Reversibility of a Phosphoroclastic Reaction

By J. WILSON, L. O. KRAMPITZ AND C. H. WERKMAN
Industrial Science Research Institute, Iowa State College, Ames, Iowa, U.S.A.

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Utter, Lipmann & Werkman (1945) using C^{13} as a tracer showed reversibility of the phosphoroclastic reaction in *Escherichia coli* according to the equation:

Esch. coli possesses an enzyme, hydrogenlyase, which reversibly performs the reaction:

Reaction 1 occurring with reaction 2 involves carbon dioxide fixation.

Koepsell & Johnson (1942) obtained a cell-free extract from *Clostridium butylicum* which converts pyruvic acid into acetic acid, carbon dioxide and hydrogen, and requires phosphate for the reaction. Later Koepsell, Johnson & Meek (1944) found that inorganic phosphate is taken up and labile phosphate appears. The overall reaction may be illustrated:

The question arises whether formic acid occurs as an intermediate (Osburn, Brown & Werkman (1937) and Stiles, Peterson & Fred (1929)). This communication deals with the reversibility of reaction 3, and evidence is presented that formic acid is not an intermediate. Reaction 3 appears to differ fundamentally from the phosphoroclastic reaction of *Esch. coli*.

METHODS

Cl. butylicum (Beijerinck) Donker was used in this investigation. The organism was grown in a medium of 1% glucose, 0.4% yeast extract, 0.4% peptone, 0.1% beef extract, 0.05% reduced iron, 10% tap water and 1.25% K_2HPO_4 and adjusted to pH 7 with HCl. The glucose and K_2HPO_4 were sterilized separately and added aseptically to the medium. The medium was inoculated with 100 ml. of an 18 hr. culture and incubated at 37° for 18–24 hr. The medium was strained through cheese cloth to remove most

of the iron. The cells were harvested with a Sharples centrifuge. The yield varied from 1.5 to 2.5 g./l. of medium. A cell-free extract was prepared from these cells by the method of Utter, Kalnitsky & Werkman (1946). Experiments with this extract were conducted in 125 ml. manometric flasks with total volumes indicated in the tables.

Reversibility was determined by incubating pyruvic acid with a properly buffered enzyme preparation in the presence of one or the other of the products containing an excess of C^{13} . The reaction was stopped short of completion and the residual pyruvic acid was recovered, degraded and analyzed for excess C^{13} .

Separation of the pyruvic acid from the acetic acid was accomplished by two methods: (1) steam distillation as carried out by Utter *et al.* (1945), and (2) ether extraction of the acetic acid in the presence of $NaHSO_3$ which forms an ether-insoluble complex with pyruvic acid. The bisulphite complex was decomposed by boiling and the pyruvic acid removed by ether extraction.

When only the carboxyl carbon of pyruvic acid was desired for isotopic analysis, it was obtained by oxidation with ceric sulphate, which oxidizes pyruvic acid to acetic acid and CO_2 . The acetic acid was separated by steam distillation and oxidized to CO_2 with $K_2S_2O_8$. For the isotopic analysis of each carbon atom in the pyruvic acid the latter was degraded first by yeast Lebedev juice which decarboxylates pyruvic acid to acetaldehyde and CO_2 . The acetaldehyde was treated with $NaOI$, when the methyl group formed iodoform and the aldehyde group formic acid. The iodoform was oxidized by dry combustion to CO_2 and the formic acid was oxidized to CO_2 by the method of Osburn & Werkman (1932).

$CH_3C^{13}OOH$ was prepared by means of the Grignard reaction. $HC^{13}OOH$ was prepared by the method of Utter *et al.* (1945). $C^{13}O_2$ was added as $NaHC^{13}O_3$.

RESULTS

Fixation of $C^{13}O_2$ in the carboxyl group of pyruvic acid. When the buffered enzyme preparation was incubated with sodium pyruvate and $NaHC^{13}O_3$ under the conditions of Table 1, the residual pyruvate contained C^{13} only in the carboxyl group. The

Table 1. *Fixation of $C^{13}O_2$ in pyruvic acid by enzyme extract from Clostridium butylicum*

(Reaction mixture: 375 mg. lyophilized *Cl. butylicum* extract, 1.6 mmol. $NaHC^{13}O_3$ containing 5.43% (atom) excess C^{13} , 2.0 mmol. pyruvate, 1.08 mmol. phosphate, pH 6.8; total volume, 30 ml. Time, 7 hr.; temp. 30°. Atmosphere, 100% N_2 .)

Pyruvic acid utilized (mmol.)	Excess of C^{13} in (atom %)		
	Residual CO_2	CH_3CO- of pyruvic acid	$-COOH$ of pyruvic acid
0.66	1.96	0.00	1.98

C^{13} contents of the carboxyl carbon and the residual CO_2 indicate that an equilibrium was established. There was no excess C^{13} in the CH_3CO- group.

