poured into 100 ml. of water covered with 50 ml. of ether and the organic layer was separated after shaking thoroughly. The aqueous solution was then acidified and extracted with two portions of 40 ml. of ether. The combined ether extracts were washed first with dilute hydrochloric acid, then with dilute sodium bicarbonate solution and finally with water. After distillation of the dried ethereal solution, diethyl N-dithiocarbobenzoxyhydroxyaspartate remained as a viscous oil which was thoroughly dried *in vacuo*. The oil was cooled in an ice-bath and 3 ml. of thionyl chloride added and the mixture frequently shaken for 10 minutes, until it became homogeneous. After 30 minutes at 0°, the excess of thionyl chloride was removed *in vacuo* at 35° and then 30 ml. of ether added. When after some shaking the residue became crystalline it was filtered off and washed with ether. It melted at 68–73°; yield 3.4 g. (70%). *Anal.* Calcd. for $C_{16}H_{20}O_4S_2NCl$: N, 3.58; S, 16.39. Found: N, 3.59; S, 16.38.

The substance loses hydrogen chloride *in vacuo* and is decomposed by water.

SÃO PAULO, BRAZIL

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

Studies on Diastereoisomeric α-Amino Acids and Corresponding α-Hydroxy Acids. I. Preparation of the Four Optical Isomers of α-Aminotricarballylic Acid and their Conversion to the Corresponding Isocitric Acid Lactones

BY JESSE P. GREENSTEIN, NOBUO IZUMIYA,[1] MILTON WINITZ AND SANFORD M. BIRNBAUM

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A mixture of the two racemic forms of α-aminotricarballylic acid (A and B) was prepared by the interaction of ethyl acetamidocyanoacetate with diethylbromosuccinate in the presence of sodium followed by acid hydrolysis of the resulting triethyl-α-acetamido-α-cyanotricarballylate. Most of the A form present was separated by crystallization from the condensed solution of the mixture at *p*H 2.9. The mixture of residual B and unprecipitated A forms was separated from the diluted filtrate as the insoluble copper salts. The washed copper salts were decomposed with hydrogen sulfide, and the aqueous filtrate refluxed for several hours to convert the amino acids quantitatively to the corresponding pyrrolidone-α,β-dicarboxylic acids. The latter mixture, isolated and dried, yielded in methanol solution with 2 moles of quinine a nearly quantitative precipitation of the salt of the A pyrrolidone and quinine with no evidence of resolution of the optical antipodes. From the mother liquor, the racemic B pyrrolidone was isolated after removal of the alkaloid. The free racemic amino acid A was resolved through the action of brucine in dilute ethanol solution. The racemic pyrrolidonedicarboxylic acid B was resolved through the action of brucine in aqueous solution, and the optical antipodes each converted to the free amino acids by HCl hydrolysis and isolated by crystallization after adjustment to *p*H 2.9. Treatment of each of the four isomeric α-aminotricarballylic acids with nitrous acid led to the preparation of the corresponding optically active isocitric acid lactones which were isolated in the crystalline state. The treatment of *l*-aminotricarballylic acid (A) led to *l*-isocitric acid and thence to the *l*-lactone, that of the *l*-isomer of the B amino acid led to *d*-isocitric acid and thence to the *l*-lactone. Only one of the four lactones so prepared was identical with the lactone of naturally-occurring isocitric acid, namely, the levorotatory (in H_2O) isomer derived from the levorotatory isomer of α-aminotricarballylic acid (B), and only this isomer of isocitric acid reacted in the isocitric acid dehydrogenase–TPN system.

Isocitric acid lactone was first synthesized in 1889 by Fittig through the decomposition of trichloromethylparaconic acid with baryta followed by dehydration.[2] Since the compound contains two centers of asymmetry, the synthesis would be expected to yield two racemic modifications. However, the greater part of the product was isolated as a single racemic form which melted at 161°. Later studies of the reaction by Pucher and Vickery indicated the presence in the mother liquors of the second racemic modification in very small amount and admixed with the first.[3] Wislicenus and Nassauer prepared isocitric acid lactone by the reduction of oxalosuccinic acid ester, followed by saponification and dehydration,[4] and this procedure appeared to yield the two racemic modifications in nearly equal amounts, although again only the form melting at 161° was isolated in the pure state.[3] A third method of preparation of isocitric acid lactone involved the prior synthesis of α-aminotricarballylic acid through the action of ammonia on triethyl aconitate followed by hydrolysis of the resulting diketopiperazine tetraamide; treatment of the purified amino acid with nitrous acid yielded a crystalline isocitric acid lactone which melted at 153°.[5] As shown below, this has turned out to be the previously expected second racemic modification of isocitric acid lactone.

The natural occurrence of optically active isocitric acid was first demonstrated by Nelson, who isolated the material as the triethyl ester and as the diethyl ester lactone from blackberries, and who found that it was by far the predominating acid of this fruit.[6] Bruce improved the isolation of the compound from this source by separating it as the readily crystallizable dimethyl ester lactone; on acid hydrolysis crystalline isocitric acid lactone with m.p. of 153–154° and $[\alpha]^{26}D = -62.0°$ (*c* 12.75, H_2O) was obtained.[7] Another rich natural source of isocitric acid was found to be *Bryophyllum* leaf tissue, and Pucher, Abrahams and Vickery employed essentially the Nelson–Bruce procedure to isolate the lactone (m.p. 153–154°) from this source.[8] No values of optical rotation were reported by these authors for the lactone or for the corresponding free isocitric acid.

Since neither of the racemic modifications of isocitric acid has been resolved into its optical antipodes, the relation of the naturally-occurring variety to the respective racemates has been neces-

(1) Visiting Fulbright and Smith-Mundt Scholar; on leave from Kyushu University, Japan.

(2) R. Fittig, *Ann.*, **255**, 47 (1889).

(3) G. W. Pucher and H. B. Vickery, *J. Biol. Chem.*, **163**, 169 (1946).

(4) W. Wislicenus and M. Nassauer, *Ann.*, **285**, 1 (1895).

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

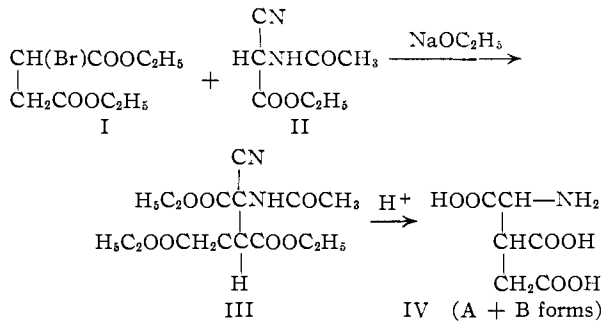
(6) E. K. Nelson, THIS JOURNAL, **47**, 568 (1925); **52**, 2928 (1930).

(7) W. F. Bruce, *ibid.*, **57**, 1725 (1935).

(8) G. W. Pucher, M. D. Abrahams and H. B. Vickery, *J. Biol. Chem.*, **172**, 579 (1948).

sarily derived by inference. Thus, when the racemic lactone with m.p. 161° was converted to isocitric acid and subjected to the action of aconitase, close to half of the compound was metabolized.[9,10] The isocitric acid dehydrogenase–triphosphopyridine nucleotide system[11] was shown by Meister and Baker in this Laboratory to be inactive toward the isocitric acid derivable from the racemic lactone with m.p. 153°, although effective toward a fraction of the racemic mixture derived by the action of nitrous acid on alkali-treated aminotricarballylic acid.[12] It would therefore appear that the lactone of the racemic modification of isocitric acid with m.p. 161° corresponded to the natural form of the compound. Inasmuch as the evidence on which this has been based has been derived from a study exclusively of racemic compounds, its validity is limited. It was considered desirable therefore to prepare in pure form all four of the possible optical isomers of isocitric acid and to subject each to metabolic study. A related problem is that of the configuration of the α- and β-carbon atoms of natural isocitric acid to which no contribution has so far been made. A possible solution to the problem of the configuration of the α-carbon seemed feasible if the analogous configuration of the precursor α-aminotricarballylic acid were known, on the generally accepted premise that the conversion of an α-amino to an α-hydroxy group in an amino acid through the use of nitrous acid is accompanied by retention of optical configuration.[13–15] Accordingly, α-aminotricarballylic acid was synthesized, the resulting two racemic forms designated arbitrarily as A and B were separated and each resolved into its optical antipodes, and finally the four isomeric amino acids were converted by nitrous acid into the corresponding isocitric acid lactones. The prefixes *d* and *l* as employed refer exclusively to direction of optical rotation.

