prisms (540 mg.), m.p. 170–172°; the analytical sample showed m.p. 177–179° (from acetone–ether), $[\alpha]^{20}$D −115°; λ_{max} 240–242 mμ, log ϵ, 4.16; λ_{max} 2.95, 6.05, 6.30 μ (reported[11] m.p. 181–182°, $[\alpha]^{20}$D −132°).

Anal. Calcd. for $C_{21}H_{30}O_2$: C, 80.21; H, 9.62; O, 10.18. Found: C, 79.98; H, 9.53; O, 10.11.

Δ^5-Androsten-3β-ol-16-one Acetate (XVIIb).—The solution of the oxime XVIc (1.3 g.) and tosyl chloride (1.3 g.) in anhydrous pyridine (30 ml.) was treated as in the previous case. Crystallization from pentane furnished needles (510 mg.), m.p. 129–130.5°, and 220 mg. more from the mother liquors, m.p. 121–123°. The analytical sample showed m.p. 129.5–130.5°, $[\alpha]^{20}$D −222°, λ_{max} 5.82 μ. (Huffman, *et al.*,[12] reported m.p. 127.5–128°; Fajkos and Sorm,[13] m.p. 134–135°, $[\alpha]^{20}$D −238° (CHCl₃).)

Anal. Calcd. for $C_{21}H_{30}O_3$: C, 76.32; H, 9.15; O, 14.53. Found: C, 76.69; H, 9.13; O, 14.28.

The free alcohol XVIIa showed m.p. 163–165°, $[\alpha]^{20}$D −235°; λ_{max} 3, 5.78 μ (reported by Huffman, *et al.*,[12] m.p. 163.5–165°, $[\alpha]^{20}$D −242° in CHCl₃; Fajkos and Sorm.[13] m.p. 168–169°, $[\alpha]^{20}$D −255° in CHCl₃).

Anal. Calcd. for $C_{19}H_{28}O_2$: C, 79.12; H, 9.79; O, 11.10. Found: C, 79.42; H, 9.82; O, 11.29.

Δ^4-Androstene-3,16-dione (XVIII).—Oppenauer oxidation of the free alcohol XVIIa (750 mg.) furnished thick prisms (410 mg.), m.p. 152–153° (from acetone–hexane), $[\alpha]^{20}$D −90°, λ_{max} 240 mμ, log ϵ, 4.22; λ_{max} 5.78, 6.05 μ (reported[13] m.p. 152–153°, $[\alpha]^{20}$D −90.5° in CHCl₃).

Anal. Calcd. for $C_{19}H_{26}O_2$: C, 79.68; H, 9.15; O, 11.17. Found: C, 79.48; H, 9.13; O, 11.45.

México, D. F., Mexico

Cellulose Ion Exchange and Rotatory Dispersion Studies with the Bacitracin Polypeptides

BY WM. KONIGSBERG AND L. C. CRAIG

Bacitracin has been shown to undergo a change at a *p*H of 4 or less which has been traced to an epimerization of the terminal isoleucine residue. The resulting stereoisomers showing different antibiotic activity have been separated by cellulose ion exchange chromatography. Anomalous rotatory dispersion behavior has been found which has been shown to be connected with the thiazoline complex. Data have been obtained which suggest an interaction of the thiazoline grouping with a ring peptide linkage.

In spite of the careful work done in a number of laboratories[1–3] on the fractionation of the mixture of polypeptides present in commerical bacitracin, a recent paper[4] has indicated that a very subtle type of heterogeneity still has persisted even in bacitracin A. The heterogeneity was correlated with small shifts in optical activity and was of particular importance to the antibiotic potency.

The sequential formula I proposed independently in two laboratories[5,6] contains a thiazoline ring structure which is unique in naturally occurring peptides. Recently, however, the possibility of such a ring being present[7,8] as one of the energy rich states in certain natural products of considerable biological interest has been raised. No relationship of these substances to bacitracin, other than the possible common occurrence of the thiazoline ring, has been shown. Irrespective of this possibility, a substance with formula I would be expected to undergo transformation easily, as indeed has been found to be the case with bacitracin A. It now appears very likely that a type of tautomeric or resonating system of linkages exists in bacitracin A which has not thus far been clearly elucidated. Chemical procedures such as those

involving direct hydrolysis with acid may act by a preliminary rearrangement or splitting as is now known to happen with the thiazoline ring and thus fail to reveal the more subtle aspects of the structure or structures. For example, there is reason to believe[6,9] that the phenylalanine residue is in some way connected to the isoleucine-cysteine residues which form the thiazoline ring system. Yet a rigid structural formula does not permit a covalent bond except by having two nitrogens linked to the carbon which emerges on hydrolysis as the carbonyl of the phenylalanyl residue. Although not accepted ordinarily in peptide chemistry as a valid linkage, unusual configurations such as those found in the ergot peptides[10,11] apparently can stabilize the linkage to the extent that it becomes a significant member of the various resonance and tautomeric forms of the amide linkage.

Often the interpretation of complex and labile structures is greatly assisted by spectroscopic methods. Indeed ultraviolet absorption measurements have been very helpful[9] in supporting the thesis of the thiazoline ring in bacitracin A. Although infrared studies have been made in this Laboratory, clear interpretation has been hindered by the multiplicity of groups present which could contribute to specific bands.

