TABLE II

KETONES

R_1—CO—R_2 R_1 = Tolyl	R_2 = Tolyl	B. p., °C. 740 mm.	Red. p.	Mm.	d^{20}	$n^{20}D$	2,4-Dinitrophenylhydrazone M. p., °C.	N,[g] %
o	*o*[a]	310 (m. p. 75)	134–135	1			194	14.31
o	*m*[b]	319	135–136	1	1.0669	1.5895	208	14.41
o	*p*[c]	316	140–141	2	1.0586	1.5924	218	14.40
m	*m*[d]	329	141–142	1	1.0681	1.5926	223	14.29
m	*p*[e]	330 (m. p. 71)	150–152	3			215	14.36
p	*p*[f]	335 (m. p. 92)	150–152	2			220	14.38

[a] *Bull. soc. chim.*, 11, 373 (1944), gives m. p. as 71°, b. p. as 308°; *Rec. trav. chim.*, 1109 (1940), gives 2,4-dinitrophenylhydrazone as 190°. [b] THIS JOURNAL, **63**, 1573 (1941), gives the 2,4-dinitrophenylhydrazone m. p. as 204–207°. [c] *Ber.*, **36**, 2025 (1903) gives b. p. as 316–318° and oxime m. p., as 122°; the oxime of our ketone melts at 121–122°. [d] *Rec. trav. chim.*, 1109 (1940), gives the 2,4-dinitrophenylhydrazone m. p. as 233°. [e] *Ber.*, **36**, 2025 (1903), gives the b. p. as 328–330° and semicarbazone m. p. as 183°. The semicarbazone of the ketone prepared in this investigation melts at 183°. [f] *Ber.*, **10**, 2174 (1877), gives the b. p. as 333° and m. p. as 92–95°. *Rec. trav. chim.*, **59**, 1109 (1940), gives the 2,4-dinitrophenylhydrazone m. p. as 220°. [g] Theoretical value, 14.35.

TABLE III

AMINES

R_1 = $CH(NH_2)R_2$ R_1 = Tolyl	R_2 = Tolyl	B. p., °C. 740 mm.	Red. p.	Mm.	d^{20}_4	$n^{20}D$	N,[a] %	Benzenesulfonamide M. p °C.	N,[b] %	Picrate M. p., °C.	N,[c] %
o	*o*	316	160–162	4	1.0449	1.5891	6.62	194	4.21	205	12.50
o	*m*	320	154–155	3	1.0380	1.5893	6.71	93	3.90	190	12.65
o	*p*	321	141–142	2	1.0286	1.5847	6.67	108	3.92	176	12.57
m	*m*	317	152–153	3	1.0264	1.5817	6.63	98	4.19	158	12.71
m	*p*	322	153–154	3	1.0297	1.5850	6.70	80	4.15	182	12.85
p	*p*[d]	318 M. p. 90°	140–141	2			6.68	128	4.11	178	12.65

[a] Theoretical values: [a] = 6.63; [b] = 4.00; [c] = 12.72. [d] *Ber.*, **24**, 2798 (1891), gives the m. p. as 93°; *Ber.*, **31**, 1773 (1898), gives b. p. as 318°.

2. The corresponding ketones were produced by hydrolysis of ketimines in 6 *N* hydrochloric acid.

3. Reduction of the ketimines at low pressure has resulted in the formation of the six dimethylbenzhydrylamines.

4. Relative rates of reduction have been determined and indicate a distinct hindrance in the case of ortho substituted ketimines.

5. Absorption spectra of the imines, ketones, and amines have been determiined.

RECEIVED MARCH 15, 1950

[CONTRIBUTION FROM THE BIOCHEMISTRY DEPARTMENT AND THE MCARDLE MEMORIAL LABORATORY, UNIVERSITY OF WISCONSIN]

Chemical Preparation of Asymmetrically Labeled Citric Acid[1]

BY PHILIP E. WILCOX,[2] CHARLES HEIDELBERGER AND VAN R. POTTER

The classic investigations of Krebs[3] on the oxidation of carbohydrates and their metabolites in minces of pigeon breast muscle led him to formulate the metabolic cycle which is presented with only minor modifications in Fig. 1. Evans and Slotin[4] with pigeon breast muscle minces and Wood, *et al.*,[5] with pigeon liver minces independently discovered the β-carboxylation of pyruvic acid (Step 1) in animal tissues. When they introduced labeled carbon dioxide into the cycle by this step, they were able to isolate α-ketoglutaric acid which carried isotopic carbon (indicated by asterisks in Fig. 1). Analysis showed that all of the isotope was in the α-carboxyl group. Weinhouse, *et al.*,[6] performed a similar experiment, introducing acetate-1-C^{13} through Step 2 in rat kidney minces, and found that in this case the isotope was predominantly in the γ-carboxyl group of the α-ketoglutaric acid.

