37.8
4_3	+	+					+(1-DNP)		+(di-DNP)	39.3
5_1		+			+(and DNP)	+(DNP)		+		33.0
6_1		+(and DNP)						+	+(δ-DNP)	26.7
6_2		+(and DNP)			+(DNP)			+	+(δ-DNP)	37.0
6_4		+			+(DNP)	+(DNP)		+		35.0
6_5			+(DNP)			+				28.2
6_6		+(and DNP)			+		+(di DNP)	+		24.7
6_7	+(DNP)				+		+(1-DNP)			21
7_4		+			+(and DNP)	+(DNP)		+	+(δ-DNP)	30
8_1		+(and DNP)			+(and DNP)			+	+(δ-DNP)	41.7
9_2					+(and DNP)			+	+(δ-DNP)	30
10_6					+		+(di DNP)			24.7
11	+	+(DNP)								35.6
12_1		+(DNP)	+	+(DNP)						34.2
12_2	+(DNP)	+	+							32.3
13_1		+(DNP)	+	+(DNP)		+				27.7
13_2			+	+(DNP)						33
14_3		+	+(DNP)		+	+		+		18.2
15_2		+(DNP)		+						33.6
15_3		+(and DNP)		+				+		34
15_4		+(DNP)		+						34
16_1		+	+(DNP)		+(DNP)	+		+	+(δ-DNP)	..
16_3		+							+(δ-DNP)	37
16_4		+(and DNP)			+(and DNP)			+	+(δ-DNP)	25.3
17					+		+(α-DNP)	+		27.2
18_3		+(DNP)			+(and DNP)			+	+(δ-DNP)	35

ing space. The number of transfers applied in each pattern is given in the legend. A perpendicular line in the central part of a pattern indicates a single withdrawal section to the right of the line.

Cut 1 appeared to have the most simple composition and seemed to be composed mainly of the dipeptide isoleucylphenylalanine previously isolated from bacitracin.[15] This was confirmed by redistribution in a different system as shown in pattern 2.

The solutes in cut 2 were set aside for study at a later date. The solutes in cut 3 were found to have a relatively high content of sulfur. They gave a strong nitroprusside reaction and were converted to DNP derivatives before fractionating further.

The mixed DNP residue from cut 3 was distributed in system SD to give pattern 3. The solute from the first band appeared to be most interesting. Total hydrolysis and investigation of the products by paper chromatography (P.C.) and paper electrophoresis (P.E.) gave two ninhydrin positive spots corresponding to leucine and isoleucine and a yellow spot behaving as DNP-phenylalanine would. The latter was definitely identified by C.C.D. in system SD and determination of the K of peak material in the phosphate system of Table I. The K's in the two systems were 0.17 and 10.6, respectively. A ninhydrin positive spot fainter than the others on the P.C. map corresponding to phenylalanine was assumed to arise from partial hydrolysis of the DNP-phenylalanine.

Although no suggestion of cystine or cysteine was derived from P.C. or P.E. a total sulfur analysis was made and 3.45% S found on the undried residue. From this it was inferred that the cysteine was present in some form perhaps originally as an S-DNP derivative.

The other DNP-peptides from pattern 3 likewise gave no spot after hydrolysis and paper chromatography which could be traced to cysteine; yet 3_3 was shown to contain sulfur though in less amount than 3_1. There was not sufficient of 3_2 for sulfur analysis. 3_1, 3_2 and 3_3 refer to the bands in pattern 3, left to right, respectively.

All the other cuts from pattern 1 were treated in a manner similar to that described above for cut 3. The result is shown in Fig. 1 and in Table III.

Discussion

The series of peptides indicated in the chart in Fig. 1 at first glance seem to present a rather confusing over-all picture. On the other hand considerable interest should be attached to their successful interpretation in view of the unusual linkages which obviously must be present in bacitracin A.

