UCRL 8832

UNIVERSITY OF CALIFORNIA

Ernest O. Lawrence Radiation Laboratory

TWO-WEEK LOAN COPY

This is a Library Circulating Copy which may be borrowed for two weeks. For a personal retention copy, call Tech. Info. Division, Ext. 5545

BERKELEY, CALIFORNIA

UCRL-8732

UNIVERSITY OF CALIFORNIA

Lawrence Radiation Laboratory
Berkeley, California

Contract No. W-7405-eng-48

CARBOXYLATIONS AND DECARBOXYLATIONS

Melvin Calvin and Ning G. Pon

April 21, 1959

Printed for the U. S. Atomic Energy Commission

CARBOXYLATIONS AND DECARBOXYLATIONS

Melvin Calvin and Ning G. Pon

Department of Chemistry and Lawrence Radiation Laboratory
University of California, Berkeley 4, California

Abstract

April 21, 1959

A brief survey of decarboxylation reactions and carboxylation reactions that are known or presumed in biological systems will be presented. While a considerable number of amino acid decarboxylations are known, their mechanisms will not be included in the present discussion but will be reserved for a later paper in the symposium.

The remaining decarboxylation reactions may be subdivided into oxidative and nonoxidative decarboxylations. In most cases, these reactions are practically irreversible except when coupled with suitable energy-yielding systems. The carboxylation reactions which are useful in the formation of carbon-carbon bonds in biological systems seem to fall into two or three groups: those which exhibit an apparent ATP requirement, those which exhibit a reduced pyridine nucleotide requirement, and those which exhibit no apparent ATP requirement. Of the first group, at least four cases, and possibly six or seven, are known, and one interpretation of them involves the preliminary formation of "active" carbon dioxide, generally in the form of a carbonic acid-phosphoric acid anhydride. Those exhibiting no apparent ATP requirement seem to be susceptible to classifications as enol carboxylations in which the energy level of the substrate compound is high, rather than that of the carbon dioxide. There appear to be at least three examples of this latter type known, amongst them being the carboxydismutase reaction of ribulose diphosphate with carbon dioxide.

Some discussion of the thermodynamics and possible mechanism of this reaction will be given. A possible alternative to the "active" carbon dioxide mechanism for the first class of carboxylation reactions which would bring them into the formal relationship with the others may be presented.

CARBOXYLATIONS AND DECARBOXYLATIONS [1]

Melvin Calvin and Ning G. Pon

Department of Chemistry and Lawrence Radiation Laboratory
University of California, Berkeley 4, California [2]

The subject for discussion, as given in the title, is carboxylations and decarboxylations. In a symposium addressed to the mechanism of enzyme action, the nature of carboxylation and decarboxylation reactions defined by these terms would naturally be those that are carried out by enzymatic means. I think it is clear that we are going to have to limit the nature of the carboxylation and decarboxylation reactions with which we will be concerned, and I shan't try to review all of the carboxylation and decarboxylation reactions that appear in the literature. We will not consider in this discussion the decarboxylation and carboxylation reactions involving the alpha carboxyl group of amino acids. This has its proper place at a later point in the symposium, in the colloquim in which Professor Snell will be discussing transamination reactions. These decarboxylations are rather closely related to the transamination reaction -- there are similar cofactors involved -- and so we will exclude from our consideration the amino acid carboxylation and decarboxylation reactions.

Furthermore, the interest in this subject, at least in our laboratory, is primarily in terms of carboxylation reactions rather than decarboxylation reactions. So I would ask you to focus your attention on reactions in which carbon dioxide is added to another carbon atom to form a new carbon-carbon bond, resulting in a carboxylic acid. This automatically will also exclude from our discussion the carboxylation reactions in which carbon dioxide reacts with atoms other than carbon, such as nitrogen, to form materials like carbamyl phosphates, urea groups and the like. We will be concerned, then, primarily with the addition of carbon dioxide to some

carbon skeleton, in which a new carbon-carbon bond is formed and in which a carboxyl group is created. Those considerations of decarboxylation reactions which we will make will be brought in rather in terms of what light they can throw on the nature of carboxylation reactions rather than for the sake of the decarboxylation reaction itself or for the sake of the completeness of the discussion.

In reviewing the mechanisms of such enzymatic carboxylation reactions, one is struck by the fact that there seems to be no unequivocal description of a primary product formed between the enzyme, or a cofactor, and carbon dioxide prior to the appearance of the new carbon-carbon linkage. For example, if we were to consider one of the longest known of carboxylation reactions (or decarboxylation reactions) the decarboxylation of pyruvic acid by the enzyme carboxylase to give acetaldehyde and carbon dioxide, there is no indication in the literature of an intermediate (a carrier of CO_2) between pyruvic acid and the liberated carbon dioxide(Green, Herbert and Subrahmanyan, '41). Another reaction of the reverse type has been studied, namely, the carboxylation of pyruvic acid to oxaloacetic acid, leading to the formation of a new carbon-carbon bond (Kaltenbach and Kalnitsky, '51). Again,

$$CH_3\text{-}\overset{O}{\overset{\|}{C}}\text{-}CO_2H \xrightarrow[\text{thiamin pyrophosphate}\ Mg^{++}]{\text{carboxylase}} CH_3\text{-}CH{=}O + CO_2$$

Reaction 4, Table 1

in this reaction, there is no described evidence of an intermediate involving CO_2 lying between the carbon dioxide and the new carbon-carbon bond that is formed in the oxaloacetic acid. That is, the carbon dioxide may or may not be bound to enzymes

$$CO_2 + CH_3\text{-}\overset{O}{\overset{\|}{C}}\text{-}CO_2H \longrightarrow HO_2C\text{-}CH_2\text{-}\overset{O}{\overset{\|}{C}}\text{-}CO_2H$$

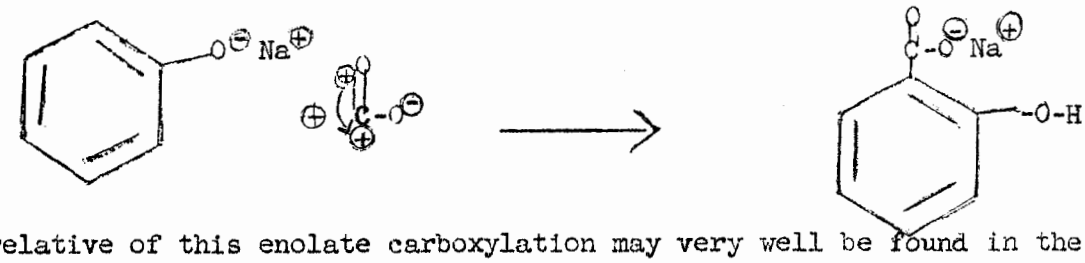
Reaction 5, Table 1

or cofactors before it is bound to the pyruvate skeleton, but if it is, we have no description of such an intermediate.

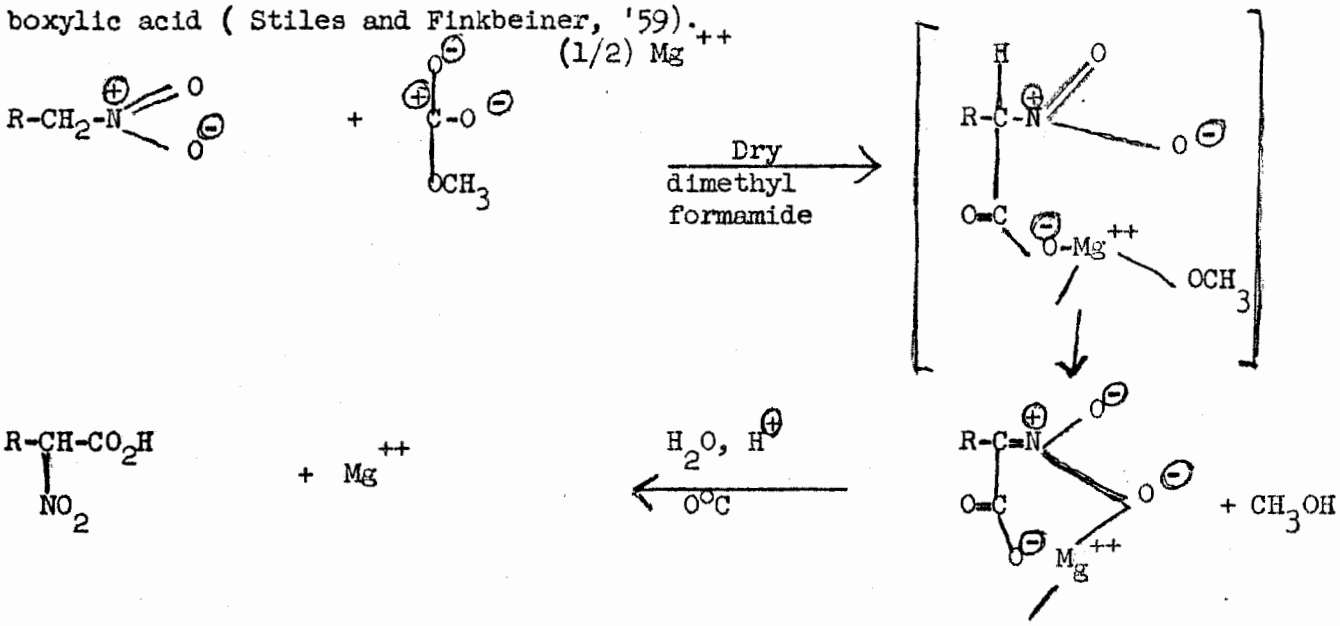
This fact is a curious thing, because in most other enzymatic reactions in which a small group is picked up and handed on to be combined with a larger one, or with another one, in general there have appeared intermediates in which the group to be transferred is found bound to either the enzyme or to a catalytic amount of cofactor which functions in conjunction with the enzyme for moving the group around before it appears in its subsequent substrate form. Therefore, in order to get some clue as to how such carboxylation and decarboxylation reactions might develop, one has to turn to the purely chemical systems and surmise from the mechanisms which are proposed for such purely chemical systems, what might be the situation in the biological carboxylation and decarboxylation reactions.

There are two purely chemical carboxylations which are very common. One of them is the reaction of an organometallic compound such as a Grignard reagent with carbon dioxide to form a new carbon-carbon bond in which the carbon-oxygen grouping of the CO_2 is presumably inserted between the carbon (carbanion) and the metal of the organometallic compound; this will work for a number of other organometallic materials as well.

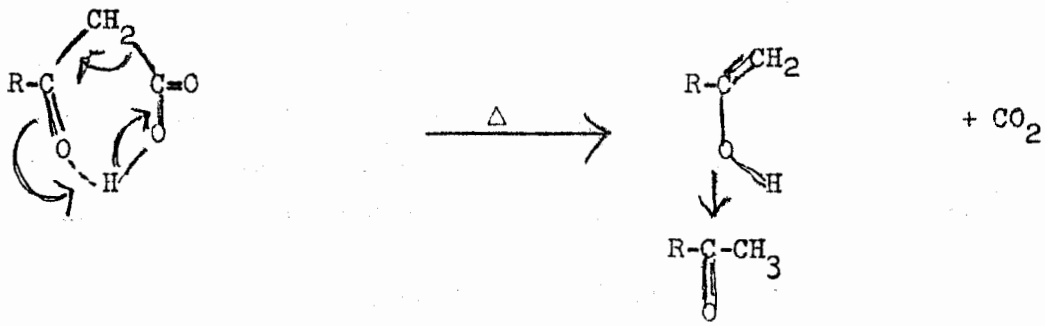
The other type of chemical carboxylation reaction with which we are familiar in organic chemistry is of quite a different sort, involving the carboxylation of the metal salt of an enol, or phenol, particularly a phenol (Brown, '51)(Pederson, '47). For example, sodium phenolate, when treated with carbon dioxide at elevated temperatures and pressures will produce the salt, sodium salicylate:



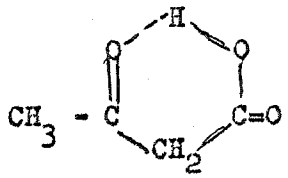
A relative of this enolate carboxylation may very well be found in the carboxylation of metal salts of nitro alkanes to form the metal chelate of the carboxylic acid (Stiles and Finkbeiner, '59).



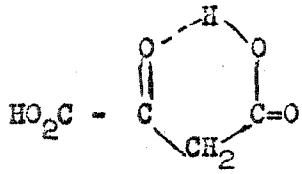
The study of deccarboxylation reactions has given a clue to the possible general character of the nature of the carboxylation reaction itself. This has been undertaken primarily in connection with the very easy decarboxylation of β-keto acids. Beta keto acids, when heated, very easily lose a molecule of carbon dioxide to give the corresponding ketone:



The R group may be an hydroxyl, as in malonic acid, or it might be a methyl group, as in acetoacetic acid, or a carboxyl, as in oxaloacetic acid, and so on. The decarboxylation reaction will, in general, lead ultimately to the corresponding carbonyl compound, and a good deal of work has been done on the mechanism of the decarbonylation, particularly of acetoacetic acid and its derivatives, and of oxaloacetic acid and its derivatives.



acetoacetic acid

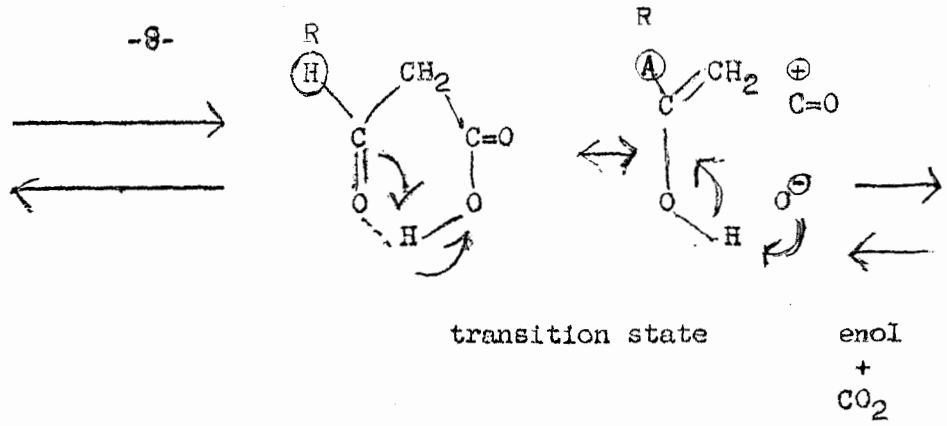


oxaloacetic acid

In both cases we have β-keto acids. The literature indicates that in some cases the primary product would appear to be an enol of the corresponding ketone, which then undergoes tautomerization. In some cases, it appears that the decarboxylationn can go as fast, or faster, when there is no enolizable hydrogen in the initial keto acid, such as the experiment of Pederson (Pederson '34) and of Westheimer (Westheimer and Jones, '41) and this has led to the opinion that the primary product is at least a hydrogen-bonded carbonyl, rather than a true enol. The truth of the matter may very well lie in a compromise between the two points of view, and on some occasions the primary product of the decarboxylation will presumably be closer to the enol and on others (sterically hindered) the primary product may very well be simply a hydrogen-bonded ketone. One can then extrapolate this notion that if an enol, or enolic type of compound is the primary residue for the breaking of the carbon-carbon in a decarboxylation reaction, one might expect that in the reverse operation, where carbon dioxide is added to make a new carbon-carbon bond, one would first have to have a system which contains a true, or at least a potential, enolic system, preparatory for the addition of the carbon dioxide to it. This is simply a statement that we may expect the same transition state to participate in the reaction in either direction.

