THE ABSOLUTE STEREOCHEMICAL COURSE OF CITRIC ACID BIOSYNTHESIS*

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In 1948 Ogston noted that when such symmetrical molecules as citric (or aminomalonic) acid are acted upon by asymmetric enzyme surfaces, pairs of identical groups may well be differentiated.[1] Shortly thereafter it was shown, by using labeled substrates, that the two —CH_2COOH groups of citric acid could, in fact, be distinguished.[2] When citrate is formed from oxaloacetate and acetyl-CoA by citrate synthase (E.C.4.1.3.7, condensing enzyme), and is then treated with aconitate hydratase (E.C.4.2.1.3, aconitase) to form *cis*-aconitate and *threo*-D_s-isocitrate, the —CH_2COOH ends of these compounds are derived from acetyl-CoA, while the other atoms are derived either from oxaloacetate or water.[3-6] The acetyl-CoA-derived group yields acetate when citrate is cleaved by citrate-lyase (E.C.4.1.3.6, citratase),[7,8] and acetyl-CoA when citrate is attacked by the citrate cleavage enzyme.[9]

The nonequivalence of the two —CH_2COOH groups is apparent when citric acid is represented as I (heavy line above, dotted lines below the plane of the paper). Carbon atoms 1 and 2 are to 5 and 4 as right arm is to left arm:[10] they cannot be superimposed except by reflection. The same molecule is shown in Emil Fischer projection as II (vertical lines below and horizontal lines above the plane of the paper). The carbon atoms are numbered according to the Hirschmann convention.[11] The major purpose of the present investigation has been to determine which —CH_2COOH group, in the absolute stereochemical sense of I, is derived from acetate and which is derived from oxaloacetate.

In order to differentiate the two groups it was necessary to obtain asymmetrically labeled citric acid from a labeled compound of known absolute configuration. The following procedure is based upon the investigations, by Dangschat and H. O. L. Fischer, of the stereochemistry of quinic acid,[12] and by Davis and his associates of the biosynthesis of this compound.[13]

In the first stage quinic acid labeled with tritium in the 6 position was obtained with the aid of an extract of *Aerobacter aerogenes* A170-143 known to contain the enzymes quinate dehydrogenase (E.C.1.1.1.24) and 5-dehydroquinate dehydratase (E.C.4.2.1.10).[13] Thus 5-dehydroshikimic acid (III) yielded 5-dehydroquinic acid-6-T (IV), which on reduction gave quinic acid-6-T (V). The symbol $\sim$ indicates that the configuration at the carbon atom bearing tritium is unknown. In the second stage V was oxidized to citric acid-4-T (VII) by the method shown.[14] Lastly, the properties of (VII) as a substrate for aconitate hydratase were studied.

Materials and Methods.—Enzyme preparations: Cells of *A. aerogenes* A170-143 were grown, with stirring at 37° for 24 hr, on minimal medium A[15] supplemented with 20 mg of yeast extract (Difco) and 600 mg of quinic acid (volume 1 liter). Mucoid growth was not observed. The post log phase cells were washed with water, suspended in 0.033 *M* Tris-HCl[16] buffer, pH 7.5, and sonically disrupted. The initial extract (42 mg of protein from 1 ml of packed cells) showed strong NADH oxidase activity. After treatment with $MnCl_2$[13] and dialysis against 0.033 *M*

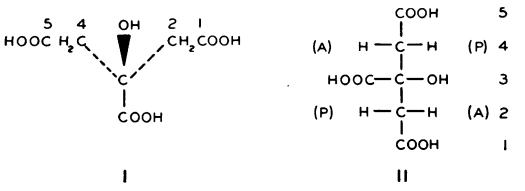

FIG. 1.

Tris-HCl buffer, pH 7.5, this activity was found to be negligible. The preparation (26 mg of protein) contained both quinate dehydrogenase and 5-dehydroquinate dehydratase. The specific activity of the former was 1.06 units/mg of protein at pH 9.4.[13] A small amount of 5-dehydroshikimic acid brought about the rapid oxidation of NADH (pH 7.4, 0.72 units/mg of protein). No appreciable losses of these activities occurred on storing the frozen solution at $-10°$ or on lyophylization. The *A. aerogenes* mutant was a gift of Dr. Bernard Davis.

