We have measured the stacking tendencies of twenty-four native DNA samples prepared from various biological sources by different methods and having different nucleotide sequences and compositions, molecular weights and extensions and traces of bound ions, proteins, etc. All of these samples, with the exception of the single-stranded ϕX DNA, show the same stacking tendency regardless of the parameter chosen as a basis of comparison. Using X-ray diffraction techniques, Langridge, *et al.*,[25] have shown that all two-stranded native DNA samples have the same molecular conformation. These data provide further support for the theory that the stacking tendency depends upon the molecular structure of the polyanion to which the dye is bound and in this sense is an intrinsic characteristic of the polyanion.

Recent theories of aggregation[20-22] express the interaction as a function of the distance and angle between the interacting chromophores. Detailed application of these theories to the spectra of the bound dye should permit the calculation of the distance between binding sites in polymer systems where, unlike the case of DNA, this information is not otherwise available. Such calculations will of course determine the distance between sites in the dye–polymer complex which may differ to a limited extent from that of the free polymer in solution.

It should be possible to use the dye method described above to determine the extent of denaturation in any given DNA sample. Under identical conditions the parameters would be expected to be within the range described above. To determine the numerical values of some of the stacking parameters of DNAs under different conditions (small changes in ionic strength, *p*H, temperature, etc., or with a different dye), those parameters for known native and fully denatured samples should be measured and used as standards.

Acknowledgments.—The authors with to express their gratitude to Drs. A. Bendich, J. Rose, R. Sinsheimer and S. H. Stone for their gifts of DNA samples and to Drs. M. K. Wolf and M. Kasha for helpful comments and discussions.

(25) R. Langridge, W. E. Seeds, H. R. Wilson, C. W. Hooper, M. H. F. Wilkins and L. D. Hamilton, *J. Biophys. Biochem. Cytol.*, **3**, 767 (1957).

[CONTRIBUTION FROM THE DEPARTMENT OF CHEMISTRY, DUQUESNE UNIVERSITY, PITTSBURGH, PENNSYLVANIA]

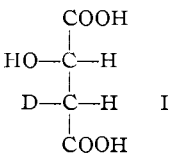
Stereochemistry of Krebs' Cycle Hydrations and Related Reactions

BY OSCAR GAWRON, ANDREW J. GLAID, III, AND THOMAS P. FONDY[1]

RECEIVED DECEMBER 23, 1960

3-Deuterio-L-malic acid obtained from the fumarase catalyzed hydration of fumaric acid in D_2O is shown to have the *erythro* configuration by n.m.r. comparison with stereospecifically synthesized *threo*-3-deuterio-DL-malic acid. The fumarase and aspartase systems thus operate by a *trans* mechanism as do the β-methylaspartase and *cis*-aconitase systems. Brewster's rules are applied to the problem of the stereochemistry of citric acid synthesized in the Krebs cycle and a configuration related to D-malic acid is arrived at. This configuration plus the α_{D_s},β_{L_s} configuration of *d*-isocitric acid permits depiction of the stereochemical pathway of the *cis*-aconitase system and of the reactions of the Krebs cycle. The possibility that *cis*-aconitase exhibits a preferred direction for addition of OH, with concomitant *trans* addition of H, is discussed.

In a previous communication[2] natural[3] 3-deuterio-L-malic acid (I) was shown to have the *erythro* configuration by n.m.r. comparison with



I

stereospecifically synthesized *threo*-3-deuterio-DL-malic acid (II). With this result the stereochemical mechanism of the fumarase and aspartase systems was unequivocally demonstrated to be *trans* and, in conjunction with previously obtained results[4,5] on the *cis*-aconitase system, the stereochemistry of the Krebs cycle from fumaric acid to α-keto-glutaric acid was elucidated.

It is the purpose of this paper to detail proof of the configuration of natural 3-deuterio-L-malic acid and to discuss the stereochemistry of Krebs cycle hydrations and the related aspartase and β-methylaspartase[6] reactions.

The stereospecific synthesis of *threo*-3-deuterio-DL-malic acid (II) was accomplished (Fig. 1) by a *trans* opening[7,8] with lithium aluminum deuteride of the oxide ring of 3,4-epoxy-2,5-dimethoxy-tetrahydrofuran (III) followed by acid hydrolysis to the dialdehyde and oxidation of the dialdehyde to *threo*-3-deuterio-DL-malic acid[9] (II).

Experimental

3-Deuterio-4-hydroxy-2,5-dimethoxy-tetrahydrofuran (IV).—The deuterated tetrahydrofuran derivative was obtained from 3,4-epoxy-2,5-dimethoxy-tetrahydrofuran[7] by the procedure of Sheehan and Bloom[7] utilizing lithium aluminum deuteride[10] in place of lithium aluminum hydride.

(1) National Science Foundation Coöperative Graduate Fellow.

(2) O. Gawron and T. P. Fondy, *J. Am. Chem. Soc.*, **81**, 6333 (1959).

(3) Obtained by the fumarase catalyzed hydration of fumaric acid in D_2O.

(4) O. Gawron and A. J. Glaid, III, *J. Am. Chem. Soc.*, **77**, 6638 (1955).

(5) O. Gawron, A. J. Glaid, III, A. LoMonte and S. Gary, *ibid.*, **80**, 5856 (1958).

(6) H. A. Barker, R. D. Smyth, R. M. Wilson and H. Weissbach, *J. Biol. Chem.*, **234**, 320 (1959).

(7) J. C. Sheehan and B. M. Bloom, *J. Am. Chem. Soc.*, **74**, 3825 (1952).