β-keto acid

(β-γ-unsaturated acid, etc.)



transition state

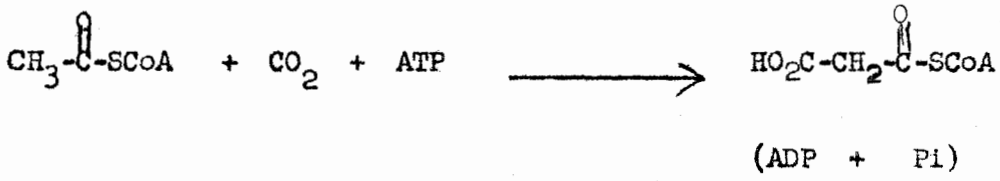
enol + CO_2

Types of Carboxylation (Decarboxylation) Reactions

An examination of those enzymatic reactions for which the requirements are fairly well established (Table 1) leads to the classification of the carboxylation, or reversible decarboxylation, reactions associated with them in to three general types. Let us formulate them in terms of carboxylation reactions rather than decarboxylation reactions, even though in some cases they are known and have been studied primarily as decarboxylation reactions. I have tried to organize the best known of the carboxylation in these terms: Those which have an energy requirement in the form of ATP clearly established; those which have a requirement for a reduced pyridine nucleotide; and those which have no apparent extra energy requirement.

Carboxylation Reactions Requiring Adenosine Triphosphate as Energy Source

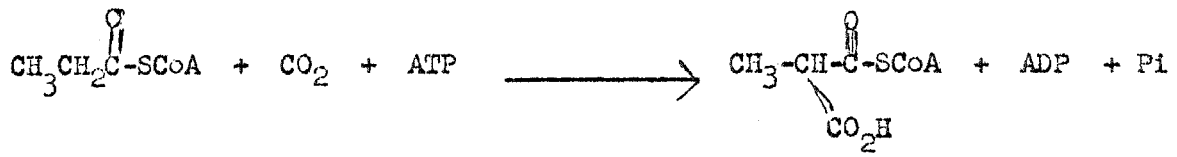
Those carboxylation reactions which have an ATP requirement are as follows: the carboxylation of acetyl thiol ester in the form of acetyl coenzyme A, to give malonyl coenzyme A (Wakil, '58; Formica and Brady, '59):



Reaction 16, Table 1

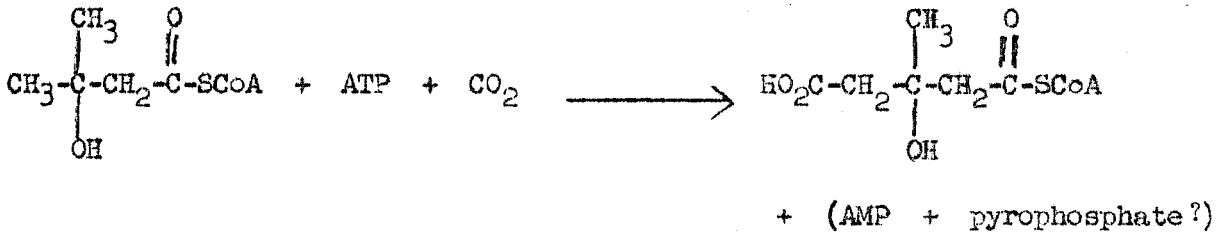
Corresponding to this in form would be the carboxylation of the next higher fatty

acid ester, propionyl coenzyme A, to give methylmalonyl coenzyme A (Tietz and Ochoa, '58) (Flavin, Castro-Mendoza and Ochoa, '57). This, again, is an alpha carboxylation.



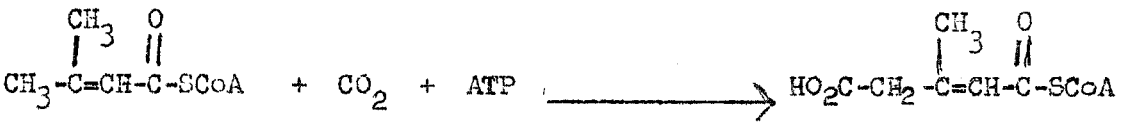
Reaction 15, Table 1

Another one for which the purely formal reaction has been well established but for which the mechanism is still a subject of some discussion is the apparent direct carboxylation not of alpha carbon atoms but of a gamma carbon atom of a thiol ester in the form of β-methyl-β-hydroxybutyryl coenzyme A (Bachhawat and Coon, '57 and '58; Bachhawat, Robinson and Coon, '56):



Reaction 14, Table 1

A reaction which appeared to be closely related to the one described above and was believed to be part of the same system is the carboxylation of β-methylcrotonyl coenzyme A (Knappe and Lynen, '58, '59):



Reaction 22, Table 1

This then would again lead to a carboxylation product in which the carboxyl group

appeared on the carbon atom gamma to the carboxyl group itself.

There is one other reaction requiring ATP which, at the beginning, we have agreed to pass over, and that would be the formation of carbamyl phosphate, which does not involve the formation of a new carbon-carbon bond and therefore falls outside the field of this discussion. These, then, are all of the reactions in which the formal creation of the carbon-carbon bond via carboxylation is known to require the presence of adenosine triphosphate.

Carboxylation Reactions Requiring Reduced Pyridine Nucleotide as Energy Source

A second type of carboxylation reaction is the one requiring no ATP but which requires reduced pyridine nucleotide (TPNH). These reactions are, in general, carboxylations beta to a carbonyl group which do not lead to the β-keto acid but rather to the β-hydroxy acid. There are at least three such clear-cut cases. The first is the carboxylation of pyruvate itself with reduced pyridine nucleotide to give, in this case, malic acid directly (Saz and Hubbard, '57)(Ochoa,Mehler and Kornberg, '58):

$$CO_2 + CH_3\overset{O}{\overset{\|}{C}}-CO_2H + H^+ + \begin{matrix}\text{TPNH(plants and animals)}\\ \text{DPNH (bacteria)}\end{matrix} \longrightarrow HO_2C-CH_2-\overset{OH}{\overset{|}{C}}H-CO_2H + \overset{\oplus}{TPN}$$

Reaction 8, Table 1

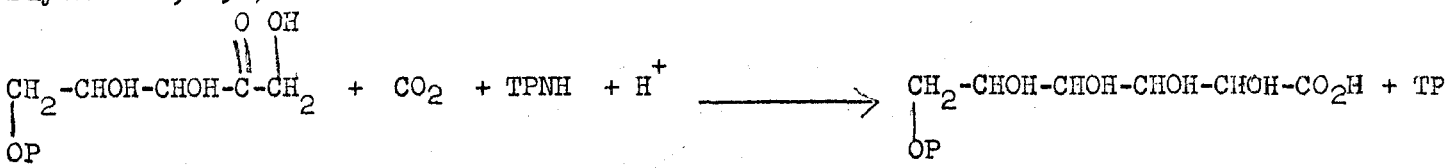
The enzyme which forms the malic acid does not make free oxaloacetic acid as a precursor to the malic acid.

Another reaction requiring TPNH which would be exactly analogous to this is the carboxylation of ketoglutaric acid to produce a β-hydroxy acid (Ochoa and Weisz-Tabori, '48)(Siebert, Carsiotis and Plaut, '57):

$$HO_2C-\overset{O}{\overset{\|}{C}}-CH_2-CH_2-CO_2H + CO_2 + TPNH + H^+ \longrightarrow HO_2C-\overset{OH}{\overset{|}{C}}H-\overset{CO_2H}{\overset{|}{C}}H-CH_2-CO_2H$$

Reaction 7, Table 1

A third reaction which may belong to this class is the reductive carboxylation of ribulose 5-phosphate to 6-phosphogluconic acid (Cohen, '54; Horecker and Smyrniotis, '52):



Reaction 9, Table 1

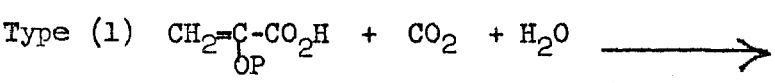
It is not certain whether an intermediate 3-keto-6-phosphogluconate free of the enzyme is involved or not. In any case, the reduction is required to show the reaction as a carboxylation.

These three reactions all require additional sources of energy in the form of reduced pyridine nucleotide.

Carboxylation Reactions Which Have No Apparent Extra Energy Requirement

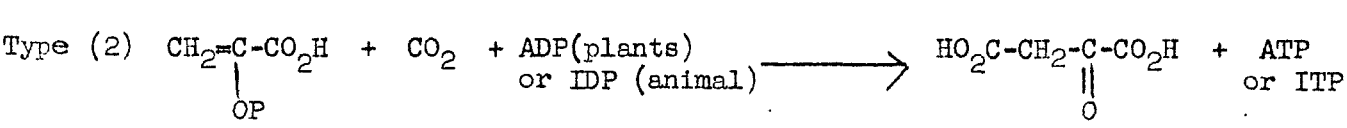
Let us have a look at those reactions in which the formation of a new carbon-carbon bond is known to take place without addition, or the direct participation so far as we can tell, of either adenosine triphosphate or reduced pyridine nucleotide. There are three of these reactions and the first is the carboxylation of phosphoenolpyruvate. This reaction can, in turn, be subdivided into two parts -- two different kinds of carboxylation: (1) The first is one in which we produce directly oxaloacetic acid and inorganic phosphate(orthophosphate) (Tchen, Loewus and Vennesland, '55; Bandurski and Greiner, '53). (2) The other subgroup of this type is the one in which the phosphate instead of appearing as orthophosphate directly is picked up by adenosine diphosphate to formnot only oxaloacetic acid but, as the other product, adenosine triphosphate (Tchen and Vennesland, '55)

Type (1)



Reaction 19, Table I

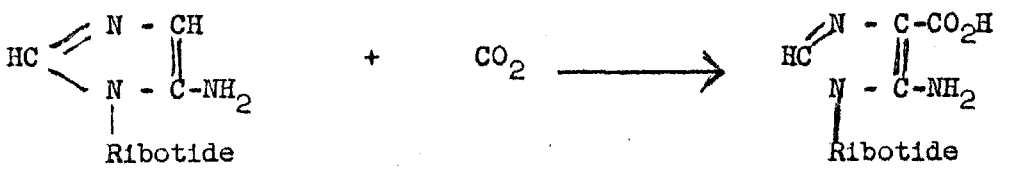
Type (2)



Reaction 18, Table 1

These are two different carboxylation reactions requiring two different carboxylation enzyme systems.

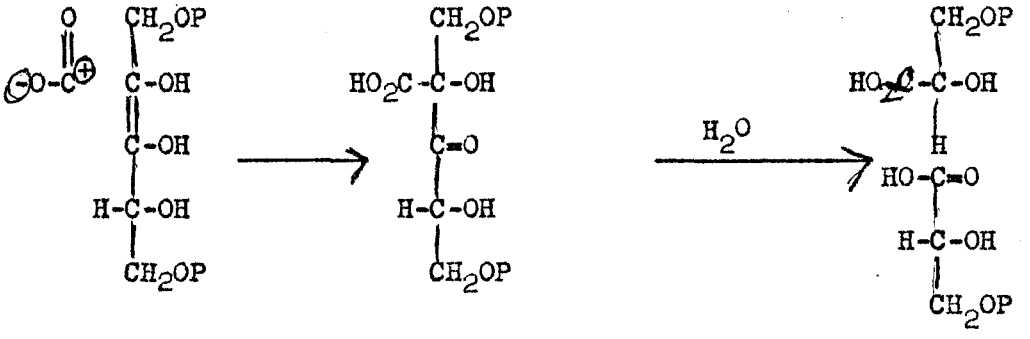
The second reaction which requires no ATP is the one in which we directly carboxylate an amino imidazole ribotide derivative of this character (Lukens and Buchanan, '57):



Reaction 20, Table 1

The carbon dioxide adds at the C_4 to give the 4-carboxy-5-amino-imidazole ribotide.

Finally, the third major carboxylation with which we are familiar which does not require ATP or reduced pyridine nucleotide(atleast <u>in vitro</u> to produce phosphoglyceric acid) is the carboxylation of ribulose diphosphate (Weissbach , Horecker and Hurwitz, '56)(Mayaudon, Benson and Calvin, '57)(Hurwitz, Jacoby and Horecker, '56).



Reaction 21, Table 1

It thus appears that we have at least three kinds of carboxylation reactions. An inspection of the three types reveals that there is a source of energy in each case which is required in order to produce the new carbon-carbon bond. This source

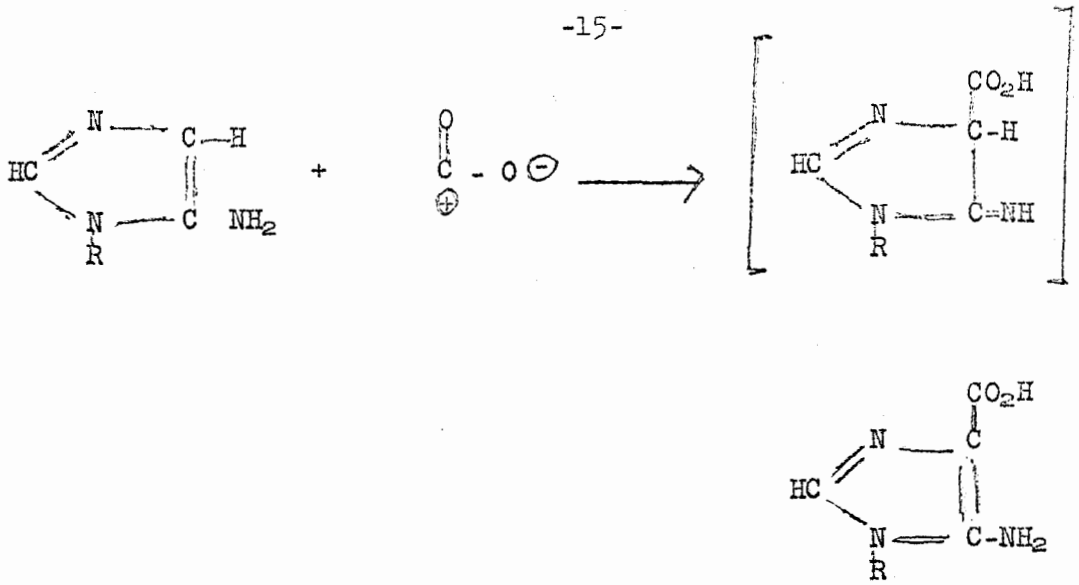
of energy, in the case of the ATP requirement, is, obviously, adenosine triphosphate itself and in the case of the pyridine nucleotide requirement is the reduced pyridine nucleotide. However, in the case of the carboxylation reaction which shows neither ATP nor reduced pyridine nucleotide requirement, the source of the energy is the substrate itself. The substrate itself is already in an "active" form in the sense that it is an unstable form with respect to the more stable isomers. For example, in the case of the phosphoenolpyruvate, the energy is stored in the form of enol phosphate. In the case of the imidazole we have again the carboxylation of an ene-amine, and in the case of the ribulose diphosphate we presumably have the carboxylation of the noncyclic form of the ribulose which is constrained to go through an ene-diol, since cyclic acetal formation is prohibited by small ring size.

