crystallization from the same ternary mixture gave analytically pure material as long needles, m.p. 221–222° dec. when placed in a bath preheated to 220°.

Reaction of X *with salicylaldehyde.* A solution of 1.22 g. of salicylaldehyde in 20 ml. of methanol was added dropwise to a solution of 1.54 g. of X in 100 ml. of 50% methanol. Almost immediately a yellow precipitate separated. After stirring for 20 min., the crystalline material (1.6 g; 95%) was collected and recrystallized first from ethanol and then from benzene–petroleum ether to give shining yellow plates of salicylaldehyde azine, m.p. 220–222°. Curtius and Jay[12] report a m.p. of 205°.

Anal. Calcd. for $C_{14}H_{12}N_2O_2$: C, 70.00; H, 5.00. Found: C, 70.01; H, 5.23.

Reaction of X *with p-[N,N-bis(2-chloroethyl)amino]-benzaldehyde.* A solution of 1.1 g. of X in 30 ml. of water was added to a solution of 1.25 g. of XII[20] in 100 ml. of methanol. After stirring for 1 hr. at room temperature, 5 ml. of concd. hydrochloric acid was added to the mixture, the color of which changed to deep orange-red. After stirring for another hour, the solid was collected and washed thoroughly with water. The filter cake was extracted with ether in a Soxhlet extractor to give 0.8 g. of shiny yellow-orange plates of XIV, m.p. 165–166°. Further recrystallization first from chloroform and then from petroleum ether (b.p. 60–75°) gave analytically pure material with no change in the melting point.

Anal. Calcd. for $C_{22}H_{26}Cl_4N_4$: C, 54.10; H, 5.32; N, 11.47; Cl, 29.09. Found: C, 54.08; H, 5.27; N, 11.59; Cl, 28.93.

p-[N,N-Bis(2-chloroethyl)amino]benzaldehyde azine (XIV). To a cold solution of 1.25 g. of XII in 20 ml. of benzene was added 0.8 ml. of 95% hydrazine. After stirring for 2 hr. at room temperature, 10 ml. of pentane was added and the precipitate was collected. Recrystallization from petroleum ether (b.p. 90–100°) gave 0.5 g. of XIV, m.p. and mixture melting point with the substance prepared from X, 164–166°. The infrared spectra of XIV prepared by the two routes were identical.

Anal. Found: C, 54.16; H, 5.39; N, 11.32.

Acknowledgment. We acknowledge the valuable assistance of James M. Hudson in the preparation of certain intermediates.

ANN ARBOR, MICH.

(20) R. C. Elderfield, I. S. Covey, J. B. Geiduschek, W. L. Meyer, A. B. Ross, and J. H. Ross, *J. Org. Chem.*, **23**, 1749 (1958).

[CONTRIBUTION FROM THE LABORATORIES OF THE ROCKEFELLER INSTITUTE]

The Oxidation and Acid Isomerization of Bacitracin A

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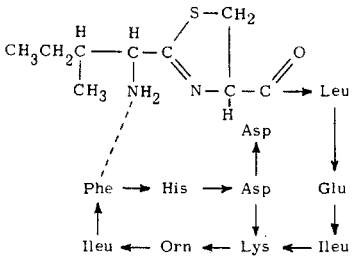
A study of the acid isomerization of bacitracin A has shown the reaction to follow first order reaction kinetics. The isomerization is reversible, and the two forms appear to be stereoisomers differing in antibiotic activity. Oxidation with performic acid has shown that the transformation results from an epimerization of the *N*-terminal isoleucine residue.

INTRODUCTION

The main structural features of bacitracin have been worked out by several investigators[1,2] and are best represented by Formula 1.



Bacitracin A

With this knowledge of the amino acid sequence and other structural considerations as a basis, a better understanding of the relationship between structure and antibiotic activity for this particular antibiotic polypeptide appeared possible.

During the study of some of the more subtle relationships connected with the structure and internal interactions of bacitracin A, we were struck, among other things, by the marked lability of this substance outside the *p*H range from 4.5 to 6.5.[3–5] The transformation leading to inactivation at *p*H's above 7.0 have been ascribed to deamination and autoxidation of the *N*-terminal amino thiazoline moiety.[6] More recently we have discovered[5] that the change which bacitracin undergoes in acidic media is related to the shift of the double bond in the thiazoline ring to the exocyclic position.

