OXALOACETATE DECARBOXYLATION AND OXALOACETATE-CARBON DIOXIDE EXCHANGE IN *ACETOBACTER XYLINUM*

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ABSTRACT

BENZIMAN, MOSHE (The Hebrew University of Jerusalem, Jerusalem, Israel), AND N. HELLER. Oxaloacetate decarboxylation and oxaloacetate-carbon dioxide exchange in *Acetobacter xylinum*. J. Bacteriol. **88**:1678-1687. 1964.—Extracts of *Acetobacter xylinum*, prepared by sonic treatment, were shown to catalyze the decarboxylation of oxaloacetate (OAA) to pyruvate and CO₂, and the exchange of C¹⁴-carbon dioxide into the β-carboxyl of OAA. Fractionation of the extracts with ammonium sulfate resulted in a 10-fold increase of the specific activity of the enzyme system catalyzing the CO₂ exchange and OAA decarboxylation reactions. The purified preparation catalyzed the exchange of pyruvate-3-C¹⁴ into OAA. Similar pH curves with a pH optimum of 5.6 were obtained for the CO₂ exchange and OAA decarboxylation reactions. Both reactions require the presence of Mn²⁺ or Mg²⁺ ions. OAA decarboxylation was more strongly inhibited than the exchange of CO₂ by dialysis or metal-chelating agents. Avidin did not inhibit either reaction. Adenosine triphosphate (ATP), adenosine diphosphate (ADP), guanosine triphosphate (GTP), guanosine diphosphate (GDP), pyrophosphate, or inorganic phosphate did not promote OAA decarboxylation and the CO₂-exchange reaction catalyzed by the purified preparation. The purified preparation failed to catalyze the carboxylation of phosphoenolpyruvate in the presence of GDP, ADP, or inorganic phosphate, and that of pyruvate in the presence of ATP or GTP, even when supplemented with an OAA-trapping system. A scheme for OAA decarboxylation which could account for the observed exchange reactions and for the failure to obtain net fixation of CO₂ is proposed. The relation between the exchange reaction and the synthesis of cellulose from pyruvate by *A. xylinum* is discussed.

Exogenous CO₂ failed to serve as a carbon source for cellulose synthesized by succinate-grown cells of *Acetobacter xylinum* in the presence of pyruvate (Benziman and Burger-Rachamimov, 1962). These cells, however, catalyze a rapid

exchange of C¹⁴O₂ into oxaloacetate (OAA) (Benziman and Abeliovitz, 1964).

The purpose of this investigation was to study the nature of the exchange reaction and its relationship to the mechanism of pyruvate conversion to cellulose. A preliminary report of this work has appeared (Heller and Benziman, 1964).

MATERIALS AND METHODS

Cells and extracts. Succinate-grown cells of *A. xylinum* were grown and harvested, and extracts were prepared as previously described (Benziman and Abeliovitz, 1964). A fraction rich in OAA decarboxylation and OAA-CO₂ exchange activities was obtained by fractional precipitation of crude extracts (containing 8 to 15 mg of protein per ml) with crystalline ammonium sulfate. Fraction AS 60-90, precipitating between 0.6 and 0.9 saturation, showed a 10-fold increase in the specific activity for both reactions, and contained 4% of the protein and 40% of both activities, relative to the crude extract. The fraction was stored in the frozen state, and was dialyzed before use for 3 hr against 3,000 volumes of 0.04 M phosphate buffer (pH 6.0), unless otherwise indicated.

OAA decarboxylation. A unit of OAA decarboxylase activity corresponds to the amount of enzyme required to decarboxylate 1 μmole of OAA in 5 min under standard conditions. Specific activity is defined as units per milligram of protein. The standard reaction mixture (1 ml) contained: 0.2 M acetate buffer (pH 5.6), 3 mM MnCl₂, 0.01 M glutathione, and enzyme (0.05 to 0.5 mg of protein, AS 60-90). Reaction was started by addition of OAA (final concentration 0.05 M), and the mixture was incubated at 30°C for 5 to 10 min as indicated. Enzyme activity was measured by determining the amount of OAA consumed. With more purified preparations, the reaction was carried out in Warburg double side-arm flasks, and the reaction was terminated by

tipping in 0.2 ml of 5 N H₂SO₄ from the side arm. Activity was determined from the amount of CO₂ evolved. The results reported were corrected for spontaneous decarboxylation of OAA which did not exceed 5% of total readings.

