+3° and at 25 hours it was about +1°. In order to know more precisely how far the B amino acid had been racemized the solution after refluxing for 25 hours was chilled and treated with concd. HCl to 5 *N* in respect to the acid and 0.4% in respect to the amino acid. The $[\alpha]^{25}D$ of the filtered solution was −31.2°, compared with the $[\alpha]^{25}D$ of *l* aminotricarballylic acid (B) in 5 *N* HCl of −48.7° (Table I), representing a decrease of about 36% in the optical rotation of the original solution. Both A and B amino acids were racemized to about the same extent by alkali in the same time interval, and the data on the B amino acid clearly demonstrate that both α and β centers in this compound were racemized at close to the same rate. Thus, if racemization at the β carbon atom of the B amino acid had not occurred, or at least had been much slower than that at the α carbon atom, there would have been a marked negative or positive shift in rotation in alkali depending on whether the *l*- or *d*-isomer of the A diastereomer with its high magnitude of rotation was being formed during the reaction.

N-Acyl Derivatives of Racemic α-Aminotricarballylic Acid (A and B).—The chloroacetyl derivatives were prepared by the interaction of chloroacetic anhydride with the amino acids in alkaline solution, and were purified by crystallization from acetone–petroleum ether. The yields were 55–60% of the theoretical. When chloroacetyl chloride was used in place of the anhydride the yields were no better than 15%; m.p. of the A derivative 180°, that of the B derivative 143°.

Anal. Calcd. for $C_8H_{10}O_7NCl$: N, 5.2; Cl, 1.23. Found (for the A): N, 5.2; Cl, 13.1. Found (for the B): N, 5.2; Cl, 12.9.

The acetyl derivative of the racemic A amino acid was obtained in crystalline form (40% of theory) by the interaction of acetic anhydride with the amino acid. The compound was crystallized from acetone–petroleum ether; m.p. 178°. The corresponding B derivative could not be obtained in crystalline form.

Anal. Calcd. for $C_8H_{11}O_7N$: C, 41.3; H, 4.8; N, 6.0. Found: C, 41.4; H, 5.0; N, 6.3.

The carbobenzoxy derivatives were prepared in the usual way, and were recrystallized from ethyl acetate and petroleum ether; m.p. of the racemic A derivative 178°, that of the corresponding B derivative 134°.

Anal. Calcd. for $C_{14}H_{15}O_8N$: C, 51.7; H, 4.6; N, 4.3; Found (for A): C, 51.8; H, 4.7; N, 4.3. Found (for B): C, 52.0; H, 4.9; N, 4.5.

Triamides of N-Acetylaminotricarballylic Acids (A and B).—The crystalline B form and sirupy A form of racemic N-acetylaminotricarballylic acid triethyl ester were treated with methanolic NH_3. The crystals of the triamide which formed were washed with methanol and water; m.p. of the A form was 259°, that of the B form 256°.

Anal. Calcd. for $C_8H_{14}O_4N_4$: C, 41.7; H, 6.1; N, 24.4. Found (for the A): C, 41.4; H, 6.3; N, 24.1. Found (for the B): C, 41.4; H, 6.1; N, 24.1.

BETHESDA, MARYLAND

[CONTRIBUTION FROM THE LABORATORY OF BIOCHEMISTRY, NATIONAL CANCER INSTITUTE, NATIONAL INSTITUTES OF HEALTH]

Studies on Diastereoisomeric α-Amino Acids and Corresponding α-Hydroxy Acids. II. Contribution of the Asymmetric α- and β-Carbon Atoms to the Molar Rotations of the Diastereomeric α-Aminotricarballylic Acids and Corresponding Isocitric Acids

