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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 three headings given in last year's Proposal:

I. Mechanism of the Carboxytransphosphorylase Reaction--J. M. Willard, M. E. Haberland, R. Singleton and H. G. Wood.

II. Catalysis and Mechanism of the Pyruvate, Phosphate Dikinase Reaction--H. J. Evans and H. G. Wood.

III. Total Synthesis of Acetate from CO_2 by Clostridium thermoaceticum--M. D. Schulman, D. Parker, T. Wu, P. Kucera and H. G. Wood.

I. The Mechanism of the Carboxytransphosphorylase Reaction--J. M. Willard, M. E. Haberland, R. Singleton and H. G. Wood.

Carboxytransphosphorylase from Propionibacterium shermanii catalyzes the following reactions:

$$\text{P-enolpyruvate} + CO_2 + P_i \overset{Mg^{2+}}{\rightleftharpoons} \text{oxalacetate} + PP_i \qquad (1)$$

and

$$\text{P-enolpyruvate} + P_i \overset{Mg^{2+}}{\rightleftharpoons} \text{pyruvate} + PP_i \qquad (2)$$

Reaction 1 is called the oxalacetate reaction and is reversible. Reaction 2 is called the pyruvate reaction and is irreversible. The enzyme is responsible for CO_2 fixation by these organisms. Three manuscripts were completed this year which deal with the mechanism of the reaction and the role of metal ions in the catalysis. Reprints are included with this report. These results have permitted the formulation of a hypothetical mechanism as illustrated below for the overall reaction and form the basis of the present studies.

DISCLAIMER

$$E\begin{matrix}\diagup \\ \diagdown\end{matrix}\begin{matrix}\\ MeII\end{matrix} + Mg^{2+} + P_i + PEP \rightleftarrows E\begin{matrix}\diagup Mg\text{-}P_i \\ \diagdown MeII\text{-}PEP\end{matrix} \rightleftarrows E\begin{matrix}\diagup Mg\text{-}PPEP \\ \diagdown MeII\end{matrix} \quad (3a)$$

$$E\begin{matrix}\diagup Mg\text{-}PPEP \\ \diagdown MeII\end{matrix} + CO_2 \rightleftarrows E\begin{matrix}\diagup Mg\text{-}PPOA \\ \diagdown MeII\end{matrix} \rightleftarrows E\begin{matrix}\diagup Mg\text{-}PP_i \\ \diagdown MeII\end{matrix} + OA \rightleftarrows E\begin{matrix}\\ \diagdown MeII\end{matrix} + PP_i + OA \quad (3b)$$

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

During the past year our principal efforts have centered on (A) Identification of the tightly bound metal of carboxytransphosphorylase, (B) Study of the subunits of carboxytransphosphorylase and, (C) Detection of enzyme bound intermediate compounds of the reaction. It was proposed last year we would make a kinetic analysis of the reaction but time did not permit this study.

A. Identification of the Bound Metal (Type II) of Carboxytransphosphorylase. The evidence is quite conclusive that there is a bound metal present in carboxytransphosphorylase which is required in addition to the freely dissociated metal (Mg^{2+}, Mn^{2+} or Co^{2+}) which also is required (see reprints). Thus far our efforts to identify the metal have failed. A major problem is the removal of contaminating metal ions from the enzyme without inactivating the enzyme and without loss of the bound metal. Emission spectroscopy requires a large amount of enzyme. We therefore have undertaken in collaboration with Dr. Michael Scrutton of Rutgers University an analysis of metals by atomic absorption. The procedure involves correlation of metal content with the enzymatic activity. If the metal is not a contaminant, but is associated directly with the enzyme, the ratio of the amount of metal and enzymatic activity should be constant throughout a fractionation procedure. The only metal that has been found to meet this requirement is Ni^{2+}. Figure 1 presents the results of one such analysis for Ni^{2+}. Mn^{2+}, Co^{2+}, Zn^{2+}, Ca^{2+} and Mg^{2+} have been excluded. Fe^{2+} and Cu^{2+} do not fully meet the requirement. Although present they were partly removed by the EDTA treatment and the amount did not completely correlate with enzymatic activity. The procedure is as follows:

A suitable sample of carboxytransphosphorylase is passed through a 25 X 1.5 cm Sephadex G-200 column equilibrated in 0.05 M K-phosphate buffer, pH 6.8 containing 0.05 M EDTA. Protein and enzymic activity are located, the latter by inclusion of 1 mM Co^{2+} in the assay to remove EDTA from the tightly bound metal and thus free the metal of EDTA and reactivate the enzyme. Suitably diluted aliquots of the enzyme also are subjected to atomic absorption analysis. As seen in Fig. 1 the specific activity (units/mg protein) and the ratio of Ni^{2+}/units were found constant through the elution peak. An aliquot of the EDTA treated enzyme from the G-200 column was passed over a second column (Sephadex G-25) to remove the EDTA. There was no longer any requirement for Co^{2+} in the assay and enzyme activity was equal to that observed with addition of Co^{2+} prior to removal of the EDTA. It must be noted that the specific activity of carboxytransphosphorylase was halved by this treatment. It was shown however that an apoenzyme, at least with respect to Ni^{2+}, was not formed by this treatment since added Ni^{2+} did not increase the enzymatic activity.

Figure 1.

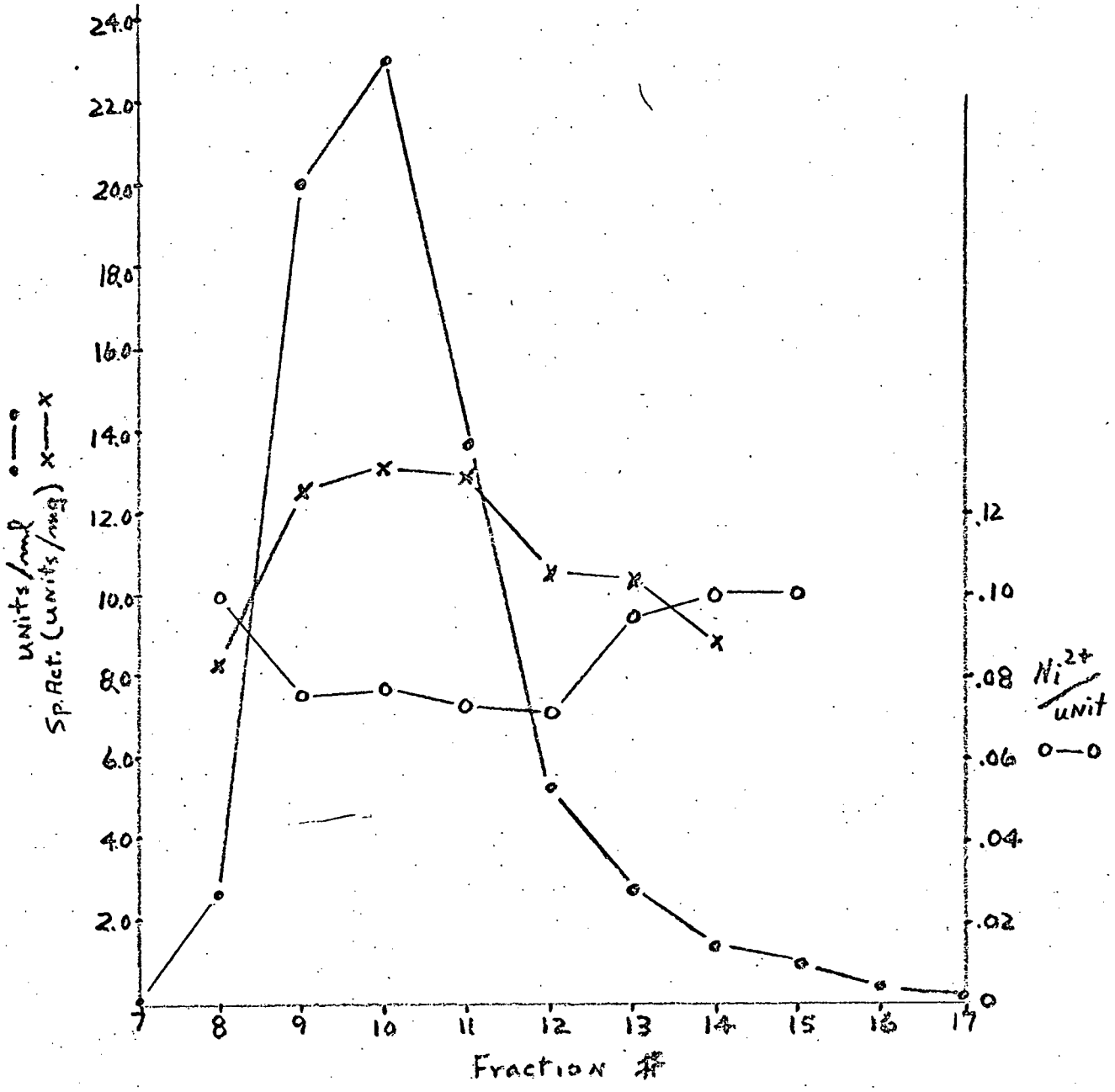

Fig. 1. Elution profile of P-enolpyruvate carboxytransphosphorylase activity and Ni^{2+} content from a column of Sephadex G-200 equilibrated in 0.05 M K-phosphate and 0.05 M EDTA, pH 6.8.

The Ni^{2+} content of the enzyme was 0.42 g-atom of Ni^{2+} per mole of carboxytransphosphorylase (molecular weight 430,000 by the Archibald method). However, other preparations have given a lower value for Ni^{2+} even prior to treatment with EDTA. This raises the question of whether or not a number of metals may serve as the bound metal of carboxytransphosphorylase and whether there is exchange of these metals during the isolation so that no one metal is present in an equal molar ratio with the enzyme. This question will be dealt with in greater detail in this year's Proposal.

B. Study of the Subunits of Carboxytransphosphorylase. It has been reported previously that carboxytransphosphorylase has two forms, one with a sedimentation coefficient of ~16S with a specific activity of ~23 and another of 8.5S with a specific activity of 7. It was proposed last year that these two forms of the enzyme be studied as to metal content, effect of chelators, K_m's, end groups and structure as observed in an electron microscope. The crystalline form of the enzyme is the larger form and the smaller form is abundant in the supernatant solution of crystallization. It has been found that the crystalline form has a sedimentation coefficient of $s_{20,w}$ = 15.2S on extrapolation to zero protein concentration.

Overnight dialysis of crystalline carboxytransphosphorylase against low ionic strength phosphate (0.03 M potassium phosphate, pH 6.8) results in conversion of about 30% of the 15.2S species to a 7.4S species as determined by centrifugation in a sucrose density gradient. The forms produced in this manner have been isolated from the sucrose gradient. It has not been determined if this 7.4S species is identical to the 8.5S form previously obtained from the spontaneous breakdown of crystalline carboxytransphosphorylase. It could be that changes which were made in the sucrose density gradients in order to handle the different experiments introduced an error in calculating S values. It is possible, however, that the two forms do exist, and that the 8.5S differs from the 7.4S species by the loss of a very small polypeptide. Unless otherwise noted, the work reported here has been performed with the 7.4S species.

