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REVIEW

Citrate Enzyme Substrates and Inhibitors: Depiction of their Absolute Configurations

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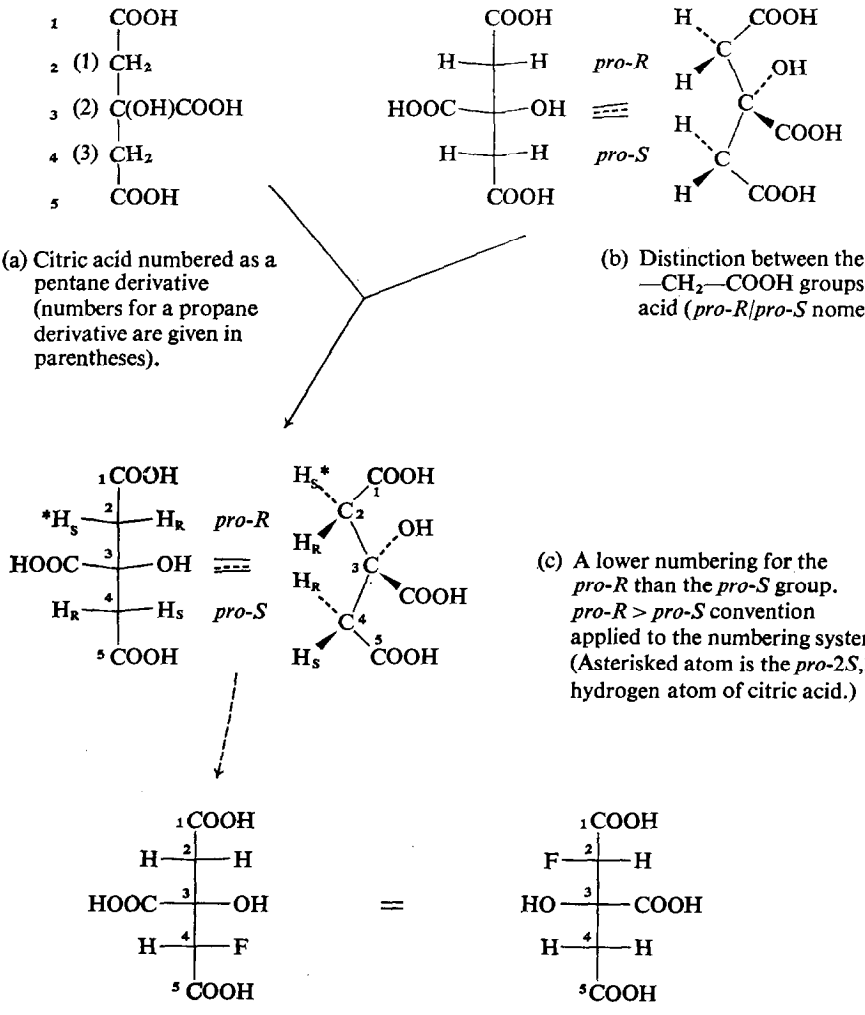
The absolute configurations of the four isomers of hydroxycitric, fluorocitric, and isocitric acids are tabulated and correlations with their interactions with some citrate enzymes are made. In order to facilitate the description of the absolute configurations of related compounds that are interacting with a given enzyme, a local numbering system, in which the compound is related to its parent compound (parent numbering), is suggested.

A study of the absolute configurations of derivatives of citric acid with their activities in the active site of a given citrate enzyme (*1*)[1] gives information on the stereochemistry of the mechanism of action of that enzyme. It is apparent from the literature [e.g., see (*2*)] that some confusion exists on the absolute configurations of certain compounds related to citric acid. It is our aim in this paper to clarify this by a description of the ways of specifying each atom in citric acid and in its derivatives. We will also introduce an *ad hoc* numbering scheme that will simplify the comparison of such derivatives. The entire plan is illustrated in Fig. 1 and will be described below.