(1) This work was supported in part by a Grant from the American Cancer Society on the recommendation of the National Research Council Committee on Growth, supplanted in part by the Research Committee of the Graduate School from funds supplied by the Wisconsin Alumni Research Foundation. A preliminary report of this work was presented at the American Chemical Society Meeting, Philadelphia, April 11, 1950.

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(3) H. A. Krebs, *Advances in Enzymology*, **3**, 191 (1943).

(4) E. A. Evans, Jr., and L. Slotin, *J. Biol. Chem.*, **141**, 439 (1941).

(5) H. G. Wood, C. H. Werkman, A. Hemingway and A. O. Nier, *ibid.*, **142**, 31 (1942).

(6) S. Weinhouse, G. Medes, N. F. Floyd and L. Noda, *ibid.*, **161**, 745 (1945).

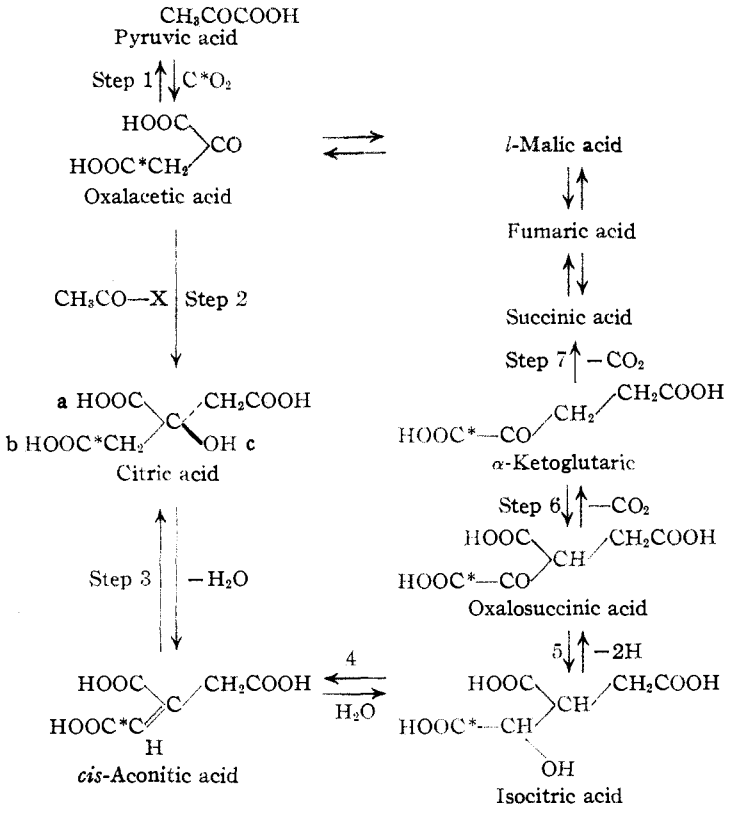


Fig. 1.—Krebs' citric acid cycle and asymmetrically labeled citric acid.

The above experiments were widely interpreted to exclude citric acid from the Krebs cycle. It was argued that the primary carboxyl groups were chemically identical and that if the symmetric molecule were an intermediate, α-ketoglutaric acid with isotopic carbon equally distributed in both of its carboxyl groups would be obtained; this was contrary to the experimental observations. However, Ogston[7] pointed out that this argument would be invalid if there were "a three-point contact" between substrate and enzyme. Under these conditions citric acid could be asymmetric in an enzyme-substrate complex and could lead to an asymmetric distribution of isotope in the carboxyl groups of α-ketoglutaric acid.

The Ogston hypothesis is a logical extension of the "polyaffinity theory" proposed by Bergmann and his co-workers[8,9] to explain the antipodal specificity of certain proteolytic enzymes. We believe that antipodal specificity of an enzymatic reaction demands the existence of (1) three distinct and specific points of interaction between enzyme and substrate and (2) some other condition, such as steric hindrance, directed forces, or another point of interaction. This latter condition can most easily be visualized as a restriction such that the catalytic complex can be formed on only one side of the plane that is defined by the three primary points of interaction.