The general procedure developed by Albertson for amino acids[16] was employed for the synthesis of the diastereoisomeric mixture of α-aminotricarballylic acid (IV). Diethyl bromosuccinate (I) was condensed with ethyl acetamidocyanoacetate (II) in the presence of sodium ethoxide to yield triethyl α-acetamido-α-cyanotricarballylate (III) which on acid hydrolysis yielded the amino acid. The reaction mixture brought to *p*H 2.9, the isoelectric point of α-aminotricarballylic acid,[17] by addition of LiOH, and evaporated *in vacuo* to low bulk, yielded a heavy crystalline precipitate of one of the racemic diastereoisomers to which the



designation of A was given. Attempts to recrystallize this material from hot water led to considerable losses, and it was suspected that under these conditions the amino acid cyclized to form the corresponding pyrrolidone-α,β-dicarboxylic acid. Quantitative studies by manometric ninhydrin–CO_2 determinations of the amino acid in refluxing aqueous solution indeed revealed an unexpectedly rapid rate of such a cyclic conversion to a practically complete extent. The isolated material was a pure pyrrolidonedicarboxylic acid which melted at 210°; on acid hydrolysis it was quantitatively and rapidly converted back to the amino acid.

The filtrate after removal of most of the A form of the amino acid contained too much salt to work up further. It was therefore diluted and treated with an aqueous solution of copper acetate to precipitate the insoluble copper salts of the B form and of any previously unprecipitated A form.[5] The washed copper salts were decomposed with H_2S and the filtrate freed of the gas. In earlier experiments the B form of the amino acid was isolated in impure condition from the foot liquors of the successive crystallizations of the A form, and purified by conversion to the crystalline N-acetyltriethyl ester derivative; on acid hydrolysis followed by adjustment to *p*H 2.9, the racemic B form of α-aminotricarballylic acid was obtained in the pure state but in relatively low yield. Under the same conditions, the N-acetyltriethyl ester derivative of the A form was a soluble, non-crystallizable oil. The method of purification of the B diastereoisomer was impractical for the preparation of the material in quantity, but the small amount thus rendered available yielded important subsequent information. On refluxing its aqueous solution, the B modification cyclized practically completely to the corresponding pyrrolidone-α,β-dicarboxylic acid, but at an appreciably slower rate than that of its A diastereoisomer. The isolated crystalline compound melted at 230°; on acid hydrolysis, it too was quantitatively converted back to the amino acid. Since the solubility of the B form of the pyrrolidone appeared to be appreciably less than that of the corresponding A form, the filtrate after the decomposition of the copper salts was refluxed for several hours to convert the mixture of diastereoisomeric amino acids to the corresponding pyrrolidonedicarboxylic acids, the solution condensed, and fractional crystallization of the compounds was attempted with the aim of separating them. Only mixtures of A and B forms in various proportions were obtained.

(9) C. Martius, *Z. physiol. Chem.*, **257**, 29 (1938).

(10) H. A. Krebs and L. V. Eggleston, *Biochem. J.*, **38**, 426 (1944).

(11) E. Adler, H. von Euler, G. Günther and M. Plass, *ibid.*, **33**, 1028 (1939).

(12) A. Meister and C. G. Baker, *Archiv. Biochem. Biophys.*, **31**, 460 (1951).

(13) A. Neuberger, *Advances in Prot. Chem.*, **4**, 298 (1948).

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

(15) An interesting exception to this rule has been noted by M. B. Watson and G. W. Youngson (*J. Chem. Soc.*, 2145 (1954)) in the action of nitrous acid on D-α-phenylglycine which yielded *d*-mandelic acid and which therefore implied an over-all inversion of configuration.

(16) N. F. Albertson, THIS JOURNAL, **68**, 450 (1946).

(17) J. P. Greenstein and N. R. Joseph, *J. Biol. Chem.*, **110**, 619 (1935).

Sometime prior to these studies, chloroacetyl-α-aminotricarballylic acid was prepared by Meister and Baker[18] and was found by one of the present authors (S. M. B.) to be resistant to the action of renal acylase I. The general enzymatic procedure of resolving racemic α-amino acids developed in this Laboratory[19] was therefore inapplicable to the resolution of α-aminotricarballylic acid, and the use of alkaloids as resolving agents was resorted to. In an attempt to follow the successful resolution of pyrrolidonecarboxylic acid through the use of quinine,[20,21] the pure racemic A and B forms of pyrrolidonedicarboxylic acid were each treated with 2 moles of various alkaloids in different solvents. It was noted that (a) when methanol was the solvent, the brucine salts of both A and B forms nearly quantitatively precipitated with no evidence of resolution of the antipodes in either case, (b) when methanol was the solvent, the quinine salt of A precipitated nearly quantitatively with no evidence of resolution of its optical isomerides, whereas the quinine salt of B was completely soluble, and (c) when water was the solvent, a clear-cut resolution into the optical antipodes of both A and of B was effected through brucine. Both a method of separation of the A and B forms of the racemic pyrrolidonedicarboxylic acids, and of a resolution of each form were thereby revealed. The mixture of A and B forms of racemic pyrrolidonedicarboxylic acid obtained as above by refluxing the filtrate from the decomposition of the copper salts was therefore isolated, dried and dissolved in absolute methanol together with quinine. Only the quinine salt of the racemic A form crystallized, and the B form was recovered from the mother liquor. After removal of alkaloid, followed by recrystallization, the pure A and B racemic diastereoisomers (m.p. 210 and 230°, respectively) were obtained.[22] This was clearly the method of choice in isolating the B form of the racemic α-aminotricarballylic acid from mixtures with the corresponding A diastereoisomer. As the free amino acids, a total of 300 g. of separate A and B forms in a state of steric and analytical purity were obtained from about 1400 g. of starting material, or a recovery of 21%. Of these 300 g., about 230 g. was A and about 70 g. was B. Much of the losses in the recovery of the B form was noted in the use of columns of Dowex-50 ion-exchange resin for the removal of the ammonium ion and traces of alkaloid following the decomposition of the quinine salt of the pyrrolidonedicarboxylic acid with ammonia. Similar losses occurred subsequently in the resolution procedure of this B form with brucine, but the ease and convenience associated with the use of this resin for the present purposes more than compensated for the diminished yields. Another procedure whereby the A and B forms of pyrrolidonedicarboxylic acid may be distinguished is to add baryta in slight excess to their aqueous solution. At 25° no precipitate appears, but at the boiling temperature the barium salt of the B form precipitates nearly quantitatively whereas that of the A form remains in solution. The respective free amino acids similarly treated in aqueous solution with baryta yield precipitates of the Ba salts on heating.

Resolution into the optical antipodes of the racemic B pyrrolidonedicarboxylic acid was readily effected by brucine in aqueous solution. The insoluble fraction was mainly the salt of the *l*-isomer of the pyrrolidone. Removal of the alkaloid from both insoluble *l*- and soluble *d*-fractions led to the isolation of optical antipodes each contaminated with varying amounts of the other. Unfortunately, the racemic pyrrolidone was more insoluble than the optically active modifications. Purification was successfully effected by the slow evaporation of the respective solutions and periodic removal of the successive crops of crystals. Each crop was examined in the polarimeter, and, when appreciable optical rotation in a crystalline deposit appeared, the material was discarded and the filtrate evaporated to dryness. Success of this tedious operation was gaged by the melting point of the residual material so obtained (187° for both isomers) and by the nearly equal and opposite rotations (−54.7 and +54.6°). Each of the optical antipodes was dissolved in HCl, the solutions were refluxed, and the free, optically isomeric aminotricarballylic acids of the B modification subsequently isolated after adjustment of the *p*H to 2.9.