Carboxylation Reaction Mechanisms

Enol carboxylations

What we would like to do is to bring into harmony at least two of these classes; I'm not certain that we can bring the third one into harmony with the other two. Let us see if we can formulate a reaction mechanism which would be common at least to two types, the enol carboxylation and the reduced pyridine nucleotide-requiring one. First of all, no further description is required for the carboxylation of the enol forms since they already represent the model types that we spoke of as being the kind of primary product of decarboxylation in ordinary chemical decarboxylation and also of the two cases of chemical carboxylation with which we are familiar.

Thus, one can describe the carboxylation of phosphoenolpyruvate, following Vennesland (Tchen, Loewus and Vennesland, '55) as a direct carboxylation of the enol, leading to the ejection of orthophosphate and the formation directly of oxaloacetic acid (see reaction Type (1), page 11). The analog of this, one in which some other acceptor than water is required for the orthophosphate, namely, inosine diphosphate or adenosine diphosphate, might be considered as a more highly evolved system in which some of the energy stored in the enol phosphate is conserved in the adenosine triphosphate or inosine triphosphate, as the case may be, for further use. In the case of the ribulose, the carboxylation is a direct carboxylation of an enol form which remains largely in the enol form because of its inability to form the furanoside ring, there being only three carbon atoms free and available for such ring formation. The amino imidazole carboxylation would correspondingly lead to the ketimine which would tautomerize because of the cyclic conjugated structure to give the carboxylated amino imidazole.



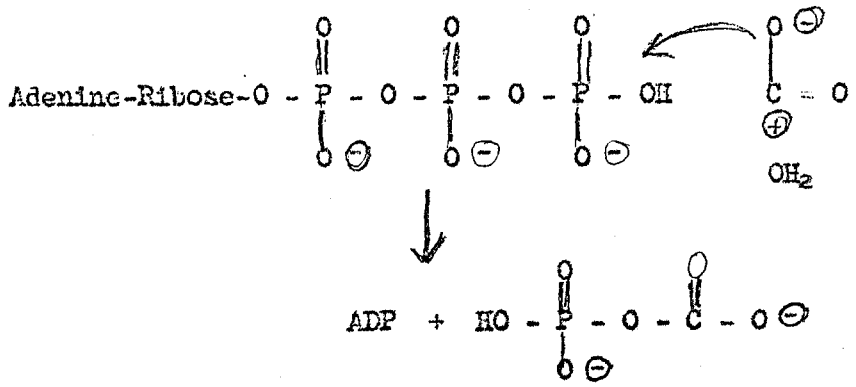
Reduced pyridine nucleotide-dependent reactions

The second type of carboxylation reaction which should be included in this classification is the one which requires reduced pyridine nucleotide as its energy source. Thus, one can carboxylate free pyruvic acid to form malic acid directly when reduced pyridine nucleotide is present (Saz and Hubbard, '57; Ochoa, Mehler and Kornberg, '48). Presumably, this will also go by way of the enol form on the enzyme, liberated only as the free malic acid after reduction by the reduced pyridine nucleotide which is required for this enzyme system. A similar arrangement could be set up for the formation of isocitric acid from ketoglutaric acid, again using reduced pyridine nucleotide as the essential energy source to complete the carboxylation reaction.

Adenosine triphosphate-dependent carboxylations

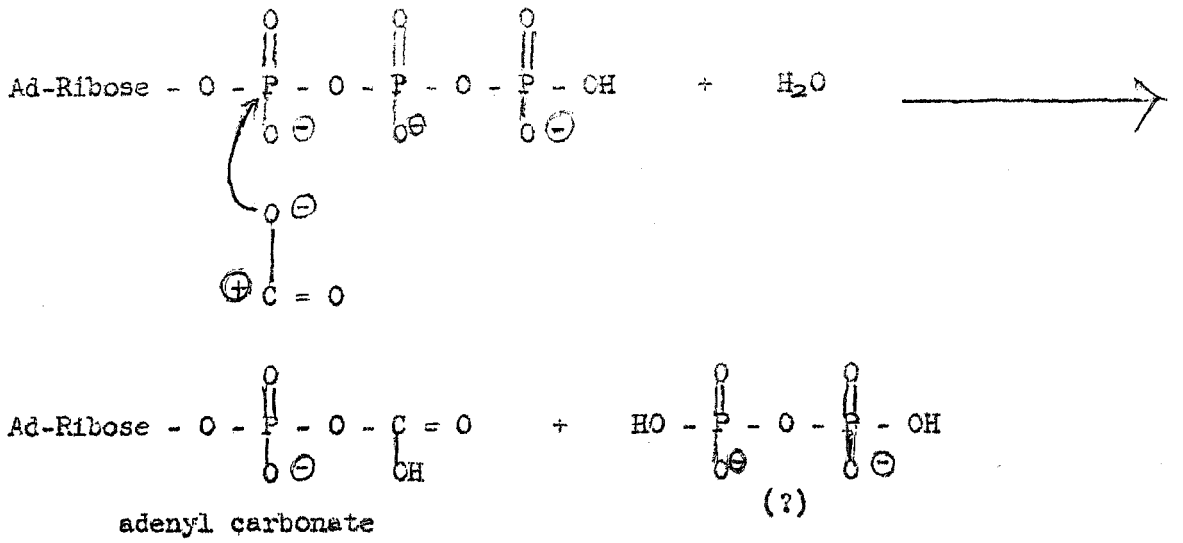
Let us now examine the class of carboxylation reactions which have an ATP requirement to see in what form this energy supplied by the ATP may actually perform its function in producing the carboxylation reaction. There have been two suggestions made with regard to this function. One was in connection with the carboxylation of propionyl coenzyme A to form a methylmalonyl coenzyme A (Flavin, Castro-Mendoza and Ochoa, '57).

This suggestion involved the primary activation of carbon dioxide to form phosphoryl carbonate. This phosphoryl carbonate would then be the 'active' CO_2 which would be able to carboxylate the alpha position of the propionyl



CoA to form methylmalonyl CoA. There is a separate enzyme whose presumed function is the 'CO_2 activation' to form the phosphoryl carbonate. Another enzyme for the carboxylation itself (of propionyl CoA) and a third for the isomerization of the product formed, methylmalonyl CoA to succinyl CoA, have also been separated (Flavin, Castro-Mendoza and Ochoa, '57; Beck, Flavin and Ochoa, '57).

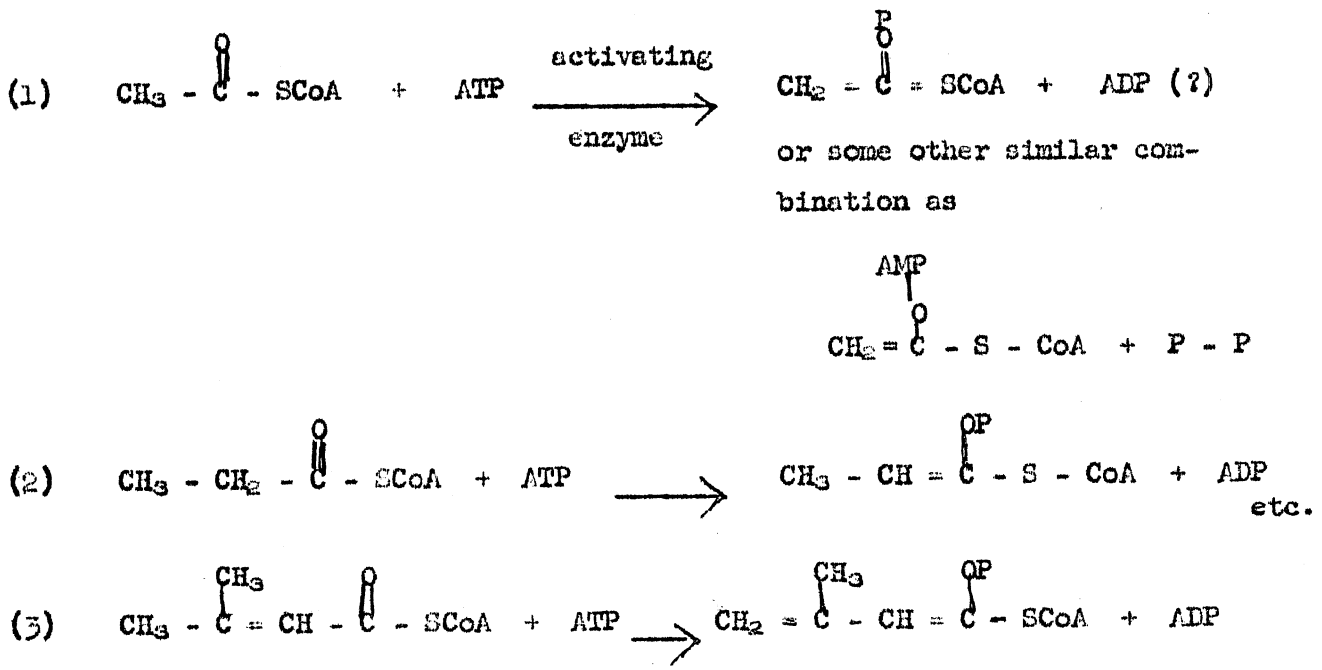
The second suggestion for the function of ATP in a carboxylation reaction has been made in connection with the carboxylation of hydroxyisovaleryl coenzyme A to form the β-methyl- β-hydroxyglutaryl CoA (Woessner, Bachhawat and Coon, '58). Here the suggestion is that the ATP reacts with the carbonate to form adenyl carbonate and pyrophosphate rather than splitting the other way, as in the first suggestion. Then the adenyl carbonate, called the 'active' CO_2, appears to proceed to carboxylate the hydroxyisovaleryl CoA to form the hydroxymethylglutaryl CoA.



In the other two carboxylation reactions, namely, that of acetyl CoA to form malonyl CoA (Wakil, '58; Formica and Brady, '59) and that of β-methylcrotonyl CoA to form glutaconyl CoA (Knappe and Lynen, '58), no direct evidence or suggestion has been made with regard to the nature of the ATP requirement.[3]

It is perhaps worth pointing out at this juncture that neither of these two 'active' CO_2 products have been isolated or demonstrated directly in the enzyme preparations. A synthesis of the adenyl carbonate ethyl ester has been performed by Coon (Bachhawat and Coon, '57; Bachhawat, Woessner and Coon, '56) using the silver salt of adenylic acid and ethylchlorocarbonate, and the crude product of that reaction has been claimed to substitute for the ATP requirement in the carboxylation of the hydroxyisovaleryl CoA. This statement was made in a brief communication and no amplification has yet appeared, so perhaps we had better reserve judgment with regard to it for the moment and see if some other unifying mechanism may be devised to account for the ATP requirement which would bring the ATP-requiring carboxylation reactions into a coherent pattern with the other two groups which we have already described as enol carboxylations.

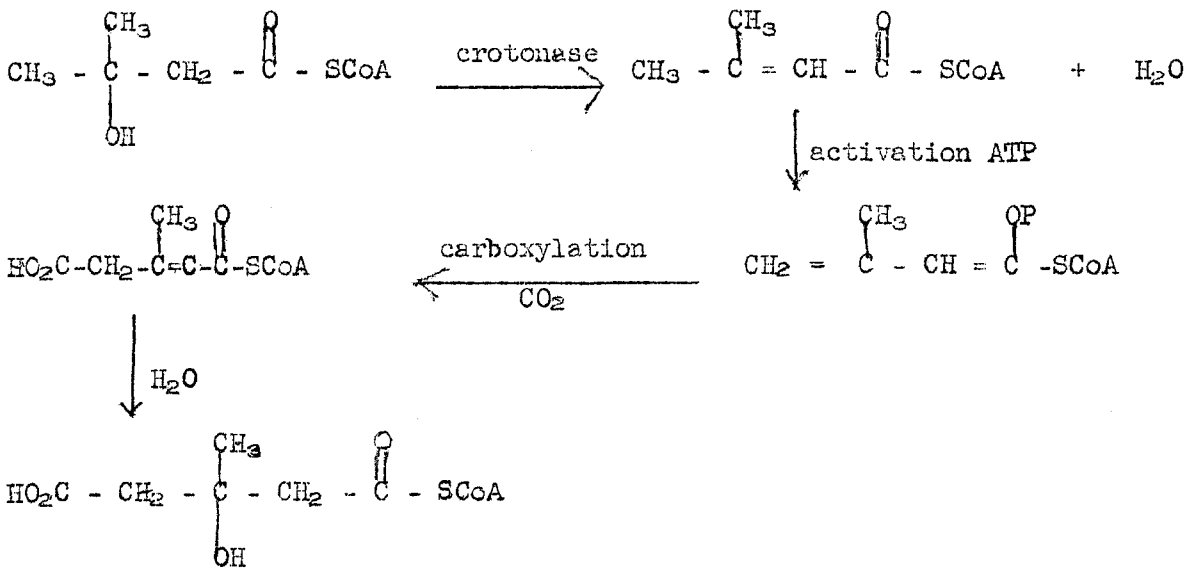
A rather obvious mode of action suggests itself, a mode of action which already has its analog in the formation of phosphoenolpyruvic acid by pyruvic kinase (Lardy and Ziegler, '45) as a preliminary step to the carboxylation of phosphoenolpyruvate (Tietz and Ochoa, '58). This is the formation of the enol phosphate of the thiol esters mentioned as those requiring ATP for their carboxylation. The formation of the enol phosphate of the thiol ester acetyl thiol CoA and propionyl CoA, as well as that of β-methylcrotonyl CoA, seems to be perfectly straightforward. The β-methylcrotonyl CoA would be a vinylogous enolization on the gamma methyl group, leading directly to a vinylogous enol which would be subject to carboxylation in the usual way.



