A NEW LOOK AT THE CITRIC ACID MOLECULE

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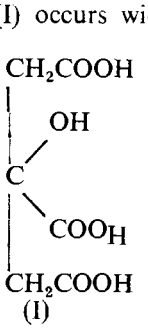
"Identical Groups" in "Symmetrical" compounds like citric acid can be differentiated by enzymes. 'Ogston effect' and analysis of the symmetry elements of such compounds as carried out by Hirschmann & Hanson leads us to an understanding of this phenomenon. These observations have led to the development of the concept of "Prochirality". It is apparent that there is nothing magical about the ability of enzymes to bring about this biological differentiation.

Citric acid (I) occurs widely in nature.



(I)

In 1937 Hans Krebs published the paper dealing with aerobic respiration in pigeon liver tissues. The sequence of reactions first formulated by him have now come to be known as the *Kreb's cycle* or the *Citric acid cycle*. The more modern version being the tricarboxylic acid or TCA cycle. It is not immediately apparent as to why this special emphasis should be laid on the tricarboxylic acid—citric acid, since the cycle also contains a few dicarboxylic acids.

One reason is that citric acid which arises out of the condensation of acetyl coenzyme A with oxalacetate provides a key link between the glycolytic pathway and the aerobic respiration. (These reactions originally discovered in the pigeon liver tissues have been found to be applicable to most other living systems as well).

OGSTON EFFECT

While formulating these reactions Krebs had also stated that if $*CO_2$ (labelled with C^{14}) was assimilated then the citric acid produced would have labelling on the carboxyls of both the CH_2COOH groups, as citric acid is a "symmetrical" compound and we would not expect the CH_2COOH groups to be differentiated by the enzymes. However, he was proved wrong very soon.

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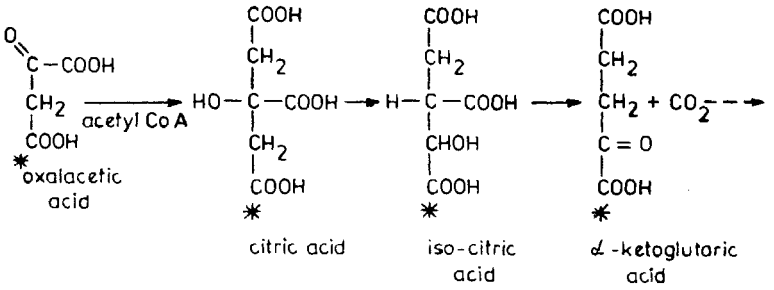
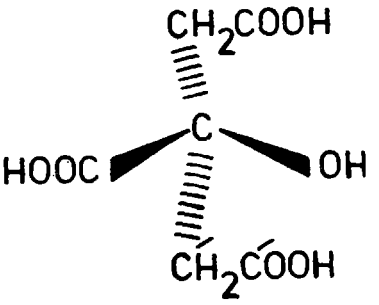
Wood *et al.* (1942) showed that Krebs prediction was wrong. They carried out experiments with labelled CO_2 and found that the α-ketoglutarate formed had the labelling preferentially on the carboxyl group adjacent to the ketonic carbonyl.



However, they interpreted their results as precluding the possible intermediacy of citric acid—A "Symmetrical" molecule. They argued that since it was an intermediate, both the CH_2COOH groups of citric acid would have been labelled and that α-ketoglutarate obtained from it would have the isotopic label on both the CH_2COOH groups and not preferentially as observed. However, these workers too were wrong so far as the interpretation of their observations are concerned and two wrongs certainly do not make a right.

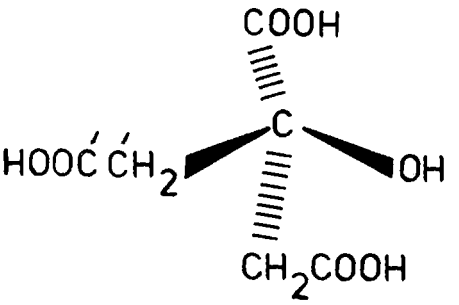
It was left to Ogston (1948) to point out the "fallacy" in their arguments. He brought this out in a short note published in '*Nature*'. This paper incidentally had no experimental section whatsoever. He pointed out that it is not correct to talk of citric acid as a symmetrical compound and he explained how the results of Wood did not preclude the possible intermediacy of citric acid in the sequence of reactions mentioned above.

He proposed a specific "3-point attachment" hypothesis. He stated that when a compound of the type of citric acid binds itself to the enzyme surface, the two possible arrangements of the substrate (described below) are not equivalent. The first arrangement of the compound is :



The second arrangement is obtained by rotating the molecule in a manner that the other "identical" CH_2COOH group now occupies the position of the original

$CH_2'COOH$ group (marked with dash to differentiate)while keeping OH group fixed as:



It should thus be clear that when we bring the CH_2COOH group to the position occupied by the $CH_2'COOH$ group originally we cannot simultaneously bring the original $CH_2'COOH$ group to occupy the position of the CH_2COOH group while keeping one of the other groups fixed.

As mentioned earlier, Ogston considered the interactions of the above two structures with the enzyme surface.

