TABLE IV

MOLAR ROTATIONS OF THE L-ANTIPODES OF AMINOTRICARBALLYLIC ACID, ISOCITRIC ACID AND RELATED DERIVATIVES

L-Isomers	$[M]_D$[a] (degrees)
Aminotricarballylic acid	−62.7,[b] −91.8,[c] +9.6[d]
Alloaminotricarballylic acid	+14.3,[b] +69.6,[c] +146.1[d]
Pyrrolidonedicarboxylic acid	− 94.6[b]
Allopyrrolidonedicarboxylic acid	+100.3[b]
Isocitric acid	+ 58.8[b,e]
Alloisocitric acid	+ 66.4[b,e]
Isocitric acid lactone	−105.7[b]
Alloisocitric acid lactone	+ 70.9[b]
Trisodium isocitrate	0[b,f]
Trisodium alloisocitrate	+ 73.7[b,f]

[a] All concentrations at 0.5–1.0%. [b] H_2O solution. [c] 5 *N* HCl solution. [d] 1 *N* NaOH solution. [e] Trisodium salt neutralized with 3 equivalents of HCl. [f] Calculated on basis of free acid.

Since the configuration of each of the alloisocitric acid isomers has already been demonstrated, the proper designation of the L,L-allo diastereomeric pair must reside in either the *l,d*-allo or the *d,d*-allo combination. Examination of the $\alpha_{H_2O} - \alpha_{salt}$ values for each of these pairs, in Table III, points to the latter combination as representative of the L,L-allo pair. Such demonstration of the L_S-configuration for *d*-isocitric acid (the natural form) permits, in turn, an assignment of configuration to the corresponding aminotricarballylic acid and related derivatives (Table IV).[32]

(32) Since isocitric acid has two asymmetric centers, sole employment of the L-designation could lead to ambiguity with regard to the asymmetric center to which such assignment refers. Employment of the Ls-designation, however, restricts such assignment to the α-asymmetric carbon atom as is conventionally employed in the amino acid series (*cf.*, H. B. Vickery[4]).

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[CONTRIBUTION FROM THE ROCKEFELLER INSTITUTE FOR MEDICAL RESEARCH]

Bacitracin A. Further Studies on the Composition

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RECEIVED JULY 15, 1954

Evidence for the presence of three isoleucine residues in bacitracin A has been derived from partial hydrolysis studies and other experimental observations. On the basis of these observations a new empirical formula $C_{66}H_{103}O_{16}N_{17}S$ has been suggested for bacitracin A which is consistent with all the experimental observations.

Bacitracin A has been studied carefully from the standpoint of its quantitative amino acid composition[1] by hydrolysis in hydrochloric acid and estimation of the amino acids by the ion-exchange chromatographic method of Moore and Stein. From the results it was evident that all the residues had not been quantitatively liberated. Even assuming a certain amount of transformation, the eleven amino acid residues shown to be present could not be joined with loss of water to give an empirical formula for a peptide which would be consistent with the over-all analytical results obtained on the intact peptide. An unstable residue was accordingly postulated. Definite information on this point has now been derived from several different types of experiment.

The first indication that a slight revision of the postulated empirical formula would be required came from partial hydrolysis studies.[2] Isoleucine residues were repeatedly found joined in three different sequences in significant amount. The three sequences were isoleucine·cysteine, isoleucine·phenylalanine and isoleucine·lysine (joined C to N as written). This prompted a reconsideration of the thesis of purity of the preparation and also of the quantitative amino acid composition. The latter seemed the most likely to be at fault.

Re-examination of the effluent pattern from the chromatographic column already published[1] showed a small discrete band emerging just before the larger band of isoleucine. The possibility that this could arise from methionine as indicated from its position on the effluent pattern from the column, had already been ruled out.[1] It then occurred to us that earlier amino acid analyses made with the starch column[3] had shown 2.45 moles of isoleucine for each leucine residue but no band in the position corresponding to methionine. Moreover, the isoleucine isolated in the earlier work[4] was partially racemic.

Upon discussing these results with Drs. Moore and Stein it was found that the starch column does not separate isoleucine and allo-isoleucine whereas with the ion-exchange column the latter forms a discrete band emerging immediately before the former.[5] Assuming the heretofore unidentified band to be from alloisoleucine arising by racemization of l-isoleucine at the α-carbon its size would indicate 0.5 mole of alloisoleucine for each mole of leucine, phenylalanine, etc., to be present. When this is added to the 1.83 moles of isoleucine in the adjoining peak a total of 2.33 moles is obtained, a value in good agreement with the result with starch.

The experience of Harfenist[6] in the hydrolysis of insulin and of others[7-9] with several proteins has definitely shown that the analytical result with isoleucine in a 24-hr. hydrolysate is likely to be low due

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

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(5) K. A. Piez, *J. Biol. Chem.*, **207**, 77 (1954).

(6) E. J. Harfenist, THIS JOURNAL, **75**, 5528 (1953).

(7) E. L. Smith and A. Stockell, *J. Biol. Chem.*, **207**, 501 (1954).

(8) E. L. Smith, A. Stockell and J. R. Kimmel, *ibid.*, **207**, 551 (1954).