OAA-C¹⁴O₂ exchange. Exchange activity was measured by determining the radioactivity of OAA after incubation of enzyme with OAA and NaHC¹⁴O₃. Reactions were carried out at 30 C in stoppered side-arm tubes (10 × 150 mm). The standard reaction mixture (1 ml) contained: 0.2 M acetate buffer (pH 5.6), 3 mM MnCl₂, 0.01 M glutathione, enzyme (0.05 to 0.2 mg of AS 60-90 protein), and 0.05 M OAA. The side arm contained 0.05 M NaHC¹⁴O₃ (10 to 20 μc). Reaction was terminated with 0.2 ml of 5 N HCl, and the mixture was centrifuged. The radioactivity of the OAA was determined after its conversion to the 2,4-dinitrophenylhydrazone derivative according to Maruyama and Lane (1962), modified as follows. To the protein-free reaction mixture, 4 ml of 2,4-dinitrophenylhydrazine saturated in 2 N HCl were added, and the mixture was allowed to stand at 30 C for 30 min. Ethyl acetate (4 ml) was added, and the mixture was shaken vigorously in a Vortex test-tube mixer; 0.5-ml samples of the ethyl acetate phase were plated on aluminum planchets and then counted in a gas-flow counter. Control experiments in the absence of enzyme gave readings that did not exceed that of the background. The enzyme concentration and the incubation periods were chosen so that the dilution of radioactive CO₂ by the CO₂ arising from OAA decarboxylation was lower than 10%.

Oxidation experiments were carried out at 30 C in a Warburg apparatus by use of standard manometric technique (Umbreit, Burris, and Stauffer, 1957). Endogenous oxidation rates accounted for less than 5% of the total readings. The values reported were corrected for endogenous rates. Substrates used were in the form of potassium salts, and the solutions were freshly prepared before each experiment.

Analytical methods. Protein was determined according to Lowry et al. (1951), and inorganic phosphate according to Fiske and SubbaRow (1925). Total keto acids were determined by the direct method of Friedman and Haugen (1943). OAA and pyruvate were determined enzymatically with malic dehydrogenase and lactic dehydrogenase, respectively, as previously de-

scribed (Benziman and Abeliowitz, 1964). Acetaldehyde was determined according to Barker and Summerson (1941). Acetate was measured as previously described (Benziman and Abeliowitz, 1964). Pyruvate kinase activity was assayed according to Cannata and Stoppani (1963a). All spectrophotometric determinations were carried out in a Zeiss spectrophotometer with cuvettes of 1-cm light path.

Radioactivity in the β-carboxyl of OAA was determined by decarboxylation with Al³⁺ (Krebs and Eggleston, 1945). The liberated CO₂ was trapped in KOH, precipitated as BaCO₃, collected on Fiberglas discs, and counted.

Radioactivity of the nonvolatile acids was determined by the method of Large, Peel, and Quayle (1962). Dinitrophenylhydrazones of OAA and pyruvate were prepared and separated, and their radioactivity was determined as described previously (Benziman and Abeliowitz, 1964).

Chemicals. Oxaloacetic acid, *p*-chloromercuribenzoate, *N*-ethyl maleimide, *o*-phenanthroline, and αα' dipyridyl were purchased from Sigma Chemical Co., St. Louis, Mo.; phosphoenolpyruvic tricyclohexylammonium salt, malic dehydrogenase (pig heart; specific activity, 36), lactic dehydrogenase (rabbit skeletal muscle; specific activity, 360), and reduced nicotinamide adenine dinucleotide phosphate (NADPH₂) were obtained from C. F. Boehringer & Soehne GmbH, Mannheim-Waldhof, Germany; avidin (2,500 units per mg), α-ketoglutaric acid, adenosine triphosphate, and adenosine diphosphate from Nutritional Biochemicals Corp., Cleveland, Ohio; guanosine diphosphate and guanosine triphosphate (sodium salts) from Pabst Laboratories, Milwaukee, Wis.; and Na₂C¹⁴O₃ and pyruvate-3-C¹⁴ from the Radiochemical Centre, Amersham, England. The latter compound was purified according to Utter and Keech (1963) and used immediately. 2,4-Dinitrophenylhydrazine, obtained from the British Drug Houses, was recrystallized twice from 2 N HCl before use. Potassium pyruvate was prepared according to Robertson (1942).