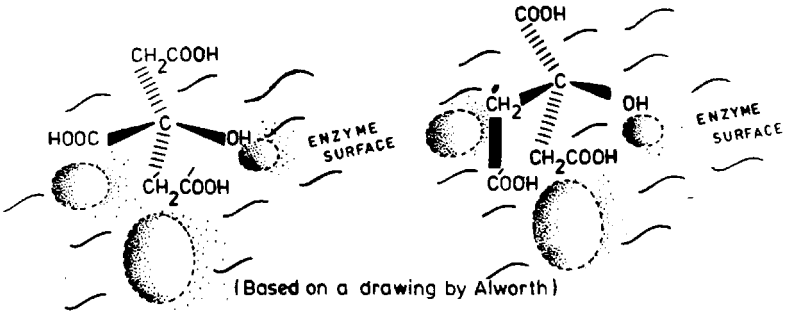


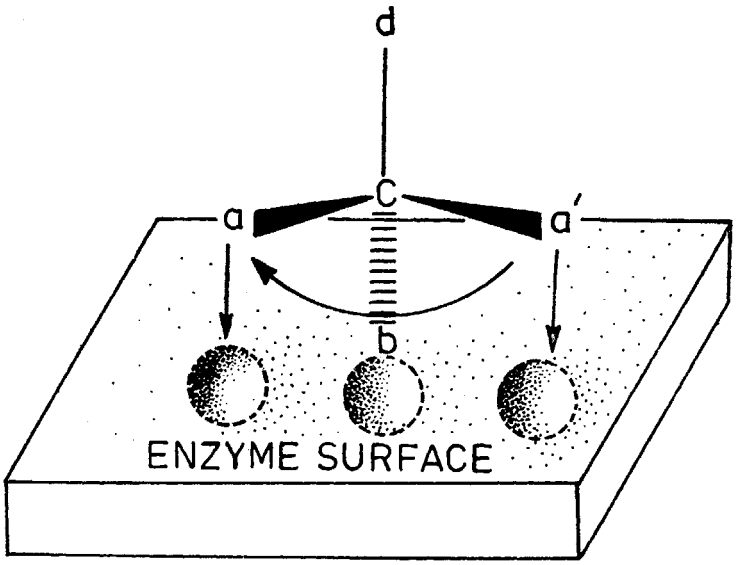
FIG. 1. cf. Alworth (1972).

The interaction of the first structure with the 'active site' of the enzyme is shown in Fig. 1. In this diagram the hydroxyl group is at the specific hydroxyl binding site on the enzyme surface, the $CH_2'COOH$ at its specific position and the COOH at the specific carboxyl binding site. This was the 3-point attachment hypothesis.

In Fig. 2, the 'identical' group (—CH_2COOH) has been brought to occupy the position originally held by $CH_2'COOH$ and the OH group kept fixed at the specific site for hydroxyl. However, this can be done only by taking the COOH group away from its specific site and this position is then occupied by the $CH_2'COOH$ group. The two arrangements described above are not equivalent and only one of these two Enzyme-Substrate complexes is effective. The enzyme is therefore able to differentiate between the 'identical' groups of a 'symmetrical' molecule like citric acid. Though in 1948 Ogston emphasised the 3-point attachment, he clearly pointed out that the enzyme merely recognises the inherent lack of symmetry in the citric acid molecule. Thus Ogston showed how the observations of Wood did not preclude the possible intermediacy of citric acid and that we can explain the preferential labelling of one of the carboxyls in α-ketoglutarate even with citric

acid as an intermediate. Thus was settled the controversy about whether citric acid was an intermediate in the citric acid cycle or not.

Citric acid is a molecule of the Caábd type. Let us call two "identical" groups as (*a*) and (*a'*) for convenience and consider the 3-point attachment of this molecule on the enzyme surface (as shown below). If one moves the group (*a'*) to the position occupied by group (*a*) while keeping the group (*b*) fixed on the enzyme surface, it is quite clear that we cannot bring simultaneously group (*a*) to the position originally occupied by group (*a'*). The two interactions with the enzymes as stated earlier are not equivalent and only one of the two interactions with the enzyme is effective for the enzyme reaction.

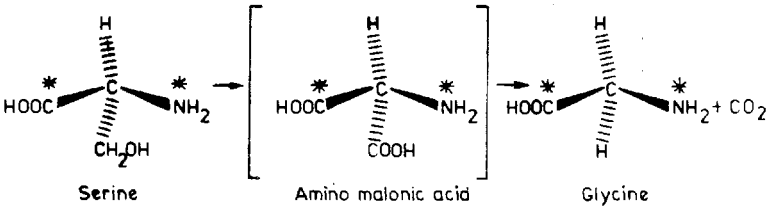


(Based on a drawing by Hirschmann.)

FIG. 2

Actually, Ogston made use of the biosynthesis of glycine from Serine to explain this concept and stated that exactly the same reasoning was applicable to the citric acid case.

Serine labelled on the N* and *CH_2OH was observed to give rise to glycine having the labelling on the carbon and nitrogen in the same ratio as in Serine. The postulated intermediate was Amino malonic acid. It was suggested by many that this observation meant aminomalonic acid could not be an intermediate. Since it was so, one would expect the labelling on the carbon to be evenly distributed on the two carboxyl groups in the "symmetrical" intermediate and the ratio C^{13} : N^{15} in the final product would be 0.5. Ogston on the basis of 3-point attachment hypothesis explained how Amino malonic acid could be an intermediate and still the glycine labelling ratio could be as observed.