We have prepared a tabulation (Fig. 2) of the absolute configurations of each of the four isomers of fluorocitric, hydroxycitric, and isocitric acids, showing (a) Fischer projections (*3*)[2], (b) perspective diagrams in extended staggered (rather than eclipsed)

[1] Enzymes utilizing citrate include (a) aconitase (EC 4.2.1.3 aconitate hydratase), (b) citrate (*si*)-synthase [(EC 4.1.3.7) citrate oxaloacetate-lyase (*pro*-3-*S*-$CH_2C00^- \rightarrow$ acetyl CoA)], (c) citrate (*re*)-synthase [EC status under consideration, (*pro*-3-*R*-$CH_2C00^- \rightarrow$ acetyl CoA)], (d) ATP citrate lyase [(EC 4.1.3.8) citrate oxalacetate-lyase (*pro*-3-S-$CH_2C00^- \rightarrow$ acetyl CoA, ATP dephosphorylating)], (e) citrate lyase [(EC 4.1.3.6) citrate oxaloacetate-lyase (*pro*-3-*S*-$CH_2C00^- \rightarrow$ acetate)], and for the purposes of this paper we will also consider (f) isocitrate dehydrogenase (EC 1.1.1.41 and EC 1.1.1.42).

[2] In Fischer projection formulae (*3*) the longest carbon chain in the compound (C-1 to C-5 in citric acid with C-1 at the top) is vertical on a page, with the C—C—C bond angle of this vertical chain bent back so that the top and bottom carbon atoms are below the plane of the paper, and with the horizontal X—C—Y angle (where X and Y are any substituents) bent up so that X and Y are above the plane of the paper. In a Newman projection a molecule is viewed along the bond between two atoms. The atoms, represented by circles, have bonds drawn to the center of the upper atom but only to the edge of the circle of the lower atom. The perspective diagrams and Newman projections, (b) and (c), show the actual configurations of the molecules and resemble the conformations found in crystal structures, and therefore *models of each isomer may be built by directly copying these diagrams* (*b*) *and* (*c*). However, in the



(a) Citric acid numbered as a pentane derivative (numbers for a propane derivative are given in parentheses).

(b) Distinction between the two $-CH_2-COOH$ groups of citric acid (*pro-R*/*pro-S* nomenclature).

(c) A lower numbering for the *pro-R* than the *pro-S* group. *pro-R* > *pro-S* convention applied to the numbering system. (Asterisked atom is the *pro*-2*S*,3*R* hydrogen atom of citric acid.)

(d) Example of parent-numbering for a substituted citric acid. (pn_{cit})-(4*S*)-4-fluorocitric acid. When F replaced by OH the product is (pn_{cit})-(4*S*)-4-hydroxycitric acid.

(e) Standard numbering system. Note the positions of the central OH and COOH groups. (2*S*,3*R*)-2-fluorocitric acid. When F replaced by OH the product is (2*S*,3*S*)-2-hydroxycitric acid.

FIG. 1. Scheme of numberings described in this paper.

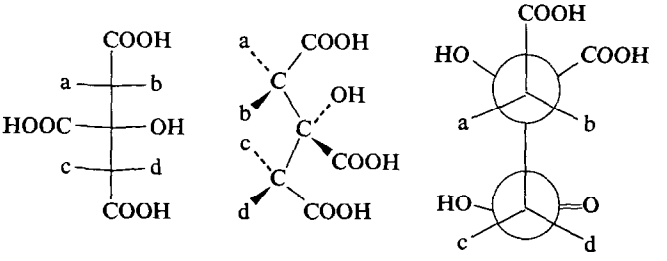
Fischer projections the situation is more complicated. The model, as initially constructed from such a projection, will have adjacent chiral or prochiral centers eclipsed and it is necessary to change the torsional angles to produce a staggered conformation. This is shown in Fig. 1 (b) and (d). Therefore in building a model from the Fischer formula of a molecule with two or more adjacent asymmetric carbon atoms the model must be turned over when the second asymmetric carbon atom is considered. This may be seen by a comparison of Fig. 2 (a) and (b).

conformations, and (c) Newman projections of the same staggered conformations, of these compounds. The acids rather than the ions are shown because the optical activity of the acid is listed for each compound. While isocitric acid is not a derivative of citric acid it is included here because it is so closely related to it metabolically and because such relationships are the subject of this article. The *erythro* and *threo* system and the configuration with respect to glyceraldehyde (subscript g) and serine (subscript s) are also listed since these systems are still in current use. Sources of the compounds and the methods used to determine the absolute configurations are listed in Table 1. In general the configurations are determined from X-ray crystallographic studies involving anomalous dispersion and from chemical syntheses with correlations of configurations.