When this resolution procedure with brucine in aqueous solution was similarly carried out with the A form of pyrrolidonedicarboxylic acid, a resolution into the optical antipodes of the compound was effected, and, as in the case of the B pyrrolidonedicarboxylic acid the insoluble salt corresponded in the main to the *l*-antipode. However, the isomers appeared as non-crystallizable oils admixed with varying amounts of crystals of the racemic variety. Subsequent studies with the pure optical isomerides of the A form of α-aminotricarballylic acid whose aqueous solutions were refluxed confirmed these findings, for only non-crystallizable oils of the optically active modifications of pyrrolidonedicarboxylic acid A were isolated. A resolution of the A form of aminotricarballylic acid was successfully accomplished through mixtures of the free racemic amino acid with brucine in dilute ethanol.[23] The salt of the levorotatory amino acid is somewhat more insoluble than that of the dextrorotatory antipode, but not greatly so, and unless both temperature and time of standing are carefully controlled, the first crop of crystals will represent largely a mixture of both optical forms. The directions cited should be closely followed. After removal of the alkaloid, purification of each of the optical isomers of the A aminotricarballylic acid is easily accomplished because the racemic modification is so much more soluble than the optically

(18) A. Meister and C. G. Baker, personal communication.

(19) J. P. Greenstein, *Advances in Prot. Chem.*, **9**, 121 (1954).

(20) S. Sugasawa, *J. pharm. Soc. Japan*, No. 537, 934 (1926).

(21) G. Hillman and A. Elies, *Z. physiol. Chem.*, **283**, 31 (1948).

(22) An analogous separation of the diastereoisomeric racemates of β-thiolbutyrine through the agency of salts with phenylethylamine has been described by H. E. Carter, C. M. Stevens and L. F. Ney, *J. Biol. Chem.*, **139**, 247 (1941).

(23) Free racemic glutamic acid has been resolved through its salt with *l*-1-hydroxy-2-aminobutane, which itself had been obtained through the interaction of the racemic base with either *d*-tartaric acid or L-glutamic acid, *cf.* R. Radke, R. B. Fearing and S. W. Fox, THIS JOURNAL, **76**, 2801 (1954).

TABLE I

Compound	M.p.,[d] °C.	$[\alpha]_D$,[e] degrees	Analyses, %,[i] found C	H	N[k]
dl-α-Aminotricarballylic acid (A)	..	.....	37.6	4.7	7.3
dl-α-Aminotricarballylic acid (B)	..	.....	37.7	4.7	7.4
l-α-Aminotricarballylic acid (A)	..	−6.8,[f] −36.5,[g] −76.3[h]	37.9	4.7	7.3
d-α-Aminotricarballylic acid (A)	..	+7.5,[f] +36.4,[g] +76.5[h]	37.9	4.7	7.4
l-α-Aminotricarballylic acid (B)	..	−33.5,[f] −48.7,[g] +5.0[h]	37.6	4.7	7.2
d-α-Aminotricarballylic acid (B)	..	+32.8,[f] +48.0,[g] −5.0[h]	37.7	4.8	7.3
dl-Pyrrolidone-α,β-dicarboxylic acid (A)	210	.....	41.8	3.9	8.0
dl-Pyrrolidone-α,β-dicarboxylic acid (B)	230	.....	41.7	4.1	8.0
l-Pyrrolidone-α,β-dicarboxylic acid (A)[a,c]	..	(−58)[f]	..	..	..
d-Pyrrolidone-α,β-dicarboxylic acid (A)[b,c]	..	(+58)[f]	..	..	..
l-Pyrrolidone-α,β-dicarboxylic acid (B)[a]	187	−54.7[f]	41.6	4.0	8.1
d-Pyrrolidone-α,β-dicarboxylic acid (B)[b]	187	+54.6	41.6	4.0	8.0
dl-Isocitric acid lactone (A)	153	.....	41.5	3.6	..
dl-Isocitric acid lactone (B)	161	.....	41.4	3.6	..
l-Isocitric acid lactone (A)[a]	143	−40.5[f]	41.4	3.6	..
d-Isocitric acid lactone (A)[b]	143	+41.0[f]	41.4	3.6	..
l-Isocitric acid lactone (B)[a]	154	−61.1[f]	41.4	3.5	..
d-Isocitric acid lactone (B)[b]	154	+60.9[f]	41.4	3.5	..
Trisodium isocitrate (A)[a]	..	−38.8[f]	..	..	..
l-Isocitric acid (A)[a]	..	−34.1[f,i]	..	..	..
Trisodium isocitrate (A)[b]	..	+38.4[f]	..	..	..
d-Isocitric acid (A)[b]	..	+34.6[f,i]	..	..	..
Trisodium isocitrate (B)[a]	..	0[f]	..	..	..
d-Isocitric acid (B)[a]	..	+30.6[f,i]	..	..	..
Trisodium isocitrate (B)[b]	..	0[f]	..	..	..
l-Isocitric acid (B)[b]	..	−30.0[f,i]	..	..	..

[a] Derived from *l*-aminotricarballylic acid. [b] Derived from *d*-aminotricarballylic acid. [c] Non-crystallizable sirups, optical rotations are approximate. [d] Corrected. [e] Optical rotations performed in a photoelectric polarimeter, using a 2-dcm. tube. All concentrations at 0.5–1.0%. [f] H_2O solution. [g] 5 *N* HCl solution. [h] 1 *N* NaOH solution. [i] Trisodium salt neutralized with 3 equivalents of HCl. [j] Calcd for aminotricarballylic acid, $C_6H_9O_6N$: C, 37.7; H, 4.7; N, 7.3. Calcd. for pyrrolidonedicarboxylic acid, $C_6H_7O_5N$: C, 41.6; H, 4.1; N, 8.1. Calcd. for isocitric acid lactone, $C_6H_8O_6$: C, 41.4; H, 3.5. [k] α-COOH–N values for all the α-aminotricarballylic acid preparations were close to that calculated for 2 moles of CO_2 per mole of compound.

active modifications. A single crystallization from water is sufficient to remove all traces of the racemate.

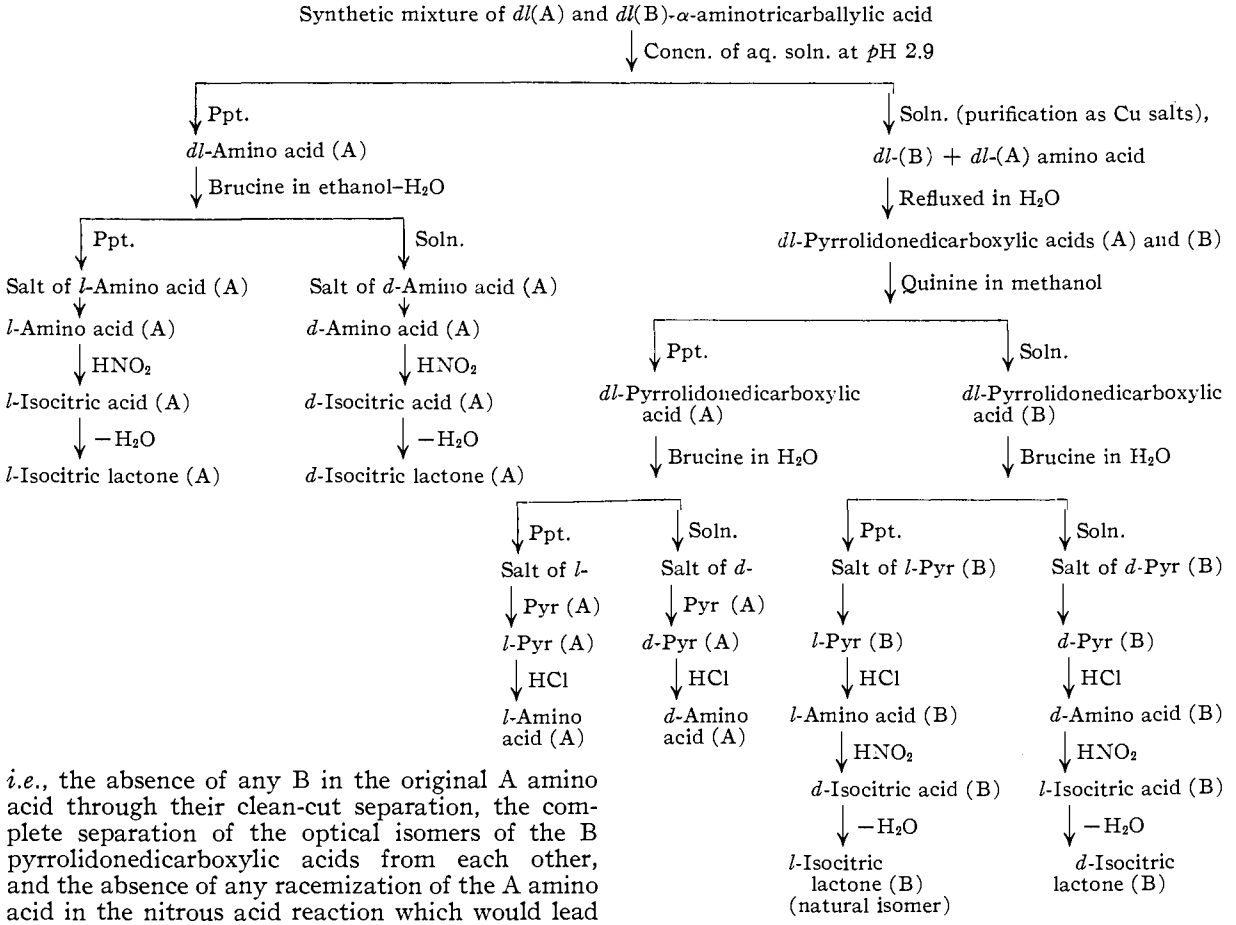
Conversion to the corresponding isocitric acid of the four optically active stereoisomers of α-aminotricarballylic acid, and of the two racemic modifications, was accomplished by dissolving the compounds in 1 *N* HCl followed by treatment with silver nitrite. The formation of the lactone revealed again a remarkable difference in the ease of the ring closure between the A and B forms. When aqueous solutions of the free amino acids were refluxed, the A modification formed the corresponding pyrrolidonedicarboxylic acid much more rapidly than did the B. Similarly, after treatment of the A form of the amino acid with nitrous acid, removal of both AgCl and solvent left an oil which rapidly crystallized within a day or two *in vacuo* at 25° to the corresponding isocitric acid lactone. The A form of isocitric acid, whether optically active or racemic, readily lactonizes at a relatively low temperature. On the other hand, the B form similarly treated, yields an isocitric acid which lactonizes much more slowly. The preparation of isocitric acid of the B variety must be heated at 100° *in vacuo* before it will lactonize in a reasonable length of time. Since the nitrous acid reaction leads to a certain amount of side products whose behavior on heating might be unpredictable, the isocitric acid of the B form obtained by this reaction was isolated as the barium salt[6]; dissolution of this salt in HCl followed by dilution with water and passage of the resulting solution through a column of Dowex-50 resin led to a solution of pure isocitric acid in HCl. Removal of the solvent and heating of the residue at 100° *in vacuo* yielded the lactone. The physical constants and analytical data of all of the compounds prepared are collected in Table I.

