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

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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).

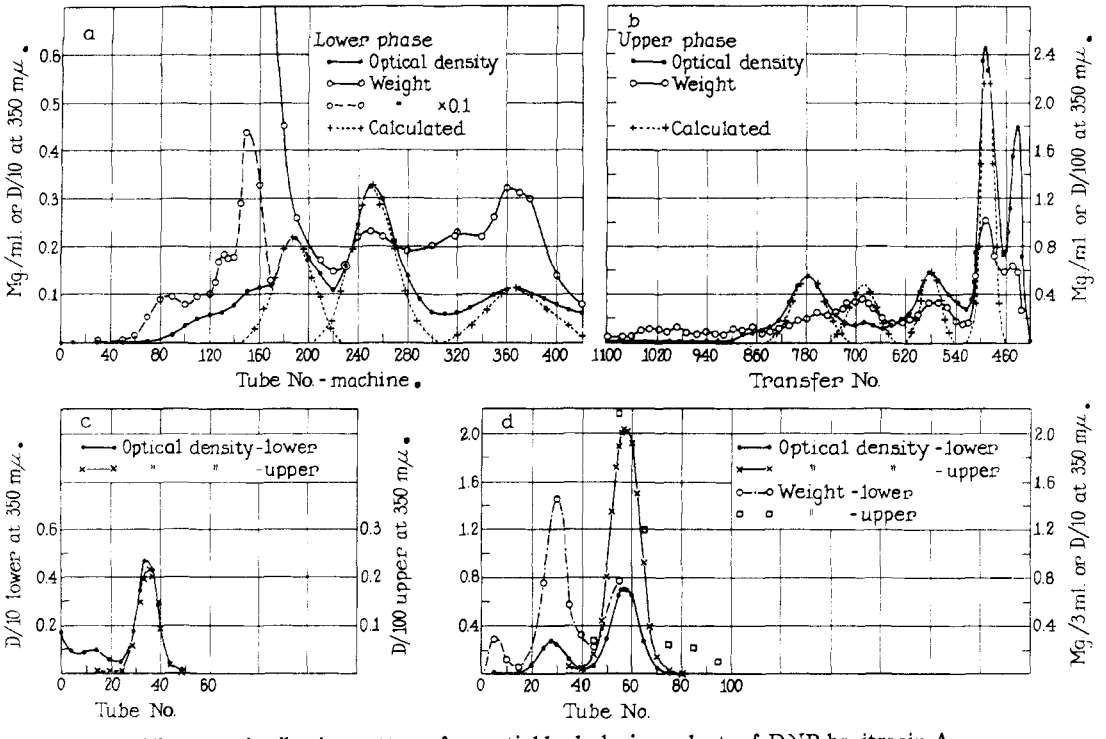


Fig. 1.—Distribution patterns for partial hydrolysis products of DNP-bacitracin A.

held at 80°. The excess acid was carefully evaporated in the rotary evaporator[5] under reduced pressure with the heating bath never above 25°.

The residue was immediately placed in the system used for distribution. This system was made by equilibrating 2-butanol with 0.1 *N* HCl. The distribution was made in an automatic countercurrent distribution apparatus of the type previously described[6] except that it contained 420 tubes. When 680 transfers had been reached no more upper phase was added but the machine was permitted to operate until no more upper phase was in the train, *e.g.*, 1103 transfers. Pattern b of Fig. 1 thus is an effluent series containing only upper phase while pattern a is also an effluent series but containing only lower phase. Both weight and optical density curves were plotted. Much weight but little absorption was found in the region of tubes 60–170, pattern a. Accordingly, for these tubes, each weight was multiplied by 0.1 for plotting purposes.

