Biochem. J. (1978) **171**, 225–230
Printed in Great Britain

Pyruvate-to-Ethanol Pathway in *Entamoeba histolytica**

By HSIN-SHENG LO and RICHARD E. REEVES
Department of Biochemistry, Louisiana State University Medical Center, 1542 Tulane Avenue, New Orleans, LA 70112, *U.S.A.*

(*Received* 25 *October* 1977)

The pyruvate-to-ethanol pathway in *Entamoeba histolytica* is unusual when compared with most investigated organisms. Pyruvate decarboxylase (EC 4.1.1.1), a key enzyme for ethanol production, is not found. Pyruvate is converted into acetyl-CoA and CO_2 by the enzyme pyruvate synthase (EC 1.2.7.1), which has been demonstrated previously in this parasitic amoeba. Acetyl-CoA is reduced to acetaldehyde and CoA by the enzyme aldehyde dehydrogenase (acylating) (EC 1.2.1.10) at an enzyme activity of 9 units per g of fresh cells with NADH as a reductant. Acetaldehyde is further reduced by either a previously identified $NADP^+$-linked alcohol dehydrogenase or by a newly found NAD^+-linked alcohol dehydrogenase at an enzyme activity of 136 units per g of fresh cells. Ethanol is identified as the product of soluble enzymes of amoeba acting on pyruvate or acetyl-CoA. This result is confirmed by radioactive isotopic, spectrophotometric and gas-chromatographic methods.

In early fermentation experiments with bacteria-grown *Entamoeba histolytica*, the end products of glucose dissimilation were found to be CO_2, H_2, acetate and ethanol (Bragg & Reeves, 1962; Montalvo *et al.*, 1971). The amoebic glycolytic pathway possesses the same Embden–Meyerhof-pathway intermediates as are found in other organisms. However, several unusual features in glycolytic enzymes extracted from this amoeba are exhibited (Reeves, 1974). First, the major enzyme that produces D-fructose 1,6-bisphosphate utilizes inorganic pyrophosphate as the phosphate donor. Secondly, the enzyme catalysing the conversion of 1,3-diphosphoglycerate to 3-phospho-D-glycerate employs GDP instead of ADP as the substrate. Thirdly, pyruvate kinase is absent and in its place is a four-enzyme system consisting of pyruvate,orthophosphate dikinase, phosphoenolpyruvate carboxykinase (PP_i), malate dehydrogenase and malate dehydrogenase (decarboxylating). These enzymes accomplish the conversion of phosphoenolpyruvate into pyruvate with the production of ATP and also provide a substrate-level transhydrogenation between NADH and $NADP^+$.

The pyruvate-to-acetate pathway has been elucidated by Reeves *et al.* (1977). Pyruvate is converted into acetyl-CoA and CO_2 by pyruvate synthase in the presence of CoA and an electron acceptor. Pyruvate synthase occurs in a variety of bacterial anaerobes (Bachofen *et al.*, 1964; Andrews & Morris, 1965; Raeburn & Rabinowitz, 1965), and is considered to be an enzyme characteristic of prokaryotes. The discovery of pyruvate synthase in a eukaryote was first demonstrated in *Tritrichomonas foetus* by Lindmark & Müller (1973). The final step is catalysed by a new enzyme, acetyl-CoA synthetase (ADP-forming), that produces ATP and acetate from acetyl-CoA and P_i. This step appears to offer a significant additional source of useful energy.

The only region in which knowledge of amoebic glycolysis was incomplete was the pathway from pyruvate to ethanol. Susskind (1976) demonstrated that, anaerobically, axenic amoebae produce mainly CO_2 and ethanol. The present work was undertaken to elucidate this pathway and thus complete the mapping of amoebic glycolysis.