Recently the introduction of the Rudolph spectrophotometric polarimeter has considerably facilitated the use of rotatory dispersion in the study of natural products. Its application to the bacitracin polypeptides together with new absorption

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

(2) G. G. F. Newton and E. P. Abraham, *Biochem. J.*, **53**, 597 (1953).

(3) J. Porath, *Acta Chem. Scand.*, **8**, 1813 (1954).

(4) L. C. Craig and Wm. Konigsberg, *J. Org. Chem.*, **22**, 1345 (1957).

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

(6) I. M. Lockhart and E. P. Abraham, *Biochem. J.*, **58**, 633 (1954).

(7) R. E. Basford and F. M. Huennekens, THIS JOURNAL, **77**, 3878 (1955).

(8) D. Cavallini, B. Mondovi and C. DeMarco, *Experientia*, **13**, 436 (1957).

(9) J. R. Weisiger, W. Hausmann and L. C. Craig, THIS JOURNAL, **77**, 3123 (1955).

(10) W. A. Jacobs and L. C. Craig, *J. Biol. Chem.*, **110**, 621 (1935).

(11) A. Stoll, A. Hoffman and Th. Petrzilka, *Helv. Chim. Acta*, 1544 (1951).

spectra data has permitted them to be much more precisely characterized. The study has stimulated further fractionation attempts and this has resulted in the isolation of isomers which are apparently stereoisomers differing in antibiotic activity.

Experimental

The rotatory dispersion measurements were made with a Rudolph photoelectric polarimeter model 200A. Absorption spectrum measurements were made with the Cary ultraviolet recording spectrophotometer model 11.

Separations of Bacitracin by Chromatography on Carboxymethyl Cellulose.—The carboxymethyl cellulose used in this work was obtained from the Brown Co., type 100. Material ordered at two different times had different neutralization equivalents, 0.8 and 0.47 meq./g. The latter gave somewhat better resolution in some cases. All the bacitracin used in this work was obtained from the Commercial Solvents Corp. We wish to thank them for the material.

Two buffers were used in the chromatography work, 0.05 M sodium acetate buffer at pH 4.52 and pyridine acetate made by combining 24 ml. of pyridine and 19 ml. of acetic acid diluted to 6 liters with distilled water. The latter had a pH of 4.88.

The column, 0.9 cm. (i.d.), was formed by pouring in a slurry of the cellulose derivative and desired buffer, 15-cm. sections at a time, with stirring to exclude air bubbles. The final solids content of the column was about 15%. The sample was put in with about 0.5 ml. of the buffer. The effluent was analyzed by ninhydrin or optical density at 255 mμ. Rates of flow were kept under 10 ml. per hour.

A column 32 cm. in length with 11.5 mg. of so-called "high potency" bacitracin A,[4] fractionated by countercurrent distribution in the 1-butanol–pyridine acetate system at pH 6.7, gave the lower pattern of Fig. 1. A column also 32 cm.
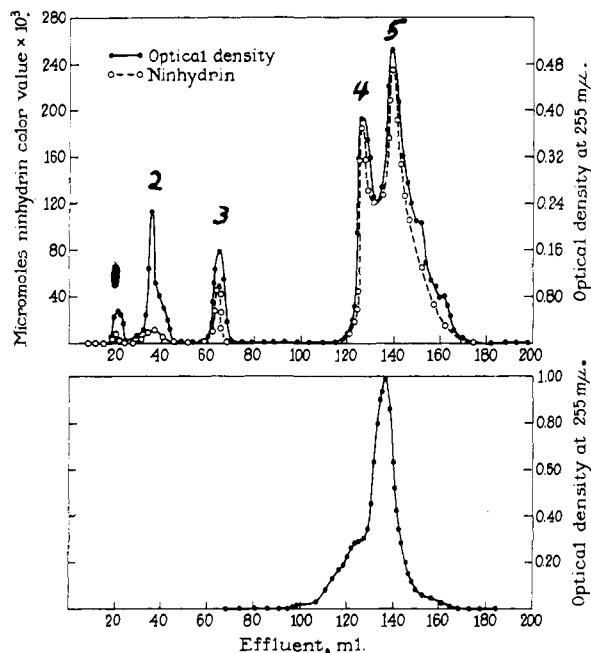

Fig. 1.—Effluent patterns obtained by fractionation of bacitracin preparations by carboxymethyl cellulose chromatography.

long with 7.3 mg. of the "low potency" bacitracin A fractionated by C.C.D. in the 2-butanol–3% acetic acid system[1] gave the upper pattern of Fig. 1. The buffer in both these cases was sodium acetate.

A column 81 cm. in length with 52 mg. of commercial bacitracin and the pyridine acetate buffer gave the pattern shown in Fig. 2.
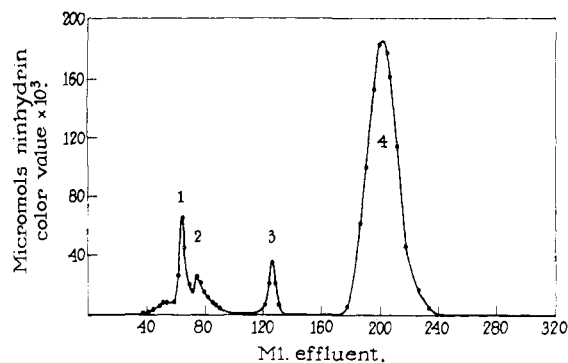

Fig. 2.—Effluent pattern obtained by fractionation of commercial bacitracin on a carboxymethyl cellulose column.

