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Institution: Case Western Reserve University, Cleveland, Ohio 44106

Department: Biochemistry

Title: A Study of Intermediary Metabolism with Isotopically Labeled Compounds

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OBJECTIVES

The objectives are to study the intermediary metabolism of carbohydrates and to determine the mechanism of these reactions using labeled compounds and purified enzymes. The role of coenzymes, metals, the properties and structures of the active sites of the enzymes are being investigated as a means of understanding catalysis and the control of metabolism. The emphasis is on the mechanisms of the utilization of carbon dioxide, the intermediate compounds involved in these utilizations and the detailed chemistry of the reactions.

MAIN RESEARCH ACCOMPLISHMENTS

I. Mechanism of the Carboxytransphosphorylase Reaction (Publications 7, 11, 12, 13)

In our last comprehensive report we described the discovery and isolation of an enzyme called carboxytransphosphorylase which occurs in propionibacteria and catalyzes Reactions 1 and 2. Reaction 1 occurs in the presence of CO_2 and we call it the oxalacetate reaction:

$$CO_2 + \text{P-enolpyruvate} + P_i \rightleftarrows \text{oxalacetate} + PP_i \qquad (1)$$

Reaction 2 occurs in the absence of CO_2. It is irreversible and we call it the pyruvate reaction:

$$\text{P-enolpyruvate} + P_i \rightleftarrows \text{pyruvate} + PP_i \qquad (2)$$

Reaction 1 resembles Reaction 3 which is catalyzes by P-enolpyruvate carboxykinase and in animals involves IDP or GDP.

$$CO_2 + \text{P-enolpyruvate} + \text{IDP (or GDP)} \rightleftarrows \text{oxalacetate} + \text{ITP (or GTP)} \qquad (3)$$

Here IDP or GDP is the phosphate acceptor rather than P_i.

DISCLAIMER

Reaction 2 resembles the reaction catalyzed by pyruvate kinase.

P-enolpyruvate + ADP → pyruvate + ATP (4)

Utter and coworkers have shown that the physiological role of carboxykinase in animal tissue is as a decarboxylase (Reaction 3 from right to left) in which it catalyzes the synthesis of P-enolpyruvate from oxalacetate and involves the utilization of one ITP. Although carboxytransphosphorylase can readily catalyze a similar synthesis of P-enolpyruvate using PP_i, (Wood et. al., J. Biol. Chem. 241, 5692 (1966)) this does not appear to be its normal role. Instead it functions as a carboxylase to supply oxalacetate by CO_2 fixation. This synthesis is accomplished in animals by pyruvate carboxylase. The propionic acid bacteria do not contain pyruvate carboxylase but do contain an enzyme called pyruvate phosphate dikinase which catalyzes the formation of P-enolpyruvate directly from pyruvate. This reaction will be duscussed in a later section.

The role of the pyruvate reaction (Reaction 2), if any, is uncertain. There are ample amounts of pyruvate kinase in propionic acid bacteria and it thus is doubtful if carboxytransphosphorylase replaces the function of this kinase in bacteria.

The studies of the last three years have dealt with the mechanism of the carboxytransphosphorylase reaction and the role of metals in the catalysis.

A. Requirement for Two Types of Metals, Type I and Type II. Carboxytransphosphorylase requires two types of metals. The Type I metal is divalent freely dissociable, with a K_m of about 10^{-3} M and its requirement is met by Mg^{2+}, Co^{2+} or Mn^{2+}. The Type II metal is bound to the enzyme. Its requirement is made evident by the use of chelators in the presence of Mg^{2+} (12 mM) as the Type I metal. Under these conditions the catalysis of both the oxalacetate and pyruvate reactions is inhibited by a wide variety of structurally unrelated metal chelators, in particular EDTA, O-phenanthroline, α, α'-dipyridyl, Tiron, diethyldithiocarbamate, 8 hydroxyguinoline, sodium cyanate and oxalate. Orthophenanthroline and α, α'-dipyridyl have very little affinity for Mg^{2+} and it is not likely that they could inhibit by complexing Mg^{2+}. In addition several of the inhibitors are effective at 10^{-5} M or less whereas the Mg^{2+} concentration is 10^{-2} M. In view of the activity by such a wide variety of metal chelators it seems quite certain that the inhibition is due to binding of a second metal (Type II) and not to a non-specific effect of the chelators. Metaphenanthroline which is not a chelator has no effect on the reaction.

B. Reversal of the Inhibition by EDTA with Divalent Metals. The inhibition by EDTA is readily reversed if Co^{2+} is added in excess of the EDTA. Mn^{2+}, Ni^{2+} and Zn^{2+} also are effective but less so than Co^{2+}. It appeared possible that the EDTA removed the bound metal yielding an apoenzyme which was reactivated by the Co^{2+} and other metals. The enzyme therefore was treated with 10^{-3} M EDTA for 24 hours and then the EDTA was removed by dialysis. This preparation of the enzyme is completely inactive in the presence of Mg^{2+} (12 mM) but addition of 10^{-5} M Co^{2+} completely reactivates the enzyme for the oxalacetate reaction. Mn^{2+}, Zn^{2+} and Ni^{2+} were less effective. It thus appeared that the Type II metal had been removed and an apoenzyme had been formed which was activated by the metals. Experiments were therefore undertaken with $^{60}Co^{2+}$ to verify that Co^{2+} was bound to the enzyme when it was reactivated. However, it was found that the EDTA-treated-dialyzed-enzyme did not bind $^{60}Co^{2+}$ even though the enzyme was reactivated by the Co^{2+}.

Clearly we were not dealing with an apoenzyme which was reactivated by binding Co^{2+}. Further study indicated that some of the EDTA remained firmly bound to the enzyme and that it was not removed by the dialysis of 10 to 12 hours. Prolonged dialyses inactivated the enzyme completely, but passage of the enzyme through a Sephadex column removed the EDTA and the enzyme was now active with Mg^{2+} alone and without addition of Co^{2+}. It appears that EDTA is bound to the Type II metal and is not readily removed by dialysis and that the Co^{2+} reactivates the enzyme by complexing the residual EDTA thus removing it from the Type II metal.

Although these studies did not yield an apoenzyme they did provide an enzyme in which the Type II metal was blocked and thus the function of the Type I metal could be studied independent of the Type II metal, as will be discussed later.