The case of the β-hydroxyisovaleryl CoA however, requires some further discussion. If, as is suggested by the work of Coon (Bachhawat, Robinson and Coon, '56) and Lynen (Knappe and Lynen, '58) these are two independent enzyme systems, and accepting Lynen's evidence that his system, beginning with hydroxyisovaleryl CoA involves at least four stages, namely, (1) dehydration to β-methylcrotonyl CoA, (2) the activation step, (3) the carboxylation step to β-methylglutaconyl CoA, and (4) a rehydration to give β-hydroxy-β-methyl-

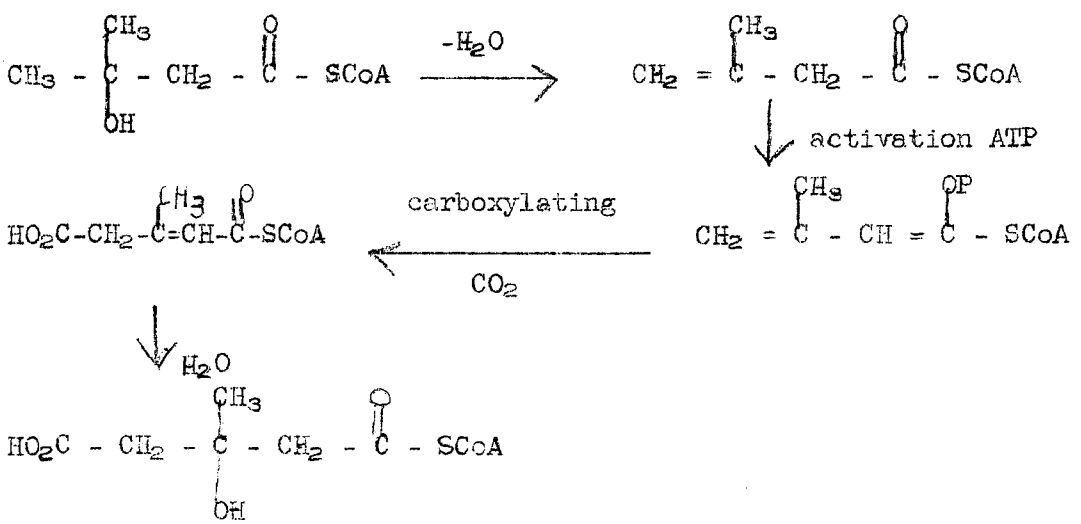
glutaryl CoA, a somewhat different route must be devised for Coon's enzyme which presumably functions in the absence of crotonase, the hydration-dehydration enzyme relating isovaleryl CoA and β-methylcrotonyl CoA.

Lynen sequence



The most obvious suggestion would be that the Coon system involves a dehydration of the hydroxyisovaleryl CoA in the opposite, or nonconjugated way, to give β-methylvinyl acetic acid rather than the crotonic acid. This would

Coon sequence



would then be followed by the activation enzyme to produced the conjugated diene-ol, the same conjugated diene-ol as would be obtained from the crotonic acid. This process would then undergo the carboxylation and hydration as before, thus bringing both systems into the same form of carboxylation reaction.

Thus, we will have brought all of the three types of carboxylation reactions which we have discussed into the same form, namely, that of the attack upon an enol by CO_2 (or bicarbonate ion) in its carbonium ion manifestation, leading directly to the formation of a carbon-carbon bond. An exactly similar formulation may be achieved for amino acid decarboxylation in which the oxygen atom of the enol is replaced by a nitrogen atom (Mandeles, Koppelman and Hanke, '54).

There remain, however, two peripheral observations in connection with the proposed 'active' CO_2 which must be accounted for. These are as follows: First, the CO_2-dependent formation of phosphoryl fluoride from ATP and fluoride ion under the influence of the fluorokinase (pyruvic kinase) enzyme (Tietz and Ochoa, '58) with the formation of ADP as the other product:

$$(1)\quad \left.\begin{array}{ll} (a) & ATP + CO_2 \rightleftharpoons ADP + P - CO_2 \\ (b) & P - CO_2 + F^{\ominus} \longrightarrow F^{\ominus} - P + CO_2 \end{array}\right\} \begin{array}{l}\text{fluorokinase}\\ \text{(pyruvic kinase)}\end{array}$$

The second peripheral observation is the apparent hydrolysis of the ATP by the CO_2-activating' enzyme of Coon (Bachhawat and Coon, '58), in the required presence of hydroxylamine and CO_2, leading to AMP and some pyrophosphate-like material, perhaps phosphoryl hydroxylamine. These reactions are presumed to take place in two stages, as follows:

$$(2)\quad (a)\quad ATP + CO_2 \rightleftharpoons AMP - CO_2 + P - P$$

$$(b)\quad AMP - CO_2 + H_2N - OH \longrightarrow HONCO_2H + AMP$$

CO_2-activating enzyme

or

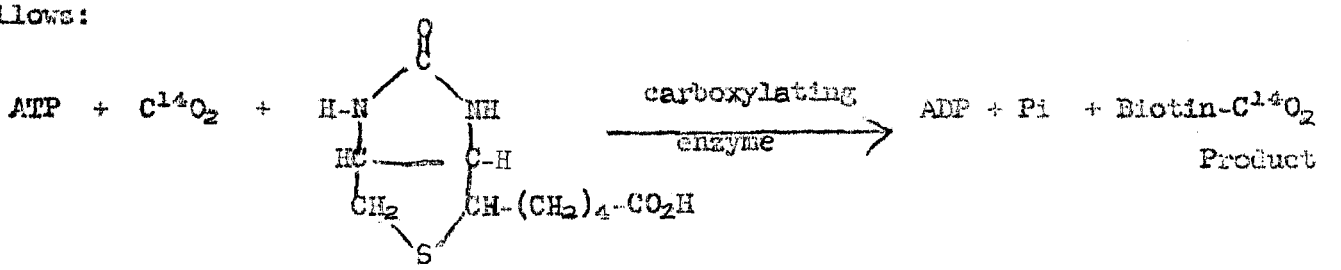
$$(2')\quad ATP + NH_2OH \xrightarrow[CO_2]{\text{enzyme}} ADP + P\text{-}NHOH$$

In both the cases just described, it would appear that the fluoride and hydroxylamine, respectively, are substitute acceptors of the 'active' CO_2 in place of the natural acceptors, namely, the thiol esters. Also, in each case, the first reaction is required to be a reversible one, and if this is so, one would indeed expect a rapid exchange of carbon-labeled ADP with ATP, at least in the fluorokinase case; this has not been observed (Tietz and Ochoa, '58). It should also be pointed out that it appears possible to separate the propionyl CoA carboxylation system from purified fluorokinase (Tietz and Ochoa, '58).

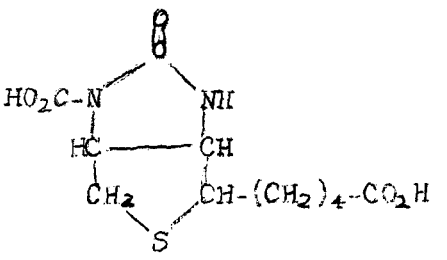
An alternative explanation for these two CO_2-dependent side reactions would be as follows: The activating enzymes, or kinases, in both cases would be conceived of as ATP-activating enzymes in which the ATP is prepared for its reaction with a suitable thiol ester substrate to form the active enol thiol ester. However, the activity of these ATP-activating enzymes would be absolutely dependent upon the presence of carbon dioxide which would presumably, in some way probably involving biotin (Lynen, Knappe, Lorch and Jutting, '59), change the configuration of the enzyme (Koshland, '58) so as to make it active in its ATP-activating function. Such a system, then, in which the primary function of these activating enzymes is to produce 'active' ATP preparatory to its transfer to the natural substrate might or might not involve the reversible fission of the pyrophosphate linkages, depending upon the nature of the activation process. It might thus be possible to find conditions giving an ADP-ATP exchange as well as not.

The specific requirement of carbon dioxide for the activation of this enzyme is understandable in evolutionary terms when we examine the nature of the proposed products formed under the influence of this enzyme, namely, the phosphoenol thiol esters. These esters might be expected to be extremely labile to spontaneous hydrolysis if CO_2 were not present and the enzyme remained fully active. This would, in effect, provide a fruitless mechanism of hydrolysis of the energy-storing compound, ATP. By requiring the mere presence of carbon dioxide for bringing the enzyme to full activity, the true substrates for the carboxylation reaction of the enzyme must always be present when the primary product is formed, thus leading to the efficient use of the ATP in the carboxylation reaction.[4]

Prof. E. Lynen has just presented evidence (Lynen, '59) for the formation of a compound between CO_2 and added free biotin under the influence of the enzyme for the carboxylation of β-methylcrotonyl CoA and using ATP.[4] The properties of the products were briefly mentioned as including very great lability to dilute acid (pH 2) but considerable resistance to neutral or slightly alkaline media (pH 7-8) at ice temperatures (lifetime 20 minutes) as evidenced by nonexchangeability of the $C^{14}O_2$ compound with nonradioactive CO_2 swept through the solution of the product of the enzymatic reaction, as follows:



The product was formulated as a carboxamidic acid. This is almost certainly a



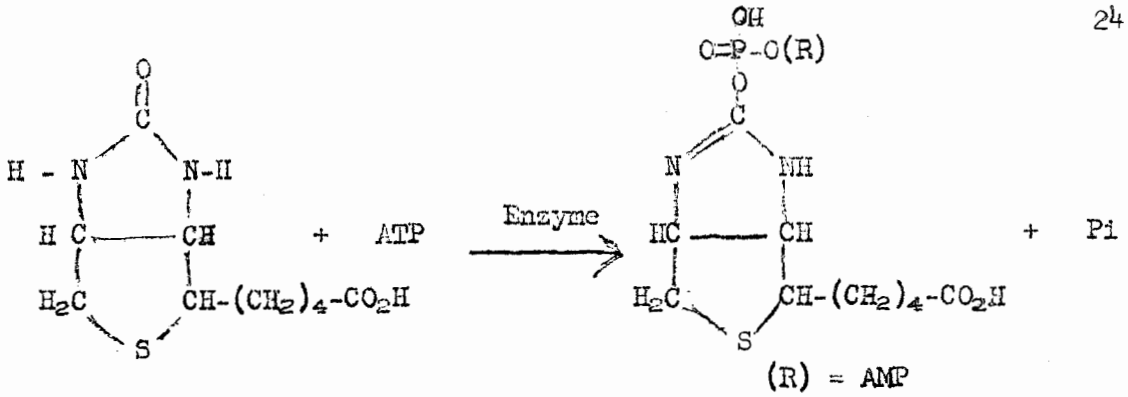
very unstable structure, having the carboxyl group free on an amide (urea) nitrogen atom. It is conceivable, however, that it would have the claimed stability as the anion. Because the activity of the carboxylation enzyme in carboxylating β-methylcrotonyl CoA is dependent upon bound biotin, it was suggested that the 'active' CO_2 in the enzyme has the same structure as that proposed for the free biotin carboxylation product, and that the latter is formed in an exchange reaction with free biotin, according to the following sequence:

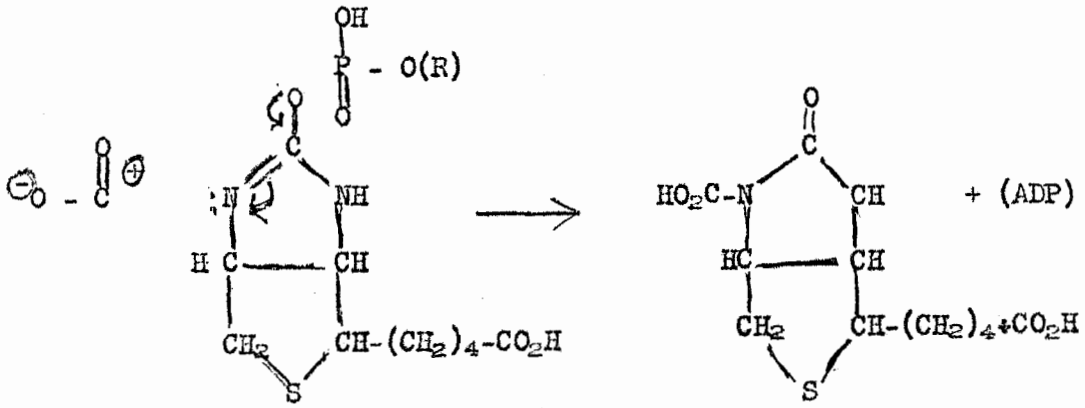
$$\text{ATP:} + \text{Biotin-Enzyme} \rightleftharpoons \text{ADP-Biotin-Enzyme} + \text{Pi}$$

$$\text{ADP-Biotin-Enzyme} + CO_2 \rightleftharpoons \text{ADP} + CO_2\text{-Biotin-Enzyme}$$

$$CO_2\text{-Biotin-Enzyme} + \text{free Biotin} \rightleftharpoons CO_2\text{-Biotin (free)} + \underset{\text{(isolated product)}}{\text{Biotin-Enzyme}}$$

Accepting the existence of such a free biotin-CO_2 compound, it is easy to formulate its formation in terms of the enol carboxylation mechanism proposed as general in the body of this paper. The free biotin would be considered as a substitute substrate for the enol phosphorylation and carboxylation as follows:





The fact that biotin is a much poorer substrate for the disappearance of ATP under the influence of this enzyme than is its natural substrate, β-methyl-crotonyl CoA, suggests that the 'active' form of either the CO_2, or the phosphate on the enzyme, is not identical with that found on the free biotin, since this would involve a relatively simple exchange reaction which might be expected to proceed rapidly. This, however, does not absolve the enzyme-bound biotin from direct implication in the CO_2-dependent ATP-activating function of the enzyme.