The specific activity of the 7.4S and 8.5S forms are about one-third that of the crystals. Studies of the 7.4S species in both the forward oxalacetate reaction and the pyruvate reaction have been initiated to determine if either the K_m or V_{max}, or both, differ from those for the 15.2S species. Apparent K_m's completed thus far are shown in Table I and are compared to those previously determined with the crystalline enzyme. 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. It is seen that the K_m values are very nearly the same for both species.

As part of these studies we determined the effect of substrate on the sedimentation characteristics of the crystalline form (15.2S) and the 8.5S form in the supernatant solution of crystallization. Surprisingly in the presence of substrates the two materials become alike in terms of S values as determined on a sucrose gradient even though they still differ in enzymatic activity. It is seen (Fig. 2A) that the bulk of the enzyme from the crystals traveled at 14.9S in a 0.05 M phosphate, pH 6.8, but sedimented predominantly at 10.8S in a gradient containing all substrates for the forward reaction in saturating amounts (Fig. 2B). Fig. 2 also shows the position of the catalase marker at 11.35S.

Table I

Comparison of the K_m's of the Two Forms of Carboxytransphosphorylase

Enzyme Form	Substrate	Apparent K_m	True K_m
		mM	mM
	Pyruvate Reaction		
7.4S	P-enolpyruvate	.017	
15.2S	P-enolpyruvate	.030	.031
7.4S	Orthophosphate	.24	
15.2S	Orthophosphate		.58
	Forward Reaction		
7.4S	P-enolpyruvate	.083	
15.2S	P-enolpyruvate		.14

The mother liquor from which two crops of crystals had been removed was predominantly 8.5S in character in phosphate buffer (Fig. 3A) but in the presence of substrate the S value was 10.2 S for the bulk of the material (Fig. 3B).

To check if sucrose was contributing to this transformation the enzyme from the supernatant solution was incubated for 8 hr with substrates in the absence of sucrose and then analyzed by Model E ultracentrifugation. A major peak sedimenting at 9.1S, accompanied by a small, broad peak of 6.0S was found; corrections for the density and viscosity of the substrates would yield $s_{20,w}$ values consistent with those observed in the sucrose density gradient experiments.

It is intriguing that in the presence of substrates the two forms of the enzyme rearrange to species with the same sedimentation characteristics yet the difference in enzymatic activity which existed prior to the transformation persists. We have no explanation of this phenomenon at present. It is possible that there is partial loss of the Type II metal from the 8.5S species and therefore it has less activity in the 11S form.

Prof. Robert Valentine of Mill Hill England has studied the 15.2S species and 8.5S species of carboxytransphosphorylase under the electron microscope. It appears that the crystalline species is a tetramer and the smaller species is a monomer. The 15.2S species dissociated to the monomer unless it was stabilized with glutaraldehyde.

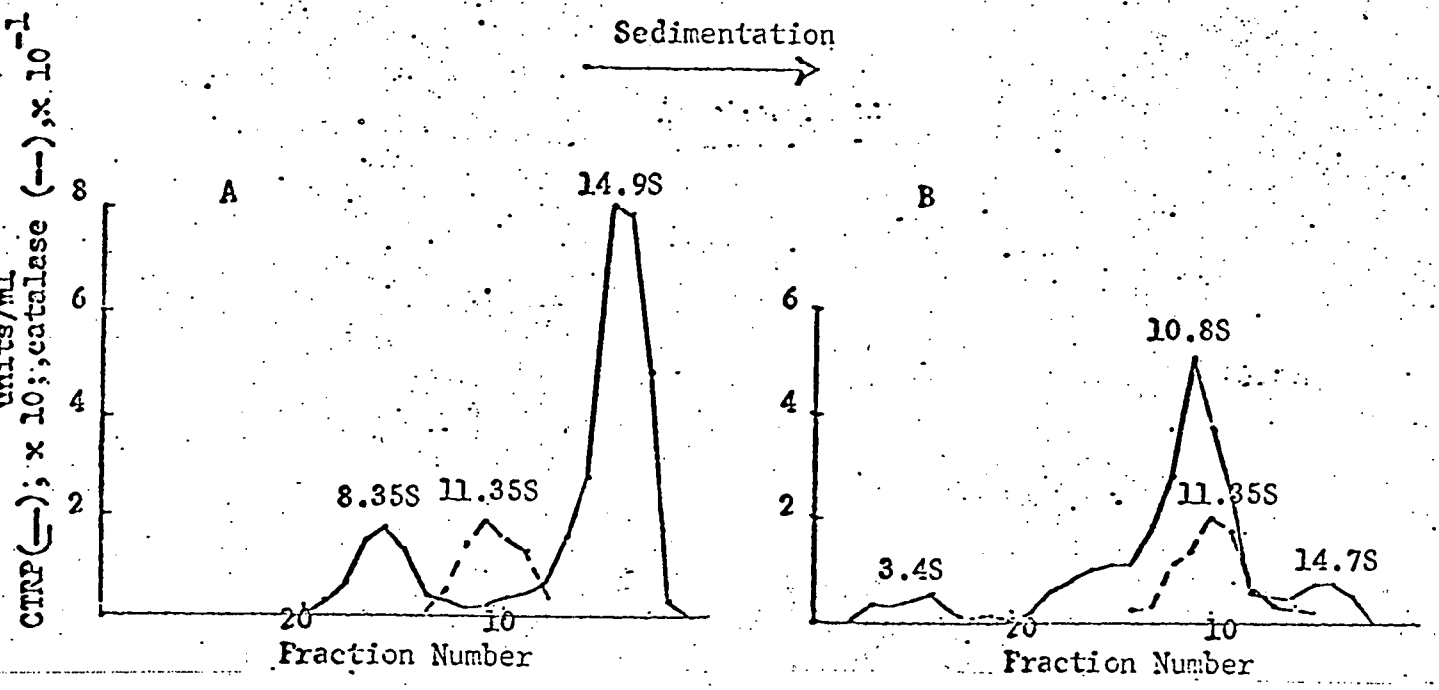

Fig. 2. Effect of substrates on the sedimentation coefficient of crystalline carboxytransphosphorylase (CTRP). 130 γ of crystalline carboxytransphosphorylase of specific activity 20, applied to a 5 to 30% sucrose density gradient. Time of centrifugation was 8-1/3 hr at 50,000 rpm and 8°C.

A. Gradient contained 50 mM potassium phosphate, pH 6.8. 85.5% of the units applied were recovered.

B. Gradient contained saturating concentrations of substrates for the forward reaction: 10 mM potassium phosphate, pH 6.8; 30 mM $KHCO_3$; 12 mM $MgCl_2$ and 2 mM P-enolpyruvate. The 5 and 30% sucrose solutions were gassed with CO_2 15 min at room temperature before the gradients were poured. 90% of the units applied were recovered.

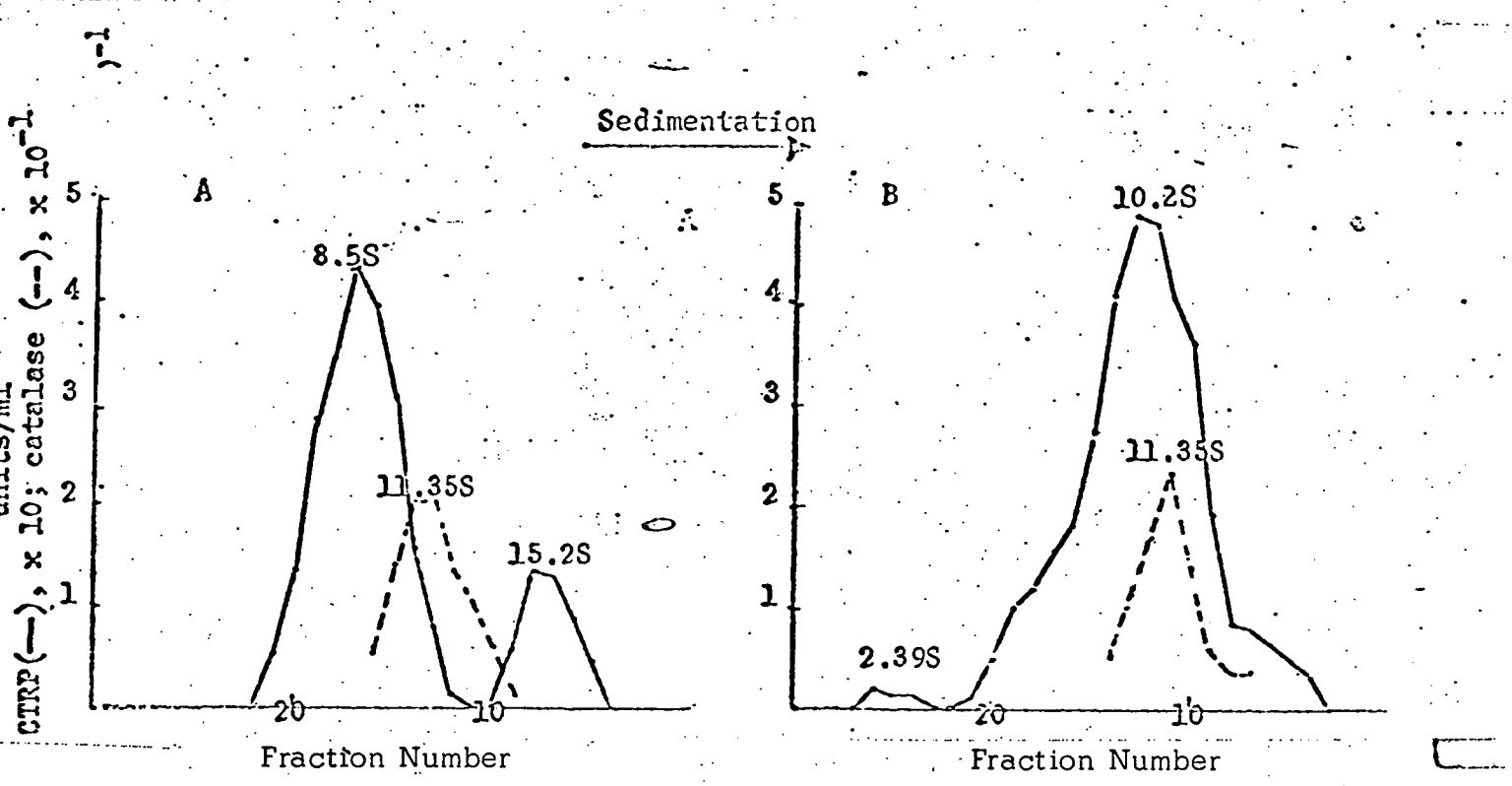

Fig. 3. Effect of substrates on the sedimentation coefficient of carboxytransphosphorylase (CTRP) in the supernatent solution of crystallization. 284 γ carboxytransphosphorylase from the mother liquor of crystallization of specific activity 7 was applied to a 5 to 30% sucrose density gradient. Time of centrifugation was 8 hr at 50,000 rpm and 4°C. Conditions for A and B as in Fig. 2.