RESULTS

End products of OAA degradation. In crude extracts OAA is broken down to pyruvate, acetaldehyde, acetate, and CO₂, but in the purified fraction only a single-step decarboxyla-

TABLE 1. *Stoichiometry of OAA degradation by Acetobacter xylinum extracts*

System*	Component measured†						Recovery of carbon %
	OAA	Pyruvate	CO ₂	O ₂	Acetaldehyde	Acetate	
Crude extract							
Net change	-18.1	+8.9	+27.0	-2.6	+3.9	+5.3	100
AS-60-90							
Net change	-8.1	+8.0	+7.6	0.0	0.0	0.0	97

* Reaction mixtures (1 ml) in Warburg double side arm flasks contained in the main chamber: 0.2 M phosphate buffer (pH 6.0), 3 mM MnCl₂, 0.01 M glutathione, and enzyme (crude extract, 10 mg of protein; AS 60-90, 0.39 mg of protein). The side arm contained 0.02 M OAA. incubated for 10 min at 30 C. Reaction was terminated by tipping in 0.2 ml of 5 N H₂SO₄ from the second side arm.

† Components were measured as described in Materials and Methods; amounts are expressed in micromoles.

tion to pyruvate occurs (Table 1). The changes observed in the crude extract were consistent with a scheme in which OAA was decarboxylated to pyruvate and CO₂, and the pyruvate formed was partly oxidized to acetate with acetaldehyde as an intermediary step. The proposed scheme was in line with earlier observations on pyruvate metabolism in *A. xylinum* extracts, namely, the decarboxylation of pyruvate to acetaldehyde and CO₂, the oxidation of acetaldehyde to acetate, and the inability of the extracts to oxidize acetate further under the experimental conditions employed (Neeman and Benziman, 1962). It was further proven by incubating AS 60-90 with pyruvate that the purified fraction is devoid of activity toward pyruvate. We found that there was no change in the level of pyruvate after 60 min of incubation. This observation was in line with the previous finding that the pyruvic decarboxylase of *A. xylinum* extracts precipitates below 50% saturation with ammonium sulfate (Neeman and Benziman, 1962).

When the crude extract and AS 60-90 were assayed for their OAA decarboxylation activity

(Materials and Methods), it was found that AS 60-90 had a specific activity 10 times that of the crude extract (1.8 and 18 μ moles of OAA decarboxylated in 5 min per mg of protein of the crude extract and AS 60-90, respectively).

Exchange of CO₂ into OAA. *A. xylinum* cells were previously shown to catalyze the exchange of C¹⁴O₂ into OAA (Benziman and Abeliowitz, 1964). Crude extracts and fraction AS 60-90 were similarly tested for their ability to mediate the exchange reaction (Table 2). As seen from the results, the OAA-CO₂ exchange activity was retained in the cell-free preparations. With both preparations practically all the labeling observed in the intact OAA molecule was found in the β -carboxyl, whereas the moiety of OAA containing the α -carboxyl was unlabeled. A similar distribution of C¹⁴ in OAA was found when the exchange reaction was catalyzed by whole cells (Benziman and Abeliowitz, 1964).

The dilution of radioactive CO₂ by unlabeled CO₂ arising from the decarboxylation reactions was negligible under the experimental conditions used (less than 10%); therefore, the exchange of CO₂ could be calculated in absolute amounts from the specific radioactivity of the NaHC¹⁴O₃ added to the reaction mixture. The values obtained by such calculations for the crude extract and AS 60-90 were, respectively, 0.018 and 0.18 μ mole of CO₂ exchanged per mg of protein per 5 min under standard conditions.

The ratio between the CO₂ exchange and OAA decarboxylation activities observed in the crude extract was 1:100. The same ratio was retained in AS 60-90.

TABLE 2. *Isotope distribution in OAA after exchange of OAA and C¹⁴O₂ by Acetobacter xylinum extracts*

Extract*	Total activity in OAA†	Activity in β -carboxyl of OAA†	Activity in $-\text{CH}_2-\text{CO}-\text{COOH}$ of OAA†
Crude extract	6,500	6,500	<10
AS 60-90	6,700	6,750	<10

* Standard exchange reaction mixture as described in Materials and Methods. Crude extract, 3.0 mg of protein. AS 60-90, 0.3 mg of protein. NaHC¹⁴O₃: 6×10^6 count/min. Reaction time was 5 min.

† Expressed as counts per minute.

