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Institution: Case Western Reserve University

Department: Biochemistry

Title: A Study of Intermediary Metabolism of Carbohydrates
with Isotopically Labeled Compounds

Principal

Investigator: Harland G. Wood

The Progress Report will be considered under the headings
given in last year's Proposal:

- I. The Mechanism of the Carboxytransphosphorylase Reaction--
R. Singleton, M. E. Haberland and H. G. Wood.
 - II. Total Synthesis of Acetate from CO₂--D. Parker, M. Schulman,
T. Wu, P. Kucera and H. G. Wood.
- I. The Mechanism of the Carboxytransphosphorylase Reaction--R. Singleton,
M. E. Haberland and H. G. Wood.

Carboxytransphosphorylase from Propionibacterium shermanii catalyzes the
following reactions:

Reaction 1 is called the oxalacetate reaction and is reversible and Reaction 2
the pyruvate reaction and it is irreversible. The enzyme is responsible for CO₂
fixation by these organisms. Past studies described in previous reports have dealt
with the mechanism of the reaction and the role of metal ions in the catalysis.
These results have permitted the formulation of a hypothetical mechanism as
illustrated below and form the basis of the present studies.

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E is enzyme, MeII is the Type II unidentified metal, PEP is phosphoenolpyruvate, PPEP is pyrophosphoenolpyruvate, OA is oxalacetate and PPOA is pyrophosphoenol oxalacetate.

In the absence of CO_2 , the pyruvate reaction is proposed to occur by combination of reaction 3 and reaction 5.

The bases of these proposals have been considered previously and are published in Biochemistry 8, 3127, 3137 and 3145 (1969).

Last year we proposed to study the following subjects in relation to carboxytransphosphorylase: (A) Identification of the Bound Metal, Type II, (B) Study of the Subunits of Carboxytransphosphorylase, (C) Detection of Enzyme Bound Intermediate Compounds of the Reaction, (D) Examination of the Mechanism of the Carboxytransphosphorylase Reaction with ^{18}O , (E) Stereochemistry of the Carboxytransphosphorylase Reaction and (F) Kinetic Analysis of the Carboxytransphosphorylase Reaction. The kinetic analysis was not attempted due to unexpected difficulties with other aspects of the problem.

A. Identification of the Bound Metal (Type II) of Carboxytransphosphorylase. These studies have been carried out in collaboration with Dr. J. M. Willard of the Department of Biochemistry, University of Vermont. Previous studies with carboxytransphosphorylase have shown that two types of metals are required. Type I metal is 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, and its requirement is made evident by the use of chelators in the presence of excess Mg^{2+} serving as the Type I metal. The catalysis of both the oxalacetate and pyruvate reactions is inhibited by a large variety of structurally unrelated chelators, some of which have a low affinity for Mg^{2+} and are effective at 10^{-5} M with the Mg^{2+} at 10^{-2} M. The effectiveness of the wide variety of chelators makes it quite certain that the inhibition is due to binding of a second metal and not to a non-specific effect of the chelators.

Thus far all attempts to identify the Type II metal by emission and atomic absorption spectroscopy have been inconclusive. It seems possible that the metals may exchange from carboxytransphosphorylase during isolation so that the metal is not present in an equimolar ratio with the enzyme. We proposed last year that the problem be approached by growing the propionic acid bacteria in a defined medium so that the metal content of the medium might be controlled. However, thus far we have not been able to prepare a medium devoid of metals to limit growth of the bacteria. It appears that with time metals leach from the glass, even though the glassware has been

rigorously cleaned with nitric acid. Thus medium which had been extensively treated with chelators to remove the metals and initially contained 10^{-9} M Zn supported growth after two days and the Zn content at this time had risen to 10^{-7} M. It appears it will be necessary to grow the bacteria in plastic bottles to remove this contamination with metals.

It is proposed to follow three procedures. First the bacteria will be grown on a metal deficient medium and carboxytransphosphorylase will be isolated under metal free conditions in the hope of obtaining an apoenzyme which will be activated by an appropriate metal. Second growth requirements for specific metals will be determined. Radioactive metals of those required will be included in the growth medium and the distribution of the radioactivity will be determined to see if it is incorporated in carboxytransphosphorylase. Third a given metal will be added to the deficient medium in ample amount (for example cobalt) and then the cobalt will be maintained in all solutions which are used for isolation of the enzyme. Other metals will be kept minimal. In this way, if there is exchange of metals during isolation of the enzyme only cobalt will be present. If carboxytransphosphorylase contains cobalt, it will be maintained in the enzyme.

Present results indicate that cobalt may in fact be the Type II metal or may replace its function. It has been found that some preparations of carboxytransphosphorylase are increased in activity by low concentrations of Co^{2+} in the presence of 10^{-2} M Mg^{2+} . The apparent K_m for this activation has been determined and is shown in Fig. 1. The K_m was 3.8×10^{-6} M which is far below that of cobalt as a

Fig. 1. Determination of the apparent K_m for Co^{2+} with enzyme showing a 33% activation of the oxalacetate reaction by Co^{2+} . $K_m = 0.0038$ mM. Specific activity of carboxytransphosphorylase, 14.0 units/mg protein.

Type I metal (10^{-3} M). Furthermore, zinc, manganese and iron were not effective in replacing cobalt in the activation. It therefore appears that studies with cobalt added to the metal deficient medium may be profitable.

Dr. J. M. Willard will carry on these studies in the future at the University of Vermont and the investigations will be discontinued at Case Western Reserve University except for such collaboration as may seem desirable on some particular phase of the work.

B. Study of the Subunits of Carboxytransphosphorylase. We have previously reported that carboxytransphosphorylase has two forms, one of $s_{20,w} = 15.2$ with a specific activity of ~ 23 I. U. and the other with a sedimentation coefficient around 7S and a specific activity of about 7. The crystalline form of the enzyme is the larger species; the smaller form is abundant in the supernatant solution of crystallization. The $\sim 7S$ component can also be produced by prolonged dialysis of the crystalline carboxytransphosphorylase against low ionic strength buffer (0.03 M potassium phosphate, pH 6.8). Separation of the components has been accomplished by sucrose density gradient centrifugation.

Electron micrographs by the late Professor Robin C. Valentine of Mill Hill, England, have indicated that the crystalline $\sim 15S$ species is a tetramer and the $\sim 7S$ form, a monomer. Stabilization of the tetramer by gluteraldehyde was required in preparing the samples for electron micrographs to prevent dissociation to the monomer.

Last year we reported the intriguing observation that the $\sim 15S$ and the $\sim 7S$ forms of the enzyme rearrange to species with the same sedimentation characteristics ($\sim 10S$) when placed in saturating concentrations of the substrates of the forward reaction, *i.e.* P-enolpyruvate, phosphate and CO_2 . Although the two forms are transformed to species with similar sedimentation coefficients, the differences in enzymatic activities persist; the $\sim 10S$ material originating from $\sim 15S$ material has a specific activity about three times that of the $\sim 10S$ material coming from the $\sim 7S$ material. Apparently the $\sim 10S$ species is the dimeric form of carboxytransphosphorylase, which R. C. Valentine had predicted should exist on the basis of the electron micrograph studies.

The past studies of the alterations of the sedimentation coefficients of the $\sim 7S$ and $\sim 15S$ species have been analyzed by sucrose density gradient ultracentrifugation (5-8 hours duration) or by sedimentation velocity on the ultracentrifuge using the Schlieren optical system (> 1 mg/ml protein required). These determinations of the sedimentation constants involve several hours and do not measure the alterations of the constants under actual conditions used in the assay of the enzyme. *i.e.* at low protein concentration and in the absence of sucrose.

We have recently applied the method described by Cohen, Giraud and Messiah (Biopolymers 5, 203 (1967)) to these studies which makes it possible to determine if only the $\sim 10S$ form or all the forms, $\sim 7S$, $\sim 10S$ and $\sim 15S$, are active in catalyzing the oxalacetate and pyruvate reactions (Reactions 1 and 2). Furthermore these measurements are made under conditions of the assay and in addition show whether the transformation is rapid or slow. The technique consists of sedimenting the enzyme through the assay mixture which contains P-enolpyruvate, P_i , $KHCO_3$ (saturated with CO_2), Mg^{2+} , DPNH and malate dehydrogenase. The moving boundary of optical density at 350 m μ is measured with the ultraviolet optical scanning system and is due to the formation of oxalacetate by the carboxytransphosphorylase and the subsequent oxidation of DPNH catalyzed by the malate dehydrogenase. Thus only the catalytically active species are observed. The carboxytransphosphorylase is layered onto the reaction mixture by the synthetic boundary cell during acceleration of the rotor and instantaneous observations at frequent time intervals are made.