Materials and Methods

Growth and harvesting of amoebae

Large lots of *E. histolytica*, strain 200:NIH, were grown axenically in 125ml flasks containing 120ml of Diamond's TP-S-1 medium (Diamond, 1968). Each flask was inoculated with 6×10^5 amoebae. Tropozoites [10×10^6 to 20×10^6 cells/flask (about 0.1 to 0.2g wet wt.)] were harvested after incubation for 96h at 36°C. Cells were washed by a balanced salt buffer, pH7.0, containing 10mM-potassium phosphate, 20mM-KCl, 0.5mM-$MgCl_2$, 100mM-NaCl and 0.1mM-$Ca(NO_3)_2$ that had been purged with N_2. Cells were then packed in this buffer by

* A preliminary report of this work was presented to the Federation of American Societies for Experimental Biology in Chicago, April 1–8, 1977 (Abstract 2003). Some of the data are from a thesis by H.-S. L. submitted to the graduate faculty of the Louisiana State University Medical Center in partial fulfilment of the requirements of the Master of Science degree, 1977.

centrifugation (2400*g*-min) and the cell volume was measured.

Amoebic cell extracts

The harvested amoebae were suspended in an equal volume of argon-purged 50mM-Mes(4-morpholine-ethanesulphonic acid)/NaOH buffer, pH6.0, containing 0.25M-sucrose and 2mM-dithiothreitol. They were ruptured under argon by 20 passes in a Teflon pestle tissue grinder cooled in an ice/water bath. The homogenates were diluted with 0.5 to 1vol. of the suspending buffer and centrifuged for 30min at 35000*g*. The supernatant solutions, called cell extracts, were placed under N_2 in septum-stoppered tubes.

Enzyme assays

All enzyme assays were carried out at 30°C with a Gilford model 240 spectrophotometer. Standard assays for pyruvate synthase (EC 1.2.7.1) and aldehyde dehydrogenase (acylating) (EC 1.2.1.10) were carried out with cuvettes made anaerobic by the techniques described by Ueyda & Rabinowitz (1971). By this technique the cuvettes were closed with a rubber septum and air was displaced by a flow of prepurified N_2 for a minimum of 5min.

The standard pyruvate synthase assay was that described by Reeves *et al.* (1977). The standard assay for amoebic aldehyde dehydrogenase (acylating) was devised for the present work. Cuvettes with an optical path length of 0.5cm containing 25μmol of potassium phosphate buffer, pH6.5, 1μmol of dithiothreitol, 50nmol of CoA, 2.5μmol of sodium pyruvate, 0.16μmol of FMN and water were made anaerobic. At least 0.1 unit of amoebic pyruvate synthase and 0.02–0.04 unit of enzyme were added through the septum with a syringe. The final volume was 0.5ml. The CoA-limited reduction of FMN was completed during thermal equilibration and reaction was started by adding 0.2μmol of NADH. The rate of reduction of FMN was monitored with $\varepsilon =$ 12200litre$\cdot$mol$^{-1}\cdot$cm^{-1} at A_{450}. One unit of enzyme is that amount causing the reduction of 1μmol of FMN/min under these conditions. The standard assays for amoebic alcohol dehydrogenase (NAD^+) or ($NADP^+$) were conducted aerobically, in the direction of acetaldehyde to ethanol. Quartz cuvettes with an optical path length of 1cm containing 20μmol of potassium phosphate buffer, pH6.5, 7.6μmol of acetaldehyde, 0.1μmol of NADH or NADPH and water were used for the assay. The final volume was 0.4ml. Reaction was started by adding 0.05–0.1 unit of enzyme. The rate of oxidation of NADH or NADPH was monitored with $\varepsilon =$ 6220litre$\cdot$mol$^{-1}\cdot$cm^{-1} at A_{340}. One unit of enzyme is that amount causing the oxidation of 1μmol of NADH or NADPH/min under these conditions. Acetaldehyde was freshly distilled and collected in ice/water. The solution was assayed with yeast alcohol dehydrogenase and NADH.

Pyruvate decarboxylase (EC 4.1.1.1) was assayed by employing cuvettes containing 25μmol of potassium phosphate buffer, pH6.8, 5μmol of sodium pyruvate, 1μmol of dithiothreitol, 0.2μmol of NADPH, 0.5μmol of thiamin pyrophosphate, 2.5μmol of $MgCl_2$ and water. Amoebic homogenate containing 0.03 unit of $NADP^+$-linked alcohol dehydrogenase was added to give a final volume of 0.5ml. The oxidation of NADPH was measured at A_{340}.