This latter reaction has been studied further and evidence has been obtained which indicates that the structure having a double bond exocyclic to the thiazoline ring is a transient intermediate which is responsible for epimerization at the alpha carbon of the *N*-terminal isoleucine.

We have also explored the possibility of separating the acid isomerized bacitracins by countercurrent distribution. These results are reported and

(1) W. Hausmann, J. R. Weisiger, and L. C. Craig, *J. Am. Chem. Soc.*, **77**, 723 (1955).

(2) I. M. Lockhart, E. P. Abraham, and G. G. F. Newton, *Biochem. J.*, **61**, 534 (1955).

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

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

(5) Wm. Konigsberg and L. C. Craig, *J. Am. Chem. Soc.*, **81**, 3452 (1959).

(6) J. R. Weisiger, W. Hausmann, and L. C. Craig, *J. Am. Chem. Soc.*, **77**, 3123 (1955).

are compared with the separations obtained using carboxymethyl cellulose.

In attempting to obtain stable derivatives so that other features of the molecule could be studied, oxidation with preformed performic acid at 0°[7] has been investigated. This reaction proved to be quite different from oxidation experiments previously reported[6] which were carried out with hydrogen peroxide in formic acid at room temperature. Fortunately, the oxidation proceeded without causing racemization of the *N*-terminal amino acid and thus made possible separation and precise characterization of the stereoisomers of acid isomerized bacitracin A.

The oxidation product also proved to be much more suited to characterization by conversion to the dinitrophenyl derivative than was unmodified bacitracin A. This contrast has further emphasized the very labile nature of the unsubstituted peptide. Bacitracin A which was first treated with fluorodinitrobenzene and then oxidized with performic acid at 0° gave a much more complicated countercurrent distribution pattern than that obtained when oxidation was carried out before conversion to the dinitrophenyl derivative.

EXPERIMENTAL

The bacitracin used in this work was a gift of the Commercial Solvents Co. We thank them for the material and also for the antibiotic assays reported in this paper.

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

Isomerization of bacitracin A and commercial bacitracin. A 0.5% solution of bacitracin was allowed to stand in 3% acetic acid overnight at 37°. The solution was then lyophilized. The freeze dried material was used for countercurrent distribution and carboxymethyl cellulose chromatography.

Rate studies on the isomerization of bacitracin A. A 1.0% solution of "high potency"[4] bacitracin A was allowed to stand in 3% acetic acid at 37°. Aliquots were taken at various times as indicated in Fig. 3 and chromatographed on 0.9 × 150 cm. carboxymethyl cellulose (0.47 meq./gm.). The eluent was 0.55*M* sodium acetate buffer, *p*H 4.54. The analysis was carried out by the ninhydrin procedure of Moore and Stein.[8]

Chromatography of bacitracin on carboxymethyl cellulose. The procedure outlined above was used but in some cases, the method previously described[5] was more suitable.

Distribution of acid isomerized bacitracin. After isomerization in 3% acetic acid the material was lyophilized and distributed in a system of 1-butanol, 0.5*M* phosphate buffer at *p*H 5.4 in equal volumes. The effluent fractions were pooled in 10-ml. fractions as they emerged from the 1000-tube (2 ml. each phase) train. These fractions were taken and placed in the 440-tube (10 ml. each phase) train, equilibrated with lower phase, and recycled in order to obtain a further fractionation. The distribution patterns obtained are given in Fig. 1.

Distribution of oxidized bacitracin. A system made up of 1-butanol–pyridine–acetic acid–water (20:5:5:30 parts by volume) was found suitable for distributing the oxidation products of bacitracin. The *p*H of the lower phase was 4.59. Analyses were carried out by the ninhydrin method of Moore and Stein[8] and by weight. The ninhydrin result is given in Fig. 4.

Cuts as indicated in Fig. 4 were recovered by evaporation, taken up in water and freeze dried. The major band, cut 2, was submitted to elementary analysis.

Anal. Calcd. for $C_{66}H_{107}O_{21}N_{17}S$: C, 52.6; H, 7.2; N, 15.8; amide, N, 0.93. Found: C, 52.52; H, 7.06; N(KJELDAHL), 15.4; amide N, 0.98.

Both cuts 1 and 2, Fig. 4, were hydrolyzed for 24 hr. in 6*N* hydrochloric acid at 110°, and the resulting amino acids were analyzed by the method of Moore, Spackman, and Stein.[9] The results are given in Table I.