The lactone derived from the racemic B aminotricarballylic acid melted at 161°, identical with that reported by Fittig.[2] On opening the ring, the isocitric acid obtained thereby reacted in the isocitric acid dehydrogenase–TPN system to exactly one-half of the molecule.[24] The lactone derived from levorotatory α-aminotricarballylic acid B, possessed a m.p. of 154° and $[\alpha]^{25}D$ −61.1° in water, values close to those reported by Bruce for natural isocitric acid lactone isolated from blackberries.[7] This synthetic lactone reacted to 100% in the isocitric acid dehydrogenase–TPN system. Its optical isomer, derived from dextrorotatory α-aminotricarballylic acid B, as well as the two optical isomers of isocitric acid lactone derived from the optically isomeric α-aminotricarballylic acids A, were completely inert in the enzyme system. The lack of any detectable enzymatic susceptibility on the part of the latter three isomeric forms indicates their essential steric purity,

(24) The authors are indebted to Dr. Alton Meister for a sample of authentic natural isocitric acid lactone employed for comparison purposes.

FIG. 1

SEPARATION OF STEREOISOMERS OF AMINOTRICARBALLYLIC ACID AND CONVERSION TO CORRESPONDING ISOCITRIC ACIDS



i.e., the absence of any B in the original A amino acid through their clean-cut separation, the complete separation of the optical isomers of the B pyrrolidonedicarboxylic acids from each other, and the absence of any racemization of the A amino acid in the nitrous acid reaction which would lead to epimerization at the α-carbon atom and partial formation of B lactone. Conversely, the practically complete enzymatic susceptibility of one of the two optical isomers of the B lactone indicates the absence of racemization of the B amino acid in the nitrous acid reaction which by epimerization would lead to partial formation of inert A lactone.

For both A and B forms, the sequence of reactions, pyrrolidonedicarboxylic acid ⇄ aminotricarballylic acid → isocitric acid lactone, is conducted without change in the sign of rotation of the aqueous solutions of the various components. However, Bruce had observed that natural isocitric acid (*i.e.*, *d*-B in the nomenclature of this paper) has a sign of rotation in water opposite to that of its lactone (*i.e.*, *l* − B).[7] On the other hand, as found in the present studies, the A form of isocitric acid has the same sign of rotation as its lactone. Bruce had also observed that the optical rotation of the trisodium salt of natural isocitric acid was close to zero, and that when this salt was converted to the free acid by treatment with 3 equivalents of HCl, the resulting $[\alpha]^{26}D$ was +17.7°. The present authors using both optical isomers corresponding to the configuration of natural isocitric acid (*i.e.*, B) could confirm Bruce's finding regarding the lack of optical rotation of the trisodium salt, but regret to take issue with the order of magnitude given in Bruce's admirable report for the rotation of free isocitric acid inasmuch as $[\alpha]^{25}D$ values of +30.6 and −30.0° were noted for the isomers corresponding to natural isocitric acid and for its antipode, respectively. The trisodium salt of isocitric acid (A) possesses an appreciable rotation. The relationships among the various optically active compounds and the sequence of reactions are described in Fig. 1. The optical configuration of these compounds is considered in subsequent papers in this series.[25]

Experimental

Synthesis of α-Aminotricarballylic Acid (Diastereomeric Mixture).—In a three-necked, five-liter round-bottom flask, equipped with reflux condenser and dropping funnel, was placed 2 liters of absolute ethanol (magnesium dried). 87.4 g. (3.8 moles) of sodium, cut into small pieces, was added and the mixture refluxed to expedite solution. After all the sodium had dissolved, the flask was cooled to room temperature, 646 g. of ethyl acetamidocyanoacetate (Winthrop–Stearns) was added, and the mixture refluxed for 15 minutes. This was followed by the dropwise addition of 1 kg. (3.95 moles) of diethyl bromosuccinate over a period of 30 minutes. The reaction was exothermic, heat liberation being sufficient to maintain a vigorous reflux. After the addition was complete, the reaction mixture was further refluxed for one-half hour. At the end of this period, the mixture was no longer alkaline to litmus. The reaction mixture was allowed to cool to room temperature and 2 liters of ether added with stirring. The precipitated salt (NaBr) was

(25) M. Winitz, S. M. Birnbaum and J. P. Greenstein, THIS JOURNAL, **77**, 716 (1955).

filtered off over suction and washed with 1 liter of ether. The precipitate was discarded and the combined filtrate and washings evaporated to an oil in a jet of dry air. The oil was taken up in 1.5 liters of chloroform and washed twice, each time with 1 liter of distilled water. The chloroform layer was dried over anhydrous sodium sulfate and concentrated to an oil in a stream of dry air. The oil was dissolved in 3 liters of concentrated hydrochloric acid, boiled for one-half hour and finally refluxed for 6 hours. The acid hydrolysate was taken to a small volume (approximately 1 liter) under reduced pressure. Two liters of water was added, the solution decolorized with activated charcoal, and the concentration repeated. The evaporation was repeated once more after the addition of water. The final volume was approximately 1.5 liters.

Two preparations, as described above, were prepared and combined. The total theoretical yield for the two preparations was 1452 g. The diastereomers were separated from the above solution without prior separation of each diastereomeric pair.

