An Energy-conserving Pyruvate-to-Acetate Pathway in *Entamoeba histolytica*

**PYRUVATE SYNTHASE AND A NEW ACETATE THIOKINASE***

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Under anaerobic conditions, cells of *Entamoeba histolytica* grown with bacteria produce H₂ and acetate while cells grown axenically produce neither. Aerobically, acetate is produced and O₂ is consumed by amebae from either type of cells. Centrifuged extracts, 2.4 x 10⁷ x g x min, from both types of cells contain pyruvate synthase (EC 1.2.7.1) and an acetate thio kinase which, together, form a system capable of converting pyruvate to acetate. Pyruvate synthase catalyzes the reaction: pyruvate + CoA → CO₂ + acetyl-CoA + 2e. Electron acceptors which function with this enzyme are FAD, FMN, riboflavin, ferredoxin, and methyl viologen, but not NAD or NADP. The amebal acetate thio kinase catalyzes the reaction acetyl-CoA + ADP + Pᵢ → acetate + ATP + CoA. For this reaction, the enzymes which might function in this area are a PP₆-dependent acetate kinase (3) and one called a new pyruvate oxidase (CoA-acetylating) (4).

**EXPERIMENTAL PROCEDURES**

**Materials**

FAD, FMN, spinach ferredoxin, acetyl-CoA, adenine nucleotides, citrate synthase, phosphotransacetylase, and bacterial acetate kinase were from Sigma. Other assay or linking enzymes were Boehringer products. Enzymes A and nucleotides other than adenosine were from P-L Biochemicals. CoA and acetyl-CoA were assayed using the Ellman reagent by the method of Tubbs and Garland (5). Acetyl phosphate was prepared by Stadtman's method (6) and assayed by the method of Lipmann and Tuttle (7). Prepurified nitrogen and argon were from Matheson.

HK9 and 200:NIH strains of *Entamoeba histolytica* were grown under axenic conditions in Diamond's TPS-1 medium (8), and these two strains plus the DKR and HM2 strains were grown with penicillin-inhibited cells of *Bacteroides symbiosus* (9). Cells were harvested under argon, and argon was placed into septum-stoppered tubes. All enzyme assays were made at 30°. The standard assay for pyruvate synthase and amebal acetyl-CoA-synthetase involved the use of cuvettes made anaerobic by the technique described by Uyeda and Rubinowitz (11).

**Cell Extracts**

Cells packed by 2,400 x g x min centrifugation were suspended in an equal volume of 50 mM Tris/HCl buffer, pH 7.4, containing 0.25 M sucrose and 2 mM dithiothreitol and ruptured under argon by 20 passes in a Teflon-pestle tissue grinder. The homogenates were diluted with 0.5 to 2.5 volumes of the suspending buffer and centrifuged for 30 min at 81,000 x g. Subsequent manipulations were conducted at reduced temperature. All buffers employed had been purged with nitrogen, suspensions in open vessels were protected from air by argon, and argon was displaced from septum-stoppered vessels by argon or nitrogen.

**Enzyme Assays**

All enzyme assays were made at 30°. The standard assay for pyruvate synthase and amebal acetyl-CoA-synthetase involved the use of cuvettes made anaerobic by the technique described by Uyeda and Rubinowitz (11).

**Pyruvate Synthase**—The standard assay for pyruvate synthase measured the rate of reduction of FMN at 450 nm. Quartz cells of 0.5-cm optical path containing 25 μmol of potassium phosphate buffer, pH 7.4, 2.5 μmol of sodium pyruvate, 1 μmol of dithiothreitol, 0.2 μmol of Co₆, 0.16 μmol of FMN, and water were made anaerobic and brought to temperature equilibrium. Reaction started by injecting 0.01 to 0.02 unit of enzyme through the septum with a Hamilton syringe. The final volume was 0.1 ml. The rate of reduction of FMN was monitored in a Gilford spectrophotometer taking ε = 12,200 cm⁻¹ x M⁻¹ at 450 nm. A cell lacking Co₆ served as control. A unit of enzyme is defined as that amount causing the reduction of 1 μmol of...
Acetyl-CoA synthetase (ADP-forming) - The standard assay for amebal acetyl-CoA synthetase was conducted in anaerobic cuvettes prepared exactly like those for the pyruvate synthase assay, above, except that they contained only 50 nmol of CoA. At least 0.15 unit of amebal pyruvate synthase and up to 0.05 unit of enzyme were added through the septum with a Hamilton syringe. The CoA-limited reduction of FMN was complete during thermal equilibration and reaction was then started by adding 1 pmol of MgCl₂ and 0.4 mmol of ADP through the septum. A cuvette without MgCl₂ and ADP served as control. The reaction was monitored as for the standard pyruvate synthase assay and a unit of acetyl-CoA synthetase is similarly defined.