The results, when crystalline carboxytransphosphorylase is sedimented through an assay solution of the forward oxalacetate reaction, are shown in Fig. 2. These data show that both the $\sim 15S$ species and $\sim 10S$ species are active.

Fig. 2

Fig. 2. Determination of the active species of carboxytransphosphorylase during sedimentation through substrates of the oxalacetate reaction.

A. Superimposed tracings at 350 m μ of the oxidation of DPNH were taken every two minutes. 1.4 μ g of crystalline carboxytransphosphorylase (Specific activity 24 units/mg) in 0.02 ml was layered during the acceleration by the valve type sedimentation boundary cell upon 0.45 ml of solution containing 0.01 M potassium phosphate buffer (pH 6.8), 0.03 M KHCO₃, 0.012 M MgCl₂, 2×10^{-4} M phosphoenolpyruvate, 2×10^{-4} M DPNH, 10^{-4} M CoCl₂ and 0.5 units malate dehydrogenase. The numbers on the curves designate the time at which the scan was begun. Direction of sedimentation is to the right. Conditions of the run are 52,000 rpm, 19.8°, and magnification factor of 16.82.

B. Difference curves were obtained by subtracting points of $x + 2$ minutes from x minutes at the same rotor distances. The curves indicate those areas in which DPNH oxidation, and therefore product release by carboxytransphosphorylase was the greatest, and represent the location of active carboxytransphosphorylase. Note that with increasing time two forms separate. The one is evident as peaks due to the activity of the $\sim 10S$ dimer and the shoulder is probably due to the 15S tetramer. The shoulder is most evident on the curve for 18-20 min at 70 on the abscissa. Direction of sedimentation is to the right.

C. A conventional analysis of the sedimentation velocity run is plotted as log radius vs. time. The slope can be related to the sedimentation coefficient of the major peak. The value is 10.0S for this experiment. The sedimentation coefficient for the shoulder could not be calculated because of the lack of discrete peaks.

When the monomer is tested under the same conditions it too is converted to the active dimer ($\sim 10S$) and the $\sim 7S$ monomer is likewise active.

When similar tests were conducted for the pyruvate reaction (Reaction 2), *i.e.* in the absence of bicarbonate and with P-enolpyruvate, P_i, Mg²⁺, DPNH and lactate dehydrogenase, only the $\sim 15S$ component has been observed to be active starting with the tetramer and only the $\sim 7S$ component beginning with the monomer. Likewise when the tetramer or monomer forms were studied by sucrose density gradients containing substrates for the pyruvate reaction about 90% of the original enzymatic activity was recovered in the $\sim 15S$ or $\sim 7S$ regions, respectively. It thus appears that there is little transformation of either monomer or tetramer to dimer in the absence of bicarbonate-CO₂. The pH of the assay mixtures for the oxalacetate reaction and pyruvate reaction were nearly the same ($\sim$ pH 6.8). The ionic strengths were maintained constant. Further study will be required to determine whether bicarbonate-CO₂ as such causes the conversion of the monomer and tetramer to dimer or whether the other substrates are required in addition. It is noted that formation of tetramer from monomer has never been observed even though dimer is formed from both the $\sim 15S$ and $\sim 7S$ forms. We propose to investigate further whether it is possible to convert the monomer to tetramer or whether some irreversible change occurs during formation of the monomer.

It is of interest that monomer formed from tetramer by dialysis at low ionic strength when converted to dimer has about 1/3 of the enzymatic activity of dimer arising directly from tetramer. At present we have no explanation for this difference in activity of the two dimers. A most intriguing result of these studies is that all three forms of carboxytransphosphorylase, monomer, dimer, and tetramer, are active as enzymes.

A comparison of the K_m values of the $\sim 15S$ and $\sim 7S$ forms in both the forward oxalacetate reaction and the pyruvate reaction is shown in Table I. It is seen that in this regard both species are similar. Obviously in the oxalacetate reaction when the $\sim 15S$ species is used both the $\sim 15S$ and $\sim 10S$ forms are present and when $\sim 7S$ is used both the $\sim 7S$ and the $\sim 10S$ forms are present.

Table I

K_m values for substrates with different forms of carboxytransphosphorylase

Enzyme Form	Substrate	Apparent K_m mM	True K_m mM
<u>Pyruvate Reaction</u>			
7S	PEP	.017	
15S	PEP	.030	.031
7S	KP _i	.243	
15S	KP _i		.583
<u>Forward Reaction</u>			
7S	PEP	.083	
15S	PEP		.143

The apparent K_m 's for P-enolpyruvate were determined at the K_m level of orthophosphate (1.0 mM) with all remaining substrates saturating; those for orthophosphate were done with all other substrates saturating.

Last year we estimated by sucrose density gradient ultracentrifugation an $s_{20,w}$ value of 8.3S for the monomer occurring in the supernatant solution from crystallization of carboxytransphosphorylase, whereas that formed from tetramer by dialysis against dilute phosphate was found to have a $s_{20,w}$ value of 7.4S. It has now been determined that there is no significant difference in the sedimentation coefficients of the monomers from the two sources and that our last year's value was in error. The results of the present determinations are shown in Fig. 3. Note the absence of any evidence of dimer under the conditions of these experiments.

Fig. 3. Comparison of sedimentation of patterns of supernatant solution (A) and of the crystalline carboxytransphosphorylase (B) which had been dissociated by dialysis at low ionic strength. Sedimentation was in 5 to 20% sucrose density gradients containing 0.05 M potassium phosphate (pH 6.8) for 7.5 hour ultracentrifugation at 50,000 rpm. Solid lines indicate location of carboxytransphosphorylase by activity; dotted lines, positions of catalase (11.3S) and aldolase (8.0S) markers by their activities. Direction of sedimentation is to the right.

- A. Supernatant solution from crystallization (150 μ g/ml, 9.3 units/mg).
- B. 229 μ g dialyzed crystals, which prior to dialysis had a specific activity of 20 units/mg.

Sedimentation velocity determinations have also been done (Table II). The sedimentation coefficients are similar for the enzyme in the supernatant solution and also from the crystalline enzyme which has been dissociated by dialysis against low ionic strength buffer.

Table II

Sedimentation coefficients of carboxytransphosphorylase in
the supernatant solution and of crystals after dialysis against
low ionic strength buffer

Sample Source	S-values	Protein concentration
Crystals after 12.5 hour dialysis	15.7S 7.6S	4.28 mg/ml
Supernatant solution	15.6S 7.3S	0.52 mg/ml

The character of the centrifugation patterns indicate that either method yields a stable mixture of tetramers and monomers with little or no dimer present.

A rapid method of separating monomers from tetramers has been effected with Sephadex G-150 column chromatography. The results of a typical column are shown in Fig. 4, as well as analysis of the peak fractions by sucrose density gradient ultracentrifugation.

Fig. 4. Separation of $\sim 15S$ and $\sim 7S$ forms of carboxytransphosphorylase on a Sephadex G-150 column. 4 units (0.98 mg) was applied to $\sim 0.9 \times 45$ cm Sephadex G-150 column and eluted with 0.05 M potassium phosphate (pH 6.8) plus 0.001 M β -mercaptoethanol. 88% of the units and 100% of the protein were recovered. Scales: units/ml $\times 10$, mg/ml $\times 10^2$, units/mg $\times 1$.

Insets A, B, and C are the sucrose density gradient characterizations of peak fractions 25, 33, and 38, respectively. Solid lines indicate activity of carboxytransphosphorylase, and dotted lines, activity of catalase marker (11.3S).

Monomers isolated as in Fig. 4 have catalytic properties identical to those collected by the density gradient method, and have an $s_{20,w}^0 = 7.0$ based on extrapolation over a protein concentration range of 0.25 to 0.06 mg/ml.

In an effort to determine the approximate size of the protein chains composing carboxytransphosphorylase, the tetrameric crystals have been subjected to 72-hour dialysis against 6 M guanidine-hydrochloride and 0.1 M β -mercaptoethanol, acylation with iodoacetic acid, further dialysis against 6 M guanidine-hydrochloride, and then examination by high-speed, meniscus-depletion sedimentation equilibrium. The log C versus r^2 plots were linear, indicating homogeneity of the solution, and gave the following molecular weights using the indicated assumed partial specific volumes:

Table III

Estimated molecular weight of subunits formed from crystalline carboxy-transphosphorylase following treatment with 6 M guanidine-hydrochloride and acylation.