Separation of Racemic A and B Forms of α-Aminotricarballylic Acid.—The HCl salt of approximately 1400 g. of the synthetic mixture of A and B forms of α-aminotricarballylic acid was treated with sufficient water to effect solution, and satd. LiOH added to bring the *p*H to 2.9. The volume was about 6 l. Treatment with Norit removed most of the color from the solution, leaving it a pale yellow. The solution was evaporated *in vacuo* to the appearance of crystals and allowed to stand at 5° for 18 hours. The crystalline mass of *dl*-α-aminotricarballylic acid (A) was filtered, washed with cold water, and dried; yield 240 g. The combined filtrate and washings was further evaporated *in vacuo* to a second crystallization amounting to 70 g., and similarly to a third crystal crop amounting to 8 g. These three crops of the crude A amino acid were combined, dissolved in 900 ml. of warm 3 *N* HCl, the solution chilled and treated with concd. NH_3 to *p*H 2.9. On evaporation *in vacuo* to 400 ml., 200 g. of pure A amino acid crystallized. A second crop of 10 g. of pure A was obtained by concentrating the filtrate, and was added to the first.[26]

The combined mother liquors from the above crystallizations, containing the B amino acid together with some unprecipitated A amino acid, was brought to 10-l. volume and *p*H 5.0 by addition of water. A hot solution of 5 lb. of copper acetate in 20 l. of H_2O was added with stirring, and the resulting heavy precipitate of copper salt allowed to stand at 25° for several hours. The precipitate was filtered, washed with water until free of adherent halide, suspended in 6 l. of H_2O, and decomposed with H_2S. The mixture was filtered through a layer of Celite and Norit, and the residue washed with H_2S–water. The volume of filtrate was 8 l. On evaporation *in vacuo* a crop of 18 g. of *dl*-α-aminotricarballylic acid (A) appeared, which, after recrystallization as above, dropped to 10 g. The combined mother liquors were then further evaporated *in vacuo* to a thick yellow sirup. There was evidence of considerable halide in this sirup, suggesting that although the original copper salt had been washed free of adherent halide it may yet have contained some halogen as part of the copper complex. This sirup consisted of a mixture of A and B forms of *dl*-α-aminotricarballylic acid, the B form predominating. The sirup was taken up in 6 l. of H_2O, and the clear solution refluxed at *p*H 2.9 for 7 hours. After decolorization with Norit, and evaporation *in vacuo* to 200 ml., crystallization of 180 g. of the mixture of A and B forms of *dl*-pyrrolidone-α,β-dicarboxylic acid occurred. Recrystallization from water yielded 160 g. of analytically pure material, which revealed less than 0.3% of free amino acid by ninhydrin–CO_2 determinations; m.p. 203°. This melting point revealed the presence of a mixture. Several crystallizations from water served to raise this melting point to 208°, but in no way effected a purification of either form. The separation of the A and B forms of the *dl*-pyrrolidonedicarboxylic acid was achieved by the different solubilities of their quinine salts in methanol as described below. It is sufficient at this point to state that from 158 g. of this pyrrolidonedicarboxylic acid mixture, 9 g. of the A, and 67 g. of the B forms were isolated in the pure state.

The total amount of analytically pure *dl*-α-aminotricarballylic acid (A) obtained was therefore 220 g. Together with the 9 g. of *dl*-pyrrolidonedicarboxylic acid (A) isolated as the quinine salt, which corresponds to 10 g. of the free α-amino acid, the total recovery of the A form was 230 g. The total amount of racemic B form of pyrrolidonedicarboxylic acid was 67 g., corresponding to some 74 g. of the amino acid. It would appear that the ratio of A to B forms in the synthetic aminotricarballylic acid is about 3 to 1, but in view of the considerable losses involved in the separation of these forms, this ratio may be deceptive. No more than about 300 g. of separated A and B forms were obtained in a state of steric and analytical purity from some 1400 g. of starting product, or a recovery of 21%.

Separation of Racemic A and B Forms of Pyrrolidone-α,β-dicarboxylic Acid.—One hundred and fifty-eight grams of the mixture of these two forms was brought into solution in 2100 ml. of boiling absolute methanol with 707 g. of quinine hydrate (2 moles). After standing at 5° for 18 hours, the crystalline quinine salt was filtered and washed with chilled methanol and ether; yield 129 g. The precipitate was recrystallized from methanol, yielding 97 g. when dried. This material was dissolved with warming in 4 l. of H_2O, the solution cooled, and treated with concd. NH_3 in slight excess. After chilling the mixture for several hours at 5°, the alkaloid was removed by filtration, and the filtrate passed over a column of Dowex-50 in the acid phase. The clear effluent was free of NH_3 and quinine. It was evaporated *in vacuo* to crystallization; yield 12 g. The optical rotation of a 1% solution in water was zero. On recrystallization from water, 9 g. of pure *dl*-pyrrolidonedicarboxylic acid (A) was obtained, m.p. 210°.

The mother liquor from the original quinine precipitation was freed of quinine and of NH_3 by the resin as described, and yielded on evaporation *in vacuo* 82 g. of the crude B form. The material was optically inactive. Condensation of the mother liquor nearly to dryness yielded 4.5 g. of crystals, also optically inactive. Both fractions were separately crystallized from water, yielding, respectively, 67 g. (m.p. 230°) and 2.5 g. (m.p. 198°). The larger fraction was pure *dl*-pyrrolidonedicarboxylic acid (B). The smaller fraction was largely the corresponding A form. On treatment of 1.7 g. of the latter with 7.6 g. of quinine hydrate in 25 ml. of methanol, 7.3 g. of insoluble quinine salt rapidly precipitated. When 0.85 g. of the B form was treated with proportionate quantities of quinine and methanol, no precipitate was evident after standing several days at 5°.

A Preparation of *dl*-α-Aminotricarballylic Acid (B).—To 6 l. of an aqueous solution containing approximately 325 g. of synthetic α-aminotricarballylic acid as the hydrochloride, and brought to *p*H 5.5 by addition of dilute NaOH, there was added consecutively 2 l. of absolute ethanol and 1050 g. of copper acetate in 14.5 l. of H_2O. The resulting copper salt, when washed free of halide and dried, weighed 425 g. It was suspended in 8 l. of H_2O and decomposed with H_2S. The filtrate was evaporated *in vacuo* to about 300 ml. and deposited 125 g. of the crude A amino acid. On further condensation of the filtrate a second crop of crystals was obtained. The final filtrate was treated with an excess of copper acetate in water, and the precipitate which formed was largely the copper salt of the B form of *dl*-α-aminotricarballylic acid. It was washed and decomposed as usual with H_2S. On evaporation *in vacuo* to a very low bulk, no crystals appeared. Alcohol and acetone were added in excess, and the semi-solid mass of crude *dl*-α-aminotricarballylic acid (B) filtered and dried; yield 65 g.

After thorough drying, 38.2 g. (0.2 mole) of this material was suspended in the 10-fold amount of absolute ethanol and treated with dry HCl to saturation. The solution was refluxed for 1 hour, and evaporated *in vacuo* to a sirup. The residue was dissolved in ethanol and the esterification repeated. When kept in a vacuum desiccator for 1–2 weeks, the residual sirup crystallized as long needles. After recrystallization from ethanol–ether, the *dl*-α-aminotricarballylic acid triethyl ester hydrochloride (B) melted at 107°. Under the same conditions, the corresponding sirupy A form failed to crystallize.

Anal. Calcd. for $C_{12}H_{21}O_6N\cdot HCl$: N, 4.5; Cl, 11.4. Found: N, 4.7; Cl, 11.7.

It was not necessary to employ the crystalline triester hydrochloride for the subsequent steps, for the sirupy residue could serve as well. The latter was dissolved in 120 ml. of H_2O, neutralized to *p*H 3 with MgO, treated with 360 ml.

(26) The α-aminotricarballylic acids, whether A or B, racemic or optically active, possessed decomposition points which varied considerably with the rate of heating, and hence these data have been omitted.

of chloroform, and the mixture shaken at −10° alternately with 28.5 ml. (0.4 mol) of acetyl chloride and 16.1 g. of MgO. After completion of the reaction, the mixture was acidified to *p*H 1.7 with HCl, and the chloroform layer removed and washed successively with water, bicarbonate solution, and again with water. The chloroform solution was dried over Na_2SO_4, the solvent was removed, and the residue dissolved in a little ethyl acetate. On careful addition of petroleum ether, the crystalline N-acetyl-α-aminotricarballylic acid triethyl ester (B) separated. It was recrystallized from ethyl acetate–petroleum ether as soft needles. The yields varied from 31 to 40 g., m.p. 78°. Treatment of the corresponding A form in the same manner invariably led to oils which failed to crystallize.

Anal. Calcd. for $C_{14}H_{23}O_7N$: C, 52.9; H, 7.3; N, 4.4. Found: C, 52.5; H, 7.3; N, 4.5.