Amino Acid Analyses.—The most recent ion exchange chromatography method of Spackman, Moore and Stein[12] was used. For analysis the peptide fraction to be hydrolyzed in some cases was first oxidized by performic acid[13] in order to prevent racemization. Otherwise standard conditions of hydrolysis (6 N HCl in a sealed evacuated tube at 108° for various times) was used. Table I gives the results for the analysis of the hydrolysates of peptides recovered from bands 4 and 5 of Fig. 1 before oxidation. Table II gives the analysis at two different hydrolysis times after oxidation.

TABLE I

AMINO ACID ANALYSES OF THE SOLUTE IN BANDS 4 AND 5 OF FIG. 1

| | Residues per mole | |
| Amino acid | (24-hr. hydrolysate) | |
	Band 4	Band 5
Cystine (half)	0.68	0.64
Aspartic acid	1.61	1.65
Glutamic acid	1.00	1.00
Allo-isoleucine	0.60	0.51
Isoleucine	1.83	2.04
Leucine	1.00	1.02
Phenylalanine	0.78	0.87
Ornithine	1.00	1.00
Lysine	0.65	0.66
NH₃	3.8	1.28
Histidine	..	..

TABLE II

AMINO ACID ANALYSES OF THE SOLUTE IN BANDS 4 AND 5 OF FIG. 1 AFTER OXIDATION WITH PERFORMIC ACID

| | Residues per mole | | | |
| | 24-hr. hydrolysate | | 96-hr. hydrolysate | |
Amino acid	Band 4	Band 5	Band 4	Band 5
Cysteic acid	0.54	0.77	0.76	0.81
Aspartic acid	1.61	1.65	1.86	2.10
Glutamic acid	1.00	1.00	1.00	1.00
Allo isoleucine	0.62	0.06	0.64	0.05
Isoleucine	1.70	2.31	2.18	2.56
Leucine	1.01	0.99	1.00	0.98
Phenylalanine	0.13	0.65	0.23	0.85
Ornithine	1.00	1.00	..	..
Lysine	0.83	0.65	..	..
NH₃	1.80	1.28	..	..
Histidine	0.99	0.87	..	..

Vapor Phase Chromatography of Fatty Acid Methyl Esters.—One gram of commercial bacitracin was dissolved in 3% acetic acid and permitted to stand at room tempera-

(12) S. Moore, D. H. Spackman and W. H. Stein, *Anal. Chem.*, **30**, 1185 (1958).

(13) E. Schram, S. Moore and E. J. Bigwood, *Biochem. J.*, **57**, 33 (1954).

ture for 24 hours. This would give a mixture containing about 200 mg. of the isomer as described in the discussion which gave the low yield of phenylalanine on oxidation and hydrolysis. The whole mixture was oxidized with performic acid at 0°,[13] lyophilized and hydrolyzed in 6 N HCl in a sealed evacuated tube at 110° for 26 hours. The hydrolysate was diluted with water and extracted with ethyl ether. After drying over sodium sulfate the ether was evaporated and the oily residue was esterified with a solution of diazomethane in ether. Removal of the solvent gave an oily residue. An aliquot of approximately $1/20$ was put in a vapor–liquid chromatographic column of the James–Martin type[14] with the Lovelock–James ionization detector.[15] The 4-foot column was vapor jacketed at 100° and packed with Celite 545 (140–200 mesh), alkali treated and acid washed. The stationary phase was Reoplex 400.[16] We are indebted to Dr. Wilhelm Stoffel for the determinations.

Discussion and Results

From the very beginning of the work with bacitracin it was realized that transformations could occur with great ease. It was also realized that the final preparations were not pure in every respect. Only recently has it been possible to explain definitely[4] the nature of some of the difficulty. Countercurrent distribution has thus far been the separation tool of choice for the bacitracins but in order to reach a different type of selectivity we were prompted to try ion exchange chromatography on carboxymethyl cellulose.[17] Although at first with crude bacitracin these columns gave a rather indefinite type of effluent pattern, a different result was obtained with bacitracin A prepared by C.C.D. in the 2-butanol–3% acetic acid system.[4] Here there was evidence of resolution into two major bands and several minor ones. After slight changes in the conditions the upper pattern in Fig. 1 was obtained. Recovery of bands 4 and 5 and rechromatography showed that each retained its identity.

Samples from bands 4 and 5 were assayed for antibiotic potency by Mr. George Craig of the Commercial Solvents Corporation. We wish to thank him for the results. The residues assayed contained sodium acetate. After correcting for moisture and salt content the material from band 4 gave 46 u/mg. and that from band 5 gave 92 u/mg. The latter is a higher assay than any preparation from this Laboratory has thus far shown.