Sample concentration	Molecular weight		
	$\bar{v} = .700$	$\bar{v} = .725$	$\bar{v} = .750$
100 $\mu\text{g/ml}$	34,690	40,395	48,365
50 $\mu\text{g/ml}$	35,226	41,040	49,440

Evaluation of the partial specific volume by comparing sedimentation equilibrium of the tetramers in 90% D_2O to those in H_2O (Edelstein and Schachman, J. Biol. Chem. 242, 306 (1967)) is currently under investigation.

The partial amino acid analysis of carboxytransphosphorylase after 20 hour hydrolysis was presented in the 1969 Progress Report. Tryptophan has now been determined as well, and is listed in the following table.

Table IV

Amino acid composition of the $\sim 15S$ form of carboxytrans-phosphorylase

Amino Acid	μmoles	μg
lysine	137	12,222
histidine	121	18,736
arginine	221	38,531
aspartic acid	365	48,636
threonine	261	31,056
serine	246	25,861
glutamic acid	341	50,092
proline	223	25,700
glycine	203	15,270
alanine	275	24,510
valine	189	22,172
methionine	50	7,417
isoleucine	126	16,530
leucine	316	41,510
tyrosine	109	19,660
phenylalanine	126	20,874
cysteic acid	35	5,922
tryptophan	40	8,078
TOTAL		432,778

A tryptic digest and peptide spot map of crystalline carboxytransphosphorylase has been prepared in collaboration with J. Mendicino and coworkers, University of Georgia, Athens, Georgia; 80-89 peptide spots have been found out of a possible 358 (combined lysyl and arginyl residues). This suggests that there are four entities of similar peptide composition in the $\sim 15S$ species. Since the sedimentation equilibrium data of the guanidinated protein indicate that there are between eight and twelve polypeptide chains of similar size (430,000/35,000 to 430,000/50,000) one could speculate that crystalline carboxytransphosphorylase is composed of but two or three different types of chains.

C. Detection of Enzyme Bound Intermediate Compounds of the Reaction. We noted previously (p. 2) that carboxytransphosphorylase is inhibited by a large variety of metal chelators including EDTA. Although the EDTA treated enzyme does not catalyze the overall reactions, the inhibited enzyme does catalyze an exchange between CO_2 and the β carboxyl of oxalacetate. This exchange requires the presence of PP_i . This observation suggests that the mechanism involves a two-step process. This and other observations led to the proposal of the mechanism shown in Fig. 5 for the mechanism of the carboxytransphosphorylase reaction. (Biochem. 8, 3145 (1969)).

Fig. 5. Possible role of the dissociable type I metal (Mg^{2+}) and the unidentified tightly bound type II metal (Mn^{2+}) of carboxytransphosphorylase (E) in the oxalacetate reaction (A-1 to A-4) and the pyruvate reaction (A-1 to B-2). Conversions A-1 into A-4 are reversible. The dotted arrows indicate electron shifts when the conversion is from right to left and the solid arrows when it is from left to right. Conversion of A-4 into A-3 requires only the type I metal (Mg^{2+}). Thus CO_2 exchanges with the carboxyl group of oxalacetate when the type II metal is blocked by chelators. On the other hand, the phosphate cleavage requires both the type I and type II metals. Thus $^{32}P_i$ exchange occurs only when both metals are free. The pyruvate reaction A-1 to B-2 is experimentally irreversible and $^{32}P_i$ is not converted into phosphoenolpyruvate by this sequence.

In last year's Proposal, we planned to synthesize the intermediate (A-2) via chemical means and study the effect of the enzyme on this compound. It was considered that the ethyl ester of this compound (1) would have greater stability than the unesterified compound, but we were not certain what effect the ethyl group might have on the reactivity of the compound. We therefore proposed to examine the effect of the enzyme on ethyl phosphate (ethyl P_i , compound 2) and ethyl phosphoenolpyruvate

Ethyl pyrophosphoenolpyruvate

Ethyl phosphate

Ethyl phosphoenolpyruvate

(ethyl P-enolpyruvate, compound 3). If these compounds were found to act as substrates, then we felt that we would be safe in assuming that ethyl pyrophosphoenolpyruvate (1) could serve as a model for the proposed intermediate in the reaction.

Ethyl P_i and ethyl P-enolpyruvate were synthesized by Dr. Dean Griffith, Department of Chemistry, Case Western Reserve University and were tested in the standard assay for the forward oxalacetate reaction, both in the presence and absence of the natural substrate. The velocity of the reaction was followed by changes in optical density at 340 $m\mu$ in the presence of malate dehydrogenase. The results are summarized in Tables V and VI. In Table V it is seen that ethyl P_i neither serves as substrate for carboxytransphosphorylase, nor does it apparently inhibit the activity. The very slight optical density change occurring in the tests with ethyl P_i apparently are due to small traces of phosphate in the mixture.

Table V

Effect of ethyl phosphate in the carboxy-
transphosphorylase reaction

P_i mM	Ethyl P_i mM	$\Delta OD/min$
10.0	0	.016
10.0	9.5	.016
0	6.3	.003
0	12.6	.003

Table VI demonstrates that ethyl P-enolpyruvate also does not serve as a substrate for the reaction. Early experiments with this compound seemed to suggest that it was undergoing reaction. In these experiments, the solution of the compound

Table VI

Effect of ethyl phosphoenolpyruvate in
the carboxytransphosphorylase reaction

PEP mM	Ethyl P-enol pyruvate mM	$\Delta O. D.$ mi
2.5	--	.124
2.5	3.0	.125
--	3.0	0
--	6.0	0
--	9.0	0

were gassed with CO_2 before use. It is believed that this caused a acidic shift in pH to around 4.5 which is very near the pH maximum of the deethylation of ethyl P-enolpyruvate (Bruice, T. G. and Benkovic, S., "Bioorganic Mechanisms, Vol. 2", W. A. Benjamin, Inc., New York, 1966, p: 4). Thus, under these conditions, the ethyl P-enolpyruvate was most likely contaminated with trace amounts of P-enolpyruvate which was the source of the activity observed in the early experiment.

The observation that ethyl P_i does not serve as substrate for the carboxy-transphosphorylase reaction most likely precludes the use of ethyl pyrophosphoenolpyruvate as a model compound for the reaction intermediate. This is apparent by examination of the structure of the proposed intermediate (A-2 in Fig. 5) and the structure of ethyl pyrophosphoenolpyruvate (1). If ethyl pyrophosphoenolpyruvate is to be formed on the enzyme surface, it must be able to bind and utilize ethyl P_i as substrate.

Recently, Dr. Griffith has informed us that he believes it is feasible to synthesize pyrophosphoenolpyruvate directly, in a non-esterified form. If this synthesis is successful, we will test this compound for activity in the formation of pyruvate and pyrophosphate (pyruvate reaction) and of P-enolpyruvate or oxalacetate (oxalacetate reaction).