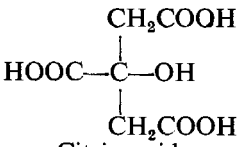
The concept of Ogston, however, was never fully understood. His "3-point attachment" was perverted to mean that the citric acid is never free and is always bound to the enzyme surface. This was soon proved wrong by Potter and Heidelberger (1949) who isolated free citric acid from such systems.

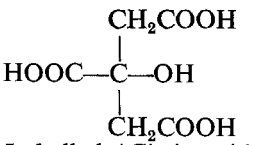
The "3-point attachment"was given undue importance. As was stated by Ogston himself in 1958, the 3-point attachment is not to be taken literally. However, whatever he meant by the word "identical" groups it created enough confusion. Most biochemists were under the impression that there is something 'magical' in the manner in which this biological differentiation of 'identical' groups was carried out by the enzymes. This feeling seems to be a carry over of the feeling prevalent among many biologists till even 1930's that completely new laws had to be discovered to explain biological phenomenon. In other words they felt that an apple had to fall on somebody's head! Further, in any case many felt: "Oh, no, not this dirty chemistry to explain our biological phenomenon".

To digress from the main stream, are the chemists absolved of all blame? Mention may be made of Berzelius and Liebig who were completely satisfied after putting down a balanced equation for fermentation and would not allow biology a quarter. More recently Martius and Knoop who had also formulated many of the reactions of the Krebs cycle but did not care to think about the significance of the same and it was left to Krebs to do the digging up operations. Krebs ascribes their reluctance to their outlook. Having been trained under Arndt (of the Arndt-Eistert Reaction fame), Martius & Knoop considered themselves as "theoretical organic chemists" interested only in reaction mechanisms.

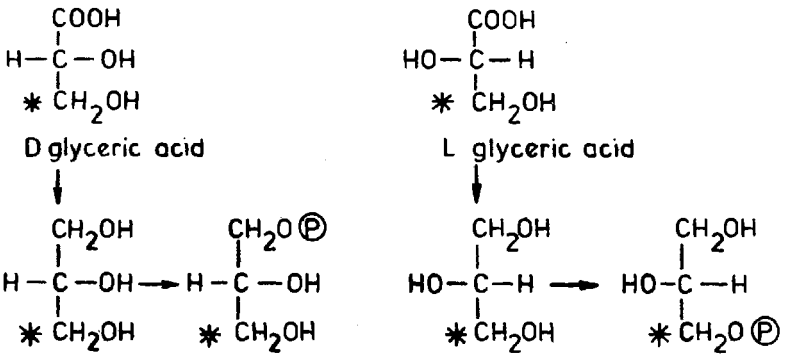
As we have seen, there is nothing magical about this ability of enzymes to bring about this biological differentiation of the so called "identical" groups. The enzyme merely recognises the inherent lack of symmetry in molecules of the type of citric acid, amino malonic acid, etc.

An element of doubt was introduced by Aronoff (1956) who argued that this differentiation by enzymes observed in the case of citric acid and amino malonic acid is simply one between enantiomers. Though this observation is clearly wrong it is good to look into the details of this argument. When we talk of the isotopically labelled citric acid, as soon as the labelling is introduced the "symmetrical" citric acid becomes an "asymmetrical" compound and therefore he argued that this differentiation between the two groups is merely a case of the usual differentiation between enantiomers.

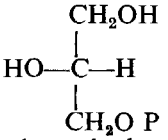

Citric acid
(Symmetrical compound)


Labelled *Citric acid
(Asymmetrical compound)

In the parallel case of glycerol this argument has even been refuted experimentally. Karnovsky (1957) prepared D & L glyceric acids labelled on the hydroxymethyl. (This was done by resolving DL Serine-3-^{14}C and then separately treating D & L Serine-3-^{14}C with HNO_2).



On carrying out enzymatic phosphorylation the glycerol obtained from L glyceric acid was phosphorylated on the *CH_2OH (labelled). On the other hand the glycerol obtained from D-glyceric acid had the phosphate on the CH_2OH (unlabelled). This is not a differentiation between enantiomers as here the enzyme reacts with both the compounds unlike the usual observations that enzymes react with only one of the two enantiomers. Further, if what Aronoff said was right i.e., if the differentiation was due to the introduction of the isotopic labelling then with ordinary glycerol ("Symmetric") no differentiation should be possible and one would expect the racemic product. When the reaction using ordinary glycerol was carried out enzymatically the product was found to be optically active—only L-α-glycerol phosphate was formed.


L-α-glycerol phosphate

Thus enzymes are able to distinguish between the so called "identical" groups in compounds like citric acid, aminomalonic acid, and glycerol because of the inherent lack of symmetry in such compounds.

This is not, however, a point that has been understood so well. One can gauge this from the following :

As pointed out by Alworth one finds in a recent biochemistry text by Lehniger (1970) : "Condensation of oxalacetate with carboxyl labelled acetate would be expected to produce citrate labelled in one carboxyl group but since citrate is a completely symmetrical molecule one would expect the two terminal carboxyl groups to be chemically indistinguishable". Another popular biochemistry text book by Conn and Stump (1966) more or less states that compounds like citric acid are symmetrical from the point of organic chemistry and asymmetrical from the point of biochemistry!

All this clearly point to the mistaken notion that citric acid is a completely symmetrical molecule.

However it is interesting to note what Van't Hoff (1874) had to say about citric acid. He stated "*The left part of citric acid bears to the right part of the molecule the relation which the left hand of a man has to the right hand*". This, no doubt, is yet another demonstration of the clarity with which Van't Hoff could visualise molecules (*vide* Plate I & Plate II).