Ten grams of the crystalline acetyl triester derivative of the B form of α-aminotricarballylic acid was refluxed for 3 hours with 150 ml. of 2 *N* HCl. After removal of solvent *in vacuo*, the residual sirup was dissolved in about 50 ml. of water and the solution brought to *p*H 2.9 by addition of triethylamine. The solution was evaporated *in vacuo* to about 15 ml. and treated with an excess of alcohol together with a little acetone. The supernatant fluid was decanted, and the gummy residue dissolved in the minimum quantity of water. To this solution, maintained for several days at 5°, alcohol was carefully added in small amounts. Crystals of pure α-aminotricarballylic acid (B) slowly appeared as small, square plates; yield 2 g.

Conversion of A and B Forms of *dl*-α-Aminotricarballylic Acid to the Corresponding Pyrrolidone-α,β-dicarboxylic Acids.—47.7 mg. each of the A and B forms of *dl*-α-aminotricarballylic acid was dissolved in 25 ml. of hot water, the solutions quickly cooled to 25°, and 2-ml. aliquots distributed among a number of Van Slyke tubes. The tubes were simultaneously immersed in a boiling water-bath, individual tubes were removed at selected time intervals, and α-carboxyl nitrogen determined by the manometric ninhydrin–CO_2 procedure. α-Aminotricarballylic acid, like aspartic acid, should yield 2 moles of CO_2 per mole of amino acid. Actually, it yields about 5% more than the theoretical 2 moles of CO_2. The data showing the rapid ring closure of the compounds are given in Fig. 2. It is evident that such ring closure occurs more rapidly for the A than for the B amino acid, and that the conversion in both cases is nearly complete. Under practically the same conditions, glutamic acid reaches an equilibrium at 14% of the amino acid in about 24 hours, α-aminoadipic acid reaches an equilibrium at about 30% of the amino acid in 2 hours, whereas aspartic acid shows no ring closure at all. The equilibrium point for aminotricarballylic acid is at less than 0.5% of the amino acid. The m.p of *dl*-pyrrolidone-α,β-dicarboxylic acid (A) is 210°, and that for the corresponding B form is 230°. Both compounds are quantitatively transformed into the free α-amino acid when their solutions in 2 *N* HCl are refluxed for 2 hours.

Resolution of *dl*-α-Aminotricarballylic Acid (A).—A mixture of 19.1 g. of pure *dl*-α-aminotricarballylic acid (A) and 93.3 g. of recrystallized brucine hydrate was dissolved in a hot solution of 150 ml. of H_2O and 150 ml. of absolute ethanol, and to this solution 1050 ml. of hot ethanol was added with stirring and continuous heating. At the end of the addition the solution was clear. It was quickly cooled to 25° and allowed to stand at this temperature for 10 hours with some agitation and scratching. Crystals rapidly formed as flat, glistening plates. At the end of this period the crystals were removed by filtration and washed with 25–50 ml. of ethanol; yield 24 g. Two repetitions of this procedure each yielded 23 g. Collectively, these yields are designated P1. In all three cases and within a few minutes after the filtration procedure, the combined filtrate and washings began to deposit a crystalline precipitate in the form of long needles. This mixture was allowed to stand for 16 hours at 25°, and the crystals then filtered and washed with a little ethanol. Combined from all three runs, the crystals weighed 112 g. (P2). The mother liquors and washings also were all combined (F1) and set in a bath at −20°. The P2 fraction (112 g.) was recrystallized by dissolving in a hot mixture of 170 ml. of H_2O and 170 ml. of ethanol to which 1530 ml. of hot ethanol was subsequently added. On standing for 18 hours at 25°, 70 g. of fine needles separated (P3). The mother liquor from this crystallization was evaporated to dryness, and yielded a solid residue which was designated P4. After several days of standing at −20°, F1 had deposited a heavy precipitate (P5) weighing 44 g. The mother liquor and washings at this step were discarded.

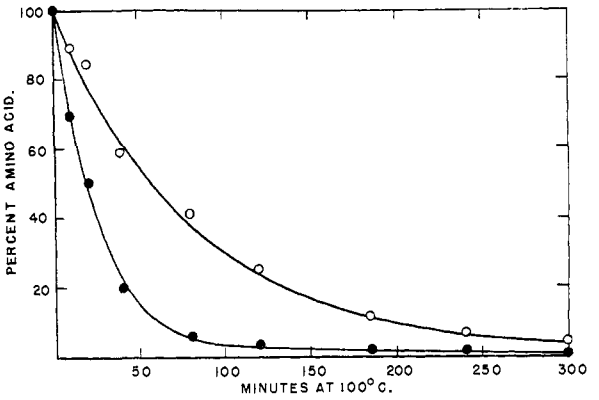


Fig. 2.—Conversion of *dl*-α-aminotricarballylic acid (A) ● and (B) ○ into the corresponding pyrrolidone-α,β-dicarboxylic acid in solutions immersed at 100° as a function of the time of heating.

Each of the crystalline P fractions was dissolved in water and treated with a slight excess of triethylamine. After removal of the liberated brucine, the filtrate and washings were passed through a column of Dowex-50 resin in the acid phase. The clear effluent, free of triethylamine and brucine was evaporated *in vacuo* until crystals appeared. After chilling at 5° for several hours, the crystalline, optically active α-aminotricarballylic acid was filtered and washed successively with cold water, alcohol, and ether. The various fractions yielded results as follows.

Brucine salt fraction	Wt. of fraction (g.)	Wt. of amino acid	$[\alpha]^{25}D$ of amino acid[a] (*c* 1, 5 *N* HCl
P1	70	18	−35°
P3	70	9.2	+35°
P4	40	3.6	+35°
P5	44	4.9	+35°

[a] These rotation values were obtained with a visual polarimeter.

Each of the fractions yielded optically pure α-aminotricarballylic acid (A), but this was largely due to the considerably greater insolubility in water of the optically active amino acid as contrasted with that of the racemic variety. The amino acid should constitute about 25% of its salt with brucine. Only in the case of the P1 fraction was that true. Among the other fractions, evaporation of the mother liquors yielded partially racemic products. From 57.3 g. of starting *dl*-α-aminotricarballylic acid (A), there was obtained 18 g. of the *l*-isomer, and 17.7 g. of the *d*-isomer, together accounting for a 62% recovery.

The influence of temperature on the initial crystallization of brucine salt was illustrated by placing the original solution of *dl*-α-aminotricarballylic acid (A) (19.1 g.) and hydrated brucine (93.3 g.) in dilute ethanol as above not at 25° but at 5° for approximately the same period of time. The crystalline precipitate which ensued weighed 51 g. On recrystallization from a mixture of 80 ml. of H_2O and 80 ml. of ethanol to which 800 ml. of boiling ethanol was added, there appeared after standing 18 hours at 25° a crop of 24 g. of the brucine salt as flat plates which yielded 6 g. of free amino acid with $[\alpha]^{25}D$ −35° (*c* 1, 5 *N* HCl); the mother liquor from this crystallization step yielded 14 g. of brucine salt which in turn yielded 3 g. of free amino acid with $[\alpha]^{25}D$ +35° (*c* 1, 5 *N* HCl). The original resolution mother liquor deposited after chilling at −20° a crystalline precipitate which weighed 27 g., and from which 2.8 g. of amino acid was obtained with $[\alpha]^{25}D$ +35° (*c* 1, 5 *N* HCl). Thus, from 19.1 g. of starting material, there was obtained 6 g. of the *l*-isomer, and 5.8 g. of the *d*-isomer, or substantially the same yield as described above.

The individual samples of each isomer were combined and

recrystallized by dissolving in warm HCl solution, filtering with aid of Norit, and adding triethylamine carefully to *p*H 2.9. The crystalline precipitate which rapidly appeared was filtered and washed successively with water, ethanol and ether. The recoveries were almost quantitative. For further purification, the amino acids were recrystallized from hot water, taking care to avoid prolonged heating of the solutions because of the ready conversion of these compounds to the corresponding pyrrolidonedicarboxylic acids.