(8) P. A. Plattner, H. Heuser and M. Feurer, *Helv. Chim. Acta*, **32**, 587 (1949); L. W. Trevoy and W. G. Brown, *J. Am. Chem. Soc.*, **71**, 1675 (1949); W. G. Dauben, R. C. Tweit and R. L. McLean, *ibid.*, **77**, 48 (1955); A. Streitweiser, Jr., R. H. Jagow, R. C. Fahey and S. Suzuki, *ibid.*, **80**, 2326 (1958).

(9) Subsequent to this work, it was found that palladium catalyzed hydrogenation of *cis*-1,2-dicarboxy-ethylene oxide proceeded in a stereospecific fashion yielding *threo*-3-deuterio-DL-malic acid by a *trans* opening. O. Gawron and T. P. Fondy, unpublished work.

(10) Metal Hydrides, Inc.

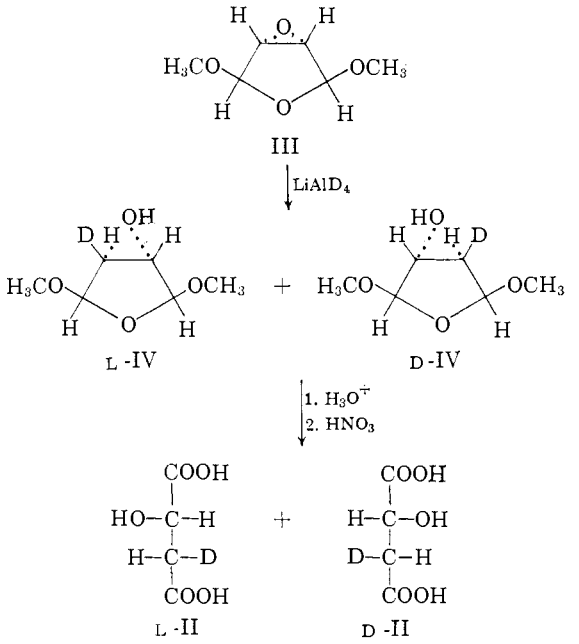


Fig. 1.—Stereospecific synthetic route to *threo*-3-deuterio-DL-malic acid.

From 12.5 g. of epoxide, 10.3 g. (83%) of product was obtained; b.p. 45–49° (0.5 mm.).

threo-**3-Deuterio-DL-malic Acid** (II).—Nine and five-tenths g. of 3-deuterio-4-hydroxy-2,5-dimethoxy-tetrahydrofuran (IV) was shaken at 65° with 25 ml. of 1 *M* nitric acid for 10 minutes. The resulting orange solution of *threo*-3-deuterio-DL-malicaldehyde was cooled to 40° and 75 ml. of concd. nitric acid was added. The solution was then heated to 62° at which temperature oxidation set in vigorously. After the reaction subsided, the reaction mixture was permitted to stand overnight at room temperature. Concentrated ammonia was added to *p*H 3.0 and subsequently a solution of 50 g. of lead nitrate in 70 ml. of water. Lead salts were then precipitated in a stepwise fashion by first adjusting the *p*H to 6.6 with concd. ammonia, filtering and then raising the *p*H to 9 to complete precipitation. The combined precipitates then were washed two times by suspending in 400 ml. of distilled water and filtering. The washed lead salts next were decomposed by adding with stirring 8.4 ml. of 18 *M* sulfuric acid in 16 ml. of water to a suspension of the lead salts in 250 ml. of distilled water. After filtering and washing of lead sulfate, the combined filtrates were vacuum concentrated to a 10-ml. volume, and this solution was absorbed on to 19 g. of Celite 535. The adsorbate then was extracted continuously with ether for 24 hours. After drying over magnesium sulfate, the ether extract was concentrated to 20 ml., 500 mg. of insoluble material was filtered off and 4.1 g. of mixed acids were obtained by precipitation with petroleum ether. This product was then chromatographed on a 250 × 32 mm. column of Celite 535 (30 g. Celite plus 15 ml. of 0.5 *N* hydrochloric acid) using a chloroform, 1-butanol, ethyl alcohol (18:2:1) solvent mixture and collecting 9 ml. fractions. From tubes 123–210 there was isolated 400 mg. (twice recrystallized) of *threo*-deuterio-DL-malic acid; m.p. 127–128.5°.

Anal. Calcd. for $C_4DH_5O_5$: D, 1.48. Found[11]: D, 1.45.

N.M.R. Studies.—The carboxyl and hydroxyl protons of twice recrystallized *threo*-deuterio-DL-malic acid were replaced by deuterium by dissolving the sample in D_2O, removing water by lyophilization and repeating the process. The sample was then dissolved in D_2O to give a 2 *M* solution.

The spectra were obtained at 40 megacycles with a Varian 4300 n.m.r. spectrometer equipped with a superstabilizer. Line separations from an external $Si(CH_3)_4$ standard were determined by a sideband resonance produced by modulation of the magnetic field. Frequency calibration was made with a Hewlett-Packard Model 522 B Electronic Counter.

(11) We are indebted to Dr. S. Englard for this determination.

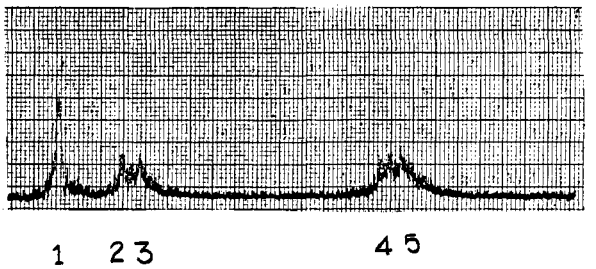
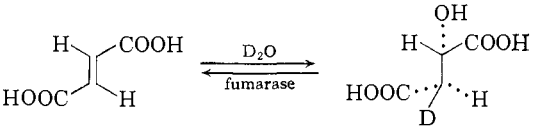


Fig. 2.—Proton spectrum at 40 megacycles of *threo*-deuterio-DL-malic acid, 2 *M* in D_2O, exchangeable protons having been removed previously. Line 1 represents water protons in the added reference capillary. The doublet represented by lines 2 and 3 results from spin interaction of the methine hydrogen with the methylene hydrogen (doublet indicated by lines 4 and 5).