D. Examination of the Mechanism of the Carboxytransphosphorylase Reaction with ^{18}O . Mechanism I (Fig. 5) for the carboxytransphosphorylase reaction involves a hydrolytic step (A-2 to either A-3 or B-1). Therefore, one would predict that if the reaction were conducted in H_2^{18}O , the ^{18}O should appear in the product, pyrophosphate. The experiment to test this hypothesis was performed as follows. A reaction mixture containing P-enolpyruvate, MgCl_2 , KHCO_3 , CoCl_2 , P_i and mercaptoethanol was made up with a composition identical to that used in the assay. Water (H_2^{18}O) was added to give a final concentration of 1.4 atom % excess H_2^{18}O (this was sufficient to give a theoretical incorporation of 0.17 atom % excess into the PP_i). The mixture was then bubbled with CO_2 for 15 minutes and then incubated at 25° . Malate dehydrogenase and carboxytransphosphorylase were then added. Small aliquots of DPNH were added (this is because it has been found that DPNH is inhibitory at high concentrations) and aliquots of the mixture were removed at various times to determine the DPNH oxidized. When the DPNH oxidation ceased at a level of around 16.5 $\mu\text{moles DPN}^+$, the reaction was terminated by 3X dilution with cold water. The mixture was then subjected to chromatography on a column of Dowex-1- H^+ , using a dilute HCl linear gradient to elute the P_i and PP_i . The recovery of PP_i was essentially quantitative (98%). The PP_i was hydrolyzed to P_i by adjusting the pH to ~ 0.7 with HCl and heating in boiling water for 20 minutes. The resulting phosphate was crystallized as KH_2PO_4 (Miller and Lane, J. Biol. Chem. 243, 6041 (1968)), then converted to CO_2 by the method of Boyer et al. (Anal. Chem. 33, 1906 (1961)). In this procedure, phosphate is pyrolyzed in the presence of guanidine hydrochloride, thereby converting the oxygen in phosphate to oxygen in CO_2 . With the assistance of Dr. Cecil Cooper of this department, the mass ratios of the CO_2 were then determined in the mass spectrometer and the percent enrichment of ^{18}O calculated. No ^{18}O (above natural abundance) was found to be present in the pyrophosphate isolated from the reaction described above. There are two possible conclusions from these results. First, the mechanism described above does not apply for carboxytransphosphorylase. Second, the mechanism does apply, however, the OH^- released in $\text{A-1} \rightarrow \text{A-2}$ is the same one attacking in $\text{A-3} \rightarrow \text{A-4}$ and possibly is sequestered in some way such that it is not free to exchange with solvent water. Experiments are currently in progress to examine these two possibilities.

In December of last year, we were contacted by Dr. S. J. Benkovic of Pennsylvania State University, proposing a mechanism involving formation of a cyclic anhydride of P-enolpyruvate, Fig. 6.

Fig. 6. Hypothetical mechanism of the carboxytransphosphorylase reaction involving a cyclic anhydride of P-enolpyruvate.

It should be noted that this mechanism meets the two step criterion as discussed by Wood *et al.* (Biochemistry 8, 3145 (1969)). The mechanism also involves a hydrolytic step and predicts that the reaction should proceed with the incorporation of ^{18}O from water into the α -carboxyl of either pyruvate in the pyruvate reaction or in oxalacetate in the oxalacetate reaction in contrast to the mechanism of Fig. 5.

The possibility was therefore tested using H_2^{18}O . It was decided to use the pyruvate reaction rather than the oxalacetate reaction due to the ease of product isolation. A reaction mix was prepared containing P-enolpyruvate, P_i and MgCl_2 . Water (H_2^{18}O) was added to give a final atom percent excess of around 2%. The mixture was bubbled with nitrogen for 30 minutes to expell any CO_2 , then maintained at 25° under nitrogen. Inorganic pyrophosphatase was added to remove the pyrophosphate and thereby pull the reaction in the direction of pyruvate. Carboxytransphosphorylase was added and aliquots were removed at various times for determination of oxalacetate, P-enolpyruvate, and pyruvate. In all cases, no oxalacetate was found, demonstrating the absence of the oxalacetate reaction. When the level of pyruvate plateaued at around $36 \mu\text{moles}$ (the theoretical amount), the reaction was terminated by the addition of EDTA and cooling. The reaction mixture was diluted 3X with cold water, then passed over a column of QAE-Sephadex (A-25). The column was eluted with a linear gradient of triethylammonium acetate. The pyruvate fractions were pooled and taken to dryness by lypolization. Before lypolization, a small amount of NaOH (approximately equal to the amount of pyruvate) was added to prevent vaporization as pyruvic acid. Around $28 \mu\text{moles}$ of pyruvate were recovered at this point. The pyruvate was then dissolved in a small amount

of water, silver nitrate was added and the silver salt was crystallized. The silver salt was decarboxylated by heating under vacuum and the resulting CO_2 was collected and analyzed in the mass spectrometer.

A control experiment (to follow possible non-enzymatic incorporation of water into the carboxyl of pyruvate) was done under similar conditions. A mixture was made up having the same H_2^{18}O content and final composition as the experimental except the carboxytransphosphorylase was omitted. The pyruvate was isolated, purified and the ^{18}O content of the carboxyl group determined as described above. Both the experimental and control CO_2 had the same ^{18}O content, corresponding to the natural abundance, thereby indicating no incorporation from H_2^{18}O . The conclusions of this experiment are identical to those discussed above, i.e. either the mechanism is not applicable or the water involved in hydrolysis is not available for exchange with solvent water.

It should be noted that Chang *et al.* (J. Biol. Chem. 241, 242 (1966)) proposed a mechanism for P-enolpyruvate carboxykinase similar to that proposed for carboxytransphosphorylase (Fig. 5). The carboxykinase reaction involves inosine diphosphate and inosine triphosphate rather than phosphate and inorganic pyrophosphate. Their proposal was based in part on the observation of an inosine triphosphate dependent exchange of CO_2 into oxalacetate. However, Miller *et al.* (J. Biol. Chem. 243, 6030 (1968)) and Miller and Lane (J. Biol. Chem. 243, 6041 (1968)) latter proposed that the reaction proceeds via a one-step concerted mechanism. This proposal was based in part on kinetic evidence and in part on the observation that ^{18}O from water (H_2^{18}O) was not incorporated into P-enolpyruvate or inosine diphosphate, as would be predicted by the multi-step mechanism of Chang *et al.* In this case as well there is a possibility of sequestered water which might be unavailable for exchange with solvent water. In this case a two-step reaction could occur without incorporation of ^{18}O from H_2^{18}O .

We intend to pursue further in this year's Proposal the problem of non-exchangeable water in the carboxytransphosphorylase reaction.

E. Stereochemistry of the Carboxytransphosphorylase Reaction. These studies have been done in collaboration with Dr. Irwin Rose at the Institute for Cancer Research in Philadelphia and with Dr. James Willard at the University of Vermont College of Medicine.

Last year the stereochemistry of the carboxylation reaction of carboxytransphosphorylase was examined by the following set of reactions (J. Biol. Chem. 244, 6130 (1969)) using specifically labeled 3- ^3H -P-enolpyruvate.

The carboxylation was shown to occur predominantly by addition of CO₂ from the si side of the P-enolpyruvate. There was some degree of non-specificity since 25 to 35% of the T was released when the B position was labeled with T and 83 to 90 when the A position was labeled. With P-enolpyruvate carboxylase and carboxykinase the specificity was nearly complete.

This year the stereochemistry of the pyruvate reaction by carboxytransphosphorylase has been examined (Reaction 2). The experiment was done by the procedures developed by Cornforth in England, Eggerr in Germany, and Luthy in Switzerland (Nature 221, 1212 and 1213 (1969)), in which they were able to determine the chirality of methyl groups.

The pyruvate reaction was conducted with both types of P-enolpyruvate (i.e. labeled with tritium in A or B of the methylene group) and D₂O was used as solvent. If the reaction is stereospecific, the pyruvate formed should have one of the two chiralities given below:

The pyruvate formed in the reaction was converted to lactate with dogfish lactate dehydrogenase, isolated and then examined for the distribution of label. The lactates were analyzed as follows:

It was observed that fumarase released 50% of the tritium with lactates from the pyruvate from either type of T labeled P-enolpyruvate. Furthermore, the specific activities of the malates had only 60% the specific activity of the H,T-P-enolpyruvate, substrate. Two different explanations of these results appear possible: (1) the addition of the proton (here deuterium) is completely random, (2) the release of pyruvate from the enzyme is so slow as to allow the hydrogens of the pyruvate to be exchanged with deuterium before release of pyruvate. For such randomization to occur the course of events might be as shown in Fig. 7 which is based on Mechanism I of Fig. 5 (and also illustrated in Reactions 3 and 5). It is proposed that enzyme bound enolpyruvate is formed but the release of pyruvate from the enzyme is slow thus allowing keto-enol equilibration. Thus replacement of the H with a D might occur before release of the pyruvate. This mechanism would permit complete randomization and yet be consistent with our isotopic studies that the pyruvate reaction is essentially irreversible. Further study of this problem is being continued by I. Rose and J. Willard but will not be dealt with in this years Proposal since our involvement will be minimal.

Fig. 7. Mechanism for the loss of chirality in the pyruvate reaction.

II. Total Synthesis of Acetate from CO₂--D. Parker, M. Schulman, T. Wu, P. Kucera and H. G. Wood

Most of this research has been done in collaboration with Dr. Lars Ljungdahl, Department of Biochemistry, University of Georgia, Athens, Georgia. The principal investigator (HGW) spent the past year in Dr. Ljungdahl's laboratory on leave of absence and Drs. M. Schulman and P. Kucera accompanied him to this laboratory.