Ogston's paper did not merely clear the confusion over the intermediacy of citric acid in the Krebs cycle. It, in fact, set into motion some very basic ideas concerning symmetry of molecules and the concept of prochirality, to be discussed shortly, is a direct outcome of these deliberations. Some of the questions which arise naturally are :

(A) How are we to convey stereochemical information about molecules of this kind in an unambiguous manner?

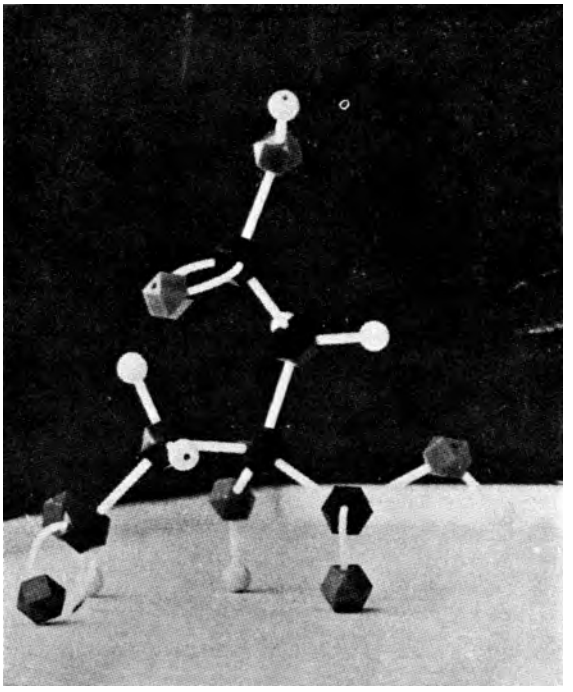


PLATE I.

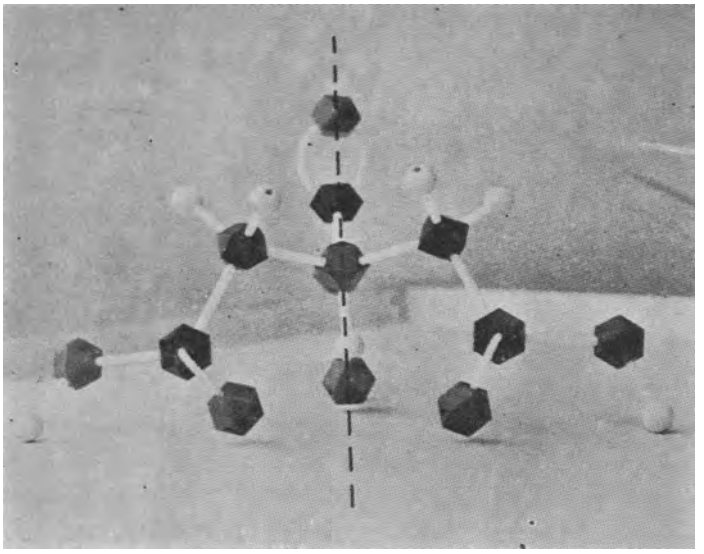
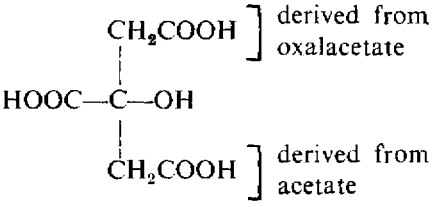


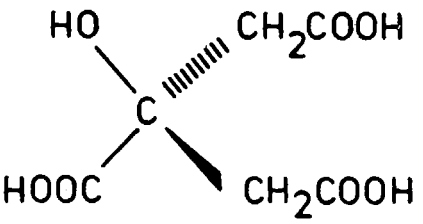
PLATE II

(B) Which kind of molecules will permit this kind of biological differentiation? What is the basic criterion by which we can decide this?

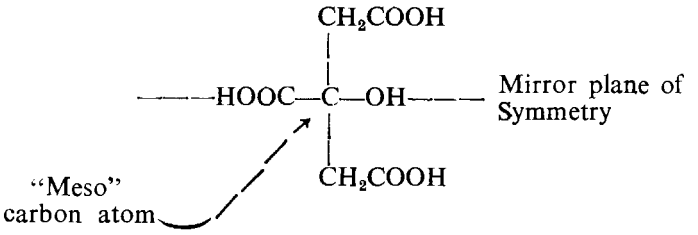
The problem of describing molecules clearly and precisely is apparent from the classic paper of Hanson (1966) where he describes his finding about which part of citric acid originates from oxalacetate and which comes from acetyl Co A.