Peptide A.—The band of pattern b with its peak at 490 transfers seemed to approach the width and shape of a theoretical distribution. During evaporation of the system crystalline material began to separate as needles. It was collected and washed with water. It proved to be the hydrochloride of a DNP-peptide. Complete hydrolysis in 6 *N* HCl and two-dimensional chromatography in the formic acid and ammonia systems for amino acids[7] indicated spots corresponding to δ-DNP-ornithine, isoleucine and phenylalanine.

Anal. Calcd. for $C_{26}H_{35}O_8N_6Cl$: C, 52.3; H, 5.94. Found: C, 52.1; H, 6.00.

The molecular weight calculated[8] from the weight *vs.* absorption at the peak of the curve in Fig. 1 is 580. The molecular weight of the formula given is 594.7.

Peptide B.—A cut from pattern b, Fig. 1, which included transfer numbers 554–606 was taken for further investigation. It gave only a single neutral spot by paper electrophoresis at *p*H 5.6. After evaporation of the solvent the residue was distributed to 50 transfers in the system methanol, chloroform, 0.01 *N* HCl (2:2:1 volume proportions). In this distribution the upper phases contained only 5 ml. as compared to 10 for the lower phases. A distribution pattern determined by absorption at 350 mμ is shown in pattern c of Fig. 1. In this pattern different ordinates are used for the upper and lower phases in order to show that for the main band the upper and lower phases agree satisfactorily and thus provide an indication of homogeneity.

After recovery of the material in tubes 30 to 40 a sample was studied by two-dimensional paper chromatography in a slight variation of a system used by Blackburn and Lowther[9]; (1) aqueous phthalate buffer 0.2 molar at *p*H 6 (short axis of Whatman No. 3 MM paper) and (2) tertiary amyl alcohol saturated with the phthalate buffer. The ascending technique was used in both cases.[10] Only a single yellow spot was obtained with the intact peptide. This gave support to the thesis of homogeneity.

After total hydrolysis two-dimensional paper chromatography gave a single yellow spot in the position of δ-DNP ornithine. This spot turned blue after the paper was sprayed with ninhydrin. In addition there were blue spots corresponding to aspartic acid, glutamic acid, phenylalanine, isoleucine, leucine and lysine. No histidine or cysteine was present. This latter indication was confirmed by a negative sulfur analysis on the intact DNP peptide.

Peptide C.—A cut of effluents 740–815, pattern b, was studied further. After evaporation the residue was distributed to 100 transfers in a system made from methanol, chloroform and 1% aqueous trichloroacetic acid (volume proportions 2:2:1). Again only 5 ml. was used for each upper phase. Pattern d gives the result. Two major bands were found, one of which was strongly yellow in color and obviously contained a DNP peptide. The coincidence of the absorption curves of upper and lower phases as well as weight with optical density suggested that both could approach homogeneity.

Two-dimensional paper chromatography in the phthalate buffered systems above gave only one yellow spot with the material from the right-hand band of pattern d. On spraying with ninhydrin the yellow spot turned blue but no other blue spots appeared. This supports the thesis of homogeneity derived from the distribution pattern.

After total hydrolysis and two-dimensional paper chromatography in the systems for amino acids seven spots of

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

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

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

(8) A. R. Battersby and L. C. Craig, *ibid.*, **74**, 4023 (1952).

(9) S. Blackburn and A. G. Lowther, *Biochem. J.*, **48**, 126 (1951).

(10) R. J. Williams and H. Kirby, *Science*, **107**, 481 (1948).

similar intensity were noted. The positions corresponded to those of leucine, isoleucine, glutamic acid, aspartic acid, lysine, phenylalanine and δ-DNP ornithine.

In the case of the smaller left hand band in pattern d paper chromatography in the phthalate systems of the intact peptide gave a large, rather pale yellow spot and a smaller yellow one. Although the material crystallized on evaporation from acetic acid it probably is not pure. The amount of the second component present is not known. On spraying with ninhydrin a blue color did not develop.