That the Ogston concept was valid in regard to citric acid was proved by Potter and Heidelberger[10,11] who isolated radioactive citric acid from a fortified rat liver homogenate into which $C^{14}O_2$ had been introduced. This labeled citric acid was enzymatically converted by another rat liver homogenate in the presence of arsenite into α-ketoglutaric acid which contained the entire radioactivity in the α-carboxyl group. The asymmetric distribution of the isotope in the carboxyl groups presumably arose from an asymmetric configuration about the tertiary carbon atom of the citric acid. Dr. R. M. McGilvery suggested the feasibility of a chemical proof of this thesis, perhaps by the resolution of a five-carbon asymmetric precursor which would then be converted into labeled citric acid by reaction with radioactive cyanide.

An attractive precursor would be a γ-halo-β-carboxy-β-hydroxybutyric acid. Attempts to reach citric acid through the bromo compound were unsuccessful. However, the following reactions did lead to the desired intermediate when the halogen was chlorine

$$ClCH_2COOEt \xrightarrow[50\%]{Mg} ClCH_2COCH_2COOEt \xrightarrow[(NaCN)]{HCN}$$

$$\left[ClCH_2\underset{CN}{\overset{OH}{C}}CH_2COOEt \right] \xrightarrow[80\%]{H_2O\ (H^+)} ClCH_2\underset{COOH}{\overset{OH}{C}}CH_2COOH$$

The resulting γ-chloro-β-carboxy-β-hydroxybutyric acid which was obtained without isolation of the intermediate nitrile, could not be crystallized but was characterized by analysis of the crystalline dimethyl ester.

The resolution of the chloro-acid was accomplished through recrystallization of the brucine salt. The acid was regenerated by dissolving the salt in 50% sulfuric acid and extracting with ether. At two stages during the resolution, samples of the partially resolved acids (sp. optical activity of −9.3° and −25.9°, resp.) were separately converted into labeled citric acid, the citric acids so obtained were degraded enzymatically to α-ketoglutaric acid, and the distribution of isotope was determined (see below). The distribution of the isotope was assumed to be a measure of the optical purity of the chloro-acid. When the theoretical specific optical activity of optically

(7) A. G. Ogston, *Nature*, **162**, 963 (1948).

(8) M. Bergmann, L. Zervas, J. S. Fruton, F. Schneider and H. Schleich, *J. Biol. Chem.*, **109**, 325 (1935).

(9) M. Bergmann and J. S. Fruton, *ibid.*, **117**, 189 (1937).

(10) V. R. Potter and C. Heidelberger, *Nature*, **164**, 180 (1949).

(11) V. R. Potter and C. Heidelberger, in preparation.

pure chloro-acid was calculated from these data (Table I), in both cases the value was near −44°. After eleven alternate recrystallizations from methanol–water and chloroform, the regenerated acid had a specific optical activity of −44.9°.

TABLE I

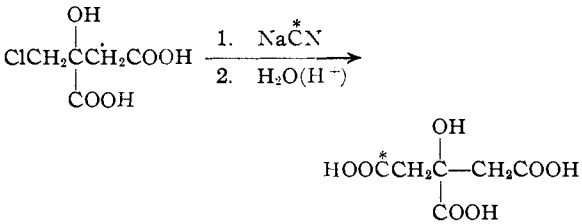
DISTRIBUTION OF RADIOACTIVITY IN α-KETOGLUTARIC ACID

Each entry relates to the degradation of the keto-acid from one typical Warburg flask which originally contained the labeled citric acid from the indicated source. In each case several such degradations of keto-acid were performed and were found to be in agreement.

Source, chloro acid of $[\alpha]^{25}D$	−9.3°	−25.9°	−44.9°
Total c./m. in α-ketoglutaric acid dinitrophenylhydrazone	3220	3150	3140
Total c./m. in carbon dioxide	1260	615	<75[a]
Total c./m. in succinic acid	1920	2520	3100
$\%C^{14}$ in CO_2/$\%$ C^{14} in succinic acid	39/61	20/80	2[a]/98

[a] There was no detectable radioactivity in the barium carbonate, but with the amount obtained, 275 mg., as much as 75 c./m. could have been present without being detected.