Aconitate hydratase was prepared from pig heart and stored as a frozen solution.[17] Before use it was activated with Fe^{2+} and cysteine. Isocitrate dehydrogenase (E.C.1.1.1.42) and glutamate dehydrogenase (E.C.1.4.1.3) in phosphate buffer were purchased from Boehringer.

Throughout this paper a unit of enzyme is defined as that amount which catalyzes the transformation of 1 μmole of substrate/min at room temperature under optimum or specified conditions.

Quinic acid-6-T: (*a*) From 5-dehydroshikimic acid: To the lyophylized enzymes (0.43 mg protein), buffer pH 7.4 (Tris-HCl, 100 μmoles in 33 μl), 5-dehydroshikimic acid (8 μmoles), tritiated water (0.25 ml), and, finally, NADH (13 μmoles) were added and the reaction mixture left at 24°. The oxidation of NADH was followed by diluting 5 μl aliquots to 1 ml with 0.1 *M* NaOH (added to terminate the reaction) and observing the decrease in absorbancy at 340 mμ. The oxidation was complete in 35 min, at which time further reaction was prevented by adding HCl (100 μmoles). The tritiated water was removed by vacuum sublimation. Water was added and then removed by distillation, and this operation was repeated until all the easily exchangeable tritium had been displaced. The specific activity of the water in the reaction mixture was 578,000 cpm/μatom. The 5-dehydroshikimic acid was a gift of Dr. P. R. Srinivasan.

(*b*) From quinic acid: The enzymes (0.43 mg of lyophylized protein) in tritiated water (0.3 ml) were added to a lyophylized mixture of Tris-HCl buffer pH 7.4 (100 μmoles), NAD^+ (1 μmole), and potassium quinate (10 μmoles). The reaction mixture was maintained at 24° for 2 hr and 37° for a further 6 hr. Samples were withdrawn at various times, and the extent of incorporation was estimated. The specific activity of the water in the reaction mixture was 1×10^6 cpm/μatom. The reaction was terminated as in experiment (*a*).

Citric acid-4-T by oxidation of quinic acid-6-T: A sample containing less than 10 μmoles of tritiated quinic acid was chromatographed on a Dowex 1 acetate column.[18] Measurements of radioactivity served to locate the quinic acid peak. The combined peak fractions were concentrated to dryness, periodic acid (100 μmoles, 0.1 ml) was added, and the solution left in the dark at 24° for 1 hr. Under these conditions quinic acid consumes exactly two molar proportions of periodate, the reaction being complete in about 10 min. Periodic and iodic acids were removed with the aid of a Dowex 1-X8 formate column (7 × 0.7 cm). The column was developed with 3 *N* formic acid and the first 16 ml of effluent were concentrated to dryness. Water (2 ml) and bromine (10 μl) were added and the solution left in the dark at 27° overnight. The excess bromine was removed with a current of air and the reaction products were chromatographed on a Dowex-1 formate column.[18] The citric acid was located by radioactivity measurements. The over-all yield, based on radioactivity, was about 50%. The yield was not improved by adding strontium carbonate to the bromine oxidation mixture.[19] The specific activity of the citric acid from quinic acid was: method (*a*) 240,000 cpm/μmole, and (*b*) 435,000 cpm/μmole.

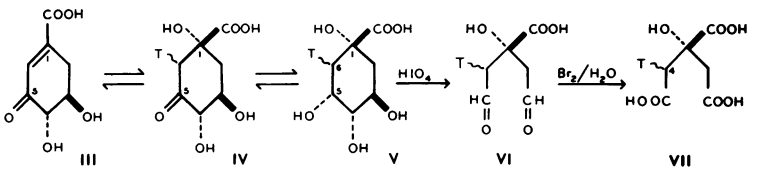

FIG. 2.