Identification of ethanol

Spectrophotometric method. Quartz cuvettes with an optical path length of 1cm containing 1mmol of Tris/HCl buffer, pH9.0, 2μmol of NAD^+, 1μmol of dithiothreitol, 0.1ml of sample and water were used. After the background readings were taken, 30 units of yeast alcohol dehydrogenase were added and the reaction was measured at A_{340}. The final volume was 1.0ml. One cuvette lacking sample served as a control.

Gas-chromatographic method. A Hewlett–Packard (Avondale, PA, U.S.A.) model 5830A gas chromatograph was used for identifying ethanol. A column (2mm$\times$1.8m) packed with Chromosorb 101 was used at a temperature of 140°C. Aqueous solutions (2μl each) of the sample were injected and ethanol in the sample was identified by comparing its retention time with that exhibited by a standard ethanol solution.

Radioactivity measurement

Radioactivity was measured with a Beckman LS-100 liquid-scintillation spectrometer in Aquasol 'cocktail'. Quenching was corrected by using an internal [^{14}C]toluene standard. All counts were corrected in terms of d.p.m.

Determination of protein

Protein in amoebic cell extracts was assayed by the method of Lowry *et al.* (1951) with bovine serum albumin as the standard, and in column fractions by the spectrophotometric method of Layne (1957).

Reagents used

Bovine serum albumin, FMN, dithiothreitol, Mes, phosphotransacetylase (phosphate acetyltransferase, EC 2.3.1.8; *Clostridium kluyveri*) and pyruvate decarboxylase (yeast) were from Sigma Chemical Co., St. Louis, MO, U.S.A. Alcohol dehydrogenase (yeast) was from Boehringer (Mannheim) Corp., New York, NY, U.S.A. NAD^+, $NADP^+$, NADH, NADPH, CoA, acetyl-CoA were from P–L Biochemicals, Milwaukee, WI, U.S.A. [2-^{14}C]Pyruvate and [1-^{14}C]acetyl-CoA were from Amersham/Searle, Arlington Heights, IL, U.S.A. Aquasol-2 was from New England Nuclear Corp., Boston, MA, U.S.A.

Argon and prepurified N_2 were from Matheson (East Rutherford, NJ, U.S.A.).

Results

Activities of enzymes involved in pyruvate-to-ethanol metabolism

Pyruvate decarboxylase activity was not observed in amoebic homogenates prepared in Mes/NaOH buffer, pH 6, or in Tris/HCl buffer, pH 7.4. The assay system was validated by the use of yeast pyruvate decarboxylase. The activities of pyruvate synthase, aldehyde dehydrogenase (acylating) and the NAD^+- and $NADP^+$-linked alcohol dehydrogenases in cell extracts are shown in Table 1. These enzymes were not sedimented in 30min at 35000*g*.

Column fractionation of amoebal enzymes

Because of the instability of the enzymes, the columns were equilibrated with freshly boiled buffers and operated under N_2 at 10°C. Fractions were collected in tubes immersed in ice and filled with argon. The tubes were immediately closed with a rubber septum and the gas was displaced by N_2. Enzyme activities were assayed by the standard methods. Four different columns were employed: Sephadex G-100, Bio-Gel P-300, DEAE-cellulose and hydroxyapatite. On all four columns the aldehyde dehydrogenase and the NAD^+-linked alcohol dehydrogenase were eluted together and their maximal activities occurred in the same fraction. The peak fractions from Sephadex G-100 column were used for most of the experiments reported in the present paper. The activities of the two alcohol dehydrogenases were resolved by DEAE-cellulose and hydroxyapatite columns. Resolution of pyruvate synthase from the other three enzymes was achieved by the hydroxyapatite column.

Table 1. *Activities of enzymes involved in pyruvate-to-ethanol metabolism in the cell extracts of axenic strain 200: NIH of E. histolytica*

Mean values are given ± S.D. Units for enzyme activity are defined or references to the assay are given under 'Enzyme assays'.