Interpretation of the results becomes much easier when the over-all amino acid residue formula of bacitracin A is kept in mind. In an earlier paper[16] the formula $Ileu_2$·Phe·Leu·Cys·Asp_2·Glu·His·Lys-Orn (unknown C_5H_8O residue) (amide) was proposed. However, the number of places in which Ileu appeared in the work shown by Fig. 1 has now forced a revision of this formula. In an earlier

(15) C. L. Barry, J. D. Gregory and L. C. Craig, *J. Biol. Chem.*, **175**, 485 (1948).

(16) L. C. Craig, W. Hausmann and J. Weisiger, *ibid.*, **200**, 705 (1953).

TABLE IV

ANALYTICAL RESULTS ON VARIOUS PEPTIDES FROM BACITRACIN A

Peptide	Found C	H	N	S	Calculated C	H	N	S	Most likely formula
11_2	56.63	5.50			56.7	5.45			DNP-Ileu·Phe
3_1				3.45				3.87	DNP-Phe·Ileu(Cys·Leu)DNP
4_1	48.08	4.68	14.22	4.20	47.75	4.89	14.4	4.71	DNP-Ileu(Cys·Leu)DNP
4_3	51.1	4.37	18.00	None	51.3	4.36	17.8		$(DNP)_2$Orn·Ileu·Phe(His)DNP
6_7	49.7	3.50	17.14	None	49.7	3.63	16.8		DNP-Phe(His·Asp)DNP
12_2	52.3	5.33			52.3	5.06			DNP-Phe·Ileu(Cys·Leu)DNP
15_2	44.7	4.64	14.0		45.1	4.79	14.1		DNP·Ileu Cys DNP Ileu Cys
15_4	44.7	4.20	14.42	5.9	44.5	3.90	14.8	5.65	DNP-Ileu(Cys)DNP
10_3	44.02	3.74			43.81	3.55			Di-DNP-Orn
13_1	47.89	5.13			47.5	4.99			DNP-Ileu(Cys·Leu·Glu)DNP
13_3	54.54	4.04			54.4	3.96			DNP-Phe
6_6	48.5	4.43	16.7	None	48.5	4.81	17.00		DNP, DNP, $His·(Asp)_2$, DNP-Ileu, Lys·Ileu

paper of this series[17] it will be shown that the analytical data for intact bacitracin A are in fact in complete accord with three isoleucines instead of two. This finding makes the assumption of an unknown residue unnecessary. Other data supporting this viewpoint are given in the paper mentioned. The discussion here will therefore be concerned with the formula $Ileu_3$·Phe·Leu·Cys·Asp_2·Glu·His·Lys·Orn (amide).

The first peptide isolated from the hydrolysis of bacitracin was obtained from an attempt to study the products resulting from supposed total hydrolysis.[15] It was a dipeptide isolated in analytically pure form and containing isoleucine and phenylalanine. The ninhydrin color yield was surprisingly low. Dr. J. D. Gregory found that treatment of the peptide with nitrous acid resulted in the disappearance of the isoleucine spot on subsequent hydrolysis and paper chromatography. This indicated the peptide to be Ileu·Phe. A Van Slyke amino nitrogen determination showed the presence of one free amino group in theoretical amount.

The same dipeptide appeared in fair yield from the present hydrolysis experiment. Cut 1 of pattern 1 was mainly this substance. After purification in a second system, pattern 2, it was converted to the DNP derivative and distributed in system SE. Pattern 11 gives the result. As shown in Table IV the analytical data for DNP peptide 11_2 are in agreement with that expected for DNP·Ileu·Phe. The molecular weight calculated from the density at 350 mμ, Table III, is 408. The theoretical mol. wt. is 444. The peptide had an optical rotation of $[\alpha]^{25}D$ +100° (*c* 0.7% in glacial acetic acid).

Hydrolysis of the DNP-peptide gave a DNP amino acid which gave a homogeneous band by C.C.D. in the $CHCl_3$–HAc system of Table I. Its *K* was 0.18. The yellow solute taken from this band gave a *K* of 4.2 in the phosphate system.