BY MILTON WINITZ, SANFORD M. BIRNBAUM AND JESSE P. GREENSTEIN

RECEIVED AUGUST 26, 1954

The contribution (partial molar rotation) of the α- and ω-asymmetric centers to the observed molar rotations of several diasymmetric amino acids in water, 5 *N* hydrochloric acid and glacial acetic acid was calculated. These calculations demonstrated that the negative shift in optical rotation exhibited by the addition of acid to an aqueous solution of *threo*-L-β-phenylserine, which was contrary to the empirical rule of Lutz and Jirgensons, was attributable to the very marked negative shift of the partial rotation of its ω-asymmetric carbon atom. This served to completely mask the lesser shift of the partial rotation of the α-asymmetric center in the positive direction. Calculation of the partial molar rotation of the α- and ω-asymmetric carbon atoms of four other diasymmetric L-amino acids, as well as their L-allo isomers, showed a similar positive water-to-acid shift in the partial rotations of their α-asymmetric centers. The specific application of this rule to the partial rotation of the α-asymmetric center served to clarify it with respect to α-amino acids with more than one center of asymmetry. On the basis of structural considerations, the rule was also clarified with regard to L-isovaline (which shows a negative water to HCl shift in optical rotation) to include only amino acids bearing a hydrogen atom on the α-asymmetric center. Since this modified rule shows no known exceptions for some fifty amino acids tested, the partial molar rotation data in water and hydrochloric acid was used for the assignment of configurations to the four optical isomers of α-aminotricarballylic acid, as well as to the four corresponding isocitric acids.

The first paper of this series[1] included the preparation of the four optically active isomeric isocitric acids which were obtained by deamination of the corresponding α-aminotricarballylic acids with nitrous acid.[2] The purpose of this work was twofold, first, to test the biological susceptibility of the four optical isomers of isocitric acid to the isocitric dehydrogenase–triphosphopyridine nucleotide system,[3] and secondly, to ascertain the configuration of natural isocitric acid by relating it to the precursor amino acid whose configuration could be determined. The first objective has been achieved[1] while the second forms the basis of the present communication. The confusion which exists with regard to the configuration of natural isocitric acid has been the subject of a recent report by Vickery.[4]

Inasmuch as the shift in the direction of optical rotation of α-amino acids in water and in acid was found to be the same for amino acids of the same configuration, the determination of the rotation of an amino acid in these two media affords a convenient physical method for determining their configuration. This effect of acid on the specific rotation of amino acids was first observed by Pasteur[5] in 1851, and was later extended by other workers.[6] In 1915, Clough[7] suggested that amino

(1) J. P. Greenstein, N. Izumiya, M. Winitz and S. M. Birnbaum, THIS JOURNAL, **77**, 707 (1955).

(2) J. P. Greenstein, *J. Biol. Chem.*, **109**, 529 (1935); **116**, 463 (1936).

(3) A. Adler, H. von Euler, G. Günther and M. Plass, *Biochem. J.*, **33**, 1028 (1939).

(4) H. B. Vickery, *Science*, **113**, 314 (1951).

(5) L. Pasteur, *Ann. chim. phys.*, **31**, 81 (1851).

(6) E. P. Cook, *Ber.*, **30**, 294 (1897); J. K. Wood, *J. Chem. Soc.*, **105**, 1988 (1914).

(7) G. W. Clough, *ibid.*, **107**, 1509 (1915).

acids which showed parallel shifts in optical rotation on the addition of acid to their aqueous solutions were configurationally related. Additional measurements on a large variety of naturally occurring amino acids, effected later by Lutz and Jirgensons,[8] established that the exhibition of a more positive optical rotation value in acid than in water solutions appeared to be a general characteristic of certain L-amino acids derived from proteins. Comparable regularities have been observed with the configurationally related α-hydroxy acids.[8-11]

Although the change in the direction of rotation from water to acid is consistent for most of them, recent tabulation[12,13] of the molecular rotations of some fifty L-amino acids revealed at least two apparent exceptions to the empirical rule of Lutz and Jirgensons,[8] notably, isovaline[14] and the *threo*-isomer of β-phenylserine.[12] The L-forms of these "anomalous" amino acids, whose configurations were determined unequivocally by enzymatic criteria, become more levorotatory on the addition of acid to their aqueous solutions. This would presume that such rules for the determination of configuration are, at best, of only limited validity in their present form. However, clarification of these exceptions, in what follows, will not only tend to define the limits within which these empirical rules are reasonably valid, but will also tend to make more reliable the configurational assignments to the antipodes of α-aminotricarballylic acid,[1] discussed later, which are based on such phenomena.

Interpretation of Optical Rotation Data and Discussion

Partial Molar Rotations in Diasymmetric Amino Acids.—One of the classical studies of C. S. Hudson[15] was concerned with the testing of the validity of the van't Hoff theory of optical superposition in the sugar group. Hudson formulated rules (rules of isorotation) to show that the difference in the rotation values between the isomeric sugars, which differ only in their configuration about a given asymmetric center, is a constant. Although they did not have a rigid general application,[16] these modified rules of optical superposition were found to hold remarkably well for many closely related structures. No similar studies have heretofore been effected with amino acids which contain more than a single asymmetric center.