The oxidation process was studied in greater detail. A sample containing quinic acid (176,000 cpm) labeled by method (*b*) was diluted with potassium quinate (250 μmoles), and the solution chromatographed as above. The amount of acid in the various dried fractions was determined by titration, and 93% of the applied radioactivity was associated with the quinic acid peak. An isotope effect was observed: the specific activity fell linearly by 26% from the front to the tail of the peak; mean 617 cpm/μmole. The sodium quinate fractions were combined and the solution was passed through a short Dowex 50-H^+ column to give the free acid which was then oxidized. When the final reaction mixture was chromatographed, a series of peaks were recorded. The fourth and last peak corresponded to citric acid (33% yield). The specific activity of the acid increased by about 10% from the front to the tail of the peak. The mean specific activity (620 cpm/μmole) was in close agreement with that of the quinic acid.

Tritiated citric acids for control experiments: From isocitrate+T_2O: *threo*-D_s-isocitric acid (from crassulacean plants) was treated with aconitate hydratase in the presence of tritiated water and the enzyme destroyed by heating. The residual isocitrate was converted to α-ketoglutarate with isocitrate dehydrogenase, and the mixture of acids separated by silica gel chromatography.[20] Tritium was not incorporated into *cis*-aconitic acid. The incorporation into citric acid was 60% of the equilibrium value assuming labeling at a single position.[21] Specific activity: 85,000 cpm/μmole.

From isocitrate-3,4,4-T: A mixture of the four possible isocitric acids was prepared by the method of Fittig and Miller[22, 23] using tritiated succinic acid (New England Nuclear). The *threo*-D_sL_s mixture was separated from the *erythro*-D_sL_s mixture [(±)-alloisocitric acid] and from succinic acid by chromatography on a Dowex 1 formate column.[18] The *threo*-isocitric acids were eluted slightly ahead of the *erythro* mixture (ratio *threo*:*erythro*, 22:1). The *threo* mixture was treated with aconitate hydratase, and the citric acid formed was isolated by silica gel chromatography. Specific activity: 386,000 cpm/μmole.

From isocitrate-2-T: A mixture of the four stereoisomers of isocitric acid in roughly equal proportions (from New England Nuclear; synthesized by the method of Wislicenus and Nassauer[24]) was treated with aconitate hydratase, and the citric acid formed was isolated by silica gel and ion exchange chromatography. Specific activity: 50,000 cpm/μmole.

In each of these preparations the treatment with aconitate hydratase was continued for at least six times the period required to establish equilibrium with untritiated isocitrate.

Results.—Two methods were adopted for the preparation of quinic acid-6-T. In method (*a*) 5-dehydroshikimate was converted to quinate in the presence of tritiated water with NADH and the *A. aerogenes* enzyme preparation. The specific activity of the citric acid finally isolated was about half that of the tritiated water. If it is assumed that the oxidation of quinic to citric acid takes place without loss of tritium and that quinic acid can be labeled at only the one position, then a relatively small (twofold) discrimination against the tritium of the medium occurred. Discriminations as high as 40-fold have been observed.[24] In order to establish that this was characteristic of the 5-dehydroquinate dehydratase reaction, it would be necessary to show that this enzyme was present in a rate-limiting amount in the reaction mixture. In method (*b*) the attempt was made to bring quinic acid into equilibrium with tritiated water through the enzyme-catalyzed cycle of oxidation, dehydration, hydration, reduction. Despite the long period of equilibration, the specific activity of the derived citric acid was only half that of the tritiated water employed.

Although the oxidation of quinic to citric acid was not quantitative, the specific activities of the two acids were in close agreement. It follows that position 4 of quinic acid was not significantly tritiated. If nonenzymatic tritiation had taken place at the 5-dehydroquinate stage, labeling on both C-4 and C-6 (adjacent to the keto group) would be expected.

