NASA TT F-10,143

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GPO PRICE $ _____

CFSTI PRICE(S) $ _____

N66 27500 (ACCESSION NUMBER)

(THRU) 1

37 (PAGES)

(CODE) 04

(NASA CR OR TMX OR AD NUMBER)

(CATEGORY)

FACILITY FORM 602

Hard copy (HC) 2.00

Microfiche (MF) .50

ff 653 July 65

Translation of "Biologicheskiye mekhanizmy dekarboksilirovaniya shchavelevouksusnoy kisloty"
Uspekhi Sovremennoy Biologii,
Vol. 52, No. 1 (4), pp. 3-18, 1961

NATIONAL AERONAUTICS AND SPACE ADMINISTRATION
WASHINGTON MAY 1966

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BIOLOGICAL MECHANISMS OF OXALACETIC ACID DECARBOXYLATION

N. K. Nagradova

ABSTRACT

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Biological mechanisms of oxalacetic acid decarboxylation are reviewed on the basis of data reported in the literature. The associated process of CO_2 fixation is also extensively discussed, particularly in terms of the role of malic enzyme, which plays a predominant part in the formation of dicarboxylic acids in both plant and animal tissues. The interrelationships of the various reactions of oxalacetic acid decarboxylation are presented and an overall diagram of these reactions is given. The action of the Utter-Kurahashi enzyme, phosphorylenolpyruvate carboxylase, and other carboxylases of oxalacetic acid involved in the formation of oxalacetic or its decarboxylation is discussed, and a mechanism of the decarboxylation is presented. The role of biotin in this process is also discussed, as well as the relation of oxalacetic acid conversions to the synthesis of glycogen.

Oxalacetic acid is an important intermediate in cellular metabolism. /3*

Author

* Numbers given in margin indicate pagination in original foreign text.

This compound has assumed particular interest in connection with the demonstration of the assimilation of CO_2 by heterotrophic bacteria (Wood and Werkman, 1938) and animal cells (Solomon, Vennesland, Klemperer, Buchanan, Hastings, 1941; Evans, Slotin, 1940). On the basis of a series of investigations devoted to the mechanism of this reaction, it was postulated (Krebs, Eggleston, 1940; Wood, 1946) that the incorporation of CO_2 in the organic molecule takes place via condensation with pyruvic acid (Wood-Werkman reaction)

$$CO_2 + CH_3COCOOH \rightleftharpoons COOHCH_2-CO-COOH \quad (1)$$

The first experimental proof of this reaction was the demonstration given by Krampitz and Werkman (1941) of the fact that there exists an enzyme which catalyzes the decarboxylation. They showed that acetone and lysine preparations of micrococcus lysodeikticus contained a thermolabile component capabable of catalyzing the decarboxylation of oxalacetic acid. Magnesium was necessary for the reaction; the products of OAA were CO_2 and the pyruvate, if the process took place under anaerobic conditions. The formation of oxalacetic acid from CO_2 in this system was soon shown by Krampitz et al. (Krampitz, Wood, Werkman, 1943) by means of an isotope technique. Similar results were obtained by Kalnitsky and Werkman (Kalnitsky, Werkman, 1944) with Escherichia

NOTE: The following designations were adopted in the article: OAA -- oxalacetic acid; PA--pyruvic acid; PPA--phosphopyruvic acid; PGA--phosphoglyceric acid; CIT--citric acid; MA--malic acid; FUM--fumaric acid; MAL--malonic acid; DPN--diphosphopyridine nucleotide; DPNN -- reduced form of DPN; TPN -- triphosphopyridine nucleotide; TPNN -- reduced form of TPN; ATP--adenosine triphosphate; ADP--adenosine diphosphate; ITP--inosine triphosphate ; IDP--inosine diphosphate; GTP--guanosine triphosphate; GDP -- guanosine diphosphate; P_{in} -- inorganic phosphate.

coli preparations. On the basis of indirect data it was assumed that reaction (1) also takes place in animal tissues.

Evans et al. (Evans, Slotin, Vennesland, 1942; Evans, Vennesland, Slotin, 1943) were the first to show experimentally that extracts of pigeon liver contain a thermolabile factor which decarboxylyzes OAA. However, the reverse conversion, i. e., the synthesis of oxalacetic acid from PA and CO_2, could not be achieved. When pyruvic acid was added as the reaction substrate, there was observed a rapid fixation of CO_2, which, as was shown by Wood, Vennesland and Evans (Wood, Vennesland, Evans, 1945), became localized in the carboxyl groups of fumaric, malic, pyruvic and lactic acids. The mechanism of the primary /4 fixation reaction of CO_2 in animal tissues remained obscure.

Soon thereafter, Utter and Wood (Utter and Wood, 1946) showed that the addition of ATP to pigeon liver extract causes a rapid incorporation of $C^{13}O_2$ in oxalacetic acid. The effect of ATP on the rate of formation of OAA from $C^{14}O_2$ in partially purified preparations of a carboxylase[1] from pigeon liver was confirmed by Vennesland et al. (Vennesland, Evans, Altman, 1947).