Enzymes (no. of preparations)	Enzyme activity (units/g of fresh cells)	Enzyme activity (units/mg of protein)
Pyruvate decarboxylase (2)	0	0
Pyruvate synthase (11)	44.5 ± 14.6	1.05 ± 0.20
Aldehyde dehydrogenase (16)	9.3 ± 3.4	0.48 ± 0.15
Alcohol dehydrogenase (NAD^+) (18)	135.7 ± 40.9	6.90 ± 1.70
Alcohol dehydrogenase ($NADP^+$) (6)	15.9 ± 4.5	0.96 ± 0.28

Some properties of enzymes of the pyruvate-to-ethanol pathway

Amoebic pyruvate synthase is rapidly inactivated by air (Reeves *et al.*, 1977). The aldehyde dehydrogenase and the NAD^+-linked alcohol dehydrogenase were also inactivated rapidly by air. When kept under N_2 and refrigerated, these enzymes retained 50 to 70% of their activities up to 24h. The apparent K_m for acetaldehyde of amoebic alcohol dehydrogenase (NAD^+) at pH 6.5 was 10mM.

Conversion of [2-^{14}C]pyruvate into labelled ethanol

The formation of ethanol from pyruvate was demonstrated by measuring the incorporation of the label of [2-^{14}C]pyruvate into ethanol. Results of these experiments are shown in Table 2. In the complete reaction 85.4nmol of labelled ethanol was found, corresponding to 20% of the pyruvate present at the

Table 2. *Requirements for the incorporation of label from [2-^{14}C]pyruvate into ethanol by column-purified enzyme*

Small tubes (10mm × 75mm) closed by rubber septa, containing 25μmol of potassium phosphate buffer, pH 6.5, 50nmol of CoA, 1μmol of dithiothreitol, 0.25μmol of FMN, 1μmol of EDTA, 1μmol of NADH and water, were bubbled with N_2 for 10min. Enzyme (280μg of protein) and 440nmol of [2-^{14}C]-pyruvate (sp. radioactivity 85d.p.m./nmol) were injected through the septum to initiate the reaction. The final volume was 0.5ml. The contents of the tubes were mixed well and incubated at 30°C for 15min. The reaction was stopped by adding 1ml of 1M-HCl. After centrifugation (800*g*, 5min), the supernatant was removed to a larger tube and neutralized to pH 7 with KOH and 1ml of 0.2M-potassium phosphate. Neutralized 2M-hydroxylamine hydrochloride (50μl) was added and 0.1μmol of ethanol as a carrier. This solution was placed in a distillation flask and diluted to a volume of 12ml. Then 8ml of distillate was collected in ice/water and the condenser was rinsed to produce a final volume of 10ml. Portions of this solution were taken for radioactivity measurements. Results are corrected for radioactivity counts found for an experiment in which acid was added before enzyme, which served as a control. The enzyme contained 0.5 unit of pyruvate synthase activity, 0.09 unit of aldehyde dehydrogenase (acylating) activity and 2.4 units of alcohol dehydrogenase (NAD^+) activity. Abbreviation used: DTT, dithiothreitol.

Conditions of experiment	Radioactivity in distillate (d.p.m.)	Ethanol formed (nmol)
Complete	7260	85.4
−EDTA	6150	72.3
−DTT	5790	68.1
−FMN	2000	23.5
−CoA	385	4.5
−NADH+NADPH	110	1.3
−NADH	60	0.7

start of the reaction. By omitting, singly, the components of the reaction mixture it was found that CoA, FMN, and NADH were required for the conversion of pyruvate into ethanol. The findings indicate that acetyl-CoA is an intermediate between pyruvate and ethanol, that an electron acceptor is required and that NADPH cannot effectively replace NADH in the reductive step.

Conversion of [1-^{14}C]acetyl-CoA into labelled ethanol

Label from [1-^{14}C]acetyl-CoA was recovered in ethanol after incubation with amoebic enzymes. Results shown in Table 3 indicate that the conversion of acetyl-CoA into ethanol requires NADH, not NADPH. That the product of this reaction was ethanol was confirmed in parallel spectrophotometric and gas chromatographic experiments.