TABLE I

AMINO ACID COMPOSITION OF CUTS 1 AND 2 FROM FIG. 4 (Low and high potency bacitracin A after performic acid oxidation)

Amino Acid	Residues per Mole	
	Cut 1, Low Potency	Cut 2, High Potency
Cysteic	1.00	0.98
Asp	1.94	1.68
Glu	1.01	0.97
Allo-ileu	0.88	0.09
Ileu	1.92	2.85
Leu	0.99	1.04
Phe	0.96	0.98
Lys + orn	1.70	1.82
His	0.97	0.99
NH_3	1.11	1.08

Experiments with paper electrophoresis on each cut showed a single neutral spot with ninhydrin at *p*H 5.6 and a single acidic one at *p*H 7.3. These results are in accord with the expected charge distribution.

Tri-dinitrophenyl derivative of oxidized bacitracin. Samples, 60 mg., of the oxidized products from CCD, Fig. 4, cuts 1 and 2, were substituted with the dinitrophenyl reagent, fluoro-2,4-dinitrobenzene, under the conditions used for bacitracin.[6] The recovered product was distributed to 100 transfers in the system benzene–chloroform–glacial acetic acid–0.1*N* hydrochloric acid (2:6:6:3) in order to remove the dinitrophenol and any possible dinitroaniline. The distribution patterns are given in Fig. 5. Assuming the molar extinction of 29,000, molecular weights of 1,900 and 1,980 were calculated from optical density to weight ratios for the tri-dinitrophenyl derivatives from the major bands (cut 1).

A sample of the dinitrophenyl derivative was hydrolyzed for 22 hr. in a mixture of equal parts of glacial acetic acid and concentrated hydrochloric acid in a sealed evacuated tube. A distribution experiment[5] indicated ε-dinitrophenyl ornithine and a good yield (0.69 mole) of a band corresponding to dinitrophenyl isoleucine.

Part of the hydrolysate was used for an amino acid analysis of the neutral and acidic amino acids. The result for a 24-hr. hydrolysis time is given in Table II.

DISCUSSION

In a previous paper[5] it has been shown that "low potency" or acid isomerized bacitracin A could be fractionated on carboxymethyl cellulose and that

(7) E. Schramm, S. Moore, and E. V. Bigwood, *Biochem. J.*, **57**, 33 (1954).

(8) S. Moore and W. H. Stein, *J. Biol. Chem.*, **211**, 907 (1954).

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

TABLE II

AMINO ACID ANALYSIS OF TRI-DNP-DERIVATIVES OF OXIDIZED BACITRACIN A ISOMERS

(50:50 Acetic acid–concentrated hydrochloric acid, 110°, 24 hr.)

Amino Acid	Residues per Mole	
	Low Potency, Cut 1, Fig. 5A	High Potency, Cut 1, Fig. 5B
Cysteic	0.92	0.95
Asp	1.40	1.37
Glu	1.00	1.01
Allo-ileu	0.08	0.04
Ileu	1.85	1.83
Leu	1.00	0.99
Phe	0.97	0.95

the two forms obtained differed from each other in their D-Allo-Ileu and L-Ileu content after performic acid oxidation. The rotatory dispersion curves of the two bands curved in opposite directions. Several possibilities for the difference in structure and mechanism of formation of these two isomers were proposed.

In order to decide which possibility was correct, greater quantities of each isomer were needed for further study. This led us to attempt countercurrent distribution fractionation of acid isomerized commercial bacitracin using a 1-butanol, phosphate buffer system.

After 1292 transfers the distribution pattern indicated the usual broad trailing edge (Fig. 1A). Rotatory dispersion curves from material on the right and left sides of the main band were found to be different. The run was, therefore, continued to 2300 transfers but by this time nearly all the solute of interest had emerged from the train as effluent. Fig. 1B is the effluent pattern. Further separation of the overlapping bands, 1550 to 1850, was undertaken. The distribution up to this point had been done in a 1000-tube train with 2/2 ml. phase volumes and collection of the effluent in 10 ml. portions (5 transfers combined). The emerging fractions were, therefore, transferred in their proper sequence to a 420-tube train of 10/10 ml. volumes and the required lower phases added. After applying 1327 additional transfers, the pattern in Fig. 1C was obtained. The additional number of transfers in 1C over 1B apparently did not improve the separation although the optical rotatory dispersions on material from each side of the band were again quite different. Analytical CMC chromatograms run on cuts 1, 2, and 3 of the band after desalting showed marked differences. The results in Fig. 2 indicate that only partial separation was achieved.