A. 76% of the units applied were recovered.

B. Recovery of units was 106.5%.

The tetramer was observed by fixation of the 15.2S species with glutaraldehyde for 0.5 hr. If the fixation with glutaraldehyde was terminated after only 5 min there was a predominance of dimers. The tetramer appears to consist of two rods, each 120A long and each composed of 2 monomers. Because this formation has 2-fold, rather than 4-fold symmetry, Prof. Valentine noted that the half molecule or dimer was to be expected and that it should have a sedimentation coefficient of 11S. It is likely that the 11S species which we observe to be formed from the 15.2S species and 8.5S species is the dimer or half molecule.

Figure 4 shows the glutaraldehyde fixed enzyme with examples of tetramer, dimer, and monomer and Fig. 5 the electron microscope picture of the 8.5S component showing the monomer.

Due to Prof. Valentine's death these collaborative studies could not be continued.

It has been observed by Lochmüller et al. (J. B. C. 241, 5678 (1966)) that carboxytransphosphorylase crystals incubated in 6 M urea and .033 M β-mercaptoethanol for 12 hr dissociates into a form with an $s_{20,w} = 7.0S$ after correction for density and viscosity of the urea. This year an effort was made to dissociate the enzyme more vigorously by dialyzing the crystals 72 hr against 6 M ultrapure guanidine hydrochloride (Heico, Inc.) and .1 M distilled β-mercaptoethanol (Eastman). Analysis by sedimentation velocity showed a sharp, well-defined peak of .91S; calculations for the density and viscosity of the guanidine-thiol solution according to K. Kawahara and C. Tanford (J. Biol. Chem. 241, 3228 (1966)) gave a corrected value of 3.0S. Thus it appears that the 7.4S form is not a single polypeptide.

As part of the comparative study between the 15.2S and smaller forms (7.4S and 8.5S), amino acid analyses are now in progress. The analysis for the 15.2S is presented in Table II; the value for tryptophan will be determined by spectral analysis. The molecular weight calculated by amino acid composition, excluding tryptophan, is 424,685 g/mole; this agrees well with that of 430,000 determined by H. Lochmüller, H. G. Wood and J. J. Davies (J. Biol. Chem. 241, 5678 (1966)) using the Archibald sedimentation method.

Table II

Amino Acid Composition of the 15.2S Form of Carboxytransphosphorylase

Amino Acid	μmoles	μg
lysine	132	11,761
histidine	132	20,480
arginine	231	40,240
aspartic acid	363	48,315
threonine	264	31,442
serine	231	24,278
glutamic acid	330	48,543
proline	231	26,588
glycine	198	14,870
alanine	264	23,522
cysteic acid	33	5,584
valine	198	23,186
methionine	66	9,847
isoleucine	132	17,318
leucine	297	38,966
tyrosine	99	17,939
phenylalanine	132	21,806
tryptophan		
Total		424,685

Fig. 4. Electron micrograph of crystalline carboxytransphosphorylase after fixation with glutaraldehyde (0.5%, pH 7.0). Note the appearance of tetramer, dimer rods, and monomers.

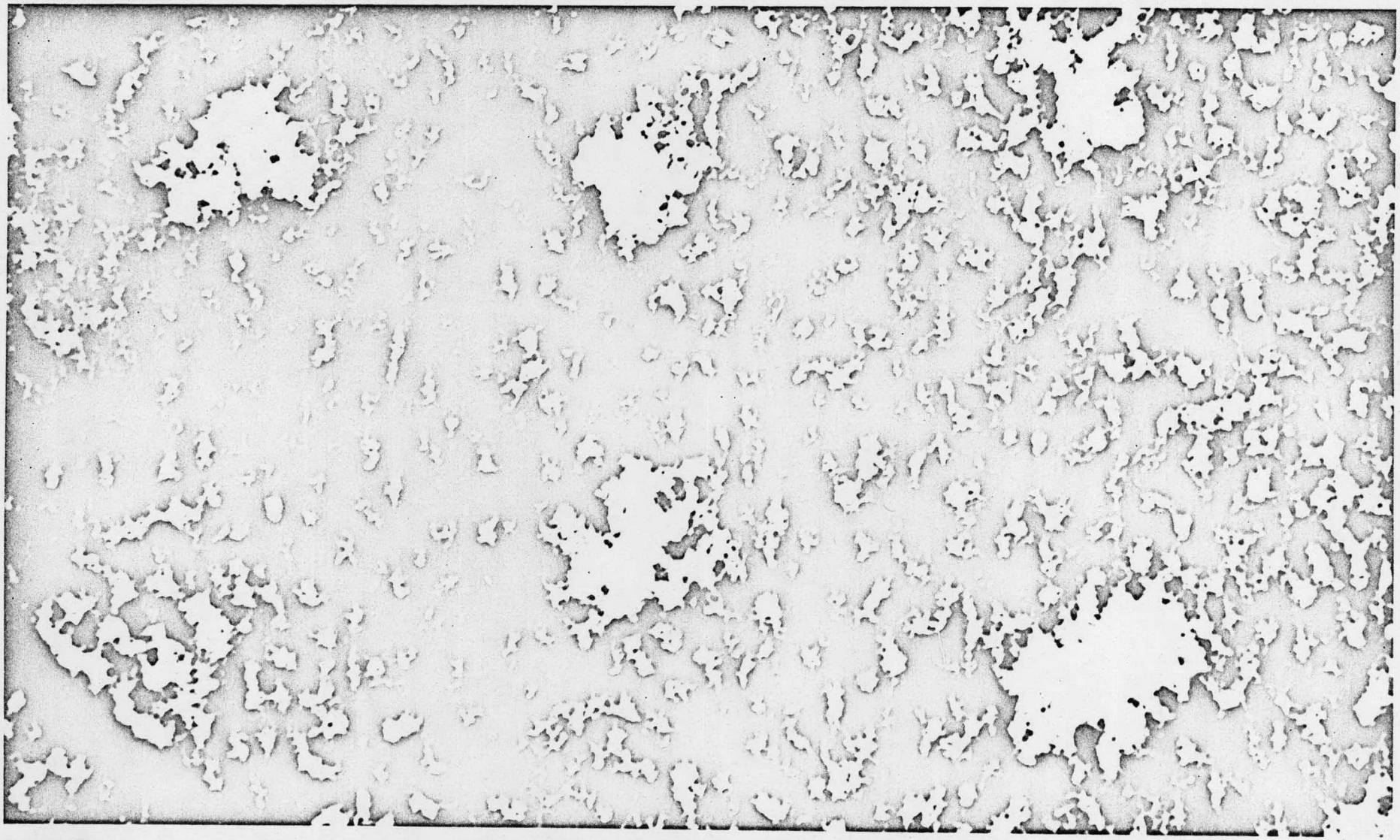

Fig. 5. Electron micrograph of 8.5S component obtained by centrifugation of supernatant solution in a partition cell.

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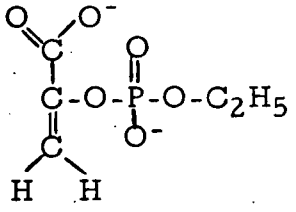
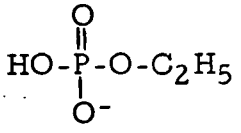
C. Detection of Enzyme Bound Intermediate Compounds of the Reaction. The mechanism which we have outlined in our previous reports as shown in Fig. 2 of the enclosed reprint of Biochemistry 8, 3145 (1969) and Reaction 3 involves intermediate compounds of pyrophosphoenolpyruvate and pyrophosphoenol oxalacetate. We proposed last year that we attempt to isolate the enzyme bound intermediates. The enzyme bound intermediate compounds were to be labeled by use of $^{32}P_i$. Since very small amounts would be present it would be necessary to add carrier compounds to permit isolation and characterization of the compounds.

The chemistry of the en pyrophate esters of pyruvate and oxalacetate is not known but it is probable that they are not very stable. It is likely that the methyl or ethyl esters would be more stable. It, therefore, was decided to investigate the reactivity of the monoethyl esters of P-enolpyruvate and of orthophosphate with carboxytransphosphorylase. If these were reactive, then the esters of pyrophosphoenolpyruvate and oxalacetate might occur as intermediate compounds. Chemical synthesis of the esters of the pyrophospho compounds is probably feasible which then could be used as carriers. In addition other substituted esters, including the di esters might be tested as probes of the mechanism.

These studies are being done in collaboration with Prof. Dean Griffith of the Chemistry Department of Case Western Reserve University. He has provided the monoethyl esters of P-enolpyruvate and of orthophosphate.





Preliminary results indicate that both compounds have some activity in the oxalacetate and pyruvate reactions as determined by coupling the reaction with malate dehydrogenase or lactate dehydrogenase respectively. Further studies with rigidly purified compounds are required to be certain the esters are not contaminated with non-esterified phosphate or P-enolpyruvate which permit the rather slow reaction. The reactions presumably are as follows:

In the presence of CO_2

$$\text{P-enolpyruvate} + \text{EtP}_i + CO_2 \rightleftharpoons \text{oxalacetate} + \text{EtPP}_i \quad (4)$$

and

$$\text{EtP-enolpyruvate} + P_i + CO_2 \rightleftharpoons \text{oxalacetate} + \text{EtPP}_i \quad (5)$$

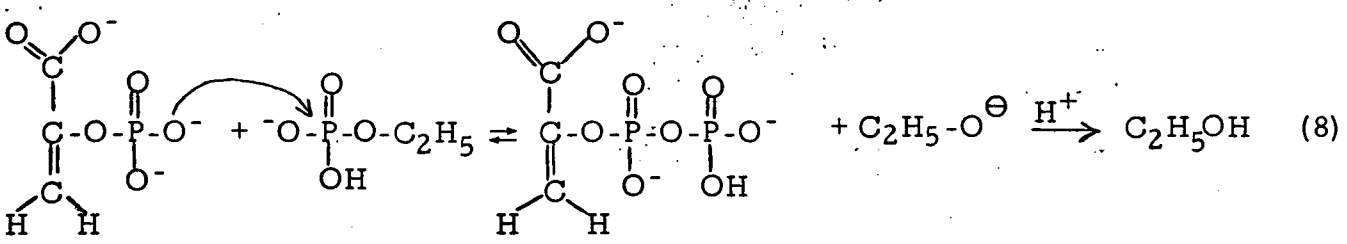
In the absence of CO_2

$$\text{P-enolpyruvate} + \text{EtP} \rightarrow \text{pyruvate} + \text{EtPP}_i \quad (6)$$

and

$$\text{EtP-enolpyruvate} + P_i \rightarrow \text{pyruvate} + \text{EtPP}_i \quad (7)$$

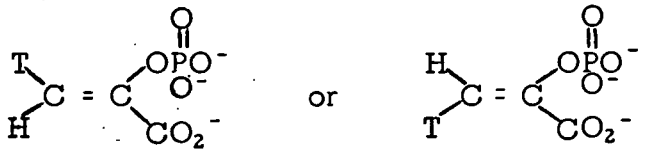
Thus far it has not been established that ethyl pyrophosphate is a product. The reactions may occur as follows since ethoxide is a better leaving group than is hydroxide.



