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Occurrence of the Coupling Reaction of Alcohol Dehydrogenase with Malate Dehydrogenase in *Schizophyllum commune**

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Abstract

The L-malate-producing pathway from ethanol and $CaCO_3$ by *Schizophyllum commune* was enzymatically studied using the crude cell-free extract. The occurrence of nicotinamide adenine dinucleotide (NAD)-dependent alcohol dehydrogenase [EC 1.1.1.1] was recognized as an enzyme responsible for ethanol oxidation. As an enzyme responsible for L-malate formation, NAD-dependent malate dehydrogenase [EC 1.1.1.37], which has been found in glucose-grown mycelia of this mold, occurred in the ethanol-grown mycelia with strong activity, while NAD-dependent malic enzyme [EC 1.1.1.38] did not. We experimentally proved that alcohol dehydrogenase reaction couples with malate dehydrogenase reaction *via* NAD(H) in the crude cell-free extract of *S. commune*. The crude enzyme solution used in this experiment did not seem to contain mitochondrial systems, because isocitrate dehydrogenase [EC 1.1.1.41] activity was not detected to any appreciable extent. Consequently, we speculated that the last step of the L-malate-producing pathway from ethanol and $CaCO_3$ by *S. commune* is the reduction of oxalacetate to which the coupling reaction of alcohol dehydrogenase with malate dehydrogenase contributes in the cytoplasm compartment.

Introduction

We reported previously that *Schizophyllum commune* produced a large amount of L-malate from ethanol and $CaCO_3$,[1,2] and that the overall reaction might be explained by the following reaction indicating CO_2-fixing fermentation.[3]

$$\text{Ethanol} + 2CO_2 \longrightarrow \text{L-Malate} \qquad (1)$$

In this paper, the first and the last steps of our proposed reaction were enzymatically studied using the crude cell-free extract of *S. commune*. Both activities of alcohol dehydrogenase (ADH) [EC 1.1.1.1] and malate dehydrogenase (MDH) [EC 1.1.1.37] were detected in the crude cell-free extract. In addition to the detection, the coupling reaction of ADH with MDH was found in the same extract.

Materials and Methods

Microorganism *Schizophyllum commune* IAM No. 9006 was used as the most preferable strain for ethanol. For the stock culture, the mold was grown on malt agar for about 10 days at 30°C, then stored at 5°C.

* Studies on CO_2-fixing Fermentation (XXX).

Cultivation The basal medium employed was composed of 1 g ethanol, 0.05 g NH_4NO_3, 0.2 g KH_2PO_4, 0.15 g $MgSO_4 \cdot 7H_2O$, 0.025 g KCl, 0.13 mg $MnCl_2$, 0.75 μg thiamine, 1 g $CaCO_3$, and 100 ml tap water. Sixty milliliters of the medium in a 500-ml shaking flask was inoculated with a few loopfuls of the stock culture. Shaking culture on a reciprocal shaker was carried out for 7 days at 30°C.

Preparation of cell-free extract After cultivation, the mycelia harvested by filtering the broth were washed well with deionized water and dried with air for 3–4 hr, then stored by freezing at −20°C. The air-dried mycelia were ground for 10 min with an equal weight of quartz sand, extracted with 10 volumes of 0.067 M Na_2HPO_4, and centrifuged at 12,000 × *g* for 20 min. The supernatant obtained was used as the crude cell-free extract and was sometime subjected to ammonium sulfate fractionation. Fresh mycelia in place of air-dried mycelia were sometime used for extraction.

Assay of enzyme activities Alcohol dehydrogenase, malate dehydrogenase, malic enzyme [EC 1.1.1.38], and isocitrate dehydrogenase [EC 1.1.1.41] activities were assayed according to the methods of Racker,[4] Ochoa,[5] Ochoa *et al.*,[6] and Sanwal *et al.*,[7] respectively, with slight modifications. Absorbance at 340 nm was measured with a Hitachi EPU-2 spectrophotometer equipped with a Hitachi SR-1 recording. Protein concentration was determined by the spectrophotometric method of Warburg and Christian[8] or Biuret method using egg albumin as a standard.[9]

Chemicals Oxalacetate, nicotinamide adenine dinucleotide (NAD), reduced nicotinamide adenine dinucleotide (NADH), and adenosine triphosphate (ATP) were from Sigma Chemical Co. Pyruvate was from E. Merck AG. Ethanol, L-malate, DL-isocitrate, adenosine monophosphate (AMP), and other chemicals were of analytical grade from Wako Pure Chemical Industries, Ltd.