Labeled citric acid was prepared from the chloro-acid by the addition of $NaC^{14}N$ under mild conditions to a concentrated and slightly alkaline solution. The nitrile was hydrolyzed directly



with hydrochloric acid, volatile acid was evaporated, and citric acid was isolated by crystallization of the calcium salt from the boiling solution. It was possible to prepare calcium citrate in a 23% yield from crude racemic chloro-acid, and in a 32% yield from the dimethyl ester by the same procedure. In the preparation of asymmetrically labeled citric acid, it was found advisable to add carrier citric acid just before the isolation of the calcium salt. Solutions of labeled sodium citrate for the enzymatic degradation and characterization were prepared from the calcium citrate by metathesis with sodium oxalate.

The characterization of the labeled compound as citric acid was strongly indicated by the method of its isolation, since the solubility properties of calcium citrate are unusual. Furthermore, a sample of synthetic calcium salt, prepared without the addition of carrier, gave a quantitative analysis by the pentabromoacetone reaction[12]; this test is very specific for citric acid and acetonedicarboxylic acid. However, to prove conclusively that we did have asymmetrically labeled citric acid in hand, it was necessary to relate unequivocally a constant specific radioactivity with a specific property of citric acid. Partition chromatography upon a silica-gel column was chosen as a highly critical method which could be applied to a micro-quantity of valuable material.[13,14] An equal quantity of authentic citric acid was added to a solution of the radioactive citric acid, and the mixture was passed through a specially prepared column. A comparison of the acidity titer and the radioactive assay of the effluent (see Fig. 3) showed that the mixture of acids behaved as a single component in the correct position for citric acid and that the radioactivity was closely proportional to the acidity. Thus the product must be citric acid and the C^{14} is unequivocally associated with the citric acid. A more complete description of the characterization of radioactive citric acid in biological material is given elsewhere.[11]

The biochemical degradation of the labeled citric acid by rat liver homogenate was carried out by procedures described elsewhere.[10,11] Recycling of the isotope was blocked at Step 7 by addition of arsenite to the fortified homogenate. The α-ketoglutaric acid which accumulated was isolated as the 2,4-dinitrophenylhydrazone, was purified by chromatography and recrystallization, and was chemically degraded by oxidation. The α-carboxyl group appears as carbon dioxide and the γ-carboxyl group appears in succinic acid. The radioactivity assays (see Table I) indicate that labeled citric acid which was prepared from the chloroacid with an optical activity of −44.9° gives an α-ketoglutaric acid which contains essentially all of the activity in the γ-carboxyl group; therefore, this citric acid can be considered to be a pure isotopic antipode (enantiomorph).

The stereochemical relationships in the enzymatic reactions which could lead to (1) the specific asymmetric incorporation of isotopic carbon into citric acid and (2) the subsequent asymmetric distribution of the isotope in the carboxyl groups of α-ketoglutaric acid may be visualized as follows: We depict the molecule of oxalacetic acid as lying in the plane of the paper (see Fig. 1). Evidently, the antipodal specificity of the enzyme which catalyzes Step 2, the addition of the activated acetate moiety (CH_3CO–X), is such that the addition is restricted to one of the two possible ways. We have arbitrarily placed the hydroxyl group above the plane and the acetate group below the plane of reference. If the oxalacetic acid carries isotopic carbon in the β-carboxyl group, an isomer of asymmetrically labeled citric acid will result. If the acetate moiety carries the label in its carboxyl group, the other isomer will result, the mirror image of the first. Since the two are not superimposable, they can be described as isotopic antipodes.

(12) S. Natelson, J. K. Lugovoy and J. B. Pincus, *J. Biol. Chem.*, **170**, 597 (1947).

(13) F. A. Isherwood, *Biochem. J.*, **40**, 688 (1946).

(14) L. M. Marshall, J. M. Orten and A. H. Smith, *J. Biol. Chem.*, **179**, 1127 (1949).

Now consider the steps which lead from citric acid to α-ketoglutaric acid. The enzyme which catalyzes the dehydration of citric acid (Step 3) must interact asymmetrically with the molecule. Suppose interaction occurs at three points; with a tertiary carboxyl group at **a**, a primary carboxyl group at **b**, and with a hydroxyl group at point **c** which is above the plane of the paper. Furthermore, assume that some fourth condition, such as steric hindrance, restricts the formation of the catalytic complex to one side of the plane, **a b c**. In the present example, the citric acid molecule is restricted so that the tertiary carbon atom lies in the plane of the paper. When dehydration occurs specifically between the hydroxyl group and the hydrogens of the acetate group at point **b**, *cis*-aconitic acid results with its double bond lying toward the labeled primary carboxyl group (designated by the asterisk) which originated in the oxalacetic acid.