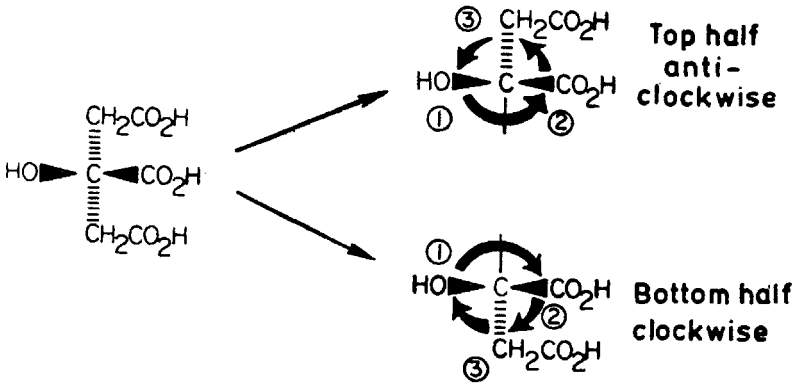
In this paper one comes across the words quoted below and the difficulties in describing the stereochemical information about molecules can be gauged from the same. He states, "*If the axis of centre carbon atom and its attached carboxyl group is equated with the axis of the body so that the carboxyl group is at the feet and if the* OH *is behind the body then the acetyl* Co-A *derived* CH_2COOH *group is to the right front, and the oxalacetate derived* CH_2COOH *is to the left front.*" However, interestingly about ten years later, the same worker developed a system to put down this kind of information unambiguously. Wilcox (1949) was the first to analyse the symmetry of molecules like citric acid and said that the CH_2COOH groups above and below the plane containing the carboxyl and the hydroxyl groups could be differentiated by enzymes.



Schwartz & Carter (1954) looked at the problem in a slightly different manner. They defined a carbon atom of the type of C-3 carbon atom in citric acid as a *Meso carbon atom* since this resembles compounds like Meso Tartaric acid.



They said in a molecule of the Caábd type one half of the molecule is the mirror image of the other and they are non-superimposable. This is particularly clear from an analysis of the ordering of the groups according to Cahn-Ingold-Prelog priority rules which shows the top and bottom halves bear an enantiomeric, mirror image, non superimposable relationship—exactly what Van't Hoff foresaw so many years ago.



However, the concept of the Meso Carbon atom was severly criticised by Jaeger (1930) and Hirschmann. Hirschmann (1964) stated : "*The place of the Meso-carbon atom in the problem of differentiation is not unlike that of the asymmetric carbon atom in the theory of optical isomerism and therefore what Jaeger said applied to it, as well. The whole concept of asymmetric atoms must be considered as an incomplete*

and rudimentary one. The fundamental truth of the general principle underlying the phenomena of optical isomerism has been disturbed by bringing to the fore a mere special case of a general principle, as if it were the fundamental and exhaustive one".

Hirschmann pointed out that the organic chemists had for far too long been preoccupied with just one aspect of symmetry of organic structures, viz., Reflective Symmetry and to the exclusion of all other aspects. Even within reflective symmetry operations the organic chemists bothered about only two :

(*i*) The mirror plane of symmetry, σ (whereever possible the organic chemist was only interested in just pushing in a mirror into the molecule!); and (*ii*) the centre of symmetry, *i*.

Organic chemists, however, did not realise that even these two elements of symmetry form a part of the very much more general reflective symmetry operation viz., 'The Improper Rotation Axis', i.e., Rotation/Reflection operation—S_n. In fact, S_n where $n = 1$ is the mirror plane of symmetry, and S_n, $n = 2$ is the centre of symmetry, *i*.

A molecule which possesses S_n i.e., Reflective Symmetry will not show optical isomerism. Sokolov (1973) infac't stated "The Van't Hoff—Le Bel tetrahedral model played a decisive role in the practical development of stereochemistry, although the detailed geometry was in some sense a retrograde step in comparison with Pasteur's general idea on molecular asymmetry as the cause of optical activity".

Organic chemists were, however, never bothered about Rotational Symmetry C_n

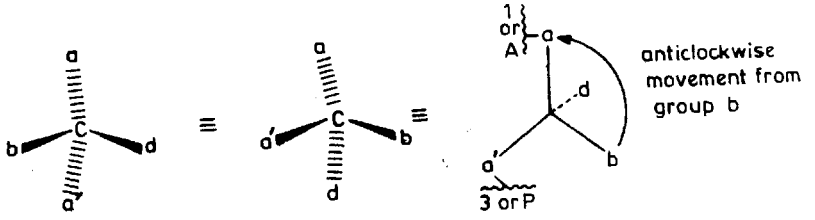
Let's look at Malonic acid :

It possesses Rotational symmetry. The two identical groups can both be superimposed upon each other by rotation of 180° i.e., it possesses a C_2-axis of Rotational Symmetry.

The two, so called, identical groups in malonic acid cannot be differentiated even by an enzyme.

Therefore, just as a molecule cannot show optical isomerism if it possesses Reflective Symmetry. Biological differentiation is not possible if a molecule possesses Rotational Symmetry.

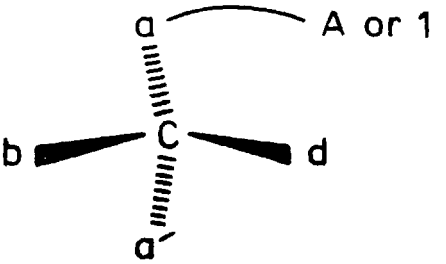
Hirschmann also proposed a system for designation of such molecules : (The Hirschmann System)



For a molecule of the type Caábd if group b has a higher priority than group d (according to the *R/S* system rules) then we keep group d pointed away from us (shown in the figure) and move from group *b* in an anticlockwise (*S*) manner (chosen on biogenetic grounds).