When *l*-α-aminotricarballylic acid (A) was dissolved in the 20-fold amount of water and the solution refluxed for 3 hours, the corresponding pyrrolidone-α,β-dicarboxylic acid was nearly quantitatively formed (∠0.5% free amino acid), and the $[\alpha]^{25}D$ for the solution diluted to 1% was −58°. Similar treatment of the *d*-isomer of the amino acid resulted in $[\alpha]^{25}D$ +58°. Removal of the solvent in each case yielded a non-crystallizable oil as residue. This is in contrast with the behavior of the racemic A amino acid which, under the same conditions, readily formed the crystalline pyrrolidonedicarboxylic acid with m.p. 210°. Hydrolysis of the oily optically active isomers in 2 *N* HCl resulted in reconversion to the respective amino acids in quantitative amount (as determined by ninhydrin–CO_2 measurements) and with no loss in optical rotation. The unfavorable physical properties of the optically active isomers of the A form of pyrrolidonedicarboxylic acid discourage the use of this racemic variety as starting material for a practical resolution, although a resolution with brucine can be readily effected. Thus, a mixture of 17.3 g. of *dl*-pyrrolidone-α,β-dicarboxylic acid (A) and 93.3 g. of recrystallized brucine hydrate dissolved in 500 ml. of water with heating yielded on chilling 50 g. of a crystalline salt. This was recrystallized from water, whereby 40 g. of the salt was obtained. Brucine was liberated through the use of triethylamine and removed by filtration, and the filtrate passed through a Dowex-50 column. Evaporation of the effluent *in vacuo* led to a thick, residual sirup which, on stirring with a little cold water, yielded a precipitation of a small amount of crystals. The crystals were removed by filtration, and the filtrate evaporated again to a sirup. The $[\alpha]^{25}D$ of the sirup could only be approximately determined but appeared to be close to −58°. Brucine was removed from the original resolution filtrate as usual, and on evaporation there was obtained a mixture of oil and crystals. The crystals were removed as above, and yielded after drying an $[\alpha]^{25}D$ 0°. The residual sirup gave an $[\alpha]^{25}D$ close to +56°. When methanol was used as solvent in place of water, no resolution of the diastereoisomeric brucine salts could be achieved for both optical forms precipitated.

Resolution of *dl*-Pyrrolidone-α,β-dicarboxylic Acid (B).—A mixture of 24 g. of *dl*-pyrrolidonedicarboxylic acid (B) and 132 g. of recrystallized brucine hydrate in 200 ml. water was heated to solution. A little Norit was added, the solution was filtered clear and allowed to stand at 25° for 8 hours. At the end of this period, the resulting mass of crystals was filtered and washed with a little cold water, yield 75 g. It was recrystallized from the minimum amount of water and the solution allowed to stand at 25° for 12 hours. The crystalline precipitate weighed 58 g. (P). It was dissolved in about 2 l. of warm water, and the brucine precipitated by a slight excess of ammonia. The original resolution filtrate and washings were treated in the same way (S). Brucine in each mixture was removed by filtration, each filtrate was passed through a Dowex-50 resin column in the acid phase, and the respective effluents evaporated *in vacuo* to about 50 ml. Each of the condensed solutions was slowly condensed further by blowing a clear stream of air over its surface. The more insoluble racemate first crystallized from each solution. Racemic pyrrolidonedicarboxylic acid appears as soft needles, the optically active isomers as quite granular precipitates. As successive crops of crystals appeared on condensation of the solutions, they were removed by filtration without washing and their rotations determined at 1% concentration in water. From S, a total of 2.6 g. of crystals with $[\alpha]^{25}D$ 0° from 4 or 5 successive fractions was removed in this way before a crop of granular crystals weighing 0.53 g. and $[\alpha]^{25}D$ +52° was obtained. Thenceforward, the filtrate was evaporated completely to dryness, the residual crystals of optically and analytically pure *d*-pyrrolidonedicarboxylic acid brought to the filter with acetone and washed with acetone and ether; yield 5.4 g., m.p. 187°, $[\alpha]^{25}D$ +54.7° (*c* 1, H_2O). In the same way, P yielded a total of 1.0 g. of crystals with $[\alpha]^{25}D$ 0° from 2 or 3 successive fractions before a crop of granular crystals weighing 0.7 g. and $[\alpha]^{25}D$ −53° was obtained. Evaporation of the filtrate to dryness yielded 6.4 g. of pure *l*-pyrrolidonedicarboxylic acid, m.p. 187°, $[\alpha]^{25}D$ −54.6° (*c* 1, H_2O). The optically impure fractions were discarded. It is evident that the fractionation procedure is time-consuming, but with care quite pure optical isomers as judged by the physical criteria can be obtained. The yields of each isomer were about 50% of the theoretical.

The total recovery of the pure isomers was 11.8 g. from 24 g. of racemic starting material, or 49%. A preliminary study of this resolution procedure resulted in a recovery of the pure isomers to 34% based on the starting material. No resolution of the diastereoisomeric brucine salts of *dl*-pyrrolidonedicarboxylic acid could be observed when methanol or ethanol was used as solvent in place of water, for, like the case of the corresponding A form in the alcoholic solvents, the precipitate of brucine salt was always that of the racemate. With quinine in methanol, the nearly quantitatively precipitated salt of the A form is that of the racemate, whereas the salt of the corresponding B form is completely soluble; this difference has been employed (see above) to separate the racemic A and B forms of pyrrolidonedicarboxylic acid.

Preparation of the Optical Isomers of α-Aminotricarballylic Acid (B).—Refluxing of the optical isomers of pyrrolidone-α,β-dicarboxylic acid B in the fifteen-fold amount of 2 *N* HCl solution for 2 hours resulted in a quantitative conversion to α-aminotricarballylic acid (B) as measured by the manometric ninhydrin–CO_2 procedure. The respective solutions were evaporated *in vacuo* to remove excess HCl, the sirupy residues taken up in H_2O, and the solutions brought to *p*H 2.9 with LiOH. With 5 g. of starting material, the volume at this stage was generally 80 ml. The solution was evaporated to dryness by blowing a stream of clean air on its surface, and the sirupy residue transformed into a slightly gummy white solid mass by addition of ethanol. The alcohol was removed and, on stirring the solid with a little cold water, solution occurred followed almost immediately by the separation of needle-like crystals. The product was filtered and washed successively with cold water, alcohol and ether. The yields were quite variable, ranging from 10 to 45% of the expected, based on the amount of pyrrolidonedicarboxylic acid taken as starting material. Better results were obtained when these products were used to seed the freshly neutralized solutions above, for during the subsequent slow evaporation, the amino acid isomers separated as large, granular crystals which were more insoluble in water than the materials used for the seeding. It is advisable occasionally to agitate the mixture of crystals and solution during the evaporation in order to obtain good yields. The evaporation was discontinued when the residual solution was condensed to about one-tenth of its original volume. The yields of washed and dried products were about 70% of the expected. *dl*-α-Aminotricarballylic acid (B) is much more soluble than the corresponding optically active forms, and thus any traces of racemic amino acid carried through from the starting pyrrolidonedicarboxylic acid would remain in the mother liquor. For complete purification the amino acids were recrystallized from hot water, taking care to avoid prolonged heating of the solutions because of ring closure of the compounds to the corresponding pyrrolidonedicarboxylic acids. The latter are very soluble in water and would not contaminate the crystals, but their formation would reduce the recovery of crystalline amino acid. As in the case of the corresponding A forms, the sign of optical rotation in water is maintained in the amino acid–pyrrolidonedicarboxylic acid transformation, for the *l*-pyrrolidonedicarboxylic acid yielded the *l*-amino acid, and similarly the *d*-isomer yielded the *d*-amino acid.

Preparation of the Stereoisomers of Isocitric Acid Lactone.—These were prepared by the action of nitrous acid on the corresponding α-amino acids. Solutions in 60 ml. 1 *N* HCl were prepared for 2 g. of *l*-α-aminotricarballylic acid (A) and for its *d*-isomer. To each of the chilled solutions was added 6.2 g. of $AgNO_2$ in portions and with shaking, and the mixture allowed to stand at 5° for 24 hours. The silver chloride was removed by filtration and washed with ice-water, and the combined filtrate and washings gassed with H_2S. The colloidal sulfur was removed by filtration with the aid of Norit, and the filtrate evaporated *in vacuo* to a sirup. On standing 2 days at 25° in a vacuum desicca-

tor over P_2O_5 and NaOH the sirup crystallized. The solid was dissolved in a little warm ethyl acetate, the solution filtered from a small amount of flocculent material, and the filtrates treated with petroleum ether. On stirring and scratching, crystals rapidly formed. These were filtered, redissolved in ethyl acetate, again filtered from a small amount of undefined material, and precipitated again with petroleum ether. Repetition of the procedure finally yielded 1.1–1.3 g. of each isomer. The *l*-isomer of the amino acid yielded the *l*-isocitric acid lactone and, similarly, the *d*-isomer yielded the *d*-lactone. *dl*-α-Aminotricarballylic acid (A) treated in the same way yielded 1 g. of pure lactone from 2 g. of starting material.