Discussion

Synthetic Proof.—The synthetic route chosen for the required stereospecific synthesis needs little discussion, the *trans* nature of lithium aluminum hydride reduction of epoxide rings having been amply demonstrated[7,8] and the use of acidic oxidizing conditions precluding racemization, particularly at the aldehyde stage.[12] Figure 2 shows the proton spectrum of *threo*-3-deuterio-DL-malic acid (II) synthesized in this fashion. The spectrum is not similar to that[13] for enzymatically synthesized[14] 3-deuterio-L-malic acid, and the coupling constants for the two non-equivalent protons are 4.3 ± 0.2 c.p.s. for the synthetic *threo* compound and 7.1 c.p.s. for the enzymatically synthesized material. The steric non-identity of these two monodeuterio-malic acids is further evidenced by the fact that the 3-deuterio-D-malic acid[15] obtained by the indirect inversion of 3-deuterio-L-malic acid (enzymatically synthesized) gives[15] a coupling constant of 4 c.p.s. It is clear then that the 3-deuterio-L-malic acid obtained by fumarase-catalyzed synthesis in D_2O has the *erythro* configuration (I), and the above 3-deuterio-D-malic acid has the *threo* configuration.

Stereochemistry of the Fumarase, Aspartase and Methylaspartase Catalyzed Reactions.—Assignment of the *erythro* configuration to 3-deuterio-L-malic acid permits the unequivocal conclusion that the fumarase-catalyzed reversible hydration of fumaric acid proceeds by a *trans* mechanism, *viz.*



It is to be noted that the protons attached to carbons 2 and 3 in the resulting 3-deuterio-L-malic acid (I) are *trans* when the carboxyl groups are

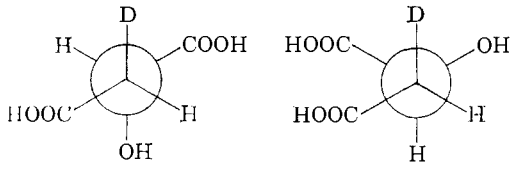
(12) An independent corroborating synthesis utilizing different oxidizing conditions was published subsequent to our work by F. A. L. Anet, *J. Am. Chem. Soc.*, **82**, 994 (1960).

(13) R. A. Alberty and P. Bender, *ibid.*, **81**, 542 (1959).

(14) H. F. Fisher, C. Frieden, J. S. M. McKee and R. A. Alberty, *ibid.*, **77**, 4436 (1955).

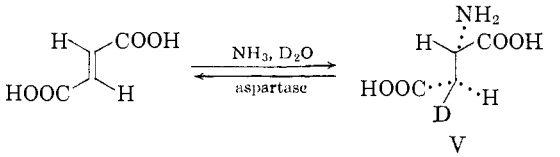
(15) A. I. Krasna, *J. Biol. Chem.*, **233**, 1010 (1958).

trans and *gauche* when the carboxyl groups are depicted in a *gauche* fashion.[16]



I

The aspartase catalyzed reaction analogous to the fumarase catalyzed reaction has been shown[15] to proceed in a stereospecific fashion and to yield 3-deuterio-L-aspartic acid. The *erythro* configuration, V, may be assigned to this 3-deuterio-L-aspartic acid[18] since it has been transformed[15] by a two step stereospecific synthesis involving one inversion to *threo*-3-deuterio-D-malic acid. Accordingly, the aspartase catalyzed reaction must proceed *via* a *trans* mechanism.



V

In connection with the *trans* nature of the fumarase and aspartase systems, the recently described[6] β-methylaspartase catalyzed reaction is of interest. This enzyme catalyzes the reversible deamination of *threo*-L-β-methylaspartic acid (VI) and *erythro*-L-β-

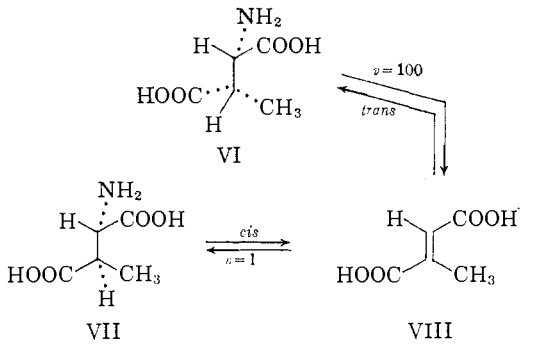
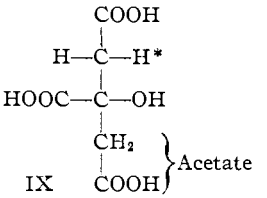


Fig. 3.—Stereochemistry of β-methylaspartase system.

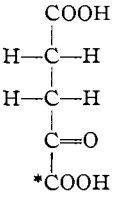
methylaspartic acid (VII) to mesaconic acid (VIII), the rate of deamination of the *threo* compound being 100 times that of the *erythro* compound. Stereochemically these reactions may be depicted as in Fig. 3, the *trans* mode of amination and deamination being the preferred route.[19]

Stereochemistry of Citric Acid, the *cis*-Aconitase System and the Krebs Cycle.—In previous work[4,5] the *trans* nature of the *cis*-aconitase system with respect to the reversible dehydration of *d*-isocitric acid to *cis*-aconitic acid was established. On the basis of this result the *trans* nature of the system with respect to the reversible dehydration of citric acid to *cis*-aconitic acid was inferred and stereochemical formula IX was suggested for citric acid

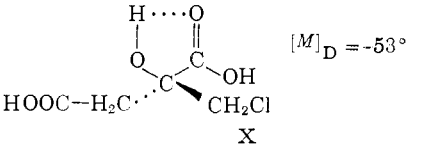


where H* participates in the *cis*-aconitase catalyzed reaction and that part of the molecule arising from acetate does not participate[20] in the *cis*-aconitase catalyzed reaction. We now wish to show that stereochemical considerations, presented below, also lead to stereochemical formula IX for citric acid.