Results

Alcohol dehydrogenase (ADH)

a) *Recognition of the occurrence*: The occurrence of NAD-dependent ADH was recognized as an enzyme responsible for ethanol oxidation in the crude cell-free extract of *S. commune*. As shown in Fig. 1, the ADH activity was revealed by the spectrographs that ethanol addition caused NAD reduction, then acetaldehyde addition did the NADH oxidation. NADP was not reduced by the extract in the presence of the same substrate. Table 1 shows that the ADH was partially purified by ammonium sulfate fractionation. The activity peak was in the 0.5–0.7 saturation fraction and the specific activity was raised approximately three folds. The fraction was subjected to subsequent experiments examining some properties of ADH of *S. commune*.

b) *Optimum pH*: The effect of pH on the ADH activity was examined by the spectrophotometric method described above. Phosphate buffer (KH_2PO_4-Na_2HPO_4) or Tris-HCl buffer was added to the reaction mixture from pH 5.5 to 9.0. Figure 2 shows that the ADH activity increased with pH up to pH 7.0 and subsequently decreased. The optimum pH was estimated to be pH 7.0, in contrast to pH 8.0 of the liver enzyme or pH 6.2 of *Cephalosporium* sp. enzyme.[10]

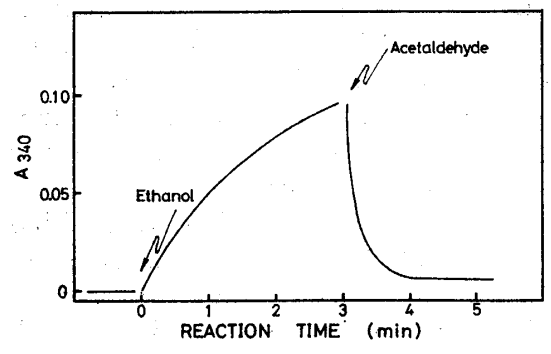


Fig. 1. Occurrence of ADH in the crude extract of *S. commune*.
The reaction mixture (2.5 ml) contained 2.1 ml of 0.1 M phosphate buffer (pH 7.0), 0.1 ml of 2.5 M ethanol, 0.1 ml of 0.0015 M NAD, 0.1 ml of 0.005 M acetaldehyde, and 0.1 ml of the crude cell-free extract. Ethanol and acetaldehyde were added at the points indicated. Reactions were started by the addition of substrate after preincubation for 2 min at room-temperature (approximately 20°C).

Table 1. Partial purification of ADH by $(NH_4)_2SO_4$ saturation fractionation.

Fraction	Total activity (units*)	Total protein (mg)	Specific activity (units/mg protein)
Crude extract	14.90	184.0	0.081
0.5 Satd. ppt	1.57	36.0	0.044
0.7 Satd. ppt	11.26	46.2	0.244
0.9 Satd. ppt	0.14	18.8	0.007

The crude cell-free extract was fractionated with solid ammonium sulfate. The resulting precipitate at each fraction was suspended in a small volume of 0.1 M phosphate buffer (pH 7.0). The reaction mixture (2.5 ml) contained 2.0 ml of 0.1 M phosphate buffdr (pH 7.0), 0.1 ml of 2.5 M ethanol, 0.1 ml of 0.0015 M NAD, 0.1 ml of 0.1 M semicarbazide, 0.1 ml of 0.2 M cysteine, and 0.1 ml of the enzyme solution. Assay conditions for ADH were the same as those described in the legend to Fig. 1.

* 1 unit: the amount of enzyme which reduces 1 μmole NAD per minute.

