[CONTRIBUTION FROM THE DEPARTMENTO DE QUÍMICA DA FACULDADE DE FILOSOFIA, CIÊNCIAS E LETRAS DA UNIVERSIDADE DE SÃO PAULO]

The Synthesis of Cystine-β,β'-dicarboxylic Acid[1]

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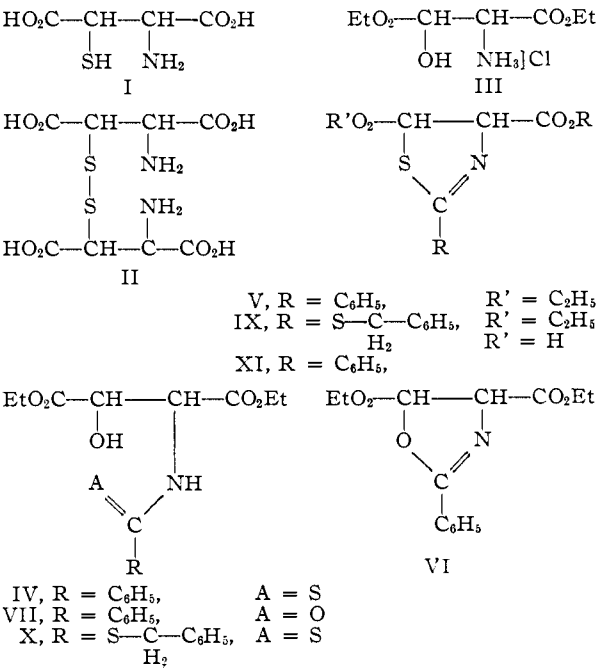
Cystine-β,β'-dicarboxylic acid was synthesized from hydroxyaspartic acid *via* 2-phenyl-4,5-dicarbethoxy-Δ²-thiazoline, 2-phenyl-4,5-dicarbethoxy-Δ²-oxazoline being obtained as a by-product. The mechanism of formation and the steric configurations of the compounds are discussed.

Mercaptoaminodicarboxylic acids or their disulfides appear to be unknown. Therefore, we investigated the possibilities of synthesizing the simplest compound of this group: cysteine-β-carboxylic acid (mercaptoaspartic acid) (I) or its disulfide, cystine-β,β'-dicarboxylic acid (II). We took advantage of Elliot's[2] observation "that replacement of the β-hydroxyl group by a thiol group occurred when N-thiobenzoylserine methyl ester was treated with thionyl chloride." This observation permitted the synthesis of cystine,[3] not only from serine but also from the N-thiobenzoyl and N-dithiobenzoxy derivatives of hydroxymethylaminomalonic ester. In an analogous manner, a thiol group should be substituted for the hydroxyl in hydroxyaspartic acid.

We prepared this compound in the form of its diethyl ester hydrochloride (III) from chloromalic acid, which, following Kuhn's directions,[4] was obtained from maleic anhydride *via* epoxysuccinic acid as the pure *erythro* isomer[4] in 47% yield. By treatment with aqueous ammonia, chloromalic acid was transformed into hydroxyaspartic acid[5] which was isolated as its monosodium salt in 65% yield. It could be shown[6] that the acid was the *erythro* isomer, already described by Dakin.[5] The sodium salt, by refluxing with alcoholic hydrochloric acid,[7] was transformed in 68% yield into diethyl hydroxyaspartate hydrochloride, which melted at 154–156°.

When this compound was allowed to react with S-thiobenzoylthioglycolic acid,[8] diethyl N-thiobenzoylhydroxy aspartate (IV) which melted at 98–99°, was obtained in 90% yield. By treatment with thionyl chloride 2-phenyl-4,5-dicarbethoxy-Δ²-thiazoline (V) was formed as a yellow oil, in 60% yield, and the corresponding oxazoline (VI) of m.p. 97.5–99°, in 40% yield. However, formation of the latter could be avoided when phosphorus pentachloride was used for cyclization instead of thionyl chloride, whereby the yield of 2-phenyl-4,5-dicarbethoxy-Δ²-thiazoline (V) was raised to 90%.