The empirical rules of Brewster[21] regarding relationships between configuration and sign of rotation may be applied to the problem of the configuration of enzymatically synthesized citric acid. Wilcox, Potter and Heidelberger[22] have synthesized and resolved α-chloromethyl-α-hydroxy-succinic acid (γ-chloro-β-carboxy-β-hydroxy-butyric acid). The levorotatory enantiomorph was obtained pure, $[M_D]$ −53.2°, and treated with NaC*N followed by hydrolysis to yield an asymmetrically labelled citric acid. This isotopic enantiomorph when subjected to the successive action of *cis*-aconitase and isocitric dehydrogenase yielded α-ketoglutaric



acid with all of the isotope in the α-carboxyl group. It is obvious then that the isotopic label in the citric acid used was not in that side chain contributed by acetate in the enzymatic synthesis of citric acid. If one applies Brewster's procedure for α-hydroxy acids to the levorotatory α-chloromethyl-α-hydroxy-succinic acid, the absolute configuration X for this enantiomorph is obtained.[23]



X

On this basis the isotopic citric acid obtained by Wilcox, *et al.*, is

(16) The previous assignment of a *cis* mechanism to the fumarase system was based on an interpretation of n.m.r. data which indicated the protons in questions to be *gauche* and on the assumption the carboxyl groups in solid *erythro*-3-deuterio-L-malic acid were *trans*.[17] Doubts as to the validity of these conclusions already have been raised, personal communication, R. A. Alberty.

(17) T. C. Farrar, H. S. Gutowsky, R. A. Alberty and W. G. Miller, *J. Am. Chem. Soc.*, **79**, 3978 (1957).

(18) A previous *threo* assignment based on *erythro* assignment to the obtained 3-deuterio-D-malic acid, led to a *cis* mechanism for the aspartase system.

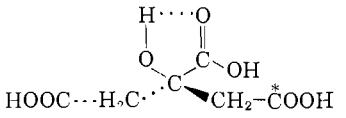
(19) Evidence for the assigned *threo* and *erythro* configurations is considered presumptive, if not conclusive, ref. 6.

(20) J. S. Fruton and S. Simmonds, "General Biochemistry," 2nd Ed., John Wiley and Sons, Inc., New York, N. Y., 1958, pp. 511, 514.

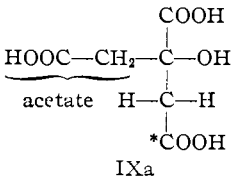
(21) J. H. Brewster, *J. Am. Chem. Soc.*, **81**, 5475 (1959).

(22) P. E. Wilcox, C. Heidelberger and V. R. Potter, *ibid.*, **72**, 5019 (1950).

(23) On the basis that the polarizability rank of the chloromethyl group is greater than that of the carboxymethyl group, the polarizability effects of the chloro group and the carboxyl group will be second-order effects. However, the polarizability rank of chlorine is 5.844 and of the carboxyl carbonyl is 3.379.

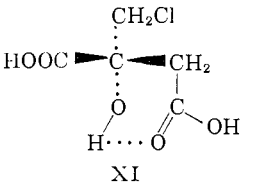


which written in projection form is IXa



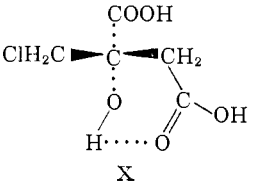
IXa

is seen to be a derivative of D-malic acid and is identical with the configuration already arrived at by considering the citric acid to *cis*-aconitic conversion to proceed by a *trans* mechanism. This same absolute configuration for the above citric acid can be arrived at by application of Brewster's first rule and the polarizability rank COOH > CH_2Cl > CH_2COOH > OH. On this basis the absolute configuration of dextrorotatory α-chloromethyl-α-hydroxy-succinic acid would be



XI

and the absolute configuration of the levorotatory enantiomorph obtained by Wilcox, *et al.*, would of necessity be



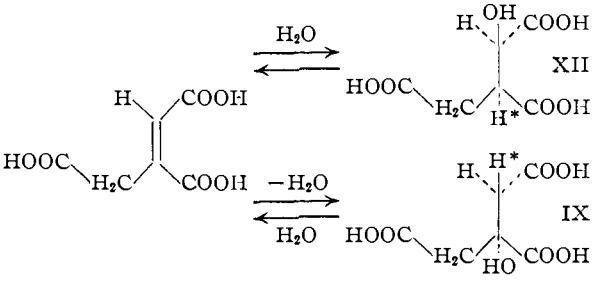
X

which again leads to the configuration IXa for citric acid.

Thus the application of Brewster's empirical rules leads to the absolute configuration IX for citric acid, synthesized enzymatically from oxaloacetic acid and acetyl-CoA.

While application of Brewster's rules leads to configuration IX for citric acid, the previous basis,[4,5] α_{L_s},β_{D_s}, configuration of *d*-isocitric acid, for assignment of configuration IX is no longer tenable since Kaneko, *et al.*,[24,25,26] have demonstrated recently by classical organic methodology that the stereochemistry of *d*-isocitric acid is α_{D_s},β_{I_s}. These results refute the Greenstein, *et al.*,[27,28] physical assignment of α_{L_s} to *d*-isocitric acid[29] (XII) but confirm our work[4,5] and the X-ray work of Glusker, *et al.*,[30] on the relative (*threo*) configurations of the α-OH and β-COOH groups. However, the original assignment,[4,5] α_{L_s},β_{D_s}, was based on the α_{L_s} assignment of Greenstein, *et al.*,[27,28] and now is seen to be incorrect. The configuration α_{L_s},β_{D_s} thus represents the enantiomorph, *threo*-L-isocitric acid.