In Fig. 2 is formulated the other type of citric acid as it would be produced biosynthetically from labeled acetate and unlabeled oxalacetate. Examination of the three-dimensional model will convince the reader that this is the antipode of the other labeled citric acid. If this is then dehydrated enzymatically, again specifically between the hydroxyl group and the hydrogens of the acetate group at point **b**, *cis*-aconitic acid is obtained with the double bond lying away from the labeled primary carboxyl group. Since this formulation is purely arbitrary, other similar sets of conditions can be conceived which would give the same asymmetric reaction. It should be noted that interaction at any point may be an attraction or a repulsion.

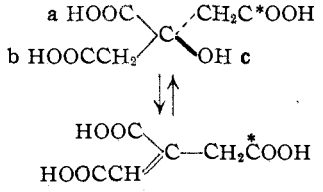


Fig. 2.—Enzymatic dehydration of the antipodally labeled citric acid

The next steps of the cycle lead to α-ketoglutaric acid in such a way that the α-carboxyl group is derived unequivocally from the carbon dioxide and the γ-carboxyl group from the acetate moiety. In the present investigation we have prepared chemically that isotopic antipode of citric acid which is predominantly formed biochemically from acetic acid-1-C^{14} and unlabeled oxalacetic acid.[11]

The following general rule has been proposed by one of the present authors[15] which includes the enzymatic reaction of asymmetrically labeled citric acid as a specific case: In a molecule which has a center of symmetry or a plane of symmetry, if one of the atoms which does not lie at the center or in any plane of symmetry is replaced by an isotopic atom, the molecule becomes asymmetric with respect to the labeled atom. In a reaction with an asymmetric reagent (chemical or enzymatic), this labeled atom (or group) may react at a rate which differs from that of its counterpart through the plane or center of symmetry, and the difference in rates will be expressed in the distribution of the isotope in the products. This effect would be superimposed on any difference in the rates which would result from the different masses of the isotopic atoms.[15a]

(15) P. E. Wilcox, *Nature*, **164**, 757 (1949).

Experimental

DL-γ-Chloro-β-carboxy-β-hydroxybutyric Acid.—Ethyl γ-chloroacetoacetate was prepared in 50% yield from ethyl chloroacetate by the procedure of Hamel.[16] Anhydrous hydrogen cyanide was made by dropping a 30% solution of sodium cyanide below the surface of hot 75% sulfuric acid, passing the evolved gas over calcium chloride, and condensing it at −5°. The addition of the hydrogen cyanide to the chloro-keto-ester was carried out with catalysis by sodium cyanide as recommended by Lapworth.[17] Ethyl γ-chloroacetoacetate (122 g., 0.75 mole) and anhydrous hydrogen cyanide (30 g., 1.1 mole) were mixed at 0° in a 250-ml. flask fitted with a reflux condenser at −5°. Sufficient sodium cyanide was added to promote spontaneous refluxing of the hydrogen cyanide (about 0.5 g.). After the reaction had subsided, the mixture was heated in a water-bath at 80° for thirty minutes, a second portion of sodium cyanide was added, and the mixture was refluxed for thirty minutes longer and allowed to cool to room temperature. About 30 ml. of 6 *N* hydrochloric acid was added and the two phases were distilled in vacuum until most of the water was removed. Two volumes of concentrated hydrochloric acid were added with cooling, and the solution was allowed to stand at room temperature for twenty-four hours. The acid was removed by vacuum distillation, followed by the addition of 100 ml. of water to the paste and concentration in vacuum to a heavy sirup. Enough water was added to the sirup to dissolve the ammonium chloride, and the solution was extracted continuously with ether for four hours. The ether was dried over sodium sulfate and, after evaporation, 108 g. (80%) of an orange sirup was obtained. All attempts to crystallize the sirup were unsuccessful.

The dimethyl ester was prepared from the crude chloro-acid (50 g.) by dissolving it in 250 ml. of absolute methanol, saturating the solution with anhydrous hydrogen chloride at 30°, and refluxing it overnight. The solvent was removed in vacuum, and the treatment with methanol and hydrochloric acid was twice repeated except that the solutions were allowed to stand at room temperature. Seed crystals were obtained from the solvent-free product by manipulation with ether–ligroin in a Dry Ice bath. The whole product was crystallized from 1:1 ethyl acetate–ligroin at −30°, m. p. 43–45°. After two recrystallizations at −5°, the melting point was 44.0–45.0° and was not raised by further recrystallization.