Effect of hydrogen on fixation of $C^{13}O_2$. Lipmann & Tuttle (1945) observed that molecular hydrogen inhibited the breakdown of pyruvic acid by the enzyme extract of *Cl. butylicum*. It was of interest to determine the effect of hydrogen on the fixation of $C^{13}O_2$ under conditions similar to those of the previous experiment except that the atmosphere consisted of 50% hydrogen and 50% nitrogen (Table 2). Fixation was substantially greater in the presence of hydrogen, although the amount of pyruvate dissimilated was less than in the presence of 100% nitrogen. Apparently the point of equilibrium in the presence of hydrogen is farther to the left, resulting in greater fixation.

without an added energy-rich source of phosphate. In order to ensure adequate separation of the pyruvic acid from the $CH_3C^{13}OOH$, both the bisulphite-ether extraction method and the double steam distillation were employed on each sample. The pyruvic acid was degraded by the yeast carboxylase and by the ceric sulphate methods, and essentially the same C^{13} content was obtained by both methods. Results in Table 3 were obtained by the ceric sulphate method. The C^{13} content of the $-CO-$ group was calculated from the content of the CH_3CO- group. The average values obtained with repeated experiments showed the same magnitude of fixation. The calculated value of C^{13} for the $-CO-$ group is nearly in equilibrium with the residual $CH_3C^{13}OOH$. Results of an experiment carried out with whole cells (when possibly greater exchange might occur)

Table 2. *Effect of hydrogen on fixation of $C^{13}O_2$ by Clostridium butylicum*

(Reaction mixture: 15 ml. enzyme extract, 1.6 mmol. $NaHC^{13}O_3$ (5.43% excess C^{13}), 2.0 mmol. pyruvate, 1.08 mmol. phosphate, pH 6.8; total volume, 30 ml. Time, 3 hr.; temp. 30°. Degradation by ceric sulphate.)

Atmosphere	Pyruvic acid utilized (mmol.)	Excess of C^{13} in (atom %)		
		Residual CO_2	CH_3CO- of pyruvic acid	$-COOH$ of pyruvic acid
100% N_2	1.19	1.90	0.00	1.59
50% N_2 + 50% H_2	0.78	1.80	0.00	1.75

Exchange between $CH_3C^{13}OOH$ and $CH_3COCOOH$. If equation (3) is reversible, then before acetic acid could participate in it, it would first need to be phosphorylated to acetyl phosphate. Results of Table 3 show the results of adding $CH_3C^{13}OOH$

did not significantly differ from those obtained with the enzyme extract.

Effect of adenosine triphosphate (ATP) and acetyl phosphate on the exchange of $CH_3C^{13}OOH$. Utter *et al.* (1945) working with cell-free extracts of *Esch. coli* found that significant fixation of $CH_3C^{13}OOH$ in pyruvate occurred only in the presence of adenosine triphosphate. The high-energy phosphate was required apparently for the phosphorylation of the acetic acid. Table 4, Exp. 1, records the results of a typical experiment carried out in the presence of ATP and $CH_3C^{13}OOH$. Exp. 2 contained acetyl phosphate and adenylic acid in addition to the $CH_3C^{13}OOH$. In the presence of acetyl phosphate, adenylic acid and $CH_3C^{13}OOH$ (Exp. 2) the C^{13} content of the CH_3CO- group of the pyruvate was significantly higher than when $CH_3C^{13}OOH$ was used alone. The C^{13} content is also greater in the presence of ATP (Exp. 1); however, it is not as high

Table 3. *Exchange between $CH_3C^{13}OOH$ and $CH_3COCOOH$*

(Reaction mixture: 6 ml. enzyme extract from *Cl. butylicum*, 1.1 mmol. $CH_3C^{13}OONa$ (4.65% excess C^{13}), 2.0 mmol. pyruvate, 1.08 mmol. phosphate buffer, pH 6.8; total volume, 30 ml. Time, 60 min.; temp. 30°. Atmosphere, 95% H_2 + 5% CO_2 . Pyruvate dissimilated, 1.39 mmol.)

Reaction product examined	Excess C^{13} (atom %)
Residual CH_3COOH	0.11
Pyruvate:	
$-COOH$	0.00
CH_3CO-	0.04
$-CO-$	0.08 (calc.)