The rotation of the diastereomeric amino acids with two optical centers of asymmetry, α-, and ω-, may be considered to be a function of the sum of the contributions (partial rotations) of each center. The α-asymmetric center of an amino acid of the L-configuration will make the same contribution to the total molar rotation as its L-allo form, but the contribution of the ω-asymmetric center will be of equal and opposite magnitude for the two L-diastereomers. The partial molar rotation of the asymmetric α-carbon atom may therefore be calculated by halving the algebraic sum of the molar rotations of the L- and L-allo forms

$$\alpha_L = \alpha_{L\text{-allo}} = 0.5([M]_L + [M]_{L\text{-allo}})$$

In a like manner, one-half of the algebraic sum of the molar rotations of the L- and D-allo forms will yield the partial molar rotation of the asymmetric ω-carbon atom

$$\omega_L = -\omega_{L\text{-allo}} = 0.5([M]_L + [M]_{D\text{-allo}})$$

The sum of $\alpha_L + \omega_L$ or $\alpha_L + \omega_{L\text{-allo}}$ will therefore be equal to the molar rotation of the L- or the L-allo form, respectively.

If, on the basis of these calculations, the α and ω values were determined in both water and hydrochloric acid, then $\alpha_{HCl} - \alpha_{H_2O}$ and $\omega_{HCl} - \omega_{H_2O}$ would be equal to the net difference of the H_2O-to-HCl shift in rotation for the α- and ω-centers, respectively. The sum of $(\alpha_{HCl} - \alpha_{H_2O}) + (\omega_{HCl} - \omega_{H_2O})$ would therefore represent the observed shift in rotation from water to acid. For amino acids with only a single asymmetric center, this shift would obviously be dependent solely upon the $\alpha_{HCl} - \alpha_{H_2O}$ value.

The β-Phenylserine "Exception."—Table I presents calculations for the partial molar rotation values, α and ω, of the L- and L-allo forms of five pairs of diastereomeric amino acids in both water and 5 *N* HCl. With the exception of the stereoisomeric hydroxyprolines, these amino acids were obtained *via* the action of stereospecific enzymes on the pure, suitably substituted DL- and DL-allo forms or on the DL-epimers which contained an ω-carbon atom of the same configuration.[13] Through the use of essentially the same procedure developed by Neuberger,[17] the L-allo stereoisomer was obtained from natural hydroxy-L-proline by C_4 inversion while the D-isomers were obtained by racemization of this same compound, followed by fractional crystallization and C_4 inversion.[18] Routine purity determinations with amino acid oxidases and decarboxylases, developed in this Laboratory,[19] demonstrated that the optical rotation values[12,13] on which these calculations are based were determined with amino acids in which the level of possible contamination of one isomer by the other was less than 1 part in 1000.

Examination of Table I indicates that the net positive shift in rotation, $(\alpha_{HCl} - \alpha_{H_2O}) + (\omega_{HCl} - \omega_{H_2O})$, obtains for all of the L-amino acids tested with the exception of L-β-phenylserine. These data would seem to reveal a very apparent lack of conformity between the configuration of β-phenyl-

(8) O. Lutz and B. Jirgensons, *Ber.*, **63**, 448 (1930); **64**, 1221 (1931); **65**, 784 (1932).

(9) G. W. Clough, *J. Chem. Soc.*, **113**, 526 (1918).

(10) All known L-α-hydroxy acids become more dextrorotatory on the addition of acid to their sodium salts. This phenomenon, in both α-amino and α-hydroxy acids, appears to be associated with the ionization of the carboxyl group.

(11) P. A. Levene, G. Mori and L. A. Mikeska, *J. Biol. Chem.*, **75**, 348 (1927).

(12) J. P. Greenstein, S. M. Birnbaum and M. C. Otey, *ibid.*, **204**, 307 (1953).

(13) J. P. Greenstein, *Advances in Protein Chem.*, **9**, 121 (1954).

(14) C. G. Baker, S-C. J. Fu, S. M. Birnbaum, H. A. Sober and J. P. Greenstein, THIS JOURNAL, **74**, 4701 (1952).

(15) C. S. Hudson, *ibid.*, **31**, 66 (1909).

(16) K. Freudenberg and W. Kuhn, *Ber.*, **64**, 703 (1931).