Influence of incubation time on CO₂ exchange and OAA decarboxylation activities. OAA decarboxylation was linear with time, under the standard conditions described for reaction periods as long as 30 min (Fig. 1). The exchange activity, as measured by the rate of C¹⁴O₂ incorporation into a pool of OAA, decreased with time. This can be explained by the progressive dilution of C¹⁴O₂ with the nonisotopic carbon dioxide formed by decarboxylation of OAA. On this basis, the labeling of OAA was taken as representative of the exchange activity only in the first minutes of incubation.

Relationship between protein concentration and enzyme activity. The relationship between protein concentration and enzyme activity was linear under the standard conditions described up to 0.25 and 0.5 mg of protein (AS 60-90) for the CO₂ exchange and OAA decarboxylation activities, respectively (Fig. 2). The deviation from linearity observed in the exchange experiment was explained as being due to the dilution of radioactive CO₂ by the CO₂ arising from OAA decarboxylation.

Effect of substrate concentration on enzyme activity. The rate of OAA decarboxylation increased with increasing concentrations of OAA

FIG. 1. OAA-CO₂ exchange and OAA decarboxylation as a function of time. Standard reaction mixtures for OAA decarboxylation and CO₂ exchange as described in Materials and Methods. Enzyme: 0.13 mg of protein, AS 60-90. NaHC¹⁴O₃: 3×10^6 count/min.

FIG. 2. OAA-CO₂ exchange and OAA decarboxylation as a function of protein concentration. Standard reaction mixtures for OAA decarboxylation and CO₂ exchange as described in Materials and Methods. NaHC¹⁴O₃: 3×10^6 count/min. Protein (AS 60-90) as indicated. Reaction time, 5 min.

(Fig. 3). The apparent Michaelis-Menten constant (K_m) of OAA (Fig. 3, insert), calculated from a Lineweaver and Burk plot, was 2.8×10^{-2} M. The OAA decarboxylases from *Micrococcus lysodeikticus* (Herbert, 1951) and rat liver mitochondria (Corwin, 1959) were reported to have much lower K_m values (2×10^{-3} and 1.1×10^{-4} M, respectively).

The data summarized in Fig. 4 and 5 show the dependence of the rate of OAA-CO₂ exchange on the concentrations of OAA and bicarbonate, respectively. When NaHCO₃ was held constant at 50 mM, the enzyme system was saturated with OAA at about 2.5×10^{-2} M. Conversely, when OAA was held constant at 50 mM the maximal rate of exchange was given by about 4.5×10^{-2} M NaHCO₃.

Optimal pH. The dependence of decarboxylation and exchange activities on the pH is shown in Fig. 6. The CO₂ exchange and OAA decarboxylation activities had similar pH curves with maximal activity given at pH 5.6. The pH optimum obtained was similar to that reported by Herbert (1955) for OAA decarboxylase of *M. lysodeikticus*. The OAA decarboxylase of rat liver mitochondria has a much higher pH optimum of 7.5 (Corwin,

1959), whereas the optimal pH for the enzyme of *Azotobacter vinelandii* ranged from 6.5 to 8.0 (Plaut and Lardy, 1949).

Requirement of divalent cations. CO_2 exchange

FIG. 3. Effect of substrate concentration on oxaloacetate decarboxylation. Standard assay system for OAA decarboxylation, as described in Materials and Methods. Enzyme: 0.7 mg of protein, AS 60-90. OAA concentration as indicated.

FIG. 4. Effect of OAA concentration on rate of exchange. Standard assay system for OAA- CO_2 exchange as described in Materials and Methods. Enzyme: 0.15 mg of protein, AS 60-90. $\text{NaHC}^{14}\text{O}_3$: 6×10^6 count/min. OAA concentration as indicated. Reaction time, 5 min.