A sample of bacitracin prepared by C.C.D. in a 1-butanol–pyridine acetate system (high potency bacitracin A) gave the lower pattern of Fig. 1. It would seem clear that distinct differences between the so-called high potency and low potency bacitracin A as already suggested in the previous paper[4] can be shown clearly by chromatography on carboxymethyl cellulose.

The effluent from the columns contained considerable sodium acetate. An easy way of removing this was found by rechromatographing on carboxymethyl cellulose but with the use of a pyridine acetate buffer. The effluent was now free of inorganic residue. In fact, the pyridine acetate buffer could be used for the initial chromatography instead of sodium acetate but here the

resolution was not as good and the two main bands of the low potency material were scarcely resolved.

When an attempt was made to separate the bacitracins directly from the commercial preparation without first fractionating by C.C.D. the pattern of Fig. 2 was obtained with pyridine acetate as the buffer. The larger one, 4, of the four different bands is apparently a mixture of bacitracin A and B and probably some of their stereoisomers.

After recovery of band 4, two bands representing A and B could be separated by re-running on another column of the carboxymethyl cellulose with the pyridine acetate buffer but only after addition of 26 mg. of NaH_2PO_4 to the sample. This changed considerably the position of both bands in the effluent.

It was concluded that the C.C.D. method was considerably better for the initial separation because of its much greater capacity without sacrifice of selectivity. In a single run with the appropriate system this method yielded bacitracin A of high purity. A could be prepared in smaller amount by carboxymethyl cellulose chromatography also in high purity but only by using more than one column.

On the other hand, for certain mixtures the carboxymethyl cellulose had a selectivity not yet achieved by C.C.D. When a sample of bacitracin A which gave the lower pattern of Fig. 1 was permitted to stand one week in the 2-butanol–3% acetic acid system, recovered and then put on the column, the two nearly separated bands, 4 and 5, on the right of the upper pattern of Fig. 1 were observed. Although it was subsequently found that these isomers could be resolved in the phosphate system[4] by C.C.D., other bands not yet revealed by C.C.D. have been shown by carboxymethyl cellulose. The latter is an excellent analytical tool ideally suited to compliment the preparative separations of C.C.D.

When a sample of bacitracin F was studied in the pyridine acetate buffer it emerged at the same position as band 2 of Fig. 2. The absorption spectrum of band 2 of Fig. 1 also indicated that it was bacitracin F.

When bacitracin A was treated under the conditions used by Newton and Abraham[2] for removing the amide group and the product fractionated by C.C.D. a series of reaction products was revealed. These will be discussed in a separate report but one of the products, perhaps a desamido derivative, was studied with the carboxymethyl cellulose column. It emerged in the same position as band 1 of Fig. 2. The third band of Fig. 2 seems to be a transformation product of A which cannot be correlated with any other fraction thus far isolated. Its behavior during paper electrophoresis may offer a clue as to its nature. It remained as an uncharged spot both in buffer at pH 5.6 and 7.3. Bacitracin A shows a difference at these two pH values due to the free imidazole group of the histidine. This would indicate that the imidazole is not free in the substance from band 3. Moreover, the single amide group in bacitracin A was found not to be present in the new substance.

(14) A. T. James and A. J. P. Martin, Biochem. J., 63, 144 (1955).

(15) J. E. Lovelock and A. T. James, Ann. N. Y. Acad. Sci., in press (1958).

(16) C. H. Orr and J. E. Callen, THIS JOURNAL, 80, 249 (1958).

(17) H. A. Sober and E. A. Peterson, ibid., 76, 1711 (1954).

If the amide had been lost by hydrolysis then three carboxyl groups would explain the lack of net charge at pH 5.6 but not as 7.3 where the free imidazole would be uncharged. Transfer of the aspartyl from the amide to the imidazole with loss of ammonia to give an aspartyl imidazole linkage would account for the observed properties.

It was known from the previous work[4] that the optical rotations of the bacitracin fractions are low. However, with the Rudolph spectrophotometric polarimeter the possibility of more precise characterization by rotatory dispersion was now offered.

Bacitracin A (high potency) proved to have a very interesting type of rotatory dispersion. A plot of the specific rotation in 3% aqueous acetic acid against wave length is shown in Fig. 3. The change
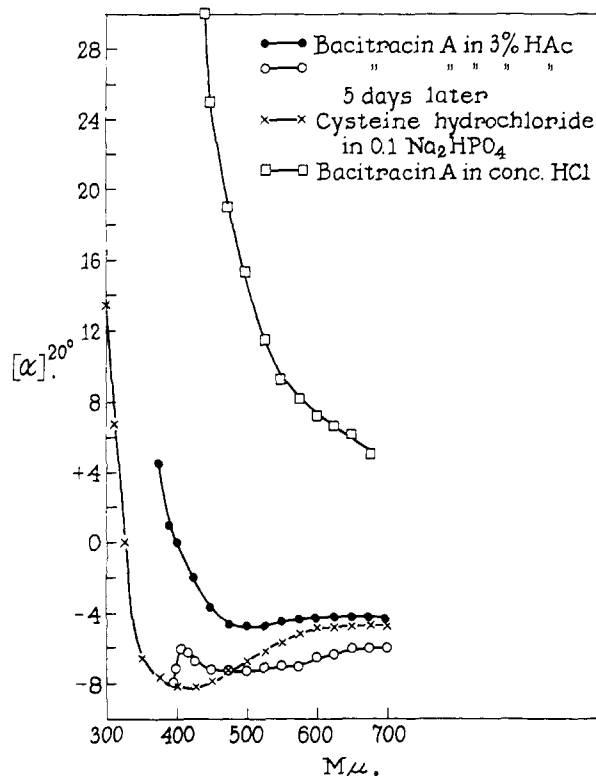