(8)

In this case the ethyl pyrophosphate ester would not be an intermediate. Further work is underway to determine whether ethyl pyrophosphate is the product or ethyl alcohol and inorganic pyrophosphate.

Another interesting aspect of the mechanism is the stereochemistry 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 stereospecifically ^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 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 counterclockwise 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 of oxalacetate should be labilized 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 of P-enolpyruvate carboxylase from peanuts and for 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 pyrophospho-enolpyruvate as an intermediate $\left(^{-}O_2C\text{-}CH{=}C(\text{-}O\text{-}P\text{-}O\text{-}P)\text{-}CO_2^{-}\right)$. This mechanism would require labilization of a proton of the methylene of P-enolpyruvate and this presumably would be a stereospecific displacement. In this case 100% of the

^3H would be lost unless there was removal of the proton and its return specifically. Such specific transfer of hydrogen is known in enzyme reactions (methylmalonyl-CoA 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 a pyrophosphoenoloxalacetate intermediate. The results, however, do not mitigate against the occurrance of pyrophosphoenolpyruvate as an intermediate.

A manuscript describing these studies has been submitted for publication to the Journal of Biological Chemistry.

II. Catalysis and Mechanism of the Pyruvate, Phosphate Dikinase Reaction--
H. J. Evans and H. G. Wood.

The conversion of pyruvate to P-enolpyruvate in propionibacteria occurs by the action of pyruvate, phosphate dikinase. The enzyme requires Mg^{2+} and a monovalent cation (K^+ or NH_4^+) for maximum activity. Reports of previous years have dealt with the discovery of the enzyme, its partial purification and investigation of the mechanism. The report submitted last year presented evidence that the reaction occurs by the following partial reactions:

$$\text{enzyme} + \text{ATP} \rightleftharpoons \text{enzyme-}\overset{\beta\gamma}{\text{P}}\text{P} + \text{AMP} \qquad (9a)$$

$$\text{enzyme-}\overset{\beta\gamma}{\text{P}}\text{P} + \text{P}_i \rightleftharpoons \text{enzyme-}\overset{\beta}{\text{P}} + \overset{\gamma}{\text{P}}\text{P}_i \qquad (9b)$$

$$\text{enzyme-}\overset{\beta}{\text{P}} + \text{pyruvate} \rightleftharpoons \text{enzyme} + \overset{\beta}{\text{P}}\text{-enolpyruvate} \qquad (9c)$$

$$\text{Sum:} \quad \text{pyruvate} + \text{ATP} + \text{P}_i \rightleftharpoons \text{P-enolpyruvate} + \text{AMP} + \text{PP}_i \qquad (9)$$

These results have been published in the Proc. Natl. Acad. Sci. U.S. 61, 1448 (1968) and reprints are included with this report.

During the past year, further purification of the dikinase has been achieved. Various properties of the enzyme have been investigated, including the sedimentation pattern, cold lability, pH optima for the forward and the reverse reactions, apparent K_m values for the forward and reverse reactions, nucleotide specificity for the forward and reverse reactions, and the requirement of a divalent metal. Attempts to further characterize the enzyme phosphate intermediate were not successful due in part to the limited amount of pure enzyme available.

A. Purification of Pyruvate, Phosphate Dikinase. An additional step involving use of DEAE-Sephadex A-50 has been added to the purification scheme, and the results are shown in Table III. The enzyme is eluted from DEAE-Sephadex by the use of a convex gradient from 0.15 M potassium phosphate (pH 6.8) to 0.2 M potassium phosphate plus 0.3 M KCl (pH 6.8), with twice the volume of the latter flowing into the former. The dikinase is precipitated by 90% saturation with ammonium sulfate and sedimented at 20,000 X g. It is dissolved in 1.0 M sucrose and stored at -15°. Over a period of months the enzyme spontaneously recovers activity which had been lost during the TEAE-cellulose step.

Table III

The Purification of Pyruvate, Phosphate Dikinase from P. shermanii (2.0 kg wet weight) Grown on a Lactate Medium

Fractions	Total units μ moles/ min.	Specific activity units/mg	Purification	Yield
Crude extract	1435	.02	1	100
Batch DEAE-cellulose--- .4 M eluate	2820	.05	2.5	196
Cellulose phosphate column--- .05 M phosphate eluate	3680	.12	6	130
Fractionation with $(NH_4)_2SO_4$ 35-55%	2790	.20	10	78
TEAE-cellulose--0.2 M phosphate plus 0.2 M KCl	900	.60	30	24
DEAE-Sephadex column	520	1.70	85	14

B. Properties of Pyruvate, Phosphate Dikinase. One of the major problems encountered in the purification of the dikinase is the cold lability of the enzyme particularly after the purification on TEAE-cellulose. An example is presented in Table IV. It is seen that a solution of the dikinase in 0.05 M potassium phosphate (pH 6.8) lost all enzymatic activity over a period of 17 hrs, when it was stored at 0°C. But an identical sample lost only 31% of its activity over the same period, when kept at 30°C. Pyruvate, at 0.7 mM concentration, afforded some protection against destruction by cold. The activity lost by exposure to cold could not be regained by incubation of the enzyme at 30° for prolonged periods.

Table IV

Effect of Temperature on the Stability of Pyruvate, Phosphate Dikinase

Temperature of Storage	Original activity remaining		
	1 hr	4.5 hr	17 hr
	%	%	%
30°C	94	83	69
0°C	85	50	0
0°C (7×10^{-4} M pyruvate)	87	74	25

Pyruvate, phosphate dikinase was passed through a Sephadex G-50 column equilibrated with 0.05 M potassium phosphate (pH 6.8), assayed and divided into three samples for storage as described. After storage for the indicated time period, the samples were warmed for 1 minute at 30°, and assayed again. The stored samples contained 1.8 mg of protein per ml.

Dikinase which had been purified through the last step of Table III was examined in an analytical ultracentrifuge. It was passed through a column of Sephadex G-50 to remove sucrose and to transfer the enzyme to 0.1 M potassium phosphate (pH 6.8). The sedimentation pattern at 19.2°C revealed two major protein peaks with sedimentation coefficients of 6.7S and 4.6S. At the end of the centrifugation only 14% of the activity was recovered. When the enzyme was transferred using a column of Sephadex G-50 equilibrated with 0.1 M imidazole-Cl, 0.05 M ammonium sulfate, 0.005 M pyruvate and 0.005 M $MgCl_2$ at pH 6.7 there was only one major protein peak with an S value of 6.9, and 94% of the activity was recovered at the end of the run. These conditions appear to stabilize the enzyme.

The optimum pH of the reaction in either the forward and reverse direction was found to be 6.5 to 7.0.

The nucleotide specificity of the forward reaction is shown in Table V. It is seen that the reaction is very specific for ATP. When both ATP and the other nucleotide were added in equimolar concentration, GTP was found to inhibit approximately 8%, but the other nucleotides showed negligible inhibition. The nucleotide specificity in the reverse reaction is shown in Table VI. The reaction is very specific for AMP. In the presence of AMP, GMP inhibited approximately 50%, CMP 6%, and the other nucleotides were without effect at concentrations equimolar with the AMP.

Table V

Nucleotide Specificity in the Forward Reaction of Pyruvate, Phosphate Dikinase

Nucleotide	Activity	Inhibition
	%	%
ATP	100	-
GTP	0	8
CTP	0	0
UTP	0	0
TTP	0	0

The activity was determined by use of the nucleotides shown in the Table and inhibition by addition to ATP of an equimolar concentration (2 mM). Each cuvette contained 1.3 μg of dikinase from the TEAE-cellulose step of Table III.

Table VI

Nucleotide Specificity in the Reverse Reaction of Pyruvate, Phosphate Dikinase

Nucleotide	Activity	Inhibition
	%	%
AMP	100	-
GMP	0	50
CMP	0	6
UMP	0	0
TMP	0	0

The activity was determined by using the nucleotides shown in the Table. The % inhibition was determined using the indicated nucleotide in addition to AMP, at equimolar concentration (2 mM). Each cuvette contained 1.7 μg of dikinase from the last step of Table I.

The apparent K_m values were determined for the substrates and activators of the enzyme in both the forward and reverse reactions. For the forward reaction, the following values were obtained; pyruvate, 1.0×10^{-4} M; ATP, 4.3×10^{-5} M; P_i, 1.0×10^{-3} M; Mg^{2+}, 4.0×10^{-3} M; and NH_4^+, 2.0×10^{-3} M. For the reverse reaction, the apparent K_m values were as follows: P-enolpyruvate, 3.6×10^{-5} M; AMP, 1.5×10^{-5} M; PP_i, 1.2×10^{-4} M; Mg^{2+}, 2.4×10^{-3} M; and NH_4^+, 2.0×10^{-3} M.

The requirement for a divalent metal was investigated by substitution of other metals for Mg^{2+} in the reverse reaction. Only the reverse reaction was studied because the forward assay uses carboxytransphosphorylase as a coupling enzyme, and this enzyme also has a requirement for a divalent metal. Mn^{2+} and Co^{2+} were able to stimulate the reaction, but not as well as Mg^{2+}. The apparent K_m for activation by Co^{2+} was 5×10^{-4} M, which is approximately four-fold lower than the apparent K_m of Mg^{2+}, although the V_{max} with Co^{2+} was also lower than that with Mg^{2+}. Ca^{2+}, Cu^{2+} and Fe^{2+} inhibited the dikinase reaction. Inhibition by Ca^{2+} is characteristic of metals which have a Type II function, as described by Cohn (Biochemistry 2, 623 (1963)). In those cases reviewed by Cohn, such metals are bound directly to the enzyme and may form a bridge between the enzyme and substrate molecules.