After total hydrolysis and two-dimensional paper chromatography for amino acids, six spots of about equal intensity could be seen. The positions of the spots corresponded to those of lysine, aspartic acid, glutamic acid, phenylalanine, isoleucine and leucine. No strong yellow spot was present.

The more polar solutes present in the tubes represented by pattern a of Fig. 1 were further fractionated by paper electrophoresis. The apparatus and procedure were essentially those given by Kunkel.[11] Pyridine acetate was the buffer most often used.

For preparative purposes a slurry of powdered cellulose (solka floc) in the appropriate buffer was poured onto waxed paper supported on the bottom and at the sides by sections of plate glass to make a bed approximately 10 × 43 cm. in size. The surface was patted with a broad spatula to provide as even distribution of the pulp as possible. Excess buffer was absorbed by a cellulose sponge. Sections of cotton toweling approximately 10 × 20 cm. were placed at the last 5 mm. of each end. They protruded from the paper into the electrolyte to provide a junction. The waxed paper was folded over the cake and toweling to prevent evaporation of solvent. The assembly was then mounted above the electrolyte baths and allowed to equilibrate for one to two hours. This preliminary equilibration served to prevent excessive water flow during the actual separation.

The cake was then removed and its surface exposed. A solution of the sample in the buffer was distributed uniformly across a narrow central section from a pipet. Again the pulp was covered with the waxed paper and voltages applied overnight at a height somewhat less than would cause the solute to migrate into the electrolyte baths (200–400 volts).

After completion of the separation a preliminary analysis of ninhydrin staining materials was made by touching the edge of a strip of filter paper (Whatman No. 3 MM.) evenly along the length of the cake. After drying and staining, the strip served to indicate the positions of the bands without necessarily providing qualitative identification. Qualitative identification of the bands was obtained by micropipetting of samples at half inch intervals where staining material was known to be present. Such samples were then separated by paper electrophoresis. These patterns served to indicate the purity of the solution at each position of the cake.

Cuts of the pure material were removed from the indicated sections of the pulp and placed in a 50-ml. syringe containing a small wad of cotton in the tip to prevent the passage of cellulose fibers. The fluid was expressed through the cotton by forcing in the barrel of the syringe. The expressed solution was concentrated to remove pyridine and acetic acid under reduced pressure in the rotary evaporator.[5] A final electrophoretic study was made of each fraction to establish the completeness of separation.

Peptide D.—A cut, tubes 225–275, from pattern a of Fig. 1 was evaporated to dryness. The residue was shown to be sulfur free by analysis. It was also shown by paper electrophoresis not to be entirely pure but to give a major yellow zone in pyridine–acetate at *p*H 5.6 which behaved at this *p*H as a neutral solute. The appropriate zone was removed from preparative paper electrophoresis and the solute recovered as a residue. It was washed with 95% ethanol, then taken up in a little water and reprecipitated with ethanol. This precipitate appeared at least partly crystalline. It gave a single yellow spot by two-dimensional paper chromatography with the phthalate systems mentioned above. A solution of the material in glacial acetic acid containing 1 mg. per ml. showed a density value of 14.9 at 350 mμ. Its absorption spectrum was similar to that of δ-DNP ornithine.

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

After complete hydrolysis and two-dimensional paper chromatography four well-formed spots were obtained. Their positions corresponded to lysine, aspartic acid, δ-DNP ornithine and isoleucine. Quantitative analysis of the hydrolysate with an ion-exchange column[12] gave four major bands in the positions of aspartic acid, isoleucine, ammonia and lysine with the molar ratios of 1.98, 1.29, 0.80 and 1.00. The δ-DNP ornithine was lost but a small band in the position of ornithine was obtained. This probably came from the breakdown of the DNP derivative. A small band, amounting to a molar ratio of 0.13 in the position of alloisoleucine, was obtained.