In 1947 Ochoa et al. (Ochoa, Mehler, Kornberg, 1947) reported that pigeon liver extracts contain an enzyme system which catalyzes the reversible oxidation of l-malic acid into CO_2 and pyruvate:

$$COOH-CHOH-CH_2-COOH + TPN \rightleftharpoons CH_3-CO-COOH + CO_2 + TPNH + H^+ \qquad (2)$$

The process takes place only upon addition of Mn^{++} ions; ATP does not affect the reaction rate. An interesting property of this enzyme was found to be its ability also to catalyze the decarboxylation of oxalacetic acid in the presence of Mn^{++}:

[1] Carboxylase is the name given to enzymes which catalyze both the carboxylation and the decarboxylation reaction (Ochoa, 1951).

$$COOH—CH_2—CO—COOH \rightarrow CH_3—CO—COOH + CO_2. \quad (3)$$

TPN showed a stimulating effect on this reaction. It was shown later that the reverse reaction, the formation of OAA, cannot take place with the participation of this enzyme (Veiga Salles, Harary, Banfi, Ochoa, 1950). As the enzyme became purer, the increase of its activity in the oxidation-reduction reaction (reaction (2)) was accompanied by a simultaneous increase in the activity of decarboxylation of OAA. The ratio of these two activities remained constant. Thus, the new enzyme, called "malic" enzyme, was found to be a bifunctional catalyst.

The separation of this enzyme made it possible to visualize the mechanism of fixation of CO_2 in pigeon liver preparations. The data of Wood et al. (Wood, Vennesland, Evans, 1945) on the localization of labiled CO_2 in the carboxyl groups of malic and fumaric acids became understandable. However, the studies made in Ochoa's laboratory did not provide any explanation for the pronounced effect of ATP on the rate of fixation of CO_2 in oxalacetic acid observed by other authors (Utter, Wood, 1946).

Malic Enzyme

A whole series of studies made in Ochoa's laboratory was devoted to this enzyme, which was also studied by other researchers. Following the establishment of the stoichiometry of the reaction of oxidative decarboxylation of malic acid, catalyzed by this enzyme (Ochoa, Mehler, Kornberg, 1947), a series of publications appeared which described in detail the properties of malic enzyme. The enzyme, isolated from pigeon liver and partly purified, displayed a strict specificity toward TPN, ℓ-malic and pyruvic acids. Triphosphopyridine nucleotide showed a distinct stimulating influence both on reaction (2)

and on the process of decarboxylation of OAA (reaction (3)), which was regarded as evidence of the fact that both activities belong to the same protein (Ochoa, Mehler, Kornberg, 1948).

This hypothesis received further experimental support in experiments with a highly purified enzyme(Veiga Salles, Ochoa, 1950). The two reactions /5 catalyzed by malic enzyme were found to have different optimum pH values. The reaction rate of the oxidative decarboxylation of malic acid was maximum at pH = 7.5 and dropped to zero at pH = 4.5. The decarboxylation of oxalacetic acid (reaction (3)), on the contrary, has an optimum pH of 4.5 and ceases completely in a neutral medium.

Ten years later Rutter and Lardy (Rutter, Lardy, 1958) obtained from pigeon liver highly active preparations of malic enzyme which did not contain any other enzymes as impurities. The authors confirmed the bifunctional role of this enzyme. The use of various methods of purification did not change the ratio of these two activities; they were both stimulated by the addition of TPN and Mn^{++}. It was noted that the action of TPN is highly specific. DPN was found to be completely incapable of accelerating either the reaction of oxidative decarboxylation of malic acid or the process of decarboxylation of OAA. On the basis of these data, the authors do not exclude the possibility that the occurrence of both reactions (2) and (3) is due to the same active center on the protein molecule. The results obtained in the study indicate an appreciable role of SH groups in the manifestation of the activity of malic enzyme.

Malic enzymes are widely distributed in both animal and plant tissues. In most cases, the ability of the enzyme to decarboxylate malic acid is associated with a decarboxylase activity relative to OAA as well. However, preparations of

malic enzymes from insect blood (Faulkner, 1956) and from Ascaris Lumbricoides (Saz, Hubbard, 1957) have been described, which are unable to catalyze the decarboxylation of OAA. It was also found that TPN is not required for the reaction in all cases. The adaptive "malic" enzyme from Lactobacillus arabinosus, described by Ochoa and forming lactic acid from malic acid, requires Mn^{++} and DPN for its activity (Ochoa, 1951; Korkes, del Campillo, Ochoa, 1950; Kaufman, Korkes, del Campillo, 1951).

The richest source of malic enzyme is the liver of birds (Ochoa, 1951), and also the brain of the bull (Ochoa, Veiga Salles, Ortiz, 1950). The enzyme was also found in the kidneys (Green, Loomis, Auerbach, 1948), the crystalline lens (van Heyningen, Pirie, 1953), and apparently is absent in muscles (Ochoa, 1951). Various higher plants (seeds of wheat, lentil, squash, green pea, the root of beet, carrot, parsley, garden radish, potato tuber, cucumber pulp, cabbage, parsnip, spinach leaves) contain a malic enzyme which has a carboxylase activity relative to OAA; i. e., it catalyzes reaction (3) (Conn, Vennesland, Kraemer, 1949; Vennesland, 1949). Preparations of malic enzyme from parsley root (Vennesland, Gollub, Speck, 1949) and wheat germ (Kraemer, Conn, Vennesland, 1951) were similarly studied. In both cases, the enzyme catalyzed reactions (2) and (3), which are characteristic of the enzyme from pigeon liver.

Apparently malic enzyme plays a substantial part in the process of CO_2 fixation, which results in the formation of dicarboxylic acids both in plant and animal tissues. In the presence of hydrogen donors, reaction (2) proceeds readily from right to left. The reduced TPN may form as a result of some oxidation-reduction reaction simultaneous with the action of malic enzyme. An example of such a conjugate reaction is the following system described

by Ochoa (Ochoa, Kaufman, 1951):

$$\text{Glucose-6-phosphate} + \text{PA} + CO_2 \xrightarrow{TPN, Mn^{++}} \text{6-phosphogluconic acid} + \text{MA} \qquad (4)$$

In the presence of malic enzyme from pigeon liver, glucose-6-phosphate /6 dehydrogenase, Mn^{++} and TPN, dismutation between glucose-6-phosphate and PA + CO_2 led to the accumulation of malic acid. CO_2, having incorporated in malic acid, becomes distributed in other dicarboxylic acids (oxalacetic, fumaric, succinic) as a result of reactions catalyzed by malic dehydrogenase, fumarase and succinic dehydrogenase (see diagram 1). It is possible that malic enzyme plays the part of a regulator of the level of dicarboxylic acids in the tissue (Ochoa, Veiga Salles, Ortiz, 1950).