From the known geometry[31] of *cis*-aconitic acid and the proved configuration of *d*-isocitric acid (XII) and the deduced configuration of citric acid (IX), the *trans* nature of the *cis*-aconitase catalyzed reversible hydration is established and the stereochemical relations between the components of the *cis*-aconitase system are



where the starred hydrogen atoms are removed. It is to be noted here that apparently OH adds to *cis*-aconitic acid from one side to give *d*-isocitric acid and from the other side to give citric acid.[32] Now this apparent mode of addition of OH is consistent with Ogston's suggestion,[34] albeit the stereochemistry of the reaction and the reactants was not known, that attack may occur at either side of the double bond so that one enzyme can form both citric acid and isocitric acid. Of course, the attack in each case is now seen to be stereospecific.

The above relations are supported by labelling studies related to the Krebs cycle and may be incorporated into a stereochemical depiction (Fig. 4) of the Krebs cycle. *erythro*-3-Deuterio-L-malic acid (I) on being carried through the reactions of the Krebs cycle to isocitric acid yields isocitric acid lacking deuterium.[35,36] Tracing *erythro*-3-deuterio-

(24) T. Kaneko, H. Katsura, H. Asano and K. Wakabayashi, *Chem. & Ind.*, 1187 (1960).

(25) T. Kaneko and H. Katsura, *ibid.*, 1188 (1960).

(26) NOTE ADDED IN PROOF.—The results of Kaneko, *et al.*, came to our attention after acceptance of the manuscript and we wisb to thank Dr. Ronald Bentley for calling these articles to our attention. The above revision of tbe stereochemistry of *d*-isocitric acid required a reinterpretation of the *cis*-aconitase system and the matter was dealt with in proof.

(27) J. P. Greensteln, N. Izumeya, M. Winitz and S. M. Birnbaum, *J. Am. Chem. Soc.*, **77**, 707 (1955).

(28) M. Winitz, J. M. Birnbaum and J. P. Greenstein, *ibid.*, **77**, 716 (1955).

(29) *d*-Isocitric acid can be conveniently named *threo*-D-isocitric acid.

(30) J. P. Glusker, A. L. Patterson, W. E. Love and M. L. Dornberg, *J. Am. Chem. Soc.*, **80**, 4426 (1958).

(31) R. Malachowski and M. Maslowski, *Ber.*, **61B**, 2521 (1928); R. Malachowski, M. Giedroyć and Z. Jerzmanowska, *ibid.*, **61B**, 2525 (1928).

(32) In our previous depiction (ref. 5) of these relations, OH was considered to add from the same side to give both *d*-isocitric acid and citric acid. This mode of addition was, of course, based, in part, on the α_{L_s},β_{D_s} configuration of *d*-isocitric acid. The protonated double bond intermediate previously used (ref. 5) to represent the common intermediate of Speyer and Dickman (ref. 33) also was based on this assignment. If application of Brewster's rules results in an incorrect assignment to citric acid, then OH must still add from the same side to give both citric acid and isocitric acid.

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

(34) A. G. Ogston, *Nature*, **167**, 693 (1951).

(35) S. Englard, *Fed. Proc.*, **18**, 222 (1959); S. Englard, *J. Biol. Chem.*, **235**, 1510 (1960). In the former paper data were interpreted on the basis of a *cis* mechanism for the fumarase system while in the latter paper a *trans* interpretation was used.

(36) Deuterium is not incorporated into the α position of isocitric acid when the *cis*-aconitase reaction is carried out in D_2O: S. Englard and S. P. Colowick, *J. Biol. Chem.*, **226**, 1047 (1957).

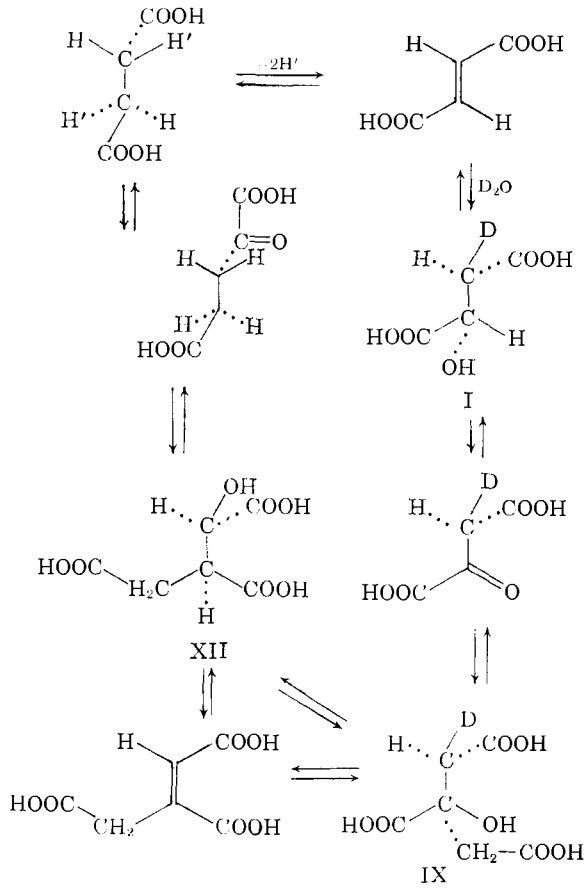
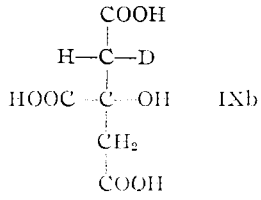


Fig. 4.—Stereochemical reactions of Krebs cycle.