Fig. 3.—Rotatory dispersion curves.

of rotation with wave length does not follow a first-order Drude equation and is therefore said to be anomalous. The shape of this curve was not markedly altered by pH changes in the range of 3 to 8 but was at extremes of pH or on standing. Thus the rotatory dispersion in concentrated hydrochloric acid is that of the upper curve in Fig. 3. This dispersion now approaches the normal type as is shown by plotting the reciprocal of the rotation against the square of the wave length according to the procedure of Lowry and Dickson[18] as shown in Fig. 4. The data give nearly a straight line.

When bacitracin A is heated in 0.5 N HCl for 20 minutes at 100° as suggested by Newton and

Abraham[19] for opening the thiazoline ring the characteristic absorption spectrum of bacitracin A disappears and a considerably higher levorotation is obtained in contrast to bacitracin dissolved in concentrated HCl which is dextro rotatory. A Lowry plot of the hydrolyzed peptide is linear as shown in Fig. 4. Glutathione under the same conditions is also levo rotatory and gives a linear Lowry plot.
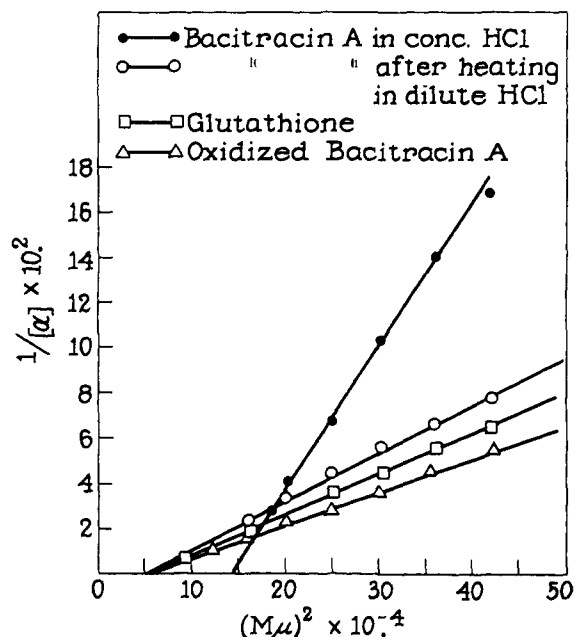

Fig. 4.—Lowry plots of rotatory dispersion curves of bacitracin preparations and glutathione.

Bacitracin A oxidized with performic acid as given in a paper to be published[20] does not show the typical thiazoline absorption. The ring is opened to give a sulfonic group as shown by the appearance of a further negative charge in the paper electrophoresis experiment. A study of the reaction with the DNP reagent indicated all the basic groups to be retained. Rotatory dispersion measurements gave data which followed a linear Lowry plot as shown in Fig. 4.

In view of the present interest in the application of rotatory dispersion data to the problem of the configuration of proteins[21,22] it seemed of interest to investigate the behavior of other cyclic antibiotic polypeptides. Rotatory dispersion data for penicillin G, polymyxin B, tyrocidin A, gramicidin S, A and subtilin were obtained. All these gave Lowry plots indicating a normal type of dispersion. Bacitracin B showed a dispersion very similar to that of A.

Cysteine at pH approximately 4 was found to give an anomalous dispersion very similar to bacitracin as the curve in Fig. 3 shows. The shape of

(18) T. M. Lowry and T. W. Dickson, *J. Chem. Soc.*, **103**, 1067 (1913).

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

(20) R. J. Hill and L. C. Craig, unpublished results.

(21) J. A. Schellman and C. G. Schellman, *Arch. Biochem. Biophys.*, **65**, 58 (1956).

(22) P. Dory and R. D. Lundberg, *Proc. Natl. Acad. Sci.*, **43**, 213 (1957).

the dispersion curve was changed by concentrated hydrochloric acid but, in contrast to bacitracin, not to a normal type.

Bacitracin A reacts rapidly with formaldehyde at neutral pH.[1] The product was found to have lost the characteristic thiazoline type of absorption spectrum and to have also lost its basic groups. The rotatory dispersion was found to be of the normal type. Cysteine is known to react with formaldehyde[23] to give a thiazolidene. In contrast to cysteine we have found the thiazolidine to give the normal type of dispersion.

Bacitracin F is derived from bacitracin A by a unique type of oxidative loss of ammonia. The amino group[9] attached to the terminal isoleucine is also known to be lost as shown by acid hydrolysis of F. In spite of the fact that the α-carbon of this residue could not be optically active, the substance shows an anomalous type of optical dispersion which is different but bears somewhat of a similarity to that of A. However, it is not converted to the normal type of dispersion by either formaldehyde or concentrated hydrochloric acid.