Thus far attempts to identify the Type II metal by emission spectroscopy and atomic absorption have been inconclusive. In part this is because it is difficult to obtain sufficient enzyme to permit extensive studies and perhaps because a number of metals may serve as Type II metals. The only metal which has been found to have a direct relationship with enzymatic activity is Ni^{2+} but only 0.42 g-atoms per mole of carboxytransphosphorylase were observed. There is some indication that Cu^{2+} and Fe^{2+} may be involved but Mn^{2+}, Co^{2+}, Zn^{2+}, Cu^{2+} and Mg^{2+} appear to be eliminated. Present studies involve the growth of the bacteria on defined medium in which the metal content may be controlled and the relationship of metal to enzyme activity determined.

C. Differences in the Requirements of the Oxalacetate and Pyruvate Reaction. Careful study of the enzyme during purification has shown that catalysis of the oxalacetate reaction and the pyruvate reaction occur in the same proportion throughout the purification, which includes crystallization of the enzyme. Thus, it appears quite certain that the same enzyme catalyzes both reactions. Nevertheless, the requirements for the two reactions differ. As shown in Fig. 1 in the absence of thiols such as β-mercaptoethanol, catalysis of the oxalacetate and pyruvate reactions occurs at about equal rates, i.e. in the presence of 30 mM bicarbonate oxalacetate is formed at about the same rate as is pyruvate when the bicarbonate concentration is zero.

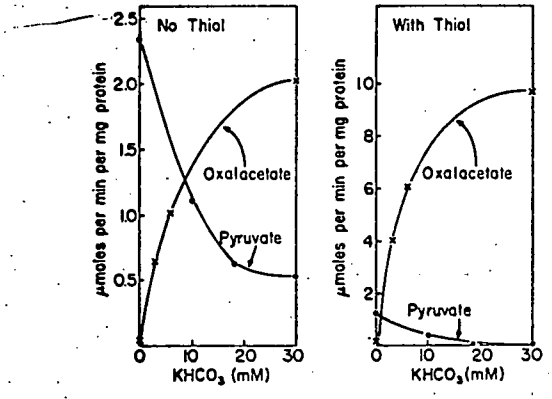


FIGURE 1: Effect of bicarbonate and mercaptoethanol on the rates of formation of pyruvate and oxalacetate by carboxytransphosphorylase

When thiol is added, however, the results are quite different. Thiols stimulate the oxalacetate reaction but inhibit the pyruvate reaction. Thus in the presence of mercaptoethanol and 30 mM bicarbonate the rate of formation of oxalacetate is about 7 times that of the pyruvate reaction in the absence of bicarbonate.

It also is shown in Fig. 1 that in the presence or absence of mercaptoethanol that bicarbonate inhibits the pyruvate reaction and that the pyruvate reaction is practically eliminated by excess bicarbonate in the presence of mercaptoethanol. We have proposed that this occurs because the same intermediate component is common to both reactions as explained below under "Role of Type I and Type II Metals in the Carboxytransphosphorylase Reaction". When CO_2 is present it competes for the intermediate and reduces the formation of pyruvate.

A further difference between the catalysis of the pyruvate and the oxalacetate reaction is observed in the response of the EDTA treated enzyme to metals. Whereas Co^{2+} effectively reverses the inhibition of catalysis of the oxalacetate reaction by EDTA, Cu^{2+} is very much more effective than Co^{2+} for the pyruvate reaction. In fact Cu^{2+} inhibits the oxalacetate reaction. We have suggested that heavy metals may be required for the pyruvate reaction and that for this reaction three metals are required. Type I, Type II and the heavy metal. The heavy metal may shift the configuration of the enzyme to a form which is more conducive to catalysis of the pyruvate reaction and less so for the oxalacetate reaction. The relationship of these forms is diagrammed in Fig. 2.

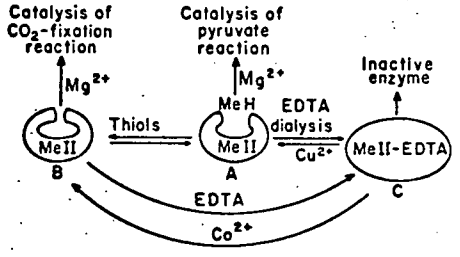


FIGURE 2: Schematic representation of the different forms of carboxytransphosphorylase. A dissociable type I metal (Mg^{2+}) is required for both the oxalacetate and pyruvate reactions. The A form catalyzes the pyruvate reaction. It is proposed to contain a heavy metal (Me H) as well as the tightly bound type II metal (ME II). Treatment with thiols converts the heavy metal into a non-functional form to yield form B which catalyzes the fixation of CO_2. Treatment of A with EDTA yields the inactive form C. Treatment of C with Cu^{2+} removes the EDTA from the type II metal and from the heavy metal or substitutes for it yielding the A form. Treatment of C with Co^{2+} removes the EDTA from the type II but since there is no functioning heavy metal it gives rise to the B form in the absence of thiols.

The heavy metal requirement may explain why sulfhyfryl compounds inhibit the pyruvate reaction. They may bind the required heavy metals and by the same action stimulate the oxalacetate reaction which is inhibited by heavy metals.

It is not likely that sulfhydryl groups stimulate the oxalacetate reaction by formation of required SH groups on the enzyme since EDTA treated dialyzed enzyme is fully active for the oxalacetate reaction in the presence of Co^{2+} and without the presence of added sulfhydryl compounds. It is suggested that the EDTA treatment removes the heavy metal and thus mercaptoethanol is no longer required for this purpose.