Acid hydrolysis which was employed successfully in the synthesis of cystine[3] was complicated in the case of 2-phenyl-4,5-dicarbethoxy-Δ²-thiazoline by the rather peculiar properties of the resulting mercaptoaspartic acid (I). Its hydrochloride is extremely hygroscopic and therefore not suitable for isolation. Furthermore, as a derivative of isocystine it has an especially labile thiol group.[9] Even by decomposition of the insoluble lead salt with hydrogen sulfide, no pure product could be obtained. Therefore, the substance resulting from acid hydrolysis was oxidized with iodine in order to obtain cystine-β,β'-dicarboxylic acid (II). The products obtained by this procedure under different conditions were apparently uniform but had always a low sulfur content.

When using 2-benzylthio-4,5-dicarbethoxy-Δ²-thiazoline hydrochloride (IX), obtained in 70% yield by the reaction of diethyl hydroxy aspartate hydrochloride with carbon disulfide and benzyl chloride and treating the resulting N-thiobenzoxyhydroxy aspartate (X) with thionyl chloride, acid hydrolysis did not yield pure cystine-β,β'-dicarboxylic acid (II) either, the yields of the crude product being considerably lower than those obtained from the phenyl derivative V.

(1) The results published in this paper are taken from a thesis to be presented by Horst Berl to the Faculdade de Filosofia, Ciências e Letras da Universidade de São Paulo in partial fulfillment of the requirements for obtaining the degree of Dr. in Science. Part of these results was presented to the 5th Congress of the Sociedade Brasileira para o Progresso da Ciência in Curitiba, November, 1953.

(2) D. F. Elliot, *Nature*, **162**, 658 (1948).

(3) J. C. Crawhall and D. F. Elliot, *J. Chem. Soc.*, 2071 (1951).

(4) R. Kuhn and Th. Wagner-Jauregg, *Ber.*, **61**, 504 (1928). There the expressions *meso* and *racemic* are used for *erythro* and *threo*.

(5) H. D. Dakin, *J. Biol. Chem.*, **48**, 273 (1921); **50**, 410 (1922).

(6) H. Hauptmann and H. Berl, previous communication to the 11th Congress of the Associação Brasileira de Química, São Paulo, July, 1954.

(7) Th. Curtius and F. Göbel, *J. prakt. Chem.*, **37**, 150 (1888); E. Fischer, *Ber.*, **34**, 436 (1901).

(8) This compound was prepared following the directions of Crawhall and Elliot.[3]

(9) A. Schöberl and H. Braun, *Ann.*, **542**, 274 (1939).

Eventually all difficulties were overcome by hydrolyzing the free 2-phenyl-Δ^2-thiazoline-4,5-dicarboxylic acid (XI) instead of its diethyl ester (V). When 2-phenyl-4,5-dicarbethoxy-Δ^2-thiazoline (VI) was saponified with potassium hydroxide, it gave the well-crystallized dipotassium 2-phenyl-Δ^2-thiazoline-4,5-carboxylate (XI). From this the free acid was prepared, which could be purified by recrystallization, melting then at 192–194°. By hydrolysis with hydrochloric acid followed by oxidation with iodine in alcohol it was transformed into cystine-β,β'-dicarboxylic acid (II) in 65% yield. This compound melted at 163–166° with slight decomposition, was hygroscopic, insoluble in organic solvents and gave, as expected, the reactions of cystine and isocystine. Several stereoisomers might be expected, and their presence may explain why attempts to obtain crystalline derivatives have failed.