(17) A. Neuberger, *J. Chem. Soc.*, 429 (1945).

(18) D. S. Robinson and J. P. Greenstein, *J. Biol. Chem.*, **195**, 383 (1952).

(19) A. Meister, L. Levintow, R. Kingsley and J. P. Greenstein, *ibid.*, **192**, 535 (1951).

TABLE I

CONTRIBUTION OF ASYMMETRIC α- AND ω-CARBON ATOMS TO THE MOLAR ROTATION OF DIASTEREOISOMERIC AMINO ACIDS

L-Amino acid	$[M]^{25}D$ In H_2O[a]	$[M]^{25}D$ In 5 N HCl[a]	Molar rotation of α in H_2O	α in 5 N HCl	$\alpha_{HCl} - \alpha_{H_2O}$	ω in H_2O	ω in 5 N HCl	$\omega_{HCl} - \omega_{H_2O}$	$(\alpha_{HCl} - \alpha_{H_2O}) + (\omega_{HCl} - \omega_{H_2O})$
Isoleucine	+16.3	+ 51.8	+18.6	+51.9	+33.3	− 2.3	− 0.1	+ 2.2	+ 35.5
Alloisoleucine	+20.8	+ 51.9	+18.6	+51.9	+33.3	+ 2.3	+ 0.1	− 2.2	+ 31.1
Threonine	−33.9	− 17.9	−11.0	+10.0	+21.0	−22.9	− 27.9	− 5.0	+ 16.0
Allothreonine	+11.9	+ 37.8	−11.0	+10.0	+21.0	+22.9	+ 27.9	+ 5.0	+ 26.0
γ-Hydroxyproline	−99.6	− 66.2	−88.8	−45.5	+43.3	−10.8	− 20.8	−10.0	+ 33.3
γ-Allohydroxyproline	−78.0	− 24.7	−88.8	−45.5	+43.3	+10.8	+ 20.8	+10.0	+ 53.3
β-Phenylserine	−60.0	− 91.1	−22.6	+28.1	+50.7	−37.5	−119.2	−81.7	− 31.0
β-Allophenylserine	+14.9	+147.3	−22.6	+28.1	+50.7	+37.5	+119.2	+81.7	+132.4
δ-Hydroxylysine	+14.9[b]	+ 28.9[b]	+16.3	+39.9	+23.6	− 1.4	− 11.0	− 9.6	+ 14.0
δ-Allohydroxylysine	+17.7[b]	+ 50.9[b]	+16.3	+39.9	+23.6	+ 1.4	+ 11.0	+ 9.6	+ 33.2

[a] All rotations performed in a photoelectric polarimeter using a 2-dcm tube. All concentrations at approx. 1.0%. [b] Calculated as free base.

TABLE II

CONTRIBUTION OF ASYMMETRIC α- AND ω-CARBON ATOMS TO THE MOLAR ROTATIONS OF DIASTEREOMERIC AMINO ACIDS

L-Amino acid	$[M]^{25}D$ In H_2O[a]	$[M]^{25}D$ In HOAc[a]	Molar rotation of α in H_2O	α in HOAc	$\alpha_{HOAc} - \alpha_{H_2O}$	ω in H_2O	ω in HOAc	$\omega_{HOAc} - \omega_{H_2O}$	$(\alpha_{HOAc} - \alpha_{H_2O}) + (\omega_{HOAc} - \omega_{H_2O})$
Isoleucine	+16.3	+ 64.2	+18.6	+60.0	+41.4	− 2.3	+ 4.3	+ 6.6	+48.0
Alloisoleucine	+20.8	+ 55.7	+18.6	+60.0	+41.4	+ 2.3	− 4.3	− 6.6	+34.8
Threonine	−33.9	− 35.7	−11.0	+ 4.8	+15.8	−22.9	− 40.5	−17.6	− 1.8
Allothreonine	+11.9	+ 45.3	−11.0	+ 4.8	+15.8	+22.9	+ 40.5	+17.6	+33.4
Hydroxyproline	−99.6	−100.9	−88.8	−70.1	+18.7	−10.8	− 30.8	−20.0	− 1.3
Allohydroxyproline	−78.0	− 39.3	−88.8	−70.1	+18.7	+10.8	+ 30.8	+20.0	+38.7
Phenylserine	−60.0	− 92.6	−22.6	+ 8.1	+30.7	−37.5	−100.7	−63.2	−32.5
Allophenylserine	+14.9	+108.7	−22.6	+ 8.1	+30.7	+37.5	+100.7	+63.2	+93.9