D. Role of Type I and Type II Metals in the Carboxytransphosphorylase Reaction.

By use of the EDTA treated enzyme it has been possible to delinate the role of Type I and Type II metals in the oxalacetate reaction and to separate the overall reaction into partial reactions. The partial reactions have been determined by exchange studies using $^{14}CO_2$, $^{32}P_i$ and $^{32}PP_i$. Certain mechanisms could be excluded by these results and the most likely mechanism is that shown in Reaction 5.

$$E\begin{matrix}\diagup \\ \diagdown\end{matrix}\begin{matrix}\\ MeII\end{matrix} + Mg^{2+} + P_i + PEP \rightleftharpoons E\begin{matrix}\diagup Mg\text{-}P_i \\ \diagdown MeII\text{-}PEP\end{matrix} \rightleftharpoons E\begin{matrix}\diagup Mg\text{-}PPEP \\ \diagdown MeII\end{matrix} \qquad (5a)$$

$$E\begin{matrix}\diagup MgPPEP \\ \diagdown MeII\end{matrix} + CO_2 \rightleftharpoons E\begin{matrix}\diagup Mg\text{-}PPOA \\ \diagdown MeII\end{matrix} \rightleftharpoons E\begin{matrix}\diagup Mg\text{-}PP_i \\ \diagdown MeII\end{matrix} + OA \rightleftharpoons E\begin{matrix}\\ \diagdown MeII\end{matrix} + PP_i + OA \qquad (5b)$$

E is enzyme, MeII is the Type II unidentified metal, PEP is P-enolpyruvate, PPEP is pyrophosphoenolpyruvate, OA is oxalacetate and PPOA is pyrophosphoenol-oxalacetate. The mechanism of course is hypothetical but forms a basis for future studies. For the details which exclude certain other mechanisms the complete account in Biochemistry 8, 3145 (1969) should be consulted, see reprints.

In brief, the following are the principal observations:

1. Exchange of $^{14}CO_2$ into the carboxyl group of oxalacetate occurs in the presence of Mg^{2+} even though the Type II metal is blocked by EDTA. Thus the overall reaction can be separated into partial reactions 5A and 5B.

2. Exchange of $^{32}P_i$ into PP_i or PEP or of $^{32}PP_i$ into PEP or P_i requires a free Type II metal as well as the Type I metal (Mg^{2+}).

3. The exchange of $^{14}CO_2$ into the carboxyl groups of oxalacetate requires PP_i in addition to Mg^{2+}. Thus it is unlikely that the reaction involves the following partial reaction since PP_i would not be required for exchange of CO_2 into the oxalacetate by this sequence.

$$E\begin{matrix}\diagup Mg \\ \diagdown MeII\end{matrix} + OA \rightleftharpoons E\begin{matrix}\diagup Mg\text{-}OA \\ \diagdown MeII\end{matrix} \rightleftharpoons E\begin{matrix}\diagup MgPyr + CO_2 \\ \diagdown MeII\end{matrix} \rightleftharpoons E\begin{matrix}\diagup Mg \\ \diagdown MeII\end{matrix} + Pyr + CO_2 \qquad (6)$$

4. Pyruvate-^{14}C does not exchange into oxalacetate or P-enolpyruvate under any conditions tested including conditions in which the overall reaction occurs rapidly.

5. Ca^{2+} inhibits the carboxytransphosphorylase reaction. This places the enzyme in the Type II classification of Mildred Cohen (Biochemistry 2, 623 (1963)), i.e. among the enzymes in which the metal (Mg^{2+}) forms a direct link with the enzyme.

6. The exchange of $^{32}P_i$ into PEP or PP_i or of $^{32}PP_i$ into P_i or PEP not only requires a Type I metal and a free Type II metal but also CO_2. Thus the mechanism is not as follows in which a high energy phosphoenzyme is formed from the PP_i.

$$E\begin{matrix}Mg\\MeII\end{matrix} + PP_i \rightleftarrows E\begin{matrix}Mg\\MeII \sim P_i\end{matrix} + P_i \qquad (6A)$$

$$E\begin{matrix}Mg\\MeII \sim P\end{matrix} + OA \rightleftarrows E\begin{matrix}MgOA\\MeII \sim P\end{matrix} \rightleftarrows E\begin{matrix}Mg\\MeII\end{matrix} + PEP + CO_2 \qquad (6B)$$

In this case there would be exchange of $^{32}P_i$ into PP_i in the absence of the other substrates.

7. The occurrance of pyrophospho intermediates is speculative and is based on the observation that no evidence of a pyruvate intermediate was obtained by exchange studies with pyruvate-^{14}C (item 4). However, Reactions 5A and 5B may occur by concerted mechanisms which do not involve free intermediate compounds such as pyruvate. Studies to determine whether either pyrophosphoenolpyruvate or pyrophosphoenoloxalacetate occur as intermediates are underway, see this year's Proposal.

8. It is shown in Fig. 1 that the pyruvate reaction is retarded by the presence of CO_2 and it is proposed that this occurs by competing for a common intermediate. In the absence of CO_2 Reaction 5B is replaced by 5C in which a proton reacts with the intermediate instead of CO_2.

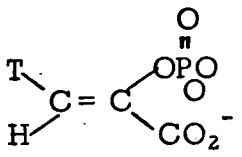
$$E\begin{matrix}MgPPEP\\MeII\end{matrix} + H^+ \rightarrow E\begin{matrix}MgPP\\MeII\end{matrix} + Pyr \rightleftarrows E\begin{matrix}Mg\\MeII\end{matrix} + PP + Pyr \qquad (5C)$$

In Reaction 5B the decarboxylation of the oxalacetate may supply enough energy to permit formation of the $E\begin{matrix}MgPPEP\\MeII\end{matrix}$ intermediate so that 5B is reversible and exchange of $^{14}CO_2$ occurs when PP_i and oxalacetate are present. However, starting with PP_i and pyruvate this energy of decarboxylation is lacking and thus the intermediate is not formed by the reverse of Reaction 5C. Therefore there is no exchange of pyruvate-^{14}C into P-enolpyruvate.

9. The Type II metal has not been identified but it is clear that Mg^{2+} (a Type I metal) cannot replace the function of the Type II metal. The exchange studies thus support the occurrance of two functions for metals.

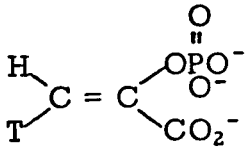
E. Stereochemistry of the Carboxytransphosphorylase Reaction. These studies were done during the past year and for that reason the statement given in this year's Progress Report covers the subject and is repeated here for the reviewer's convenience.