Seven and a half mg. of the peptide was treated with excess fluorodinitrobenzene in 66% ethanol at room temperature for 15 min. The *p*H was adjusted to 8 with triethylamine. After removal of the excess reagent by extraction with ether, the aqueous layer was evaporated to dryness and distributed to 50 transfers in a system made from chloroform, glacial acetic acid and 0.1 *N* HCl in the volume proportions of 2:2:1. The phase volumes were 5 ml. upper, 10 ml. lower. The pattern shown in Fig. 2 was obtained. On evaporation the lower phase of tube 5 which had shown an optical density of 1.9, gave a residue weighing 0.0585 mg. per ml.

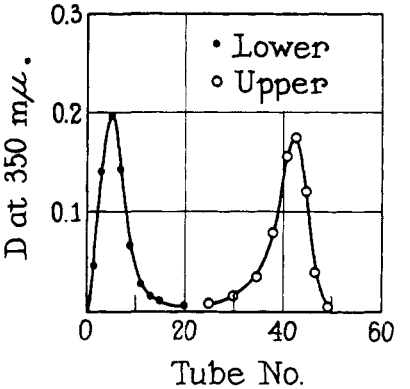


Fig. 2.—Distribution pattern for the DNP derivative of the hexapeptide derivative.

The solute from tubes 1 to 10 was recovered and completely hydrolyzed in concentrated HCl, glacial acetic acid 1:1, heated at 108° for 24 hr. Two-dimensional paper chromatography of the hydrolysate in the formic acid and ammonia systems for amino acids showed strong spots in the positions corresponding to aspartic acid, isoleucine, lysine and δ-DNP ornithine. A fainter yellow spot in the position of DNP-aspartic acid was present and a strong yellow spot which traveled with the solvent front in both directions as would be expected from DNP isoleucine. Isoleucine is the only amino acid shown by paper chromatography and the ion exchange column after hydrolysis to be present in the peptide which would give such a DNP derivative.

Peptide E.—Solute from cuts 65–100 and 101–139, pattern a Fig. 1, was studied by paper electrophoresis at *p*H 5.6 (pyridine acetate). One of the solutes from both appeared to migrate slowly as an acid in this buffer. Since it appeared to be the same in both cuts, preparative zones containing this material from each were combined and rerun at *p*H 4 (pyridine acetate). At this *p*H the main part of the peptide still appeared to migrate as an acidic solute but more slowly than at *p*H 5.6. The zone was eluted to give approximately 12 mg. of residue.

A sample of the residue was hydrolyzed in 6 *N* HCl at 108° for 24 hr. and analyzed by ion-exchange chromatography.[12] The effluent pattern was very clean cut with 5 sharp bands and nothing else appearing. These bands were in the positions of aspartic acid, glutamic acid, isoleucine, lysine and ammonia with molar ratios of 2.02, 1.00, 0.99, 1.05 and 2.73, respectively. A direct amide determination of the hydrolysate by diffusion from potassium metaborate in a Conway diffusion cup showed only 2 moles of ammonia to be present. The cause of the high value from the column is not known.

An amount of the peptide roughly approximating 20 mg.

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

was converted to the DNP derivative as above except that the reaction time was 1 hour. The DNP residue was distributed to 100 transfers in a system made from chloroform, glacial acetic acid and 0.1 *N* HCl in the volume proportions of 2:2:1, respectively. The phase volumes were 5 ml. upper and 10 ml. lower. Analysis by optical density at 350 mμ gave the pattern shown in Fig. 3. Determination of the weight of the solute in the upper phase of tubes 45 and 47 gave 0.0404 and 0.043 mg. per ml., respectively.