Comparison of citric acid-4-T with tritiated citric acids prepared by other methods:

Investigations in a number of laboratories into the action of aconitate hydratase have shown that three types of isotopic labeling of the four methylene hydrogens of citric acid may be distinguished: (1) Of the two hydrogens on the oxaloacetate-derived methylene group of citrate, one remains attached to the carbon atom and appears on C-2 of *cis*-aconitate and *threo*-D_s-isocitrate when citrate is acted upon by aconitate hydratase. When the isocitrate is oxidized by $NADP^+$ in the presence of isocitrate dehydrogenase to give α-ketoglutarate, this hydrogen is transferred to the 4*P* position (the *A* side)[11, 26] of the coenzyme-reduced nicotinamide ring.[6, 27, 28] (2) The other hydrogen of this methylene group passes to water when the substrate is brought to isotopic equilibrium with aconitate hydratase. Conversely, hydrogen from the water passes to this position of citrate, but none is incorporated into *cis*-aconitate. In the interconversion of citrate and isocitrate, not all of the molecules pass through the free *cis*-aconitate stage. Hydrogen from the hydratase-exchangeable position of citrate may pass to the C-3 position of isocitrate, and vice versa.[21, 29] The total replacement of hydrogen observed on isotopic equilibration is thus the result of repeated cycling of the substrates. (3) The hydrogens on the acetate-derived methylene group appear on C-4 of α-ketoglutarate when citrate is acted upon by the coupled system aconitate hydratase and isocitrate dehydrogenase.[5]

The results of comparing samples of citric acid-4-T (tests), prepared from quinic acid-6-T, with tritiated citric acids labeled in the above three ways (controls) are shown in Table 1. Both test samples behaved almost precisely as the citric acid prepared from unlabeled *threo*-D_s-isocitrate and tritiated water: nearly all of the tritium was released to the water on prolonged incubation with aconitate hydratase. The citric acids prepared from isocitrate-2-T or -3,4,4-T behaved otherwise: less than 10 per cent of the radioactivity was so released. When the residues from the equilibrium reaction were enzymatically converted to α-ketoglutarate and the α-ketoglutarate converted to glutamate, 79 and 86 per cent of the remaining radioactivity terminated in NADPH and glutamate, respectively. It follows that the maximum fraction of the test citric acids labeled in the acetate-derived methylene is 0.02 per cent and in the oxaloacetate-derived methylene, at the position not associated with the aconitate hydratase exchange reaction, is 2 per cent.

A further test was performed on a sample of citric acid-4-T (from 5-dehydroshikimic acid) with use of the coupled aconitate hydratase–isocitrate dehydrogenase system. The dehydrogenase was present in such a quantity that the rate of increase in absorbancy at 340 mμ was determined by the aconitate hydratase reaction. Throughout the reaction the ratio of tritium released to the water to NADPH formed was essentially constant. This result was typical of experiments performed on citric acid obtained by exchange labeling with tritiated water and aconitate hydratase. The same result has also been observed with various preparations of the hydratase. The fraction of tritium appearing on the C-3 position of α-ketoglutarate when test and control samples were acted upon by the coupled system varied from 40 to 17 per cent for reasons which are not at present understood.

Discussion.—The above results may be summarized as follows: The tritium of citric acid-4-T, obtained from enzymatically labeled quinic acid-6-T, is released to the water when the citric acid is acted upon by aconitate hydratase. Since the portion of the molecule derived from oxaloacetate is known to bear the hydrogen

TABLE 1

EQUILIBRATION OF TRITIATED CITRIC ACIDS WITH ACONITATE HYDRATASE

Tritiated citric acids	Total radioactivity (water + residue), cpm	Distribution of Counts: % Into water	% Into residue: Total	% Into residue: Into NADPH	% Into glutamic acid
Tests: citric acid-4-T derived from					
(*a*) 5-Dehydroshikimate	23,323	97	3	0	0.01
(*b*) Quinate	36,640	95	5	1.5	0.015
Controls: tritiated citric acids prepared from					
(1) Isocitrate + T_2O	24,439	95	5	0	0.65
(2) Isocitrate-2-T	37,043	5	95	75	0.30
(3) Isocitrate-3,4,4-T	8,849	8	92	0	79.0

Samples of quinic acid-6-T (V) were prepared from the compounds indicated (methods *a* and *b*) with the aid of 5-dehydroquinate dehydratase and quinate dehydrogenase, and these samples were oxidized to citric acid-4-T (VII). The three types of tritiated citric acids employed as controls were prepared from labeled or unlabeled isocitrate by prolonged incubation with aconitate hydratase. Samples 1 and 2 bear tritium at the oxaloacetate-derived end of the citric acid molecule. Most of the tritium on C-3 of isocitrate-3,4,4-T should have passed to the water, giving citric acid labeled at the end of the molecule not involved in the aconitate hydratase reaction.