When bacitracin A is reduced[24] with Adams PtO$_2$ catalyst it loses the characteristic absorption band at 255 mμ. The product has a very weak dextrorotation in contrast to bacitracin which has a weak levorotation. The rotatory dispersion of the reduced form, although very weak, appeared to be anomalous. It proved to have poor stability in cold concentrated hydrochloric acid. Unlike bacitracin A the dispersion did not seem to be of the normal type in acid of this strength.

The rotatory dispersion of components 4 and 5 from a column similar to that seen in the upper pattern of Fig. 1 was measured directly on the effluent from the column by using a longer (40 cm.) light path to compensate for the dilution. The result given in Fig. 5 was obtained. Both bands were anomalous, but that of component 5 corresponded to high potency bacitracin A. This result confirms the fact that bands 4 and 5 are distinctly different substances. In further support of the findings a sample of high potency A permitted to stand in 3% acetic acid for five days gave the changed dispersion curve shown in Fig. 3 and now also gave bands corresponding to 4 and 5 of Fig. 1 on a cellulose column.

A recent paper[25] by Leonard, Adamcik, Djerassi and Halpern has shown that transannular nitrogen–carbonyl interaction can produce an anomalous type of rotatory dispersion. A form of nitrogen–carbonyl interaction similar to this has been considered for some time in bacitracin.[26] It undoubtedly influences the type of dispersion found but as will be seen in the later discussion other influences must be considered also.

The most conclusive evidence to show that the subtle transformation occurring in bacitracin A when it is dissolved in 3% acetic acid involves the terminal isoleucine has been derived by oxi-

(23) S. Ratner and H. T. Clarke, THIS JOURNAL, 59, 200 (1937).
(24) P. Marfey and L. C. Craig, results to be published.
(25) N. J. Leonard, J. A. Adamcik, C. Djerassi and O. Halpern, THIS JOURNAL, 80, 4858 (1958).
(26) L. C. Craig, "Proc. 3rd Intern. Cong. Biochem., Brussels," 1955, p. 2.

dation and hydrolysis experiments. Material from bands 4 and 5 of Fig. 1 were separately oxidized by performic acid at 0°[13] before hydrolysis. The data in the unpublished paper[20] show that this clearly oxidizes the sulfur to a sulfonic acid derivative. This oxidation should prevent the racemization to be expected in the strong acid used for hydrolysis. That such is the case is clearly shown by the results in Table II. The peptide from band 4 gave 0.64 mole of allo-isoleucine while that from band 5 gave only 0.05 mole.

A number of the peptide linkages in bacitracin are very difficult to hydrolyze and for a reliable estimate of the quantitative amino acid composition different hydrolysis times must be studied since too long a time can lead to destruction of certain residues. The isoleucine and aspartyllysine bonds are known to be very resistant.[27] Table II gives the results with 24- and 96-hour hydrolysis times.

In results obtained by the direct hydrolysis of bands 4 and 5, the expected amount of allo-isoleucine appeared as shown in Table I but with a slightly higher figure in band 4 than in band 5. Otherwise the result was in good agreement with the earlier analyses of bacitracin A.

In order to obtain the results in Table II, samples from bands 4 and 5 of Fig. 1 were first oxidized with performic acid and then hydrolyzed. Here 0.62 and 0.64 mole of allo-isoleucine was found in band 4, but 0.06 and 0.05 found in band 5. Clearly band 4 is the one from which the allo-isoleucine residue is derived. A further striking point in this analysis concerns the phenylalanine value which is somewhat low in material from band 5 but very low in the material from band 4.

When material from bands 4 or 5 was permitted to stand in solution at a pH of 4.52 and a temperature of 4° for a month it slowly changed. Table III shows the analysis of this material after oxidation. The alloisoleucine figure from band 4 had decreased while that of 5 had increased showing a rearrangement back to an intermediate mixture.

TABLE III

AMINO ACID ANALYSES OF THE SOLUTE FROM BANDS 4 AND 5 OF FIG. 1 AFTER STORAGE FOR A MONTH AT 4° AND THEN OXIDIZED WITH PERFORMIC ACID

Amino acid	Residues per mole (24-hr. hydrolysate)	
	Band 4	Band 5
Cysteic acid	0.46	0.49
Aspartic acid	1.93	1.90
Glutamic acid	1.03	1.00
Allo-isoleucine	0.23	0.25
Isoleucine	1.54	1.60
Leucine	0.97	0.98
Phenylalanine	0.07	0.46
Ornithine	1.00	1.01
Lysine	0.52	0.48
NH$_3$	1.38	1.63
Histidine	..	..

Ultraviolet absorption spectrum measurements were made on fractions from various sources. It was found that a fresh effluent from the cellulose
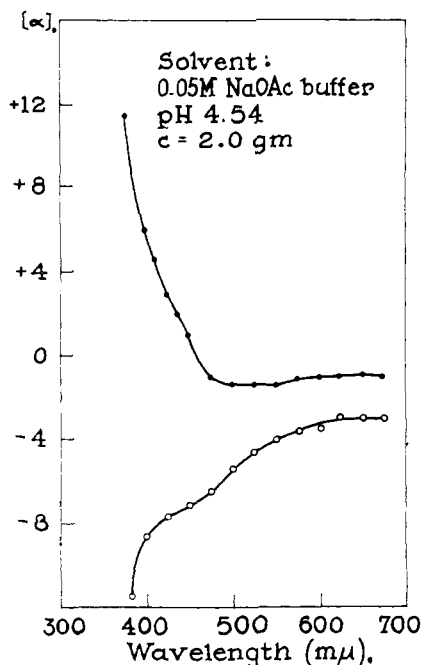
(27) I. M. Lockhart and E. P. Abraham, Biochem. J., 62, 645 (1956).