The antibiotic activities on each cut from Fig. 1C were determined. Cut 1 had a specific activity of 41 units per mg., cut 2 gave 59 units/mg. and cut 3 gave a response of 70 units/mg. These activities are in good agreement with the relative amounts of "low" and "high" potency material in each cut as estimated from the carbomethoxy cellulose patterns.

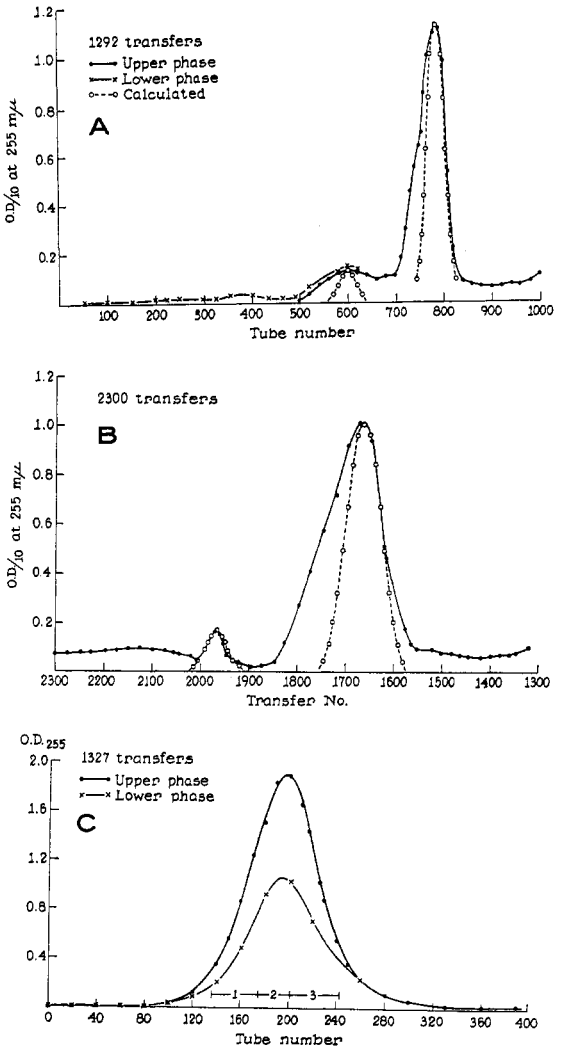


Fig. 1. Countercurrent distribution patterns of acid isomerized bacitracin: 1A at 1292 transfers; 1B effluent pattern after 2300 transfers; 1C after 1327 additional transfers

Failure to obtain complete separation of the two forms is probably due to the interconversion of the two isomers which occurs even at *p*H 5.45 although at a much slower rate than in acid solutions. This was demonstrated when "high potency" bacitracin A was allowed to stand for four days at 37° in the lower phase of the 0.5*M*, *p*H 5.4, phosphate system. After desalting and chromatographing on carbomethoxy cellulose, a pattern was obtained which clearly indicated that interconversion had occurred. As, because of a mechanical failure of the robot, the time required for the entire countercurrent distribution of Figs. 1A, 1B, and 1C was over two weeks, it was not surprising that we failed to get either isomer in an entirely pure form.

To gain further insight into the exact nature of the transformation, it seemed worth while to study the rate of isomerization and the amount of each isomer present at the end of the reaction. By chromatographing aliquots of an isomerization reaction

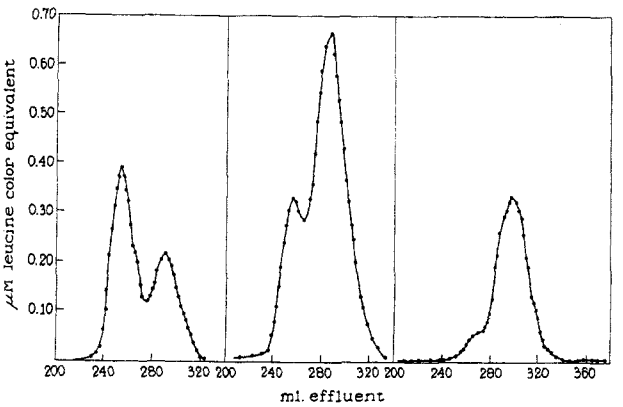


Fig. 2. Analytical carbomethoxycellulose chromatograms run on the cuts from Fig. 1C, from left to right , 2, and 3, respectively