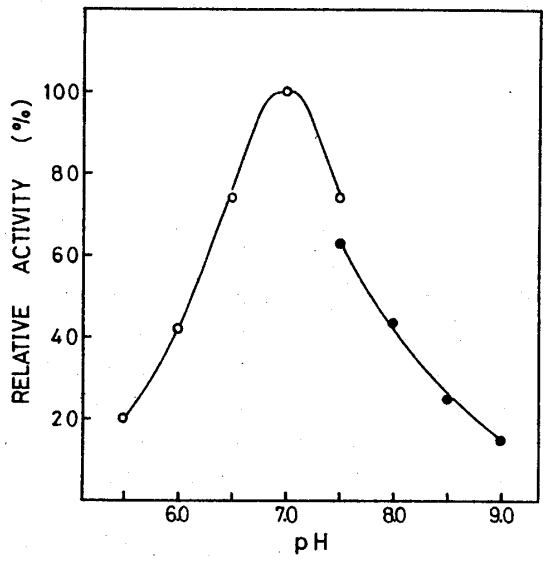


Fig. 2. Effect of pH on the ADH activity.
The reaction mixture and assay conditions for ADH were the same as those described in the legend to Table 1 and Fig. 1, respectively. ∘—∘, 0.1 M phosphate buffer; ·—·, 0.1 M Tris-HCl buffer.

c) *Substrate specificity*: The substrate specificity of ADH was determined by measuring the reduction rate of NAD linked to the oxidation of each alcohol. As shown in Table 2, ADH was most active on ethyl alcohol and oxidized lower aliphatic primary alcohols except for methyl alcohol. However, secondary or polyhydric alcohols were not oxidized to any observable extent. This tendency resembled that of the yeast enzyme.[11]

d) *Effect of inhibitors*: Some inhibitors for ADH were added to the reaction mixture, as shown in Table 3. Pyrazole, which gives a compound with free NAD and *o*-phenanthroline, a metal-complexing agent, showed about 80 and 40% inhibition, respectively. Of the thiol reagents, *p*-chloromercuribenzoate caused complete inhibition while iodoacetate-(mono) scarcely at all, like the ADH of horse liver.[12] The ADH of *S. commune* seems to be sulfhydryl enzyme which is quite sensitive to *p*-chloromercuribenzoate.

Malate dehydrogenase (MDH) The occurrence of NAD-dependent MDH was recognized again as an enzyme responsible for L-malate formation in the crude cell-free extract from ethanol-grown mycelia of *S. commune*, as was that from glucose-grown mycelia.[13] As shown in Fig. 3, the MDH activity was revealed to be so high that the

Table 2. Substrate specificity of ADH.

Substrate (10 mM)	ADH activity (units/ml enzyme soln.)	Relative activity (%)
Methyl alcohol	<0.002	—
Ethyl alcohol	0.142	100
n-Propyl alcohol	0.109	77
n-Butyl alcohol	0.097	68
iso-Propyl alcohol	0.008	6
sec-Butyl alcohol	0.006	4
Ethylene glycol	<0.002	—
Glycerol	<0.002	—

The reaction mixture (2.5 ml) contained 2.0 ml of 0.1 M phosphate buffer (pH 7.0) 0.1 ml of 0.25 M substrate, 0.1 ml of 0.0015 M NAD, 0.1 ml of 0.1 M semicarbazide, 0.1 ml of 0.2 M cysteine, and 0.1 ml of the enzyme solution. Assay conditions for ADH were the same as those described in the legend to Fig. 1. The relative activity was expressed as a percentage of that in the control experiment (ethyl alcohol).

Table 3. Effect of inhibitors on ADH activity.

Inhibitor (mM)		ADH activity (units/ml enzyme soln.)	Inhibition (%)
None		0.119	0
Pyrazole	1.0	0.029	76
o-Phenanthroline	1.0	0.067	44
PCMB*	0.1	0.001	100
PCMB	0.01	0.004	97
Iodoacetate(mono)	1.0	0.107	10

The reaction mixture (2.5 ml) contained 2.0 ml 0.1 M phosphate buffer (pH 7.0), 0.1 ml of 2.5 M ethanol, 0.1 ml of 0.0015 M NAD, 0.1 ml of 0.1 M semicarbazide, 0.1 ml of the inhibitor indicated above and 0.1 ml of the enzyme solution. Assay conditions for ADH were the same as those described in the legend to Fig. 1. The inhibition was expressed as the percent activity which disappeared from the control ADH activity.

* PCMB, *p*-Chloromercuribenzoate.