Thus, reaction (2) is the one through which CO_2 can become incorporated in oxalacetic acid. Considering that the process of decarboxylation of OAA, catalyzed by malic enzyme, is irreversible, Ochoa and coworkers picture the occurrence of the Wood-Werkman reaction in tissues containing malic enzyme only in an indirect manner, via the formation of malic acid. They showed

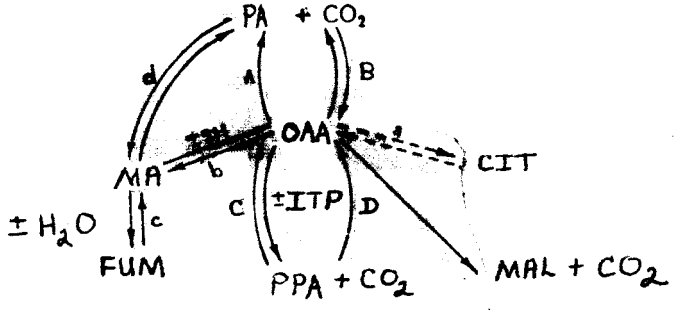


Diagram 1. Interrelationship of various reactions of oxalacetic acid decarboxylation: A, irreversible decarboxylation of OAA (reaction 3); B, reversible decarboxylation of OAA (reaction 1); C, reaction catalyzed by Utter-Kurahashi enzyme (reaction 7); D, reaction catalyzed by phosphorylenolpyruvate carboxylase (reaction 8); a, condensing enzyme; b, malic dehydrase; c, fumarase; d, malic enzyme.

experimentally that the incorporation of labeled CO_2 in oxalacetic acid occurs with the participation of malic enzyme via the formation of malic acid labeled at the carboxyl group, this being followed by its oxidation, catalyzed by malic dehydrogenase:

$$CH_3\text{-}CO\text{-}COOH + \dot{C}O_2 + TPNN + H^+ \underset{\text{"malic" enzyme}}{\overset{Mn^{++}}{\rightleftharpoons}}$$

$$\rightleftharpoons \dot{C}OOH\text{-}CH_2\text{-}CHOH\text{-}COOH + TPN \qquad (5a)$$

$$\dot{C}OOH\text{-}CH_2\text{-}CHOH\text{-}COOH + TPN \underset{\text{malic dehydrogenase}}{\rightleftharpoons} \dot{C}OOH\text{-}CH_2\text{-}CO\text{-}\underset{1}{C}OOH + TPNN + H^+ \qquad (5b)$$

As is evident from the overall equation

$$CH_3\text{-}CO\text{-}COOH + \dot{C}O_2 \overset{Mn^{++},TPN}{\rightleftharpoons} \dot{C}OOH\text{-}CH_2\text{-}CO\text{-}COOH, \qquad (5c)$$

malic acid acts catalytically, and the reaction described constitutes a reversible decarboxylation of OAA, or the Wood-Werkman reaction (Veiga Salles, Harary, Banfi, Ochoa, 1950). This mechanism of CO_2 fixation by prepa- /7
rations of pigeon liver was supported by the following experimental data: in the presence of "malic" enzyme alone, an insignificant inclusion of isotopic CO_2 in OAA was observed; it increased upon addition of malic dehydrogenase. If no malic acid was added to the reaction mixture, the CO_2 fixation did not occur.

In view of the data of Utter (Utter, Wood, 1946) on the influence of ATP on the reaction of condensation of CO_2 with pyruvic acid, the authors checked the action of this nucleotide on their system. They found that the addition of ATP did not affect the rate of inclusion of CO_2 in OAA, and that the addition of malic acid played a decisive part. However, in unpurified prepa-

1. As was shown by Mehler et al. (Mehler, Kormber, Grisolia, Ochoa, 1948), malic dehydrogenase can react not only with DPN, but also with TPN.

rations from pigeon liver, ATP was found capable of replacing malic acid. Ochoa and coworkers attribute to ATP only an indirect role, which amounts either to an acceleration of the synthesis of malic acid or to its activation (Veiga Salles, Harary, Banfi, Ochoa, 1950).

Having studied this problem in detail, Utter (1951) came to the conclusion that the action of ATP, which accelerates the inclusion of CO_2 in oxalacetic acid in pigeon liver preparations, is the result of its direct participation in the carboxylase reaction, and that malic acid cannot be considered a precursor of OAA. Pigeon liver extracts contain two enzymes which act independently: malic enzyme, which requires the presence of TPN for its activity, and OAA carboxylase, for which adenosine triphosphate is necessary. In Utter's experiments it was found possible, by adding one of these compounds to the liver extracts, to cause the inclusion of $C^{14}O_2$ in malic or oxalacetic acid. When ATP was added, an intensified formation of $C^{14}OOH\text{-}CH_2\text{-}CO\text{-}COOH$ took place. The decomposition of TPN, which was present in the extract, also caused the accumulation of OAA. Discussing the mechanism of the reactions in both cases, Utter suggests two possibilities: (1) the enzymatic preparations from pigeon liver catalyze two independent series of reactions which lead to formation of oxalacetic and malic acid; (2) both series of reactions have a common link, the hypothetical precursor of OAA and malic acid (Utter, Wood, 1951).