"Another interesting aspect of the mechanism is the sterochemistry of the addition of the CO_2 to P-enolpyruvate. These studies have been done in collaboration with Irwin Rose at the Institute of Cancer Research in Philadelphia as part of his studies of a number of carboxylation reactions with P-enolpyruvate. Two types of sterospecifically ^3H-labeled P-enolpyruvate may be formed enzymatically; one from 1-^3H (1R) fructose-6-P which is obtained by the glucose-6-P isomerase reaction with ^3HOH and the second from 1-^3H (1S) fructose-6-P which is obtained by the isomerase reaction with 1-^3H glucose-6-P (Rose, I. A. and O'Connell, E. L., Biochem. Biophys. Acta 42, 159 (1960) and unpublished work). The two types may be represented as follows:



Re

or



Si

They may be used to determine whether the CO_2 approaches the C-3 carbon of P-enolpyruvate from the side of the plane in which the three substituents of carbon-2 (phosphate, carboxyl, and vinyl carbon appear in a clockwise or counter-clockwise sequence. These two sides of the plane of the carbon skeleton are designated Re and Si (Hanson, K. R., J. Amer. Chem. Soc. 88, 2731 (1966)). The experiment is done by converting the oxalacetate, which results from the carboxylation of the two types to malate with malate dehydrogenase and then to fumarate with fumarase. The stereochemistry of fumarase is well established. The ^3H from one of the two types should be labilized by fumarase as a proton and from the other should be retained, depending on whether the addition is from the Re or Si side. The results indicate that the addition is from the Si side. This was also found to be true with P-enolpyruvate carboxylase from peanuts and P-enolpyruvate carboxykinase from pigeon liver.

With carboxytransphosphorylase, however, there was some evidence of non-stereospecificity. The specific radioactivity of the malate was 20 to 30% less than that of the starting P-enolpyruvate. This is of interest since the mechanism which we have proposed included the possibility of pyrophosphoenolpyruvate as an intermediate ($^-O_2C-CH=C(-O-P-O-P)-CO_2^-$). This mechanism would require labilization of a proton of the O-P-O-P methylene of P-enolpyruvate. This presumably would be a stereospecific displacement and in this case 100% of the ^3H sould be lost unless there was removal of the proton and its return specifically. Such specific transfer of hydrogen is know in enzyme reactions (methylmalonyl mutase) so the incomplete loss of ^3H does not exclude the proposed mechanism. Nevertheless, the partial loss of ^3H is most readily explained by a partial ketonization $\rightleftharpoons$ enolization reaction prior to product formation and the weight of the evidence is probably not in favor of the occurrance of pyrophosphoenoloxalacetate as an intermediate. The results, however, do not mitigate against the occurrance of pyrophosphoenolpyruvate as an intermediate."

F. Subunits of Carboxytransphosphorylase. Carboxytransphosphorylase in the crystaline form has an $s^{\circ}_{20,w} = 15.2S$ and a molecular weight of 430,000 + 30,000 as determined by the Archibald method. The specific activity in terms of μmoles oxalacetate per mg protein is about 23 under optimum conditions.

The supernatant solution from the crystallization has been found to contain a protein with an $s_{20,w} = \sim 8S$. This component has been obtained free from the 15.2S species by sedimentation in a partition cell and has been found to have an activity in both the oxalacetate and pyruvate reaction of about one-third that of the crystalline enzyme.

Overnight dialysis of the 15.2S crystalline enzyme against low ionic strength phosphate (0.03 M potassium phosphate, pH 6.8) results in conversion of about 30% to a species with an $s_{20,w} = 7.4S$. The 15.2S and 7.4S species have been isolated by sucrose density gradient centrifugation. The 7.4S form, thus obtained, appears to be similar to the species that occurs in the supernatant solution from the crystallization.

The 15.2S and 7.4S froms have been compared in terms of their K_m values for P-enolpyruvate and phosphate in both the oxalacetate and pyruvate reaction and they are nearly the same for both substrates and both reactions. The small form is inhibited by metal chelators just as is the crystalline enzyme. In collaboration with Prof. Robert Valentine of England the two forms have been viewed under the electron microscope. It appears that the crystalline form is a tetramer and the smaller species is a monomer. The 15.2S species dissociated to the monomer unless it was stabilizes by gluteraldehyde.

The effect of substrates on the sedimentation characteristics of these two forms has been determined and it has been found that there is shift in the sedimentation properties of both forms to a form with an $s_{20,w} = \sim 10S$. These values have been determined by sedimentation in a sucrose gradient in the presence of saturating amounts of substrates for the forward reaction. Even though the two forms give rise to a component with about the same S value the difference in specific activity persists when tested in either the oxalacetate or pyruvate reactions.

It seems possible that the breakdown of the crystals to the 7.4S form may be accompanied by the loss of a small subunit. This change might account for the difference in specific activities of the two forms inspite of the fact that both have about the same S values in the presence of substrate. The amino acid composition of the two forms is now under study to obtain information on this question. When the crystalline form is treated with 6M quanidine in the presence of 0.1 M mercaptoethanol a single sharp peak is observed of $s_{20,w} = .91S$ which on correction for density and viscosity of the quandine and mercaptoethanol gives an $s_{20,w} = 3.0S$. Thus it appears that the 7.4S form is not a single polypeptide.

II. Catalysis and Mechanism of the Pyruvate Phosphate Dikinase Reaction (Publications 3 and 5)

The propionic acid bacteria grow readily on pyruvate or lactate and clearly they synthesize P-enolpyruvate from these compounds for construction of cellular components. Utter and coworkers ahve shown in animal tissues that this synthesis occurs as follows:

$$\text{Pyruvate} + \text{ATP} + CO_2 \overset{\text{Pyruvate Carboxylase}}{\rightleftharpoons} \text{oxalacetate} + \text{ADP} + P_i \qquad (7)$$

P-enolpyruvate carboxykinase

$$\text{Oxalacetate} + \text{ITP} \rightleftarrows \text{P-enolpyruvate} + \text{IDP} + P_i + CO_2 \quad (3)$$

$$\text{Pyruvate} + \text{ATP} + \text{ITP} \rightleftarrows \text{P-enolpyruvate} + 2P_i + \text{ADP} + \text{IDP} \quad (8)$$

When carboxytransphosphorylase was found in the propionic acid bacteria it seemed possible these bacteria might synthesize P-enolpyruvate from pyruvate by a similar set of reactions but substitute the carboxytransphosphorylase reaction for carboxykinase and use PP_i as an energy source instead of the ITP. A search for pyruvate carboxylase was therefore undertaken and it was found that crude extracts did catalyze the conversion of pyruvate, ATP and CO_2 to oxalacetate but there was a requirement for P_i for this conversion, which could not be accounted for by Reaction 7. Further study lead to the discovery that these bacteria contain pyruvate phosphate dikinase which catalyzes Reaction 9. The enzyme is called a dikinase since both pyruvate and P_i are phosphorylated by the ATP.