C. Demonstration of Inorganic Pyrophosphatase in Crude Extracts of P. shermanii. The major function of pyruvate, phosphate dikinase in propionic acid bacteria is considered to be the conversion of pyruvate to P-enolpyruvate, and, together with carboxytransphosphorylase, to bring about the synthesis of oxalacetate from pyruvate. It is seen from the reactions below that synthesis of P-enolpyruvate, as well as of oxalacetate, would be more readily accomplished if there were cleavage of PP_i by a phosphatase.

$$\text{pyruvate} + \text{ATP} + P_i \rightleftharpoons \text{P-enolpyruvate} + \text{AMP} + PP_i \qquad (10)$$

$$\text{P-enolpyruvate} + CO_2 + P_i \rightleftharpoons \text{oxalacetate} + PP_i \qquad (11)$$

$$2\ PP_i + 2\ H_2O \rightleftharpoons 4\ P_i \qquad (12)$$

$$\text{Sum: pyruvate} + CO_2 + \text{ATP} + 2\ H_2O \rightleftharpoons \text{oxalacetate} + \text{AMP} + 2\ P_i \qquad (13)$$

Tests for pyrophosphatase enzyme have shown that it is present in a crude extract from P. shermanii grown on lactate. The extract was found to contain pyrophosphatase with a specific activity of approximately 6×10^{-3} μmoles of PP_i cleaved per minute per mg protein. This figure is approximately one-third of the specific activity of dikinase found in the crude extract. No attempt was made to find optimum conditions for the pyrophosphatase and it may be much more potent than observed in this test.

According to Reeves and coworkers, neither the amoeba Entamoeba histolytica (Reeves, J. Biol. Chem. 243, 3202 (1968), nor the bacterium Bacteroides symbiosus (Reeves et al., J. Biol. Chem. 243, 5486 (1968)) has been found to contain pyruvate kinase but both organisms contain pyruvate, phosphate dikinase. Because of the absence of pyruvate kinase, Reeves and coworkers proposed that the dikinase in these organisms replaces the glycolytic function of pyruvate kinase, i.e., converts P-enolpyruvate to pyruvate with formation of ATP. However, it appears that the major role of dikinase in propionibacteria is the opposite, i.e., to synthesize P-enolpyruvate. Crude extracts of propionibacteria contain an abundant amount of pyruvate kinase. Furthermore, the dikinase is induced approximately ten-fold by growing propionibacteria on lactate rather than on glycerol, presumably because the dikinase is essential for net formation of P-enolpyruvate from lactate.

These studies are being discontinued at least for the coming year since H. J. Evans has received his Ph. D. degree and is joining Robert Hill's group at Duke University as a postdoctoral fellow.

III. Total Synthesis of Acetate from CO_2 by Clostridium thermoaceticum--M. Schulman, D. Parker, T. Wu., P. Kucera and H. G. Wood.

Certain clostridia have the ability to ferment a variety of substrates anaerobically and use CO_2 as a hydrogen acceptor in their oxidation-reduction processes. The reduction of CO_2 takes place by a mechanism which leads to the total synthesis of acetate. Of these organisms C. thermoaceticum has been studied the most extensively. Mainly from the results of our laboratory and those of Earl Stadtman a general outline of the mechanism of this total synthesis of acetate from CO_2 has been formulated as shown in Reactions 14 to 21 for the fermentation of pyruvate. Corrinoid-E is an abbreviation for an enzyme-corrinoid complex and THF is tetrahydrofolate.

$$4\ \text{Pyruvate} + 4\ \text{ADP} + 4\ P_i + 4\ \text{NADP} \rightarrow 4\ \text{acetate} + 4\ CO_2 + 4\ \text{ATP} + 4\ \text{NADPH} + 4\ H^+ \quad (14)$$

$$CO_2 + \text{NADPH} + H^+ \rightleftarrows \text{HCOOH} + \text{NADP}^+ \quad (15)$$

$$\text{THF} + \text{HCOOH} + \text{ATP} \rightleftarrows \text{formyl-THF} + \text{ADP} + P_i \quad (16)$$

$$\text{Formyl-THF} + 2\ \text{NADPH} + 2\ H^+ \rightarrow\rightarrow \text{CH}_3\text{-THF} + 2\ \text{NADP}^+ \quad (17)$$

$$\text{CH}_3\text{-THF} + \text{corrinoid-E} \rightarrow \text{THF} + \text{CH}_3\text{-corrinoid-E} \quad (18)$$

$$CO_2 + \text{CH}_3\text{-corrinoid-E} + \text{ATP} \rightarrow \text{HOOC-CH}_2\text{-corrinoid-E} + \text{ADP} + P_i \quad (19)$$

$$\text{HOOC-CH}_2\text{-corrinoid-E} + \text{NADPH} + H^+ \rightarrow \text{acetate} + \text{corrinoid-E} + \text{NADP}^+ \quad (20)$$

$$\text{Sum: 4 pyruvate} + 2\ \text{ADP} + 2\ P_i \rightarrow 5\ \text{acetate} + 2\ CO_2 + 2\ \text{ATP}. \quad (21)$$

Reaction 21 gives the overall stoichiometry and shows 4 moles of pyruvate yielding 5 of acetate, one of which is by total synthesis from CO_2. Evidence for the mechanism has been reviewed in previous reports and publications. The evidence includes the demonstration that ^{14}C is found in both carbons of acetate from $^{14}CO_2$, that CO_2 is converted to a methyl group which is linked to the cobalt of corrinoids, that this methyl is converted to the methyl of acetate in fermentations of pyruvate by extracts of C. thermoaceticum, that $^{14}CH_3$-THF is converted to the methyl of acetate under similar conditions and that carboxyl methyl B_{12} is reduced to acetate. Nevertheless, none of the enzymes involved in these transformations have been isolated from C. thermoaceticum and purified extensively except for formyltetrahydrofolate synthetase and many aspects of this very interesting synthesis are still tentative.

During the past year our major efforts have been concerned with the role of folates in this synthesis and determination of optimum condition for preparing active enzymes from C. thermoaceticum.

A. Evidence that Methyltetrahydrofolate is an Intermediate in the Conversion of CO_2 to Acetate. It was pointed out in last year's Proposal that studies of the role of folates were needed, first to strengthen the evidence that folate derivatives are on the direct path of acetate synthesis and second to determine the type of folate derivative which is the natural precursor or cofactor in C. thermoaceticum. Some doubt about the role of folate existed because the methyl group of methionine and the β-carbon of serine had been found by Ljungdahl and Wood (J. Bact. 89, 1055 (1965)) to have a low specific radioactivity whereas that of acetate was high from $^{14}CO_2$. Since methionine and serine are known to be formed via formyltetrahydrofolates they might also have been expected to be highly labeled from C-1 folate derivatives if the latter were involved in acetate synthesis. It seems probable that there was little net synthesis of the amino acids and therefore very little incorporation of ^{14}C. However, it was desirable to determine which form of folate is the natural cofactor in C. thermoaceticum. Our studies with $^{14}CH_3$-tetrahydrofolate show that it reacts slowly but Rabinowitz and Himes using other clostridia have shown that the natural cofactor is a triglutamate derivative of folate. For our studies it seems likely it will be necessary to use the natural cofactors in order to obtain the optimum reactivity.

It was proposed last year that we investigate this problem using whole cells of C. thermoaceticum fermenting glucose with $^{14}CO_2$ present and isolate the folate derivatives, the Co-CH_3-corrinoids and acetate from these cells. The radioactivity of each would be determined to ascertain whether or not they were in agreement with the predicted precursor product relationships. For this purpose methods of separating and identifying the folate derivatives were necessary.

Since the amounts of folate are small (mμg to μg) the best method of detection is the microbiological assay using standard growth curves of Lactobacillus casei and Streptococcus feacalis (Bakerman, Anal. Biochem. 2, 588 (1961)) and relating turbidity to mμg of folate. These bacteria differ in their response to various folate derivatives as is shown in Table VII. This difference in growth response may be used to partially identify the different types of folate especially when used in conjunction with conjugase from hog kidney (Bird et al., J. Biol. Chem. 237, 2530 (1961)) which hydrolyzes the polyglutamates to the monoglutamate. Thus S. feacalis will not respond to the triglutamate ($PtGlu_3$) whereas L. casei does, but S. feacalis would respond after treatment with conjugase. If it were CH_3PtGlu, S. feacalis would not respond even after treatment with conjugase.

Table VII

Relative Activity of Various Folate Derivations for S. faecalis and L. casei

Compounds	S. faecalis	L. casei
Folic acid (Pt GLU_1)*	+	+
Pt GLU_2	+	++
Pt GLU_3	–	+
Pt $GLU_{4\ to\ 7}$	–	–
H_4 Pt GLU_1**	+	+
5 CHO H_4 Pt GLU_1	+	+
10 CHO H_4 Pt GLU_1	+	+
5 H_3CH_4 Pt GLU_1***	–	+
Pteroic acid	+	–

Note: diglutamates (except methyl) supports growth of S. faecalis but tri or higher glutamates do not. L. casei responds to diglutamates much more than does S. faecalis.

* Pt GLU_1 equals Pteroylmonoglutamate

**H_4 Pt GLU_1 equals tetradropteroylmonoglutamate

***5 H_3CH_4 Pt GLU_1 equals 5-methyltetrahydropteroylmonoglutamate

The chromatographic method of Silverman et al. (J. Biol. Chem. 237, 2530 (1961)) was found unsatisfactory for separation of the folates and a method employing QAE-Sephadex column chromatography was devised. The folate containing samples are placed on a 1 X 16 cm column of QAE-Sephadex A-25 which has been equilibrated with 0.01 M NH_4HCO_3. The column is eluted with a linear bicarbonate gradient using a mixing chamber containing 400 ml of 0.01 M NH_4HCO_3 and a reservoir chamber containing 400 ml of 0.7 M NH_4HCO_3. Fractions containing 3.5 ml are collected. β-Mercaptoethanol was added to retard air oxidation of tetrahydrofolate compounds. The various fractions were pooled, freeze dried, desalted by gel filtration (G-25 coarse) and concentrated to 1-2 ml. Elution patterns were determined by the folate assay with L. casei and S. feacalis.

The standard folate compounds were supplied by Dr. W. Sakami and the results of chromatography are shown in Fig. 6. The folate monoglutamates are completely resolved, and the formyl triglutamates are well separated from the methyl triglutamates (Fig. 6). The unidentified peaks located at fraction 100-110 are most likely the 7, 8 H_2 Pt GLU_1 and Pt GLU_1 forms of 5 $CHOH_4$ Pte Glu. Fractions 175-180 and 220-230 are unidentified.

Cells of C. thermoaceticum (12.5 g wet weight) were harvested after 44 hrs growth at 55° and washed twice in 0.1 M phosphate, pH = 6.8 containing 0.05 M glucose and 0.05 M mercaptoethanol. A 50% suspension in this solution was allowed to ferment glucose under a N_2 stream for 10 min at 55°. Then the N_2 was shut off, and 25.5 μmole of $NaHC^{14}O_3$ (8.83×10^7 dpm/μmole) was introduced by syringe. In 30 sec the reaction mixture was removed from the 55° bath and placed in a 100° bath for 5 min. It was centrifuged at 17,000 rpm for 10' giving 30 ml of solution. The folates were separated from an aliquot by chromatography.