Acetyl-CoA synthetase was also assayed by its catalysis of the arsenolysis of acetyl-CoA. Quartz cuvettes of 2-mm optical path contained 150 mm sodium arsenate, pH 7, 10 mm MgCl₂, 0.3 mm acetyl-CoA and enzyme. Reaction was monitored at 232 nm taking Δε = 4,500 M⁻¹ cm⁻¹. The enzyme was assayed in the direction of acetyl-CoA formation by the malate dehydrogenase-citrate synthase system in the presence of NAD and L-malate (12). The initial substrate concentrations were 0.2 mm CoA, 1 mm ATP, and 0.13 mm acetylacetate.

Other Assays

Hydrogenase was assayed spectrophotometrically by a method adapted from that of Lindmark and Müller (13) in which the initial methyl viologen concentration was reduced to 10 mm and the Triton X-100 was omitted. In calculating moles of H₂ oxidized, the extinction coefficient of the dye was taken at 50 nm as 8275 M⁻¹ cm⁻¹ (14) and this value was doubled to allow for it being a one-electron acceptor.

The presence of phosphotransacetylase in amebal extracts was made evident by an increased rate of reduction of FMN prior to the addition of MgCl₂ and ADP in the standard assay for acetyl-CoA synthetase.

Phosphotransacetylase was assayed by substituting 4 mm acetyl phosphate for the acetate and the ATP specified in the malate dehydrogenase-citrate synthase system (15). Phosphotransacetylase was also assayed by the formation of acetyl-CoA from CoA and acetyl phosphate, monitored at 232 nm (16).

Protein in amebal extracts was assayed by the method of Lowry et al. (16) with bovine serum albumin as the standard; and in column fractions, by the spectrophotometric method of Layne (17).

**RESULTS**

Acetate and Gas Metabolism in Axenic Whole Cells

Axenic cells of the 200: NIH strain, 0.2 g, were placed in an arm of a Warburg vessel containing 2.2 ml of axenic culture medium in the main compartment and KOH in the center well. After 10 min of equilibration at 37°C, the amebae were tipped into the medium and gas volume changes were monitored by standard manometric techniques. After incubation for a suitable interval, the reaction was stopped by the addition of perchloric acid from a second arm. In the control vessel acid was added to the main compartment before the amebae. Following centrifugation and neutralization with KOH the precipitated salt was removed and steam-volatile acids were recovered, titrated, and characterized by gas chromatography (2). In an atmosphere of air, average values were as follows (micromoles per g of fresh cells per h): oxygen consumed, 57 and acetate produced, 42. In a nitrogen atmosphere no gas was produced and no acetate was found.

**Conversion of Pyruvate to Acetate by Amebal Extract**

The reaction mixture in a septum-stoppered tube contained 25 mmol of potassium phosphate, pH 7.4, 0.62 mmol of FMN, 0.1 mmol of CoA, 1 mmol of dithiothreitol, 1 mmol of MgCl₂, 0.4 mmol of AMP, 0.4 mmol of [2-¹⁴C]pyruvic acid (1 µCi/µmol), and water to a volume of 0.475 ml. Nitrogen was bubbled through the solution for 5 min. The tube was then placed in a 30°C bath and 25 µl of enzyme (350 µg of protein) from the extract of axenic HK9 strain was added through the septum. Incubation was for 5 min at which time reaction was stopped by adding 0.1 ml of 10 N sulfuric acid. To a control tube, the sulfuric acid was added prior to the enzyme. To both tubes were added unlabeled carriers, 10 µmol of acetic acid and 45 µmol of pyruvic acid, and 50 µmol of solid 2,4-dinitrophenylhydrazine. The stoppered tubes were heated to 70°C for 45 min with frequent agitation, chilled for 0.5 h, and centrifuged from the difficulty soluble hydrazone. An aliquot of the supernatant solution was steam-distilled for the recovery of volatile acid. After correcting for label in the control distillate, the experimental tube contained label from 306 mmol of pyruvate. That the labeled product was acetate was confirmed by chromatography of the product on a Celite column by the method of Bueding and Yale (18).