A solution of 2 g. of *dl*-pyrrolidone-α,β-dicarboxylic acid (B) in 30 ml. of 2 *N* HCl was refluxed for 2 hours, the solution diluted with 30 ml. of H_2O, chilled, and treated with 6.7 g. of $AgNO_2$ as above. Silver chloride and solvent were removed, and the residual mixture after standing 8 days at 25° in the desiccator was heated at 100° *in vacuo* for 30 minutes. The solid residue was dissolved in hot ethyl acetate, the solution decolorized with Norit, and the filtrate evaporated to a small volume and treated with a little chloroform. The resulting crystals were removed, and the procedure repeated. Yield of pure lactone was 0.25 g.

The low yield of the lactone from the racemic B compound prompted the use of baryta in order to isolate the optically active lactone isomers. One gram of either the *l*- or *d*-isomer of α-aminotricarballylic acid (B) was dissolved in 30 ml. of 1 *N* HCl, the solution was chilled, and treated portionwise with 3.5 g. of $AgNO_2$ over a period of 30 minutes. The mixture was allowed to stand at 25° for another 30 minutes, the AgCl removed by filtration and washed with a little cold water. The combined filtrate and washings were evaporated to a thick yellow sirup by blowing a stream of air over the solution, and the flask with its contents placed on a desiccator and dried over P_2O_5 and NaOH *in vacuo* for 48 hours. At the end of this time, the semisolid residue was dissolved in 50 ml. of H_2O and the solution treated with a few drops of phenolphthalein in alcohol. Saturated baryta solution was added to a pink color and the mixture placed in a boiling water-bath. As the lactone opened the dye decolorized, and therefore a few drops of baryta solution were added from time to time in order to maintain the *p*H at about 8.5. After the pink color was maintained, the mixture was further heated for 30 minutes in the boiling water-bath, with exclusion of atmospheric CO_2. The barium isocitrate was filtered and washed with water, alcohol and ether, and finally dried in the pistol at 100° for several hours. The yields of barium salt were rather variable, two preparations of the *l*-isomer yielding 60 and 70%, and two preparations of the *d*-isomer yielding 60 and 85%, of the theoretical. The barium salts were dissolved in warm 0.5 *N* HCl, and the solutions diluted with water so that the final normality of the HCl was 0.075. These solutions were run through a column of Dowex-50 resin in the acid phase, and the resin subsequently washed with water. The combined effluent and washings, now free of Ba^{++}, was evaporated to dryness *in vacuo* and heated in the vacuum at 100° for 24 hours. The flask with its crystalline contents was transferred to a vacuum desiccator and heated at 105° over P_2O_5 and NaOH for 2 hours. After cooling, the crystalline residue was dissolved in a little hot dry ethyl acetate, the solution decolorized with the aid of Norit, and the filtrate heated on the hot plate to a low bulk. Chloroform was carefully added to the hot solution to a slight turbidity. On cooling, the lactone rapidly crystallized. After standing for several hours at 25°, the crystals were brought to the filter with a 50:50 mixture of ethyl acetate and chloroform, and were washed with chloroform. The yield was generally 50–70% of the theoretical, based on the amount of barium salt taken. The lactones were recrystallized in the same manner, but the melting point (154°) did not change.

The optically active isocitric acid lactones of both A and B varieties were each dissolved in 3 equivalents of 0.5 *N* NaOH and the resulting solutions kept in a boiling water-bath for 30 minutes. The solutions were then cooled to 25° and the optical rotations of the trisodium salts measured. To each solution exactly 3 equivalents of 1 *N* HCl were added, and the rotations of the resulting free isocitric acid isomers measured again. The optical data are given in Table I.

Action of Isocitric Acid Dehydrogenase on the Isomeric Isocitric Acids.—The four optically-active stereoisomers of isocitric acid lactone and the two racemic modifications were each converted to isocitric acid by heating with 3 moles of NaOH for 30 minutes in a boiling water-bath. The enzymatic assay was that of Grafflin and Ochoa,[27] using an acetone powder prepared from pig heart. A Cary recording spectrophotometer was used at 340 mμ, the cells containing 0.5 μmole of TPN (ox.) and 0.25 μmole of isocitrate in a volume of 3 ml. A known preparation of natural isocitric acid lactone served as standard. Only two of the six preparations studied reacted in the enzymatic system, namely, *l*-isocitric acid lactone B to 100%, and *dl*-isocitric acid lactone B to 50%. The others were completely inert. Also inert in this system were the *l*- and *d*-aminotricarballylic acids (B).

Barium Salts.—When 10 ml. of saturated baryta solution was added to 20 ml. of a 1% solution in water of *dl*-aminotricarballylic acid A or B at 25° there occurred an immediate precipitate of the barium salt of the B amino acid whereas the solution of the A amino acid remained clear. No further change occurred in the latter solution after several hours of standing at 25°, but when it was brought to the boiling temperature and maintained there for 2–3 minutes a dense white precipitate of the barium salt separated. Both precipitates were filtered at the pump and washed with a little water. The salt of the B amino acid dissolved in the wash water, that of the A amino acid did not. The combined mother liquor and washings related to the B amino acid was brought to the boiling temperature and maintained there for 2–3 minutes when, as in the case of the A form, a dense white precipitate formed during the heating and separated from solution. Again, like the A form, this precipitate was not soluble in water and could be washed with large volumes of fluid. When a more dilute solution of the B amino acid was treated with baryta at 25° no precipitate appeared, but when the solution was brought to a boil a dense precipitate separated. A more concentrated solution than 1% of A was not advisable to attempt because of the danger of pyrrolidonedicarboxylic acid formation being brought about by the necessary heating to bring the amino acid into solution. It will appear that heating of the solutions of the amino acids with baryta produced an insoluble modification of the barium salts. The salts so formed of the A and B amino acids were washed not only with water but also with alcohol and ether. They were dried at 100° *in vacuo* for several hours but still retained water of crystallization. The yields from 200 mg. each of A and B amino acids were, respectively, 325 and 300 mg. Analysis revealed the presence of 1.5 molecules of water of crystallization in a compound of 3 molecules of barium to 2 of amino acid.

Anal. Calcd. for $C_{12}H_{12}O_{12}N_2Ba_3 + 1.5H_2O$: Ba, 50.6; N, 3.4. Found (for the A form): Ba, 50.7; N, 3.2. Found (for the B form): Ba, 50.6; N, 3.4.

Under the same conditions (*i.e.*, 1% solution in water), pyrrolidonedicarboxylic acids A and B gave no precipitate with baryta at 25°. When the solution of the B form was rapidly brought to a boil the Ba salt precipitated from the hot solution. The yield was 570 from 400 mg. of starting material. It was dried at 100° *in vacuo* for several hours.

Anal. Calcd. for $C_6H_5O_5NBa + 0.5\ H_2O$: Ba, 43.3; N, 4.4. Found: Ba, 43.1; N, 4.2.

The solution of the A stereoisomer with the same relative amount of baryta remained clear even after it had been boiled for several minutes; on chilling for several days at 5° a small amount of precipitate appeared.

Racemization of α-Aminotricarballylic Acid with Alkali.—Solutions of 1% concentration in 3 *N* NaOH were prepared for the *l*-isomers of the A and B forms of aminotricarballylic acid. The $[\alpha]^{25}D$ values noted were very close to those of −76.3 and 5.0° which, respectively, had been observed in 1 *N* NaOH (Table I). On refluxing the solutions up to 25 hours, the diminution in the rotation of the A amino acid appeared to follow a linear course, in that at 3 hours the $[\alpha]^{25}D$ was −71.0°, at 10 hours it was −62.5°, and at 25 hours it was −46.3°. The last-mentioned figure represented about a 38% diminution in the optical rotation of the original solution. The change in the rotation of the B form was more difficult to follow because of the small value of the angular rotation, but at 9 hours the $[\alpha]^{25}D$ appeared to be

(27) A. L. Grafflin and S. Ochoa, *Biochem. Biophys. Acta*, **4**, 205 (1950).

+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.

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[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

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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

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