The elution pattern is shown in Fig. 7. Peaks II, III and IV contained ^{14}C. None of the peaks supported growth of S. feacalis. Peaks III and IV were freeze dried and desalted. A portion of each was incubated with conjugase and rechromatographed. The elution patterns revealed only one major peak containing all the ^{14}C in each case (Fig. 8). These peaks supported growth of L. casei but not of S. feacalis. Moreover, each chromatographed at the same position as peak II of Fig. 7 and 5 CH_3H_4 PteGlu of Fig. 6.

Peak IV of Fig. 7 was incubated with homocysteine and methyltransferase which was obtained from Dr. Warwick Sakami. This enzyme catalyzes the transfer of the methyl from methyltetrahydro-di or tri-glutamate to homocysteine forming methionine (Taylor and Weissbach, J. Biol. Chem. 242, 1502 (1967)). The reaction mixture and the control (without homocysteine) were passed separately through a Dowex column to separate the methionine from the folates which are retained by the column. It is seen in Fig. 9 that the ^{14}C activity which passed through the column was much greater when homocysteine was present in the reaction mixture. This is quite conclusive evidence that the ^{14}C was present in the methyl group of the isolated folate and was converted to methionine.

The specific radioactive activities of the peaks from Fig. 8A (IV of Fig. 7) and of Fig. 8B (III of Fig. 7) were determined and are given in Table VIII. Peak II of Fig. 7 could not be rechromatographed after the conjugase treatment because of insufficient material and its radioactivity was determined on material from the peak of Fig. 7. There was little change in its activity for S. feacalis after treatment with conjugase. The material of Peak II of Fig. 7 therefore is tentatively identified as $H_3C \cdot H_4$ Pt Glu.

Fig. 6. Chromatography on QAE-Sephadex column (1 X 16 cm) of a mixture of standard folate derivatives. mμg of folate (by L. casei growth) via Fraction #

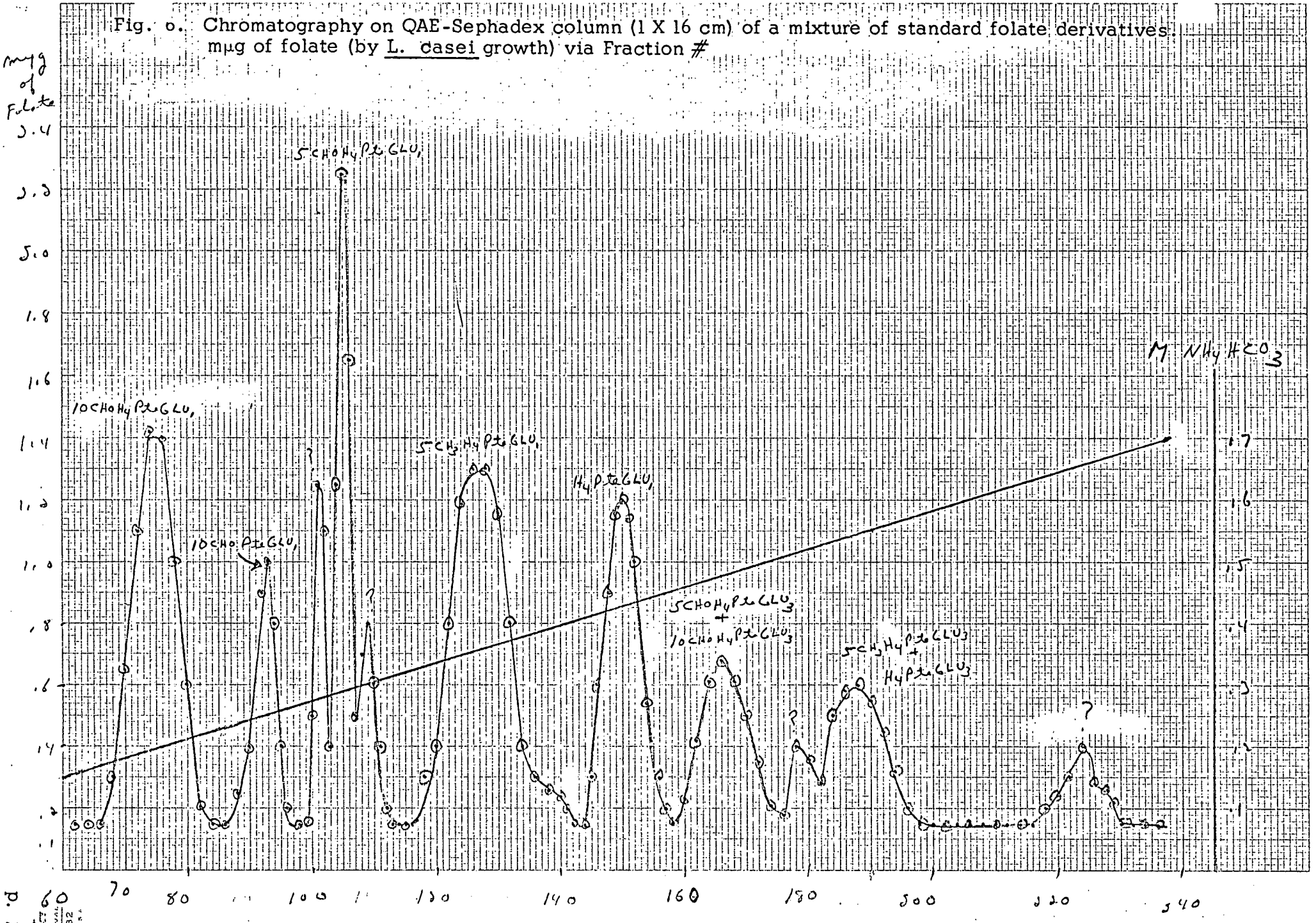
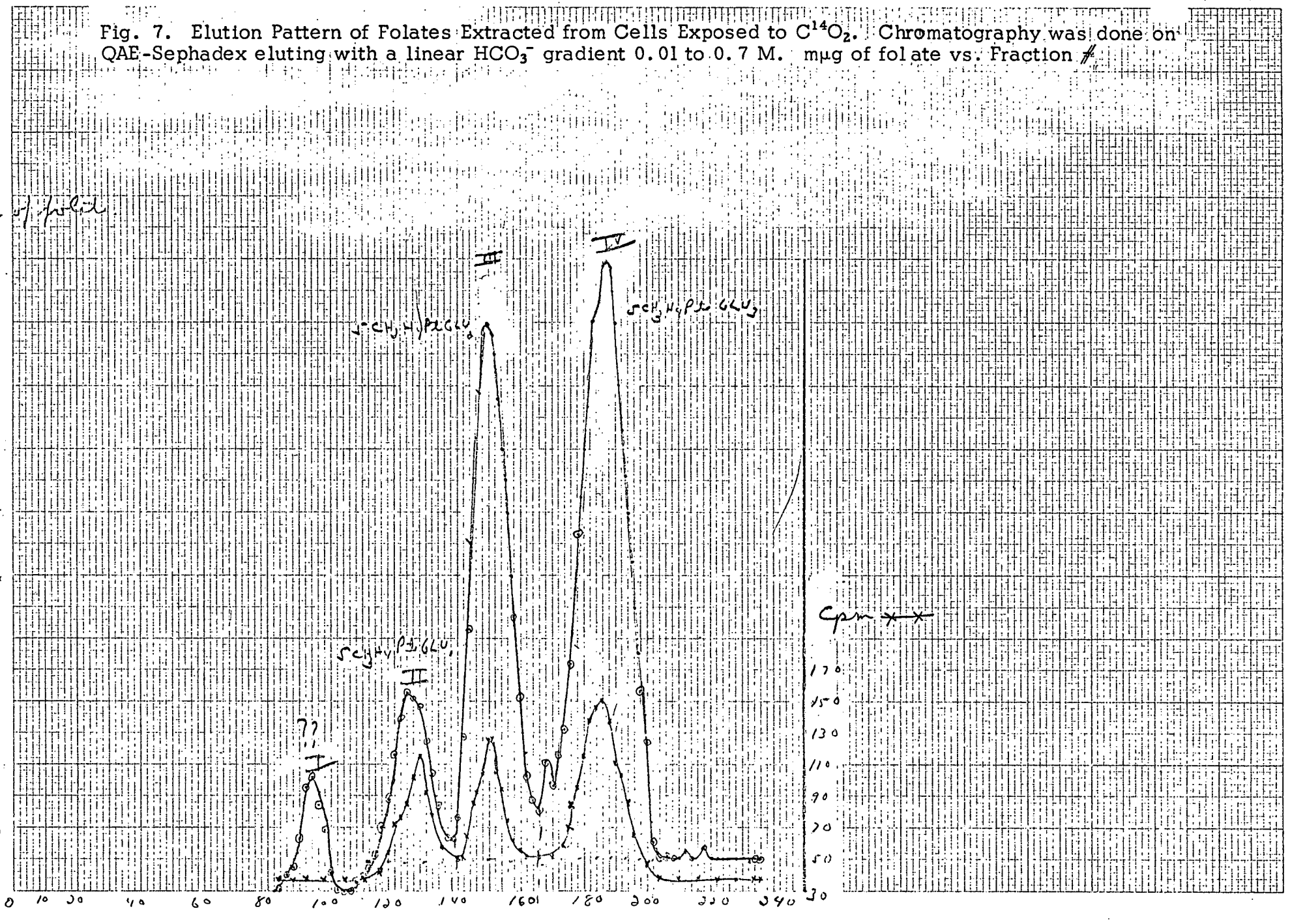


Fig. 7. Elution Pattern of Folates Extracted from Cells Exposed to $C^{14}O_2$. Chromatography was done on QAE-Sephadex eluting with a linear HCO_3^- gradient 0.01 to 0.7 M. mμg of folate vs. Fraction #



COO-1783-2

Fig. 8A. Chromatography on QAE-Sephadex of Peak IV of Fig. 6. after conjugase treatment. mμg folate vs. Fraction #.