It seems that such a proposal as this will account for the experimental observations that have so far been reported and for which the phosphoric-carbonic anhydride systems have been devised. In some earlier work (Weissbach, Horecker and Hurwitz, '56; Racker, '57) the high K_m values of the carboxydismutase system, when calculated on total carbonate added, as observed in <u>in vitro</u> systems, seemed to require some form of CO_2 activation in the <u>in</u>

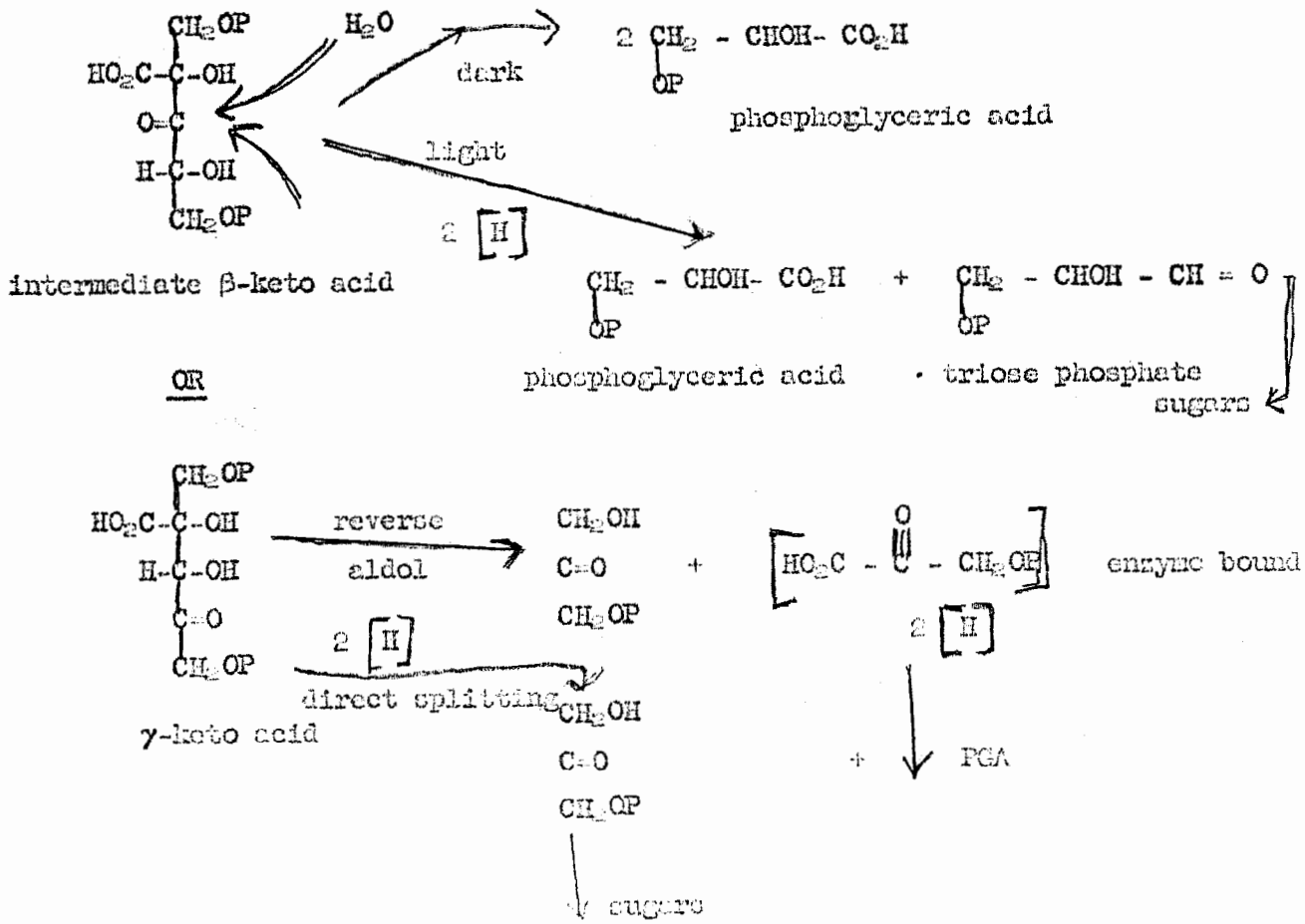
vivo systems in order to account for the extremely rapid rate of carboxylation of ribulose diphosphate observed in the in vivo systems. At first, some evidence for such a CO_2-activation in the form of extremely labile compounds seemed apparent (Metzner, Simon, Metzner and Calvin, '57; Metzner, Metzner and Calvin, '58). However, further investigation has failed to confirm any evidence for such a product (Kasprzyk and Calvin, '59). In Figs. 1 and 2 we see that the evidence indicates the absence of any product more stable than bicarbonate lying between CO_2 and the relatively stable materials that can withstand plating, i.e., phosphoglyceric acid, etc. The chromatographic evidence for such a product has been accounted for otherwise (Bassham, Kirk and Calvin, '58).

However, an examination ofthe in vitro carboxydismutase enzyme system has revealed a dependence of the activity of the enzyme on the preliminary presence of carbon dioxide in addition to Mg^{++}. Preliminary incubation of the enzyme with bicarbonate in the presence of magnesium does indeed induce a greater carboxylation activity than preliminary incubation with any other component of the reaction system; see Table 2 and Figs. 3 and 4 (Pon, '59).

It is perhaps worth noting at this point that the precise investigations of the kinetics of carbon flow in the in vivo systems through the carboxydismutase reaction seem to indicate that whereas in the dark the primary product of carboxylation does indeed split into two molecules of phosphoglyceric acid, in the light it may be otherwise. In the light there is an indication that the primary product of carboxylation may be splitting by a reductive reaction, leading to only one molecule of phosphoglyceric acid and one molecule at the triose phosphate oxidation level (Bassham, '59).

Such an alternative, of two possible modes of action, was proposed when the carboxylation of ribulose diphosphate was first recognized (Calvin and Massini, '52; Wilson and Calvin, '55; Bassham and Calvin, '57). The best evidence that it might be so has only recently been produced and is in the form of more precise measurements of the rate of approach to carbon-14 saturation of the pools of PGA and ribulose diphosphate in algae in a more nearly true steady state of photosynthesis than has heretofore been achieved (see Fig. 5). From this it is possible to show that only if a single molecule of PGA is liberated for each molecule of CO_2 entering the algae does the specific activity (C^{14}) of the RuDP remain higher than that of the α- and β-carbon atoms of PGA. The remaining three carbon atoms in the reaction go directly to the sugar level of oxidation, when the light is on.

Two possible sequences which would fulfill these requirements are as follows:



Much more remains to be done, however, before we will know all the intimate details of this reaction, and it is not impossible to conceive that this reductive splitting reaction of the intermediate carboxylation product (β- or γ-keto acid) might very well require a reducing system as yet either unknown or, at best, unsurmised and undetermined.

LITERATURE CITED

AXELROD, B., R. S. BANDURSKI, C. M. GREINER AND R. JANG 1953 The Metabolism of hexose and pentose phosphates in higher plants. J. Biol. Chem., 202:619-634.

BACHHAWAT, B. K. AND M. J. COON 1957 The role of adenosine triphosphate in the enzymatic activation of carbon dioxide. J. Am. Chem. Soc., 79:1505-1506

_____ 1958 Enzymatic activation of carbon dioxide. I. Crystalline carbon dioxide-activating enzyme. J. Biol. Chem., 231:625-635.

BACHHAWAT, B. K., W. G. ROBINSON AND M. J. COON 1954 Carbon dioxide fixation in heart extracts by β-hydroxyisovaleryl Coenzyme A. J. Am. Chem. Soc., 76:3098-3099.

_____ 1956 Enzymatic carboxylation of β-hydroxyisovaleryl Coenzyme A. J. Biol. Chem., 219:539-550.

BACHHAWAT, B. K., J. F. WOESSNER AND M. J. COON 1956 Role of adenosine triphosphate in the enzymatic activation of carbon dioxide. Federation Proc., 15:214.

BANDURSKI, R. S. AND C. M. GREINER 1953 The enzymatic synthesis of oxalacetate from phosphoryl-enolpyruvate and carbon dioxide. J. Biol. Chem., 204:781-786.

BASSHAM, J. A. 1959 Photosynthesis. In Division of Chemical Education Abstracts, No. 22, p. 11F. 135th Meeting of American Chemical Society, Boston, Massachusetts. April 1959.

BASSHAM, J. A. AND M. CALVIN 1957 The Path of Carbon in Photosynthesis Prentice-Hall, Inc., Englewood Cliffs, New Jersey.

BASSHAM, J. A., M. R. KIRK AND M. CALVIN 1958 Effect of hydroxylamine on $C^{14}O_2$ fixation patterns during photosynthesis. Proc. Nat. Acad. Sci., 44:491-493.

BECK, W. S., M. FLAVIN AND S. OCHOA 1957 Metabolism of propionic acid in animal tissues. III. Formation of succinate. J. Biol. Chem., 229:997-1010.

BROWN, B. R. 1951 The mechanism of thermal decarboxylation. Quart. Rev., 5:131-146.

BURTON, K. AND H. A. KREBS 1953 The free-energy changes associated with the individual steps of the tricarboxylic acid cycle, glycolysis and alcoholic fermentation and with the hydrolysis of the pyrophosphate groups of adenosine triphosphate. Biochem. J., 54:94-105.

CALVIN, M. AND P. MASSINI 1952 The path of carbon in photosynthesis. XX. The steady state. Experientia, 8:444-457.

COHEN, S. 1954 Other pathways of carbohydrate metabolism (Chapter 5) in Chemical Pathways of Metabolism, Vol. 1 ed. by D. M. Greenberg, Academic Press, Inc., New York, New York, p. 173.

CRAWFORD, I., A. KORNBERG AND E. S. SIMMS 1957 Conversion of uracil and orotate to uridine-5'-phosphate by enzymes in Lactobacilli. J. Biol. Chem., 226:1093-1101.

FLAVIN, M., H. CASTRO-MENDOZA and S. OCHOA 1956 Bicarbonate-dependent enzymic phosphorylation of fluoride by adenosine triphosphate. Biochim. et Biophys. Acta, 20:590-593.

_____ 1957 Metabolism of propionic acid in animal tissues. II. Propionyl Coenzyme A carboxylation system. J. Biol. Chem., 229:981-996.

FORMICA, J. V. AND R. O. BRADY 1959 The enzymatic carboxylation of acetyl Coenzyme A. J. Am. Chem. Soc., 81:752.

GREEN, D. E., D. HERBERT AND V. SUBRAHMANYAN 1941 Carboxylase. J. Biol. Chem., 138:327-339.

GRISOLIA, S. AND P. P. COHEN 1953 Catalytic role of glutamate derivatives in citrulline biosynthesis. J. Biol. Chem., 204:753-757.

GUNSALUS, I. C. 1954 Group transfer and acyl-generating functions of lipoic acid derivatives in The Mechanism of Enzyme Action, ed. by W. D. McElroy and B. Glass, Johns Hopkins Press, Baltimore, Maryland, p. 545.

HANKES, L. V. AND L. M. HENDERSON 1957 The mechanism of carboxyl-labeled 3-hydroxyanthranilic acid in the rat. J. Biol. Chem., 225:349-354.

HORECKER, B. L. AND P. Z. SMYRNIOTIS 1952 The fixation of carbon dioxide in 6-phosphogluconic acid. J. Biol. Chem., 196:135-142.

HURWITZ, J., W. B. JAKOBY AND B. L. HORECKER 1956 On the mechanism of carbon dioxide fixation leading to phosphoglyceric acid. Biochim. et Biophys. Acta, 22:194-195.

JAGANNATHAN V, AND R. S. SCHWEET 1952 Pyruvic oxidase of pigeon breast muscle. I. Purification and properties of the enzyme. J. Biol. Chem., 196:551-562.

JONES, M. E., L. SPECTOR AND F. LIPMANN 1955 Carbamyl phosphate: The carbamyl donor in enzymatic citrulline synthesis. J. Am. Chem. Soc., 77:819-820.

KALTENBACH, J. P. AND G. KALNITSKY 1951 The enzymatic formation of oxalacetate from pyruvate and carbon dioxide. J. Biol. Chem., 192: 629-639, 641-649.

KASPRZYK, Z. AND M. CALVIN 1959 Search for unstable CO_2 fixation product in algae using low temperature scintillators. Proc. Nat. Acad. Sci., in press.

KAUFMAN, S. C. GILVARG, O. CORI AND S. OCHOA 1953 Enzymatic oxidation of α-ketoglutarate and coupled phosphorylation. J. Biol. Chem., 203:869-888.

KNAPPE, J. AND F. LYNEN 1958 Enzymatische Carboxylierung von β-Methylcrotonyl-Coenzyme A. IV International Congress of Biochemistry, Abstracts, 49, Sept. 1958.

KORKES, S. A. DEL CAMPILLO, I. C. GUNSALUS AND S. OCHOA 1951 Enzymatic synthesis of citric acid. IV. Pyruvate as acetyl donor. J. Biol. Chem., 193:721-735.

_____ A. DEL CAMPILLO AND S. OCHOA 1952 Pyruvate oxidation system of heart muscle. J. Biol. Chem., 195:541-547.

KOSHLAND, D. E. 1958 Application of a theory of enzyme specificity to protein synthesis. Proc. Nat. Acad. Sci., 44:98-104.

KUN, E., J. M. DECHARY AND H. C. PITOT 1954 The oxidation of glycolic acid by a liver enzyme. J. Biol. Chem., 210:269-280.

KURAHASHI, K., R. J. PENNINGTON AND M. F. UTTER 1957 Nucleotide specificity of oxalacetic carboxylase. J. Biol. Chem., 226:1059-1075.

LARDY, H. A. AND J. A. ZIEGLER 1945 The enzymatic synthesis of phosphopyruvate. J. Biol. Chem., 159:343-351.

LIEBERMAN, I., A. KORNBERG AND E. S. SIMMS 1955 Enzymatic synthesis of pyrimidine nucleotides. Orotidine-5'-phosphate and uridine-5'-phosphate. J. Biol. Chem., 215:403-415.

LUKENS, L. N. AND J. M. BUCHANAN 1957 Further intermediated in the biosynthesis of inosinic acid de novo. J. Am. Chem. Soc., 79:1511-1513.

LYNEN, F., H. J. KNAPPE, E. LORCH AND G. JUTTING 1959 Carboxylation of β-methylcrotonyl Coenzyme A. American Chemical Society National Meeting, Boston, Massachusetts, April 1959. Division of Biological Chemistry, Abstract No. 83.

MANDELES, S., R. KOPPELMAN AND M. E. HANKE 1954 Deuterium studies on the mechanism of enzymatic amino acid decarboxylation. J. Biol. Chem., 209:327-335.

MAYAUDON, J. 1957 Study of association between the main nucleoprotein of green leaves and carboxydismutase. Enzymologia, 18:343-354.

_____ A. A. BENSON AND M. CALVIN 1957 Ribulose-1,5-diphosphate from and CO_2 fixation by Tetragonia expansa leaves extract. Biochim. et Biophys. Acta, 23:342-351.

MEHLER, A. H. 1956 Formation of picolinic and quinolinic acids following enzymatic oxidation of 3-hydroxyanthranilic acid. J. Biol. Chem., 218:241-254.

MEISTER, A. 1957 in The Biochemistry of the Amino Acids, Academic Press,

METZNER, H., B. METZNER AND M. CALVIN 1958 Labile products of early CO_2 fixation in photosynthesis. Arch. Biochem. and Biophys., 74:1-6.

METZNER, H., H. SIMON, B. METZNER AND M. CALVIN 1957 Evidence for an unstable CO_2 fixation product in algal cells. Proc. Nat. Acad. Sci. 43:892-895.

NEUBERGER, A., J. J.SCOTT AND L. SHUSTER 1956 Synthesis and metabolism of some substances related to δ-aminolevulinic acid. Biochem. J., 64:137-145.