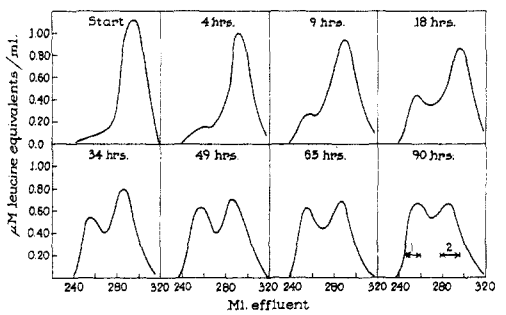


Fig. 3. Carbomethoxy cellulose chromatograms of bacitracin A after different times of acid exposure

mixture of bacitracin A in 3% acetic acid on carbomethoxy cellulose, patterns as shown in Fig. 3 were obtained. The amount of the "low potency" form was nearly equal to the "high potency" form after two days. When the amount of isomer in each band was estimated using the method for overlapping curves[10] and plotted against time on a semilog scale, a straight line was obtained indicating first-order kinetics. This is consistent with a simple racemization process.

The activities of cuts 1 and cut 2 obtained from the final reaction mixture, Fig. 3, lower right, were 23 and 79 units/mg., respectively. The amino acid analyses of cut 1 and cut 2 after performic acid oxidation were essentially the same as those given in Table I and indicate that the "low potency" form has D-Allo-Ileu as the predominant end group while the "high potency" bacitracin A has Ileu in the L configuration.

The reversibility of the process was demonstrated by exposing the separated low and high potency forms to 3% acetic acid and rerunning the reaction mixtures on carboxymethyl cellulose. The curves showed that the transformation can proceed in either direction probably with an approach to true equilibrium.

The activities of the low and high potency forms

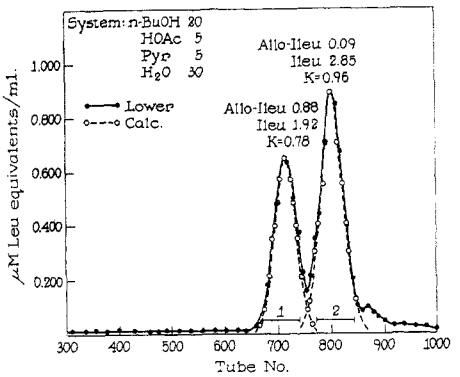


Fig. 4. Countercurrent distribution pattern of acid isomerized oxidized bacitracin A after 1629 transfers

from Fig. 3 were measured after re-isomerization and found to be 39 and 54 units/mg., respectively. Thus the low potency material had regained a fair percentage of its activity.

One of the disadvantages of working with the intact bacitracin was that complete separation of each isomer could not be achieved. A degree of overlapping always occurred even on carboxymethyl cellulose under the most favorable conditions. For this reason it was desirable to find a derivative that could be formed without destroying the optical activity of the *N*-terminal Ileu residue and that could be fractionated without undergoing interconversion. Of particular interest in this connection was an observation that the oxidation of the low and high potency forms of bacitracin with performic acid[7] gave products which differed in their D-Allo and L-Ileu contents, thus indicating that the oxidation proceeded with retention of configuration.

It was then found that the A component from commercial bacitracin, partially isomerized by 3% acetic acid and subsequently oxidized with performic acid, could be cleanly resolved by countercurrent distribution into several bands in an *n*-butyl alcohol–pyridine–acetic acid–water system as shown in Fig. 4. A small band on the left because of ammonia and perhaps F components is not shown. The material from cuts 1 and 2 was then recovered and amino acid analyses made. The results (Table I) showed the cysteine to be almost completely oxidized to cysteic acid in both cases. The only difference found was in the isoleucine content. Cut 1 showed 0.88 residue of allo-isoleucine and 1.92 residues of isoleucine; Cut 2 showed 0.09 and 2.85 of these residues, respectively. All the other residues were found in the expected amount. This indicated that the only effect of the oxidation was to split the thiazoline ring to give the corresponding cysteic acid residue which would occur between the terminal isoleucine and the leucine.

Dinitrophenylation of the oxidized isomers now proceeded smoothly under the usual basic conditions in contrast to bacitracin A.[11] The tri-dinitro-

(10) W. H. Stein and S. Moore, *J. Biol. Chem.*, **176**, 337 (1948).

