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REGULATORY AND PHYSICOCHEMICAL PROPERTIES OF TWO ISOENZYMES OF MALATE DEHYDROGENASE FROM *SCHIZOSACCHAROMYCES POMBE*

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SUMMARY

In *Schizosaccharomyces pombe* two isoenzymes of malate dehydrogenase (EC 1.1.1.37) were found which differ markedly in their response to glucose. One isoenzyme is synthesized only in glucose-repressed cells and disappears during respiratory derepression. The synthesis of the other form starts after glucose has been reduced by assimilation to a concentration of approx. 1.0 g/l. Fully derepressed cells contain exclusively this second isoenzymic form, which is rapidly inactivated after addition of glucose, probably by an enzymatic catalysed chemical modification. Inhibition of derepression by antibiotics indicates that this isoenzyme is synthesized by cytoplasmic and not mitochondrial ribosomes.

Both isoenzymes were purified 600-fold with about the same yield to electrophoretic homogeneity. 3 mg of pure enzyme were isolated from glucose-repressed as well as derepressed cells of this fission yeast. Thus the intracellular concentration of the enzymes is about the same in both physiological states. The glucose repressible isoenzyme is therefore 20-fold as active as the isoenzyme synthesized in presence of glucose.

Both isoenzymes possess a molecular weight of 60 000, are composed of two subunits identical in molecular weight and show the same sensitivity to inhibition by high concentrations of oxaloacetate, corresponding to the cytoplasmic forms of malate dehydrogenase from mammalian cells. The apparent Michaelis constants, the pH and temperature optima are similar for both forms. The isoenzymes are demonstrated to differ in their isoelectric points and their amino acid compositions.

INTRODUCTION

The existence of at least two molecular distinct forms of malate dehydrogenase in a variety of mammalian tissues is well established [1–4]. Subcellular distribution studies have indicated that one of the two main forms is localized in the mitochondria

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and the other in the cytoplasm [5, 6]. These two isoenzymes are separable by anion-exchange chromatography [1], differing in their electrophoretic mobility and demonstrating different substrate inhibition by high concentrations of oxaloacetate [4, 7]. Although several authors reported isoenzymes of malate dehydrogenase in yeasts the results are somewhat confusing: in one strain of *Saccharomyces cerevisiae* three isoenzymes were reported, one is localized in the mitochondria and the other two in the cytoplasm. One cytoplasmic form, which is associated with the glyoxylate cycle is repressed in presence of glucose, whereas the other and the mitochondrial form are synthesized constitutively [8, 9]. On the other hand, further detailed studies indicated that one of these isoenzymic forms was probably an artifact caused by unwanted proteolysis [10]. Based on their instability during purification, these isoenzymes have not been isolated in pure form and their physicochemical properties are not completely known. On the other hand Vary et al. [11] found only one form of malate dehydrogenase in *S. cerevisiae* and it has been shown that this single form is localized in the cytoplasmic as well as in the mitochondrial fraction. In *Neurospora crassa* one cytoplasmic form which is sensitive to repression by glucose and three mitochondrial subforms were found. The cytoplasmic isoenzyme seems to be quite instable and was not isolated in sufficient quantity and purity for physicochemical analyses [12]. Very little is known about isoenzymes of malate dehydrogenase in *Schizosaccharomyces pombe* and their sensitivities to the "glucose effect". This fission yeast oxidizes ethanol but, in opposition to other yeast strains, is unable to utilize this substrate as carbon source for growth [13]. It has previously been reported that *S. pombe* is a "petite-negative" yeast because no viable cytoplasmic respiratory-deficient mutants can be induced [14]. Nevertheless, respiration of this yeast is repressed by glucose [15]. Preliminary observations have shown that glucose represses the synthesis of some mitochondrial enzymes [16].

This study describes some results concerning the metabolic regulation, purification and the physicochemical properties of two isoenzymes of malate dehydrogenase from this fission yeast.