$$\text{Pyruvate} + \text{ATP} + P_i \rightleftarrows \text{P-enolpyruvate} + \text{AMP} + PP_i \quad (9)$$

It thus became clear that the P_i was required for this conversion and the oxalacetate resulted from coupling of this enzyme with carboxytransphosphorylase which was also present in the crude extract. This sequence is shown below.

$$\text{Pyruvate} + \text{ATP} + P_i \rightleftarrows \text{P-enolpyruvate} + \text{AMP} + PP_i \quad (9)$$

$$CO_2 + \text{P-enolpyruvate} + P_i \rightleftarrows \text{oxalacetate} + PP_i \quad (I)$$

$$\text{Pyruvate} + \text{ATP} + 2P_i + CO_2 \rightleftarrows \text{oxalacetate} + \text{AMP} + 2\ PP_i \quad (10)$$

Thus when the propionic acid bacteria grow on pyruvate they use the dikinase reaction to form P-enolpyruvate and the dikinase linked to carboxytransphosphorylase supplies the oxalacetate. Thus the mechanism of formation of P-enolpyruvate and oxalacetate differs a great deal from that in animals. The hydrolysis of the PP_i by the propionibacteria would serve to pull both reactions. It seems likely that PP_i serves as a control point for pyruvate, P-enolpyruvate, and oxalacetate interplay in these bacteria.

The dikinase enzyme has been discovered independently by Hatch and Slack (Biochem. J. 106, 141 (1968)) in tropical grasses and by Reeves (J. Biol. Chem. 243, 3202 (1968)) in Entamoeba histolytica.

We have been very interested in the mechanism of the dikinase reaction and have obtained evidence that it occurs as follows:

$$\text{Enzyme} + \text{ATP} \rightleftarrows \text{enzyme-}\overset{\beta\gamma}{P}P + \text{AMP} \quad (11)$$

$$\text{Enzyme-}\overset{\beta\gamma}{P}P + P_i \rightleftarrows \text{enzyme-P} + PP_i \quad (12)$$

$$\text{Enzyme-P} + \text{pyruvate} \rightleftarrows \text{enzyme} + \text{P-enolpyruvate} \quad (13)$$

$$\text{Pyruvate} + \text{ATP} + P_i \rightleftarrows \text{P-enolpyruvate} + \text{AMP} + PP_i \quad (9)$$

Most of the evidence has been obtained by exchange studies using labeled $^{32}P_i$, $^{32}PP_i$, ^{14}C-AMP and ^{14}C-pyruvate. The following observations have been made:

1. There is exchange of ^{14}C-AMP into ATP and there is no requirement for P_i or pyruvate. This is in accord with Reaction 11.

2. The exchange of $^{32}P_i$ into PP_i requires either the presence of ATP or of P-enolpyruvate. This exchange presumably occurs by Reaction 12 which requires that either enzyme-PP_i or enzyme-P be formed so that the exchange is possible. ATP makes possible the formation of enzyme-PP and P-enolpyruvate of enzyme-P.

3. There is exchange of ^{14}C-pyruvate with P-enolpyruvate and there is no requirement for other substrates or products. This is in accord with Reaction 13.

4. ^{32}P-enolpyruvate yields β-labeled ATP by the overall reaction from right to left as predicted from the mechanism.

5. $^{32}PP_i$ yields $^{32}P_i$ and γ labeled ATP in the overall reaction from right to left as predicted from the mechanism.

6. Enzyme-^{32}P is formed when ^{32}P-enolpyruvate is incubated with the enzyme. The enzyme-^{32}P may be separated from the products by Sephadex filtration. Treatment of the ^{32}P-enzyme with 0.4 N HCl at 100° for 10' converts it to ^{32}P-orthophosphate. The label is removed from the ^{32}P-enzyme and converted to products when incubated in the overall reaction.

7. Thus far we have been unable to obtain enzyme-^{32}PP from the enzyme plus $AT^{32}P$. This intermediate enzyme complex may be very label and require special techniques to isolate it. It is not known whether it is a pyrophospho-enzyme complex. It is quite possible that the enzyme is phosphorylated at two sites by the ATP.

Studies are underway to determine whether or not the phosphate is linked to a histidyl group of the enzyme. If pyruvate is to be phosphorylated by Reaction 13 a very high energy complex is required. The linkage in the enzyme is of considerable interest.

A mechanism which is very similar to that shown above has previously been proposed by Cooper and Kornberg of England (Biochem. Biophys. Acta 114, 214 (1967)) for P-enolpyruvate synthase of E. coli which catalyzes Reaction 14.

$$\text{Pyruvate} + \text{ATP} \rightleftharpoons \text{P-enolpyruvate} + \text{AMP} + P_i \qquad (14)$$

In this reaction pyrophosphate is not a product and Reaction 12 becomes a hydrolytic instead of a phosphorlytic step in the mechanism.

III. Species of CO_2 Invdved in Carboxylation Reactions (Publication 4)

It is important in elucidating the mechanism carboxylation or decarboxylation to know whether free CO_2 or HCO_3^- is the reactant. Krebs and Roughton in the

1940's using carbonic anhydrase and respirometer techniques showed that free CO_2 is the primary product of the decarboxylation of pyruvate by yeast decarboxylase. It then was generally assumed that free CO_2 is the reactant in all carboxylation reactions. In 1962 by use of O^{18} Kaziro et al. (J. Biol. Chem. 237, 1460 (1962)) with propionyl carboxylase and Maruyama and Lane with P-enolpyruvate carboxylase (Biochem. Biophys. Res. Commun. 9, 461 (1967)) showed that HCO_3^- is the reactant with these enzymes. They found that three atoms of ^{18}O were incorporated in the products from HCO_3^- rather than two as would be expected if $C^{18}O_2$ was the reactant. The trend of thought then shifted to the view that HCO_3^- is the general reactant in fixation reactions.

With carboxytransphosphorylase there were indications that the reactant might be free CO_2. At the same time T. G. Cooper, T. T. Tchen and C. R. Benedict of Wayne State University became interested in this question with the enzyme, P-enolpyruvate carboxykinase, from Rhodospirillum rubrum (Reaction 3).