Last year we proposed to study the following subjects: (A) Formyl, Methylene and Methylfolate Derivatives as Intermediates in the Total Synthesis of Acetate. (B) Acetate Synthesis by Cell Free Extracts of C. thermoaceticum, (C) Isolation of Active Corrinoid Proteins Involved in the Synthesis of Acetate from CO₂, and (D) General Occurrence of Corrinoid Dependent Total Synthesis² of Acetate from CO₂. These subjects will be considered below following a short description² of the fermentation.

C. thermoaceticum has the ability to ferment a variety of substrate anaerobically and to use CO₂ as a hydrogen acceptor in its oxidation reduction processes. The reduction takes place by a mechanism which leads to the total synthesis of acetate from CO₂. Mainly from the results of our laboratories and those of Earl Stadtman a general outline of the mechanism of this total synthesis of acetate has been formulated as shown in Reactions 6 to 13 for the fermentation of pyruvate. Corrinoid-E is an abbreviation for an enzyme-corrinoid complex and THF is tetrahydrofolate. Reaction 13 gives the overall stoichiometry with 4 moles of pyruvate yielding 5 of acetate one of which is totally synthesized from CO₂.

A. Formyl Methylene and Methyl Folate Derivatives as Intermediates in the Total Synthesis of Acetate. Although the reactions for the conversion of formate to methyltetrahydrofolate have been well documented in various clostridia, none of the enzymes involved have been isolated from *C. thermoaceticum* and purified extensively except for formyltetrahydrofolate synthetase. Purification of methylene tetrahydrofolate dehydrogenase is underway in Dr. Ljungdahl's laboratory. Even if the enzymes are shown to be present it does not prove that they are directly involved in acetate synthesis since the same enzymes are involved in methionine synthesis and other metabolic processes. More direct evidence of their probable role in acetate synthesis would be a demonstration in pulse labeling experiments with $^{14}\text{CO}_2$ that the folate derivatives become highly labeled in accordance with predictions for a precursor relationship to acetate.

Last year we reported on the isolation of methyltetrahydrofolate (mono-, di- and triglutamates) from whole cell preparations of *C. thermoaceticum* fermenting glucose following 15 second exposure to $^{14}\text{CO}_2$. The ^{14}C was found exclusively in the 5-methyl position of the folates with a specific activity very close to that of the $^{14}\text{CO}_2$ and greater than that of the methyl acetate. To further clarify the sequence of events from $^{14}\text{CO}_2$ to methyltetrahydrofolate we proposed that formyl and methylene tetrahydrofolate derivatives be isolated from similar large scale experiments and their specific activities be determined. The details of these experiments will not be presented since the methods were essentially the same as given in last year's Proposal. They included chromatography on QAE Sephadex, analysis of the various fraction by microbial assay using *S. faecalis* and *L. casei*, treatment with conjugase from hog kidney and then separation of the products by a second chromatography on QAE Sephadex followed by microbial assay of the fractions. The results of the experiments were not as successful as those reported last year, partly because the cells (for reasons unknown) failed to synthesize acetate effectively from the $^{14}\text{CO}_2$. The formyl derivatives were identified however, and they had a high specific activity. The overall evidence of these pulse labeling experiments does support the proposal that the folate derivatives are intermediates in the synthesis of acetate. A decision as to which form of folate (mono-, di- or triglutamate) is active was not possible from these experiments. All three folates were highly labeled.

These studies have been done exclusively in the Department of Biochemistry at Case Western Reserve University by Dr. Donald Parker.

B. Acetate Synthesis by Cell Free Extracts of *C. thermoaceticum*. The fermentation of pyruvate by extracts with accompanying total synthesis of acetate from CO_2 requires the combined action of a large battery of enzymes; those involved in the oxidation of pyruvate to acetate and CO_2 and reduction of CO_2 to formate, the series of enzymes involved in the formation of the methyltetrahydrofolate from formate and then transmethylation of the methyl to the corrinoid enzyme complex and carboxylation to yield acetate (Reactions 6-13). Synthesis of acetate from CO_2 has been observed in the fermentations of pyruvate with crude extracts in our laboratory and in Stadtman's, but we have never obtained a consistent

formation of substantial amount of acetate from CO_2 . Table VII shows some typical results with extracts. Measurements were made on a respirometer and the pyruvate fermented, acetate produced, total CO_2 fixed and acetate formed from CO_2 were determined. Although a considerable amount of pyruvate was fermented the yield of acetate was low (about 0.2 μmoles per mole of pyruvate) and very little acetate was synthesized from the $^{14}\text{CO}_2$. Further studies showed that crude extracts of frozen cells were a suitable source of enzymes for the conversion of $^{14}\text{CH}_3\text{-B}_{12}$ or $^{14}\text{CH}_3\text{-THF}$ to the methyl of acetate in the fermentation of pyruvate although the extracts did not catalyze effectively the total synthesis of acetate from CO_2 .

It is seen (Table VIII) that ferrous ions and pyruvate were required for the conversion of ^{14}C -methyltetrahydrofolate to acetate and this was also true for the utilization of $^{14}\text{CH}_3\text{-B}_{12}$. Table IX shows a comparison of the utilization of $\text{CH}_3\text{-B}_{12}$ and $\text{CH}_3\text{-THF}$. Both substrates were utilized at about the same rate. The same enzyme preparation also ferments α -ketobutyrate and converts $^{14}\text{CH-B}_{12}$ to acetate, Table X.

Table VII

Fermentation of Pyruvate with Extracts of *C. thermoaceticum* in Presence of $^{14}\text{CO}_2$

Time 160 min

Description of Extract	Protein mg	Pyruvate Fermented μmoles	Respiro- meter CO_2 μmoles	Total CO_2 Fixed μmoles	Total Acetate Produced μmoles	Acetate per Pyruvate μmoles	Acetate from $^{14}\text{CO}_2$ μmoles	Acetate from $^{14}\text{CO}_2$ per pyruvate μmoles
¹ Extract from washed cells	18	77	53	11	11	.14	0.17	.002
² Extract after being frozen 6 days	36	77	50	7	14	.18	0.04	.0005
³ Extract from frozen cells	16	69	25	19	14	.20	0.06	.0009
⁴ Extract from unwashed cells and precipitated with $(\text{NH}_4)_2\text{SO}_4$	11	37	14	15	7	.19	0.15	.004

Reaction mixture contained 0.1 M phosphate (pH 6.8), 0.1 M $\text{NaH}^{14}\text{CO}_3$, 0.018 M ATP, 0.001 M DPN, 0.035 M β -mercaptoethanol, 0.05 M pyruvate, and protein as indicated. In addition No. 4 contained 0.025 M glucose. Temperature 50°C under CO_2 , CO_2 measured in a Warburg respirometer.

Table VIII

Effect of Pyruvate and Ferrous Compound on Synthesis of ^{14}C Acetate From Methyl-tetrahydrofolate (CH_3THF) by an Ammonium Sulfate Precipitate of an Extract of
C. thermoaceticum

Conditions	Acetate from $^{14}\text{CH}_3\text{-THF}$ μmoles	Conversion of $^{14}\text{CH}_3\text{-THF}$ to acetate %
Complete reaction mixture	0.178	118
minus pyruvate	0.016	10
minus ferrous ammonium sulfate	0.008	5
minus pyruvate and ferrous ammonium sulfate	0.008	5

Reactions were conducted in test tubes under argon with pyruvate and $^{14}\text{CH}_3\text{-THF}$ as substrates. The total volume was 0.5 ml containing 7.25 mg of enzyme, temperature 57° , time of conversion of $^{14}\text{CH}_3\text{-THF}$ was 3 min. Yields are expressed per ml of reaction and % conversion on the basis of 50% of $^{14}\text{CH}_3\text{-THF}$ being a utilizable isomer. The mixture contained in mM concentrations: pyruvate, 30; freshly prepared $\text{Fe}(\text{NH}_4)(\text{SO}_4)_2$, 3; phosphate buffer (pH 6.8), 50; cysteine, 2; $^{14}\text{CH}_3\text{-THF}$, 0.3; and the enzyme, 7.25 mg. All components except the $^{14}\text{CH}_3\text{-THF}$ were placed in 1 X 6 cm test tube with serum stoppers and flushed with argon 5 times. The mixture was then incubated for 10 min at 57° and then the $^{14}\text{CH}_3\text{-THF}$ was injected and the incubation was continued for the stated 3 min. The enzyme was prepared from frozen cells under argon using a French press. It was precipitated with 90% saturated $(\text{NH}_4)_2\text{SO}_4$ and dialyzed against phosphate cysteine buffer for 10 hrs under argon.