MATERIALS AND METHODS

Reagents

Special reagents used were oxaloacetic acid, L-malic acid, EDTA, 2-mercaptoethanol (Fluka AG, Buchs); NAD (Grade II), NADH (Grade II), cytochrome *c*, chymotrypsinogen, ovalbumin, catalase (Boehringer, Mannheim); Sephadex G-100 (Pharmacia, Fine Chemicals, Uppsala); bovine serum albumin (Fraction V), sodium dodecylsulfate, nitrobluetetrazolium, phenazine methosulfate, MTT-tetrazolium, DEAE-cellulose with a capacity of 0.84 mequiv/g, calcium phosphate gel (Sigma Chemical Co., St. Louis); acrylamide, *N,N'*-methylenebisacrylamide, *N,N,N',N'*-tetramethylethylenediamine, ammonium peroxodisulphate, amidoblack 10, coomassie brilliant blue R 250 (Serva, Heidelberg); ampholine pH 5–8 (LKB Produkter, Bromma).

All other chemicals were obtained from the usual commercial sources.

Organism

The strain (wild type) *S. pombe* 972h$^-$ (heterothallic minus) was obtained from

Dr U. Leupold, Laboratory of General Microbiology, University of Berne, Switzerland. The strain was maintained on yeast extract agar slants at 4 °C.

Production of cell material and growth measurements

Cells were transferred from the agar slants to 2-l Erlenmeyer flasks, each containing 250 ml of the synthetic medium described by Leupold [17] with 3% glucose as sole carbon source. After incubation on a shaker at 30 °C for 36 h, 400 ml of the cell suspension was transferred to a 10-l chemostat (Chemap AG, Männedorf), containing 7 l of the above medium. The culture conditions were the following: temperature 33 °C; pH 5.0 (kept constant during cultivation by automatic addition of 4 M NaOH); aeration rate 0.5 l/min per l of medium (mixing with flat-blade stirrer at 900 rev./min); 3 ml of Dow Corning Antifoam Y-30 Emulsion were added to the culture medium to prevent foaming. After 18 h growth, the cells were transferred to a 150-l fermenter containing 100 l of the above medium. The culture conditions were as described above.

For purification of malate dehydrogenase from glucose-repressed cells, the cultivation was stopped when the glucose concentration had been decreased to 2 g/l, the cell material was separated from the medium by centrifugation in a Cepa Rapid Centrifuge Model Z 81 (Carl Padberg, Lahr, Germany), washed three times with 0.1 M phosphate buffer (pH 7.5) and stored at −20 °C. Approx. 1.5 kg of cells (wet weight) were obtained per 100 l of culture medium.

For purification of the isoenzyme from derepressed cells, cells were collected 10 h after the exhaustion of the hexose, washed and stored as described above. Approx. 1.7 kg of cells were obtained per 100 l of culture medium.

Dry weight (X), glucose (S) and ethanol concentration (A) were determined as previously described [18].

Oxygen and carbon dioxide were determined continuously in the exit airstream by use of a gas analyser system (Hartmann and Braun, Frankfurt, Germany). Respiratory quotient was calculated according to Fiechter and von Meyenburg [19].

Enzyme assays

L-Malate:NAD oxidoreductase (EC 1.1.1.37, malate dehydrogenase) activity was routinely assayed using the reverse reaction. The reaction mixture contained 0.1 M potassium phosphate (pH 7.5), oxaloacetate $1.0 \cdot 10^{-3}$ M, NADH $3.75 \cdot 10^{-4}$ M, and enzyme yielding a final volume of 3.0 ml. Oxaloacetate was dissolved in the assay buffer at 4 °C and stored in the dark at the same temperature. NADH was dissolved in distilled water and also stored at 4 °C. Fresh solutions of oxaloacetate and NADH were prepared daily. Enzyme solutions were routinely diluted in the assay buffer. The reaction was started by the addition of enzyme after the other components had been equilibrated at 30 °C. Continuous recording of the absorbance at 366 nm was made with a Beckman model Acta III recording spectrophotometer. One unit of enzyme activity is equivalent to the formation of 1 μmole NAD^+ per min at 30 °C ($\varepsilon_{NADH} = 3.3 \cdot 10^6$ cm²/mole). Specific activity is defined as units/min per mg protein.

Isocitrate lyase (EC 4.1.3.1) and malate synthase (EC 4. 1.3.2) were determined according to the method of Dixon and Kornberg [20].

Protein determination

Protein was routinely assayed by the method of Lowry et al. [21]. Bovine serum albumin was used as a reference standard.