**Activity of Enzymes Involved in Pyruvate to Acetate Metabolism**

Table I summarizes the activities of pyruvate synthase and amebal acetyl-CoA synthetase in extracts of cells grown axenically or with bacteria. Neither of these enzymes was sedimented in 30 min at 81,000 × g. Assays on pyruvate synthase made by the aerobic method agreed with those of the standard assay, within experimental error. Activities per mg of protein were 12- to 25-fold greater than that reported for amebal pyruvate oxidase by Takeuchi et al. (9). Pyruvate synthase and amebal acetyl-CoA synthetase in cell extracts were stable for a week while kept under nitrogen in the refrigerator.

Assays on the acetyl-CoA synthetase by the arsenolysis method proceeded at about one-sixth the rate of the standard assay. The acetate to acetyl-CoA assay with malate dehydrogenase-citrate synthase system gave rates about 50% less than the standard assay.

Neither hydrogenase nor phosphotransacetylase was detected in any extract derived from axenic amebae. On the other hand, phosphotransacetylase was defined in the extracts of bacteria-grown amebae.
other hand, hydrogenase was regularly present in extracts from amebae grown with bacteria, and phosphotransacetylase was occasionally found in these extracts. Activities of hydrogenase observed in cells grown with bacteria were 0.2 to 1.4 μmol/min/mg of protein and for phosphotransacetylase the activities were zero to 0.1 μmol/min/mg of protein.

Column Fractionation of Amebal Enzymes

By excluding air from columns and column buffers, operating columns at reduced temperature, and collecting fractions in tubes filled with argon, we were able to recover the major portion of applied enzyme activities. Fig. 1 shows the results of a fractionation experiment employing a Bio-Gel P-300 column. A Sephadex G-100 column also separated adenylate kinase from pyruvate synthase and acetyl-CoA-synthetase. Less successful separations were made on columns containing hydroxylapatite, Bio-Gel A-0.5 m, or DEAE-cellulose. The last gave pyruvate synthase of specific activity 10, but its activity was not resolved from the acetyl-CoA-synthetase or adenylate kinase. Unfortunately, column-purified enzymes were distinctly unstable upon storage, often losing 50 to 95% of their activity in 24 h.

Characterization of Amebal Pyruvate Synthase

Both CoA and an electron acceptor were required for the functioning of amebal pyruvate synthase. In the aerobic assay system the addition of 50 units/ml of catalase halved the rate of oxygen consumption and doubled the amount of CoA utilized per mol of oxygen consumed indicating that H₂O₂ is a product of the aerobic assay.

Optimum rates of reaction catalyzed by pyruvate synthase occurred between pH 7.4 and 8 in Tris/HCl buffer or in potassium phosphate buffer; rates were about 10% greater in the latter. In the aerobic assay system the enzyme of cell extracts is very unstable in the absence of the electron acceptor. When the FMN was added last, the half-life of enzyme in the presence of air, pyruvate, and CoA was less than 10 s. This rapid decay of activity depended upon the presence of both pyruvate and CoA.

Electron Acceptors—Some electron acceptors which function with amebal pyruvate synthase are listed in Table II. The concentrations listed in the table are optimal for each acceptor. Other acceptors observed to be reduced by pyruvate in the presence of the enzyme and CoA included spinach ferredoxin and 2,3,5-triphenyltetrazolium chloride.

Products—Identification of acetyl-CoA as a product of the reaction catalyzed by amebal pyruvate synthase was accomplished by linking the reaction with citrate synthase as is shown by the experiment in Fig. 2. In this work, trace amounts of CoA and citrate synthase were substituted for the amount of CoA specified in the aerobic assay system. Oxygen uptake was initially restricted by the limiting amount of CoA. Then, upon addition of oxalacetate, CoA, regenerated by citrate synthesis, served to sustain a continued utilization of oxygen. The inset of the figure indicates that the apparent Kᵣ for CoA is 2 μM.