L-malic acid (I) through the Krebs cycle (Fig. 4) to isocitric acid, it is seen that this result leads to the *erythro* configuration (D and OH, IXb) for



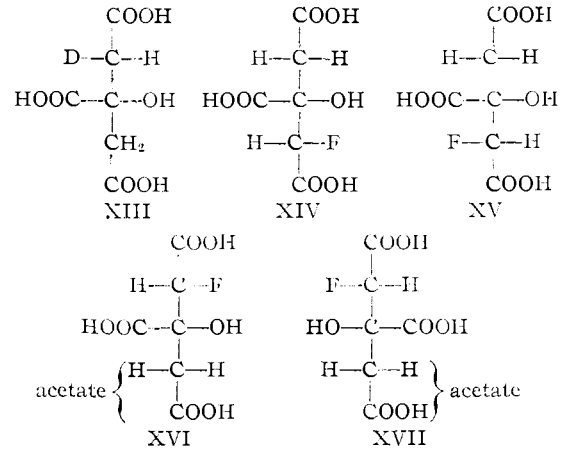
IXb

mono-deuteriocitric acid via a stereospecific condensation of acetate with oxaloacetic acid and to *d*-isocitric acid lacking deuterium through a *trans* operation of the *cis*-aconitase system.[37] The stereospecificity of the reaction between acetyl CoA and oxaloacetate must be such that the newly introduced carboxymethylene side chain occupies the same position in citric acid as did the original hydroxyl group in L-malic acid and citric acid (IXa) may thus be thought of as a substituted D-malic acid.

Included in Fig. 4 is a depiction of the recently, seemingly conclusive, ascertained *trans*-succinic dehydrogenase catalyzed dehydrogenation of succinic acid.[41,42] In addition to the fact that this reaction also proceeds *via* a *trans* stereoselectivity, it is of interest to note that if any α,α'-dideuteriosuccinic acid were to be operated on by the enzymes of the Krebs cycle, monodeuterio-isocitric acid would be obtained[43] since dideuterio-fumaric acid already has been shown[35] to yield monodeuterio-isocitric acid.

Enzyme Mechanisms.—Of particular interest with regard to mechanistic interpretation are the *cis*-aconitase, fumarase and aspartase systems[44] since the reaction type and stereochemical mechanism is the same for all three. A detailed study of

or *cis* nature of the aconitase catalyzed reversible dehydration of citric acid.



(38) C. Martius and G. Schorre, *Ann.*, **570**, 140, 143 (1950).

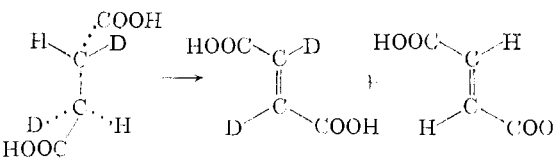
(39) R. A. Peters, P. Buffa, R. W. Wakelin and L. C. Thomas, *Proc. Roy. Soc.* (*London*), **140B**, 497 (1953).

(40) R. A. Peters, *Faraday Soc. Disc.*, 189 (1955).

(41) T. T. Chen and H. Van Milligan, *J. Am. Chem. Soc.*, **82**, 4115 (1960).

(42) Further evidence indicates the enzyme has a steric preference for those hydrogen atoms of succinic acid which in the enzyme substrate complex would have the D-configuration. O. Gawron, A. J. Glaid, III, T. P. Fondy and M. M. Bechtold, unpublished work and *Nature*, **189**, 1004 (1961).

(43) Albeit only half of the isocitric molecules would be so labelled from *meso*-α,α,-dideuterio-succinic acid, since



(44) The stereochemistry of the histidase-catalyzed reaction[45] the enzyme-catalyzed reversible formation of adenylosuccinic acid from fumaric acid and adenylic acid[46] and the enzymatic cleavage of L-argininosuccinic acid to arginine and fumaric acid[47] would be of interest in this connection.

(45) A. Meister, "Biochemistry of the Amino Acids," Academic Press, Inc., New York, N. Y., 1957, p. 162.

(46) Ref. 41, p. 261.

(47) Ref. 41, p. 284.

(37) Since the stereochemistry of citric acid (IX) is inferred, the possibility exists that condensation of the deuterioöxaloacetic acid with acetate yields citric acid with the enantiomorphic configuration at the β-carbon. This would necessitate a *cis* reversible dehydration to yield *cis*-aconitic acid. An unequivocal solution would require mono-deuterio-citric acid of known configuration, either IXb or XIII, and submission of these to the action of *cis*-aconitase.

It might also be mentioned that the assigned configuration IX to citric acid permits spatial depiction of the various synthetic[22-33] isotopic antipodes of citric acid and also suggests that enzymatically synthesized fluorocitric acid[39] has the configuration XIV or XV. In this latter connection synthetic fluorocitric acid is more inhibitory to soluble *cis*-aconitase than is natural fluorocitric acid.[40] Synthetic fluorocitric acid would be a configurational mixture possessing in addition to configurations XIV and XV the configurations XVI and XVI and the latter configurations, or one of the configurations XIV or XV, undoubtedly would show different properties to the enzyme than does natural fluorocitric acid. Elucidation of the configuration of natural fluorocitric acid would also provide information regarding the *trans*

the fumarase system has been carried out by Alberty, *et al.*,[48,49] and the mechanism presented by these workers may be taken as a basis for discussion. In this mechanism[50] a specific carbon atom of the double bond of fumarate is initially attacked by a proton from a specific group of the enzyme. This attack results in a carbonium ion at the adjacent carbon to which in turn a water molecule attaches itself. A proton then is removed from the attached water molecule by a specific group on the protein and L-malic acid results. This mechanism which is ably supported and documented by the accumulated evidence of Alberty and coworkers has been modified[49] to accord with the stereochemical evidence. That is, that fumaric acid must sit in the enzyme so that the site of one of the two specific groups involved is on one side of the molecule and the site of the other specific group is on the other side of the molecule, thus giving the mechanism a *trans* stereochemistry. While no evidence has been brought to bear on whether one or both α-carbons of fumaric acid may be hydroxylated in the enzyme substrate complex, either α-carbon may be hydroxylated if the enzyme has some flexibility.[51] This modified scheme is presented in Fig. 5, and we have again utilized the protonated double bond intermediate. The protonated double bond intermediate may, of course, be in equilibrium with all possible carbonium ions. While evidence is not available for the participation of specific protein groups in the *cis*-aconitase and aspartase catalyzed reactions, it would not seem unlikely a similar situation prevails in these systems.