Anal.[18] Calcd. for $C_6H_{11}O_5Cl$: C, 39.92; H, 5.27. Found: C, 39.89; H, 5.12.

Resolution of DL-γ-Chloro-β-carboxy-β-hydroxybutyric Acid.—Crude γ-chloro-β-carboxy-β-hydroxybutyric acid

(15a) NOTE ADDED IN PROOF:—Very recently the biochemical experiments[10] have been confirmed by V. Lorber, M. F. Utter, H. Rudney and M. Cook, *J. Biol. Chem.*, **185**, 689 (1950), and C. Martius and G. Schorre, *Z. Naturforsch.*, **5b**, 170 (1950), have reported experiments with α,α-dideuteriocitric acid similar in scope to those of the present paper.

(16) J. Hamel, *Bull. soc. chim.*, [4] **29**, 390 (1921).

(17) A. Lapworth, *J. Chem. Soc.*, **83**, 1000 (1903).

(18) Microanalyses, courtesy of Prof. A. L. Wilds.

(53 g.) was dissolved in 53 ml. of water, and to it was added 138 g. of brucine in 170 ml. of hot methanol, followed by 170 ml. of water. The mixture was cooled immediately to 0° and allowed to stand in the refrigerator for 18 hours. The crystalline salt was filtered, washed, and dried in vacuum, m. p. 175° (d.), wt. 63 g. This salt was dissolved in 63 ml. of hot methanol containing 10 g. of brucine, 126 ml. of water was added, and the salt was allowed to crystallize for eighteen hours at 0°. To the 38.5 g. of brucine salt was added 77 ml. of chloroform, and 29.0 g. of salt was obtained by crystallization. Alternate recrystallizations were carried out from methanol–water and from chloroform with the proportions indicated above, and after 11 recrystallizations 5.9 g. of salt was obtained.

Anal. Calcd. for $C_{28}H_{33}O_9N_2Cl$: C, 58.28; H, 5.76; Cl, 6.04; $C_{29}H_{36}O_{10}N_2$: C, 60.83; H, 6.34; Cl, 0.00. Found: C, 60.19; H, 6.41; Cl, 2.11.[18a]

We deduce from the analysis that the salt cannot be a simple combination of brucine and chloro-acid. The acid component could not contain more than one-third chloro-acid. Loss of chlorine is also indicated by the fact that further treatment of the first mother liquor gave a brucine salt which was devoid of halogen. The analysis seems to indicate that the chlorine was replaced by a methoxyl group.

The eleven-times recrystallized salt (5.0 g.) was dissolved in 27 ml. of warm 50% sulfuric acid (by weight), and the cooled solution was extracted 15 times with 20-ml. portions of ether. The combined extracts were dried over sodium sulfate, the ether was distilled, and the residual sirup was dissolved in 25 ml. of water. After the solution was adjusted to *p*H 7 with bicarbonate, it was extracted three times with 25-ml. portions of chloroform and finally with ether. The aqueous phase was acidified to *p*H 2 with 6 *N* hydrochloric acid and 2 ml. excess was added. After the water had been removed in vacuum, the residue was extracted with ether and the extract was dried over sodium sulfate. The ether was evaporated and the residual oil was heated to 60° for four hours in vacuum. The yield of liquid acid was 0.71 g., $[\alpha]^{25}D$ −44.9° (15% in water). This acid could not be crystallized.

Conversion of Levorotatory γ-Chloro-β-carboxy-β-hydroxybutyric Acid into Sodium Citrate-1-C^{14}.—γ-Chloro-β-carboxy-β-hydroxybutyric acid (0.71 g., $[\alpha]^{25}D$ −44.9°) was dissolved in 5.0 ml. of water, neutralized with sodium bicarbonate, and 0.20 g. of $NaC^{14}N$[19] was added. The solution was heated for five minutes on the steam-bath, diluted with 25 ml. of water, saturated with hydrogen chloride gas at 30°, and refluxed for five hours on the steam-bath. Water was added and the volatile acid was distilled in vacuum. The volume was brought to 30 ml. with water, 0.25 g. of carrier citric acid was added, and the hot solution was treated with excess precipitated calcium carbonate until the *p*H reached 5.5. After the mixture was boiled for five minutes, the precipitate was filtered hot and washed with boiling water. The crude calcium citrate was dissolved in 20 ml. of 6 *N* hydrochloric acid, and the solution was neutralized with concentrated ammonium hydroxide and boiled for ten minutes. After the crystalline calcium citrate was filtered and washed with boiling water, it was dried at 70° in vacuum; a yield of 230 mg. was obtained.