Fig. 2. Various depictions of citric acid, of its derivatives and of isocitric acid.

1. *Citric acid and some of its derivatives*

(*a*) *Fischer* (*b*) *Perspective* (*c*) *Newman*

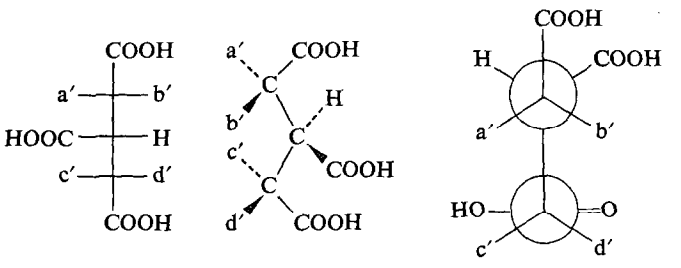


Formula number in text	Substituent in above formulae				Compound	*R*/*S* system	(pn_{clt})-system
	a	b	c	d			
1	H	H	H	H	citric acid		
2	H	F	H	H	fluorocitric acids	(2*R*,3*S*)-2-	(pn_{clt})-(2*R*)-2-
3	H	H	H	F		(2*S*,3*R*)-2-	(pn_{clt})-(4*S*)-4-
4	F	H	H	H		(2*S*,3*S*)-2-	(pn_{clt})-(2*S*)-2-
5	H	H	F	H		(2*R*,3*R*)-2-	(pn_{clt})-(4*R*)-4-
6	H	OH	H	H	(+)-hydroxycitric acid [lactone (−)]	(2*R*,3*R*)-2-	(pn_{clt})-(2*R*)-2-
7	H	H	H	OH	(−)-hydroxycitric acid [lactone (+)]	(2*S*,3*S*)-2-	(pn_{clt})-(4*S*)-4-
8	OH	H	H	H	(+)-allohydroxycitric acid [lactone (+)]	(2*S*,3*R*)-2-	(pn_{clt})-(2*S*)-2-
9	H	H	OH	H	(−)-allohydroxycitric acid [lactone (−)]	(2*R*,3*S*)-2-	(pn_{clt})-(4*R*)-4-

FIG. 2—*continued*

2. *Isocitric acids*

(*a*) *Fischer* (*b*) *Perspective* (*c*) *Newman*



Formula number in text	Substituent in above formulae				Compound	*R*/*S* system	Former systems
	a′	b′	c′	d′			
10	H	OH	H	H	(+)-isocitric acid [lactone (−)]	(2*R*,3*S*)-	*threo*-D_s-, D_sL_g-
11	H	H	H	OH	(−)-isocitric acid [lactone (+)]	(2*S*,3*R*)-	*threo*-L_s-, L_sD_g-
12	OH	H	H	H	(+)-alloisocitric acid [lactone (+)]	(2*S*,3*S*)-	*erythro*-L_s-, L_sL_g-
13	H	H	OH	H	(−)-alloisocitric acid [lactone (−)]	(2*R*,3*R*)-	*erythro*-D_s-, D_sD_g-

1. DESCRIPTION OF CITRIC ACID

a. *Citric acid numbered as a pentane derivative.* Citric acid is described in standard chemical nomenclature (*4*) as 2-hydroxy-1,2,3-propanetricarboxylic acid. However, our main interest has been in active site–substrate interactions. Therefore, in order to refer to the terminal carboxyl groups by number it is more convenient to have a numbering system in which the five carbon atoms in the long backbone of citric acid are numbered 1 to 5 as shown in Fig. 1a (i.e., the compound is considered as a derivative of pentane rather than of propane).