Fig. 5.—Rotatory dispersion curves of the isomeric bacitracin A forms: upper, component 5; lower, component 4.

Fig. 6.—Absorption spectrum curves.

column containing the major band in the lower pattern of Fig. 1 had a slightly sharper band than did the material from the best C.C.D. work after recovery. The ratio of absorption at 255 and 290 mμ was 40. Apparently a little bacitracin F or something similar is formed from A even under the best conditions of recovery.

Figure 6 shows the ultraviolet absorption curve of bacitracin A and that of thiazoline-4-carboxylic acid[7] for comparison. The absorption of A was not altered immediately on standing. However, when A was dissolved in concentrated hydrochloric acid it gave curve 1 of the right hand series of curves of Fig. 6. Formylcysteine[7] gave a very similar curve in concentrated hydrochloric acid while glutathione also apparently formed the thiazoline ring but somewhat more slowly and with a lower extinction. Penicillin K gave this type of absorption, but the yield probably was low. It was a type of absorption similar to the penillic acids.[28]

Glutathione[7] did not form the thiazoline ring as well in 6 N hydrochloric acid as in concentrated acid. Bacitracin A in this solvent gave the immediate shift from the absorption on the left to that of the right of Fig. 6 but was less stable than in concentrated acid. Both the absorption spectra and rotatory dispersion showed a steady shift. On the other hand, in concentrated acid these measurements were reasonably steady over a period up to one hour at room temperature. On recovery by dilution with glacial acetic acid and lyophilization a residue was obtained which showed the absorption curve with the maximum at 255 mμ and the low anomalous rotatory dispersion. The latter was slightly altered and more

like that which had stood in acetic acid for a time.

It would seem that the anomalous dispersion in bacitracin stems primarily from the optically active β-carbon atom of the terminal isoleucine residue. The α-carbon atom of this residue is also involved, but preservation of the optical activity of this carbon is not essential for the anomalous dispersion since F[29] has a structure in which the α-carbon has lost its asymmetry. The weak optical rotations found can be observed because the remainder of the molecule has a low net optical rotation due to the balance of dextro and levo amino acid residues in the whole molecule.

There now seems to be sufficient evidence to suggest two of the theoretically possible interchangeable structural arrangements of the thiazoline ring system. These are shown in the partial formulas 2 and 3. A good review of the evidence for 2 has been given by Abraham.[30]
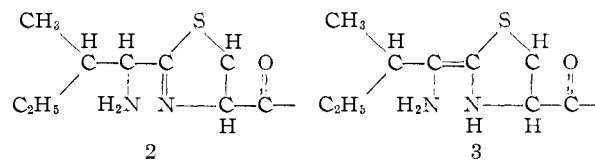
The epimerization behavior with respect to the terminal isoleucine presented in this paper would appear to show definitely that 3 is one of the tautomeric forms. This conclusion is supported by the finding that one of the smaller bands obtained by countercurrent distribution of the reaction products of bacitracin A with FDNB at pH 4.66 is in fact a tetra-DNP derivative.[29] Hydrolysis and quantitative amino acid analysis showed the cysteine residue to have been destroyed.

There now remains to be considered the interesting connection to the phenylalanine residue. The α-carbon atom of phenylalanine is known[26] to emerge on acid hydrolysis in the partially racemized dextrorotatory form. This indicates that a double bond can exist, at least momentarily, between the α-carbon atom and the carbon of the amide carbonyl in acid solution as shown in

(28) H. T. Clarke, J. R. Johnson and R. Robinson, "The Chemistry of Penicillin," Princeton University Press, Princeton, N. J.

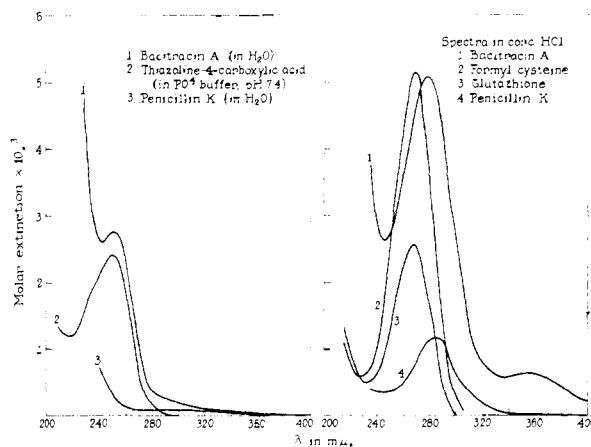(29) W. Konigsberg and L. C. Craig, to be published.