The amino acid remaining after extraction of the DNP amino acid was also converted to the DNP derivative. It gave a homogeneous band by C.C.D. in the benzene system, SE, $[\alpha]^{25}D$ +42 ± 4° (*c* 0.7 in glacial acetic acid). The peptide is unquestionably isoleucylphenylalanine, but the phenylalanine appears to be partly racemized during hydrolysis since the rotation of the DNP derivative of phenylalanine is 110°.[18] A strange property of the unsubstituted peptide is the failure to give a good ninhydrin color under the standard conditions. Less than 10% of the color expected was obtained.

Peptide 10_6 can be considered next. 10_6 is the 6th band from the left of pattern 10. It was not obtained in sufficient amount for satisfactory analytical study of the intact DNP derivative but the result given in Table III indicated it to be di-DNP·His·Asp. Peptide 6_7 from the data of Table III must therefore be DNP·Phe(i·DNP)His·Asp. The C, H and N values in Table IV are in good agreement with this conclusion.

Peptide 4_3 behaved on hydrolysis as if it were di-

(17) W. Hausmann, J. R. Weisiger and I. C. Craig, THIS JOURNAL, **77**, 721 (1955).

(18) A. Paladini and I. C. Craig, *ibid.*, **76**, 688 (1954).

DNP·Orn·Ileu·Phe(i.DNP)His and the analytical data on the intact peptide, Table IV, supported this conclusion. The molecular weight calculated from the density at 350 mμ was 840 assuming three DNP groups, one of which is on the imidazole. Here the optical density at 350 mμ of a solution of di-DNP-histidine was used as a basis (ϵ = 18,500); theory, 1037. The sequence Orn·Ileu·Phe·His·Asp would now appear strongly suggested as a result of the composition of 6_7 and 4_3. The less securely established data on 16_3, apparently an incompletely DNP substituted peptide, Table III, are in accord with the sequence Orn·Ileu, but do not prove it.

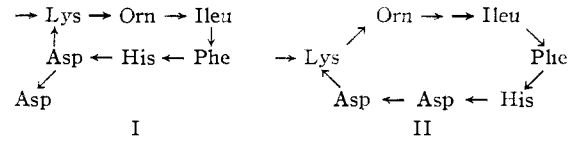
Peptide 17 is next of interest. Interpretation of this peptide rests mainly on the data of Table III. When fraction 9_3 was studied by zone electrophoresis it was found to be neutral at *p*H 5.6 but acid at *p*H 8. A strong yellow spot did not turn blue on spraying with the ninhydrin reagent indicating the absence of a free α-amino group. The intact peptide gave a good Pauly color test for the free imidazole group. The position of the band in pattern 9 indicated the presence of a strong basic group. The electrophoresis behavior at *p*H 8 was consistent with two COOH groups, one imidazole group and one amino group. Hydrolysis and paper chromatography indicated a tripeptide of α-DNP-histidine, lysine and aspartic acid. These spots were of equal intensity. However, there were two other quite faint spots. Therefore, part of 9_3 was redistributed to give pattern 17 which on hydrolysis and paper chromatography gave no faint spots but only the three strong spots.

An absorption spectrum curve in the ultraviolet agreed well with the α-N-DNP type of absorption. A minimum molecular weight in the range of 530 was indicated. DNP·His·Asp·Lys would have a molecular weight of 564. Such an interpretation would permit lysine to be added to the aspartic acid end of the pentapeptide sequence above.

The results with peptide 9_2 give an indication of the amino acid joined to the carboxyl of the lysine. This peptide gave three clear cut spots on hydrolysis and analysis by paper chromatography and electrophoresis. The three spots corresponded to lysine, aspartic acid and δ-DNP-ornithine. The aspartic spot seemed weaker than the other two. In addition there was a fainter spot in the position of DNP-aspartic. This is indicated in Table III. The presence in the free state of any of these amino acids in the peptide preparation, 9_2, was excluded by paper electrophoresis. The intact peptide migrated as an acid. The exact analytical composition of the peptide is not certain because it apparently was hygroscopic and melted down as an oil not suitable for precise analysis. The uncertainty involves whether or not 2 aspartic residues instead of 1 are present. Bacitracin A contains two aspartic acid residues but only one lysine and one ornithine.