Table IX

Rate of Synthesis of ^{14}C -Acetate from $^{14}\text{CH}_3\text{B}_{12}$ and $^{14}\text{CH}_3$ Tetrahydro-
folate in Fermentations of Pyruvate

Period of Incubation	Acetate from $\text{CH}_3\text{-B}_{12}$ μmoles	Conversion of $\text{CH}_3\text{-B}_{12}$ to acetate %	Acetate from $\text{CH}_3\text{-THF}$ μmoles	Conversion of $\text{CH}_3\text{-THF}$ to acetate %
3'	0.112	22	0.08	27
6'	0.198	40	0.20	68
9'	0.268	54	0.32	108
12'	0.311	62	--	--

Reactions were as described in Table VIII. The $^{14}\text{CH}_3\text{-B}_{12}$ was 0.5 mM (2.2×10^5 dpm/ μmole) and $^{14}\text{CH}_3\text{THF}$ 0.59 mM (6.55×10^5 dpm/ μmole). Total volume was 1 ml. Yields of acetate are per ml and conversion of $^{14}\text{CH}_3\text{-THF}$ on basis of 50% being utilizable by the enzyme.

Table X

Rate of Acetate Synthesis from $^{14}\text{CH}_3\text{B}_{12}$ in a Fermentation of α -Ketobutyrate

Time of Incubation min	α -Ketobutyrate utilized μmoles	Acetate from $\text{CH}_3\text{-B}_{12}$ μmoles	Conversion of $\text{CH}_3\text{-B}_{12}$ to acetate %
10	3.7	0.053	
20	6.9	0.234	48
30	8.0	0.388	79
40	9.0	0.435	89

Five tubes were set up (one for zero time) containing 14.5 mg each of enzyme, sodium α -ketobutyrate, 30 mM; ferrous ammonium sulfate, 3 mM; ^{14}C -methyl B_{12} , 0.49 mM containing 1.01×10^5 dpm; potassium phosphate (pH 7), 50 mM; and cysteine, 2 mM in a total volume of 1 ml. At the end of each time period, 0.20 ml aliquots were withdrawn from a tube and transferred to 0.04 ml of 6 N H_2SO_4 for termination of the reaction prior to determination of the α -ketobutyrate by assay with lactate dehydrogenase. The remainder of 0.80 ml was used to assay the other products.

It is characteristic of reactions involving methylcorrinoids that the cobalt is alkylated by alkylhalides and the corrinoid enzyme is inactivated. Brot and Weissbach have studied this reaction with homocysteine transmethylase. They showed that the cobalt-propyl bond is cleaved by photolysis yielding an active enzyme. Wood and Wolfe have conducted similar experiments in studies of methane formation by *M. omelianski*. Bauchop and Wood and his collaborators have also shown that low concentrations of methylene chloride, chloroform and carbon tetrachloride inhibit methane formation. If the total synthesis of acetate from CO_2 by *C. thermoaceticum* involves transmethylation from methyltetrahydrofolate to form a methyl corrinoid enzyme complex (Reaction 10) then the synthesis of acetate from $^{14}\text{CH}_3\text{-THF}$ should be inhibited by the alkylhalides. If the inhibition were reversed by light it would be strong indication that the inhibition is by alkylation of the Co of the corrinoid enzyme rather than by inhibition of some other enzyme of the complex, for example an SH enzyme.

The effect of propyl iodide and carbon tetrachloride on conversion of $^{14}\text{CH}_2\text{-B}_{12}$ and $^{14}\text{CH}_3\text{-THF}$ to acetate is shown in Table XI. It was found that both propyl iodide and carbon tetrachloride at 1 mM concentration substantially inhibit the conversion of $^{14}\text{CH}_3\text{-THF}$ to acetate after a 2 min preincubation with the halide. This is the same concentration of propyl iodide that inhibited the fixation of $^{14}\text{CO}_2$ into acetate during fermentations of glucose with cell suspension (see below), but more carbon tetrachloride was required than with cells since 2.5×10^{-5} M was effective with cells. There was less inhibition with 1 mM alkyl halides of the conversion of $\text{CH}_3\text{-B}_{12}$ to acetate than of $\text{CH}_3\text{-THF}$ (Table XI). In part this may be due to differences in the experiments since the experiment with $^{14}\text{CH}_3\text{-B}_{12}$ was for 24 min and that with $^{14}\text{CH}_3\text{-THF}$ for 3 min.

Table XI
Inhibition by Propyl Iodide and Carbon Tetrachloride of the Synthesis of Acetate from $^{14}\text{CH}_3\text{-B}_{12}$ and $^{14}\text{CH}_3\text{-THF}$ during Fermentations of Pyruvate

Inhibitor	Concentration of inhibitor	Acetate from $\text{CH}_3\text{-B}_{12}$		Acetate from $\text{CH}_3\text{-THF}$	
		Yield 24 min	Inhibition	Yield 3 min	Inhibition
	mM	μmoles	%	μmoles	%
Propyl iodide	10	0.019	92	0.012	89
"	1	0.15	38	0.034	68
"	0.1	0.21	19	0.118	0
None	0	0.26	--	0.106	--
Carbon tetrachloride	10	0.018	94	0.010	91
"	1	0.26	28	0.016	85
"	0.1	0.35	3	0.076	28
None	0	0.36		0.106	--

The reaction mixtures contained 15 mg enzyme, sodium pyruvate, 30 mM. $\text{Fe}(\text{NH}_4)_2 (\text{SO}_4)_2$, 3 mM; potassium phosphate buffer, 50 mM (pH 6.8); cysteine, 2 mM and $^{14}\text{CH}_3\text{-B}_{12}$ 0.5 mM (101,000 dpm) or 0.3 mM $^{14}\text{CH}_3\text{-THF}$ (160,000 dpm). Propyl iodide or carbon tetrachloride was added in 0.02 ml of 95% ethanol after 10 min preincubation and 2 min later the $^{14}\text{CH}_3\text{-B}_{12}$ or $^{14}\text{CH}_3\text{-THF}$ was

injected. 0.02 ml of ethanol was injected in the control. The volume was 1 ml under argon and was at 57° in the dark. Time of incubation after $^{14}\text{CH}_3\text{-B}_{12}$ was injected was 24 min and after $^{14}\text{CH}_3\text{-THF}$ was injected 3 min.

Table XII shows the results of experiments on reactivation with light. Enzyme was treated with propyl iodide or carbon tetrachloride in the presence of the complete reaction mixture containing pyruvate. Fermentation was considered favorable for the combination with halide since the cobalt of the enzyme would be maintained in a reduced state. Taylor and Weissbach have shown that reduction is required for propylation of the cobalt of the corrinoid of homocysteine transmethylase.

After removal of the excess halide by dialysis, the photolysis was done in the presence of the complete reaction mixture so that the enzyme would be maintained in the reduced state and perhaps be stabilized by methylation. Following the photolysis the $^{14}\text{CH}_3\text{-THF}$ or $^{14}\text{CH}_3\text{-B}_{12}$ was added to the reaction mixture and the conversion to acetate was determined. It was found (Table XII) that both halide treated enzymes when protected from light gave reduced conversion of either $\text{CH}_3\text{-THF}$ or $\text{CH}_3\text{-B}_{12}$ to acetate when compared with the controls. The yield of acetate was about 50% of that of the control, with $\text{CH}_3\text{-THF}$ or $\text{CH}_3\text{-B}_{12}$ and was 9 and 16% with carbon tetrachloride. On exposure to light the enzyme treated with propyl iodide was fully reactivated whereas those treated with carbon tetrachloride showed little change. This result may indicate that the complex of carbon tetrachloride with the Co-atom of the corrinoid is not cleaved by light. Taylor and Weissbach have shown that the ^{14}C of $^{14}\text{CH}_3\text{-Co}$ homocysteine transmethylase was not cleaved by light unless the pH was lowered to about 2 whereas the propylated enzyme was readily cleaved at neutral pH. The carbon tetrachloride complex may have characteristics similar to the methylated enzyme. On the other hand the carbon tetrachloride complex may be cleaved from the corrinoid enzyme by light but the enzyme complex may remain inactive due to effects on other enzymes of the system. Wood *et al.* have shown that the methyl is cleaved photolytically from the bacterial enzyme catalyzing the synthesis of methane. Although further study is required, the results with propyl iodide lend substantial support for the proposed role of corrinoids in the total synthesis of acetate.