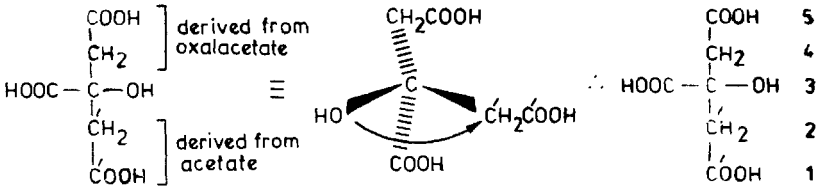
The group *a* or *a'* whichever one comes across first is then called '*A*' (Ante, front) or given the lower number, The other group is then designated as *P* (Post, behind). In the above example therefore $a = 1$ or A; $a' = 3$ or P

The same designation may be done using Fischer projections as well. If $b > d$ (i.e., *b* has a higher priority) and if group *b* is on the left and group *d* on the right side, both pointing towards us in the Fischer projection then the "identical" group-a on top in this projection is designated *A* or given the lower number. In the above case one reaches the same conclusion as before. (Another variant of this method is useful for Fischer projections in which group *b* is on top and group *c* at the bottom, then group *a* on the left is called *A* or given the lower number).



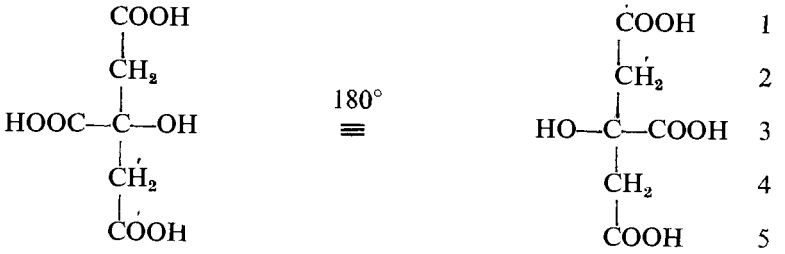
Let's now use the above rules to designate the citric acid molecule according to the Hirschmann system :

Here OH > COOH i.e., hydroxyl group has a higher priority. The two 'identical' groups are CH_2COOH and $CH_2'COOH$ (marked with dash only to differentiate). We keep the COOH group pointed away from us. On moving from the hydroxyl group in an anticlockwise manner one comes across $CH_2'COOH$ first and hence it is given the lower number.

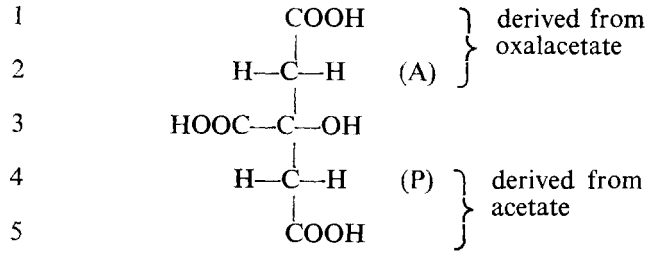


The same designation can be done using the Fischer projection. By doing a 180° rotation of both the sets of groups in the Fischer projection one obtains an equivalent Fischer projection with the hydroxyl group (higher priority group) on the left and the carboxyl group on the right. Therefore, the CH_2COOH which is on

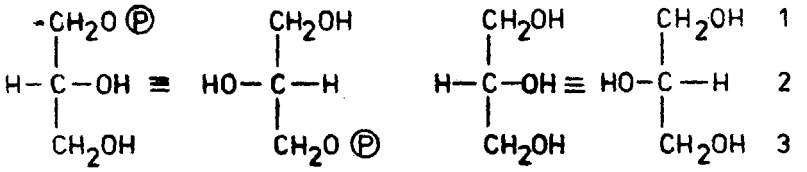
top is given the lower number. The hydrogens of the methylenic groups at positions 2 and 4 of citric acid can be designated similarly by this system.



The complete designation of the citric acid molecule is shown below :

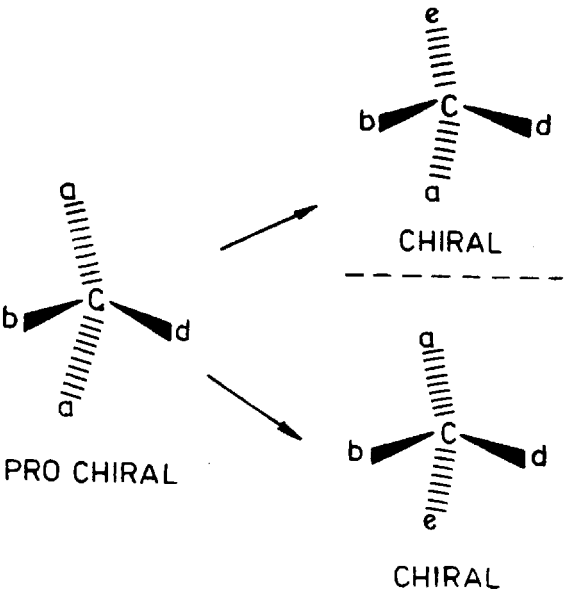


Though the *A/P* designation of Hirschmann is no longer in use, the idea of stereospecific numbering contributed by him is really a very significant one. In fact it forms the basis of the stereospecific numbering (Sn) system advovated by the joint IUPAC-IUB Committee. Glycerol having a single phosphate on a hydroxymethyl group may be called both *D* or *L*. However, using the above system of numbering it will be called Sn-glycerol-3-phosphate, thus removing all confusion.