Collaborative work was undertaken with these investigators using these two enzymes. Two methods were employed: (1) a spectrophotometric assay, and (2) a radiochemical assay. The methods utilize the fact that the hydration of CO_2 is a relatively slow reaction, the velocity constant equals 0.01 per second at 0°. The dehydration of H_2CO_3 is much faster, 11 per second. The ionization reaction ($H_2CO_3 \rightleftarrows H^+ + HCO_3^-$) is very rapid and may be considered instantaneous. The CO_2 in solution is about 99 per cent as free CO_2 as 1 per cent as H_2CO_3.

$$H_2O + CO_2 \underset{k = 11 \text{ sec}^{-1}}{\overset{k = .01 \text{ sec}^{-1}}{\rightleftharpoons}} H_2CO_3 \rightleftarrows HCO_3^- + H^+ \qquad (14a)$$

The spectrophotometric assay involved coupling the enzymatic reaction with malic dehydrogenase, and the rate of the reaction was followed by DPNH disappearance, measured by absorption at 340 mμ. The CO_2 or HCO_3^- were used at concentrations (2.5×10^{-3} M) substantially below the K_m values for total "CO_2". The reaction rate then should be directly proportional to the active species. If CO_2 was added and CO_2 was the active species the initial rate should be high. After about a minute the equilibrium betweeen the 3 species of Reaction 14a should be reached and the rate should become linear. With carbonic anhydrase present the equilibrium between the species is very rapidly attained and the enzymatic fixation of CO_2 should proceed at a constant rate from time 0. If HCO_3^- is the added species and CO_2 is the active species the reverse of the above should hold, but the rate of conversion of $H^+ + HCO_3^-$ to H_2CO_3 and of H_2CO_3 to CO_2 and H_2O is rapid compared to that of $CO_2 + H_2O$ to H_2CO_3 so the concentration of CO_2 would rapidly increase. Therefore the initial slow rate would be of short duration and then become linear.

If HCO_3^- were the active species in the situation discussed above, the results would be reversed but will not be discussed here in detail.

It was found when CO_2 was the added species that the rate was rapid at first and after about 2 min reached a linear rate. When carbonic anhydrase was present the initial rapid rate with CO_2 was not observed. The rate with HCO_3^- was linear and identical with those obtained with CO_2 plus carbonic anhydrase. The results showed clearly that CO_2 is the active species.

The radiochemical assay was done with a mixture of the two species each at $5X10^{-3}$ M (total $1X10^{-2}$ M) only 1 of which contained ^{14}C. The reaction was linked with malate dehydrogenase and the reaction was followed by the incorporation of ^{14}C into malate. The radiochemical procedure is superior, particularly for study of the HCO_3^-. The addition of the non-radioactive alternate species serves to slow down the attainment of isotopic equilibrium and thereby increases the sensitivity of the technique. Even though the rate of conversion of $H^{14}CO_3^-$ is rapid the rate of the reverse conversion, i.e. CO_2 to HCO_3^-, is slow. Thus the specific activity of the $H^{14}CO_3^-$ is not diluted rapidly by the $^{12}CO_2$ and isotopic equilibrium is reached slower than the chemical equilibrium.

If the active species is CO_2, a high initial rate of ^{14}C-malate formation should be observed when the label is in CO_2 and a low initial rate when the label is in HCO_3^-. The results showed that CO_2 is the active species. With carbonic anhydrase present the results were identical with both labeled species.

The results with P-enolpyruvate carboxykinase were similar to those with carboxytransphosphorylase. Thus it too uses CO_2 as the reactant. The radiochemical assay was also applied to pyruvate carboxylase, a biotin enzyme. In this case HCO_3^- was found to be the active species which is in conformity with the results of Kaziro et al. who found that the biotin enzyme, propionyl carboxylase, utilizes HCO_3^- as the active specied.

Maruyama and Lane found that P-enolpyruvate carboxylase utilizes HCO_3^-. This enzyme catalyzes the following reaction:

$$\text{P-enolpyruvate} + HCO_3^- \rightarrow \text{oxalacetate} + P_i \qquad (16)$$

It is noted that Reaction 16 involves P-enolpyruvate just as do Reaction 1 (carboxytransphosphorylase) and Reaction 3 (carboxykinase). Nevertheless they differ in the forms of CO_2 which are utilized. In unpublished results we have confirmed that HCO_3^- is the reactant in the P-enolpyruvate carboxylase reaction. Thus even though the reactions are quite similar the machanisms differ fundamentally.

IV. Total Synthesis of Acetate from CO_2 (Publications 1, 2, 6, 10)

The problem of how certain heterotrophic bacteria (particularly clostridia) catalyze a total synthesis of acetate from CO_2 has been a subject of long interest to us. The bacteria use the CO_2 as an electron acceptor during the fermentation and form acetate from the CO_2. If the fermentations are done in the presence of $^{14}CO_2$, acetate is formed in which ^{14}C is present in both the methyl and carboxyl carbons. Of these organisms Clostridium thermoaceticum has been studied the most extensively and there is quite conclusive evidence that the overall stiochiometry is as follows:

$$C_6H_{12}O_6 + 2\ H_2O \rightarrow 2\ CH_3COOH + 2\ CO_2 + 8\ H \qquad (17)$$

$$8\ H + 2\ CO_2 \rightarrow CH_3COOH + 2\ H_2O \qquad (18)$$

$$C_6H_{12}O_6 \rightarrow 3\ CH_3COOH \qquad (19)$$

It has been shown by Stadtman's group and our own that the synthesis of acetate occurs by a unique process involving formation of an organametalic complex arising from CO_2. The CO_2 apparently is reduced to formate and then is converted to methyltetrahydrofolate and the methyl is transferred to the cobalt of a corrinoid enzyme complex where it in turn reacts with a second molecule of CO_2 forming acetate with regeneration of the corrinoid enzyme complex. The broad outline of the mechanism is shown in Fig. 3, but confirmation with purified enzymes is yet to be accomplished. The steps involving the cobalt complex are of particular interest since they represent a type of synthesis from CO_2 which is different from those previously described and it may have broader implications than is presently recognized. The background and present concepts of the mechanism have been reviewed in publication 1 and reprints are included to provide more details.