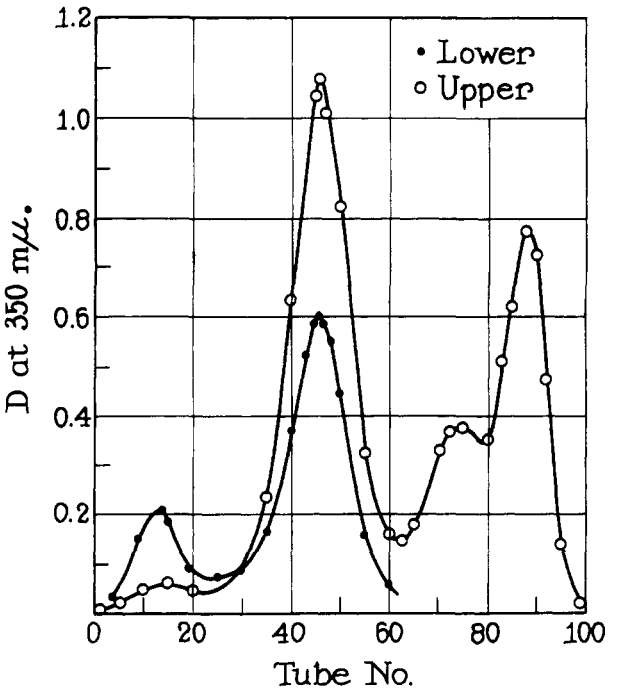
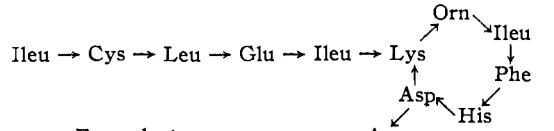


Fig. 3.—Distribution pattern for the DNP derivative of the pentapeptide E.

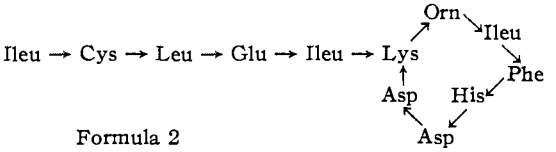
The solute in tubes 38–55 was recovered and a portion of it studied by complete hydrolysis and paper electrophoresis. Spots in the positions of DNP-aspartic, DNP-glutamic, aspartic acid, isoleucine and lysine were obtained. In addition an unknown spot was obtained in a region midway between the lysine position and the origin.

Discussion

Direct partial hydrolysis of bacitracin A with hydrochloric acid[1] results in the formation of many peptides which can be separated by a variety of fractionation techniques, mainly countercurrent distribution. The over-all amino acid sequence which appears to rationalize most satisfactorily the peptides obtained is that shown in formula 1 or 2



Formula 1



Formula 2

It is now of interest to learn if the peptides obtained by partial hydrolysis of DNP-bacitracin A could arise from such an over-all sequence.

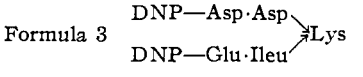
The tripeptide containing δ-DNP ornithine, isoleucine and phenylalanine which was isolated in good yield would be expected to arise from partial hydrolysis of the above sequence. This peptide was not studied further since good evidence for the Orn.Ileu and Ileu.Phe sequences had been previously given.[1]

The pentapeptide, peptide E, from the region of tubes 65 to 139 is of great interest. It was isolated as a hygroscopic resin from a zone obtained by electrophoresis fractionation. Complete hydrolysis and amino acid analysis by ion-exchange chromatography showed aspartic acid, glutamic acid, lysine and isoleucine to be present in molar ratios of 2:1:1:1, respectively. Ammonia was also present perhaps in part as amide nitrogen but in too high an amount for one amide.

The behavior of this peptide toward the DNP reagent appears to be interpretable on the basis of a single pure peptide. Three yellow bands, Fig. 3, were obtained two of them probably due to incomplete substitution. The small band on the extreme left was due to dinitrophenol from the reagent. The shape of the major band and the agreement of curves from upper and lower phases suggested homogeneity. Following hydrolysis, paper chromatography clearly indicated two basic end groups to have been present in the unsubstituted peptide, glutamic and aspartic acids. In addition to these DNP amino acids, aspartic acid, lysine and isoleucine, a basic spot was obtained.