Conversion of acetaldehyde into ethanol

On addition of amoebic cell extracts or column-purified fractions to buffered solutions containing acetaldehyde and NADH, the latter was rapidly oxidized in a reaction that was dependent on the acetaldehyde. That the product of this reaction was ethanol was confirmed by spectrophotometric and gas-chromatographic methods.

Conversion of acetaldehyde into acetyl-CoA

The synthesis of acetyl-CoA from acetaldehyde and CoA catalyzed by the amoebic enzymes was measured by the formation of acetyl phosphate in the presence of NAD^+, P_i and phosphotransacetylase (Table 4). In these experiments a dismutation system between amoebic aldehyde dehydrogenase (acylating) and alcohol dehydrogenase (NAD^+) was involved in the reaction. CoA and NAD^+ were regenerated in the system. The large amount of phosphate provided by the buffer favoured the formation of acetyl phosphate. In the complete reaction 2.5 μmol of acetyl phosphate was formed corresponding to 33% of the acetaldehyde present at the start of the reaction. The requirements for this reaction were deduced by omitting, singly, the components of the reaction mixture, and were found to be CoA, NAD^+ or NADH, P_i and phosphotransacetylase. $NADP^+$ was not capable of replacing NAD^+ in the reaction.

Stoicheiometry of the conversion of acetyl-CoA into ethanol

In the conversion of acetyl-CoA into ethanol, acetaldehyde dehydrogenase (acylating), in the first step, reduces acetyl-CoA to acetaldehyde with

Table 3. *Nucleotide requirement for the incorporation of label from [1-^{14}C]acetyl-CoA into ethanol by column-purified enzyme*

The experiments were run under anaerobic conditions as described for Table 2. Several tubes each contained 25 μmol of potassium phosphate buffer, pH 6.5, 1 μmol of dithiothreitol, 1 μmol of EDTA, 98 nmol of NADH or NADPH, 142 nmol of [1-^{14}C]acetyl-CoA (144 d.p.m./nmol) and water. Enzyme (240 μg of protein) was added to initiate the reaction. The final volume was 0.5 ml. The reaction mixture was incubated at 30°C. At specific times, the reaction was stopped by adding 1 ml of 1 M-HCl to one of the tubes. The distillation and counting procedures were as described for Table 2. An experiment in which acid was added before enzyme served as a control. The enzyme contained 0.15 unit of aldehyde dehydrogenase (acylating) activity and 1.1 units of alcohol dehydrogenase (NAD^+) activity.

Incubation time (min)	Radioactivity in distillate (d.p.m.)	Ethanol formed (nmol)
NADH		
2	3220	22.4
5	3750	26.0
10	4610	32.0
NADPH		
2	0	0
5	0	0
10	260	1.8

Table 4. *Requirements for the dismutation reaction catalysed by column-purified enzyme*

Small septum-stoppered tubes containing 30 μmol of potassium phosphate buffer, pH 7.4, 1 μmol of dithiothreitol, 50 nmol of CoA, 0.2 μmol of NAD^+ and water were made anaerobic. Then 7.6 μmol of acetaldehyde and 12 units of phosphotransacetylase were added. Enzyme (260 μg of protein) was added to initiate the reaction. The final volume was 0.6 ml. The reaction mixture was incubated at 30°C for 30 min. The reaction was stopped by adding 0.2 ml of neutralized 2 M-hydroxylamine hydrochloride. After 10 min, the acetohydroxamic acid was assayed by the method of Lipmann & Tuttle (1945). Results were corrected for an acid-before-enzyme experiment in which acid was added before enzyme, which served as a control. The enzyme contained 0.24 unit of aldehyde dehydrogenase (acylating) and 1.5 units of alcohol dehydrogenase (NAD^+). Abbreviations used: DTT, dithiothreitol; PTA, phosphotransacetylase.

Conditions of experiment	Acetyl phosphate formed (μmol)
Complete	2.50
−NAD+NADH	2.40
−DTT	0.35
−CoA	0.20
−P_i+Tris	0.12
−PTA	0.12
−NAD^+	0.09
−NAD^++$NADP^+$	0.08

the concomitant production of NAD^+ and CoA. The CoA generated then couples with pyruvate synthase to reduce FMN. In the second step, the acetaldehyde formed is further reduced to ethanol by alcohol dehydrogenase (NAD^+) and a second molecule of NADH is oxidized. Results in Table 5 show that, when a limiting amount of NADH is added, the ratio between reduced FMN (i.e., acetyl-CoA formed) and NADH added is approx. 1:2. This shows that 2mol of NADH is oxidized in the conversion of 1mol of acetyl-CoA into ethanol.