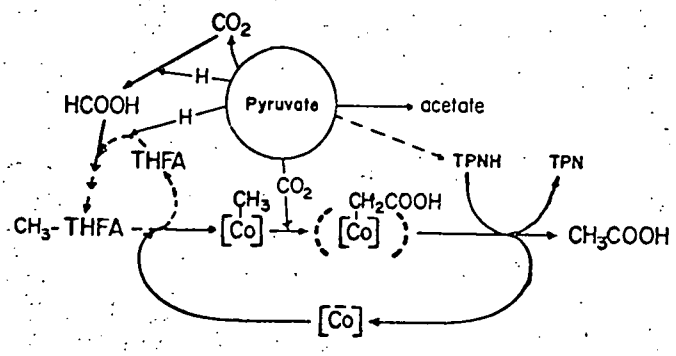


FIG. 3. Summary of present concept of the total synthesis of acetate from CO_2 as it occurs in *C. thermoaceticum*. CH_3-THFA is *N*-5-methyltetrahydrofolate and [Co] signifies a corrinoid. Pyruvate is required for the conversion of *N*-5-methyltetrahydrofolate and Co-methylcorrinoids to the methyl group of acetate. TPNH can replace pyruvate in the cleavage of the Co-carboxymethyl-corrinoid to acetate by a crude cell-free extract. In the over-all conversion of pyruvate, it is oxidized to acetate with CO_2 serving as the hydrogen acceptor and yields energy for the acetate synthesis.

Our present efforts are toward purification of the enzymes so that the system may be studied step by step. The entire synthesis is obviously a complex process involving many enzymes which catalyze both the breakdown of the carbohydrates and the transport of electrons coupled with the CO_2 reduction and synthesis of the acetate. When sufficient information is at hand it is hoped to substitute a well defined ATP generating system and electron donor system for the more complex fermentation and to link these with the purified corrinoid enzyme which catalyzes the synthesis of acetate from the derivatives arising from CO_2.

The studies have been directed to three parts of the scheme of Fig. 3. The first is concerned with the Co-carboxymethyl-corrinoid, the second with the isolation of the corrinoid-enzyme complex or complexes and the third with the mechanism of conversion of CO_2 to methyl tetrahydrofolates.

A. Experiments relating to carboxymethyl-corrinoids as an intermediate--The evidence that a Co-carboxymethyl-corrinoid is an intermediate is inconclusive. When pulse labeling experiments are done with $^{14}CO_2$ using whole cells, Co-methylcorrinoids which are highly labeled are readily isolated and when these labeled corrinoids are added to fermentations of pyruvate by crude extracts of

C. thermoaceticum the ^{14}C is converted to the methyl of acetate. In the same experiments qualitative evidence was obtained that a labeled Co-carboxymethyl-corrinoid was formed. A labeled corrinoid was isolated which on photolysis yielded some acetate, glyoxalate, oxalate and succinate as well as formaldehyde, formate, methanol and CO_2. The photolysis of Co-carboxymethyl-B_{12} yields similar products. The yield of products varies with conditions. At high concentrations of Co-carboxymethyl-B_{12} under anaerobic conditions the yield is about 50 per cent as acetate, while almost no acetate is formed at low concentrations under aerobic conditions. A more precise method for the identification of the carboxymethyl ligand was necessary. It was found that sodium borohydride reductively cleaves Co-carboxymethyl-B_{12} with almost a 100 per cent yield of acetate (Ljungdahl and Irion, Biochemistry 5, 1846 (1966)).

The stability of carboxymethyl-B_{12} was investigated to determine the best conditions for isolation of such compounds. Sulfhydryl compounds such as cysteine, mercaptoethanol and thioglycolic acid are used to maintain the reduced conditions required for growth or for use of extracts. It was found that Co-carboxymethyl-B_{12} is rapidly decomposed at acid pH in the presence of sulfhydryl reagents. Under alkaline conditions Co-carboxymethyl-B_{12} is stable. However, when we applied these conditions – positive identification of carboxymethyl derivatives failed in pulse labeling experiments with either whole cells or extracts. It is possible that the amount of this derivative is extremely small in the cells and it is cleaved enzymatically or chemically in spite of our efforts to prevent the cleavage.

The mechanism of formation of Co-carboxymethylcorrinoids may occur as illustrated in the following reaction:

$$\text{[Co]}\text{-}CH_3 \rightarrow \left[\text{[Co]}\text{-}CH_2^- H^+\right] \xrightarrow{CO_2} \text{[Co]}\text{-}CH_2COOH \qquad (20)$$

In the first step a proton dissociates from the methyl and is replaced in the second step by CO_2.

Wagner and Bernhauer (Ann. N. Y. Acad. Sci. 112, 580 (1964)) reported that the hydrogen atoms of the Co-methyl group are active and exchange with tritiated water. Our own tests failed to confirm such an exchange with Co-methyl-B_{12} and Dr. R. H. Abels (personal communications) likewise obtained no exchange. The enzyme may of course catalyze such activation of the hydrogen.

However acetate might be formed from Co-methyl corrinoids by a Grignard type of reaction.

$$\text{[Co]}\text{-}CH_3 \rightarrow \left[\text{[Co]}\text{-}CH_3^- + \right] \xrightarrow{+CO_2} \left[\text{[Co]}\text{-}O\text{-}C(=O)\text{-}CH_3\right] \rightarrow \text{[Co]} + CH_3COOH \qquad (21)$$

In the mechanism of Reaction 20 a hydrogen of the methyl group would be lost during the conversion to acetate whereas by Reaction 21 it would not. Therefore

tritium and ^{14}C-labeled Co-methyl-B_{12} were prepared. In preliminary experiments using crude enzyme preparations it has been found that the ratio $T/^{14}C$ was lower in the methyl of acetate than in the methyl of the B_{12} derivative from which it was derived. Our present opinion is that these experiments should be performed using a more purified enzyme system. There is a possibility that the ratio was lowered through secondary exchange catalyzed by enzymes utilizing acetate or alteratively through reversible reactions of the Co-methyl group. There also may be a large isotope effect by the ^{3}H which make its conversion slow compared to that of the ^{14}C-labeled methyl group.