Table 4. *Effect of ATP and acetyl phosphate on $CH_3C^{13}OOH$ fixation*

(Each experimental mixture contained 5 ml. *Cl. butylicum* extract, 2 mmol. pyruvate, 1.06 mmol. $CH_3C^{13}OONa$ (4.65% excess C^{13}), 0.1 mmol. $MgCl_2$, and 1.08 mmol. phosphate buffer, pH 6.8. In addition, Exp. 1 contained 34 mg. adenylic acid and 75 mg. acetyl phosphate. Exp. 2 contained 195 mg. ATP. Atmosphere, 95% H_2 and 5% CO_2 . Time, 1.5 hr.; temp. 30°.)

Grouping of pyruvate molecule which was estimated	Exp. 1, with adenylic acid + acetyl phosphate		Exp. 2, with adenosine triphosphate	
	Quantity (mmol.)	Excess C^{13} (atom %)	Quantity (mmol.)	Excess C^{13} (atom %)
$-COOH$	0.64	0.00	0.36	0.00
CH_3CO-	1.18	0.12	2.20	0.06
$-CO-$	—	0.24 (calc.)	—	0.12 (calc.)

as in Exp. 2. Assuming that the CH_3 group of the pyruvate contains no C^{13} , as indicated by degradation by yeast carboxylase and the iodoform reaction, calculation of the C^{13} content of the $-\text{CO}-$ group gives values which are significant. The presence of the C^{13} in the $-\text{CO}-$ group is expected from the fixation of $\text{CH}_3\text{C}^{13}\text{OOH}$.

Exchange between HC^{13}OOH and CH_3COCOOH . Since it was questionable whether formic acid was an intermediate in the reaction, an experiment was carried out with pyruvic acid and HC^{13}OOH . If the reaction is similar to that in *Esch. coli*, exchange should occur with pyruvic acid. Since the occurrence of formic acid in the dissimilation of pyruvic acid by *Cl. butylicum* was in question, HC^{13}OOH was used in an experiment with pyruvic acid. If the reaction with *Cl. butylicum* is similar to that of *Esch. coli*, C^{13} should be found in the residual pyruvic acid. The results (Table 5) show conclusively that formate

Table 5. *Absence of exchange between HC^{13}OOH and CH_3COCOOH*

(Reaction mixture: 8 ml. *Cl. butylicum* extract, 2.0 mmol. phosphate buffer, pH 6.8; 1 mmol. HC^{13}OOH (2.31% excess C^{13}), 2.0 mmol. pyruvate, 25 mg. ATP; total vol., 37 ml. Time, 60 min.; temp. 30° . Atmosphere, 50% H_2 and 50% N_2 . Pyruvate dissimilated, 0.88 mmol.)

Reaction product examined	Excess C^{13} (atom %)
Residual NaHCO_3	0.00
Pyruvate:	
$-\text{COOH}$	0.00
$\text{CH}_3\text{CO}-$	0.00
Formate	2.32

does not exchange with pyruvate, and from the C^{13} content of the residual NaHCO_3 it is evident that the formate does not break down to H_2 and CO_2 .

DISCUSSION

The phosphoroclastic reaction in *Cl. butylicum* has been shown to be reversible. Formic acid was not found to be an intermediate. This appears to be the

first demonstration of C_2 and C_1 addition where carbon dioxide, *per se*, is the C_1 compound fixed. Such a fixation clarifies the data of Brown, Wood & Werkman (1944) who found an excess of C^{13} in the carboxyl group of lactic acid formed by *Cl. butylicum* from glucose in the presence of C^{13}O_2 . Since no appreciable amounts of C_4 compounds were detected, no significant fixation of the $\text{C}_2 + \text{C}_1$ type could have occurred.

Rigorous proof that acetyl phosphate is an intermediate in the reaction remains to be demonstrated; however, the presence of an energy-rich phosphate source together with $\text{CH}_3\text{C}^{13}\text{OOH}$ does enhance the reversibility of the reaction. In the phosphoroclastic reaction of *Esch. coli*, reduction of carbon dioxide to formic acid occurs prior to the C_2 and C_1 addition. With *Cl. butylicum* there is either a simultaneous reduction with fixation, or fixation occurs with subsequent reduction. It is difficult to visualize the latter possibility with acetic acid or acetyl phosphate as the C_2 compound concerned in the fixation. The possibility remains that some C_1 reduced compound other than formic acid is a component of the system.

The observation that an atmosphere of hydrogen enhanced fixation of carbon dioxide in pyruvic acid strengthens the evidence for reversibility of the reaction.

SUMMARY

1. The conversion of pyruvic acid into acetic acid, carbon dioxide and hydrogen by *Clostridium butylicum* which requires phosphate has been demonstrated by the use of $\text{NaHC}^{13}\text{O}_3$ to be reversible.

2. It is shown that molecular hydrogen enhances fixation of carbon dioxide in this reaction.

3. Formic acid is not an intermediate and it is suggested that acetyl phosphate might be a component of the system, although no rigorous proof for this hypothesis has as yet been obtained.

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