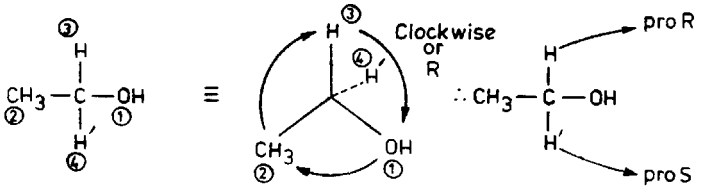


Sn – glycerol – 3 – phosphate

Pro-Chirality—The study of Ogston effect and the understanding provided by it that molecules like citric acid are not completely symmetrical led to the development of the concept of prochirality, Hanson(1966) defined molecules of the type Caabd as pro-chiral. Replacement of one of the "identical groups" by a new group (e) gives rise to a chiral molecule. The replacement of the other "identical group" gives an enantiomeric molecule of opposite chirality.

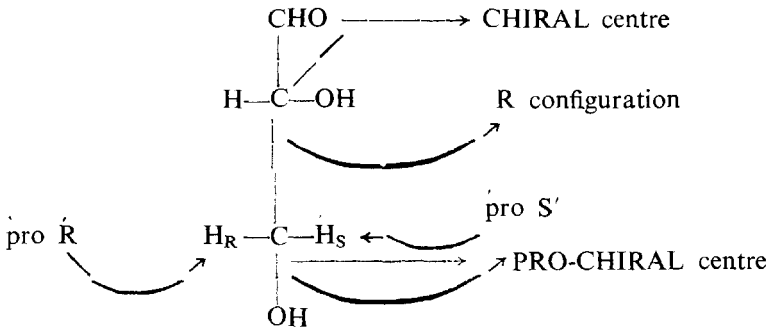


Hanson also proposed a system for designation of such molecules, which essentially is an extension of the now well established *R* & *S* system. It consists of giving arbitrarily, a higher priority to one of the two 'identical' groups at a time and then applying the rules of the *R*/*S* system. Let's look at the molecule of ethanol.



It contains one prochiral centre the methylene group. For convenience, we label one of the hydrogens H′ and give a higher priority to the hydrogen H first. The order of priority, therefore, is : OH> CH_3 > H > H′. Applying the *R*/*S* system rules we get a clockwise or *R* arrangement. Group H is accordingly designated as a pro-*R* group in this system. Replacement of this group leads to a chiral compound of *R* configuration. Similarly the H′ group can, in turn, be given a higher priority and the *R*/*S* system rules applied once again. It can easily be seen that the arrangement will then be '*S*' or anticlockwise. The group H′ is therefore called pro-*S*. The two hydrogens H & H′ of the methylene group in ethanol are therefore pro-*R* and pro-*S*, respectively. Since, their replacement leads to the production of enantiomers, these two hydrogens are Enantiotopic.

D(+)-Glyceraldehyde has the *R* configuration at the chiral centre in Cahn-Ingold-Prelog system. It contains one pro-chiral centre—the methylene group.

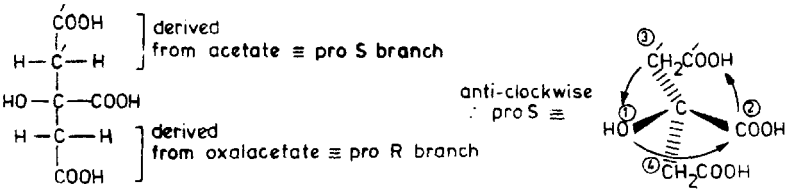


According to Hanson's designations one can easily put down the two hydrogens H and H′ as pro-*R* and pro-*S*, respectively. These two hydrogens are, however, not enantiotopic. Replacement of the pro-*R* hydrogen gives us a molecule of configuration (2*R*, 3*R*), while replacement of the pro-*S* hyrdogen gives rise to (2*R*, 3*S*) configuration. These two products are clearly diastereomeric and hence the methylenic hydrogens of *R* glyceraldehyde bear a diastereotopic relation to each other.

One can apply the same rules to citric acid. As should be obvious, it contains three pro-chiral centres, (the C-2, C-3, C-4 carbon atoms).

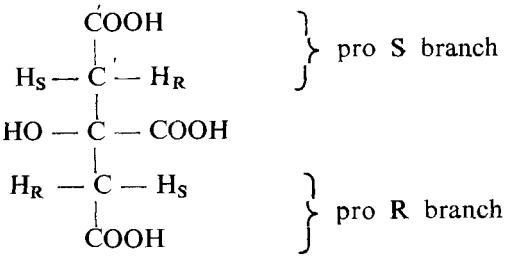
To start with, we analyse the C-3 pro-chiral centre. The two identical groups are the CH_2COOH groups. The oxalacetate derived CH_2COOH is at the bottom and the acetate derived CH_2COOH group at the top. Giving arbitrarily a higher priority to the CH_2COOH group, one gets the priority order as :

OH > COOH > CH_2COOH > CH_2COOH and therefore an anticlockwise or *S* arrangement. The CH_2COOH group on top (derived from acetate) can therefore be designated as the 'pro-*S* carboxymethyl branch'.



The oxalacetate derived CH_2COOH similarly will be designated the "pro-*R* branch".

Similarly one can designate the four hydrogens of the two methylene groups. The complete designation of citric acid by the Hanson system is given below.



Using this system of designation one can easily convey stereochemical information in an unambiguous manner. Thus the "pro-*R* hydrogen of the pro-*R* branch" of citric acid is a precise statement and refers to only one of the four methylene hydrogens.