The calcium citrate-1-C^{14} (230 mg.) was placed in 3 ml. of water, and 0.4 ml. of 6 *N* hydrochloric acid was added to dissolve the salt. A solution of oxalic acid equivalent to the calcium citrate was added, the *p*H was adjusted to 5.5 with sodium hydroxide, and the calcium oxalate precipitate was removed by centrifugation. The solution of sodium citrate was then made up to 10.0 ml.

Proof of the Identity of the Sodium Citrate-1-C^{14}.—The concentration of sodium citrate in the above solution was determined colorimetrically by the method of Natelson, *et al.*,[12] which is specific for citric acid and acetonedicarboxylic acid. The assay, 14.3 mg. of citric acid per ml., agreed well with the amount of calcium citrate which had been used. A radioactivity assay showed that the specific activity of the material was 11,000 c./m./mg. of citric acid.

Authentic citric aid (5.6 mg.) was added to 0.31 ml. of the radioactive sodium citrate solution, the material was converted to free acid in amyl alcohol–chloroform solution, and the mixture was chromatographed on a column of silica gel according to the method of Isherwood[13] and Marshall, *et al.*[14] Two-hundred drop fractions of the effluent were assayed for acid and for radioactivity. The results are plotted in Fig. 3.

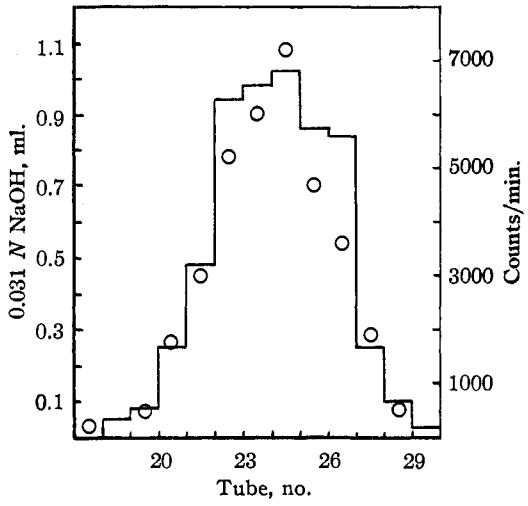


Fig. 3.—Titration and radioactivity of silica-gel chromatogram of citric acid-1-C^{14}. The bar graph represents the titration, and the circles represent the total radioactivity in each tube.

Biochemical Degradation of Sodium Citrate-1-C^{14}.—The radioactive sodium citrate solution was adjusted to *p*H 7, and an aliquot was added to a fortified rat liver homogenate, containing 0.003 *M* arsenite, as described by Potter and Heidelberger.[11] The reaction was stopped with perchloric acid, and the 2,4-dinitrophenylhydrazones of the keto-acids were prepared and were separated by chromatography on Celite in ether solution by the method of LePage.[20] The dinitrophenylhydrazone of α-ketoglutaric acid was obtained by evaporation of its ether solution, a known amount of carrier dinitrophenylhydrazone was added, and the compound was recrystallized five times from 10% ethanol. After the product was assayed for radioactivity, carrier succinic acid was added and the mixture was oxidized with acid permanganate according to Krebs.[21] The carbon dioxide was trapped in sodium hydroxide and precipitated as barium carbonate. The succinic acid was recovered by exhaustive ether extraction and was purified by recrystallization from water. Radioactivity assays of these oxidation products are given in Table I.

Summary

Asymmetric sodium citrate-1-C^{14} has been prepared by the reaction of $NaC^{14}N$ with levorotatory γ-chloro-β-carboxy-β-hydroxybutyric acid, a new compound.

The labeled citric acid was carefully characterized and subjected to the enzymatic action of a fortified rat liver homogenate. The α-

(18a) Determined by the method of C. W. Bacon, THIS JOURNAL, **31**, 49 (1909).

(19) Purchased from Tracerlab. Inc., on allocation from the U. S. Atomic Energy Commission.

(20) G. A. LePage, *Cancer Research*, **10**, 393 (1950).