The intact peptide 9_2 gave no ninhydrin color test. This would indicate that a free α-amino group is not present although the unusual experience with Ileu·Phe must be kept in mind. A free ε-amino group would not give such a test. On the basis of one DNP group the extinction–weight ratio from Table III would indicate a molecular weight of 484 but 968 for two. The calculated figure for a di-DNP-tripeptide of aspartic acid, lysine and ornithine with 1 HCl group is 743. DNP-aspartic acid peptides are known to give poor yields of DNP-aspartic acid or hardly any on hydrolysis,[19,20] a result we have confirmed. This would account for the faint spot obtained corresponding to DNP-aspartic acid. Ion-exchange chromatography showed that very little free aspartic was in the hydrolysate.

The formation of an Asp·Lys·Orn peptide would indicate a Lys·Orn sequence. One of two ring systems given in formula I or II could now come



under consideration. In this formula a $-\overset{\overset{O}{\|}}{C}-\overset{H}{N}-$ linkage is indicated by an arrow with the head of the arrow indicating the nitrogen. Although the molecular weight found in peptide 17 indicates a tripeptide and thus formula I, it is felt that other supporting evidence must be forthcoming before a second aspartic acid residue in 17 and formula II can be ruled out.

Bacitracin A gives a result with the Van Slyke amino nitrogen analysis which indicates two NH_2 groups. It also gives a di-DNP derivative when the imidazole group of the histidine[3] has not reacted. Yet on hydrolysis a good yield of δ-DNP-ornithine can be obtained but only a small yield of the second DNP amino acid. It has been suggested[4] and in a later paper from this Laboratory it will be shown conclusively that the amino acid involved in the second site of the DNP reaction is isoleucine. Therefore peptide 15_4 could be the end of a chain.

This peptide gave good analytical data, Table IV, for di-DNP-Ileu·Cys or di-DNP·Leu·Cys. The absorption spectrum was in good agreement with that of N·DNP·S·DNP·cysteine. Using the molecular extinction coefficient of the latter a molecular weight of 622 could be calculated for 15_4. The molecular weight of di-DNP-Ileu·Cys is 566. The decision that isoleucine was involved rather than leucine comes from the definite indication of another position for the single leucine of bacitracin A, *e.g.*, peptides 13_1, 13_2 and 4_1 as discussed further on.

Peptide 4_1 behaved as a single substance by P.C. and P.E. On hydrolysis it gave a definite spot with ninhydrin which corresponded to leucine and a yellow spot in the position of di-DNP-cystine. N-DNP-cysteine would form di-DNP-cystine in the ammonia system used for P.C. Another yellow spot was in the DNP-isoleucine position. The analytical data, Table IV, were in agreement with the di-DNP derivative of the tripeptide Ileu·Cys·

(19) F. Weygand and R. Junk, *Naturwissenschaften*, **13**, 1 (1951).

(20) R. R. Porter, "Methods in Medical Research," Vol. 3, Year Book Publishers, Inc., Chicago, Ill., 1950, p. 261.

Leu. The absorption spectrum agreed closely with that of N-DNP-cysteine with somewhat higher absorption in the 250–290 mμ range. From the molecular extinction of the latter a molecular weight of 570 could be calculated. The di-DNP derivative of the peptide has a molecular weight of 679. If the molecular weight were calculated on the basis of the expected extinction for a di-N-DNP derivative a value of 770 would have been obtained. Either derivative is possible.

Irrespective of the finer points of the structure, the data are quite satisfactory for a tripeptide Ileu·Cys·Leu. Paper chromatography showed conclusively that the one unsubstituted amino acid emerging on hydrolysis was leucine. Since there is only a single leucine in bacitracin A the DNP derivative must be DNP-isoleucine and not DNP-leucine.