(30) E. P. Abraham, "Biochemistry of Some Peptide and Steroid Antibiotics," John Wiley and Sons, Inc., New York, N. Y., 1957.

formula 5. Several tautomeric forms could be as shown in partial formulas 4 to 7.

The very striking observation in Table III that one of the isomeric forms of bacitracin A, following oxidation with performic acid and complete hydrolysis, gives a half mole of phenylalanine while with the other the phenylalanine is almost totally destroyed can now be discussed. The double bond in the isoleucine residue of formula 3 would permit cis–trans isomerism. If an N to carbonyl interaction, as pictured in formulas 4 to 7, should be sterically possible then a definite difference between the two isomers due to the double bond might be expected. The amino group of the cis isomer would approach the α-carbon of the phenyl-alanine residue and promote enolization driving the equilibrium toward formula 5. Performic acid would be expected to attack this double bond to destroy the phenylalanine residue.

On the other hand, the trans isomer might not be so strong an inducer of enolization and the phenyl-alanine residue would thus not be oxidized and destroyed.

An odor similar to that of phenylacetic acid was noted in the reaction products. Ether extraction of the acidified reaction products gave a small amount of an oily residue which was esterified with diazomethane and fractionated by gas partition chromatography. Several bands were obtained, one of which corresponded in position to that of the methyl ester of synthetic phenylacetic acid. It
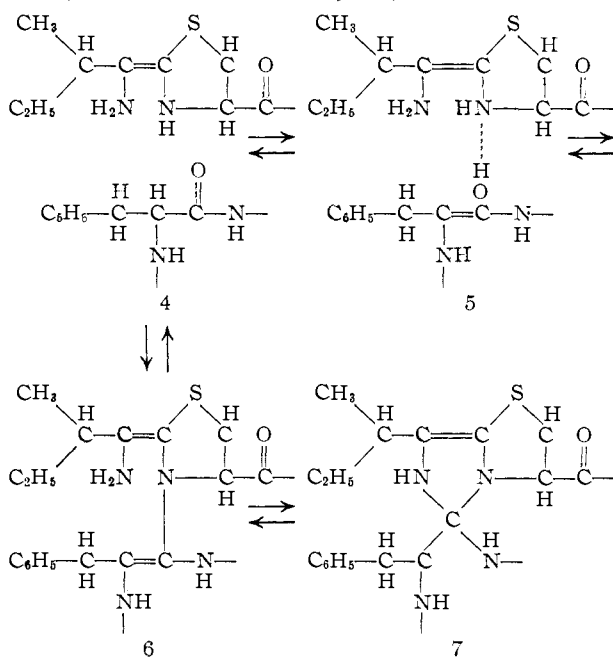
was obtained in too small an amount for further identification. Another band in the gas chromatograph corresponded to that of the methyl ester of isovaleric acid. This could arise from the enamine form of the terminal isoleucyl residue in formula 3.

Some destruction occurs as is shown by the low total recovery of isoleucine in every analysis of oxidized bacitracin. Formula 7 could be one of the further possible forms resulting from the cis attraction.

The tetrapeptide Phe.Ileu.Cys.Leu isolated on partial hydrolysis[5] of bacitracin A could easily arise by transfer through the intermediate form of formula 7.

An alternative possibility is that the isomer in band 5 of Fig. 1 has the double bond in the position shown in formula 2 while that in band 4 differ by the double bond position of formula 3. With either this possibility or the previously discussed one the liberation of the sulfonic group during the performic acid oxidation step may have a bearing on the quantitative liberation of this isoleucine as either L: or allo-isoleucine. Formula 2 should give the L-isoleucyl peptide with subsequent nearly quantitative liberation of L-isoleucine. Here the data found are quite satisfactory.

On the other hand, the liberation of the sulfonic group from formula 3 might be expected to give a peptide with the configuration of the α-carbon atom of the isoleucine partly racemized but predominantly one form. The yield of 0.64 mole of allo-isoleucine found from the isomer in band 4 is in line with this theory.

Assuming that band 4 has the structure shown in formula 3, then the interaction between the phenyl-alanine residue and the amino thiazoline grouping as pictured in formula 6 would be favored over the structure formulated in 2 since both nitrogens are fixed in a plane and can form a six-membered ring as shown in 6, while free rotation is permissible in formula 2.

A structural analogy is apparent between part of the formula of 6 and the ketopiperazine structure. Ketopiperazines are known to racemize easily.

We are well aware of the general similarity of some of the proposed interactions and those suggested elsewhere to recently considered enzyme mechanisms. The problem from this standpoint is being continued. In this connection it was interesting to find that bacitracin did not appear to react with diisopropyl fluorophosphate even though it has been shown[31] that certain Δ²-oxazoline derivatives are attacked by this reagent.

ADDENDUM.—Just prior to the receipt of proof for this paper, it was found that the destruction of the phenylalanine residue during performic acid oxidation was unexpectedly catalyzed by a small amount of sodium acetate. It then proved possible to trace the difference in behavior of peptide material from bands 4 and 5 to a different content of sodium acetate from the exchange column. The isomers do not have significantly different stabilities to performic acid oxidation.

NEW YORK 21, N. Y.

(31) G. R. Porter, H. N. Rydon and J. A. Schofield, Nature, 182, 927 (1958).