As pointed out by Crawhall and Elliot[10] the conversion of N-thiobenzoylhydroxyaspartate (IV) into 2-phenyl-4,5-dicarbethoxy-Δ^2-thiazoline (V) by the action of thionyl chloride is accompanied by inversion at the carbon atom which bears the hydroxyl group in analogy to the cyclization of N-benzoylallothreonine.[11] Inversion also occurs when phosphorus pentachloride is used. A chlorosulfinic ester containing the group –O–SOCl was considered as a possible intermediate in the cyclization with thionyl chloride.[10,11] Therefore, transient formation of a compound with a –O–PCl_4 or a similar grouping[12] may be assumed in the reaction with phosphorus pentachloride. This idea is supported by Bergmann's observation that during cyclization of 1-benzamido-2-hydroxy-3-benzoxypropane with phosphorus oxychloride a phosphorus-containing intermediate was formed, which with pyridine gave readily the corresponding oxazoline.[13]

Crawhall and Elliot[10] postulate oxazolines as intermediates in order to explain certain side reactions which occur during cyclizations in the thiazoline field, without however having been able to isolate them. The simultaneous formation of a thiazoline and an oxazoline derivative in our case represents definite evidence for their idea that, during the cyclizations with thionyl chloride two mechanisms may be operating. Either rear attack of the sulfur atom on the carbon which bears the hydroxyl group occurs or the oxygen of the nucleophilic hydroxyl group adds to the carbon of the thiocarbonyl group.[14] The hypothesis that in this case the thionyl chloride reacts with the sulfur atom holds also for our reaction and is strengthened by the observation that oxazoline formation is accompanied by appearance of elementary sulfur. Since in the second type of reaction the carbon–oxygen bond is not affected, 2-phenyl-4,5-dicarbethoxy-Δ^2-oxazoline (VI) is formed with retention of configuration and differs from the thiazoline as for its configuration about carbon 5. Indeed, it could be obtained by reaction of diethyl hydroxyaspartate hydrochloride with benzimido ethyl ether, a reaction in which no inversion occurs.[15]

We started with *erythro*-hydroxyaspartic acid and no other inversion is likely to occur during the reactions which lead to cystine-β,β'-dicarboxylic acid, through mercaptoaspartic acid. This compound should, therefore, have the *threo* configuration.

Work on these compounds as well as on other mercaptoaminodicarboxylic acids is being continued.

Acknowledgment.—We are indebted to the Rockefeller Foundation for a grant supporting this work and wish to express our gratitude to the Orquima S. A., São Paulo, Brazil, for a fellowship granted to one of us (H.B.). All microanalyses were performed by Miss Maria Luiza Miranda Valle, whose efficient collaboration we gratefully acknowledge.

Experimental[16]

Diethyl Hydroxyaspartate Hydrochloride. (a) **Monosodium Hydroxyaspartate.**—A mixture of 10 g. (0.06 mmole) of chloromalic acid and 100 ml. of ammonia was heated in a pressure bomb to 100° for 10 hr. The solution was then added to a solution of 2.5 g. of sodium hydroxide in 10 ml. of water and concentrated *in vacuo* to a volume of 20 ml. Then 2 ml. of acetic acid was added and the mixture dried at 80°. After washing twice with ice-water there remained 6.6 g. (65%) of a white crystalline salt which was redissolved in water and reprecipitated with methanol, m.p. 265–268° with dec. *Anal.* Calcd. for $C_4H_6O_5NNa$: N, 8.18; Na, 13.5. Found: N, 7.74; Na, 14.

(b) **Diethyl Hydroxyaspartate Hydrochloride.**—To 20 g. of the monosodium salt 100 ml. of 10% alcoholic hydrochloric acid was added and the mixture shaken until it became milky. Then it was saturated with gaseous hydrogen chloride and refluxed gently for 3 hours. After saturating again with gaseous hydrogen chloride, the mixture was kept in the ice-box overnight. The crystalline precipitate was filtered off and washed with acetone. The mother liquors were diluted with ether (two times its volume) whereby another crop of the ester hydrochloride was obtained; yield 19 g. (68%), m.p. 154–156°. *Anal.* Calcd. for $C_8H_{16}O_5NCl$: N, 5.80; HCl, 15.12. Found: N, 5.73; HCl, 14.93 (by titration in presence of formol). The substance is not hygroscopic, soluble in water, methanol, ethanol, slightly soluble in acetone and insoluble in ether.