b. *Distinction between the two —CH_2COOH groups of citric acid.* Ogston (*5*) has pointed out that, although citric acid does not contain an asymmetric carbon atom (chiral center), the two —CH_2COOH groups are not equivalent. They may be distinguished by a chiral agent such as an enzyme. An inspection of the perspective diagram in Fig. 2b shows that when the central carboxyl group is above the plane of the paper and the hydroxyl group is below this plane and both are to the right of the backbone, one —CH_2—COOH may be designated the "upper" group and the other the "lower" group, i.e., they may be distinguished immediately in any model by this criterion. The distinction between these "upper" and "lower" groups has been formalized (*6*, *7*) by an

TABLE 1

SOURCES OF CITRIC ACID DERIVATIVES AND THE METHODS USED TO DETERMINE THEIR ABSOLUTE CONFIGURATIONS

Formula Number[c]	Name	Source	Absolute configuration method[a]
1	Citric acid	All cells	
2	Fluorocitric acid	(*15*) Synthesis	?
3		(*15*) Synthesis	?
4		(*15*) Synthesis	c (*16*), x (*9*)
5		(*15, 17*)[b]	b
6	(+)-Hydroxycitric acid		k
7	(−)-Hydroxycitric acid lactone = "garcinia acid"	(*18*) *Garcinia Cambogia* rinds	c (*19*), x (*20*)
8	(+)-Allohydroxycitric acid (lactone = "hibiscus acid")	(*18*) *Hibiscus cannabinus* leaves	c (*19*), x (*20*)
9	(−)-Allohydroxycitric acid	?	k
10	(+)-Isocitric acid	Crassulacean plants, all cells	c (*21*), x (*22*)
11	(−)-Isocitric acid	?	c (*21*)
12	(+)-Alloisocitric acid	*Penicillium* fermentation products (*23*)	c (*21*)
13	(−)-Alloisocitric acid	?	c (*21*)

[a] c = chemical correlations, x = X-ray studies, k = known because that for the enantiomer known, b = additional biochemical information used, ? = no information.

[b] Formed in the body from fluoroacetate present in the leaves of the plant *Dichapetalum cymosum* (*16*).

[c] The formula numbers are those in Fig. 2.

extension of the *R*/*S* system (*8*), by referring to them as the *pro*-3*R* and *pro*-3*S* groups, respectively, as the groups are *pro-R* and *pro-S* to the prochiral center C-3 (see footnote 3 for further details on the *R*/*S* and *pro-R*/*pro-S* nomenclature).

c. *A lower numbering for the* pro-R *than for the* pro-S *group.* The numbering system and the *pro-R*/*pro-S* nomenclature may then be combined. This is done by the use of the general principle that a *pro-R substituent at a prochiral center should receive a lower number than a pro-S substituent* (*13*). This is referred to as the "*pro-R*>*pro-S* convention" where the symbol > indicates the importance in numbering. Thus the *pro*-3*R* —CH_2COOH group of citric acid would be numbered 1 and 2 and the *pro*-3*S* group would be numbered 4 and 5 (see Fig. 1c). At this stage all carbon and hydrogen atoms of citric acid now have a designation as shown in Fig. 1c.

2. DESCRIPTION OF SUBSTITUTED CITRIC ACIDS

a. *Standard chemical numbering.* While citric acid itself does not contain an asymmetric carbon atom the substitution of one hydrogen atom of a methylene group of citric acid by a different atom or group results in two asymmetric carbon atoms in the derivative and hence four isomers. The standard chemical numbering system will always assign

numbers to the principal chain of a singly substituted citric acid in such a manner that the methylene carbon atom bearing the substituent has the lower number, whatever the configuration of the compound. This follows because a hydrogen atom has the lowest atomic number of any possible atom attached to a carbon atom. Thus all four monofluorocitrates will be, in the standard chemical numbering system, 2-fluorocitrates and never 4-fluorocitrates (see Fig. 1e). The two asymmetric carbon atoms are then given designations by the standard *R*/*S* system (*8*).[3]