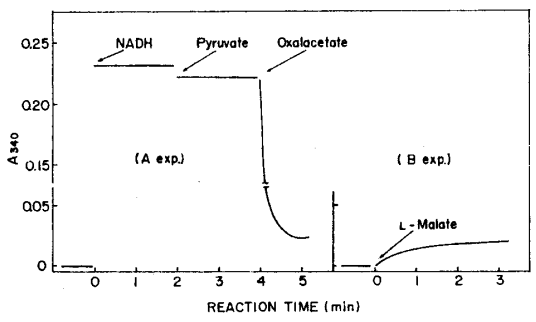


Fig. 3. Occurrence of MDH in the crude extract of *S. commune*.

Reaction mixture A (2.5 ml) contained 2.0 ml of 0.1 M phosphate buffer (pH 7.5), 0.1 ml of 0.1 M pyruvate, 0.1 ml of 0.01 M oxalacetate, 0.1 ml of 0.001 M NADH, 0.1 ml of 0.1 M $KHCO_3$ and 0.1 ml of the crude cell-free extract. Reaction mixture B (2.5 ml) contained 2.2 ml of 0.1 M phosphate buffer (pH 7.5), 0.1 ml of 0.1 M L-malate, 0.1 ml of 0.0015 M NAD, and 0.1 ml of the same extract. NADH, pyruvate, oxalacetate, and L-malate were added at the points indicated. Assay conditions were the same as those described in the legend to Fig. 1.

oxidation rate of NADH per min per 0.1 ml of extract by oxalacetate addition could not be determined with the spectrographs (A experiment), while the reverse reaction, NAD reduction linked to L-malate oxidation, was very weak (B experiment). In addition, Fig. 3 indicates that neither NAD-dependent malic enzyme nor lactate dehydrogenase activities were present, because the addition of pyruvate in place of oxalacetate did not cause NADH oxidation.

The effect of pH on the MDH activity of NADH oxidation with oxalacetate was examined using phosphate buffer (pH 5.5–8.0) and Tris-HCl buffer (pH 7.5–9.0). Figure 4 shows that the MDH activity was estimated to be maximal at pH 7.5 in Tris-HCl buffer.

Coupling reaction of ADH with MDH The above experiments demonstrated that ADH and MDH requiring NAD as a coenzyme occurred in the crude cell-free extract

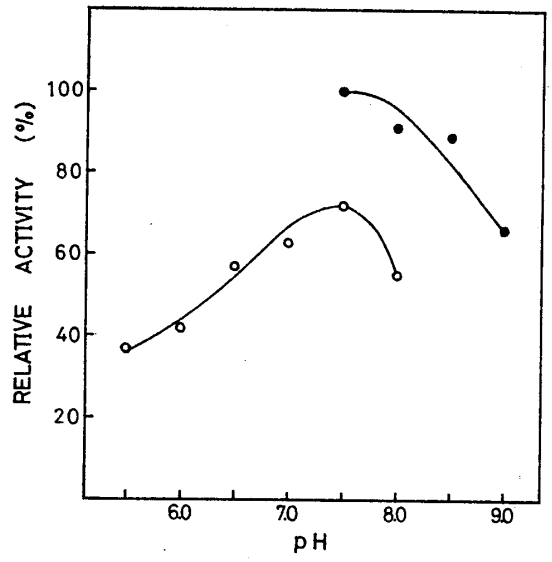


Fig. 4. Effect of pH on the MDH activity.

The reaction mixture (2.5 ml) contained 2.2 ml of 0.1 M buffer, 0.1 ml of 0.01 M oxalacetate, 0.1 ml of 0.001 M NADH, and 0.1 ml of the crude cell-free extract. Assay conditions for MDH were the same as those described in the legend to Fig. 1. ∘—∘, phosphate buffer; •—•, Tris-HCl buffer.

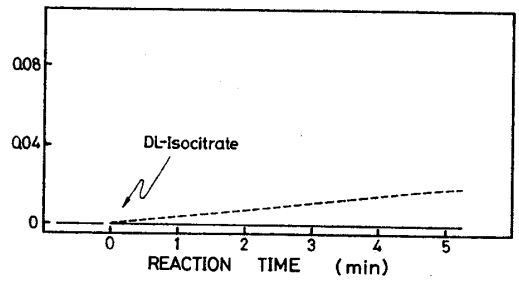


Fig. 5. Absence of isocitrate dehydrogenase in the crude extract of *S. commune* prepared from fresh mycelia.