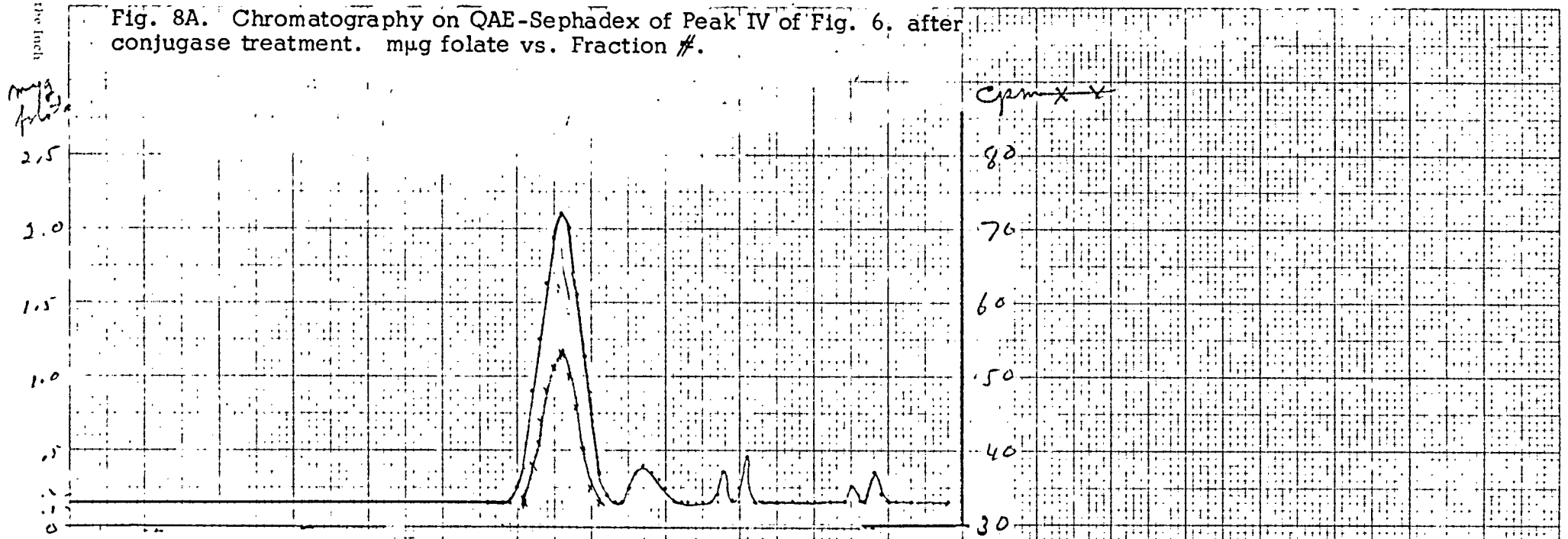


Fig. 8B. Chromatography of Peak III of Fig. 7 after conjugase treatment. mμg folate vs. Fraction #.

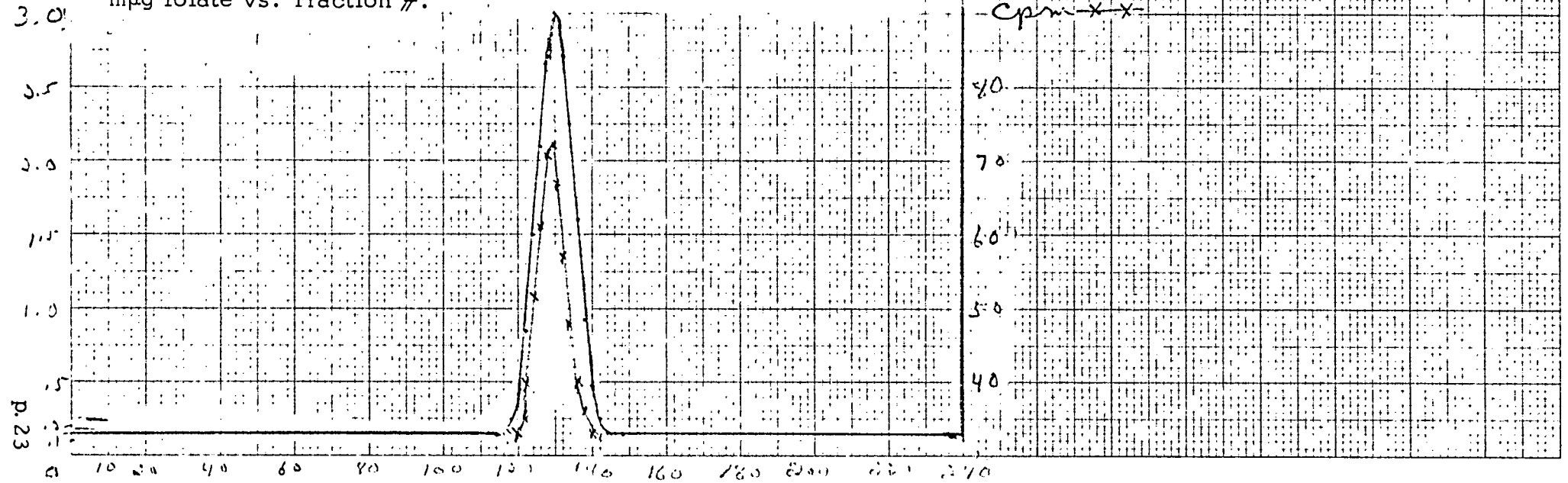


Fig. 9. ^{14}C Activity passing through a Dowex column after incubation of Peak IV of Table 5 with methyltransferase and homocysteine. cpm ^{14}C vs. Fraction #.

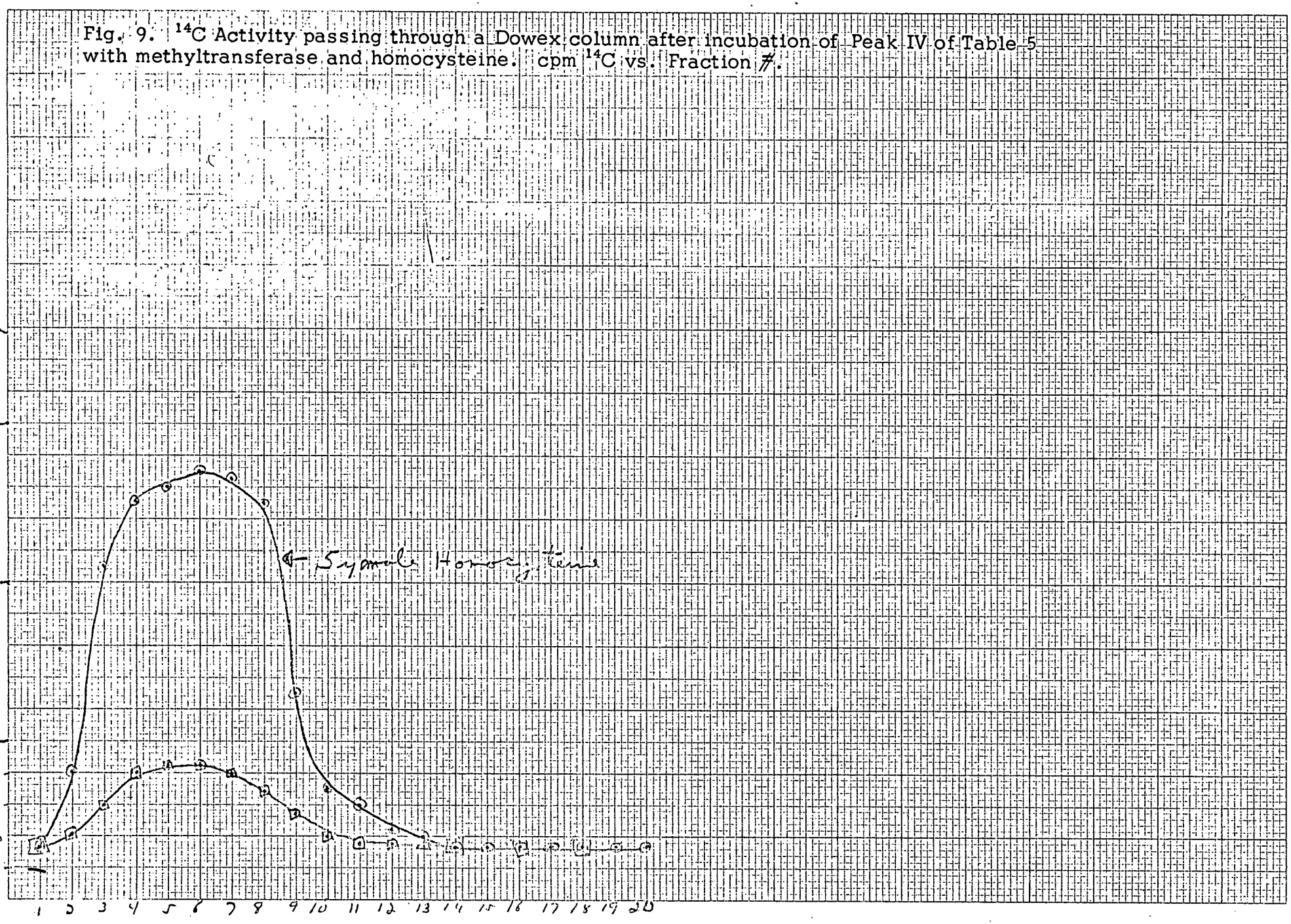


Table VIII

Specific Radioactivities of Folates Isolated from C. thermoaceticum after Exposure to $^{14}CO_2$ for 30 Seconds

Compound	Peak of Fig. 7	Total μmoles per 12.5 g of cells	Total dpm	dpm/μmole
$HC^{14}O_3$		25.5	2.25×10^9	8.84×10^7
$H_3C^{14}H_4$ Pt Glu_1	II	0.056	4.38×10^6	7.82×10^7
$H_3C^{14}H_4$ Pt Glu_2	III	0.12	1.09×10^6	0.91×10^7
$H_3C^{14}H_4$ Pt Glu_3	IV	0.14	1.02×10^6	0.73×10^7

It is to be noted that the procedure used for the methyl transfer is specific for methyl di or triglutamate. This proves that the radioactivity of peak IV of Table VI is in the 5 methyl position and it is apparently a triglutamate. The determined specific activity of the methyl folate derivatives suggests that methyltetrahydrofolate is indeed on the pathway to acetate. The high specific activity of the monoglutamate (Peak II) may be misleading, since this fraction was not rechromatographed after conjugase treatment. It remains to be proven which methyl folate derivative is the best precursor of acetate.

In extracting folates from C. thermoaceticum the methylene and methyl folates would be converted to formyl folates and folates respectively since they are unstable at pH values greater than 2 or less than 9 (May, J. Amer. Chem. Soc. 73, 3067 (1951)). Likewise any formimino folate derivatives would have been converted to the formyl compounds. No radioactivity was found at positions which would correspond to the formyl compounds (Fig. 7). Further study with larger quantities of bacteria apparently will be required to detect these compounds.

B. Further Studies of Acetate Synthesis by Crude Extracts of C. thermoaceticum. Last year we pointed out that the preparation of active extracts of C. thermoaceticum which fix $^{14}CO_2$ into acetate while fermenting pyruvate is not consistently successful. A means of preparing uniformly active cell free extracts is essential for continued progress in our studies. We therefore have undertaken a systematic study of this problem starting with standardization of the conditions for growth of cells which ferment glucose or pyruvate rapidly after being centrifuged and washed several times.

Cultures were harvested every 24 hr. The cell yield (g. fresh wght/liter), the pH and glucose concentration of the medium and the rate of glucose fermentation and acetate synthesis by the cells were determined. These studies showed that greater cell growth and stability could be achieved by increasing both the glucose and $NaHCO_3$ concentration of the culture media. Large yields of highly active cells of C. thermoaceticum have been routinely obtained after 48 hr growth in the standard culture medium (Ljungdahl and Wood, J. Bacter. 89, 1055 (1965) altered to contain 0.1 M glucose and 0.2 M $NaHCO_3$. Typical results are shown in Table IX.