[a] Rotations performed in 2-dcm. tube, solutions at 0.25 to 2.0%.

serine, determined enzymatically,[20] on the one hand, and as interpreted from the effect of ionization on molecular rotation,[12] on the other. Further examination of Table I reveals, however, that with the exception of L-phenylserine and its L-alloisomer, the shift in the partial molar rotation of the α-asymmetric center from water to acid is of greater magnitude than that encountered with the ω-asymmetric center. Thus, although the partial molar rotation of the ω-asymmetric center of these amino acids contributes to the magnitude of the rotation in any given direction, it nonetheless is not sufficiently large to determine the direction of rotation. The seemingly anomalous behavior of β-phenylserine becomes readily explicable on the basis of the very large negative H_2O-to-HCl shift in the partial molar rotation value of its ω-carbon atom, which serves to completely mask the lesser contribution of the α-asymmetric center in the positive direction.

The concordance of β-allophenylserine with the empirical rule of Lutz and Jirgensons[8] is completely understandable, since the H_2O-to-HCl shift of the partial molar rotation of the ω-asymmetric carbon atom is in the same direction as that of the asymmetric α-carbon atom. In fact, it becomes almost a corollary that for an amino acid with two centers of asymmetry, either one or both of its diastereomeric L-forms can be expected to be more dextrorotatory in acid than in water.

Since Table I presents molar rotation data for the L- and L-allo forms of four diastereomeric pairs of amino acids which contain a hydroxyl group on the ω-asymmetric carbon atom, it is of interest to briefly examine their relative behavior in water and acid. With the L-forms of all of these diastereomeric pairs, the H_2O-to-HCl shift in the partial rotation of the ω-carbon, *i.e.*, $\omega_{HCl} - \omega_{H_2O}$, is negative, whereas the L-allo forms assume a positive shift. The ω-asymmetric center of three of these L-forms, namely, L-threonine,[21] L-hydroxyproline[17] and L-phenylserine[22] have been shown to be of the D-configuration, whereas, at the present writing, no demonstration of the configuration of the ω-asymmetric carbon of L-hydroxylysine appears in the literature.

Partial Molar Rotations in Glacial Acetic Acid.—Calculations for the partial molar rotations in glacial acetic acid of the L-forms of several diastereomeric amino acids whose molecular rotations were reported earlier,[12] are given in Table II. The previous observation was made[12] that practically the same molecular rotations are shown for L-threonine and L-hydroxyproline in both water and glacial acetic acid. Comparison of the $(\alpha_{HOAc} - \alpha_{H_2O})$ and $(\omega_{HOAc} - \omega_{H_2O})$ values for each of these compounds reveals that they are approximately equal in magnitude but opposite in sign. The sum of $(\alpha_{HOAc} - \alpha_{H_2O}) + (\omega_{HOAc} - \omega_{H_2O})$ therefore gives a value that is not far from zero and thus reflects a negligible shift in the observed molar rotation from water to glacial acetic acid. It should be noted, however, that the value of $\alpha_{HOAc} - \alpha_{H_2O}$ is quite definitely positive in both instances and is thereby in agreement with previously offered

(20) W. S. Fones, *J. Biol. Chem.*, **204**, 323 (1953).

(21) C. E. Meyer and W. C. Rose, *ibid.*, **115**, 721 (1936).

(22) W. S. Fones, *Arch. Biochem. Biophys.*, **36**, 486 (1952).

data[12] in regard to the parallel optical behavior of amino acids, in glacial acetic acid and in 5 *N* HCl relative to their behavior in water. It is perhaps noteworthy that the H_2O-to-HOAc shift in the partial molar rotation of the ω-asymmetric center of L-threonine, L-hydroxyproline, and L-phenylserine in the negative direction, and of L-isoleucine in the positive direction, is in agreement with the direction of rotation obtained in the H_2O–HCl system (Table I). Due to its sparing solubility in glacial acetic acid, the molar rotation values for the hydroxylysines in this solvent are, unfortunately, unavailable for comparison.