The reaction mixture (2.5 ml) contained 1.9 ml of 0.1 M Tris-HCl buffer (pH 7.5) 0.1 ml of 0.25 M DL-isocitrate, 0.2 ml of 0.0015 M NAD, 0.1 ml of 0.01 M AMP, 0.1 ml of 0.1 M $MgCl_2$ and 0.1 ml of the crude cell-free extract. DL-isocitrate was added at the point indicated. Assay conditions were the same as those described in the legend to Fig. 1. —, extract from fresh mycelia; ---, extract from airdried mycelia.

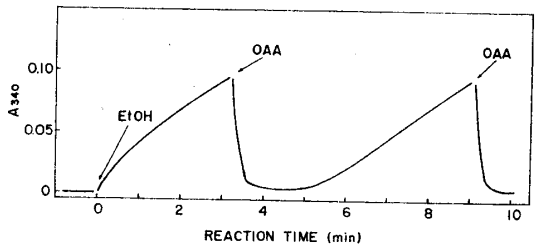


Fig. 6. Coupling reaction of ADH with MDH in the crude extract from fresh mycelia of *S. commune*.

The reaction mixture containing 2.0 ml of 0.1 M phosphate buffer (pH 7.0) 0.1 ml of 0.0015 M NAD, 0.1 ml of 0.2 M cysteine, and 0.1 ml of the crude cell-free extract, was preincubated for 2 min at room temperature (approximately 20°C). The coupling reaction was started by the addition of 0.1 ml of 2.5 M EtOH, then 0.1 ml of 0.001 M OAA* was added at the points indicated.

* OAA, oxalacetate.

of *S. commune*, and that the optimum pH of both reactions were nearly equal. Therefore, we examined whether both reactions act as an enzymatic coupling system.

a) *The coupling reaction in the crude extract from fresh mycelia*: In this experiment, fresh mycelia of *S. commune* were used in order to avoid contamination by mitochondrial enzymes (Fig. 5). Figure 6 shows that the coupling reaction of ADH with MDH was performed *via* NAD(H) in the crude cell-free extract. That is, the spectrographs of an increase or decrease in absorbance at 340 nm indicates that NAD linked to ethanol oxidation was reduced to NADH by ADH, then oxalacetate addition caused the NADH oxidation by MDH, and that if ethanol was added in excess, the NAD generated was reduced again to NADH with time, then the reoxidation of the NADH proceeded very smoothly by further addition of oxalacetate.

b) *Effect of pH on the coupling rate*: The coupling systems were compared around the optimum pH of the ADH and the MDH reactions. As shown in Fig. 7, the coupling reaction proceeded smoothly in the range of physiological pH. When a large portion of oxalacetate added had been reduced, the gradual increase in absorbance occurred. The time lag was longer at pH 6.5 than at pH 7.0 or at pH 8.0. In other words, the order of the coupling reaction rate was as follows: pH 7.0>pH 8.0>pH 6.5.