Peptide 13_1 behaved as a single substance by P.C. and P.E. After hydrolysis it showed a spot corresponding to glutamic acid in addition to the three spots shown by peptide 4_1. It was assumed to be the di-DNP derivative of –Ileu·Cys·Leu·Glu–. The C and H values of Table IV were consistent with this view. An absorption spectrum curve and the molecular weight calculated from the extinction-weight ratio were also consistent with the postulated composition.

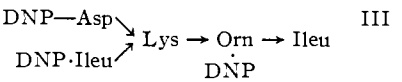
Peptide 6_5 gave spots by P.C. and P.E. which indicated it to be DNP·Leu·Glu. This would support the sequence proposed for peptide 13_1.

Peptide 6_4 gives an indication of the sequence at the carboxyl of the single glutamic acid present in bacitracin A. A sulfur analysis of this peptide was completely negative and showed cysteine to be absent. Paper chromatography and electrophoresis gave two strong spots with ninhydrin following total hydrolysis. These corresponded to isoleucine and lysine. This indicates that isoleucine is attached to one of the basic nitrogens of lysine since the carboxyl of the single lysine is accounted for in the interpretation given above of peptide 9_2. If the interpretation of peptide 17 above is correct, an aspartic acid is attached to the α-amino group of the lysine. This leaves only the ε-amino group for the isoleucine.

In addition to the two ninhydrin spots, P.C. of the hydrolysate of 6_4 gave a yellow spot in the position of glutamic or aspartic acid. P.E. gave a somewhat elongated spot suggesting that both could be present. C.C.D. of an ether extract of the hydrolysate in system SD, indicated the yellow material to be mostly DNP-glutamic acid with less DNP-aspartic being present. The hydrolytic products of DNP-aspartic acid, mostly dinitro aniline, were indicated. Both P.C. and P.E. of the hydrolysate gave two faint ninhydrin positive spots in the positions of glutamic and aspartic acids. Such would be expected from the breakdown of the postulated DNP derivatives.

The rate of migration of the intact DNP peptide in P.E. indicated more than one carboxyl group. The yellow spot did not change color with ninhydrin. The extinction–weight ratio of Table III indicated a molecular size of 415 if a single DNP were present. The molecular weight of DNP-Glu·Ileu·Lys is 574. On the basis of two DNP groups a molecular weight of 830 is indicated. This is entirely consistent with the molecular weight 853 of DNP-Glu·Ileu(DNP·Asp)Lys.

Peptide 6_2 gives a suggestion regarding whether or not an aspartic acid could be attached to the carboxyl of lysine rather than as discussed above. This peptide was not isolated in sufficient amount for satisfactory study of purity although when studied in the intact form by P.C. and P.E. it behaved as a single solute. Following hydrolysis both 6_1 and 6_2 gave strong spots corresponding to lysine, δ·DNP-ornithine, isoleucine and DNP-isoleucine. Peptide 6_2 gave in addition a fainter spot in the position of DNP-aspartic acid and a still fainter ninhydrin spot in the position of aspartic acid which could come from the breakdown of DNP-aspartic acid. The result with both peptides and 16_1 would seem to speak against an aspartic acid residue except the one covered by a DNP group. A peptide sequence

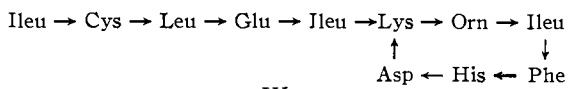


III

would be consistent with the result with peptide 6_2. This would have a molecular weight of 1099. With 3 DNP groups the molecular weight calculated from the extinction–weight ratio of Table III would be 1170.

Peptide 6_1 could differ from 6_2 by loss of the aspartic acid residue but this is not consistent with the higher molecular weight for 6_1 calculated from the extinction-weight ratio unless only 2 DNP groups are present. If the latter is true one of the basic groups of the lysine is curiously unreactive to the DNP reagent.