Diethyl N-Thiobenzoylhydroxyaspartate.—To a solution of 3 g. (12.5 mmoles) of diethyl hydroxyaspartate hydrochloride in 15 ml. of pyridine, first 5 ml. (3.6 g., 35 mmoles) of triethylamine was added and after cooling to 10° 2.8 g. (13 mmoles) of S-thiobenzoylglycolic acid.[8] After standing at room temperature for one day, the mixture was poured into 180 ml. of 10% sulfuric acid covered with 100 ml. of ether. After extracting thoroughly the ethereal layer was separated, washed once with water, twice with dilute sodium bicarbonate solution and again with water, dried with sodium sulfate and evaporated to dryness. The yellow crystalline substance after recrystallizing from 90% ethanol melted at 98–99°, yield 3.6 g. (90%). *Anal.* Calcd. for $C_{14}H_{19}O_5SN$: N, 4.30; S, 9.85. Found: N, 4.13; S, 10.20.

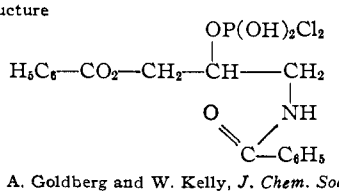
2-Phenyl-4,5-dicarbethoxy-Δ^2-thiazoline and Diethyl 2-Phenyl-4,5-dicarbethoxy-Δ^2-oxazoline. (a) **With Thionyl**

(10) J. C. Crawhall and D. F. Elliot, *J. Chem. Soc.*, 3094 (1952).

(11) J. Attenburrow, D. F. Elliot and J. F. Penny, *ibid.*, 311 (1948).

(12) (a) W. Hückel and H. Pietrzok, *Ann.*, **540**, 250 (1939); (b) L. Anschütz and W. Broeker, *Ber.*, **59**, 2848 (1926).

(13) M. Bergmann and E. Braud, *ibid.*, **56**, 1280 (1923). This intermediate has the molecular formula $C_{17}H_{18}O_6NPCl_2$ and is most likely to have the structure



(14) See also A. Goldberg and W. Kelly, *J. Chem. Soc.*, 1919 (1948).

(15) D. F. Elliot, *ibid.*, 589 (1949).

(16) All melting points are uncorrected. The method of A. Schöberl, R. Jaczynski and P. Rambacher, *Angew. Chem.*, **50**, 334 (1937), was used for sulfur analysis.

Chloride.—Four g. of diethyl N-thiobenzoylhydroxyaspartate (12.5 mmoles) was added at 5° in small portions during 20 min. to 10 ml. of thionyl chloride. The mixture was kept 30 min. at 5° and 30 min. at room temperature and then the excess of thionyl chloride removed *in vacuo*. The residue was poured into 200 ml. of a saturated sodium bicarbonate solution covered with 100 ml. of ether. After shaking throughly the aqueous phase was extracted once again with 30 ml. of ether, the ether extracts dried with sodium sulfate and the ether distilled off. The remaining mixture of oil and crystals was treated with petroleum ether (b.p. 60–80°) and the resulting white crystals separated. The yellow oily residue was distilled *in vacuo* boiling at 164°, 0.3 mm.; yield 1.6 g. (60%). *Anal.* Calcd. for $C_{15}H_{17}O_4NS$: N, 4.56; S, 10.41. Found: N, 4.18; S, 10.11.

The white crystals were recrystallized first from petroleum ether (b.p. 60–80°) then from a mixture of 9 volumes of petroleum and 1 volume of benzene; yield 1 g. (40%), m.p. 97.5–99°. *Anal.* Calcd. for $C_{15}H_{17}O_5N$: N, 4.81; C, 61.84; H, 5.88. Found: N, 4.67; C, 61.75; H, 5.66.