The Isovaline "Exception."—Since isovaline contains only a single asymmetric carbon atom, this molecule affords a somewhat different situation than that presented by β-phenylserine. Thus, although it was shown by Ehrlich and Wendel[23] that the biological behavior exhibited by (+) isovaline resembles that of the L-amino acids, a later report[12] demonstrated that, contrary to expected behavior, an aqueous solution of this isomer showed a small negative shift in optical rotation on the addition of acid. The resolution of DL-isovaline *via* its chloroacetyl derivative by the action of the optically specific acylase I[14] is compatible with the earlier observation[23] that (+)isovaline exhibits the same metabolic susceptibility as the naturally occurring L-amino acids.

On the basis of the foregoing discussion, there would seem to be a very real and apparent discrepancy between an assignment of configuration to (+)isovaline based on the interpretation of enzymatic data,[14] on the one hand, and the effect of ionization on molecular rotation,[12] on the other. Examination of Fig. 1 will readily reveal how such ambiguity could arise.

In Fig. 1, the configuration of L-butyrine[24] and D-alanine are written in the conventional Fisher diagram. (+)Isovaline is so represented to conform to the recent finding[25] that its α-methyl group occupies the same position as the α-hydrogen atom of an L-amino acid.

Figure 1 demonstrates that (+)isovaline can be assigned either an L- or a D-configuration depending upon whether it is considered a derivative of L-butyrine or D-alanine. Thus, it could arbitrarily be designated as α-methyl-L-butyrine or as α-ethyl-D-alanine. When viewed in this light, the selection of a D- or an L-designation for amino acids, in which an alkyl substituent replaces the α-hydrogen, becomes meaningless when based on optical rotation data and emphatically indicates that the interpretation of such data be confined to α-amino acids which contain an α-hydrogen atom.[26]

(23) F. Ehrlich and A. Wendel, *Biochem. Z.*, **8**, 438 (1908).

(24) L-α-Aminobutyric acid.[13]

(25) S-C. J. Fu and S. M. Birnbaum, THIS JOURNAL, **75**, 918 (1953).

(26) A further inconsistency in regard to physical data was found when the hydantoin of (+)isovaline (m.p. 178°; calcd. for $C_8H_{10}O_2N_2$: C, 50.7; H, 7.1; N, 19.4. Found: C, 50.8; H, 7.3; N, 19.7) showed the negative shift of molar rotation ($[M]^{25}D = -34.5°$, *c* 1% in ethanol) characteristic of an L-amino acid[8] whereas the negative shift in rotation from water to HCl exhibited by the free amino acid showed the behavior characteristic of a D-amino acid.[9] It was previously noted[12] that the molar rotation of (+)isovaline in glacial acetic acid is more positive than in water, typical of the behavior of an L-amino acid.

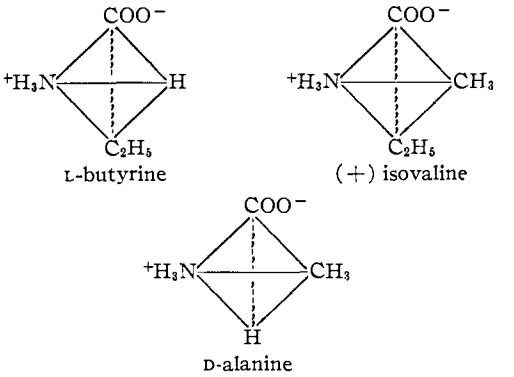

Fig. 1. Configuration of isovaline.

In order to avoid ambiguities in the assignment of configuration, Neuberger[27] has suggested that the D- or the L-designation be restricted to those amino acids in which a hydrogen atom is attached to the α-asymmetric carbon atom. Although much can be said in favor of this proposal from the physico-chemical point of view, the assignment of a D- or an L-configuration to amino acids which contain an alkyl group in lieu of hydrogen can nonetheless assume significance when viewed in the light of their biological behavior. Thus, the designation of a D- or an L-configuration for isovaline becomes quite meaningful since its behavior in biological systems of known stereochemical specificity can be related to the behavior of other amino acids of known configuration with these same biological systems. On this basis, the D- or L-designation for amino acids of the isovaline type could justifiably be retained.

Configuration of Alloaminotricarballylic Acid.[28]—The diastereomeric DL-aminotricarballylic acids were resolved by means of their brucine salts after preliminary separation of the racemic diastereomers.[1] Since an alkaloid resolution was employed, no direct information could be obtained with respect to the configuration of the optical enantiomorphs. Attempts to determine the configuration of the antipodes by the stereospecific action of L-amino acid oxidase (*Crotalus adamanteus*), aspartic acid oxidase, and *Neurospora* oxidase[29] on the free amino acids, and via the action of acylase I on the chloroacetyl derivatives, proved unsuccessful. Resort was therefore made to non-enzymatic methods.