Electrophoresis

Electrophoresis was carried out according to Ornstein and Davies [22, 23], using 5% polyacrylamide gels in Tris–glycine buffer (pH 8.9). Experiments were performed at 4 °C with a current of 3 mA/gel until the marker dye was about 5 mm from the end of the gel. After electrophoresis gels were stained with coomassie brilliant blue and destained in 7.5% acetic acid. Enzyme activity was stained using the tetrazolium test described by Laycock et al. [24]. Electrophoresis of polyacrylamide gels containing 0.1% sodium dodecylsulfate was performed according to Weber and Osborn [25]. Prior to electrophoresis, the proteins were denatured for 4 min at 95 °C and dissociated at 37 °C for 2.5 h in 1% sodium dodecylsulfate containing 1% 2-mercaptoethanol. Bovine serum albumin, catalase, ovalbumin, and chymotrypsinogen A were used as reference proteins.

Molecular weight estimations

Molecular weight estimations were carried out according to the method of Andrews [26], using a 2.6 cm × 55 cm column of Sephadex G-100 previously equilibrated with 50 mM Tris–HCl (pH 7.5) containing 100 mM KCl and 1 mM EDTA. The column was calibrated with bovine serum albumin, ovalbumin, chymotrypsinogen and cytochrome *c*.

Isoelectric focusing

Isoelectric focusing of malate dehydrogenase (both the crude and purified enzyme) was carried out in a LKB 110-ml column in 1% ampholite (pH 5–8) [27–29]. The anode (at the bottom of the column) consisted of 1.5% H_2SO_4, the cathode of 2% ethylenediamine. A sucrose gradient (0–45%) was used for stabilizing the liquid system against convection. Electrophoresis was carried out for 40 h at 500 V, 2.5 ml fractions then being collected and tested for activity and for pH.

Amino acid analyses

10 nM of dry enzyme were hydrolysed in 6 M HCl for 24 h at 110 °C and analysed in a Beckman Spinco Analyser model 121 [30]. The values of threonine and serine were corrected to zero time of hydrolysis.

RESULTS AND DISCUSSION

Malate dehydrogenase pattern during growth of S. pombe on glucose in batch culture

The growth of *S. pombe* in synthetic glucose-limited medium under aerobic conditions is characterized by a single growth phase (Fig. 1A). Glucose is metabolized to biomass, ethanol and carbon dioxide (respiratory quotient = 10–15). The maximal specific growth rate (μ_{max}) under these conditions is 0.39 (corresponding to a generation time of 110 min), the yield (Y_G) being 0.16. After the exhaustion of glucose the respiratory quotient decreased to a value of 0.7. The cellular metabolism is thus directed from aerobic glycolysis to respiration (respiratory derepression). In contrast to *S. cerevisiae* [31] no growth on ethanol occurs during this respiratory phase. However the ethanol is catabolized to carbon dioxide. The carbon balance was determined 100% under both physiological regimes. Enzymatic studies indicated the absence of the glyoxylate cycle in *S. pombe*: isocitrate lyase and malate synthase