Each type of citric acid (0.08–0.25 μmoles) together with buffer (triethanolamine-HCl, 0.1 *M*, pH 8.1) and aconitate hydratase (0.01 units) was maintained at 30° for 9 hr (final volume 1.0 ml). The water of each reaction mixture was separated by sublimation *in vacuo* and the dried residue freed of easily exchanged tritium by adding water and performing a further sublimation. The residue from each incubation was treated with additional aconitate hydratase (0.01 units) and isocitrate dehydrogenase (0.1 mg) in the presence of NADP (0.3 μmoles) and in a final volume of 1.0 ml. The reduction of NADP was followed by measuring the change in absorbancy at 340 mμ. When the reaction was complete, the radioactivity of a sample was counted, and another sample was filtered through a column of octanol-treated charcoal (1.5 × 0.6 cm) which was subsequently washed with excess water. Since the combined effluent and washes showed no absorbancy at 340 mμ, all the NADPH had been adsorbed by the charcoal. The difference in radioactivity between this material and the residue is taken as the NADPH-associated radioactivity. The remainder of the charcoal-treated solution was incubated with glutamate dehydrogenase (1.5 units), NADH (0.2 μmoles), and ammonium acetate (150 μmoles) in order to convert the α-ketoglutarate into L-glutamate (final volume 3 ml). Additional glutamic acid (10 μmoles) was added and the entire reaction mixture was chromatographed on a Dowex 1 acetate column (elution with 0.5 *N* acetic acid). Quantitative recoveries of glutamic acid were obtained. The radioactivity in this material represents the tritium derived from the methylene group of citrate not involved in the aconitate hydratase reaction. The significant fraction of the radioactivity which cannot be assigned to any of the C-H positions of citric acid in the control samples 2 and 3 is considered to represent contamination with the isomers of isocitric acid that are not substrates for aconitate hydratase. The preparations were shown to be free from the *threo*-D$_S$-isomer by assay with isocitrate dehydrogenase. Thus, the radioactivity found in NADPH and glutamic acid was derived from the citric acid present.

exchangeable by aconitate hydratase,[6] the stereochemical course of citric acid biosynthesis is established in absolute terms. The assignment may be expressed in several alternative ways: (*a*) Carbon atoms 6, 3, 4, and 5 of citric acid are derived from oxaloacetate and 1 and 2 from acetyl-CoA (see formulas I and II). (*b*) If the axis of the center carbon atom and its attached —COOH group is equated with the axis of the body so that the —COOH group is at the feet, and if the —OH is behind the body, then the acetyl-CoA-derived —CH_2COOH group is to the right front, and the oxaloacetate-derived —CH_2COOH group to the left front. (*c*) In the formation of citrate from oxaloacetate, the side of the carbonyl group attacked by acetyl-CoA is opposite to the side attacked by NADH in the formation of L-malate.[30]

This conclusion is based on a direct correlation with D(+)-glyceraldehyde: The configuration of quinic acid relative to D-glyceraldehyde is firmly established (the evidence has been reviewed elsewhere[31]). Although the study involves enzymatic labeling of quinic acid, the position of labeling is known from the known chemistry of the enzymatic substrates. It follows that, in setting forth the sequence of reactions involved in the correlation, no assumptions are required concerning the steric course of any reaction. The absolute configuration follows, since the configuration of D-glyceraldehyde is believed to be identical with that adopted in the Emil Fischer convention.[32]

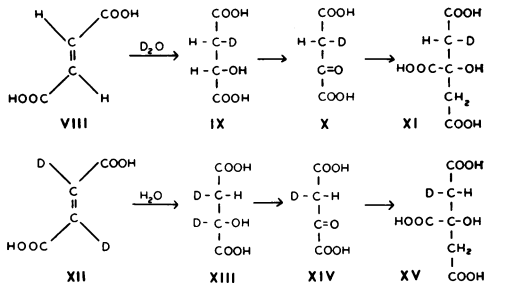

FIG. 3.