Further the 'H_R of the pro-*R* branch' and 'H_S of the pro-*S* branch' bear an enantiotopic relationship. The 'pro-*R* hydrogen of the pro-*R* branch' is, however, diastereotopic with respect to both the other two methylene hydrogens (viz., H_R of the pro *S* branch as well as H_S of the pro-*R* branch). Differentiation between the diastereotopic hydrogens is possible even with physical methods. To differentiate between enantiotopic groups one requires a chiral reagent e.g., an enzyme. That the four methylene hydrogens of citric acid are stereochemically distinct is also shown by the following evidences :

1. Reaction of citric acid with aconitase Englard (1960) involves the preferential picking up of the pro-*R* hydrogen of the pro-*R* branch by the enzyme. Thus the enzyme differentiates even between the four methylene hydrogens.

2. The NMR spectrum of citric acid Lowenstein & Roberts (1960) in the methylene region consists of a doublet with two weak lines symmetrically spaced on both sides of the doublet. This quadruplet would be expected only if the four hydrogens did not have an identical environment and are therefore non-equivalent. (Here it is interesting to note that both the biologists and the NMR spectroscopists have been aware of the distinct nature of such groups from their own separate experiences. However, the two groups have not cared to join hands).

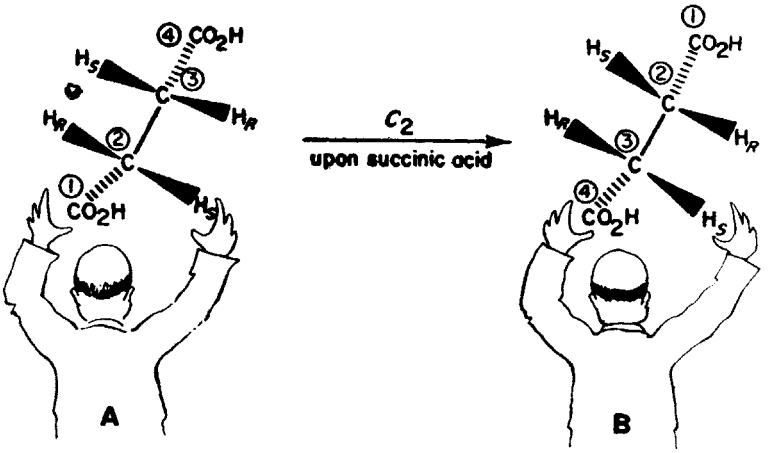
3. Replacement of a hydrogen of citric acid by one Fluorine atom gives Monofluoro Citric acid. In all four optical isomers are possible (Mono fluoro citric acid is a chiral molecule). All the four forms are known (Dummel 1969; and Fanshier *et al.* 1962, 1964)and they form two pairs of enantiomers.

Hirschmann's Superpositioning Test

Coming back to the biological differentiation between "identical" groups, a simple test has been proposed by Hirschmann. In essence, it states that those groups in a molecule which cannot be superimposed by a rotational symmetry operation can be distinguished while those groups which are interchanged by such an operation cannot be distinguished. The succinic acid molecule with the pro-chiral designations is shown here before and after a C_2 rotation about an axis perpendicular to the plane of the paper and bisecting the C-2, C-3 carbon-carbon bond :

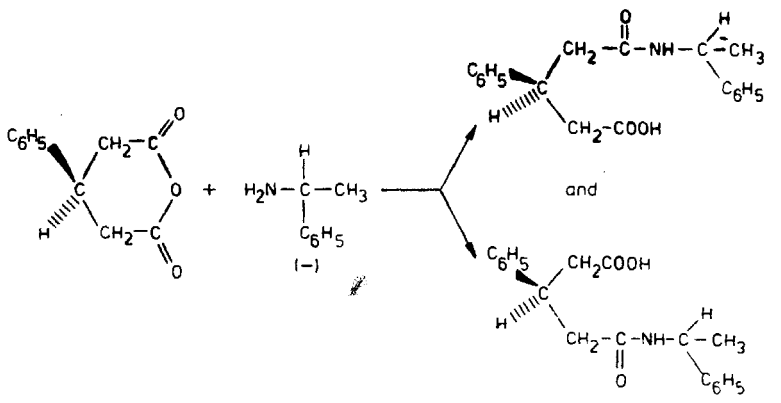
It is thus seen that the two Hs hydrogens (as also the Hr hydrogens) are interchanged by this operation. The two Hs hydrogens cannot be distinguished even by an enzyme. Enzymes can, however, differentiate the Hr hydrogens from the H_S hydrogens since they are not superimposed by a rotational symmetry operation.

Let us now look at a reaction carried out by Schwartz and Carter. They treated β-phenylglutaric acid (a molecule which possesses no rotional symmetry) with an optically active amine (a chiral reagent) and found that one of the two products



Attack on a Succinic acid molecular model by a biochemist as imagined by Alworth.

was formed in a higher yield preferentially. Today we recognise it as an example of a partial asymmetric synthesis.



The above example clearly demonstrates that for this type of differentiation the important criterion is simply the creation of a diasteremeric interaction. Even an enzyme is not essential for this purpose and one only requires a chiral environment. Thus it is clear that there is no magic about the way an enzyme is able to differentiate, between the so called "identical" groups in molecules like citric acid which possess no rotational symmetry. Thus, enzymes merely recognise the inherent lack of symmetry in such molecules.

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