$$CO_2 + C_3\text{-compound} \qquad (6)$$

precursor

ATP TPN

OAA malic acid

The Utter-Kurahashi Enzyme

The subsequent substantial step forward was the discovery by Utter and Kurahashi (Utter, Kurahashi, 1953) that the decarboxylation of oxalacetic acid by purified liver extracts of chicks can be completely separated from the action of malic enzyme and requires the presence of ATP and ITP. The enzyme, active at pH 5.8 - 7.8, catalyzes the reversible reaction

$$\text{OAA+ATP (or ITP)} \quad \text{PPA+ADP (or IDP)+}CO_2 \qquad (7)$$

It was found that the product of the decarboxylation reaction of OAA is phosphoenolpyruvic acid, which was identified by chromatographic and enzymatic methods. In the presence of adenosine diphosphate, CO_2 and phosphoenolpyruvate readily entered into the reaction leading to the synthesis of OAA.

Proof of the fact that phosphoenolpyruvic acid is the product of an enzymatic version of OAA was extremely interesting in connection with the interpretation of Kalckar's data (Kalckar, 1939) and also those of Leloir and Muñoz /8 (Leloir, Muñoz, 1944), who observed the formation of phosphoenolpyruvic acid during incubation of enzymatic liver and kidney preparations with various di- and tricarboxylic acids (malic, fumaric, succinic, citric). Similar results were obtained by Bartley and Avi-Dor (Bartley, Avi-Dor, 1955), who showed that whereas in an oxygen atmosphere a whole series of di- and tricarboxylic acids form phosphoenolpyruvate, under anaerobic conditions the source of this compound is only oxalacetic acid. The results of these studies can be readily explained by considering the reaction catalyzed by the Utter-Kurahashi enzyme.

Subsequent studies of these authors led to a thorough purification of the enzyme and a detailed study of its properties. Although the presence of OAA carboxylase, which requires a nucleotide for its activity, was first shown in

pigeon liver (Utter, Wood, 1946), a more convenient source of this enzyme was the liver of chicks (Utter, Kurahashi, 1954). This tissue also contains active malic enzyme, which, when appropriate purification methods are employed, can be completely separated from the protein catalyzing the decarboxylation of oxal - acetic acid with the formation of phosphoenolpyruvate.

The reversibility of this reaction was demonstrated by two kinds of experiments: in one series of experiments, the incorporation of $C^{14}O_2$ in OAA was studied (Utter, Kurahashi, 1954a), and in the other, the decarboxylation of oxalacetic acid (Utter, Kurahashi, Rose, 1954) was investigated. It was found that the enzyme isolated from the liver of chicks catalyzes both reactions in the presence of a nucleotide. In contrast to $C^{14}O_2$, the labeled pyruvate does not become incorporated in oxalacetic acid; only phosphoenolpyruvate was a participant in the reaction. This also showed that the action of OAA carboxylase is independent of the "malic" enzyme, which forms pyruvic acid as the reaction product. The enzyme has an optimum pH at 6.0 and contains SH groups, which are important for the activity. An interesting property of the carboxylase described, which distinguishes it from many analogous enzymes,is the fact that it can manifest its activity without the addition of metal ions.

The mechanism of reaction (7) was studied in experiments with a purified enzyme obtained from acetone powders of chick livers (Utter, Kurahashi, 1954b). Upon addition of pyruvic acid to the enzymatic preparation of phosphoenolpyruvic acid, IDP and $NaHCO_3$, it was possible to observe the synthesis of oxalacetic acid. The reaction rate increased if the ITP formed at the same time was removed (by adding hexokinase and glucose) and also OAA (by adding malic dehydrase and DPNN). Even in their early publications (Utter, Kurahashi, 1953; Utter, Kurahashi, 1954a), the authors pointed out that ATP, which is required

for the reaction, can be replaced with ITP. Subsequently it was found that the latter possesses a high activity and that adenosine triphosphate apparently acts indirectly (Utter, Kurahashi, Rose, 1954). Since even highly purified preparations of the Utter-Kurahashi enzyme from acetone powders of the liver were not free of nucleoside diphosphate kinase (Krebs, Hems, 1953; Berg, Joklik, 1953), it was possible to assume that ATP transfers its phosphate residue to IDP and thus (via ITP) influences the reaction.

Indeed, Bandurski and Lipmann at first (Bandurski, Lipmann, 1956), then Kurahashi et al. (Kurahashi, Pennington, Utter, 1957), having obtained preparations of the nucleotide-specific OAA carboxylase from liver mitochondria, completely free from nucleoside diphosphate kinase, showed that: (1) crystalline ATP is completely inactive in reaction (7), whereas amorphous, chromatographically pure ATP always contains about 5 percent of ITP impurity or another nucleotide and can therefore have a partial effect; (2) only inosine and guanosine polyphosphates can participate in the reaction, whereas derivatives /9 of adenosine, cytosine, uridine and xanthosine are inactive. Low concentrations of guanosine derivatives (GTP and GDP) are considerably more effective than derivatives of inosine taken in the same quantities, but as the concentration rises, the effect of ITP increases faster than that of GTP, and at high concentrations of nucleotides ITP becomes more effective.

In addition to the liver of birds, the Utter-Kurahashi enzyme was isolated from the liver mitochondria of lambs and from rabbit kidneys; pig and rabbit liver are poor sources of this carboxylase (Bandurski, Lipmann, 1956). Extracts from Neurospora Crassa also possess OAA-carboxylase activity associated with ATP (Strauss, 1957). Tchen and Vennesland (Tchen, Vennesland, 1954) isolated this enzyme from higher plants.