b. *Statement of the problem.* It is immediately obvious that when a hydrogen atom in citric acid is replaced by a fluorine atom, the product may not have the same numbering as the parent, citric acid. Furthermore, on the replacement of one substituent by another, the designation of one or both carbon atoms in the standard *R*/*S* system may change although no inversion has actually occurred. Let us take as an example (2*S*,3*R*)-2-fluorocitric acid (formula **3**, Fig. 2). Because of the differing priorities of fluorine and oxygen in the *R*/*S* system, substitution of fluorine by hydroxyl results in (2*S*,3*S*)-2-isomers (formula **7**, Fig. 2), i.e., the configuration at C-3 has apparently (but not actually) been altered from 3*R* to 3*S*. Thus, when describing the activities of such derivatives of citric acid in the active site of an enzyme we have to use different designations for compounds that are sterically related. This is a dilemma to which we will now address ourselves.

c. *The use of the "parent-numbering" system for such problems.* Our solution to this problem, since we are interested in discussing biogenetic relationships and enzyme mechanisms, is to have the same numbering scheme for citric acid and for such simple substituted derivatives as the hydroxycitric and fluorocitric acids. Therefore, after extensive discussion with Drs. Kenneth R. Hanson and Hans Hirschmann, and as suggested in (*1*), we will use the "*pro-R* > *pro-S* convention" for citric acid itself, and extend this system to labeled and substituted citric acids by a "parent numbering" system indicated by the prefix (pn_{cit}). In this *ad hoc* system each chiral center is named in the same manner as the parent compound (which must be carefully defined). As the numerical locant for the label or substituent (1, 2, 4, or 5), together with the prefix (pn_{cit}), unambiguously specifies the configuration at C-3, it is convenient to omit the *R*/*S* specification of this center. An example is given in Fig. 1d.

The advantage of such a system is that the possible confusion about the configuration at C-3 that arises because of the different sequence rule priorities of fluorine and hydroxyl in substituted citric acids is avoided. For example, as listed in Fig. 2, the name

[3] In the *R*/*S* system (*8*) each of the four atoms attached to an asymmetric carbon atom is assigned a priority with the highest priority (1) given to the atom with the highest atomic number, an isotope with higher atomic mass taking precedence over one with a lower atomic mass. If two atoms are of equal priorities the next neighbors are considered until a priority is finally established. Then the four atoms are viewed with the lowest priority atom (priority 4) directly behind the asymmetric carbon atom. If the sequence of priorities 1 to 3 is clockwise the carbon atom is designated R (*rectus*) and if it is anticlockwise it is designated *S* (*sinister*). In the *pro-R*/*pro-S* system (*7*) the like (paired) substituents that are to be distinguished have identical priorities. If one of these groups, chosen for convenience, is given a higher priority than the other identical group, then it may be designated *pro-R* if it is apparently *R*, or *pro-S* if it is apparently *S*, by following the rules listed above. The *re*/*si* system (*7*) names the faces of a trigonal atom such as the carbon atom in a carbonyl group. For example, for the *re* face the sequence of priorities 1 to 3 is clockwise. The enzyme citrate (*si*)-synthase catalyzes the interaction of acetyl-CoA with the *si* face of the carbonyl carbon atom of oxaloacetate. In citrate (*re*)-synthase the other face is involved. For more details, on these systems see references (*6–8*, *13*).