In the previous section of this paper, it has been pointed out that the stereochemical relation between the reactants of the *cis*-aconitase system require addition of OH, and concomitant *trans* addition of H, to one side of *cis*-aconitic acid to yield *d*-isocitric acid (XII) and addition of OH to the other side to yield citric acid (IX), provided, of course, that *cis*-aconitic acid and the other reactants fit the enzyme in only one way. This view of the reaction necessitates replacement of H with OH and *vice versa*, both with retention of configuration. While Fe^{++} is an essential component of the enzyme, no evidence is available that it participates in an oxidation–reduction reaction and, indeed, it is more likely it participates in enzyme–substrate complex formation since it forms[52] a one to one Michaelis-Menten complex with the enzyme. Since no definitive evidence is available for the nature of the *cis*-aconitase catalyzed reactions, it is appropriate to consider an alternative possibility for the apparent opposite side addition of OH with concomitant *trans* addition of H.

It is to be noted that *cis*-aconitic acid might fit the enzyme in two ways.[53,54] If such be the case

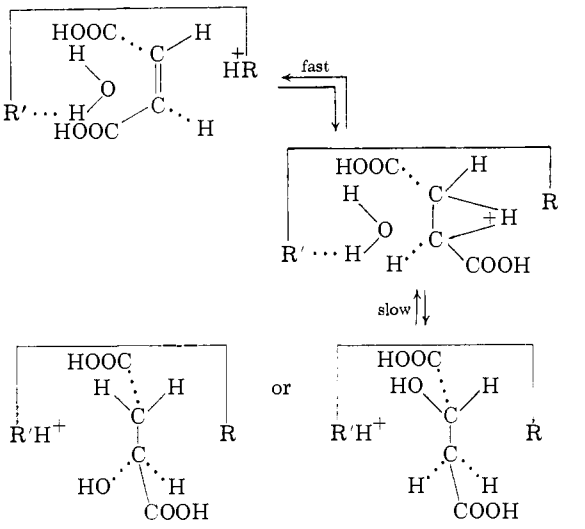
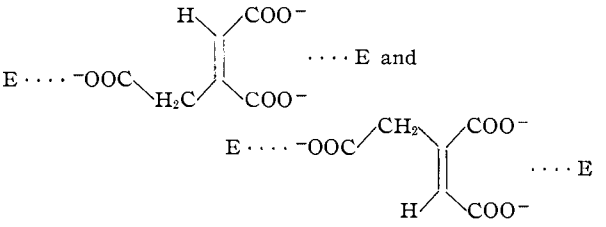
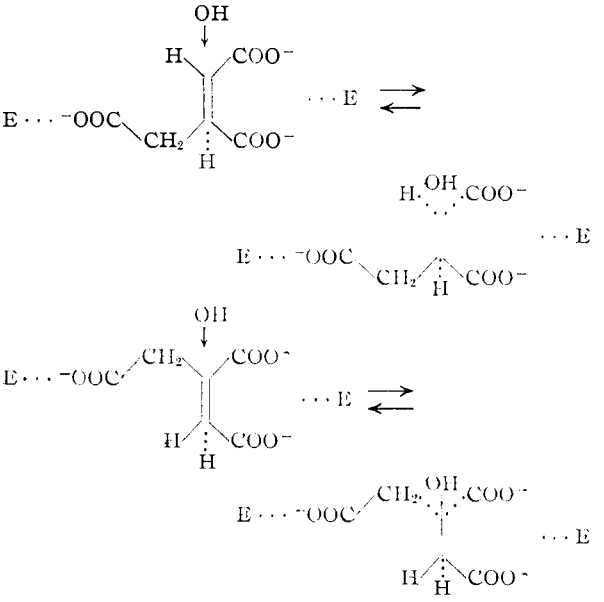


Fig. 5.—Stereochemical depiction of fumarase catalyzed reversible hydration of fumaric acid.

then addition of OH from top and addition of H



from underneath would in one case lead to *d*-isocitric acid and in the other case to citric acid, *viz.*



OH and H adding to the same carbon with respect to the enzyme in both cases. This view of the reaction permits enzyme control of the addition of water without requiring two water sites and does not require any oxidation–reduction considerations. This concept of the reaction permits simultaneous

(48) R. A. Alberty, W. G. Miller and H. F. Fisher, *J. Am. Chem. Soc.*, **79**, 3973 (1957).

(49) P. W. Wigler and R. A. Alberty, *ibid.*, **82**, 5482 (1960).

(50) Fig. 3, ref. 44, albeit D-malic acid inadvertently results.

(51) D. E. Koshland, Jr., *J. Cell. Comp. Physiol.*, **54**, 245 (1959).

(52) J. F. Morrison, *Biochem. J.*, **68**, 685 (1954).

(53) By turning the compound 180° around a horizontal axis in the plane of the paper midway between the carbon atoms of the double bond.