Phosphorylenolpyruvate Carboxylase

In 1953, Bandurski and Greiner (Bandurski, Greiner, Bonner, 1953; Bandurski, Greiner, 1953) isolated from acetone powder of spinach leaves an enzyme catalyzing the carboxylation of phosphoenolpyruvic acid with the formation of OAA and inorganic phosphate:

$$PPA + CO_2 \quad OAA + H_3PO_4. \tag{8}$$

In the initial studies, the incorporation of CO_2 was investigated by using an isotopic technique, but the use of thoroughly purified preparations of the enzyme (Bandurski, 1955) made it possible to achieve the accumulation of an appreciable amount of oxalacetic acid as a result of reaction (8) and to determine it by chemical methods. The reaction product was identified by separating crystalline 2,4 dinitrophenylhydrazone of OAA. The enzyme had an optimum activity in the pH range of 7.5 - 9.5 and required the addition of Mg^{++} and Mn^{++} ions for its action. Glutathione and cysteine also had an activating effect.

Since the enzyme isolated from spinach leaves was incapable of catalyzing the Utter-Kurahashi reaction, and the addition of nucleotides (ATP and ITP) not only was not necessary but had a depressing effect, it must be admitted that we are dealing here with a representative of a special group of carboxylases which Bandurski referred to (Bandurski, 1955) as phosphorylenolpyruvate carboxylases, in contrast to OAA carboxylases, which require the presence of nucleotides for the manifestation of their activity. A characteristic feature of this enzyme is also the fact that the reaction which it catalyzes is irreversible; this in all probability permits a rapid synthesis of dicarboxylic acids in plant tissue (Bandurski, 1955). The enzyme catalyzing

reaction (8) was also isolated from wheat germ (Tchen, Vennesland, 1955). However, unpurified enzymatic preparations from this source also had the ability to effect the process of reversible decarboxylation of oxalacetic acid (the Utter-Kurahashi reaction). Treatment of the material with manganese chloride led to a substantial inactivation of the nucleotide-specific carboxylase without affecting phosphorylenolpyruvate carboxylase. Dialysis of the enzymatic preparation against a collidine buffer, however, caused a complete inactivation of the enzyme catalyzing reaction (8). These observations provide additional evidence of the independent action of the two groups of carboxylases.

In the view of Tchen and Vennesland, the presence in plant tissue of an enzyme catalyzing the formation of oxalacetic acid from phosphoenolpyruvate and CO_2 is of great biological importance. Because of the irreversibility of the reaction, the synthesis of the dicarboxylic acid may take place at a very low vapor pressure of CO_2, while the achievement of this process /10 with the participation of the Utter-Kurahashi enzyme requires fairly high concentrations of the starting reactant. "It is known that some plants accumulate organic acids (mainly, malic acid) during the night. Such accumulation proceeds at a very low CO_2 content. The formation of malic acid in the dark may be regarded as a measure aimed at preserving the CO_2 arising from respiration, and the irreversible synthesis of OAA from phosphoenolpyruvate can serve as a very efficient trap for CO_2." (Tchen, Vennesland, 1955)

Other Carboxylases of Oxalacetic Acid

The OAA carboxylase from Micrococcus lysodeikticus was first described by Krampitz and Werkman (Krampitz, Werkman, 1941). As was shown by these authors, it catalyzes the decarboxylation of oxalacetic acid, and also the

condensation of $C^{13}O_2$ with pyruvic acid (reaction 1) (Krampitz, Wood, Werkman, 1943). Highly purified preparations of this enzyme, obtained by Herbert (Herbert, 1950) displayed their activity only in the presence of metal ions. An activating effect was shown by Mn^{++}, Mg^{++}, Cd^{++}, Co^{++} and Ni^{++}. McManus (McManus, 1951) thoroughly studied the effect of this enzyme, because after the description of malic enzyme the following question arose: Does Micrococcus lysodeikticus actually possess an OAA carboxylase? Are not the oxalacetic acid decarboxylation and CO_2 fixation the results of a manifestation of the activity of malic enzyme? It was observed that the process of incorporation of CO_2 in OAA in this microorganism occurs without the participation of malic enzyme, by means of a direct reaction with pyruvic acid. In contrast to the data of Utter and Wood (Utter, Wood, 1946), obtained with preparations from pigeon liver, the addition of ATP had no effect on the rate of CO_2 fixation. Apparently, Micro - coccus lysodeikticus contains an OAA carboxylase whose activity is independent of the nucleotide.

Similar properties are also displayed by an enzyme isolated from Azobacter Vinelandii (Plaut, Lardy, 1949). It catalyzes the irreversible reaction of OAA decarboxylation which takes place without the participation of nucleotides. Metal ions (Mn^{++}, Co^{++}, Zn^{++}) have a pronounced activating effect. The same group of carboxylases should apparently include an enzyme from rat liver mitochondria recently described by Corvin (Corvin, 1959), which also catalyzes the irreversible decarboxylation of oxalacetic acid with the formation of CO_2 and pyruvate. Magnesium and glutathione activate the enzyme; the addition of nucleotides (ATP, ITP, GTP) does not accelerate the reaction. The decarboxylating activity of the preparation should not be attributed to the effect of malic enzyme, since the latter is completely inactive at pH 7.5,

which is the optimum for the reaction observed. The author notes that this enzyme insures most of the activity of OAA decarboxylase in rat liver mitochondria, since this tissue apparently lacks other carboxylases.