OCHOA, S., A. H. MEHLER AND A. KORNBERG 1948 Biosynthesis of dicarboxylic acids by carbon dioxide fixation. I. Isolation and properties of an enzyme from pigeon liver catalyzing the reversible oxidative carboxylation of l-malic acid. J. Biol. Chem., 174:979-1000.

_____ AND E. WEISZ-TABORI 1948 Biosynthesis of tricarboxylic acids by carbon dioxide fixation. II. Oxalosuccinic carboxylase. J. Biol. Chem., 174:123-132.

PEDERSEN, K. S. 1934 The decomposition of α-nitrocarboxylic acids with some remarks on the decomposition of β-ketocarboxylic acids. J. Phys. Chem., 38:559-571.

_____ 1947 The decomposition of nitroacetic acid in concentrated aqueous solutions of some non-electrolytes. Acta Chem. Scand., 1:437-447.

PON, N. G. 1959 Thesis, University of California, Berkeley. Studies on the carboxydismutase system and related materials.

QUAYLE, J. R., R. C. FULLER, A. A. BENSON AND M. CALVIN 1954 Enzymatic carboxylation of ribubse diphosphate. J. Am. Chem. Soc., 76:3610.

RACKER, E. 1957 The reductive pentose cycle. I. Phosphoribulokinase and ribulose diphosphate carboxylase. Arch. Biochem. and Biophys., 69:300-310.

REICHARD, P. 1957 Ornithine carbamyl transferase from rat liver. Acta Chem. Scand., 11:523-536.

SANADI, D. R., J. W. LITTLEFIELD AND R. M. BOCK 1952 Studies on α-ketoglutaric oxidase. II. Purification and properties. J. Biol. Chem., 197:851-862.

SAZ, H. J. AND J. A. HUBBARD 1957 The oxidative decarboxylation of malate by Ascaris lumbricoides. J. Biol. Chem., 225:921-933.

SIEBERT, G., M. CARSIOTIS AND G.W.E. PLAUT 1957 The enzymatic properties of isocitric dehydrogenase. J. Biol. Chem., 226:977-991.

STILES, M. AND H. L. FINKBEINER 1959 Chelation as a driving force in synthesis. A new route to α-nitro acids and α-amino acids. J. Am. Chem. Soc., 81:505-506.

TCHEN, T. T., F. A. LOEWUS AND B. VENNESLAND 1955 The mechanism of enzymatic carbon dioxide fixation into oxalacetate. J. Biol. Chem., 213:547-555.

_____ AND B. VENNESLAND 1955 Enzymatic carbon dioxide fixation into oxalacetate in wheat germ. J. Biol. Chem., 213:533-546.

TIETZ, A. AND S. OCHOA 1958 'Fluorokinase' and pyruvic kinase. Arch. Biochem. and Biophys., 78:477-493.

VENNESLAND, B., M. C. GOLLAB AND J. B. SPECK 1949 The β-carboxylases of plants. I. Some properties of oxalacetic carboxylase and its quantitative assay. J. Biol. Chem., 178:301-314.

WAKIL, S. J. 1958 A malonic acid derivative as an intermediate in fatty acid synthesis. J. Am. Chem. Soc., 80:6465.

WEISSBACH, A., B. L. HORECKER AND J. HURWITZ 1956 The enzymatic formation of phosphoglyceric acid from ribulose diphosphate and carbon dioxide. J. Am Biol. Chem., 218:795-810.

WESTHEIMER, F. H., AND W. A. JONES 1941 The effect of solvent on some reaction rates. J. Am. Chem. Soc., 63:3283-3286.

WILSON, A. T., AND M. CALVIN 1955 The photosynthetic carbon cycle: CO_2-dependent transients. J. Am. Chem.Soc., 77:5948-5957.

WOESSNER, J. F., B. K. BACHHAWAT AND M. J. COON 1958 Enzymatic activation of carbon dioxide. II. Role of biotin in the carboxylation of β-hydroxyisovaleryl Coenzyme A. J. Biol. Chem., 233:520-523.

ZELITCH, I. AND S. OCHOA 1953 Oxidation and reduction of glycolic and glyoxylic acids in plants. I. Glycolic acid oxidase. J. Biol. Chem., 201:707-718.

FOOTNOTES

1 Presented at Oak Ridge National Laboratory Twelfth Annual Research Conference on Enzyme Reaction Mechanisms, Gatlinburg, Tennessee, April 1, 1959.

2 The work described in this paper was sponsored by the U.S. Atomic Energy Commission.

3 See footnote 4 on page 22, discussing results presented in the previous paper at this meeting and after this paper was written.

4 Paragraph 2, page 22, page 23, and formulas and paragraph 1, page 24 were added subsequent to presentation of foregoing paper by Lynen.

Table 1

CARBOXYLATION (DECARBOXYLATION) REACTIONS

Reaction	Enzyme	Substrate	Product	Metabolic Pathway	Cofactors or Additions
1	Pyruvic Dehydrogenase (Pyruvic oxidase)	Pyruvic Acid	Acetyl CoA	Glycolysis to TCA	DPN^+, CoA, Thioctic Acid, TPP, Mg^{++}
2	α-Ketoglutaric Dehydrogenase (α-Ketoglutaric Acid Oxidase)	α-Ketoglutaric Acid	Succinyl CoA	TCA	DPN^+, CoA, TPP, Thioctic Acid, Mg^{++}
3a	α-Ketoacyl Dehydrogenase	α-Keto, β-Methyl-valeric Acid	α-Methyl-butyrl CoA	Isoleucine Metabolism	CoA, DPN^+, Thioctic Acid, TPP
3b	" " "	α-Ketoisocap-roic Acid	Isovaleryl CoA	Leucine Metabolism	CoA, DPN^+, Thioctic Acid, TPP
3c	" " "	α-Ketoiso-valeric Acid	Isobutryl CoA	Valine Metabolism	Same as Above
4	Pyruvic Carboxylase	Pyruvic Acid	Acetalde-hyde	Glycolysis	TPP, Mg^{++}
5	Oxalacetic Decarboxylase (Oxalacetic Carboxylase)	Oxalacetic Acid	Pyruvic Acid	TCA to Glycolysis	Mn^{++}
6	α-Amino, β-Ketoadipic Decarboxylase	α-Amino, β-Ketoadipic Acid	δ-Aminolevu-linic Acid	Porphyrin Metabolism	
7	Isocitric Dehydrogenase	D-Isocitric Acid	α-Ketoglutaric Acid (α-KG)	TCA	TPN, Mn^{++}

Table 1 (continued)

	Enzyme	Substrate	Product	Metabolic Pathway	Cofactors or Additions
8	Malic Enzyme (TPN-Malic Dehydrogenase)	Malic Acid	Pyruvate	TCA to Glycolysis	TPN^+, (plants & animals), DPN^+ (bacteria) Mn^{++}
9	Phosphogluconic Dehydrogenase	6-Phospho-gluconic Acid	Ribulose-5-phosphate	HMP(Shunt)	Mg^{++}, or Mn^{++}, TPN^+
10	Acetoacetic Acid Decarboxylase	Acetoacetic Acid	Acetone	Fatty Acid Metabolism	
11	Glycolic Acid Oxidase	Glycolic Acid	Formic Acid	- - -	
12a	Picolinic Carboxylase	3-Hydroxan-thranilic Acid	Picilinic Acid	Tryptophan Metabolism	Fe^{++}, O_2 ?
12b	Nicotinic Carboxylase	3-Hydroxyan-thranilic Acid	Nicotinic Acid	Tryptophan Metabolism	" "
13	Orotidylic Decarboxylase	Orotidine-5'-phosphate	Uridine-5'-phosphate	Pyrimidine Synthesis	----
14	Hydroxyisovaleryl-CoA	β-Hydroxyiso-valeryl CoA	β-Hydroxy-β-Methyl Glutaryl CoA	Leucine Metabolism	ATP, HCO_3^-, Zn^{++}
15	Propionyl CoA Carboxylase	Propionyl CoA	Succinyl CoA	Fatty Acid Metabolism	ATP, HCO_3^-, Mg^{++}
16		Acetyl CoA	Malonyl CoA	Fatty Acid Metabolism	Mn^{++}, ATP, HCO_3^-
17		Ammonia	Carbamyl phosphate	Urea Cycle	Mg^{++}, ATP, Acetyl-glutamic Acid
18	Phosphoenolpyruvic Carboxylase	Phosphoenol-pyruvic Acid	Oxalacetic Acid	Glycolysis to TCA	IDP(animal), ADP(plant), Mn

Table 1 (Continued)

	Enzyme	Substrate	Product	Metabolic Pathyway	Cofactors or Additions
19	Phosphoenolpyruvic Carboxylase	Phosphoenol-pyruvic Acid	Oxalacetic Acid	Glycolysis to TCA	None
20		5-Aminoimidazole Ribotide	5-Amino,4-Carboxyimi-dazole Ribotide	Purine Synthesis	
21	Carboxydismutase	Ribulose-1,5 diphosphate	3-Phosphogly-ceric Acid	Photosynthesis	Mg^{++}
22		β-Methyl-crotonyl CoA	β-Methyl- glutaconyl-CoA	Leucine Metabolism	

Table 1 (continued)

	REFERENCES
Reaction 1	Jagannathan and Schweet, 1952. Korkes, del Campillo, Gunsalus and Ochoa, 1951. Gunsalus, 1954.
Reaction 2	Sanadi, Littlefield and Bock, 1952. Burton and Krebs, 1953. Kaufman, Gilvarg, Cori and Ochoa, 1953. Gunsalus, 1954.
Reactions 3a-3b	Meister, 1957.
Reaction 4	Green, 1954.
Reaction 5	Kaltenbach and Kalnitsky, 1951. Vennesland, Gallab and Speck, 1949.
Reaction 6	Neuberger, Scott and Shuster, 1956.
Reaction 7	Ochoa and Weisz-Tabori, 1958. Siebert, Carsiotis and Plaut, 1957.
Reaction 8	Saz and Hubbard, 1957. Ochoa, Mehler and Kornberg, 1948.
Reaction 9	Axelrod, Bandurski, Greiner and Jang, 1953. Cohen, 1954. Horecker and Smyrniotis, 1952.
Reaction 10	Davies, 1943.
Reaction 11	Zelitch and Ochoa, 1953. Kun, Dechary and Pitot, 1954.
Reaction 12a-12b	Mehler, 1956. Hankes and Henderson, 1957.
Reaction 13	Lieberman, Kornberg and Simms, 1955. Crawford, Kornberg and Simms, 1957.
Reaction 14a Reaction 14b	Bachhawat and Coon, 1958. Woessner, Bachhawat and Coon, 1958.
Reaction 14c	Bachhawat and Coon, 1957. Bachhawat, Robinson and Coon, 1954. Bachhawat, Woessner and Coon, 1956. Bachhawat, Robinson and Coon, 1956.
Reaction 15	Flavin, Castro-Mendoza and Ochoa, 1956, 1957. Tietz and Ochoa, 1958.
Reaction 16	Wakil, 1958. Formica and Brady, 1959.
Reaction 17	Jones, Spector and Lipmann, 1955. Grisolia and Cohen, 1953. Reichard, 1957.

Table 1 (continued)

	REFERENCES
Reaction 18	Kurahashi, Pennington and Utter, 1957. Tchen and Vennesland, 1955.
Reaction 19	Tchen, Loewus and Vennesland, 1955. Bandurski and Greiner, 1953.
Reaction 20	Lukens and Buchanan, 1957.
Reaction 21	Weissbach, Horecker and Hurwitz, 1956. Quayle, Fuller, Benson and Calvin, 1954. Mayaudon, Benson and Calvin, 1957. Mayaudon, 1957. Hurwitz, Jakoby and Horecker, 1956.
Reaction 22	Knappe and Lynen, 1958. Lynen, Knappe, Lorch and Jutting, 1959.

Table 2

PREINCUBATION OF CARBOXYDISMUTASE WITH SUBSTRATES AND COFACTOR

	Total counts/min
$E + M \longrightarrow EM \xrightarrow{S_1} EMS_1 \xrightarrow{S_2} PGA$	24,000
$EM \xrightarrow{S_2} EMS_2 \xrightarrow{S_1} PGA$	13,000
$E + S_1 \longrightarrow ES_1 \xrightarrow{M} ES_1M \xrightarrow{S_2} PGA$	24,000
$ES_1 \xrightarrow{S_2} ES_1S_2 \xrightarrow{M} PGA$	13,000
$E + S_2 \longrightarrow ES_2 \xrightarrow{S_1} ES_2S_1 \xrightarrow{M} PGA$	9,400
$ES_2 \xrightarrow{M} ES_2M \xrightarrow{S_1} PGA$	10,000

1st — Preincubation (each 10 min, 0°C) — 2nd — Incubation (5 min, 25°C)

	Total counts/min
$M + S_1 \longrightarrow MS_1 \xrightarrow{E} MS_1E \xrightarrow{S_2} PGA$	23,000
$MS_1 \xrightarrow{S_2} MS_1S_2 \xrightarrow{E} PGA$	11,000
$M + S_2 \longrightarrow MS_2 \xrightarrow{E} MS_2E \xrightarrow{S_1} PGA$	10,000
$MS_2 \xrightarrow{S_1} MS_2S_1 \xrightarrow{E} PGA$	12,000
$S_1 + S_2 \longrightarrow S_1S_2 \xrightarrow{E} S_1S_2E \xrightarrow{M} PGA$	11,000
$S_1S_2 \xrightarrow{M} S_1S_2M \xrightarrow{E} PGA$	11,000

1st — Preincubation (each 10 min, 0°C) — 2nd — Incubation (5 min, 25°C)

E: Carboxydismutase S_1: $NaHC^{14}O_3$ S_2: Ribulose-1,5-diphosphate

M: Mg^{++} PGA: 3-Phosphoglyceric Acid-1-C^{14}

MU-16949

FIGURE LEGENDS

Figure 1. Labeled Carbon in Suspension after Ethanol Killing of Algae.

Figure 2. Labeled Carbon in Suspension after Acetone Killing of Algae.

Figure 3. Preliminary Incubation of Carboxydismutase with the Substrates.

Figure 4. Preliminary Incubation of Carboxydismutase with Metal Ions.

Figure 5. Rate of Incorporation of Labeled Carbon from Labeled Bicarbonate into Phosphoglyceric Acid (PGA) and Ribulose Diphosphate (RuDP).