Discussion

Since pyruvate decarboxylase is not found in the amoeba, the only route for ethanol production appears to be via acetyl-CoA. Evidence substantiates the role of acetyl-CoA as an intermediate in the pyruvate-to-ethanol pathway. The activities of the enzymes involved in this pathway are sufficient to account for the observed glycolytic flux (Montalvo *et al.*, 1971).

In amoebic glycolytic catabolism of glucose to pyruvate, 2mol of reduced nicotinamide nucleotide is formed per mol of glucose. The further catabolism of pyruvate proceeds via acetyl-CoA and produces additional reducing equivalents. In total, 8 reducing equivalents are generated in converting 1mol of glucose into 2mol of acetyl-CoA. Since the terminal cytochrome oxidase transport system is not present in *E. histolytica* (Hilker & White, 1959; Montalvo *et al.*, 1971; Weinbach & Diamond, 1974), some other pathway must regenerate oxidized nicotinamide nucleotides for glycolysis to proceed under anaerobic conditions. This role is taken by the aldehyde dehydrogenase and alcohol dehydrogenases.

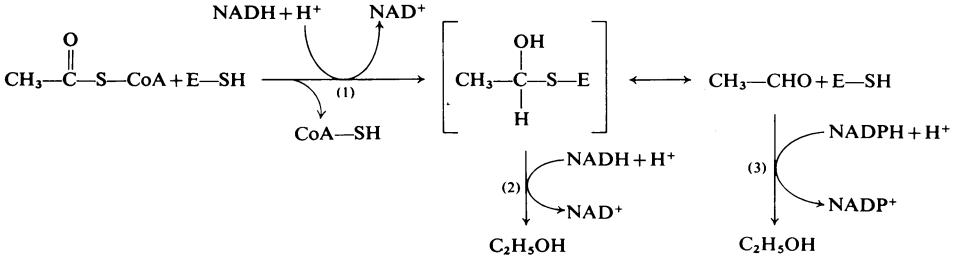
The amoebic enzyme that reduces acetyl-CoA to the aldehyde requires NADH. A similar enzyme was characterized and purified from *C. kluyveri* by Burton & Stadtman (1953). Dawes & Foster (1956) demonstrated its activity in *Escherichia coli* and reported it to be the key enzyme for ethanol production by this bacterium. Although the mechanism of the reduction of acetyl-CoA has not so far been elucidated, it is reasonable to hypothesize that an enzyme-bound hemithioacetal is the substrate for the aldehyde dehydrogenase (Burton & Stadtman, 1953). We have not been able to separate the activity of amoebic aldehyde dehydrogenase from that of the NAD^+-linked alcohol dehydrogenase. The two activities were congruent in numerous column fractionations. It seems possible that these two activities reside on a single protein. Rudolph *et al.* (1968) made a similar suggestion for the activities of the aldehyde dehydrogenase and the alcohol dehydrogenase from E. *coli*. They also proposed an enzyme-bound hemithioacetal intermediate.

There are two alcohol dehydrogenases in *E. histolytica*. The NAD^+-linked enzyme characterized

Table 5. *Stoicheiometric determination of FMN reduced with respect to NADH added*

Cuvettes with an optical path length of 0.5cm containing 25μmol of potassium phosphate buffer, pH6.5, 2.5μmol of sodium pyruvate, 1μmol of dithiothreitol, 50nmol of CoA, 0.16μmol of FMN and water were made anaerobic. Column-purified enzyme (70μg of protein) was injected through the septum with a syringe. The CoA-limited reduction of FMN was completed during thermal equilibration. Then, known limiting amounts of NADH were added through the septum to bring the final volume to 0.5ml. Reduction of FMN was monitored at A_{450} until reaction was complete. A volume of water equal to the volume of added NADH was added to the control cuvette for each experiment to correct for O_2 dissolved in the NADH solution. The amount of reduction of FMN was calculated against each control by taking $\varepsilon = 12200 \text{ litre} \cdot \text{mol}^{-1} \cdot \text{cm}^{-1}$ at A_{450}. The enzyme contained 0.12 unit of pyruvate synthase, 0.02 unit of aldehyde dehydrogenase (acylating) and 0.5 unit of alcohol dehydrogenase (NAD^+). Mean value is given ± S.D.