Table IX

Fermentation of Glucose by C. thermoaceticum Grown in Media Containing 0.1 M Glucose and 0.2 M $NaHCO_3$

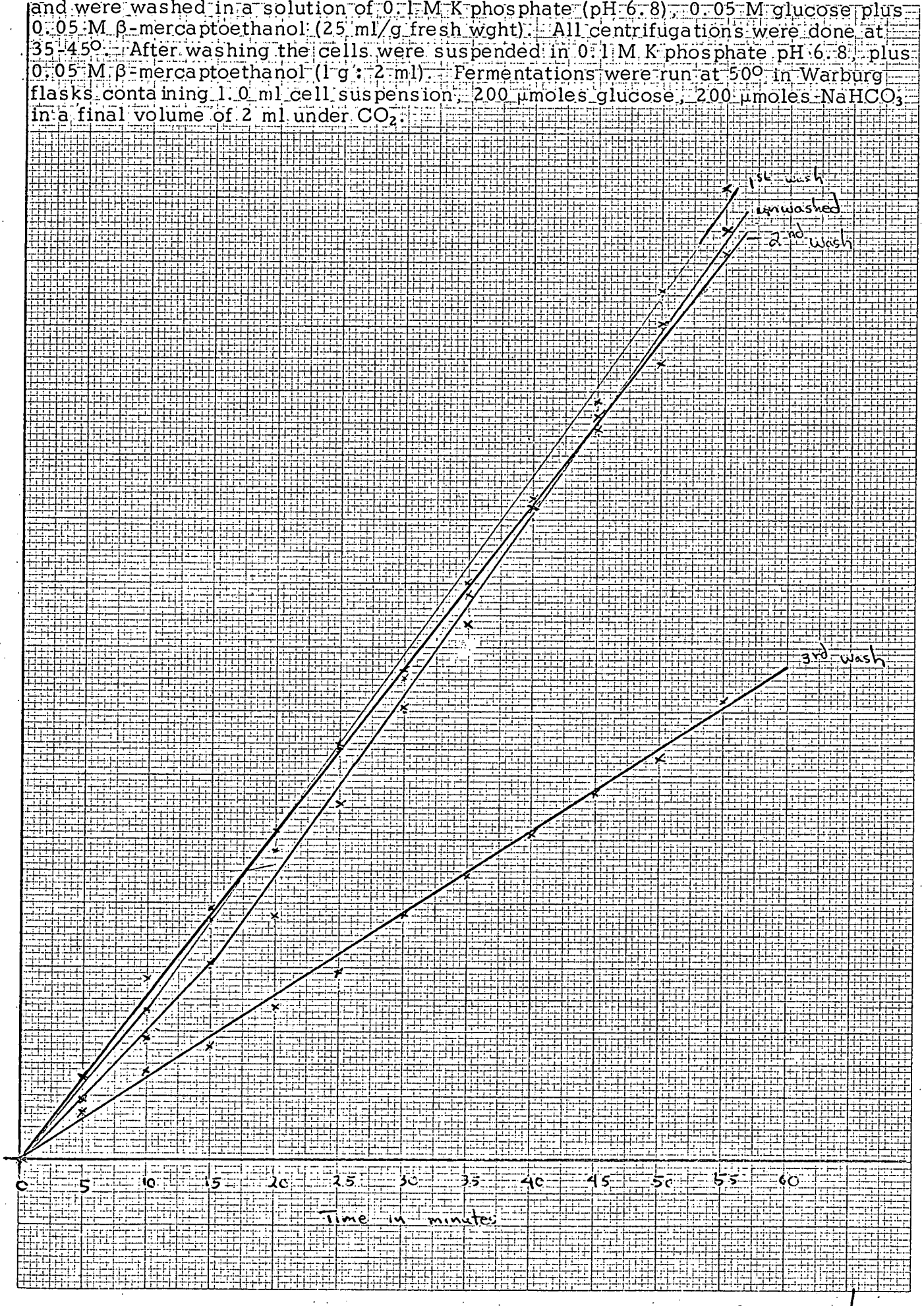
Culture Age	pH of the media	Glucose concentration in the media	Cell yield	Rate of fermentation per gram cells
hrs	pH units	μmoles/ml	Fresh Wght/liter	μmoles/ minute
0	7.4	100	-	-
24	7.2	82	5.5	0.664
48	7.15	64	9.3	0.674
72	6.75	53	10.9	0.127

Fermentation were carried out at 50°C for 2 hr in Warburg flasks which contained 1 ml of a 50% suspension of C. thermoaceticum (fresh wght/vol) in 0.1 M K phosphate pH 6.8 plus 5.0×10^{-3} M cysteine, 200 μmoles glucose and 200 μmoles $NaHCO_3$ in a final volume of 2 ml under CO_2. The rates were constant during the two hrs.

The cells can be washed twice in a solution containing 0.1 M K phosphate (pH 6.8), 0.05 M glucose and 0.05 M β-mercaptoethanol without losing any activity (Fig. 10). When stored under CO_2 at 5° the cells retain 50-60% of their activity for 72 hrs. Cells washed in the phosphate buffer only lost activity rapidly and the addition of both glucose and mercaptoethanol to the phosphate solution gave the most consistently active cells.

We have only recently begun work with extracts prepared from these cells. The extracts must be prepared anaerobically in dim light. To date extracts have been prepared by rupturing the cells in a french pressure cell under an atmosphere of CO_2. These extracts are capable of fermenting both glucose and pyruvate. Although reasonably high rates of glucose fermentation have been routinely obtained (0.5 μmoles/min/100 mgs protein) only very low rates of acetate synthesis from the $^{14}CO_2$ have been observed thus far (0.005 μmoles/μmoles glucose). Much higher rates of synthesis of acetate from the $^{14}CO_2$ have been obtained during the fermentation of pyruvate (Table X). These rates though variable are quite good. It should be noted that the theoretical value of 0.25 μmoles of acetate synthesized per μmole pyruvate fermented has been obtained. Further work is needed to optimize the conditions for preparations of extracts which will synthesize acetate from CO_2 at high rates routinely.

Fig. 10. Fermentation of glucose by washed cells of C. thermoaceticum. Cells were harvested after 48 hr growth and were washed in a solution of 0.1 M K phosphate (pH 6.8), 0.05 M glucose plus 0.05 M β-mercaptoethanol (25 ml/g fresh wght). All centrifugations were done at 35-45°. After washing the cells were suspended in 0.1 M K phosphate pH 6.8, plus 0.05 M β-mercaptoethanol (1 g : 2 ml). Fermentations were run at 50° in Warburg flasks containing 1.0 ml cell suspension, 200 μmoles glucose, 200 μmoles $NaHCO_3$ in a final volume of 2 ml under CO_2.



20 Squares to the Inch

Table X

Fermentation of Pyruvate and Synthesis of Acetate from $^{14}CO_2$ by Extracts from Washed Cells of Clostridium thermoaceticum

Additions	Time	Pyruvate Fermented	Acetate Synthesized from $^{14}CO_2$	μmoles ^{14}C-acetate synthesized / μmoles pyruvate fermented
	min	μmoles	μmoles	
None	60	0	0	
Pyruvate	30	2.70	0.381	0.141
	60	5.01	0.735	0.147
None	60	0	0	--
Pyruvate	60	9.71	2.37	0.244
Pyruvate ATP, DPN	60	18.34	4.90	0.268

Cells were harvested after 48 hr growth, washed (2X) in 0.1 M K phosphate pH 6.8, 0.05 M glucose, 0.05 M β-mercaptoethanol, suspended in 0.1 M K phosphate (pH 6.8), 0.05 M β-mercaptoethanol and broken in a french press under CO_2 in dim light. The homogenate was centrifuged at 15,000 rpm for 15 min and the supernatant solution used. Incubations were carried out at 50° in Warburg flasks which contain 1.0 ml extract (26 mgs protein for lines 1 and 2 and 23 mgs protein for lines 3, 4 and 5), 100 μmoles 0.1 M K phosphate pH 6.8, 2.0 μmoles β-mercaptoethanol, 200 μmoles Na $H^{14}CO_3$ (Sp. Act. 2×10^5 dpm (μmole) and as indicated 200 μmoles sodium pyruvate, 20 μmoles ATP and 1.0 μmoles DPN in a final volume of 2.0 ml under an atmosphere of CO_2.

It was proposed last year that we would attempt to isolate the corrinoid-enzymes which are involved in the synthesis of acetate by labeling them with ^{14}C-propyl iodide. Propyl iodide combines with the corrinoid enzyme forming (Co-$CH_2 \cdot CH_2CH_3$)-corrinoid-enzyme which are inactive but may be regenerated by treatment with light. The light cleaves the propyl group from the cobalt. These studies have been postponed until we are able to obtain an enzyme preparation which is consistently active in fixing CO_2 into acetate.

For our future studies it is almost certain that we will need TPNH and ATP generating systems and perhaps a system which reduces ferredoxin to replace the pyruvate requirements shown in Reaction 14. The enzymes which are commercially available are not stable at 55-60° (the optimum temperature of the enzymes of C. thermoaceticum) and they will not be useful. Consequently, we have recently begun an attempt to isolate these enzymes from C. thermoaceticum. These enzymes also are of interest inherently because of their thermophylic properties.

To date we have observed both lactic dehydrogenase and pyruvate kinase in extracts of C. thermoaceticum and have begun to purify the latter. The crude extracts contain enzymes which break down TPNH and ATP and this clearly demonstrates the need of an ATP and TPNH generating system for future studies.

For our studies a more rapid method for the determination of the amount of $^{14}CO_2$ incorporated into acetate was desirable. A method has been developed which requires only 2-3 hr for the assay of 24-30 samples. It may be used to determine the ^{14}C-acetate in the presence of large pools of carboxy labeled pyruvate, parapyruvate, formate and lactate which also are formed by extracts of C. thermoaceticum when fermenting pyruvate in the presence of $^{14}CO_2$.

The assay involves oxidation of the deproteinized extract with acid permangate. Formate is oxidized to CO_2; and pyruvate, parapyruvate and lactate to acetate and CO_2. $^{14}CO_2$ is fixed only in the carboxyl groups of the compounds so their effect on the determination is removed. The acetate is separated from the residue of the oxidation by extraction with ether and then the total ^{14}C content is determined on an aliquot part. The overall recovery of ^{14}C-acetate is approximately 60% and is constant over a wide range of concentrations (0.05-15.0 μmoles). Consequently, this assay can be used to measure the time course of acetate formation from $^{14}CO_2$ and its relation to enzyme concentration. The isolated acetate can be chemically degraded to determine the distribution of isotope in its carbon atoms. This assay does not allow determination of the specific activity of the acetate as such, as does the method which was described last year.

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3. Davis, J. J., Willard, J. M. and Wood, H. G.
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