The evolution of CO₂ from carbon 1 of pyruvate was demonstrated by incubating 0.7 mg of enzyme protein from an extract of axenic HK9 cells for 32 s with 1 mM [1-¹⁴C]pyruvate under conditions otherwise identical with the aerobic assay (see "Experimental Procedures"). After acidification with trichloroacetic acid and bubbling with gas, the remaining label, 330 nmol of CO₂, from the labeled pyruvate. Under identical conditions, the aerobic assay showed consumption of 320 nmol of O₂ in 32 s.

Exchange Reaction—A pyruvate-CO₂ exchange reaction was conducted as described by Lindmark and Müller (13) except that the CoCl₂ and Triton X-100 were omitted. The exchange of label from ¹⁴C-pyruvate to pyruvate was constant for 8 min at a rate of 0.52 μmol/min/mg of enzyme protein. The enzyme used for this experiment was from an extract of axenic amebae.

### Table II

<table>
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<th>Acceptor, mm</th>
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<td>FMN, 0.3</td>
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<td>100</td>
</tr>
<tr>
<td>FAD, 0.3</td>
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<td>Riboflavin, 0.4</td>
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<td>Methyl viologen, 6</td>
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<tr>
<td>NADP, 0.5</td>
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</table>

*Activities were calculated using the following extinction coefficients, m⁻¹ cm⁻¹ (wavelength in nm): FMN, 12,200 (450); FAD, 11,200 (450); riboflavin, 12,200 (450); methyl viologen, 8,250 (600); NAD and NADP, 6,220 (340).*
The results calculated as nanomoles of bicarbonate fixed were: complete reaction, 205; minus amebal enzyme, 29; minus bacterial hydrogenase, 1.4 mg of protein from an amebal cell extract of the axenic 200 strain. The final volume was 0.6 ml and incubation was under anaerobic conditions. The Bio-Gel P-300 column yielded enzyme purified about 6-fold with respect to protein and free from adenylate kinase. Enzyme eluted with 20 mM sodium Mes, pH 6, lost only about 30% of its activity in 24 h. Enzyme activity was less stable when elution from the columns was with Tris/HCl, pH 7.4.

Requirements - Experiments shown in Fig. 3 establish the requirement of column-purified amebal acetyl-CoA-synthetase for P$_i$, a divalent cation, and ADP. In these experiments the enzyme is linked with pyruvate synthase in the presence of pyruvate, FMN, and a limiting amount of CoA. When the requirements of the acetyl-CoA-synthetase are fulfilled, as in Curve D, it regenerates CoA from acetyl-CoA and permits the continued reduction of FMN. That P$_i$ is required is shown by the lack of reaction in Tris buffer in Curve A. Addition of 4 mM P$_i$ to the cuvette permitted the reaction to proceed (not shown). That a divalent cation is required is shown by the absence of reaction in Curve B. Divalent cations which permitted reaction to take place were those of magnesium, iron, and manganese, but not those of nickel, calcium, cadmium, or zinc. That AMP did not substitute for ADP with column-purified enzyme is shown by Curve C, however, reaction began when adenylate kinase (21). The amebal enzyme resembles pyruvate kinase in utilizing AMP in lieu of ADP in the presence of magnesium, iron, and manganese, but not those of nickel, calcium, cadmium, or zinc. That AMP plus 1 mM P$_i$ do not substitute for ADP in sustaining reaction by the column-purified enzyme. The amebal enzyme resembles pyruvate kinase in utilizing AMP in lieu of ADP in the presence of adenylate kinase (21).

Nucleotide Specificity - The nucleotide specificity of amebal acetyl-CoA-synthetase was explored using the malate dehydrogenase-citrate synthase system (12). Enzyme, 25 μg of protein from a Bio-Gel P-300 column fraction, was added to the system containing 1 mM nucleotide triphosphate, 0.4 mM potassium acetate, 0.1 mM CoA, and 2 mM MgCl$_2$. The results were as follows (nanomoles of acetyl-CoA formed per min): ATP, 17; GTP, 0.4; ITP, trace; UTP and CTP, none. Acetyl phosphate was unable to substitute for ATP.

Nucleotide specificity was studied in the direction acetyl-CoA to acetate by linking the acetyl-CoA-synthetase with pyruvate synthase as in the reaction mixture described for

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**Fig. 2.** The apparent $K_m$ for CoA of pyruvate synthase. The assay system contained all components of the aerobic assay (see "Experimental Procedures") except that micromolar concentrations of CoA, indicated by numbers above each curve, were employed and each reaction vessel contained 10 units of citrate synthase. The enzyme was 0.65 mg of protein from an extract of axenic amebae. The initial oxygen consumption (not shown) was limited by the amount of CoA present, and was complete by the time recording was started. One min later 1 μmol of oxalacetic acid (OAA) was added through the vent, and the rate of oxygen consumption was monitored by the oxygen electrode. The inset is a double reciprocal plot of rate versus CoA concentration presuming that all the CoA was maintained in the deacetylated form by the excess of citrate synthase.