(pn_{cit})-(4*S*)-4-fluorocitric acid fully specifies the configuration of the compound formally derived from citric acid by replacing the 4(*pro*-4*S*,3*S*)-hydrogen atom of citric acid with fluorine [also designated (2*S*,3*R*)-2-fluorocitric acid]. Substitution of this fluorine atom by a hydroxyl group gives (pn_{cit})-(4*S*)-4-hydroxycitric acid (formula 7), i.e., the correlation between the two compounds is now emphasized. When the fluorine atom or hydroxyl group is replaced by tritium the compound becomes (pn_{cit})-(4*S*)-citric-*4-t* acid. It is only important to know the designation of the compound undergoing substitution in the "parent numbering" system. Thus substituted compounds may be considered in the same manner as the parent compound so that nomenclature series, related by single substitutions, are formed, as shown in Fig. 2. Members of the (pn_{cit})-(4*S*)-series are enantiomers of members of the (pn_{cit})-(2*R*)-series. When a substituted citric acid is numbered according to the "parent numbering" system the numbering agrees with that of normal chemical practices when (pn_{cit})-C-2 is substituted but not when (pn_{cit})-C-4 is substituted, e.g., (pn_{cit})-(4*R*)-4- and (2*R*,3*R*)-2-fluorocitric acid are both names for one compound (formula **5**) and (pn_{cit})-(2*R*)-2- and (2*R*,3*S*)-2-fluorocitric acid for another (formula **2**).

The "parent numbering" system is a general idea that may be extended, if necessary for convenience, to any discussion of the absolute configurations of related compounds (although the standard *R*/*S* name should also be included in such a discussion and is generally preferable unless there is a real problem, such as that discussed above, that requires a different numbering system). However, unless the parent is carefully defined confusion may result and so it is essential that the formula of each compound be unambiguous when the "parent-numbering" system is used. For example, the (pn_{cit})-system cannot be applied to compounds such as the isocitric acids because citric acid is not a parent for these compounds. However, if required a (pn_{isocit})-series could be used for substituted derivatives of isocitrate. It must be stressed that such systems should *only* be used to solve a local problem, such as that found for citric acid derivatives.

3. THE USES OF THE (pn_{cit})-SYSTEM IN DESCRIBING ENZYME SUBSTRATES AND INHIBITORS

The parent numbering system is particularly useful when the effect of enzymes such as citrate (*si*)-synthase or aconitase on citrate is being discussed. In the biosynthesis of citric acid from oxaloacetate and acetate, *via* citrate (*si*)-synthase, by the *si* attack of acetate on the carbonyl carbon atom of oxaloacetate, the oxaloacetate-derived (*pro*-3*R*) —CH_2COOH group of the citrate ion is numbered 1 and 2 while the acetate-derived (*pro*-3*S*) —CH_2COOH group is numbered 4 and 5 (see Fig. 1). Carbon atoms 4 and 5 are derived from the methyl and carboxyl groups of acetate, respectively. In the action of the enzyme aconitase the dehydration of citrate occurs at C-2 and C-3 with the *anti*-(*trans*-) addition or elimination of the elements of water.

The absolute configurations listed in Fig. 2 may now be examined with respect to the action of the citrate-utilizing enzymes. For example, if the steric course of the enzymatic condensation of derivatives of either oxaloacetate or acetate is the same as observed for the parent compounds, it will be found that all citrate derivates substituted in the (pn_{cit})-(2*R* or *S*) positions are derived from a substituted oxaloacetate by the action of

citrate (*si*)-synthase and those substituted in (pn_{cit})-(4*R* or *S*) positions from a substituted acetyl CoA. The reverse is true for citrate (*re*)-synthase. Also dehydration by aconitase, which involves elimination of a hydroxyl group and of a *pro*-2*R*,3*R*-hydrogen, or a (pn_{cit})-2*R*-deuterium or tritium atom, will affect only these positions. Dehydration may possibly occur for citrate derivatized in the (pn_{cit})-(2*S*,4*R* or 4*S*) positions, depending on the nature of the substituent and the restrictions of the enzyme active site, but these positions will not be involved in the enzymatic reaction. Therefore if, by chance, (pn_{cit})-(4*R*)-4-fluorocitric acid were dehydrated by aconitase (there is no evidence for this) then the —CHF—COO^- group would not be affected by the dehydration. In fact, the inhibition of aconitase by (pn_{cit})-(4*R*)-4-fluorocitric acid implies either that the fluorine has interacted with a group not directly involved in the active site of the enzyme, or that the chelation to the enzyme-bound ferrous iron has been drastically altered, i.e., the interaction between the compound and enzyme is very different from that for citrate itself, a more likely possibility (*9*).