Table XII

Photolytic Reactivation of Enzyme Inhibited by Halides

Inhibitor	Condition before assay	^{14}C -substrate	Acetate from ^{14}C -substrate *	Yield Compared with control %
Propyl iodide	Exposed to light	$\text{CH}_3\text{-THF}$	0.130	120
"	Protected from light	$\text{CH}_3\text{-THF}$	0.066	52
"	Exposed to light	$\text{CH}_3\text{-B}_{12}$	0.33	95
"	Protected from light	$\text{CH}_3\text{-B}_{12}$	0.17	58
Carbon tetrachloride	Exposed to light	$\text{CH}_3\text{-THF}$	0.022	20
"	Protected from light	"	0.012	9
"	Exposed to light	$\text{CH}_3\text{-B}_{12}$	.060	17
"	Protected from light	"	.046	16
Control	Exposed to light	$\text{CH}_3\text{-THF}$	0.104	
"	Protected from light	"	0.128	
"	Exposed to light	$\text{CH}_3\text{-B}_{12}$	.346	
"	Protected from light	"	.294	

* 10 min incubation with $^{14}\text{CH}_3\text{-THF}$ and 20 min with $^{14}\text{CH}_3\text{-B}_{12}$

Treatment of enzymes with halide: The mixtures contained 90 mg enzyme, potassium pyruvate, 0.048 M; ferrous ammonium sulfate, 5 mM; potassium phosphate buffer, 50 mM (pH 7.0); cysteine, 4 mM; and where indicated propyl iodide, 1.6 mM or carbon tetrachloride, 1.6 mM, in a total volume of 3.84 ml. All components minus the inhibitor were preincubated with argon and in the dark for 10 min. Then the appropriate inhibitor in .12 ml of ethanol was injected and incubation continued for 15 min. A control containing 0.12 ml ethanol in place of the inhibitor solution was similarly prepared.

Removal of excess inhibitor by dialysis: All the preparations were dialyzed twice in the dark for 4 hrs each time against 100 ml of the dialysis fluid retained from the dialysis of the enzyme following precipitation with 90% saturated $(\text{NH}_4)_2\text{SO}_4$.

Assay of dialyzed enzymes with and without exposure to light: The mixtures contained 15 mg enzyme per ml of reaction mixture: potassium pyruvate, 30 mM; ferrous ammonium sulfate, 0.3 mM; potassium phosphate buffer, 60 mM (pH 7.0); cysteine, 2 mM; and either $^{14}\text{CH}_3\text{THF}$ 0.3 mM (containing 99,200 dpm) or $^{14}\text{CH}_3\text{C}_{12}$ 0.5 mM (containing 108,800 dpm). Reaction

(Table XII, continued)

volume was 0.50 ml with CH₃-THF and 1.0 ml with CH₃-B₁₂.

Exposure of the enzyme to light: One of the duplicate tubes of the above reaction mixture under argon was exposed to light and the other was kept dark. The ¹⁴CH₃-B₁₂ and ¹⁴CH₃-THF were added to each after the light treatment. Exposure was to a 200 W lamp for 10 min at a distance of about 6 cm at room temperature. The tubes were then removed to the dark room, the labeled compounds injected and incubation allowed for 10 min with CH₃-THF and 20 min with CH₃-B₁₂ at 57°. Values are in μ moles per ml.

Further evidence that the propylation involves a corrinoid has been obtained using ¹⁴C-propyl iodide. For this purpose ¹⁴C-propylated protein was treated with light and the controls were protected from light. The proteins were then extracted with ethanol as described by Taylor and Weissbach to obtain the "corrinoid" and protein fractions. The radioactivity of the corrinoid and protein fractions were then compared from light treated ¹⁴C-propylated protein and propylated protein which had not been treated with light. Two experiments were done, (A) in which cyanide was added and (B) without cyanide. It was considered that the cleavage in the presence of cyanide might be more complete, and that the cyanide would prevent the propyl radicle formed during the photolysis from reacting with the protein. This would prevent ¹⁴C-propyl groups from remaining attached to the protein although no longer attached to the Co-atom of the corrinoid. The results of Table XIII show that light treatment with or without cyanide caused about 60% decrease in the ¹⁴C of the corrinoid fraction and little change in the protein fractions. In addition the "corrinoid" fraction obtained by ethanol extraction of ¹⁴C-propylated protein was treated with borohydride. This treatment reduces the Co-propyl group to propane and thus cause a loss of ¹⁴C. There was a 71% loss of ¹⁴C following this treatment (Table XIII). Thus these results are in complete accord with the view that the propyl iodide inhibits synthesis of acetate by combination with cobalt of a corrinoid enzyme.

Table XIII
Cleavage of ^{14}C -Propyl from ^{14}C -Propylated Cell Free
Extract of *C. thermoaceticum*

Condition Employed	Fraction	^{14}C in	^{14}C released
		Fraction dpm	by light or borohydride %
A. CN and exposed to light "	Corrinoid fraction	11,600	66
	Precipitate fraction	6,200	10
	CN and protected from light	31,200	-
	"	Precipitate fraction 6,900	-
B. Exposed to light "	Corrinoid fraction	9,480	61
	Precipitate fraction	11,500	0
	Protected from light	24,630	-
	"	Precipitate fraction 10,500	-
C. Treated with borohydride	Corrinoid fraction	7,180	71
Untreated control	Corrinoid fraction	24,880	-

The protein was treated with ^{14}C propyl iodide in the presence of pyruvate and dialyzed to remove the excess halide as described in Table XII.

A. 0.005 ml of 1 M sodium cyanide was added to 0.5 ml in duplicate tubes containing 13 mg of ^{14}C -propylated protein (80,000 dpm). One of the duplicates was exposed to a 300 W lamp bulb for 10 min at a distance of 6 cm at room temperature. The other was kept dark.

Each sample was then extracted with hot ethanol and the precipitated proteins were removed by centrifugation. The supernatant solution was evaporated under vacuum on a rotary evaporator. The residue was dissolved in about 3 ml H_2O , and re-evaporated. The residue was dissolved in 2 ml H_2O and formed the "corrinoid fraction". The precipitated protein obtained on centrifugation of the alcohol denatured material was suspended in 2 ml 70% ethanol and centrifuged. The precipitate was dissolved in 0.2 M sodium hydroxide and constituted the precipitate fraction.

B. Treatment as in A except that cyanide was not added.

C. Two 0.50 ml samples of the ^{14}C -propylated protein were extracted with hot ethanol as outlined in A to obtain the corrinoid fraction; each fraction was taken up in 1 ml H_2O . To one fraction was added 20 mg sodium borohydride and left overnight in the dark; the other fraction formed the untreated control. The borohydride treated sample was then neutralized

(Table XIII continued)

with 1 N H_2SO_4 to pH 7.0. Both the experimental and control samples were then lyophilized to dryness, and after adding 1 ml of H_2O lyophilized again. Each sample was then taken up in 0.50 ml H_2O and assayed for radioactivity.

C. Isolation of Corrinoid Protein Involved in the Synthesis of Acetate from CO_2 . It was proposed last year that attempts be made to isolate the corrinoid proteins by use of their reaction with ^{14}C -propyl iodide. For this purpose it was decided to investigate the inhibition with whole cells. If the inhibition was observed, extracts of these cells might be used for isolation of the corrinoid protein which had reacted with the ^{14}C -propyl iodide. The results of the inhibition studies with different halides are shown in Table XIV.