(b) **With Phosphorus Pentachloride.**—A solution of 2 g. (6 mmoles) of diethyl N-thiobenzoylhydroxyaspartate in 40 ml. of chloroform was cooled in an ice-bath and then 3 g. (14.5 mmoles) of phosphorus pentachloride added in small portions shaking after each addition until solution was completed. After standing for 30 min. in an ice-bath and 30 min. at room temperature, the chloroform solution was washed with saturated sodium bicarbonate solution, the chloroform dried with sodium sulfate and evaporated to dryness. There remained a yellow oil but no white crystals were obtained; yield 1.7 g. (90%) with the same properties as the oil obtained with thionyl chloride.

2-Phenyl-4,5-dicarbethoxy-Δ^2-oxazoline.—The solution of 2 g. of diethyl hydroxyaspartate hydrochloride in 8 ml. of water was brought to *p*H 3 with ammonia. An ether solution of benzimido ethyl ether (prepared from 1.5 g. of benzimido ethyl ether hydrochloride) was added and mixture stirred for 12 hours, whereby it turned red. The ether layer was separated, the aqueous solution washed with 10 ml. of ether, the combined ether solutions washed with water and evaporated to dryness. The remaining oil crystallized. The crystals (1.1 g., 45%, m.p. 96–98.5°) were recrystallized from 50% alcohol melting then at 98–99.5°. This m.p. was not depressed by admixture of diethyl 2-phenyl-4,5-dicarbethoxy-Δ^2-oxazoline, m.p. 97.5–99°, obtained by cyclization of diethyl N-thiobenzoylhydroxyaspartate with thionyl chloride.

Dipotassium 2-Phenyl-Δ^2-thiazoline-4,5-dicarboxylate.—Two and five-tenths ml. of 20% potassium hydroxide in abs. alcohol (11 mmoles) was added to a solution of 1 g. (3.3 mmoles) of 2-phenyldicarbethoxy-4,5-Δ^2-thiazoline. After about 30 sec. a white precipitate appeared from the yellow solution which became warm at the same time. After standing for 1 hour the precipitate was centrifuged, washed with small portions of alcohol until no yellow color appeared any more and dried; yield 0.90 g. (85%) of white very hygroscopic needles which were purified by liquefying them with a few drops of water and reprecipitating with absolute alcohol. They decomposed at 252–258° without melting. *Anal.* Calcd. for $C_{11}H_7O_4NSK_2$: N, 4.28; K, 23.84. Found: N, 4.08; K, 23.60.

The same substance was obtained by adding stoichiometric amounts of alcoholic potassium hydroxide to an alcoholic solution of 2-phenyl-Δ^2-thiazoline-4,5-dicarboxylic acid.

2-Phenyl-Δ^2-thiazoline-4,5-dicarboxylic Acid. (a).—To the chilled solution of 1 g. of potassium 2-phenyl-Δ^2-thiazoline-4,5-dicarboxylate in 10 ml. of water concd. hydrochloric acid was added until the *p*H was 2. After 3 hours in the ice-box the white precipitate was filtered off and washed with ice-water; yield 0.7 g. (90%), m.p. 192–194° with dec.

(b).—The aqueous solution of 1 g. of potassium 2-phenyl-Δ^2-thiazoline-4,5-dicarboxylate in 10 ml. of water was covered with 15 ml. of ethyl acetate and then acidified with concd. hydrochloric acid to *p*H 2. After thorough shaking the ethyl acetate was separated, the aqueous layer extracted again with 10 ml. of the same solvent. The joined ethyl acetate solutions were dried with sodium sulfate, treated with charcoal and evaporated to dryness. The residue (0.6 g., 80%) was washed with ether in order to remove a small yellow impurity then dissolved in 15 ml. of alcohol, treated with charcoal and reprecipitated with 35 ml. of ice-cold water, m.p. 192–194°. *Anal.* Calcd. for $C_{11}H_9O_4NS$: N, 5.57; S, 12.74. Found: N, 5.38; S, 12.47.

The acid is insoluble in benzene and chloroform, slightly soluble in cold water and ether, soluble in alcohol, acetone and ethyl acetate. Hot water dissolves it fairly well but decomposes it, hydrolyzing the thiazoline ring as shown by the blue color which appeared with $FeCl_3$ in HCl solution.