FIG. 5. Effect of NaHCO_3 concentration on rate of exchange. Standard assay system for OAA- CO_2 exchange as described in Materials and Methods. Enzyme: 0.15 mg of protein AS 60-90. NaHCO_3 added in concentrations as indicated. Reaction time, 5 min. The values in the ordinate were calculated from the average specific activity of $\text{NaHC}^{14}\text{O}_3$ during the reaction period (initial specific activity, corrected for isotope dilution by OAA decarboxylation).

and OAA decarboxylating activities in the crude extract were increased 40% by addition of Mn^{2+} (Table 3). Mg^{2+} caused only a slight increase in the activities. Dialysis reduced both activities, though to a different extent. Whereas the decarboxylating activity was 90% reduced, the exchange activity was reduced by only 35%. In both cases activity was restored by addition of Mn^{2+} or Mg^{2+} , but not by Fe^{2+} , Cd^{2+} , Co^{2+} , or Cu^{2+} . The optimal concentration for Mn^{2+} and Mg^{2+} was approximately 3×10^{-3} M. These observations differ from the cation requirements reported for the OAA decarboxylation and OAA- CO_2 exchange system of *M. lysodeikticus*. With extracts of this organism, decarboxylation of OAA was much more rapid with Mn^{2+} than with Mg^{2+} , though the OAA- CO_2 exchange was more rapid in the presence of Mg^{2+} than of Mn^{2+} (Utter, 1961). In addition, the OAA decarboxylase of *M. lysodeikticus* was activated by Co^{2+} and Cd^{2+} to the same extent as by Mn^{2+} (Herbert, 1951). The exchange of C^{14}O_2 with OAA was much less affected by ethylenediaminetetraacetic acid (EDTA) than was OAA decarboxylation (Table 3). At 10^{-3} M, this metal-chelating

reagent completely inhibited OAA decarboxylation without affecting the exchange activity at all. Addition of Mn²⁺ partially restored the decarboxylation activity. Of other metal-chelating agents tested, 8-hydroxyquinoline (10⁻³ M) inhibited the decarboxylation and exchange activities 100 and 60%, respectively. *o*-Phenanthroline, potassium fluoride, and $\alpha\alpha'$ dipyridyl had no effect on either activity even at 10 mM concentrations.

Inhibition of the reactions. Ammonium ions at 10 mM concentration inhibited the OAA decarboxylating activity of crude extracts approximately 50% without affecting their exchange activity. Preincubation of crude extracts with 10⁻³ M *p*-chloromercuribenzoate or *N*-ethylmaleimide inhibited both the exchange and decarboxylation reactions approximately 35%. Avidin (25 units), preincubated with the crude extract or with AS 60-90, did not inhibit CO₂ exchange activity or OAA decarboxylation. The exchange system of *M. lysodeikticus* was also found to be unaffected by avidin (Utter, 1961). In contrast to the OAA decarboxylase of *A. vinelandii* (Plaut and Lardy, 1949), malate did not inhibit OAA decarboxylation by *A. xylinum* extracts.

Substrate specificity. Fraction AS 60-90 did not decarboxylate pyruvate, α -ketoglutarate,

TABLE 3. Effect of divalent cations on CO₂ exchange and OAA decarboxylation^a

Enzyme treatment	Cations added ^b	Per cent of control	
		Ex-change	Decarboxylation
None	None	100	100
	Mn ²⁺	140	140
	Mg ²⁺	110	110
Dialysis ^c	None	65	10
	Mn ²⁺	140	140
	Mg ²⁺	110	100
	Fe ²⁺ , Cd ²⁺ , Co ²⁺ or Cu ²⁺	65	10
EDTA ^d (10 ⁻³ M)	None	100	0
EDTA (10 ⁻³ M)	Mn ²⁺ (10 ⁻² M)	—	60
EDTA (7 × 10 ⁻² M)	None	70	—

^a Assayed as described in Materials and Methods. Enzyme: AS 60-90 (0.4 and 0.15 mg of protein for decarboxylation and exchange experiments, respectively). Reaction time was 5 min.

^b Enzyme was preincubated with cations given as the chlorides at 10⁻³ M, for 10 min.

^c Enzyme was dialyzed in the cold for 16 hr against 3,000 volumes of 0.04 M phosphate buffer (pH 6.0).

^d Enzyme was preincubated with ethylenediaminetetraacetic acid (EDTA) for 10 min.

FIG. 6. Effect of pH on CO₂ exchange and OAA decarboxylation. Standard assay systems for OAA decarboxylation and CO₂ exchange as described in Materials and Methods, with 0.2 M phosphate or acetate buffer at pH values as indicated; pH values in exchange experiments are after addition of the bicarbonate. Enzyme: 0.2 and 0.4 mg of protein, AS 60-90, for exchange and decarboxylation experiments, respectively. NaH¹⁴C₃: 7.5 × 10⁶ counts per min. Reaction time, 5 min.

acetoacetate, and dihydroxymaleate, nor did it exchange CO₂ with these acids.