(9) C. H. W. Hirs, W. H. Stein and S. Moore, *ibid.*, in press.

to the difficulty of completely hydrolyzing isoleucine peptides. In fact the isoleucine–phenylalanine peptide first reported from bacitracin[3] was isolated during an attempt to isolate in preparative amount the amino acids following what was thought to be complete hydrolysis. The partial hydrolysis experiments[2,10] further show an isoleucine·lysine sequence difficult to hydrolyze. The quantitative studies mentioned[1,3] indicate that 2.3–2.4 moles of isoleucine would be the amount expected when 3-isoleucine residues are present in the intact peptide.

The difficulty of conclusively settling this problem by studying different hydrolysis times as was done with insulin[6] can be appreciated from Table I. The 18- and 42-hour hydrolysates were studied before the ion-exchange column had been developed. The data indicate destruction of certain of the residues, isoleucine included, by longer heating times. The cause of this is not known.

TABLE I

AMINO ACID ANALYSES EXPRESSED AS G. RESIDUES PER 100 G. PEPTIDE

	Ion-exchange chromatography 24-hr. hydrol.	Starch chromatography[a] 18-hr. hydrol.	Starch chromatography[a] 42-hr. hydrol.
Phenylalanine	10.8	9.1	8.82
Leucine	8.2	7.66	6.88
Isoleucine + alloisoleucine	14.6	18.13	15.62
Cysteine	7.4		
Glutamic acid	8.1	8.24	8.96
Aspartic acid	14.7	13.92	13.66
Histidine	7.7	8.35	7.07
Lysine	8.1	7.78	6.84
Ornithine	6.5		
NH_3	1.3	1.25	1.97

[a] We are greatly indebted to Dr. S. Moore and Dr. W. H. Stein for the results with starch.

Further evidence in regard to the origin of the alloisoleucine band comes from studies on the nature of bacitracin F,[11] a transformation product of bacitracin A, and from a study of the desulfurization of bacitracin A with Raney nickel.

Quantitative amino acid analysis of bacitracin F by ion-exchange chromatography following total hydrolysis in acid gave a pattern similar to that of bacitracin A except that the alloisoleucine and cystine (cysteine) bands were completely missing. Bacitracin F contains about the same percentage of sulfur as does bacitracin A. However, the sulfur does not emerge as cysteine during hydrolysis as in the case of bacitracin A. Instead a sublimable crystalline fragment of molecular weight approximating 210 can be isolated. The exact nature of this fragment has not been settled as yet but it contains one sulfur and only one nitrogen and shows the characteristic absorption spectrum of bacitracin F.[1] It probably is a thiazole derivative with 9 carbon atoms.

Following treatment of bacitracin A with Raney nickel in boiling alcohol, material was extracted which gave a discrete band in a countercurrent distribution of 600 transfers. This material was free of sulfur. Again quantitative amino acid analysis by ion-exchange chromatography indicated the presence of the amino acids except cysteine and alloisoleucine. Only a trace of a band in the position of alloisoleucine was obtained. This work will be treated in a separate paper. It is mentioned here because of the support it gives to the thesis of the presence of three isoleucine residues.

If three residues of isoleucine are present in bacitracin A as the data now quite definitely indicate a revision of the empirical formula proposed[1] becomes necessary. The amino acid formula phenylalanine·leucine·(isoleucine)$_3$·cysteine·glutamic acid·(aspartic acid)$_2$·histidine·lysine·ornithine·(amide), gives an empirical formula $C_{66}H_{103}O_{16}N_{17}S$ if two additional molecules of water are removed to form 2 rings.

Anal. Calcd. for $C_{66}H_{103}O_{16}N_{17}S$: C, 55.65; H, 7.3; N, 16.7; S, 2.25. Found: C, 55.2; H, 7.3; N, 15.8; S, 2.2.

The standard preparation of bacitracin A is a residue obtained by freeze-drying a solution of the material which is taken from the countercurrent distribution machine. The solution contains acetic acid. The white residue gives a solution when dissolved in water with a *p*H approximating 4. Since the isoelectric point of bacitracin A is 6.8 the white residue obviously contains acetic acid in appreciable amount. Material dried at 100° *in vacuo* still gave a solution with an acid *p*H (4.78) and required for each 1500 mg. approximately 1.2 mmoles of NaOH to adjust to 6.8. The exact value was difficult to determine because of precipitation.

In order to determine the amount of acetic acid retained after drying at 100° for analysis such a dried sample was placed in the apparatus for determination of C-methyl. In this apparatus the acetic acid was quantitatively removed by distillation and the distillate was titrated. In order to eliminate the possibility of acetyl formation during drying the dried sample was subjected to hydrolysis before distillation. This gave no increase in the acetic acid titer; 77.7 mg. of sample required 5.14 ml. of 0.01 *N* sodium thiosulfate; calculated for 1 mole of acetic acid, 5.24 ml.

Addition of 1 molecule of acetic acid to the empirical formula given above gives an over-all empirical formula of $C_{68}H_{107}O_{18}N_{17}S$.

Anal. Calcd. for $C_{68}H_{107}O_{18}N_{17}S$: C, 55.04; H, 7.27; N, 16.05; S, 2.16. Found: C, 55.2; H, 7.3; N, 15.8; S, 2.2.

We are indebted to Mr. D. Rigakos for the analytical results in this paper.

NEW YORK, N. Y.

(10) J. R. Weisiger, W. Hausmann and L. C. Craig, THIS JOURNAL, **77**, 731 (1955).

(11) J. R. Weisiger, W. Hausmann and L. C. Craig, paper in preparation.