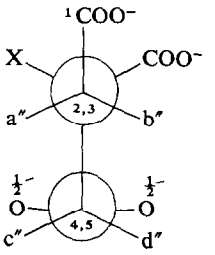
It would be interesting to test the action on aconitase of the (pn_{cit})-(4*R*)-4-hydroxycitrate (formula **9**). This is the hydroxycitrate that is derived by substitution of hydroxyl for fluorine in the isomer of fluorocitrate that is an inhibitor and inactivator of aconitase (formula **5**). The results of this experiment might demonstrate whether the inactivating effect of fluorocitrate depends on its mode of chelation to the ferrous ion, a situation that could be mimicked by hydroxycitrate causing, for example, an interaction of the central carboxyl group with the active site of the enzyme, or whether the fluorine interacts in some other way with the enzyme during enzyme inactivation, for example, by cleavage of the C—F bond with alkylation of the active site (*9*), in which case the hydroxycitrate might behave differently from the fluorocitrate.

4. THE USES OF THE (pn_{cit})-SYSTEM IN PREDICTING THE REACTION OF A CITRATE DERIVATIVE WITH A GIVEN CITRATE ENZYME

It is possible that the stereochemical relations among citrate derivatives can be used to predict the reactions expected with the different citrate enzymes. Thus, Klinman and Rose (*10*) have already pointed out (assuming that the steric course of the condensation of fluoroacetyl-CoA and oxaloacetate with citrate (*si*)-synthase is the same as that for acetyl-CoA) that only the *pro-S* hydrogen atom of fluoroacetyl-CoA is activated in the enzymic condensation. (pn_{cit})-(4*R*)-4-fluorocitrate (formula **5**), made in the citrate (*si*)-synthase reaction [and also a substrate with CoA for this enzyme (*14*)], has a proton corresponding to the 4(*pro*-4*S*-H) of citrate while the (pn_{cit})-(4*S*)-4-hydroxycitrate (formula **7**), an inhibitor for ATP citrate lyase (*11*), lacks such a proton, the position being occupied by a hydroxyl group (Z′ in Table 2). Since citrate (*si*)-synthase and ATP citrate lyase have the same stereochemical features (*12*) one would predict that the biosynthetic (pn_{cit})-(4*R*)-4-fluorocitrate (formula **5**) would be a substrate for ATP citrate lyase while the (pn_{cit})-(4*S*)-4-hydroxycitrate (formula **7**) would not be a substrate for citrate (*si*)-synthase. We have tested the latter postulate and find it to be true in that (pn_{cit})-(4*S*)-4-hydroxycitrate (with CoA) cannot be cleaved by citrate (*si*)-synthase but is a competitive inhibitor for citrate (and oxalacetate) in this reaction.

TABLE 2

ENZYMATIC ACTIVITIES OF SOME CITRATE ANALOGS AND DERIVATIVES



a″ → (pn_{cit})-(2*S*)-2-series
b″ → (pn_{cit})-(2*R*)-2-series
c″ → (pn_{cit})-(4*R*)-4-series
d″ → (pn_{cit})-(4*S*)-4-series
} for substituted citrate acid

a. Citrate (*si*)-synthase and ATP citrate lyase

X	a″	b″	c″	d″	Compound[c]	[b]
OH	H	H	H	H	**1**	**S**
OH	H	H	H	OH	**7**	**I**
OH	H	H	F	H	**5**	**S**

b. Aconitase

X	a″	b″	c″	d″	Compound	[b]
OH[a]	H	H[a]	H	H	**1**	**S**
H[a]	H	OH[a]	H	H	**10**	**S**
OH[a]	CH_3	H[a]	H	H	α-methylcitrate	**S**

c. Isocitrate dehydrogenase

X	a″	b″	c″	d″	Compound	[b]
H	H[a]	OH	H	H	**10**	**S**
OH	H[a]	OH	H	H	**6**	**S**

[a] Known to be involved directly in the enzymatic reaction.
[b] Substrate (**S**) or inhibitor (**I**).
[c] The numbering for compounds is given in Fig. 2.

ACKNOWLEDGMENTS

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