Two indirect correlations, based on optical rotation data, have been reported. Unfortunately, the arguments employed lead to opposite conclusions. Thus, in the one study, the enantiomeric forms of oxalocitramalic acid lactone-3,3-D were assigned configurations on the basis of Hudson's lactone rule. Oxidation of the free acids gave the corresponding deuterated citric acids.[5] In the other, a configuration was assigned to (−)-2-chloromethyl-2-hydroxysuccinic acid by means of Brewster's rules.[33] It had previously been shown[3] that asymmetrically labeled citric acid could be obtained from this compound with the aid of sodium cyanide-C^{14}. Both groups of investigators arrived at the assignment reached in the present paper. However, the latter group base their conclusion on a misreading of the experiments of Wilcox *et al.*[3] Given the premise that Brewster's rules apply, their conclusion must be reversed. We are indebted to Dr. Hirschmann for drawing our attention to this error.

The configuration at the oxaloacetate-derived methylene group of citric acid has been correlated with the fumarate hydratase (E.C.4.2.1.2, fumarase) reaction by Englard.[6] The *trans*-nature of this reaction has been established by Gawron *et al.*[33] and by Anet.[34] The conclusion that the C-4 methylene group of citric acid is derived from oxaloacetate leads to the summary of Englard's experiments (Fig. 3). In terms of the anterior (*A*), posterior (*P*) convention of Hirschmann,[11] citric acid-4(*A*)-D (XI) arises from fumaric acid-2,3-D (VIII) by way of L-malic acid-2,3(*A*)-D and oxaloacetic acid-3(*A*)-D, etc. When the two citric acids were treated with aconitate hydratase and isocitrate dehydrogenase, the deuterium from fumaric acid-2,3-D (VIII) appeared in NADPH, while the deuterium from D_2O did not. The hydrogen which exchanges with the hydrogen of the medium in the presence of aconitate hydratase is therefore attached to C-4 in the posterior position (see II and XVI). The stereochemistry of the aconitate hydratase reaction follows: The addition of T_2O to *cis*-aconitate (XVII) to give citrate-4(*P*)-T (XVI) or to give *threo*-D_s-isocitrate-3-T (XVIII) is *trans* in both cases. A given double-bond carbon atom is always attacked from the same side. Gawron *et al.*[33] have already noted that these conclusions follow if C-1 and C-2 of citrate are acetate-derived.

The stereochemistry of the 5-dehydroquinate dehydratase reaction may also be inferred. Thus, if the enzymatic hydration of 5-dehydroshikimate (III) is *cis*, then citric acid-4(*P*)-T (XX) is obtained via the intermediates 5-dehydroquinic

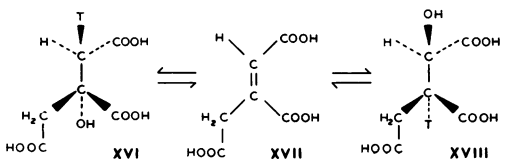

FIG. 4.

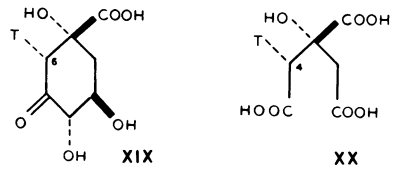

FIG. 5.

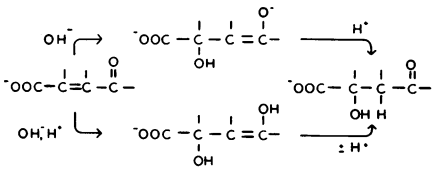

FIG. 6.

acid-6(*P*)-T (XIX) and quinic acid-6(*A*)-T. This appears to be the first example of enzymatic *cis* addition to a double bond.