This basic spot has often been observed when DNP-peptides containing the lysine and aspartic acids from bacitracin were hydrolyzed. It has been isolated in sufficient amount to study further by rehydrolysis. When this was done spots in the position of lysine and aspartic acid again appeared. Presumably some compound containing lysine and aspartic acid is indicated and since the compound is basic an amide nitrogen must also be involved. This amide nitrogen is indicated to be associated with one of the aspartic acid residues from the data obtained with the peptide just discussed and the peptide containing only aspartic acid, lysine, isoleucine and DNP-ornithine discussed further on.

The molecular weight of the amide of the peptide in formula 3 is 931. The experimental value 1100



Formula 3

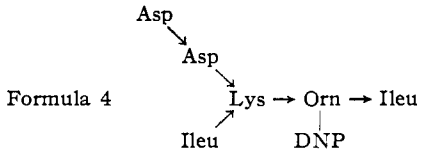
determined from weight–extinction is slightly high but this cannot be considered as evidence against formula 3 until more experience with this type of peptide is at hand.

The only real discrepancy in the present data appears to be the amount of ammonia found in the hydrolysate. However, a hint that all of this ammonia cannot be bound as amide nitrogen comes from paper electrophoresis. At *p*H 5.6 and at *p*H 4 the peptide migrates slowly as an acid, much more slowly than aspartic or glutamic acid. It migrates more slowly at *p*H 4 than at 5.6. On the basis of our experience with other peptides the rate of migration is entirely consistent with the presence of one excess carboxyl group in a pentapeptide. In the structure of formula 3 without the DNP groups

there would be a total of two NH_2 groups and four carboxyl groups. A faster rate of migration would be expected if there were two carboxyls in excess of the basic groups. One carboxyl certainly is covered by an amide group. However, if a second amide were present a net negative charge would not result. The high ammonia value from the column must therefore either arise from contamination with ammonia during manipulation or from the presence of some as yet unknown fragment in the molecule.

Support for formula 3 can be derived from peptide D isolated from the region of tubes 225–275, pattern a of Fig. 1. It contained aspartic acid, isoleucine, lysine and δ-DNP-ornithine. After complete hydrolysis ion-exchange chromatography[12] showed the molar ratios to be 1.98, 1.29, 1.00 and 0.80, respectively, for aspartic acid, isoleucine, lysine and ammonia. δ-DNP-ornithine could not be estimated on this column but the weight–extinction ratio at 350 mμ indicated one DNP group on the intact peptide per molecular weight of 1000. A small band in the effluent pattern from the ion-exchange column in the position of alloisoleucine was noted. It amounted to 0.13 mole. Assuming this to arise from the isoleucine of the peptide a total of 1.42 moles of isoleucine was found.

A formula consistent with formulas 1, 2 and 3 would be that of formula 4

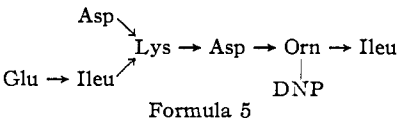


If one carboxyl were covered with an amide a peptide with two carboxyl groups and two NH_2 groups would result. This would not be expected to migrate in paper electrophoresis at *p*H 5.6 which is in agreement with the experimental finding. The molecular weight of such a peptide would be 863, a value consistent with the figure of 970 obtained by optical density–weight ratio since the residue could well contain a few percentage of weight from the buffer or cellulose.

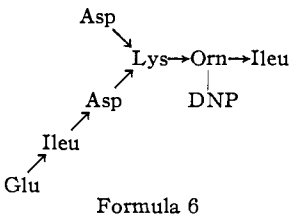
Support for formula 4 was obtained by conversion of the peptide to the DNP derivative and by separation of the products by C.C.D. The product obtained from the left-hand band of Fig. 2 gave an optical density–weight ratio which would indicate a molecular weight of 1330 on the basis of three DNP groups. The calculated molecular weight for a di-DNP derivative of formula 4 is 1213. The three DNP groups were indicated to be present by the data obtained on complete hydrolysis and two-dimensional paper chromatography. Clear spots in the position of lysine, aspartic acid, isoleucine, DNP-aspartic acid, δ-DNP-ornithine and DNP-isoleucine were obtained. Paper electrophoresis of the hydrolysate supported the findings. In addition the same unknown basic spot mentioned earlier was noted.