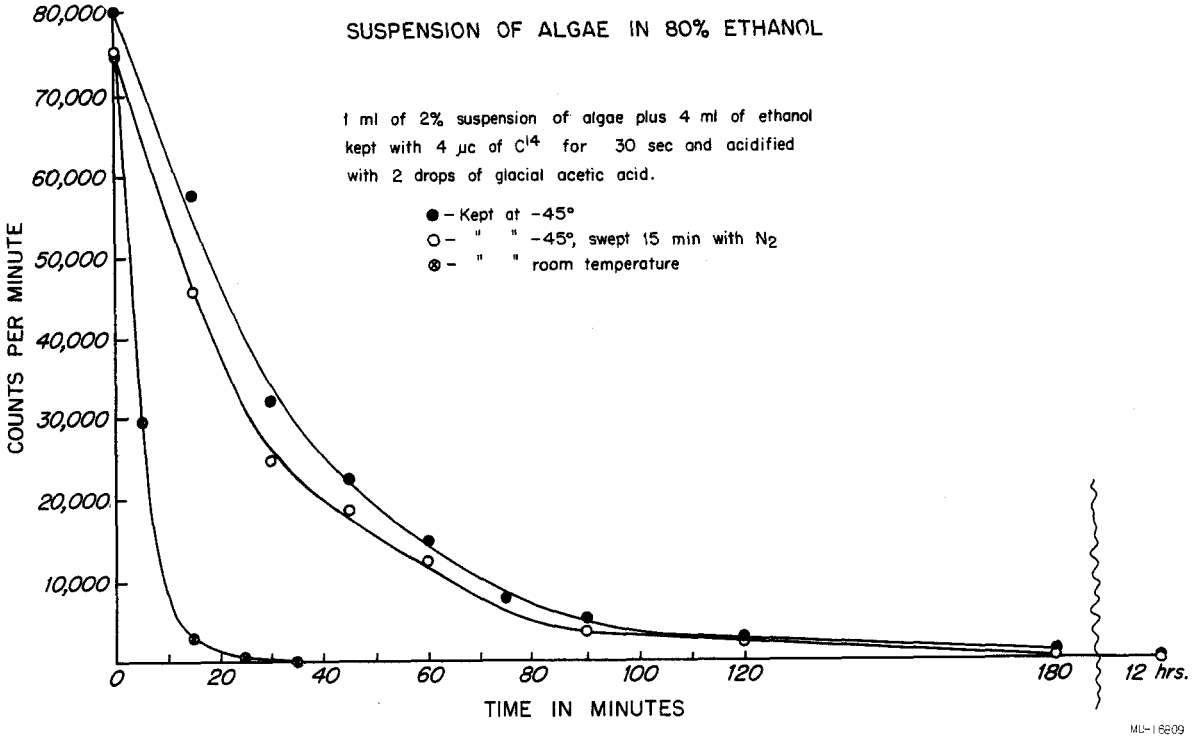

SUSPENSION OF ALGAE IN 80% ETHANOL
1 ml of 2% suspension of algae plus 4 ml of ethanol kept with 4 μc of C14 for 30 sec and acidified with 2 drops of glacial acetic acid.
● – Kept at -45°
○ – " " -45°, swept 15 min with N2
⊗ – " " room temperature
COUNTS PER MINUTE
80,000
70,000
60,000
50,000
40,000
30,000
20,000
10,000
0
20
40
60
80
100
120
180
12 hrs.
TIME IN MINUTES
MU-16809

Fig. 1a

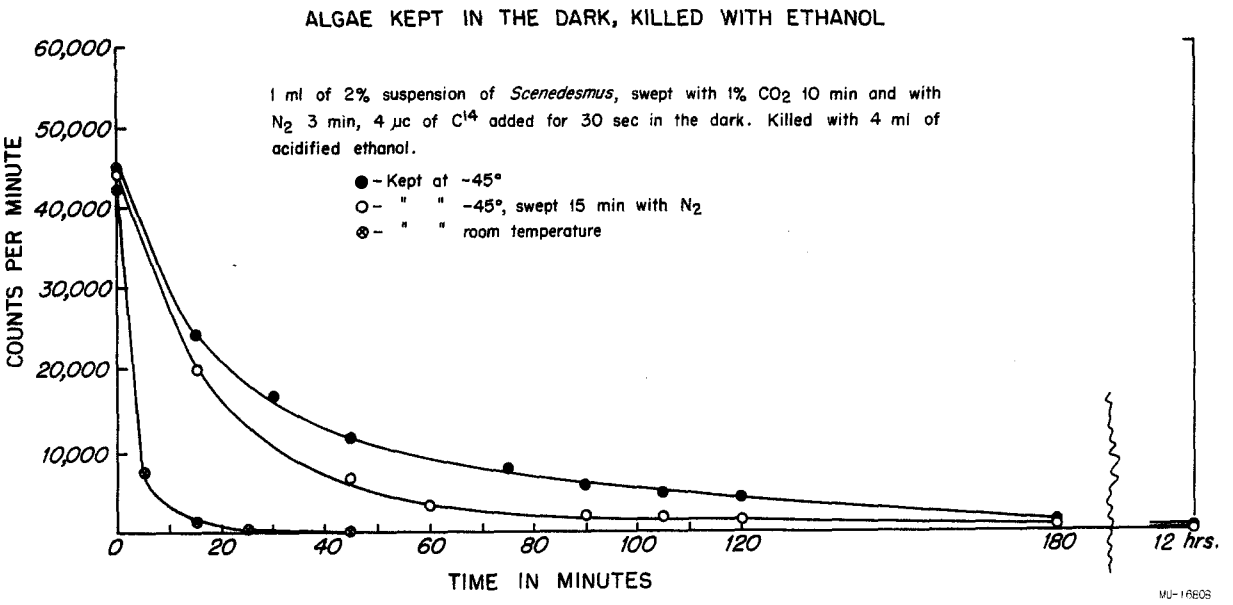

ALGAE KEPT IN THE DARK, KILLED WITH ETHANOL
1 ml of 2% suspension of *Scenedesmus*, swept with 1% CO_2 10 min and with N_2 3 min, 4 μc of C^{14} added for 30 sec in the dark. Killed with 4 ml of acidified ethanol.
● - Kept at -45°
○ - " " -45°, swept 15 min with N_2
⊗ - " " room temperature
COUNTS PER MINUTE
60,000
50,000
40,000
30,000
20,000
10,000
0
20
40
60
80
100
120
180
12 hrs.
TIME IN MINUTES
MU-16608

Fig. 1b

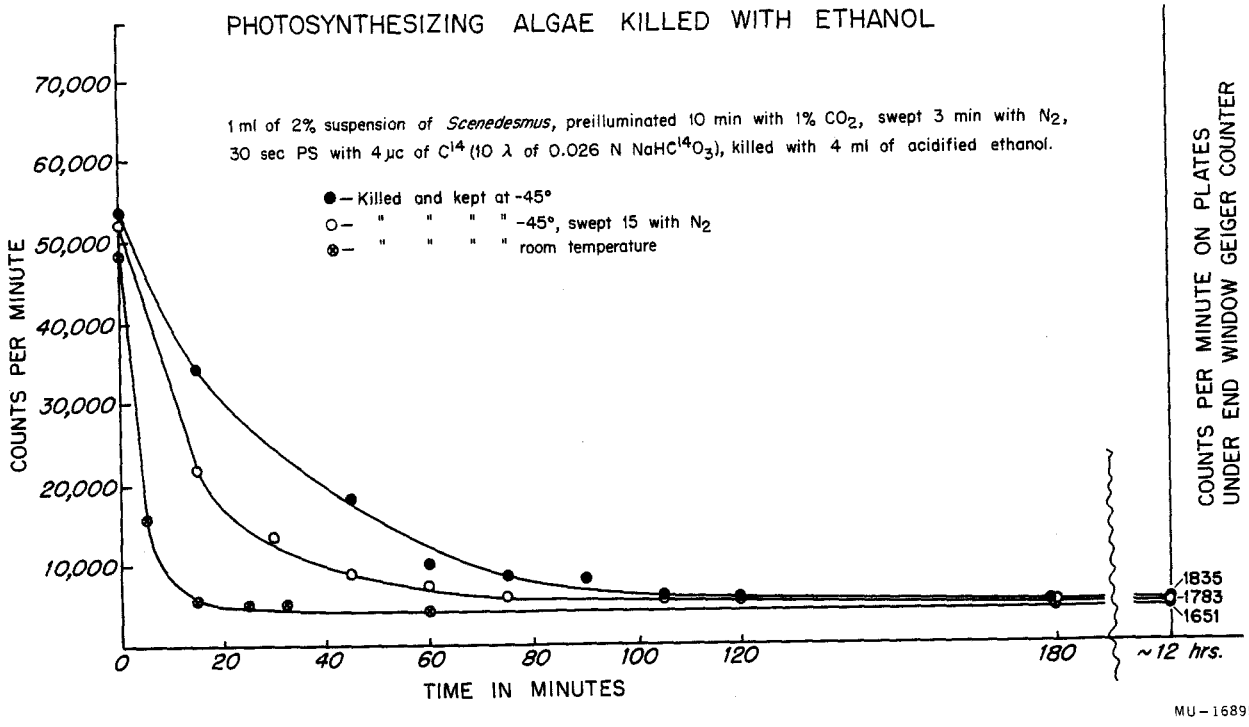

PHOTOSYNTHESIZING ALGAE KILLED WITH ETHANOL
1 ml of 2% suspension of Scenedesmus, preilluminated 10 min with 1% CO2, swept 3 min with N2, 30 sec PS with 4 µc of C14 (10 λ of 0.026 N NaHC14O3), killed with 4 ml of acidified ethanol.
●— Killed and kept at -45°
○— " " " " -45°, swept 15 with N2
⊗— " " " " room temperature
COUNTS PER MINUTE
70,000
60,000
50,000
40,000
30,000
20,000
10,000
0
20
40
60
80
100
120
180
~12 hrs.
TIME IN MINUTES
COUNTS PER MINUTE ON PLATES UNDER END WINDOW GEIGER COUNTER
1835
1783
1651
MU-16895

Fig. 1c

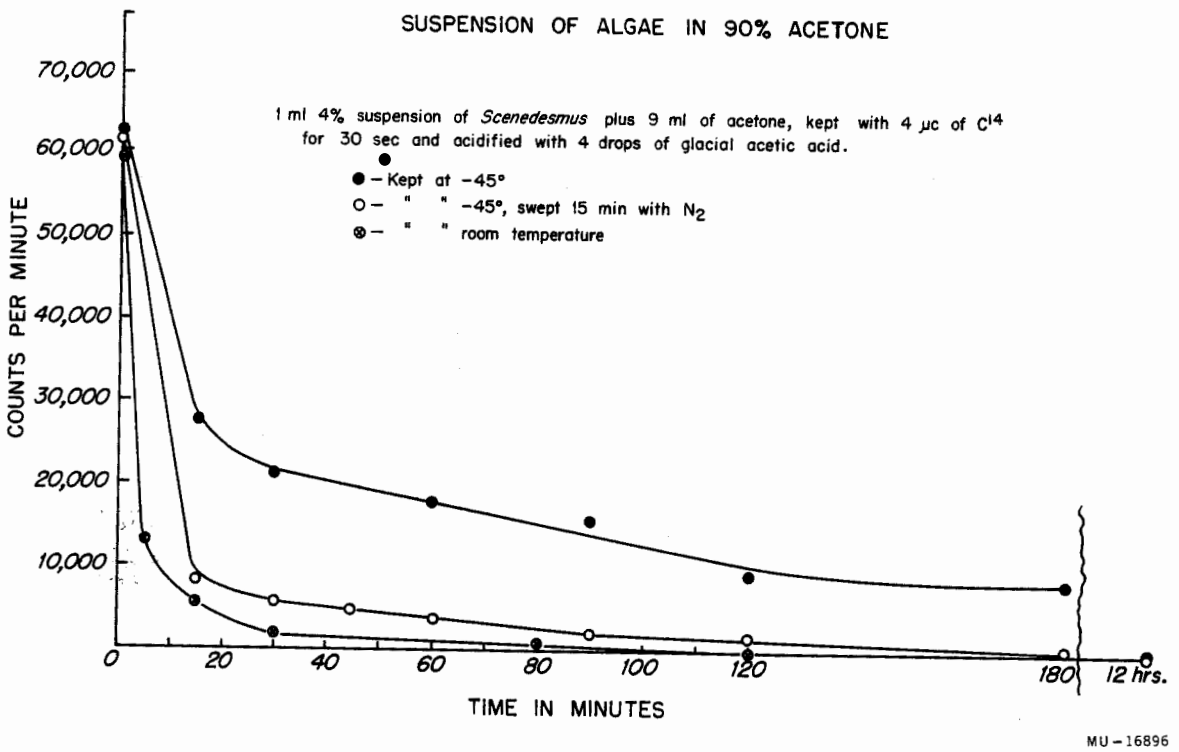

SUSPENSION OF ALGAE IN 90% ACETONE
1 ml 4% suspension of Scenedesmus plus 9 ml of acetone, kept with 4 μc of C14 for 30 sec and acidified with 4 drops of glacial acetic acid.
● – Kept at –45°
○ – " " –45°, swept 15 min with N2
⊗ – " " room temperature
COUNTS PER MINUTE
70,000
60,000
50,000
40,000
30,000
20,000
10,000
0
20
40
60
80
100
120
180
12 hrs.
TIME IN MINUTES
MU-16896

Fig. 2a

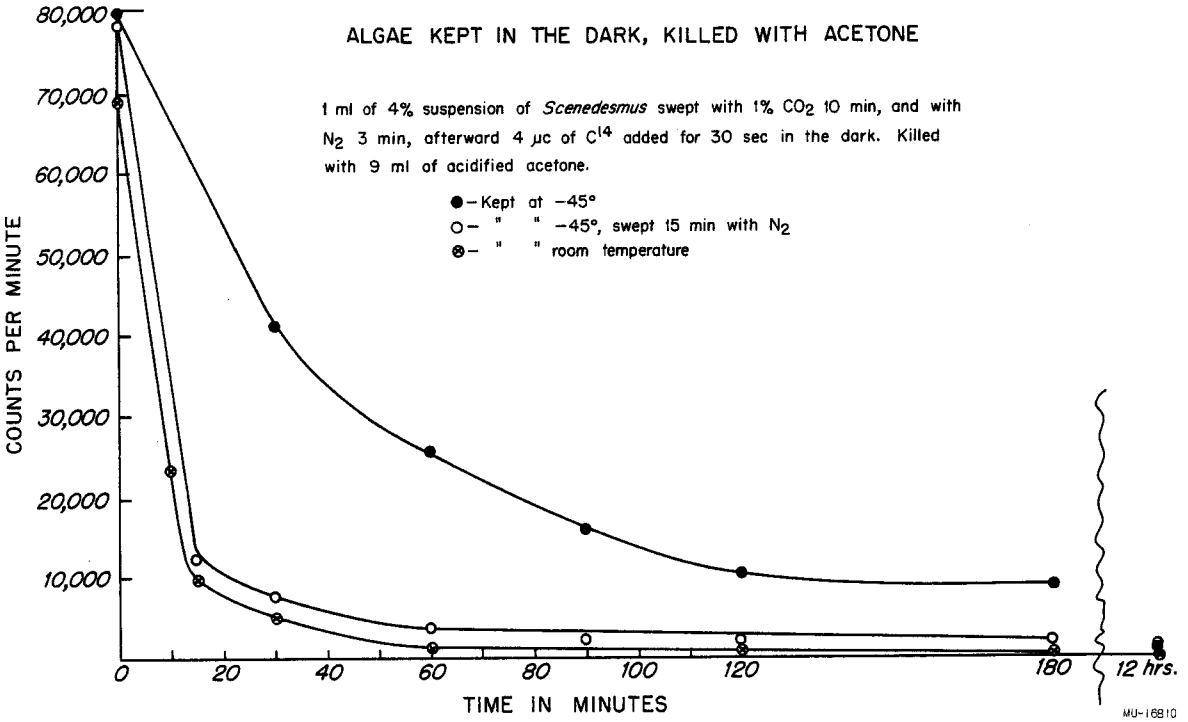

ALGAE KEPT IN THE DARK, KILLED WITH ACETONE
1 ml of 4% suspension of *Scenedesmus* swept with 1% CO_2 10 min, and with N_2 3 min, afterward 4 μc of C^{14} added for 30 sec in the dark. Killed with 9 ml of acidified acetone.
●– Kept at –45°
○– " " –45°, swept 15 min with N_2
⊗– " " room temperature
COUNTS PER MINUTE
80,000
70,000
60,000
50,000
40,000
30,000
20,000
10,000
0
20
40
60
80
100
120
180
12 hrs.
TIME IN MINUTES
MU-16810