In considering the mechanism of the 5-dehydroquinate dehydratase reaction, it may first be noted that the position of OH^- attack on 5-dehydroshikimate (III) is β to the C-5 keto group. Such an addition is in accordance with experience for nonenzymatic reactions: the keto group shows a stronger directing influence than the carboxyl ion. The addition process need not be formulated as a concerted addition to the double bond. By analogy with the behavior of unconjugated double bonds, an addition of this type might be expected to be *trans*. If the addition to the α and β positions takes place in two separate stages, then the stereochemistry will depend entirely upon the arrangement of acceptor-donor groups on the enzyme. Examples of such two stage mechanisms are shown in Figure 6. The nucleophylic addition of methoxy to *trans*-dibenzoylethylene has been formulated in terms analogous to the first mechanism. The methoxyl addition is here the rate-determining step.[35]

Summary of Conclusions.—(1) If a molecule of biosynthetically formed citric acid is viewed from the side opposite the middle COOH group, then moving from —OH in a counterclockwise direction the first $—CH_2COOH$ is derived from acetyl-CoA, and the second from oxaloacetate. (2) The enzymatic addition of water to *cis*-aconitate to give citrate is *trans*. (3) The enzymatic addition of water to 5-dehydroshikimate to give 5-dehydroquinate is *cis*.

Nomenclature: The Hirschmann rules[11] for designating the various atoms about a *meso* carbon atom have been used in this paper wherever appropriate. The various compounds in which tritium or deuterium has replaced protium, however, are asymmetric in the conventional sense and the configurations at the asymmetric centers may be fully specified by the *R/S* system.[36] Since the *A/P* system is linked to the *R/S* system, and since protium has the lowest priority of any substituent, *replacement of protium in the* P *position by deuterium or tritium necessarily gives* R *asymmetry, and replacement in the* A *position gives* S *asymmetry*. It follows that citric acid-4(*P*)-T is (2*R*,3*R*)-2-tritiocitric acid (sub-rules 1 and 4) and quinic acid-6(*A*)-T is (6*S*)-6-tritioquinic acid or (1*R*,2*S*,3*R*,4*S*,5*R*)-1,3,4,5-trihydroxy-2-tritio*cyclo*hexane-1-carboxylic acid (sub-rules 1 and 4). Quinic acid itself is (1*R*,3*R*,4*S*,5*R*)-1,3,4,5-trihydroxy*cyclo*hexane-1-carboxylic acid (C-3 and C-5 by sub-rule 1, C-1 and C-4 by sub-rule 2). (We are indebted to Dr. Cahn for his comments on the application of the *R/S* system to the quinic acids.) *Threo*-D_s-isocitric acid is (2*R*,3*S*)-isocitric acid and tritium substitution does not alter the *R/S* labels. The fluorocitric acid derived biosynthetically from fluoroacetate is either (2*S*,3*S*)- or (2*R*,3*S*)-2-fluorocitric acid.[37]

The asymmetric centers of citric acid-4(*P*)-T may also be defined by extending carbohydrate-amino acid nomenclature in the manner used for the isocitric acids.[37] The procedure leads to the names D_sD_g-2-tritiocitric acid or *erythro*-D_s-2-tritiocitric acid (the COOH attached to C-3 is here disregarded).

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[1] Ogston, A. G., *Nature*, **162**, 963 (1948).

[2] Potter, V. R., and C. Heidelberger, *Nature*, **164**, 180 (1949).

[3] Wilcox, P. E., C. Heidelberger, and V. R. Potter, *J. Am. Chem. Soc.*, **72**, 5019 (1950).

[4] Lorber, V., M. F. Utter, H. Rudney, and M. Cook, *J. Biol. Chem.*, **185**, 689 (1950).

[5] Martius, C., and G. Schorre, *Liebigs Ann. Chem.*, **570**, 140, 143 (1950); *Z. Naturforsch.*, **5B**, 170 (1950).

[6] Englard, S., *J. Biol. Chem.*, **235**, 1510 (1960).

[7] Gillespie, D. C., and I. C. Gunsalus, Proc. 53rd General Meeting, Soc. Am. Bacteriologists, *Bacteriol. Proc.*, **53**, 80 (1953).

[8] Wheat, R. W., and S. J. Ajl, *J. Biol. Chem.*, **217**, 909 (1955).

[9] Bhaduri, A., and P. A. Srere, unpublished results.