Clarification of the Lutz–Jirgensons rule with regard to diasymmetric amino acids makes it possible to assign a configuration to the antipodes of aminotricarballylic acid with a good measure of reliability. The specific rotations of these antipodes have been reported in both water and 5 *N* HCl.[1] For the present purposes, their respective configurational designations can be considered unknown. In the absence of such information, the correct designation for the L,L-allo diastereo-

(27) A. Neuberger, *Advances in Protein Chem.*, **4**, 297 (1948).

(28) The aminotricarballylic and alloaminotricarballylic acid so designated here are identical, respectively, with the B- and A-forms of aminotricarballylic acid described in a previous paper of this series.[1] Specific rotations were used for these compounds in the previous publication[1] whereas molar rotations are used here.

(29) The authors are indebted to Dr. E. Ohmura for providing the *Neurospora* oxidase data.

TABLE III

CONTRIBUTION OF ASYMMETRIC α- AND β-CARBON ATOMS TO THE MOLAR ROTATION[a] OF THE DIASTEREOISOMERIC α-AMINOTRICARBALLYLIC ACIDS AND ISOCITRIC ACIDS

	L-Amino acid	[M]^{25}D In H_2O	[M]^{25}D In 5 N HCl	Molar rotation of α in H_2O	Molar rotation of α in 5 N HCl	Molar rotation of $\alpha_{HCl} - \alpha_{H_2O}$	Molar rotation of β in H_2O	Molar rotation of β in 5 N HCl	Molar rotation of $\beta_{HCl} - \beta_{H_2O}$	$(\alpha_{HCl} - \alpha_{H_2O}) + (\beta_{HCl} - \beta_{BrO})$
A	*l*-Aminotricarballylic acid	−62.7	−91.8	−38.5	−80.7	−42.2	−24.2	−11.1	+13.1	−29.1
A	*l*-Alloaminotricarballylic acid	−14.3	−69.6	−38.5	−80.7	−42.2	+24.2	+11.1	−13.1	−55.3
B	*d*-Aminotricarballylic acid	+62.7	+91.8	+24.2	+11.1	−13.1	+38.5	+80.7	+42.2	+29.1
B	*l*-Alloaminotricarballylic acid	−14.3	−69.6	+24.2	+11.1	−13.1	−38.5	−80.7	−42.2	−55.3
C	*l*-Aminotricarballylic acid	−62.7	−91.8	−24.2	−11.1	+13.1	−38.5	−80.7	−42.2	−29.1
C	*d*-Alloaminotricarballylic acid	+14.3	+69.6	−24.2	−11.1	+13.1	+38.5	+80.7	+42.2	+55.3
D	*d*-Aminotricarballylic acid	+62.7	+91.8	+38.5	+80.7	+42.2	+24.2	+11.1	−13.1	+29.1
D	*d*-Alloaminotricarballylic acid	+14.3	+69.6	+38.5	+80.7	+42.2	−24.2	−11.1	+13.1	+55.3

	L-Hydroxy acid	[M]^{25}D In H_2O	[M]^{25}D As Na salt in H_2O	α in H_2O	α_{salt} in H_2O	$\alpha_{H_2O} - \beta_{salt}$	β in H_2O	β_{salt} in H_2O	$\beta_{H_2O} - \beta_{salt}$	$(\alpha_{H_2O} - \alpha_{salt}) + (\beta_{H_2O} - \beta_{salt})$
A	*l*-Isocitric acid	−58.8	0	+ 3.8	+36.9	−33.1	−62.6	−36.9	−25.7	−58.8
A	*d*-Alloisocitric acid	+66.4	+73.7	+ 3.8	+36.9	−33.1	+62.6	+36.9	+25.7	− 7.4
B	*d*-Isocitric acid	+58.8	0	+62.6	+36.9	+25.7	− 3.8	−36.9	+33.1	+58.8
B	*d*-Alloisocitric acid	+66.4	+73.7	+62.6	+36.9	+25.7	+ 3.8	+36.9	−33.1	− 7.4

[a] Optical rotations performed in a photoelectric polarimeter, using a 2-dcm. tube. All concentrations at 0.5–1.0%.

meric pair must reside in one of the following four possible combinations: *l*-, *l*-allo; *d*-, *l*-allo; *l*-, *d*-allo; or *d*-, *d*-allo, where *d* and *l* refer to the dextro- and levorotatory forms, respectively.