Fig. 2b

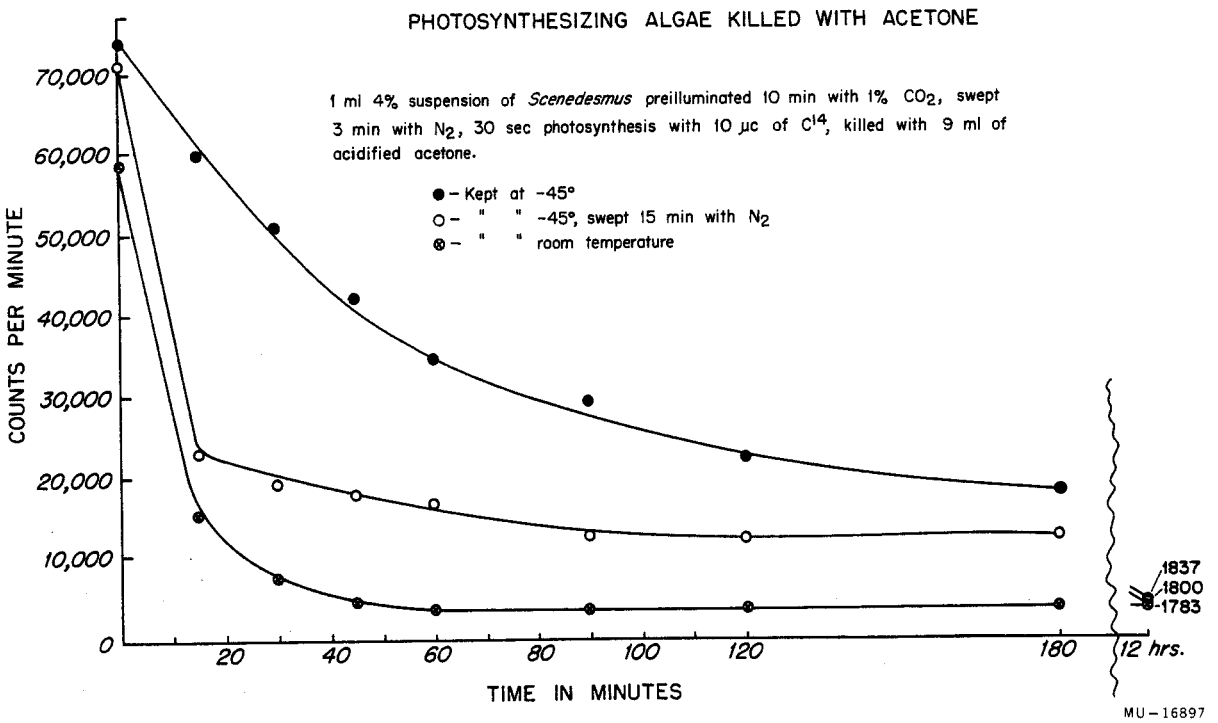
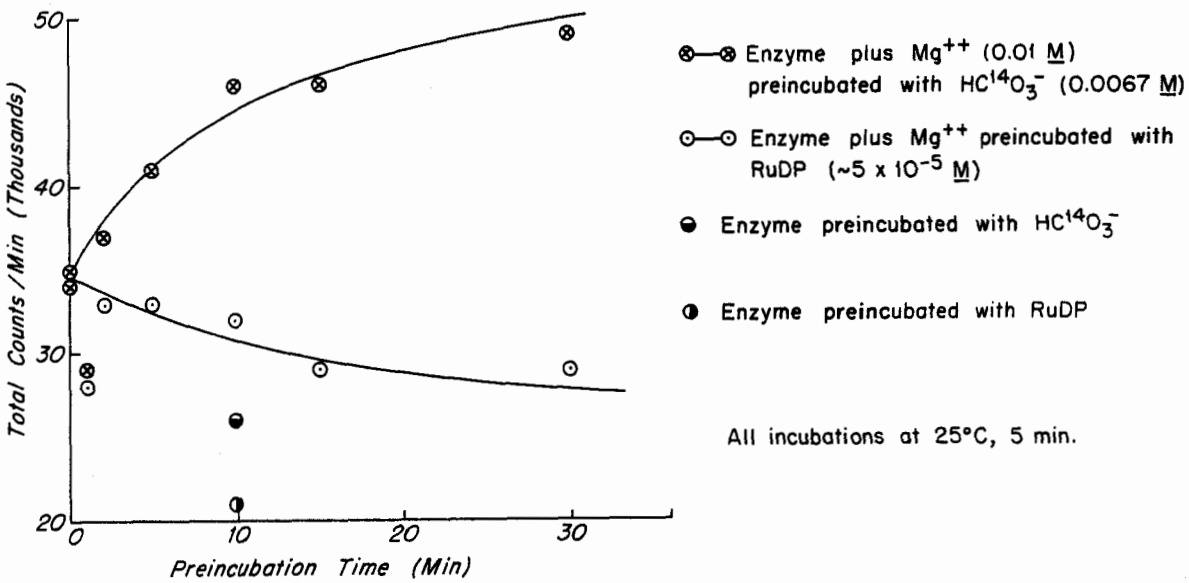

PHOTOSYNTHESIZING ALGAE KILLED WITH ACETONE
1 ml 4% suspension of Scenedesmus preilluminated 10 min with 1% CO2, swept 3 min with N2, 30 sec photosynthesis with 10 µc of C14, killed with 9 ml of acidified acetone.
● – Kept at -45°
○ – " " -45°, swept 15 min with N2
⊗ – " " room temperature
COUNTS PER MINUTE
70,000
60,000
50,000
40,000
30,000
20,000
10,000
0
20
40
60
80
100
120
180
12 hrs.
1837
1800
1783
TIME IN MINUTES
MU-16897

Fig. 2c

THE PREINCUBATION OF CARBOXYDISMUTASE WITH SUBSTRATES AT 0°C



MU-16943

Fig. 3

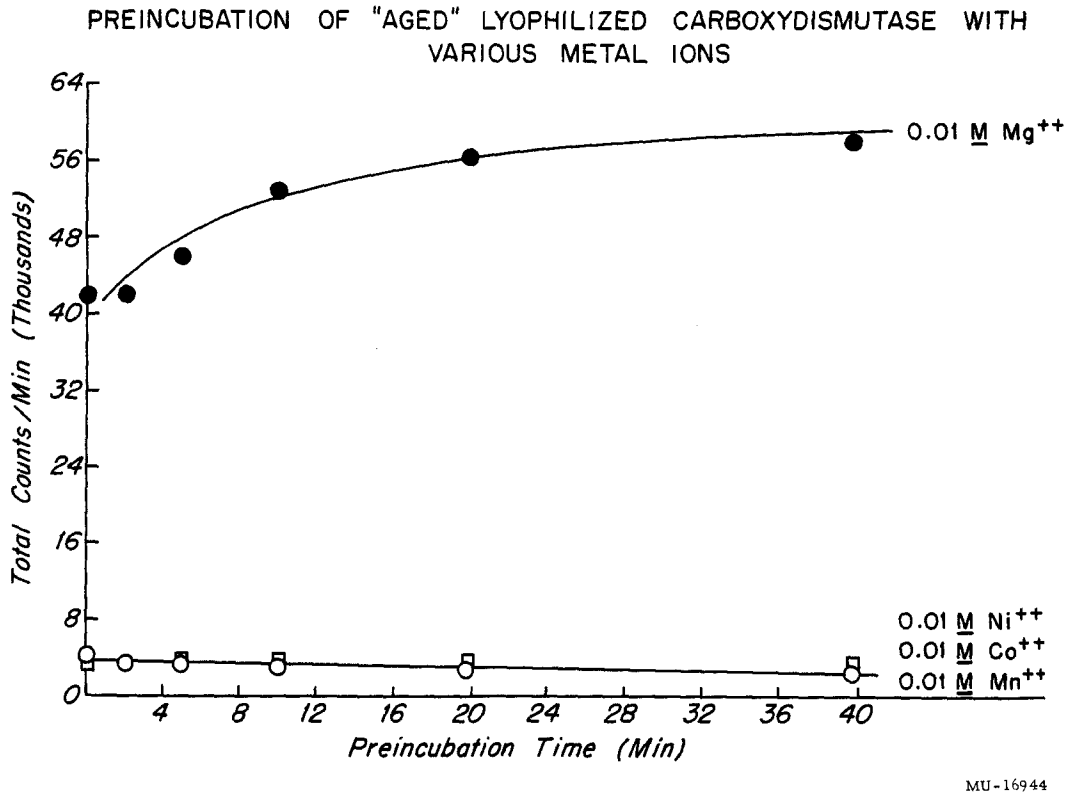

PREINCUBATION OF "AGED" LYOPHILIZED CARBOXYDISMUTASE WITH VARIOUS METAL IONS
Total Counts/Min (Thousands)
64
56
48
40
32
24
16
8
0
4
8
12
16
20
24
28
32
36
40
Preincubation Time (Min)
0.01 M Mg++
0.01 M Ni++
0.01 M Co++
0.01 M Mn++
MU-16944

Fig. 4

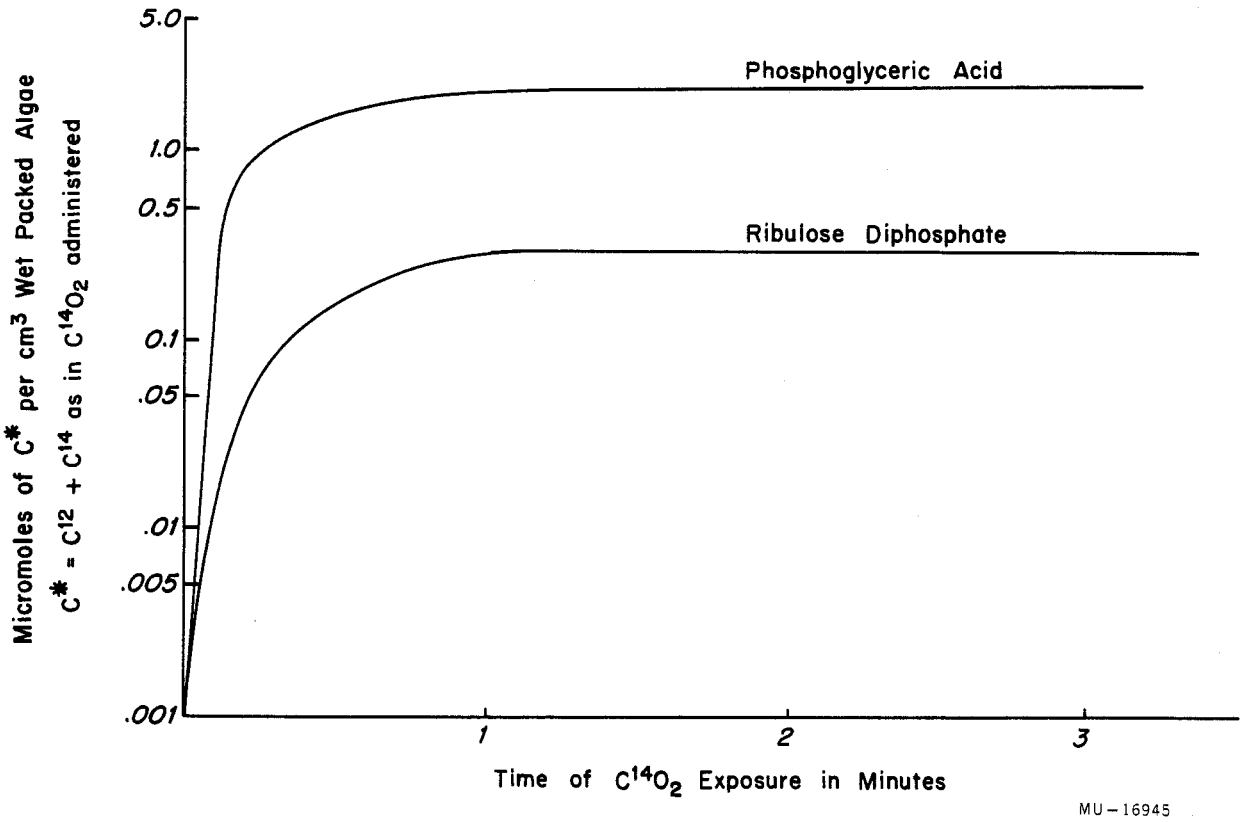

5.0
1.0
0.5
0.1
.05
.01
.005
.001
Micromoles of C* per cm3 Wet Packed Algae
C* = C12 + C14 as in C14O2 administered
Phosphoglyceric Acid
Ribulose Diphosphate
1
2
3
Time of C14O2 Exposure in Minutes
MU-16945

Fig. 5

This report was prepared as an account of Government sponsored work. Neither the United States, nor the Commission, nor any person acting on behalf of the Commission:

A. Makes any warranty or representation, expressed or implied, with respect to the accuracy, completeness, or usefulness of the information contained in this report, or that the use of any information, apparatus, method, or process disclosed in this report may not infringe privately owned rights; or

B. Assumes any liabilities with respect to the use of, or for damages resulting from the use of any information, apparatus, method, or process disclosed in this report.

As used in the above, "person acting on behalf of the Commission" includes any employee or contractor of the Commission, or employee of such contractor, to the extent that such employee or contractor of the Commission, or employee of such contractor prepares, disseminates, or provides access to, any information pursuant to his employment or contract with the Commission, or his employment with such contractor.