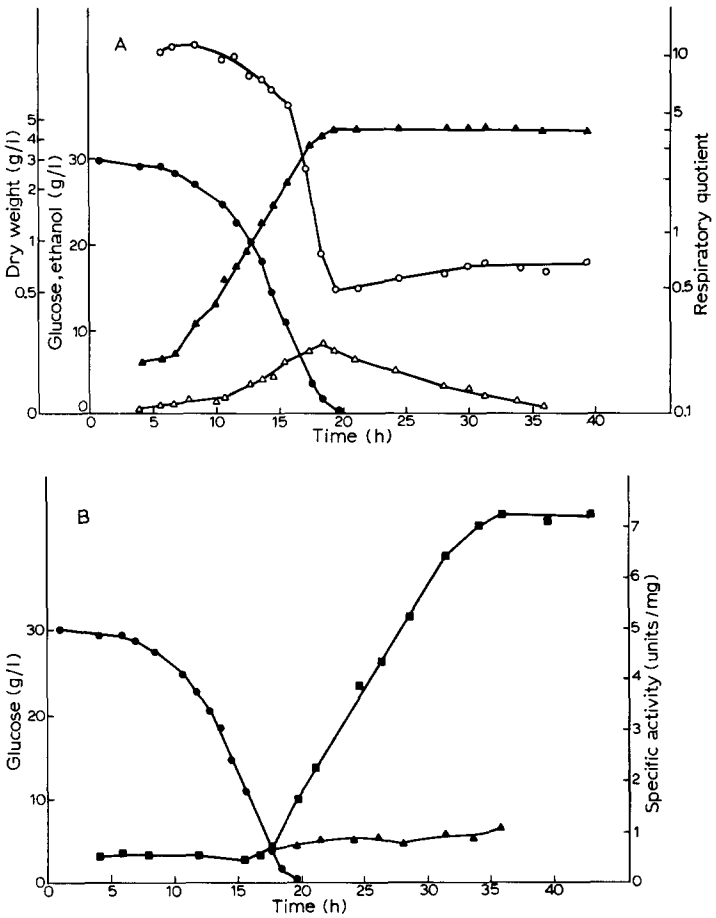

Fig. 1. Malate dehydrogenase pattern during batch growth of *S. pombe*. (A) Time course of growth. Dry weight (▲—▲); glucose concentration (●—●); ethanol concentration (△—△); respiratory quotient (○—○). (B) Derepression of malate dehydrogenase as a function of time; specific activity expressed as μmoles of NADH oxidized per mg of protein per min at 30 °C. ■—■, specific activity without inhibitor; ▲—▲, specific activity in presence of 20 μg/ml cycloheximide, inhibitor added at 17 h. Samples of 20 ml were collected at timed intervals and centrifuged at 3500 × *g*. Glucose and ethanol concentrations were determined in the supernatants. One part of the sediment was used for dry weight measurements. The remaining cell material was homogenized with glass beads for 10 min, centrifuged at 3500 × *g* and 45 000 × *g*; the supernatants were assayed for enzyme and protein as described in Materials and Methods.

activity are not detectable in crude extracts from derepressed cells. On the other hand, the ability to grow on ethanol or acetate as sole carbon sources is linked to the anaplerotic function of the glyoxylate cycle [32, 33]. Therefore respiratory derepression occurred in *S. pombe* in the absence of cellular division.

Fig. 1B illustrates the time course of derepression of malate dehydrogenase after the exhaustion of glucose. The specific activity increases by a factor of 20 within a period of 10 h. Critical glucose concentration for derepression is 1.0 g/l. Under repression specific activity remains constant in a wide span for repressor concentration

(1.0 g/l up to 50 g/l). No increase occurs in a medium containing glucose plus ethanol or glucose plus acetate, indicating that glucose (or metabolites thereof) is a repressor rather than ethanol or acetate being inducers. Of some interest is the fact that this derepression of malate dehydrogenase occurs in non-dividing cells. Nevertheless, the kinetics of derepression is about the same as in *S. cerevisiae* [31]. The increase of activity is prevented by cycloheximide (20 μg/ml, a concentration which decreases the specific growth rate by a factor of 70% (Fig. 1B)). Cycloheximide is known as an inhibitor of the synthesis of proteins on cytoplasmic ribosomes [34, 35] in yeast. Chloramphenicol (a specific inhibitor of synthesis of proteins on mitochondrial ribosomes [36, 37] doesn't affect the increase of activity. These observations suggest that protein synthesis on cytoplasmic ribosomes interferes with the increase of malate dehydrogenase activity. Similar results were obtained with *S. cerevisiae* [38] and *N. crassa* [12].

Electrophoresis

Extracts from cells grown on glucose as the sole carbon source (glucose-repressed cells) and from cells incubated 10 h in ethanol- or acetate-containing medium (derepressed cells) were subjected to polyacrylamide gel electrophoresis. Only one band of malate dehydrogenase activity was found in extracts from both types of cells (Figs 2A, 2B). However electrophoresis of a mixture of both extracts disclosed