Table III contains the calculated partial molar rotation values, in both water and 5 N HCl, of the α- and ω-asymmetric centers for the four possible L,L-allo combinations indicated above. Since the $\alpha_{HCL} - \alpha_{H2O}$ values are negative for the hypothetical *l*,*l*-allo and *d*,*l*-allo combinations (A and B, Table III), it is possible to reject them as the correct designations of the L,L-allo pair. Conversely, the $\alpha_{HCL} - \alpha_{H2O}$ values for both the *l*,*d*-allo and *d*,*d*-allo pairs are positive and neither can, for the movement, be rejected (C and D, Table III). It should be noted, however, the *d*-allo appears in both these pairs and is therefore representative of the L-allo configuration with an $[M]^{25}D = +14.3°$ (*c* 0.5% in water), whereas the levorotatory form, *l*-allo, is its D-allo enantiomorph. These calculations, as yet, permit no assignment of configuration to the *d*- and *l*-antipodes of aminotricarballylic acid. However, calculations based on the isomers of isocitric acid, below, will permit a proper choice.

Configuration of the Isomers of Isocitric Acid and Related Derivatives.—The interpretations based on optical data are concerned exclusively with the behavior of the α-asymmetric center and are not applicable to all asymmetric centers that reside within the molecule. On the basis of the calculations developed above, the behavior of the α-asymmetric center of α-amino or α-hydroxy acids with two centers of asymmetry can be ascertained if reliable optical rotation values are available for at least one antipode in each diastereomeric pair. This is exemplified with the aminotricarballylic acids, where such knowledge led to an assignment of configuration to the α-asymmetric carbon atom of each of the antipodes of the allo pair. This, coupled with comparable information obtained from the corresponding α-hydroxy acids, permit a similar assignment of configuration not only to the antipodes of the other diastereomeric pair, but to the four isomers of isocitric acid as well.

The configuration of natural isocitric acid relative to that of L-serine in the amino acid series, or D-glyceraldehyde in the carbohydrate series, has not yet been demonstrated. Choice of the nitrous acid procedure for the conversion of the four optical isomers of α-aminotricarballylic acid to the corresponding isomers of isocitric acid[1] was deemed practicable in view of the generally accepted premise that such conversion of an α-amino acid to the corresponding α-hydroxy acid proceeds with retention of configuration and without appreciable racemization.[27,30,31] Complete lack of susceptibility of three of the four isomeric isocitric acids to the isocitric dehydrogenase–triphosphopyridine nucleotide system[3] indicated the absence of detectable racemization during the nitrous acid treatment. The configuration of each of the isocitric acid isomers can therefore be considered identical to that of the amino acid from which it was derived.

Since the configuration of the antipodes of alloaminotricarballylic acid was demonstrated earlier, a configurational designation can be given to each of the corresponding antipodes of alloisocitric acid as well as to the known derivatives[1] related thereto (Table IV).

Calculations similar to those effected with the aminotricarballylic acids apply equally well to the L- and L-allo stereoisomers of isocitric acid, the molar rotation values (in water) of which are known for the free acid and its trisodium salt[1] (Table III). In this instance, a positive $\alpha_{H_2O} - \alpha_{salt}$ value is indicative of an L-configuration.[10,27]

(30) Recent kinetic studies (P. Brewster, F. Hiron, E. D. Hughes, C. K. Ingold and P. A. D. S. Rao, *Nature*, **166**, 178 (1950)) have shown that, except where an α-phenyl substituent is present, the deamination of α-amino acids with nitrous acid proceeds *via* an S_N1 mechanism wherein no inversion of configuration occurs due to the presence of the configuration-holding α-carboxylate ion.

(31) C. K. Ingold, "Structure and Mechanism in Organic Chemistry," Cornell University Press, Ithaca, N. Y., 1953.

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]).

BETHESDA, MARYLAND

[CONTRIBUTION FROM THE ROCKEFELLER INSTITUTE FOR MEDICAL RESEARCH]

Bacitracin A. Further Studies on the Composition

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

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

(1) L. C. Craig, W. Hausmann and J. R. Weisiger, *J. Biol. Chem.*, **199**, 865 (1952).

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

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

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

(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.