We should also mention still another type of conversion of oxalacetic acid, described by Vennesland et al. (Vennesland, Evans, 1944). In the presence of manganese ions, dialyzed aqueous extracts of pig heart catalyzed the decarboxylation of OAA, which was associated with the absorption of molecular oxygen. The reaction proceeded in accordance with the following equation:

$$COOH\text{-}CH_2\text{-}CO\text{-}COOH + 1/2\,O_2 \quad COOH\text{-}CH_2COOH + CO_2. \qquad (9)$$

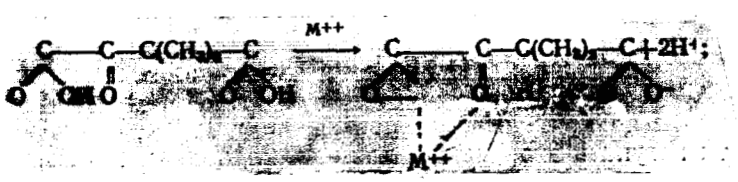
The product of the oxidative decarboxylation, malonic acid, was /11
isolated in the form of di-p-nitrobenzyl malonate. Further research (Vennesland, Evans, Francis, 1946) showed that the factor catalyzing reaction (9) is metmyoglobin. This substance, isolated from horse heart and recrystallized four times, displayed an activity equal to that of freshly dialyzed aqueous extracts of heart muscle. Hemoglobin and cytochrome C were unable to replace metmyoglobin in the decarboxylation reaction. Diagram 1 illustrates the possible pathways of the enzymatic decarboxylation of oxalacetic acid.

Mechanism of Decarboxylation of Oxalacetic Acid

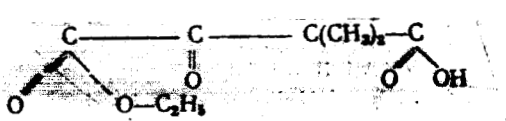
Like other β-keto acids, oxalacetic acid is unstable in aqueous solutions and undergoes a spontaneous decarboxylation to form CO_2 and pyruvic acid. The rate of this reaction depends on the pH; it is maximum at pH 4.0 and negligible in strong acids and alkalies (Krebs, 1942). The decarboxylation of OAA accelerates substantially in the presence of aniline (Ostern, 1933), amino acids and amides (Bessman, Layne, 1950). The most active were found to be histidine, lysine and glycine. The stimulation of nonenzymatic decarboxylation

by metal ions was first described by Krampitz (Krampitz, Werkman, 1941). Krebs studied this phenomenon more fundamentally and found (Krebs, 1942) that the greatest activity is manifested by Co^{++}, Zn^{++}, Cu^{++}, Fe^{++}, Fe^{+++}, and that Al^{+++}, Ca^{++}, Ba^{++}, Mg^{++} and Mn^{++}, taken in the same concentrations, are much less effective. As indicated above, some of these ions also catalyze the enzymatic decarboxylation of oxalacetic acid. This effect of metal ions is due to their ability to form labile complexes with keto acids or their enol forms (Ochoa, 1951), which undergo a rapid decarboxylation. In the case of the enzymatic process, a complex is presumably formed between protein, OAA and the metal ion.

The catalysis of spontaneous decarboxylation of oxalacetic acid and its derivatives by metal ions serves as a model system for understanding the mechanism of the enzymatic process. Steinberger and Westheimer (Steinberger, Westheimer, 1951) studied the decarboxylation of α,α-dimethyl-OAA and its monoethyl ester, catalyzed by metal ions, and found that Cu^{++} and Al^{+++} substantially accelerate the decarboxylation of the first compound without affecting conversions of the second. These observations support the authors' mechanism of formation of a complex of the metal ion with the carboxyl group at the α position relative to the carbonyl group (reaction 10a).



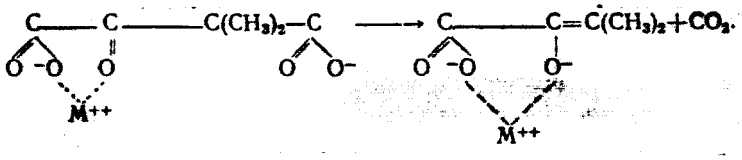
α,α - dimethyl-OAA



monoethyl ester α,α -dimethyl-OAA. M^{++}-metal ion.

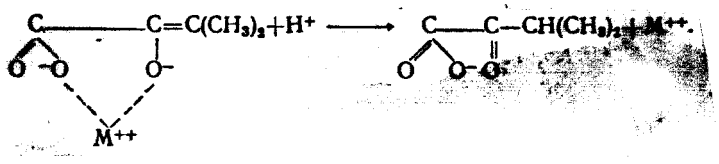
In the case of the monoethyl derivative, catalysis by metal ions /12 does not take place, because the α carboxyl group is tied up. Such a reaction mechanism has also been adopted by other investigators (Utter, Kurahashi, Rose, 1954; Ochoa, 1951), who are inclined to extend it to enzymatic decarboxylation as well.

According to the view developed by Westheimer (Seltzer, Hamilton, Westheimer, 1959), the decarboxylation of the metal-dimethyl-OAA complex is accomplished as follows:



(10b)

The enol compound formed adds a proton and converts into the keto form (dimethyl-PAO):



(10c)

On the basis of a series of experiments, Westheimer and coworkers came to the conclusion that the catalytic action of metal ions extends to reaction 10b), which is the decarboxylation proper (rupture of carbon chain); the effect of the enzyme, however, is apparently directed toward accelerating reaction (10c), which represents the conversion of the enol form of the pyruvate into its keto form.