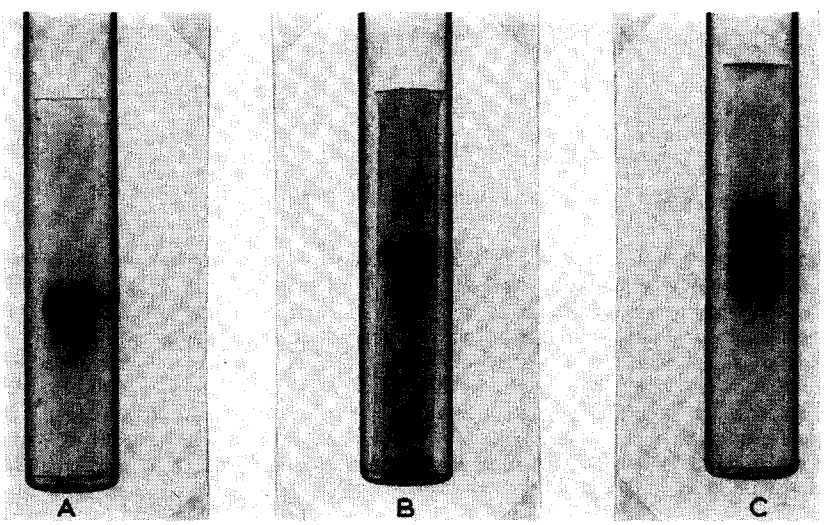

Fig. 2. Polyacrylamide gel electrophoresis. (A) Extract from cells grown on glucose (= glucose-repressed cells). (B) Extract from cells incubated for 10 h in an ethanol-containing medium (= derepressed cells). (C) Mixture of the two extracts from repressed and derepressed cells. Electrophoresis was carried out as described in Materials and Methods. For activity staining see Laycock et al. [24].

two bands of activity, indicating that the two enzymes differ markedly in their charges (Fig. 2C). On the other hand Atzpodien et al. [9] reported three isoenzymes in derepressed cells of *S. cerevisiae*. Benveniste and Munkres [12] found four isoenzymes of malate dehydrogenase in *N. crassa* incubated in acetate medium. Both isoenzymes

from the fission yeast differ markedly in their response to glucose. One isoenzyme is synthesized only in its presence. Approx. 4 h after the exhaustion of the hexose this isoenzymic form disappears from the cells [39]. This result indicates that the synthesis of this isoenzyme stops after the glucose concentration has been dropped to zero and growth has been terminated. Furthermore the enzyme is very sensitive to catabolism (protein turnover) or is inactivated by a mechanism not known at present. Some obervations on the kinectics of inactivation of this isoenzyme and its biological significance are discussed elsewhere [39]. In accordance to these findings the activities of some other enzymes e.g. aldolase and phosphofructokinase decrease likewise after the cessation of growth (Flury, U., unpublished results).

In opposition to our results several authors [9, 12] reported that in other yeast strains the mitochondrial form of malate dehydrogenase (the isoenzyme which is not repressed by glucose) is synthesized constitutively and is therefore existing also in derepressed cells. At present it is not clear if the disappearance of this isoenzyme during respiratory derepression in *S. pombe* has a special metabolic function or is only a consequence of the inability of this yeast strain to grow on ethanol or acetate. The synthesis of the other isoenzymic form is repressed at glucose concentrations higher than 1 g/l. It is not possible at present to decide if glucose itself or metabolites thereof repress the synthesis of this isoenzyme.

Substrate inhibition by high concentrations of oxaloacetate

The enzyme from glucose-repressed as well as from derepressed cells show the same sensitivity to inhibition by high concentrations of oxaloacetate. The extent of this substrate inhibition corresponds to that found for the cytoplasmic isoenzyme from different mammalian [4] sources and yeast [8, 40]. In contrast malate dehydrogenase from glucose-repressed cells (mitochondrial form) of various yeasts is relatively more sensitive than the isoenzyme from derepressed cells (cytoplasmic form) to inhibition by high concentrations of oxaloacetate [8, 40].

Inactivation of malate dehydrogenase from derepressed cells by glucose

If glucose at a final concentration of 56 mM was added to cells incubated 6 h in an ethanol or acetate medium, the rise of malate dehydrogenase activity was stopped within about 5 min. By about 10 min after glucose addition a decline of specific as well as total activity (activity per ml medium) could be detected. The activity decreased to approx. 20% of the original value within 1 h (Fig. 3). This decline was more rapid than the increase after the disappearance of glucose. Growth on glucose starts after a lag period of 30 min after the addition of glucose, the generation time being 110 min. These results indicate that the enzyme is inactivated after the repression of its synthesis by a mechanism induced by glucose (or metabolites thereof). When all glucose has been metabolized, the activity increases again at a normal rate. The inhibitory effect of glucose is therefore transient. This "inactivation repression" has also been described by other authors [9, 40, 41].