The only discrepancy found thus far with this peptide is the quantitative value for isoleucine residues. However, low values for recovery of isoleucine[13–16] residues have now been noted so often that they are not necessarily disturbing. Evidence that such is the case with this peptide and that a second isoleucine residue is indeed present comes from the data with the tri-DNP derivative. Although an isoleucine is covered by DNP a good spot of isoleucine still is obtained by paper chromatography of the hydrolysate.

It would be possible to write two other sequences, formulas 5 and 6, which would be consistent with the data given in this paper. These, however, can be ruled out on the basis of the data given elsewhere.[1]



Formula 5



Formula 6

The main peptides isolated from patterns c and d of Fig. 1 do not throw much light on the sequences present in bacitracin A because of their size. The only amino acids missing from them are cysteine and histidine although the latter may be present as an imidazole DNP-derivative and could have escaped detection. Splitting the sequence given in formula 1 or 2 at the Cys–Leu linkage and elimination of the histidine could give such a peptide. The difference between the two could arise from loss of an amide or one of the two aspartic acids. In any case the data with these peptides thus far are not inconsistent with formula 1 or 2.

The peptide fraction isolated from the middle band of pattern d of Fig. 1 is lacking in ornithine. Cysteine is not present as shown by a negative sulfur analysis. This fragment cannot be used as yet for structural evidence because paper chromatography clearly showed it to be a mixture. The small left-hand band of pattern d arises from dinitrophenol.

It is obvious from the data given in this paper as compared to those with the unsubstituted bacitracin A[1] that the DNP-substituted peptide breaks up in a different way than the unsubstituted one. This can have advantages for the study of structure. Fractionation of the hydrolysis products of the DNP derivative has not been carried as far as was that for free bacitracin A and many peptides still remain buried in various fractions. It appeared somewhat easier to handle the hydrolysate of the DNP-derivative.

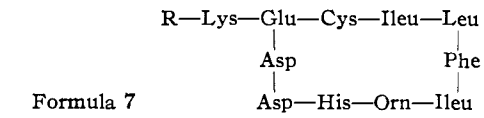
No peptides containing cysteine were isolated. Whether this is due to the effect of the nitro-groups on the sulfur is not known. Other data[17] suggest

(13) E. J. Harfenist, THIS JOURNAL, **75**, 5528 (1953).
(14) E. L. Smith and A. Stockell, *J. Biol. Chem.*, **207**, 501 (1954).
(15) E. L. Smith, A. Stockell and J. R. Kimmel, *ibid.*, **207**, 551 (1954).
(16) C. H. W. Hirs, W. H. Stein and S. Moore, *ibid.*, in press.
(17) Unpublished results from this Laboratory.

that the isoleucine connected to the cysteine is covered with a DNP group in tri-DNP-bacitracin A. Only a poor yield of DNP-isoleucine results from complete hydrolysis. Apparently the linkages around the sulfur do not present sufficient stability to strong acid to give good yields of degradation products.

A short time ago Porath[18] suggested a very tentative sequence for the amino acids present in bacitracin A which is given in formula 7. A tripeptide containing Phe, Ileu and Orn such as peptide A could arise from the sequence of formula 7. Indeed peptides clearly showing the sequence Orn → Ileu → Phe were reported in a previous paper.[1]

(18) J. Porath, *Nature*, **172**, 871 (1953).