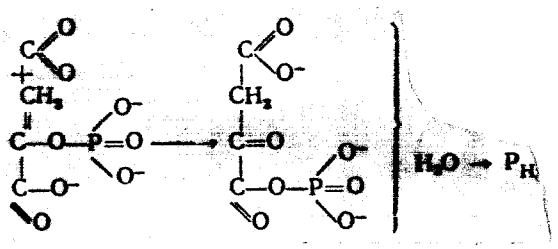
The problem of the primary reaction product arising from the conversion of oxalacetic acid into phosphoenolpyruvate has been extensively discussed by various authors. Lipmann (Lipmann, 1941) advanced the view that the precursor of phosphoenolpyruvate is phosphoenoloxalacetic acid, which undergoes decarboxylation. The formation of the phosphorylated enol form of OAA was also advanced

by Bandurski (Bandurski, Greiner, 1953) to explain the reaction mechanism of the synthesis of oxalacetic acid from phosphoenolpyruvate and CO_2 by enzymatic preparations from spinach leaves.

Although no one has been able to isolate this hypothetical product, and the schemes of its formation have been purely theoretical, evidence has gradually accumulated to support the existence of some labile compound of OAA containing phosphorus. A series of experiments have shown that if the decarboxylation of oxalacetic acid labeled at the carboxyl group under the influence of pigeon liver enzymes is studied in the presence of ATP, then the OAA separated from the reaction mixture at various time intervals turns out to be nonuniform in its specific activity (Utter,1951). Under these conditions it was possible to distinguish at least two OAA fractions which differed in their ability to enter into the decarboxylation reaction. The authors postulate that the more active part of the oxalacetic acid molecule is the enol form (Utter, Wood, 1951).

However, along with the studies supporting this view (Kornberg, Ochoa, Mehler, 1948) investigations appeared whose results indicated a greater probability of enzymatic transformations of the keto form of oxalacetic acid without its preliminary enolization. This applies primarily to the data of /13
Steinberger and Westheimer (Steinberger, Westheimer, 1951); the dimethyl derivative of OAA which they synthesized cannot convert into the enol form and yet it undergoes decarboxylation catalyzed by metal ions. Similar results were obtained by Vennesland and coworkers, who studied the mechanism of enzymatic synthesis of oxalacetic acid from phosphoenolpyruvate and CO_2 by preparations of carboxylases from plant tissue (Vennesland, Tchen, Loewus, 1954). Experiments with the use of deuterium showed that as a result of the addition of CO_2

to phosphoenolpyruvate, the keto form of oxalacetic acid is formed. In dis - cussing the report of Utter (Utter, 1951) concerning the nonuniformity of OAA formed in the course of the enzymatic reaction, the authors (Tchen, Loewus, Vennesland, 1955) postulate that the fraction possessing the highest specific activity could have been a phosphorus derivative of oxalacetic acid. However, since the latter participates in the reaction in the keto form, the phosphate is apparently linked to one of the carboxyl groups:



(11)

In the reaction catalyzed by phosphorylenolpyruvate carboxylase (Tchen, Loewus, Vennesland, 1955), the phosphate-enol bond of phosphoenolpyruvate splits simultaneously with the fission of CO_2. At the same time, the phosphate group may either immediately detach itself hydrolytically to form inorganic phosphate, or be carried to one of the two carboxyl groups of oxalacetic acid. The phosphorylated OAA formed undergoes two kinds of conversions: (1) it splits hydrolytically (this takes place under the influence of phosphorylenol-pyruvate carboxylase) and (2) it reacts with the phosphate acceptor (with the participation of the Utter-Kurahashi enzyme). In experiments with this enzyme, isolated from bird liver, Vennesland, Utter et al. (Graves, Vennesland, Utter, Pennington, 1956) also showed that the keto form of oxalacetic acid is metabolically active. The phosphorus derivative of OAA can thus be regarded as a common intermediate products of the two carboxylation reactions, the reversible and the irreversible one. It should be noted that there is no direct experimen-

tal evidence for the existence of a phosphorus derivative of oxalacetic acid; the authors merely contend that if such a compound is formed, the phosphate should be linked to one of the carboxyl groups of the dicarboxylic acid (Graves, Vennesland, Utter, Pennington, 1956).

Role of Biotin

The relation of biotin to the process of oxalacetic acid decarboxylation and CO_2 fixation was noted a long time ago. The growing of certain microorganisms on a medium poor in biotin caused an impairment of the process of condensation of CO_2 and pyruvate (Lardy, Potter, Burris, 1949; Lardy, Peanasky, 1953) the decarboxylase activity of Escherichia coli, directed toward OAA and lost after standing in acid medium (pH 4), was restored after the addition of biotin (Lichstein, Umbreit, 1947); in the liver of turkeys deprived of biotin, /14 the activity of malic enzyme decreased sharply (Ochoa, Mehler, Blanchard, Jokes, Hoffman, Regan, 1947). However, attempts to detect biotin in the composition of purified enzymes have thus far been unsuccessful (Ochoa, Mehler, Blanchard, Jokes, Hoffman, Regan, 1947; Plaut, Lardy, 1949; Vennesland, Gollub, Speck, 1949), which prompted Ochoa to advance a hypothesis on the role of this vitamin in the synthesis of enzymes catalyzing the decarboxylation, i. e., a hypothesis of its indirect influence (Ochoa, 1951). A different point of view is held by Lichstein (Lichstein, 1951), who believes that biotin can act as the coenzyme of the decarboxylation reaction. In 1955 he was able to show the presence of bound biotin in purified preparations of Utter-Kurahashi enzyme obtained from bird liver (Lichstein, 1955). Bandurski and Lipmann (Bandurski, Lipmann, 1956) observed that as the OAA carboxylase from lamb liver mitochondria was purified, the content of biotin bound to the enzyme increased. These data support the direct participation of biotin in the enzymatic reaction.