In the presence of cycloheximide (20 μg/ml) no inactivation occurs; on the other hand the enzyme is inactivated if protein synthesis is blocked by cycloheximide 15 min after addition of glucose. These data indicate that protein synthesis on cytoplasmic ribosomes interferes with the inactivation of the enzyme. We conclude from this that the inactivation is caused by an enzyme catalysed alteration rather than by a rapid

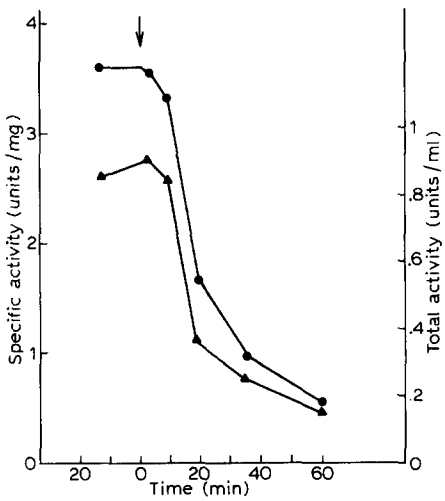

Fig. 3. Inactivation of malate dehydrogenase by glucose. Yeast cells (2.5 g wet weight per l) incubated 8 h in ethanol medium. Glucose added to 56 mM as indicated by the arrow. Cells were collected at timed intervals, washed and disrupted. Extracts were assayed for enzyme and protein as described in Materials and Methods. ●—●, specific activity; ▲—▲, total activity (per ml medium).

catabolism of the enzyme (protein turnover). We were not successful in demonstrating this inactivation in vitro. No significant decrease of activity could be detected, if a mixture of extracts from derepressed cells and from cells containing this "inactivating agent" was incubated for 2 h at 30 °C. The inactivated enzyme exhibits the same sensitivity to inhibition by high concentrations of oxaloacetate as the malate dehydrogenase from glucose-repressed cells.

Purification of the isoenzymes

All of the following steps were carried out at 4 °C, unless otherwise indicated. The values for percentage saturation with $(NH_4)_2SO_4$ are for 25 °C.

Step 1: Preparation of crude extracts. 1.0 kg of glucose-repressed or 1.5 kg of derepressed cells were suspended in 1.500 ml of 70 mM phosphate buffer (pH 7.5) containing 1 mM EDTA and 200-ml portions vibrated together with 200 g of glass beads (diameter 0.45–0.50 mm) for 10 min (Vibro mixer E 1, Chemap AG, Männedorf, Switzerland). After disruption, the resulting slurry was centrifuged at 3500 × *g* for 10 min to sediment cell debris. The supernatant was then centrifuged at 45 000 × *g* for 30 min. The two sediments (3500 × *g* and 45 000 × *g*) were disrupted once more for 5 min with 200 g of glass beads in 70 mM phosphate buffer (pH 7.5) containing 1 mM EDTA and 0.1 % Triton X-100, and centrifuged at 45 000 × *g*. The two supernatants comprised the crude extract.

Step 2: $(NH_4)_2SO_4$ precipitation. The supernatant was brought to 35 % saturation by the slow addition of 209 g of solid $(NH_4)_2SO_4$ per l of extract and stirred in the cold for 1 h. The 35 % saturated solution was centrifuged at 45 000 × *g* for 30 min and the pellets discarded. The supernatant was adjusted to 75 % saturation by the addition of 278 g of solid $(NH_4)_2SO_4$ per l. After 1 h of stirring in the cold, the turbid solution was centrifuged at 45 000 × *g* for 30 min. The precipitate was suspended in

70 mM phosphate buffer (pH 7.5) containing 1 mM EDTA, 5 mM L-malate and 20% (w/v) $(NH_4)_2SO_4$ and dialysed for 4 h against the same buffer.

Step 3: Ethanol fractionation. The dialysed extract was chilled to 0 °C in a dry ice–acetone bath. Absolute ethanol (−20 °C) was slowly added to the stirred enzyme solution. The temperature was gradually lowered to −10 °C until 25% ethanol was added. The solution was stirred at this temperature for 30 min and then centrifuged (20 000 × *g* for 10 min at −10 °C). The precipitate was discarded. The supernatant was made up to 60% ethanol, while the temperature of the solution was lowered to −20 °C. After 30 min the precipitate (obtained by centrifugation (10 min at 20 000 × *g*)), was taken up in 10 mM phosphate buffer (pH 7.5) containing 1 mM EDTA and dialysed overnight against the same buffer. The precipitate was removed by centrifugation and discarded.