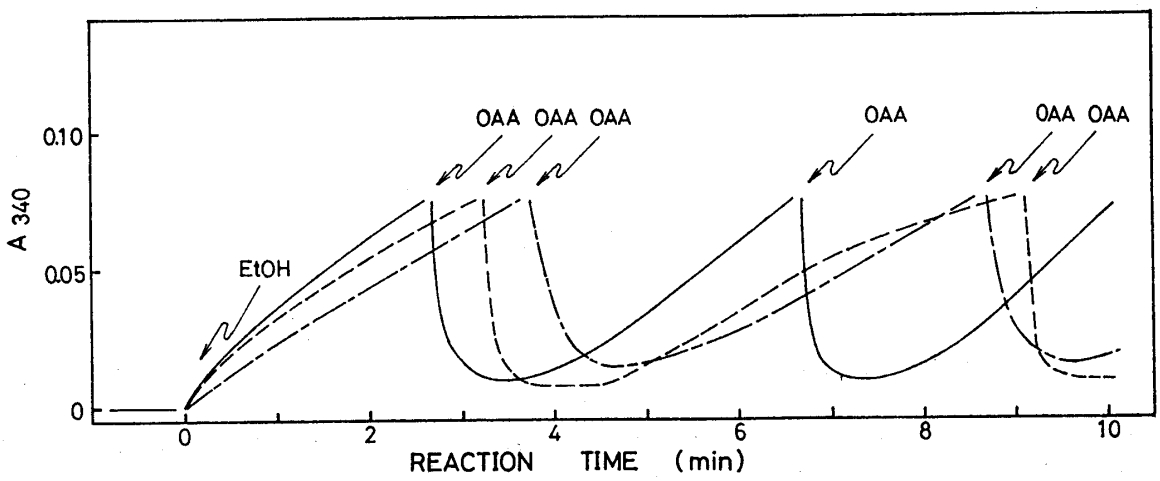


Fig. 7. Effect of pH on the coupling rate of ADH with MDH.
The reaction mixture and assay conditions were the same as those described in the legend to Fig. 6. The pH of the coupling reaction was varied from 6.5 to 8.0, using 0.1 M phosphate buffer. ---, pH 6.5; —, pH 7.0; —, pH 8.0.

Discussion

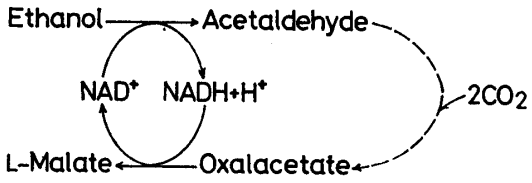
The L-malate-producing pathway from ethanol and $CaCO_3$ by *S. commune* was studied using the crude cell-free extract. This paper is the first communication to prove at enzymatic levels the scheme which had been proposed as follows:[3]

$$\text{Ethanol} \xrightarrow{CO_2} C_3 \text{ compound} \xrightarrow{CO_2} \text{L-Malate} \qquad (2)$$

Of enzymes responsible for ethanol oxidation, NAD-dependent ADH was found in the crude cell-free extract of *S. commune* (Fig. 1). As the ADH of this mold has not been described previously, some of its properties were compared with those of other origins (Fig. 2, Tables 2 and 3). These findings seem to be regarded as an attribute of the common ADH. ADH has been reported to be localized in the cytoplasm compartment.[14]

Of enzymes responsible for L-malate formation, NAD-dependent MDH activity was detected in the crude cell-free extract of *S. commune,* in spite of the absence of NAD-dependent malic enzyme activity (Fig. 3). MDH has been shown to exist in several isozymes such as mitochondrial MDH (M-MDH) and cytoplasmic MDH (C-MDH) in mammalian[15] or plant[16] tissues. The marked differences in *Km* values and turnover numbers suggested that M-MDH is better suited for the oxidation of L-malate and C-MDH for the reverse reaction.[17] The MDH of *S. commune* seems to be an isozyme which is localized in the cytoplasm compartment, judging from the preparation procedure of the crude extract (see *Materials and Methods*). In order to clarify this, the activity of isocitrate dehydrogenase, whose localization is similar to that of M-MDH, was examined as a marker of the mitochondrial enzyme. As shown in Fig. 5, isocitrate dehydrogenase seemed to have very little activity in the crude cell-free extract from air-dried mycelia, and none in the extract from fresh mycelia.

From the above discussions, both the ADH and the MDH found in this experiment appear to be localized in the cytoplasm compartment of *S. commune.* Therefore, these findings could make it indicate that the coupling reaction of both enzymes proved experimentally in Figs. 6 and 7, functions advantageously toward L-malate formation *in vivo* as follows:



Furthermore, the comparison of oxidation-reduction potentials (*Eo'*) supports the fact that the coupling system is theoretically valid, because *Eo'* of ADH and MDH reactions are −0.16 and −0.10 volt, respectively. We concluded that the last step of the L-malate-producing pathway from ethanol and $CaCO_3$ by *S. commune* is the reduction of oxalacetate to which the coupling reaction of the ADH with the MDH contributes in the cytoplasm compartment. Such a conclusion was consistent with our previous findings that neither the tricarboxylic acid cycle nor the glyoxylate bypath through which L-malate is oxidatively produced, plays a major role in the L-malate fermentation.[3]

A number of enzymatic coupling systems *via* NAD(H) are known to play important roles *in vivo.* Their significance is ascribed to the supplementation of extramitochondrial NAD(H) which can not permeate through mitochondrial membrane. Although many communications have described ADH or MDH isozymes, none has ever presented experimental proof of the occurrence of the coupling reaction of ADH with MDH *in vivo.* Therefore, our present study is noteworthy in connection with alcohol metabolism as well as L-malate production.

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