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

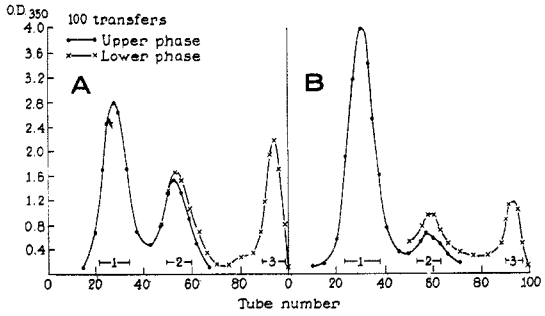


Fig. 5. Countercurrent distribution patterns of the dinitrophenylfluorobenzene reaction products with the isomers of oxidized bacitracin A. A = allo isomer; B = L isomer

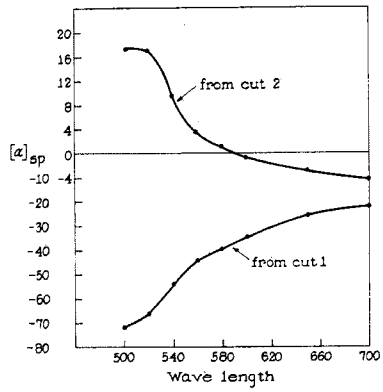


Fig. 6. Rotatory dispersion curves of dinitrophenyl derivatives from cuts 1 and 2 of Fig. 4

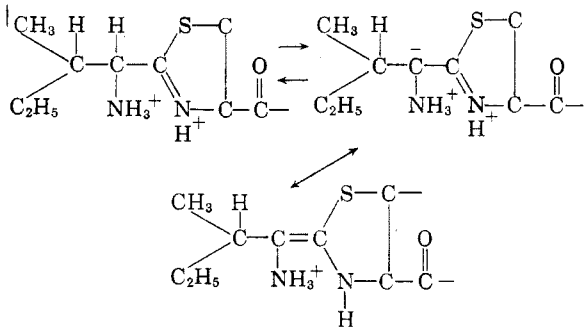
phenyl derivative of each isomer was easily purified by countercurrent distribution (Fig. 5) and the amino acid analysis shown in Table II definitely confirmed the fact that in the active antibiotic isomerization is concerned with the *N*-terminal isoleucine rather than one of the other isoleucines present in the chain as the D-Allo isoleucine has disappeared completely and the isoleucine content of both oxidized isomers was nearly equal.

As a final proof of retention of the configuration of the amino acid residues during the oxidation and dinitrophenylation, the optical rotatory dispersions of the tri-dinitrophenyl derivatives were measured and are shown in Fig. 6. The curves have opposite slopes because the specific rotation of the terminal isoleucine is greatly enhanced as expected by placing a chromophoric group adjacent to an optical center, and this effect overrides the net rotational contributions of the other residues.

That carboxymethyl cellulose chromatography like countercurrent distribution would not completely resolve the unoxidized antibiotically active epimers was shown by taking cuts from a preparative column under optimal conditions, separately oxidizing them with performic acid, and subjecting them to countercurrent distribution. From 10 to 20% of the opposite isomer in both cases was thus revealed as a contaminant.

The oxidation results along with the kinetic data enable us to rule out the *cis-trans* isomerism earlier considered.[5] If *cis-trans* isomerism had been involved, then oxidation of the exocyclic form required for *cis-trans* isomerism would give rise to approximately equal amounts of the D-Allo and L forms of the sulfonic acid derivatives.

In the most likely mechanism, lowering the *p*H below *p*H 2.5 permits protonation of the thiazoline nitrogen which facilitates the shift of the double bond and removal of a proton from the alpha carbon by a transfer of electrons as shown in Formula 2.



The ionization of a proton from the *alpha* isoleucine carbon thus opens a pathway for epimerization since stereospecific readdition of a proton would not be expected.

The amount of the ionized form would certainly be increased by the possibility of two resonance forms which would also make a significant contribution to the stability of the transient intermediate in acid solution.

The transformation, we believe, is unique in the chemistry of the polypeptide antibiotics in the sense that it is able to bring about, in a controlled fashion, inversion at one asymmetric center in a molecule of 1430 molecular weight, thereby causing a dramatic decrease in biological activity. This relatively minor modification of the bacitracin molecule should not affect its ability to permeate membranes. It demonstrates how delicate a type of structural specificity can be required for activity. Studies are under way now in this laboratory to determine how other chemical modifications will affect the antibiotic activity of bacitracin.

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