Step 4: Calcium phosphate adsorption and elution. Calcium phosphate gel (5% in 10 mM phosphate buffer (pH 7.5)) was slowly added to the dialysed enzyme solution from Step 3 (protein concentration 3.0 mg/ml); a treatment of one part of calcium phosphate per one part of supernatant solution was found to be optimal. The suspension was stirred in the cold for 30 min and then centrifuged at 3500 × *g* for 5 min. The supernatant contained no malate dehydrogenase activity and was discarded. The gel was dissolved in 30 mM phosphate buffer (pH 7.5), centrifuged and the supernatant discarded. The enzyme was eluted from the gel with 140 mM phosphate buffer (pH 7.5) containing 1 mM EDTA. After concentration in a Amicon UF cell equipped with a PM-10 membrane at a pressure of 4 atm with nitrogen the enzyme solution was dialysed for 4 h against 10 mM phosphate buffer (pH 7.8) containing 1 mM EDTA.

Step 5: DEAE-cellulose chromatography. (a) Chromatography of the enzyme from derepressed cells. DEAE-cellulose (0.84 mequiv/g) was treated with NaOH and HCl as described by Peterson and Sober [42], washed with distilled water, equilibrated with 10 mM phosphate buffer (pH 7.8), 1 mM EDTA and freed of fine particles by flotation. Columns (2.6 cm × 30 cm) were packed and washed with an excess of buffer. The enzyme from Step 4 was applied to the column and washed with starting buffer until the eluant exhibited negligible absorbance at 280 nm. The flow rate was 30 ml/h, fractions of 6 ml being collected. The enzyme was eluted from the column using a linear gradient consisting of 500 ml of starting buffer and 500 ml of 0.3 M NaCl. Only one symmetrical peak of enzyme activity could be eluted (Fig. 4B). The most active fractions were pooled, concentrated as described above and dialysed against 50 mM Tris–HCl (pH 7.5), 100 mM KCl, 1 mM EDTA. (b) Chromatography of the enzyme from glucose-repressed cells. The enzyme of repressed cells was very weakly adsorbed to the DEAE-cellulose at pH 7.8; chromatography was therefore carried out at pH 8.0. All other conditions were the same as described above. The enzyme was eluted from the column in one single peak of activity using a gradient (Fig. 4A). The most active fractions were pooled, concentrated and dialysed as described above.

Step 6: Sephadex G-100 gel filtration. The enzyme solutions from Step 5 were fractionated in a column (2.6 cm × 50 cm) of Sephadex G-100 equilibrated with 50 mM Tris–HCl (pH 7.5) containing 100 mM KCl plus 1 mM EDTA. Flow rate was 25 ml/h, fractions of 4 ml were collected. The most active fractions were pooled, concentrated as described above and stored at −20 °C. Both enzymes could be stored at this temperature for 3 months without loss of activity.