Positive evidence favoring a carboxymethyl derivative includes the fact that extracts of C. thermoaceticum catalyze the formation of acetate from Co-carboxy-methyl-B_{12} (Ljungdahl, Irion and Wood, Biochemistry 4, 2771 (1965)) as follows:

$$\begin{matrix} CH_2COOH \\ | \\ [Co] \end{matrix} \xrightarrow{TPNH} CH_3COOH + B_{12r} \qquad (22)$$

Fractionation of the crude extract by use of a DEAE-cellulose columns has yielded an active protein fraction which is eluted with 1 M phosphate buffer (pH 6.5). This fraction catalyzes Reaction 22. The fraction appears to correspond to fraction B isolated by Poston, Kuratomi and Stadtman (J. Biol. Chem. 241, 4209 (1966)) which they found to be needed for the formation of acetate from Co-methyl-B_{12}.

The isolation of this fraction has not always been successful, furthermore the rate of the reaction is not linear and decreases rapidly. This behavior may indicate that the Co-carboxymethyl-B_{12} binds to the enzyme and after cleavage of the carboxymethyl moiety to yield acetate the corrinoid remains on the enzyme inhibiting the reaction with additional carboxymethyl-B_{12}.

B. Isolation of Corrinoid Enzyme Complexes. It seems probably that the corrinoid involved in acetate synthesis is bound to an enzyme and that the methyl is transferred to it from methyl tetrahydrofolate, carboxylation then occurs, acetate is formed, and the corrinoid enzyme complex is regenerated as shown in Fig. 3. In this case the corrinoid enzyme would become labeled from $^{14}CO_2$ and it might be distinguished from other proteins on the basis of radioactivity. Evidence has been obtained that this is the case but thus far the results have not lead to purification of highly active enzymes.

For this purpose extracts were incubated with pyruvate and $^{14}CO_2$ and fractionated on a DEAE cellulose column to obtain fractions A with 0.1 M phosphate and with B with 0.5 M phosphate as described by Poston, Kuratoni and Stadtman. Both fractions contained radioactivity with the highest amount in Fraction A. The radioactivity of Fraction B was rapidly lost even at 0° while that of Fraction A was more stable. Both fractions were further purified by ammonium sulfate fractionation and by passage through Sephadex G-100. Fraction B lost its radioactivity during this treatment. It contained only one corrinoid, which was identified as Factor III_m. It catalyzed the TPNH reductive cleavage of Co-carboxymethyl-B_{12} to acetate (Reaction 22).

Several corrinoids were isolated from Fraction A and some of these contained photolabile methyl groups. Acetate labeled in the methyl group was formed when the labeled protein A was incubated with pyruvate and a crude extract. Moreover the amount of radioactivity converted to acetate was significantly lower after protein A was exposed to light. These results are consistent with the occurrence of a Co-methyl-corrinoid protein since Co-methyl-corrinoid lose the Co-methyl group when exposed to light.

C. Mechanism of Conversion of CO_2 to Methyltetrahydrofolate. When methyl B_{12} or carboxymethyl B_{12} is added to the crude enzyme the overall reaction is slow probably because the corrinoid must dissociate from the protein before the enzyme can react with another molecule of B_{12} derivative. It appears unlikely that rapid and linear activity of the corrinoid enzymes can be demonstrated until a tetrahydrofolate methyl generating system is obtained which can be coupled to the corrinoid enzymes. Furthermore, the carboxylation of the methyl group will be required and cleavage to acetate so that regeneration of the original corrinoid enzyme is accomplished completing the cycle. Towards this end we have begun to work on the folate system of C. thermoaceticum.

The evidence for folate participation in acetate synthesis is as follows.

1. ^{14}C-formate is converted preferentially to the methyl of acetate.

2. A TPN dependent formic dehydrogenase has been isolated from C. thermoaceticum which catalyzes the conversion of formate to CO_2 and TPNH. The reverse reaction has not been demonstrated thus far.

3. A formyltetrahydrofolate synthetase has been purified from C. thermoaceticum which catalyzes the conversion of formate to 10-$CHOH_4$PtGlu (10 formyltetrahydropteroylmonoglutamate).

4. There is conversion of 5-$^{14}CH_3H_4$PtGlu to the methyl of acetate by cell extracts in the presence of pyruvate.

5. ^{14}C-methyltetrahydrofolates with high radioactivity has been isolated from whole cells of C. thermoaceticum which were exposed to a short pulse of $^{14}CO_2$. Future studies will involve purification of enzymes required in the conversion of CO_2 to methyltetrahydrofolate and the transfer of the methyl to the Co of the corrinoid enzyme. The natural form of the folate probably will be required for these studies. Rabinowitz and Himes (Fed. Proc. 19, 963 (1960)) have reported that the natural cofactor of C. acidi urici and C. cylindosporum is tetrahydropteroyltriglutamate (H_4PtGlu$_3$) and the K_m for formyltetrahydrofolate synthase is 10 fold lower with it than with the monoglutamate. Our studies likewise indicate that the triglutamate is the natural cofactor in C. thermoaceticum.

PLANS FOR CONTINUATION OF PRESENT OBJECTIVES AS WELL AS POSSIBLE NEW OBJECTIVES

The present objective--the study of the mechanism of enzyme action, the control, the properties and the structure of enzymes at the active site will continue to be our objective in the coming years. Carboxytransphosphorylase catalyzes

an interesting type of fixation of CO_2 with an involvement of two types of divalent metals and a unique involvement of inorganic pyrophosphate. It is well suited for such studies. The corrinoid enzymes which catalyze the total synthesis of acetate from CO_2 involves organometallic compounds and provide a challenging subject for future studies.

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STATE OF KNOWLEDGE IN THIS AREA OF RESEARCH, ITS SIGNIFICANCE IN THE FIELD OF BIOLOGY AND MEDICINE, AND NEEDED FUTURE INVESTIGATIONS

There has been a great deal of progress in the understanding of the mechanism of enzyme catalysis but as yet it is impossible to duplicate the function of an enzyme. Enzymes are of course fundamental to all life processes and research on the mechanism of action of enzymes is of great importance to all fields of biology and medicine. The catalytic role of metaloenzymes relates to the understanding of the function of the trace metals in the body. Information on the role of corrinoids in the total synthesis of acetate from CO_2 may provide a clue to a new type of autotrophic synthesis. It seems unlikely that all autotrophic forms are limited to a single mechanism for the autotrophic utilization of CO_2.

FEDERAL SUPPORT FOR OVERALL RESEARCH OF PROGRAMS OF PRINCIPAL INVESTIGATION

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