Formula 7

However, peptide D containing only Lys, Asp, Orn and Ileu could not arise from formula 7 since it lacks a glutamic acid and a histidine residue. Nor could the pentapeptide E with two N-terminal residues, glutamic and aspartic acids, arise from the sequence in formula 7.

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Growth Inhibition of *Escherichia coli* by New Thymidine Analogs

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The preparation of 3-methylthymidine, 5-hydroxydeoxyuridine, and 5-bromodeoxyuridine and an improved method for the deamination of deoxycytidine are described. 5-Hydroxydeoxyuridine has been shown to suppress completely the growth of *Escherichia coli* K-12 at 18 hours at a level of 20 μg. per ml. 5-Bromodeoxyuridine is about $^1/_{30}$th as effective, while 3-methylthymidine, thymidine, deoxycytidine and deoxyuridine produce no inhibition. It is of interest that 5-substituted deoxyribosylpyrimidines are the only substituted nucleosides which inhibit growth of microörganisms which require neither purine nor pyrimidine compounds for growth.

Studies with totally-labeled cytidine[1] have indicated that the incorporation of this nucleoside into DNA and RNA pyrimidine nucleosides takes place without prior cleavage of the ribosyl bond, suggesting the existence of a mechanism for the conversion of ribose to deoxyribose without disruption of the glycosyl linkage. That the reverse of such a reaction does not take place is indicated by the observation of Reichard and Estborn[2] that N^{15}-labeled deoxycytidine is incorporated to a small but significant extent only into DNA cytosine and thymine, while N^{15}-labeled thymidine functions exclusively as a precursor of polynucleotide thymine. In neither case was the isotope detected in the RNA pyrimidines.

The synthesis and testing of a series of substituted deoxyribosylpyrimidines was undertaken in view of the foregoing findings with the possibility in mind that the nucleoside derivatives might block DNA biosynthesis specifically. A further incentive for synthesis of substituted deoxyribosylpyrimidines was based on the previous findings that various ribosylpyrimidine derivatives inhibit growth of microörganisms, *Neurospora*, and Theilers GD VII virus *in vitro*, and that the inhibitory effects are overcome by the addition of selected hydrolytic derivatives of RNA.[3-7] It seemed reasonable that the methods reported for the synthesis of substituted ribosylpyrimidines[3,7] might be adapted to the synthesis of substituted deoxyribosylpyrimidines, and that the latter compounds, should they prove to be inhibitory, could then be utilized by the technique of inhibition analysis[8] as a means of gaining information concerning intermediary reactions, particularly those concerning DNA biosynthesis about which relatively little is known. Furthermore, because of the unique properties ascribed to DNA, antimetabolites capable of blocking DNA biosynthesis specifically might be of value in chemotherapy.

Deoxyuridine was chosen as the nucleoside to be substituted since deoxyuridine derivatives may be considered as analogs of thymidine, a specific precursor of DNA thymine, and because methods for substitution of the uracil moiety of nucleosides are available.[3,7] The commercial scarcity of deoxyuridine and the relatively low yield of this nucleoside obtained by isolation from a DNA hydrolysate by the method of Anderson, *et al.*,[9] made it necessary to undertake the development of a method for the deamination of deoxycytidine. Modifications of the procedures described for the synthesis of uridine derivatives[3,7] were required for the synthesis of the analogous deoxyribosylpyrimidines because the latter are relatively more unstable under the conditions employed. Considerable difficulty was encountered in initiating crystallization of 5-bromodeoxyuridine and 5-hydroxydeoxyuridine. Allowing the solutions to stand uncovered

(1) I. A. Rose and B. S. Schweigert, *J. Biol. Chem.*, **202**, 635 (1953).
(2) P. Reichard and B. Estborn, *ibid.*, **188**, 839 (1951).
(3) T. Kay Fukuhara and D. W. Visser, *ibid.*, **190**, 95 (1951).
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(9) W. Anderson, C. A. Dekker and A. R. Todd, *J. Chem. Soc.*, 2721 (1952).