From the foregoing discussion a tentative sequence shown in formula IV would appear to be

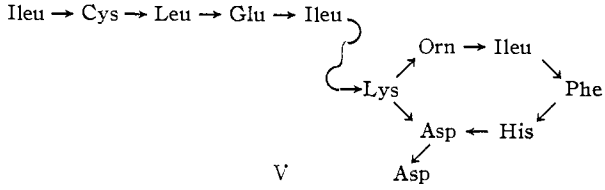


IV

strongly suggested. This would account for all the amino acids in bacitracin A except one of the two aspartic acid residues. The amide nitrogen also must be accounted for.

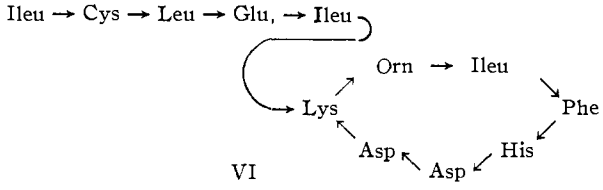
In another paper soon to be presented for publication a study of the partial hydrolysis of DNP-bacitracin A will be described. One of the peptides isolated appeared to be of special interest to the above problem. It was hydrolyzed and a quantitative amino acid analysis made by ion-exchange chromatography. The peptide was found to contain glutamic acid, isoleucine, lysine and aspartic acid in the molar ratios of 1:1:1:2 to a precision of ±3%. Unless the second aspartic is connected to a nitrogen already carrying another amino acid residue, a possibility which may have to be considered in the end, there are only two points for it to be attached, namely, the second carboxyl of either the glutamic or the aspartic acid. The other possibilities are excluded by the tripeptide His·Asp·Lys, the dipeptide Leu·Glu and others already discussed. The problem of explaining both a His·Asp·Lys sequence and a Asp·Asp·Lys sequence could be

met by assuming the extra aspartic residue to be attached to the γ-carboxyl of aspartic acid in the His·Asp·Lys sequence. The amide group might be attached by way of either of the two carboxyls of the extra aspartic acid or the δ-carboxyl of the glutamic acid. The partial formula



V

could rationalize all the partial hydrolysis data obtained thus far with the exception of 12_2. Formula VI must also be considered until stronger evidence against it is forthcoming.



VI

Peptide 12_2 came from the redistribution of the material in band 3_1. It gave analytical figures, Table IV, in satisfactory agreement with the formula shown. There was not sufficient material for a sulfur analysis. However, a sulfur analysis was made on peptide 12_1 and found to be 4.4%. Since 12_1 and 12_2 comprise all the solute in band 3_1 on which a sulfur analysis was made, 3.45%, Table IV, it is obvious that peptide 12_2 contains sulfur and thus the cysteine residue is bound in some way in the molecule. 12_1 is the smaller component of the mixture in 3_1.

As indicated in Table III, on hydrolysis and paper chromatography, peptide 12_1 gave spots indicating leucine, DNP-isoleucine and DNP-cysteine. There was not sufficient of this material for carbon and hydrogen analysis but the sulfur analysis mentioned above was low for a tripeptide of Ileu·Cys·Leu containing two DNP residues; calculated 5.5%. In spite of this finding it must be closely related to peptide 4_1 discussed previously but the exact difference is not apparent.

Peptide 12_2 as indicated in Table III gave clear spots for isoleucine and leucine but nothing clearly traceable to a DNP-cysteine or cystine. DNP-phenylalanine was definitely identified (K's in $CHCl_3$ and PO_4 systems of Table I are 0.18 and 12, respectively). The paper chromatogram also showed a faint spot in the position of the phenylalanine which probably resulted from the breakdown of the DNP derivative. The fact that a spot corresponding to a DNP derivative of cysteine did not show up should not be disturbing since these have been found to be largely transformed under the conditions of hydrolysis, depending somewhat on the other components of the molecule. The molecular weight calculated from the absorption at 350 mμ was 900, calculated for Phe·Ileu·Cys·Leu with two DNP groups attached, 826.

Bacitracin A contains only a single phenylalanine residue and a number of peptides have been isolated in fair yield from the hydrolysate which contain the sequence Phe·His. These are clearly major hydrolytic products. Only one peptide with the sequence Phe·Ileu has been isolated in a much poorer yield than those containing the Phe·His sequence. In spite of this the Phe·Ileu sequence emerging from the hydrolysate cannot be dismissed without explanation.