Relation of Conversions of Oxalacetic Acid to the Synthesis of Glycogen

The decarboxylation of oxalacetic acid, during which phosphoenolpyruvate accumulates, is an important link in the chain of reactions leading to the formation of this compound from various di- and tri-carboxylic acids which participate in the Krebs cycle. The aerobic formation of phosphoenolpyruvic acid by enzyme systems of the kidneys was described in 1939 by Kalckar (Kalckar, 1939), and later by several other authors (Leloir, Munoz, 1944; Bartley, 1954). A study of this phenomenon performed with purer preparations ("cyclophorase" from the kidneys and liver) Bartley, Avidor, 1955) and also with liver mitochondria (Bandurski, Lipmann, 1956) showed that whereas in the presence of oxygen, phosphoenolpyruvate is formed from citric, succinic, malic, and fumaric acids, under anaerobic conditions its accumulation takes place only when it is formed from OAA. Hence, this dicarboxylic acid is a direct source of phosphoenolpyruvate, whereas the other participants of the citric acid cycle act indirectly, after being converted into oxalacetic acid.

The aerobic formation of phosphoenolpyruvic acid in the liver is of considerable interest in connection with the problem of synthesis of glycogen from pyruvic acid. This process is accomplished by the reversal of glycolysis reactions, but the phosphorylation of pyruvate in the phosphoenolpyruvate kinase reaction

$$PA + ATP \rightleftarrows PPA + ADP \qquad (12)$$

although demonstrated (Lardy, Ziegler, 1945), takes place very slowly in liver tissue (Hiatt, Goldstein, Lareau, Horecker, 1958) or is completely lacking (Bandurski, Lipmann, 1956). Experiments by Lorber et al. (Lorber, Lifson, Wood, Sakami,

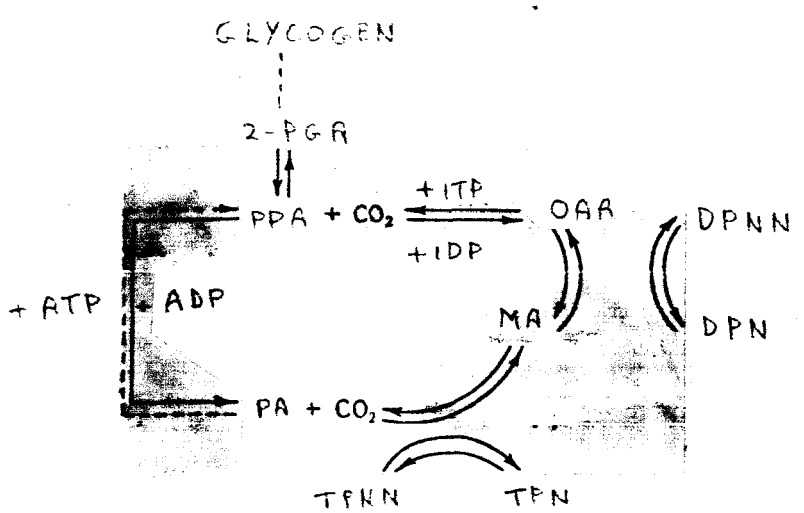


Diagram 2. Sequence of reactions leading to the synthesis of glycogen from pyruvic acid in the liver.

Shreeve, 1950) established that less than 1/6 of the labeled lactate fed to rats was converted into liver glycogen by direct reversal of the glycolysis reactions; its major portion first entered into the Krebs cycle. Similar data were obtained from a study of the synthesis of glycogen from rabbit liver sections in the presence of labeled pyruvate (Topper, Hastings, 1949). Thus, the conversions of oxalacetic acid turn out to be closely related to the main pathway of glycogen synthesis in the liver. Krebs (Krebs, 1954) discussed the possible mechanisms of this process and proposed the following system of reactions.[1]

Under the influence of malic enzyme, pyruvic acid carboxylates into malic acid, which oxidizes into OAA (with the participation of malic dehydrogenase). The nucleotide-specific carboxylase of oxalacetic acid catalyzes the formation of phosphoenolpyruvate, whose further conversions lead to the synthesis of a carbohydrate.

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[1]The system is given with certain changes.

Such an indirect path of formation of phosphoenolpyruvic acid is not more convenient energetically than the direct phosphorylation of pyruvate in reaction (12). However, thanks to the presence of several conjugate reactions promoting the removal of intermediate products, the equilibrium of the overall process is substantially shifted toward the accumulation of phosphoenolpyruvic acid. An example of such conjugate exergonic reactions is the dehydrogenation of glucose-6-phosphate and phosphogluconic acid, which supply large quantities of TPNN for the action of malic enzyme, or the oxidation of DPNN, which shifts the equilibrium of the malic dehydrogenase reaction (Krebs, 1954). Kreb's point of view concerning the mechanism of glycogen synthesis in the liver is shared by other authors (Weigman, Strisower, Chaikoff, 1957; Utter, Kurahashi, 1954b). The existence of a similar process is also postulated in plants (Tchen, Vennesland, 1955).

However, muscle tissue is characterized by a different type of conversion. As was shown by experiments on the inclusion of labeled pyruvate in the glycogen of rat diaphragm (Hiatt, Goldstein, Lareau, Horcker, 1958), in this case the process takes place without the participation of dicarboxylic acids: a direct phosphorelation of pyruvate takes place (reaction 12). Results obtained by Krimsky (Krimsky, 1959), who used purified enzymatic glycolysis systems in studying the conditions necessary for reversing the phosphoenolpyruvate kinase reaction in muscle tissue, showed that when a high ATP level is maintained, a direct phosphorylation of pyruvic acid becomes entirely possible. At the present time, the existence of two paths of glycogen synthesis from pyruvate should apparently be accepted: a direct path taking place in muscle tissue, and an indirect one, requiring the participation of decarboxylic acids and characteristic of the liver.

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