**Pyruvate Synthesis** - The synthesis of pyruvate from bicarbonate and acetyl-CoA, catalyzed by amebal pyruvate synthase, was measured by the incorporation of carbon from $^{14}$C$\text{KHCO}_3$ into an acid-stable product. The electron donor was H$_2$, mediated by bacterial hydrogenase and a carrier. The considerable thermodynamic barrier to reaction in the reverse direction was partially overcome by trapping the pyruvate formed either as alanine (13) or as lactate. A set of experiments employing the lactate trap was conducted as follows. The complete reaction mixture in a septum-stoppered tube under an atmosphere of H$_2$ contained 24 μmol of imidazole/HCl, pH 7, 5 μmol of methyl viologen, 3 μmol of acetyl phosphate, 1 μmol each of diithiothreitol and EDTA, 3 μmol of NADH, 0.1 μmol of lithium CoA, 20 μmol of bicarbonate (5070 cpm/μmol), 11 μg of phosphaenolpyruvate transferase, 30 μg of lactate dehydrogenase, 0.5 unit of Bacteroides symbiosus hydrogenase, and 1.4 mg of protein from an amebal cell extract of the axenic 200 strain. The final volume was 0.6 ml and incubation was for 15 min at 30°. Reaction was stopped with 0.5 ml of 10% of trichloroacetic acid. After sedimentation of the protein the solution was bubbled for 15 min with a N$_2$/CO$_2$ mixture. It was then added to 10 ml of Aquasol in a vial and counted. Counts were corrected for those found in an acid-before-enzyme control. The results calculated as nanomoles of bicarbonate fixed were: complete reaction, 205; minus amebal enzyme, 29; minus bacterial hydrogenase, 23; minus methyl viologen, 16; minus CoA, 17; minus acetyl phosphate, 9. In an experiment parallel to the complete reaction above, perchloric acid was employed to stop the reaction. Ninety per cent of the stable counts were extracted into ether during a 20-h continuous extraction.

Upon removal of the solvent 92% of these counts migrated with lactate upon paper chromatography with an alcohol/NH$_3$/water (80/5/15) irrigating solvent.

When 20 mM glutamate and glutamate-pyruvate transaminase replaced NADH and lactate dehydrogenase as the trap for pyruvate in the reaction described above, the labeled acid-stable product was alanine. It was identified by a split stream amino acid analysis (20) in which the label exactly tracked the alanine peak. In other similar experiments it was found that no bicarbonate is fixed when N$_2$ replaces H$_2$ as the atmosphere. This observation indicates that the exchange reaction between CO$_2$ and pyruvate is not responsible for the fixation of CO$_2$ in the pyruvate synthesis experiments.

**Characterization of Acetyl-CoA-synthetase(ADP-forming)**

**Purification** - Amebal acetyl-CoA-synthetase is rapidly inactivated by air. Column purification was successful only under anaerobic conditions. The Bio-Gel P-300 column yielded enzyme purified about 6-fold with respect to protein and free from adenylate kinase. Enzyme eluted with 20 mM sodium Mes, pH 6, lost only about 30% of its activity in 24 h. Enzyme activity was less stable when elution from the columns was with Tris/HCl, pH 7.4.

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**Nucleotide Specificity** - The nucleotide specificity of amebal acetyl-CoA-synthetase was explored using the malate dehydrogenase-citrate synthase system (12). Enzyme, 25 μg of protein from a Bio-Gel P-300 column fraction, was added to the system containing 1 mM nucleotide triphosphate, 0.4 mM potassium acetate, 0.1 mM CoA, and 2 mM MgCl$_2$. The results were as follows (nanomoles of acetyl-CoA formed per min): ATP, 17; GTP, 0.4; ITP, trace; UTP and CTP, none. Acetyl phosphate was unable to substitute for ATP.