Effect of nucleotides, pyrophosphate, and phosphate on the decarboxylation and exchange reactions. To establish whether reactants other than CO₂, pyruvate, and OAA participate in the exchange and decarboxylation reactions, the effects of various compounds known to participate in OAA decarboxylation systems on the activity of a dialyzed AS 60-90 preparation were examined (Table 4). Long period dialysis did not diminish the activity of the enzyme preparation for both reactions when assayed in the presence of Mn²⁺. The addition of adenosine triphosphate (ATP), adenosine diphosphate (ADP), guanosine triphosphate (GTP), inosine triphosphate (ITP), pyrophosphate (PP), or inorganic phosphate (Pi) did not stimulate OAA decarboxylation or the exchange reaction. It was still possible that ATP did participate in these reactions, with

phosphoenol pyruvate (PEP) as one of the products, and that the observed lack of ATP stimulation was due to the activity of pyruvic kinase, generating ATP, which was present in small amounts in the preparation. This possibility was examined by testing for pyruvic kinase activity in the dialyzed preparation, but no such activity was detected.

Noncarboxylation of pyruvate or PEP to OAA. Attempts to show carboxylation of pyruvate or PEP to OAA in our system were carried out in several ways. In one set of experiments, AS 60-90 was incubated with pyruvate or PEP, and high specific activity $C^{14}O_2$, in the presence of various factors known to participate in enzymatic systems catalyzing the carboxylation of pyruvate or PEP to OAA (Wood and Stjernholm, 1962). At the end of the incubation period, carrier OAA was added, the mixture was treated with 2,4-dinitrophenylhydrazine, and the radioactivity in the hydrazone fraction was examined. In all systems tested, practically no radioactivity could be detected in the hydrazone fraction (Table 5). On the other hand, under similar conditions, the exchange into OAA of 20 μ moles of CO_2 could be demonstrated. Under the experimental conditions used, the presence of at least 0.25 μ moles of OAA arising from carboxylation could have been detected.

TABLE 4. *Effect of various factors on CO_2 exchange and OAA decarboxylation*

Enzyme prepn*	Addition	OAA decar- boxyl- ated	CO_2 exchange
	μ moles	μ moles	count/min
Undialyzed	None	3.5	2,200
Dialyzed	None	3.4	2,250
	1 ATP	3.1	2,300
	3 ATP	3.1	2,200
	3 ADP + 3 P_i	3.0	2,200
	3 GTP	3.2	2,200
	3 ITP	3.5	2,350
	3 PP	3.2	2,200
	3 P_i	3.4	2,200

* Standard assay systems for OAA decarboxylation and CO_2 exchange, as described in Materials and Methods. Enzyme: AS 60-90, 0.2 mg of protein. AS 60-90 was dialyzed in the cold against 3,000 volumes of 0.04 M acetate buffer (pH 5.6). $NaHC^{14}O_3$: 3×10^6 counts per min. Reaction time was 5 min.

TABLE 5. *Carboxylation of pyruvate or PEP*

System*	Radioactivity of 2,4-dinitro- phenylhy- drazone fraction
	count/min
Pyruvate + $C^{14}O_2$	<50
Pyruvate + ATP + $C^{14}O_2$	<50
Pyruvate + GTP + $C^{14}O_2$	<50
Pyruvate + ITP + $C^{14}O_2$	<50
PEP + GDP + $C^{14}O_2$	<50
PEP + ADP + $C^{14}O_2$	<50
PEP + IDP + $C^{14}O_2$	<50
PEP + P_i + $C^{14}O_2$	<50
OAA + $C^{14}O_2$	40,000

* Reaction mixtures (1 ml) contained: 0.2 M acetate buffer (pH 5.6), 3 mM $MnCl_2$, 10 mM glutathione, 0.01 M $NaHC^{14}O_3$ (1.5×10^7 count/min), and 1 mg of AS 60-90 protein. Other additions included: 3 mM pyruvate; PEP and OAA; 5 mM ATP, GTP, ITP, GDP, ADP, IDP, and P_i . Incubation time was 5 min. Reaction was terminated with 0.2 ml of 5 N HCl, followed immediately by addition of 25 μ moles of OAA. Radioactivity was determined as in the standard exchange assay method (Materials and Methods).