Table XIV
Inhibition by Halides of the Conversion of $^{14}\text{CO}_2$ to Acetate by
Suspensions of *C. thermoaceticum* Fermenting Glucose

Halide	Glucose Fermented μmoles	Total $^{14}\text{CO}_2$ Fixed μmoles	Acetate from $^{14}\text{CO}_2$ μmoles	Respirometer CO_2 μmoles
$\text{CH}_3\text{CH}_2\text{CH}_2\text{I}$	20	11	0.4	35
None	172	202	67	260
CHCl_3	29	21	0.8	54
None	164	331	141	293
CCl_4	88	121	0.4	24
None	171	136	56	136

The reaction mixture contained 1.5 ml of a cell suspension (unwashed) consisting of 1 part wet weight of cells and 2 parts by volume of a glucose, phosphate mercaptoethanol solution in a final volume of 3 ml and where indicated 0.05 ml of the halide. The mixtures contained 0.5 g cells, and in μmoles glucose 200; phosphate, 100; mercaptoethanol, 50; NaHCO_3 , 300; $\text{CH}_3\text{CH}_2\text{CH}_2\text{I}$, 510; CHCl_3 , 620; or CCl_4 , 520. Atmosphere CO_2 , temperature 50°C and time 3 hr. The total CO_2 fixed was determined on a portion of the fermentation after bubbling with $^{12}\text{CO}_2$ to remove unfixed $^{14}\text{CO}_2$. The ^{14}C in the acetate was determined by KMnO_4 oxidation and ether extraction. The μmoles of acetate formed from CO_2 was estimated by dividing the total dpm in the acetate by twice the dpm per μmole of $^{14}\text{CO}_2$. The latter was determined on $^{14}\text{CO}_2$ collected from the fermentation.

It is seen that in the absence of halide there was a substantial amount of acetate formed from CO_2 . If the fermentation occurred according to proposed mechanisms 3 moles of acetate would be formed per mole of glucose, one of which would arise from CO_2 . Accordingly the μmoles of acetate formed from $^{14}\text{CO}_2$ should be equal to the μmoles of fermented glucose. The observed yield was substantially lower than this. Of the total CO_2 fixed the majority was present in acetate but there was some fixation into other products. The CO_2 observed by manometric measurement should equal the acid produced and theoretically be three times the acetate formed from CO_2 . There was considerable variation from the theoretical value.

Each halide inhibited the fixation of CO_2 into acetate almost completely and there was a strong inhibition of the fermentation of glucose. This inhibition might be because fermentation of glucose is dependent on the coupled reduction of CO_2 to acetate or that fermentation of glucose is inhibited by the halide non-specifically and thus CO_2 fixation is inhibited indirectly. For example propyl iodide might react with SH groups of enzymes. Only with carbon tetrachloride was there substantial utilization of glucose even though there was substantial inhibition of CO_2 fixation into acetate. This fact indicated that carbon tetrachloride might be a specific inhibitor of CO_2 fixation and that coupling of CO_2 reduction to acetate was not essential for fermentation of glucose.

The inhibition with time is shown in Table XV. It is seen that the inhibition occurs very rapidly and was almost complete.

Table XV

Inhibition with Time by 9×10^{-3} M Propyl Iodide or Carbon Tetrachloride of the Conversion of $^{14}\text{CO}_2$ to Acetate by Suspension of *C. thermoaceticum* Fermenting Glucose

Time	No Inhibitor	Plus CCl_4		Plus $\text{CH}_3\text{CH}_2\text{CH}_2\text{I}$	
min	dpm/ml	dpm/ml	% Inhibition	dpm/ml	% Inhibition
0	2,410	960	-	515	-
5	20,500	1,760	91	1,480	93
10	37,700	1,970	95	3,160	92
15	62,200	2,430	96	3,510	94
30	102,800	2,540	97	3,740	96

The cells were suspended without washing in the proportion of 1 g of cells (wet weight) to 2 ml of 0.05 M glucose, 0.1 M potassium phosphate buffer (pH 6.8) and 0.05 M β -mercaptoethanol. 4.5 ml of this suspension was added to 4.4 ml of solution containing 900 μmoles of NaHCO_3 in test tubes closed with serum caps and fitted with syringe needles for gassing with CO_2 . The mixture was gassed with CO_2 for 10 min at 50° . Gassing was terminated and 0.06 ml of $\text{NaH}^{14}\text{CO}_3$ (5×10^9 dpm/ml) was added followed immediately by 0.15 ml of 95% ethanol in tube 1, 0.15 of 5% (V/V) of CCl_4 in 95% ethanol in tube 2 and 0.15 ml of 5% (V/V) $\text{CH}_3\text{CH}_2\text{CH}_2\text{I}$ in 95% ethanol in tube 3. Samples were removed at the indicated time for determination of the ^{14}C fixed in the acetate.

The concentration of propyliodide or carbon tetrachloride which is required to inhibit fixation of CO_2 in acetate during fermentation of glucose is shown in Table XVI. The halide was added to the fermentation and 5 min later the labeled $\text{NaH}^{14}\text{CO}_2$. The fermentations were continued for 10 min more and then stopped by the addition of acid. It is seen that carbon tetrachloride was more effective than propyl iodide, 5×10^{-4} M propyl iodide was required for 65% inhibition and 1×10^{-4} was not inhibitory whereas with CCl_4 2.5×10^{-5} M produced 90% inhibition.

Table XVI

Concentration of Halide and the Inhibition of Conversion of $^{14}\text{CO}_2$ to Acetate by Suspensions of *C. thermoaceticum* Fermenting Glucose

Molarity of Halide	Inhibition by $\text{CH}_3\text{CH}_2\text{CH}_2\text{I}$	Inhibition by CCl_4
	%	%
None	0	0
1×10^{-2}	98	-
5×10^{-3}	93	-
1×10^{-3}	82	99
5×10^{-4}	65	97
1×10^{-4}	0	94
5×10^{-5}	-	91
2.5×10^{-5}	-	90
1×10^{-5}	0	10

Cells from 48 hr growth were harvested and suspended in the proportion of 1 g (wet weight) to 2 ml of 0.05 M glucose, 0.1 M phosphate (pH 6.8) and 0.05 M β -mercaptoethanol under argon. 1.5 ml of the suspension was added to 1.49 ml of solution containing 300 μmole of NaHCO_3 which was in a 10 ml test tube. A series of tubes were prepared and closed with serum caps and gassed with CO_2 from 10 min at 50° . Then the proper concentration of inhibitor was added in 0.05 ml of 95% ethanol. Five min later 0.01 ml of $\text{NaH}^{14}\text{CO}_3$ (1.65×10^8 dpm) was added. After 10 min the reaction was stopped by the addition of 0.5 ml of 6 N H_2SO_4 and the ^{14}C in the acetate was determined.

On the basis of these experiments it seemed that a rapid and perhaps preferential inhibition of the corrinoid enzyme might occur under these conditions. Therefore experiments were conducted to determine the distribution of ^{14}C -labeled halides in extracts of the cells and to demonstrate that the halides combine with the corrinoids and thus cause the inhibition of fixation of CO_2 in acetate. These results will not be dealt with in detail but may be summarized as follows. It could be shown that both ^{14}C -carbon tetrachloride and ^{14}C -propyl iodide combine with the corrinoids but there was only about 5% of the fixed halide in this form. Therefore this did not prove to be a satisfactory method for specifically labeling the corrinoid proteins for isolation. The results with crude extracts were more satisfactory, Table XII, and further studies are planned using such preparations. 32

The studies reported in this section (C) and those under (B) were done in Athens, Georgia in collaboration with Lars Ljungdahl and Roy Ghambeer. Only a portion of the support of this research came from the present contract.

D. General Occurrence of Corrinoid Dependent Total Synthesis of Acetate from CO₂. C. Thermoaceticum is only one of a number of anaerobic heterotrophic microorganisms that incorporates CO₂ into both carbons of acetate. In accordance with last years Proposal we have initiated studies of several of these organisms. It will be determined whether or not these organisms totally synthesize acetate from CO₂ or whether they make a mixture in which the methyl group of part of the molecules is formed from CO₂ and the carboxyl group of other molecules is from CO₂ but none are totally from CO₂. This will be done by carrying out fermentation in the presence of ¹³CO₂ containing a high concentration of ¹³C. The mass of the resulting acetate will be determined in a mass spectrometer. Mass 60, 61 and 62 will be measured, 60 representing unlabeled acetate from the fermented substrate, 61 acetate containing carbon from one ¹³CO₂ and 62 acetate containing carbon from two ¹³CO₂, i.e. acetate totally synthesized from CO₂.

The following organisms are under study Clostridium acetium, Clostridium cylindrosporum, Clostridium acidurici, Peptococcus glycinophilus and Butyribacterium rettgeri. Thus far ¹³C acetate has been isolated from fermentations by all these organisms but due to difficulties with the mass spectrometry the mass ratios have not been determined. These experiments are being done by Dr. Marvin Schulman.

Publications

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