[10] Van't Hoff, J. H., cited by H. R. Levy, P. Talalay, and B. Vennesland, in *Progress in Stereochemistry 3*, ed. P. B. D. de la Mare and W. Klein (London: Butterworths, 1962), p. 300.

[11] Hirschmann, H., *J. Biol. Chem.*, **235**, 2762 (1960).

[12] Dangschat, G., and H. O. L. Fischer, *Naturwiss.*, **26**, 562 (1938); *Biochim. Biophys. Acta*, **4**, 199 (1950).

[13] Mitsuhashi, S., and B. D. Davis, *Biochim. Biophys. Acta*, **15**, 54, 268 (1954).

[14] Fischer, H. O. L., and G. Dangschat, *Helv. Chim. Acta*, **17**, 1196 (1934).

[15] Davis, B. D., and E. S. Mingioli, *J. Bacteriol.*, **60**, 17 (1950).

[16] Abbreviation: Tris for tris(hydroxymethyl)aminomethane.

[17] Morrison, J. F., *Biochem. J.*, **56**, 99 (1954).

[18] Palmer, J. K., *Conn. Agr. Expt. Sta. Bull.*, **589** (1955).

[19] Jackson, E. L., and C. S. Hudson, *J. Am. Chem. Soc.*, **59**, 994 (1937).

[20] Bulen, W. A., J. E. Varner, and R. C. Burrell, *Anal. Chem.*, **24**, 187 (1952).

[21] Rose, I. A., E. L. O'Connell, and Z. B. Rose, *Fed. Proc.*, **21**, 245 (1962).

[22] Fittig, R., and H. E. Miller, *Liebigs Ann. Chem.*, **255**, 43 (1889).

[23] Vickery, H. B., and K. R. Hanson, *J. Biol. Chem.*, **236**, 2370 (1961).

[24] Wislicenus, W., and M. Nassauer, *Liebigs Ann. Chem.*, **285**, 1 (1895).

[25] Flavin, M., and C. Slaughter, *J. Biol. Chem.*, **235**, 1112 (1960).

[26] Cornforth, J. W., *Biochem. J.*, **86**, 7P (1963).

[27] Nakamoto, T., and B. Vennesland, *J. Biol. Chem.*, **235**, 202 (1960).

[28] Englard, S., and S. P. Colowick, *J. Biol. Chem.*, **226**, 1047 (1957).

[29] Speyer, J. F., and S. R. Dickman, *J. Biol. Chem.*, **220**, 193 (1956).

[30] P. F. V. Ward and R. A. Peters, [*Biochem. J.*, **78**, 661 (1961)] have shown that a single fluorocitric acid accumulates *in vitro* in rabbit kidney poisoned with monofluoroacetate. Two diastereoisomers are theoretically derivable through the action of citrate synthase. Such an accumulation would arise if the enzyme is able to catalyze the replacement of only one of the two nonequivalent α-hydrogens of fluoroacetyl-CoA in the condensation with oxaloacetate.

[31] Hanson, K. R., *J. Chem. Educ.*, **39**, 419 (1962).

[32] Bijvoet, J. M., A. F. Peerdeman, and A. J. van Bommel, *Nature*, **168**, 271 (1951).

[33] Gawron, O., A. J. Glaid, III, and T. P. Fondy, *J. Am. Chem. Soc.*, **83**, 3634 (1961).

[34] Anet, F. A. L., *J. Am. Chem. Soc.*, **82**, 994 (1960).

[35] Crowell, T. I., G. C. Helsley, R. E. Lutz, and W. L. Scott, *J. Am. Chem. Soc.*, **85**, 443 (1963).

[36] Cahn, R. S., C. K. Ingold, and V. Prelog, *Experientia*, **12**, 81 (1956).

[37] Vickery, H. B., *J. Biol. Chem.*, **237**, 1739 (1962).

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*A STOCHASTIC MODEL FOR THE INTERPRETATION OF CLINICAL TRIALS**

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1. *Introduction.*—There are several diseases in which possibly recurrent phases may be distinguished. Different investigators have proposed Markovian models to describe data dealing with the time-dependent phenomena associated with these diseases. We mention in particular the work of Fix and Neyman on cancer,[1] the