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* The abbreviation used is: Mes, 2-(N-morpholino)ethanesulfonic acid.
Acetyl-CoA to Acetate Experiment—Column-purified acetyl-CoA synthetase, 0.73 mg of protein, was added to make a final volume of 2.5 ml in a tube containing 4 mM MgCl₂, 40 mM Tris/HCl, pH 7.4, and the substrates for the reaction. Incubation was for 16 min at 30° in which time reaction was stopped by the addition of 2.5 ml of cold 0.6% perchloric acid. The acid was added to the unincubated control before the enzyme. The acidified samples were centrifuged, neutralized with KOH, centrifuged from precipitated salts, and made to a measured volume. Assays in duplicate were made immediately thereafter CoA and acetyl-CoA, as under "Experimental Procedures." ATP by hexokinase and glucose-6-P dehydrogenase in the presence of glucose and NADP, ADP by pyruvate kinase and lactate dehydrogenase in the presence of P-enolpyruvate and NADH, P, by the method of Lowry and Lopez (22), acetate by the enzymatic method of Rose (23).

Acetyl-CoA to Acetate Experiment—Column-purified enzyme, 1.7 mg of protein, was added to make a final volume of 2.5 ml in a tube containing 2 mM MgCl₂, 50 mM Tris/HCl, pH 7.4, 100 mM potassium acetate, and the other two substrates for the reaction. Incubation, preparation of the unincubated control, and assays were as described for the preceding experiment. Small portions of each reaction mixture were monitored in a quartz cuvette of 0.1-cm light path and changes in absorbance at 232 nm during 16 min at 30° were calculated as changes in amounts of acetyl-CoA, taking Δε = 4,500 M⁻¹ cm⁻¹.

![Graph](image-url)

**Figure 3. Requirements for the amebal acetyl-CoA-synthetase reaction.** Each cuvette contained 50 mM buffer, pH 7.4, 5 mM sodium pyruvate, 0.3 mM FMN, 0.1 mM CoA, 42 μg of pyruvate synthase from Fraction 4 of the Bio-Gel column of Fig. 1, and 13 μg of acetyl-CoA-synthetase from Fraction 10 of the same column fractionation plus any components mentioned below. Cuvettes of 0.5-cm optical path were made anaerobic with N₂ prior to the addition of the enzymes. At 0 min (arrow at left) 400 nmol of an adenine nucleotide was added through the septum. The final volume was 0.5 ml and reaction was monitored at 450 nm taking the extinction coefficient of FMN to be 12,200 cm⁻¹ × M⁻¹. A, the cuvettes contained 2 mM MgCl₂, the buffer was Tris/HCl, and ADP was added at 5 min. B, the buffer was potassium phosphate and ADP was added at 3 min. C, the cuvettes contained 2 mM MgCl₂, the buffer was potassium phosphate and AMP was the added nucleotide; at 14 min (arrow at right) adenylate kinase was added. D, like B except the cuvette contained 2 mM MgCl₂. The inflections at each addition reflect the amount of oxygen introduced with the added fluid.

**Curve D** in the legend of Fig. 3. In these experiments the enzyme was 16 μg of protein from a Bio-Gel P-300 fraction, and the linking pyruvate synthase was from another fraction from the same column. When various nucleoside phosphates were substituted for ADP the results were as follows (nanomoles of FMN reduced per min): ADP, 18; GDP, 4.8; IDP, 2.5; AMP, UDP, and CDP, none.

Acyl Specificity—Amebal acetyl-CoA-synthetase was incubated with 0.4 M solutions of the potassium salts of a number of organic acids in the presence of 6 mM ATP, 3 mM MgCl₂, 0.12 mM CoA, and 0.5 M neutralized hydroxylamine. After 30 min at 30°, hydroxamic acid colors were developed by the usual method (7). The relative amounts of hydroxamic acid derivatives found were as follows: acetate, 100; propionate, 47; n-butyrate, 3. No reaction was seen with isobutyrate, succinate, fumarate, or with 0.2 M benzoate.