(21) H. A. Krebs, *Biochem. J.*, **32**, 108 (1938).

ketoglutaric acid which was isolated from the homogenate was found to carry the isotope entirely in the γ-carboxyl group. These results prove that the asymmetric distribution of isotope in the carboxyl groups of α-ketoglutaric acid which is found in studies of Krebs' citric acid cycle arises from an asymmetric configuration (of the isotope) in the citric acid and an antipodal specificity of the enzymes which catalyze the formation and the dehydration of citric acid.

The enzymatic reactions are discussed in terms of the stereochemical properties of symmetric molecules which are asymmetrically labeled.

MADISON 6, WIS. RECEIVED MARCH 29, 1950

[CONTRIBUTION FROM THE DIVISION OF PLANT NUTRITION, COLLEGE OF AGRICULTURE, UNIVERSITY OF CALIFORNIA, AND THE DEPARTMENT OF PLANT PATHOLOGY, COLLEGE OF AGRICULTURE, UNIVERSITY OF WISCONSIN]

The Structure of Crown-Gall Polysaccharide. I

BY E. W. PUTMAN, A. L. POTTER, ROLAND HODGSON[1] AND W. Z. HASSID

The most abundant polysaccharides in nature, cellulose, starch and glycogen possess the 1,4-glucosidic linkage as their chief linkage; however, polysaccharides with glucose residues united through 1,3- and 1,6-linkages also occur.[2] Granicshstädten and Percival[3] reported a hemicellulose isolated from Iceland moss in which some of the glucose residues are joined through 1,2-glucosidic linkages.

McIntire, Peterson and Riker[4] showed that the crown-gall organism, *Phytomonas tumefaciens*, when grown on sucrose as a source of carbon, produces a low molecular weight water-soluble polysaccharide consisting entirely of D-glucose and having a specific rotation in water, $[\alpha]_D$ -9 to $-10°$. An upward shift in rotation during hydrolysis indicates a predominance of β-linkages, while the rate of hydrolysis suggests that the glucose residues have a pyranose structure. Hodgson, *et al.*,[5] demonstrated that when the crown-gall organism is grown on D-glucose or D-fructose, a polysaccharide is also produced, which is probably identical with the one formed when the bacterium is grown on sucrose.

Reeves[6] found that the shift in optical rotation of this polysaccharide in water and cuprammonium solution closely resembles that of methyl 2-methyl-β-D-glucopyranoside; he therefore suggested that the D-glucose units in the polysaccharide are most likely linked chiefly through the 2-position. It was therefore of interest to examine the structure of this polysaccharide and to ascertain the unique linkage.

The crown-gall polysaccharide was acetylated and the product methylated with methyl sulfate and sodium hydroxide by simultaneous deacetylation and methylation. It was found that methylation of the compound proceeded with difficulty and that it could not be completely methylated. However, its methoxyl content was sufficiently high to attempt the isolation of a trimethyl monosaccharide derivative. On methanolysis of the incompletely methylated polysaccharide a methyl trimethylglucoside was obtained which on hydrolysis of the glucosidic group gave rise to trimethylglucose. The rotation of this trimethylglucose agreed with that of the 3,4,6-trimethylglucopyranose synthesized by Haworth, *et al.*[7] Oxidation of the trimethylglucose with hypoiodite yielded a trimethylglucono lactone. The rate of hydrolysis of this lactone and its final specific rotation showed that it was 3,4,6-trimethylglucono lactone and that it belonged to the δ-lactone series.

On treatment of the trimethylglucose with phenylhydrazine hydrochloride and sodium acetate, an osazone was produced which was identical with that obtained from trimethylfructofuranose derived from hydrolyzed methylated inulin.[8] This constitutes further proof that positions 2 of the trimethyl glucose residues are predominately free and that some of the glucose residues in the crown-gall polysaccharide are united through 1,2-glucosidic linkages. Figure 1 represents the structure of a segment of the polysaccharide chain, consisting of β-glucopyranose units joined through 1,2-glucosidic linkages.

These methylation data confirm Reeves'[6] observation that the direction and magnitude of shift in specific rotation of the D-glucopyranose polysaccharides determined in aqueous and cuprammonium hydroxide solution can furnish information regarding the position of the linkage.

Experimental

Isolation of Polysaccharide.—The polysaccharide was isolated from cultures of *Phytomonas tumefaciens* (Smith and Townsend) Bergey, *et al.*, grown on a sucrose, mineral-salts medium. The techniques used in culturing the or-

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