(54) The α-carboxyl (acetate carboxyl) is necessary for substrate activity, ref. 33.

formation of citric acid and isocitric acid from *cis*-aconitic acid since part of the enzyme molecules would be occupied by *cis*-aconitate in one configuration and part by *cis*-aconitate in the inverted configuration. Considering the reverse reaction, the direct formation of *cis*-aconitic acid from citric acid and from isocitric acid is apparent and the conversion of citric acid to isocitric acid and *vice versa* seemingly requires *cis*-aconitic acid as an intermediate. While the kinetic and isotopic data[55] show that in part the isocitrate to citrate conversion passes through *cis*-aconitate as an intermediate, they also show that in part the isocitrate to citrate conversion occurs directly. This latter fact is an apparent objection to this new view of the *cis*-aconitase reaction. Conceivably, it may be overcome if in the isocitrate to citrate conversion an intermediate (or *cis*-aconitate) in some instances does not leave the enzyme but rather pivoting on the acetate carboxyl is inverted. On this basis, then, the initial rate of isocitrate to citrate conversion is slow and direct but as the *cis*-aconitate concentration increases return of *cis*-aconitate to the enzyme in the proper configuration is increased and the rate of the isocitrate to citrate conversion would increase. This would account for both the lag period in the isocitrate to citrate (and *vice versa*) conversion and also for the deuterium incorporation into citrate from isocitrate.

If the enzyme–substrate complex is formed as indicated

$$E \cdots \text{Substrate} \cdots Fe^{++} \cdots E$$

then it would be possible for substrate to disengage itself from the complex without leaving the enzyme, for example, by a loosening of the bonds between substrate and Fe^{++}. As a matter of interest, if isocitric acid and citric acid are attached to Fe^{++} as typical α-hydroxy acid 5-membered chelate rings, then transformation of these to *cis*-aconitic acid might loosen the attachment to Fe^{++} and permit *cis*-aconitic acid to assume the other configuration.

Of course, the available experimental evidence does not permit either definitive conclusions or a detailed mechanistic picture of the reaction.

When it is considered that all three enzyme systems operate by a *trans* mechanism, it seems likely that the three enzymes in question would have, at least, common architectural features. Such features might well be a polypeptide chain, which when in combination with substrate is folded in such a way that at the active site there is a *trans* juxtaposition of those specific groups which participate in the reaction and that the substrate when in combination with the enzyme is not at the surface of the protein but rather within, to a greater or lesser extent, the protein. Such an arrangement would be in line with the induced-fit hypothesis of Koshland,[51] would provide a role for tertiary protein structure[56] in maintaining enzyme activity and would also be similar to the accepted picture of the myoglobin molecule in which a heme residue is imbedded in the protein molecule between the adjacent sides of a protein fold.[57]

Acknowledgment.—This investigation was supported by Public Health Service research grant, RG-6245, from the Division of General Medical Sciences, Public Health Service.

(55) See ref. 33 and references contained therein and H. A. Krebs and O. Holzach, *Biochem. J.*, **52**, 527 (1952).

(56) F. M. Richards, *ibid.*, **54**, 207 (1959).

(57) J. C. Kendrew, *Federation Proc.*, **18**, 740 (1959).

[CONTRIBUTION FROM THE DIVISION OF NUCLEOPROTEIN CHEMISTRY, SLOAN-KETTERING INSTITUTE FOR CANCER RESEARCH, SLOAN-KETTERING DIVISION OF CORNELL UNIVERSITY MEDICAL COLLEGE, NEW YORK, NEW YORK]

Nucleotides. II.[1] A New Procedure for the Conversion of Ribonucleosides to 2′,3′-*O*-Isopropylidene Derivatives[2]

BY ALEXANDER HAMPTON

RECEIVED MARCH 1, 1961

Di-*p*-nitrophenyl hydrogen phosphate is an efficient catalyst for the conversion of purine and pyrimidine ribonucleosides to their 2′,3′-*O*-isopropylidene derivatives, and 2,2-dimethoxypropane is highly effective for maintaining the anhydrous conditions necessary for such conversions. The two agents in combination caused quantitative conversion of all nucleosides examined to the respective 2′,3′-*O*-isopropylidene derivatives; previous procedures have been of more restricted usefulness. The products were isolated by a method applicable to all and obtained in purified form in *ca.* 90% yield. The procedure can simplify conversion of ribonucleosides to 5′-phosphate derivatives since the intermediate isopropylidene nucleosides can often be phosphorylated *in situ*.

Purine and pyrimidine ribonucleoside 5′-monophosphates are starting materials for recent chemical syntheses of nucleotide coenzymes,[3-8] and a number of non-natural 5′-nucleotides have become of biological and chemotherapeutic interest as anabolites of anti-neoplastic purine and pyrimidine derivatives,[9] *e.g.*, of 6-mercaptopurine, 2-amino-6-mercaptopurine or 8-azaguanine. The synthesis of nucleoside 5′-phosphates[8] involves conversion

(1) Part I, A. Hampton and M. H. Maguire, *J. Am. Chem. Soc.*, **83**, 150 (1961).

(2) This investigation was supported in part by funds from the National Cancer Institute, National Institutes of Health, Public Health Service (Grant CY-3190) and from the Atomic Energy Commission (Contract AT(30-1)-910).

(3) V. M. Clark, G. W. Kirby and A. Todd, *J. Chem. Soc.*, 1497 (1957).

(4) R. W. Chambers and H. G. Khorana, *J. Am. Chem. Soc.*, **80**, 3749 (1958).

(5) J. G. Moffatt and H. G. Khorana, *ibid.*, **81**, 1265 (1959).

(6) A. M. Michelson, *Chemistry & Industry*, 1267 (1960).

(7) R. W. Chambers, P Shapiro and V. Kurkov, *J. Am. Chem. Soc.*, **82**, 970 (1960).

(8) Reviewed by H. G. Khorana, *Federation Proc.*, **19**, 931 (1960).

(9) Reviewed by H. G Mandel, *Pharmacol. Rev.*, 11, 743 (1959).