**Stoichiometric Experiment**—The results of experiments summarized in Table III reflect the stoichiometric relationships among substrates and products of the reaction catalyzed by the acetyl-CoA-synthetase. Enzyme for this experiment was fractionated on Sephadex G-100 column (16 × 400 mm) which has been preconditioned with boiled 20 mM Tris/HCl buffer, pH 7.4, without mercaptoethanol. During the preconditioning of the column, a change of reduced methyl viologen and sodium dithionite had been passed through the column to scavenge oxygen. The column fraction employed in the acetyl-CoA to acetyl experiment contained enzyme of specific activity 0.18. It was free from the activity of adenylate kinase. That

<table>
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<tr>
<th>Substrate or product</th>
<th>Unincubated control</th>
<th>Incubated experiment</th>
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<tr>
<td>AcCoA to acetate experiment</td>
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* Monitored spectrophotometrically versus a control lacking one of the substrates.
lated to represent the reaction catalyzed by amebal acetyl-CoA-synthetase as the following: acetyl-CoA + ADP + P$_i$ = acetate + CoA + ATP.

**DISCUSSION**

Montalvo *et al.* (2) had shown that *Entamoeba histolytica* grown with bacteria produce acetate and consume oxygen in approximately equimolar amounts when metabolizing glucose in the presence of air. We now find that this is also true of axenic amebae. However, under anaerobic conditions axenic and bacteria-grown amebae behave differently. Cells grown with bacteria then produce acetate and hydrogen in approximately equimolar amounts (2) while axenic cells produce neither hydrogen (1) nor acetate.

Centrifuged extracts from amebae are capable of converting pyruvate to acetate when supplemented by appropriate cofactors. The enzyme which initiates this reaction is now identified as pyruvate synthase (EC 1.2.7.1) by the following three criteria: its spectrum of electron acceptors, its catalysis of a pyruvate-CO$_2$ exchange reaction, and its catalysis of pyruvate formation from bicarbonate and acetyl-CoA in the presence of hydrogen, hydrogenase, and an electron carrier. This enzyme may be the one previously recognized in amebae by Takeuchi *et al.* (4) but described by them as a pyruvate oxidase. The second enzyme of the pyruvate-to-acetate pathway converts acetyl-CoA, P$_i$, and ADP to acetate, CoA, and ATP. It is selective for acetate and for adenine nucleotides; guanine nucleotides function with it poorly. It functions with ADP instead of AMP and is not identical with acetate thiokinase (EC 6.2.1.1) nor with the mitochondrial enzyme which functions selectively with guanine nucleotides (24). An enzyme from *Tririchomonas foetus* with similar stoichiometry is shown in a schematic diagram by Muller (25) and it is mentioned briefly in an abstract (26), but its nucleotide selectivity seems not to be resolved.

Amebal pyruvate synthase, like those from *Clostridium acidii-urtici* (11) and *T. foetus* (13), is unstable in the presence of air. This has limited purification of the amebal enzyme, but sufficient separation from interfering activities was achieved to permit characterization of its catalyzed reactions. Unlike that from *T. foetus* the amebal enzyme seems not to be contained in an osmotically sensitive subcellular particle. Our rationale for employing an osmotically balanced solution during cell breakage was to maintain the integrity of the amebal digestive vacuoles which were then sedimented and removed from the soluble enzymes.

It appears possible that the hydrogenase regularly encountered in amebae grown with bacteria is of bacterial origin, and the same may be true of the phosphotransacetylase occasion-ally found. The bacterium, *D. symbiosus*, is known to possess both of these enzymes. Neither hydrogenase nor phosphotransacetylase was encountered in extracts of axenic cells. If axenic amebae lack a means of converting acetyl-CoA to acetyl phosphate then the recently discovered pyrophosphate-dependent acetate kinase (3) may not be involved in acetate production by these organisms. In amebae feeding on bacteria the acetate kinase might have a role in scavenging the energy of acetyl phosphate produced by bacterial metabolism.

Work by Guthrie (7) had shown that amebae grown either axenically or with bacteria contain low molecular weight iron sulfur protein. A report of a similar finding for axenic amebae was recently made by Weinbach *et al.* (27). This suggests that a ferredoxin-like molecule may be the electron carrier which functions in vivo between synthase and hydrogenase or between the enzyme and oxygen.

Although several unusual features have been recognized in amebal glycolysis it is likely that catabolism of glucose to pyruvate produces two net high energy bonds per mol of glucose catabolized (28). The conversion of pyruvate to acetate via amebal acetyl-CoA-synthetase now appears to offer a significant additional source of useful energy, one available to amebae growing with bacteria under anaerobic conditions, and to axenic amebae when some oxygen is present. This concept supports a view expressed by several investigators that *E. histolytica* is not a strict anaerobe (1, 27, 29).

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