An obvious explanation raises the question of the purity of the preparation of bacitracin A hydrolyzed. Is it a mixture of two substances, one containing a Phe·His sequence and the one present in much smaller amount, a Phe·Ileu sequence? Although at present no unequivocal answer can be given to this question the over-all data at hand on bacitracin speak against the possibility and suggest a more likely explanation.

Bacitracin A is not stable[21] in aqueous solution at a pH above 7 and is slowly transformed with nearly total loss of antibiotic activity. One of the transformation products is called bacitracin F. In this transformation ammonia is lost with the disappearance of one of the two basic amino nitrogens. Quantitative amino acid analysis of F reveals all the amino acid residues to be present except cysteine and the small peak which is now indicated to be alloisoleucine. Hydrolysis of fully DNP substituted bacitracin F gave only one DNP amino acid, δ-DNP-ornithine. Not even a trace of the DNP-isoleucine found in the case of bacitracin A (bacitracin A gave a poor yield of DNP-isoleucine) was found. As will be shown in a forthcoming paper the ammonia which is lost in the transformation of A to F therefore comes from the isoleucine residue which gives rise to alloisoleucine on hydrolysis of bacitracin A.

Further evidence that this isoleucine is the one attached to the cysteine residue came from examination of the hydrolytic products of bacitracin F. A sharply crystalline acid fragment could be isolated by countercurrent distribution which showed the same absorption spectrum as F. It contained sulfur and nitrogen in 1:1 ratio. The nitrogen analysis (6.77%) indicated a molecular weight in the range of 207. The acid had no basic properties and could be sublimed under high vacuum. It gave a satisfactory C.C.D. pattern for a pure substance and after crystallization from pentane melted at 93–95°. The C and H analytical figures were in best agreement with a formula $C_9H_{11}O_3NS$.

The experimental data at present available do not conclusively show the nature of this fragment and further discussion must be left until more data are available. However, it is thought to be a thiazole derivative derived from the isoleucine–cysteine residues. It is stable to acid and not sensitive to ammonia.

These data and a number of other observations to be reported in a forthcoming communication are best explained on the basis of a thiazoline ring system as suggested by Newton and Abraham[4] but the cross-linking of this grouping to the phenylalanine carboxyl is an interesting point to be considered. Some of the suggestions of Wieland and

(21) L. C. Craig, J. D. Gregory and G. T. Barry, *J. Clin. Invest.*, **28**, 1014 (1949).

co-workers[6] in regard to the reactivity of peptides of cysteine are of interest to the problem.

Still another explanation of the Phe·Ileu sequence could arise from the theory that the carboxyl of the phenylalanine does not form a conventional peptide bond but in fact is joined both to the nitrogen of the histidine and the isoleucine. No unequivocal evidence for such type of bond is available as far as we are aware although it has been postulated by Stoll and collaborators[22] to account for the formation of the *d*-proline arising from hydrolysis of the ergot alkaloids.[23] Although such a coincidence does not constitute proof it is interesting that the phenylalanine arising from hydrolysis of bacitracin A has the *d*-configuration. If this observation should be more than a coincidence then the formation of a *d*-amino acid on hydrolysis may indicate that the particular carboxyl is joined in a manner more complicated than in simple amide linkage. Bacitracin A then becomes particularly interesting since in addition to *d*-phenylalanine it gives *d*-glutamic acid, *d*-ornithine and racemic aspartic acid (two residues present).

While the work reported here was in progress two reports from other laboratories appeared in the literature[24,25] which suggested partial sequences. The studies of Lockhart, Newton and Abraham suggested the sequence →Ileu → Cys → Leu → Glu → which would be in complete agreement with the results given above. However, a number of the sequences reported by Porath are not consistent with our results. The reason for these discrepancies cannot be properly discussed until a full account of his studies have appeared. A preliminary communication on our own studies has also been published.[26]

(22) A. Stoll, A. Hoffmann and Th. Petrzilka, *Helv. Chim. Acta*, **XXXIV**, 1544 (1951).