Cystine-β,β'-dicarboxylic Acid.—A solution of 0.5 g. of 2-phenyl-Δ^2-thiazoline-4,5-dicarboxylic acid in 15 ml. of 3*N* hydrochloric acid was refluxed for 3 hours.

The solution was extracted twice with 10 ml. of benzene. The aqueous solution was diluted with 80 ml. of absolute alcohol and then a 5% solution of iodine in alcohol added until the iodine color persisted. Ethylene oxide[17] was then passed through the solution until it became turbid. It was then kept in the ice-box for 24 hours, and the precipitate formed was separated by centrifugation and washed 3 times with alcohol.

After drying *in vacuo* there remained 0.22 g. (65%) of a white substance which melted at 163–166° with slight decomposition. It is very soluble in water and insoluble in organic solvents. *Anal.* Calcd. for $C_8H_{12}O_8N_2S_2$: C, 29.25; H, 3.66; N, 8.54; S, 19.50. Found: C, 29.36; H, 3.76; N, 8.53; S, 19.29.

When heated with alkaline plumbite solution to 50° lead sulfide was formed after 30 sec.[9] With ninhydrin a reddish violet color appeared. Bromine in water was immediately reduced.

Hydrolysis of 2-Phenyl-4,5-dicarbethoxy-Δ^2-thiazoline with Hydrochloric Acid.—The general procedure was as follows. The thiazoline derivative was refluxed with 3 *N* hydrochloric acid for the time indicated in Table I. The acid solution was then extracted with benzene in order to remove benzoic acid and then evaporated to dryness *in vacuo*. The substance thus obtained proved, when developed with ninhydrin, to be a mixture of at least 4 substances in a paper chromatogram (solvent acetic acid:butanol:water 1:4:5). It was treated with lead acetate, the resulting lead salt being washed and dried. *Anal.* Calcd.: Pb, 38.8. Found: Pb, 38.7. The lead salt was suspended in water and a stream of hydrogen sulfide passed through. The lead sulfide formed was filtered off and the solution evaporated to dryness *in vacuo*. The resulting white substance was extremely hygroscopic and could not be purified by recrystallization. It gave the following reactions. With ferric chloride in hydrochloric acid a deep blue color was developed, whereas in ammonia a reddish violet color appeared. Alcoholic iodine solution was immediately reduced. With copper sulfate a brown precipitate was immediately formed.[9]

The substance was then redissolved in alcohol and 5% alcoholic iodine solution added until a straw-yellow color persisted. Then a stream of ethylene oxide was passed through the solution, until a white precipitate appeared and then kept in the ice-box. The precipitate was filtered off, washed with alcohol, dried and analyzed. The values obtained and the melting points can be seen from Table I. The substances gave the reactions of cystine-β,β'-dicarboxylic acid as indicated in the former experiment.

TABLE I

Hydrolyzed, hr.	Spots on paper	Yield, %	S, %	M.p. dec., °C.
1.25	4	28	13.8	150–165[a]
1.25	1	28	15.5	161–165[b]
4.5	1	56	17.5	155–159
12	1	49	18.3	170–172

[a] Without purification. [b] Purified as Pb salt.

2-Benzoylmercapto-4,5-dicarbethoxy-Δ^2-thiazoline Hydrochloride.—To a solution of 3 g. of diethyl hydroxyaspartate hydrochloride (12.5 mmoles) in dry pyridine first 3.3 ml. of triethylamine (2.4 g. 24 mmoles) and after precipitation of the triethylamine hydrochloride, 1.5 ml. (1.9 g., 25 mmoles) of carbon disulfide were added in an ice-bath. The mixture was kept at 0° for 4 hours and then 1.5 ml. of benzyl chloride (1.65 g., 13 mmoles) added. After keeping at 0° for 18 more hours the reaction mixture was

(17) H. Hauptmann, P. T. Adams and B. Tolbert, THIS JOURNAL, **74**, 2962 (1952). The procedure had been suggested by Prof. M. Calvin.

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

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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).