Fraction AS 60-90 used for the carboxylation experiments has a strong OAA decarboxylating activity; therefore, it was possible that OAA formed by carboxylation could not be detected, because it was rapidly decarboxylated. Thus, in other experiments the carboxylation systems were coupled to an OAA-trapping reaction, constituted by an excess of exogenous malic dehydrogenase and reduced nicotinamide adenine dinucleotide ($NADH_2$). The carboxylation reaction mixtures (Table 5) were supplemented with 7.2 units of malic dehydrogenase and 5 μ moles of $NADH_2$, and, at the end of the incubation period, the radioactivity of the nonvolatile-acids fraction was determined (Materials and Methods). No labeling was detected in the non-volatile-acids fraction obtained from all reaction systems. In still other experiments, the carboxylation systems supplemented by malic dehydrogenase and $NADH_2$ were incubated in spectrophotometric cuvettes, and changes in absorbancy at 340 m μ were determined spectrophotometrically. The results obtained did not show any measurable formation of nicotinamide adenine

dinucleotide (NAD), which would have indicated synthesis of OAA.

Exchange of pyruvate into OAA. Table 6 compares the exchange of pyruvate-3-C¹⁴ with OAA, and the exchange of C¹⁴O₂ with OAA. The results demonstrated that pyruvate exchange was catalyzed at a rate which was of the same order of magnitude as the rate of the CO₂ exchange.

DISCUSSION

Though OAA decarboxylation to pyruvate and CO₂ has been observed with enzyme preparations from various sources (Utter, 1961), it is only the *M. lysodeikticus* system that also catalyzes, similar to our system, the exchange of CO₂ and OAA. By comparing the rate of CO₂ exchange with that of OAA decarboxylation observed in our system, a ratio value of 0.01 was obtained. This value is approximately five times higher than the respective value in the *M. lysodeikticus* system as calculated from the data of McManus (1951).

OAA decarboxylation and CO₂ exchange activities were found to differ in their response to dialysis and to the action of metal-chelating agents (Table 3). Whereas dialysis of the enzyme preparation resulted in almost complete loss of its decarboxylating activity, the loss of exchange activity amounted only to 35%. Low concentration of EDTA completely inhibited OAA decarboxylation, but had no effect on CO₂ exchange. Addition of Mn²⁺ relieved the effect of both dialysis and EDTA. From these observations, it can be concluded that the two activities differ in their requirements for this ion. A difference in metal ion requirements for OAA decarboxylation and for CO₂ exchange was also noted by Utter (1961) in the purified OAA decarboxylase system of *M. lysodeikticus*. In this system, Mn²⁺ was more effective than Mg²⁺ in the decarboxylation reaction; in the exchange reaction the reverse was true. The role of metal ions in the decarboxylation and exchange reactions has been discussed by Utter (1961) in the light of the model system provided by the chemical studies of Steinberger and Westheimer (1951) on the metal ion-catalyzed decarboxylation of OAA.

In the exchange reaction in *A. xylinum*, several pathways are known which may lead to an exchange of CO₂ and OAA. Though both mammalian and bacterial malic enzyme do not pro-

TABLE 6. *Exchange of pyruvate into OAA*

System*	Activity of OAA count/min
OAA + pyruvate-3-C ¹⁴	2,300
OAA + C ¹⁴ O ₂	3,800

* Reaction mixtures (1 ml) contained: 0.2 M acetate buffer (pH 5.6), 3 mM MnCl₂, 10 mM glutathione, 0.05 M OAA, and 0.35 mg of AS 60-90 protein. Other additions included: 0.05 M pyruvate-3-C¹⁴ (1.5 × 10⁶ counts per min), or 0.05 M NaHC¹⁴O₃ (1.5 × 10⁶ counts per min). Controls without OAA and without enzyme were included for each experiment. Incubation time was 5 min. For both exchange reactions, OAA was separated chromatographically and its radioactivity was determined (Materials and Methods).