Expt.	NADH added (nmol)	FMN reduced (nmol)	FMN/NADH ratio
1	36.9	15.4	0.42
2	43.0	20.8	0.48
3	61.0	32.8	0.53
4	98.4	41.0	0.42
5	123.0	59.7	0.49
		Mean	0.47 ± 0.05



Scheme 1.

in this work, and an $NADP^+$-linked enzyme previously described (Reeves *et al.*, 1971). The apparent K_m of the NAD^+-linked enzyme for acetaldehyde was much greater than that of the $NADP^+$-linked enzyme. It seems plausible that the former enzyme selectively acts on the hemithioacetal substrate, whereas the latter, owing to its low K_m, would reduce any free acetaldehyde formed.

The production *in vivo* of ethanol from acetyl-CoA in *E. histolytica* can be schematically represented as shown in Scheme 1 (brackets indicate an enzyme-bound intermediate), where reaction (1) is catalysed by aldehyde dehydrogenase (acylating), reaction (2) is catalysed by alcohol dehydrogenase (NAD^+), reaction (3) is catalysed by alcohol dehydrogenase ($NADP^+$) and E represents enzyme.

This work was supported in part by grants A1 02951 and GM 14023 from the National Institutes of Health, United States Public Health Service. H.-S. L. received a fellowship from the National Science Council, Republic of China. We express our appreciation to Mrs. Betty West for the cultivation of amoebae.

References

Andrews, I. G. & Morris, J. G. (1965) *Biochim. Biophys. Acta* **97**, 176–179

Bachofen, R., Buchanan, B. B. & Arnon, D. I. (1964) *Proc. Natl. Acad. Sci. U.S.A.* **51**, 690–694

Bragg, P. D. & Reeves, R. E. (1962) *Exp. Parasitol.* **12**, 393–400

Burton, R. M. & Stadtman, E. R. (1953) *J. Biol. Chem.* **202**, 873–890

Dawes, E. A. & Foster, S. M. (1956) *Biochim. Biophys. Acta* **22**, 253–265

Diamond, L. S. (1968) *J. Parasitol.* **54**, 1047–1056

Hilker, D. M. & White, A. G. C. (1959) *Exp. Parasitol.* **8**, 539–548

Layne, E. (1957) *Methods Enzymol.* **3**, 447–454

Lindmark, D. G. & Müller, M. (1973) *J. Biol. Chem.* **248**, 7724–7728

Lipmann, F. & Tuttle, L. C. (1945) *J. Biol. Chem.* **159**, 21–28

Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265–275

Montalvo, F. E., Reeves, R. E. & Warren, L. G. (1971) *Exp. Parasitol.* **30**, 249–256

Raeburn, S. & Rabinowitz, J. C. (1965) *Biochem. Biophys. Res. Commun.* **18**, 303–307

Reeves, R. E. (1974) *Arch. Invest. Med.* **5**, *Suppl.* **2**, 411–414

Reeves, R. E., Montalvo, F. E. & Lushbaugh, T. S. (1971) *Int. J. Biochem.* **2**, 55–64

Reeves, R. E., Warren, L. G., Susskind, B. & Lo, H. (1977) *J. Biol. Chem.* **252**, 726–731

Rudolph, F. B., Purich, D. L. & Fromm, H. J. (1968) *J. Biol. Chem.* **243**, 5539–5545

Susskind, B. (1976) M.Sc. Thesis, Louisiana State University Medical Center, New Orleans

Ueyda, K. & Rabinowitz, J. C. (1971) *J. Biol. Chem.* **246**, 3111–3119

Weinbach, E. C. & Diamond, L. S. (1974) *Exp. Parasitol.* **35**, 232–243