(23) W. A. Jacobs and L. C. Craig, *J. Biol. Chem.*, **110**, 521 (1935).

(24) I. M. Lockhart, G. G. F. Newton and E. P. Abraham, *Nature*, **173**, 536 (1954).

(25) J. Porath, *ibid.*, **172**, 871 (1953).

(26) L. C. Craig, W. Hausmann and J. R. Weisiger, THIS JOURNAL, **76**, 2839 (1954).

NEW YORK, N. Y.

[CONTRIBUTION FROM THE ROCKEFELLER INSTITUTE FOR MEDICAL RESEARCH]

On the Partial Hydrolysis of DNP-Bacitracin A

BY J. R. WEISIGER, W. HAUSMANN AND L. C. CRAIG

RECEIVED OCTOBER 1, 1954

A study of the partial hydrolysis products of bacitracin completely substituted with fluoro-2,4-dinitrobenzene reagent has been made. A number of peptides have been isolated and characterized. The data obtained can be satisfactorily rationalized by the amino acid sequence previously proposed for bacitracin A.

In a recent communication[1] part of the studies of this Laboratory on the partial hydrolysis of bacitracin A have been reported. The results indicated that the histidine, aspartic acid, lysine and ornithine residues occur near each other in the molecule. The lysine residue appeared to be joined at three different positions and probably is a site of cross-linking of the chain. In order to confirm this interesting and unique linkage a study of the partial hydrolysis of the dinitrophenyl (DNP) derivative of bacitracin A was undertaken.

Since in DNP-bacitracin A the δ-amino group of the single ornithine is covered, it was hoped that a mixture of peptides would be found in the hydrolysate which would be easier to separate.

When bacitracin A is fully substituted with the FDNB reagent three DNP groups are attached.[2] One covers the δ-amino group of the ornithine and a second is attached to the imidazole group of the histidine. The exact point of attachment of the third DNP group has been open to question[3] but was thought to be on either a leucine or isoleucine residue. The data reported in the partial hydrolysis studies[1] excludes the single leucine as a point of attachment. On the other hand further data to be published soon will definitely show that the isoleucine which gives rise to the alloisoleucine on hydrolysis is the one involved. On total hydrolysis of the DNP derivative the corresponding derivatives of ornithine and histidine can be easily detected in good yield but DNP-isoleucine can be isolated only in poor yield. The reason for the instability of this DNP linkage is not obvious from data published thus far but it was hoped that the present partial hydrolysis study together with a quantitative analysis of the amino acids resulting from total hydrolysis of the Tri-DNP derivative would shed light on this problem. No attempt will be made in the present study to determine the complete sequence in each peptide isolated. Where a certain sequence has been well established in the previous paper[1] it is assumed in the interpretation of the peptides isolated in the present work.

Experimental

Two grams of bacitracin A[4] was converted to the DNP-derivative by treatment for 80 minutes at room temperature with a solution containing 1 g. of fluoro-2,4-dinitrobenzene in 60 ml. of 66% ethanol. The *p*H was maintained at approximately 8 by addition of triethylamine. The ethanol was removed by concentrating the solution under reduced pressure and after dilution with water the excess FDNB was removed by extraction with ethyl ether. The aqueous solution was evaporated to dryness and hydrolyzed in the dark for 4 hr. under an atmosphere of N_2 in 400 ml. of 12 *N* HCl

(1) W. Hausmann, J. R. Weisiger and L. C. Craig, THIS JOURNAL, **77**, 723 (1955).

(2) L. C. Craig, W. Hausmann and J. R. Weisiger, *J. Biol. Chem.*, **200**, 765 (1953).

(3) I. M. Lockhart, G. G. F. Newton and E. P. Abraham, *Nature*, **173**, 536 (1954).

(4) L. C. Craig, J. R. Weisiger, W. Hausmann and E. J. Harfenist, *J. Biol. Chem.*, **199**, 259 (1952).