mote an exchange of OAA and CO₂ (Salles et al., 1950; Korkes, del Campillo, and Ochoa, 1950), it was possible that the labeling in OAA originated in malate which was subsequently oxidized to OAA. This possibility seems to be excluded, because AS 60-90 has no malate-oxidizing activity (*unpublished data*). Even for the crude extract which oxidizes malate, the isotope distribution data (Table 3) are not compatible with malate as the labeled precursor of OAA, considering the high fumarase activity present in the extract. The other pathways to be considered include the carboxylation of pyruvate to OAA by pyruvate carboxylase (Utter and Keech, 1963; Seubert and Remberger, 1961), and carboxylation of PEP to OAA by pyruvate carboxykinase (Utter and Kurahashi, 1954; Cannata and Stoppani, 1963*b*) or by PEP carboxytransphosphorylase (Siu and Wood, 1962). These reactions are reversible and include as reactants compounds like ATP, ADP, GDP, ITP, pyrophosphate, and P_i. It was possible that the exchange reaction in *A. xylinum* was accomplished by one of these pathways and was independent of the decarboxylation reaction. In this case, the presence of only catalytic amounts of some of the above-mentioned reactants, in addition to OAA and CO₂, would have been required for the exchange reaction to occur. Moreover, it could be visualized that the decarboxylation reaction results from the coupling of pyruvate carboxylase with an adenosine triphosphatase regenerating ADP and P_i, or from PEP carboxykinase coupled with pyruvic kinase regenerating ATP, assuming the presence of these cofactors in the enzyme preparation in

catalytic amounts. However, the failure of nucleotides, inorganic phosphate, or pyrophosphate to stimulate the decarboxylation and exchange activities of dialyzed preparations (Table 4), in addition to the observed absence of adenosine triphosphatase and pyruvic kinase activities in such preparations, make it very unlikely that either of these pathways plays a role in the decarboxylation and exchange activities of our system. This conclusion is further strengthened by the finding that enzyme preparations active in the exchange reaction fail to catalyze any carboxylation of pyruvate or PEP to OAA, even when supplemented by an OAA-trapping system (Table 5).

Failure to promote net synthesis of OAA was also noted with the OAA-CO₂ exchange system of *M. lysodeikticus*. Herbert (1951), Mehler et al. (1948), and Ochoa, Mehler, and Kornberg (1948) have tried to couple that system with malic dehydrogenase to obtain net fixation of CO₂, but the results were negative. The thermodynamic considerations underlying these experiments were extensively discussed by Wood and Stjernholm (1962), who accordingly proposed a scheme for the exchange of CO₂ catalyzed by OAA decarboxylase. Inasmuch as the exchange of CO₂ and pyruvate into OAA in *A. xylinum* are parts of the decarboxylation reaction, our findings could be explained by a mechanism of OAA decarboxylation as outlined in Fig. 7. In this scheme, the complex of the enzyme with both the pyruvate and β -carboxyl moieties of OAA may be reversibly dissociated either to free pyruvate and enzyme-bound CO₂, or to free CO₂ and enzyme-bound pyruvate. According to the proposed reaction mechanism, formation of enzyme-bound pyruvate or enzyme-bound CO₂ occurs much faster from OAA than from free pyruvate or CO₂, respectively. Thus, an exchange of CO₂ or pyruvate with OAA would be expected, but the synthesis of OAA from these compounds

would be very much slower. The difference between the exchange and decarboxylation in their response to EDTA (Table 3) could be explained according to this scheme if the last steps of the decarboxylation, in which the free enzyme is released, are more sensitive than previous steps to the action of EDTA. Similarly, these steps may be those inhibited by ammonium ions, explaining the observation that these ions inhibited OAA decarboxylation, but not CO₂ exchange.

Incorporation of CO₂ carbon into carbohydrates, synthesized from substrates at the level of pyruvate or lactate, is generally believed to arise from the carboxylation of pyruvate to OAA, which is equilibrated with a symmetrical 4-carbon compound, like fumarate, prior to its conversion to PEP (Shargo et al., 1963). In earlier work (Benziman and Burger-Rachamimov, 1962), however, it was observed that exogenous CO₂ fails to serve as a carbon source for cellulose, synthesized by *A. xylinum* cells in the presence of pyruvate. This observation may be explained by the findings reported here on the inability of *A. xylinum* extracts to carboxylate pyruvate or PEP (Bloom and Foster, 1962). However, it can still be argued that pyruvate carboxylation activity of intact cells was destroyed during the extraction procedures, and synthesis of cellulose from pyruvate, demonstrable only with whole cells, may still occur via a carboxylation mechanism. In this case, the CO₂-cellulose data could be explained, as suggested earlier (Benziman and Abeliowitz, 1964), as due to the inability of the cells to equilibrate OAA with fumarate as a result of the irreversibility of the malate oxidation system present in *A. xylinum*.

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FIG. 7. Scheme for OAA decarboxylation and exchange of pyruvate and CO₂ into OAA. Dotted lines indicate very slow reactions.

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