Tables I and II summarize the purification procedures. If enzyme solutions

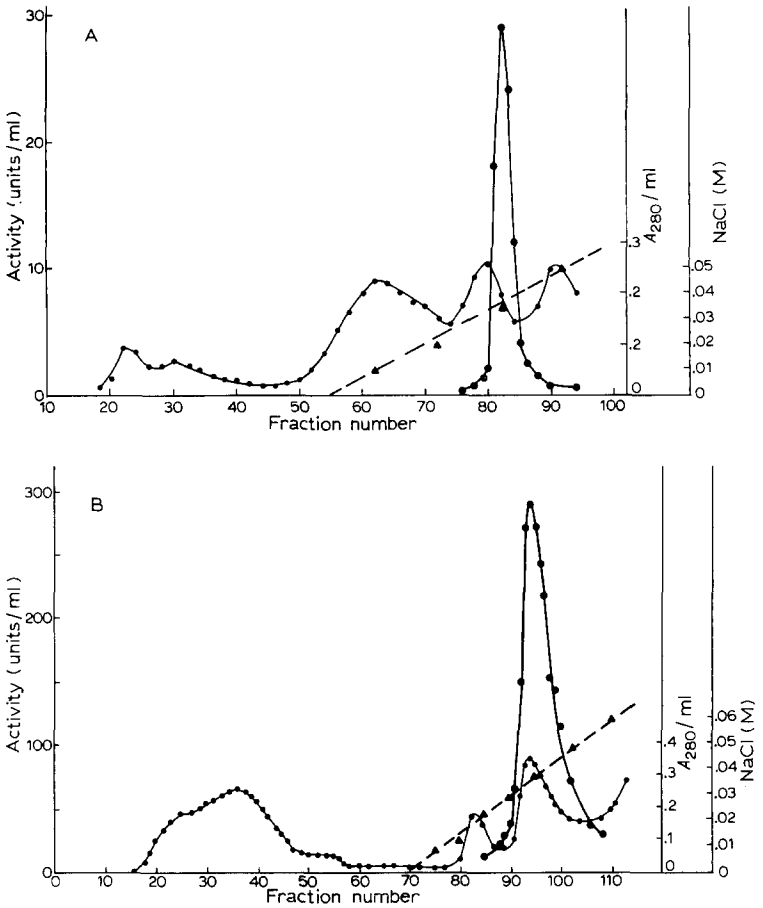

Fig. 4. Chromatography of malate dehydrogenases on DEAE-cellulose. The column (2.6 cm × 30 cm) was previously equilibrated with 1 mM EDTA in 10 mM phosphate buffer pH 8.0 (A) and pH 7.8 (B). Purification Step 5. Flow rate: 30 ml/h, fractions of 6 ml being collected and assayed for activity (●—●); protein (•—•); and NaCl concentration (▲—▲). A, isoenzyme from glucose-repressed cells; B, isoenzyme from derepressed cells.

from Step 2 ($(NH_4)_2SO_4$ precipitation) were directly applied to the DEAE-cellulose, a loss of activity of about 80% was observed during chromatography. Therefore a modification (e.g. by a proteolytic attack) of the enzymes leading to this loss of activity during chromatography cannot be ruled out; in fact Pringle [10] was able to demonstrate that one of the two "isoenzymes" of malate dehydrogenase from *S. cerevisiae* which could be separated by DEAE-cellulose chromatography is a purification artifact caused by proteolytic degradation. It is possible that also a proteolytic activity is present in crude extracts from *S. pombe* which leads to this marked instability of both isoenzymes during DEAE-cellulose chromatography. On the other hand using our new purification procedure both enzymes behaved very stably on DEAE-cellulose: approx. 90–100% of the activity could be recovered after chromatography. It is possible that this postulated proteolytic activity is removed from the extracts in the ethanol fractionation and calcium phosphate adsorption steps.

TABLE I

PURIFICATION OF MALATE DEHYDROGENASE FROM GLUCOSE-REPRESSED CELLS OF *S. POMBE*

Cells were harvested when glucose concentration has been reduced to 2 g/l. The purification outlined is from 1 kg wet weight of cells.

Step	Total units*	Total protein (mg)	Specific activity (units/mg)	Purification factor	Recovery (%)
1. Crude extract	3100	8700	0.36	1	100
2. $(NH_4)_2SO_4$ (35–75%)	2635	3875	0.68	1.9	85
3. Ethanol (25–60%)	2325	1785	1.3	3.6	75
4. Calcium phosphate	1800	620	2.9	8.0	58
5. DEAE-cellulose	1270	15	84.7	235	41
6. Sephadex G-100	619	3.0	206	573	20

*μmoles NADH oxidized per min at 30 °C.

However chromatography of a mixture of both enzymes on DEAE-cellulose disclosed two distinct peaks of activity indicating that both isoenzymes differ markedly in their net charges.

Homogeneity of the purified isoenzymes

Electrophoretic purity of both isoenzymes is evidenced by Fig. 5. The band obtained by staining of malate dehydrogenase activity corresponds perfectly with the protein band. Only one protein band was found likewise after sodium dodecylsulfate electrophoresis.

Intracellular concentrations of malate dehydrogenase

It is shown in Tables I and II that both isoenzymes are purified 600-fold with about the same yield. During purification the specific activity of malate dehydrogenase from repressed cells increases from 0.36 to 200, that of malate dehydrogenase from

TABLE II

PURIFICATION OF MALATE DEHYDROGENASE FROM DEREPRESSED CELLS OF *S. POMBE*

Cells were incubated 10 h in an ethanol-containing medium. The purification outlined is from 1.5 kg wet weight of cells.

Step	Total units* ($\times 10^{-3}$)	Total protein (mg)	Specific activity (units/mg)	Purification factor	Recovery (%)
1. Crude extract	83.5	10 600	7.8	1	100
2. $(NH_4)_2SO_4$ (35–75%)	62.6	5 350	11.7	1.5	75
3. Ethanol (25–60%)	52.1	2 430	21.4	2.7	62
4. Calcium phosphate	43.3	390	111	14.2	52
5. DEAE-cellulose	33.4	59	566	72.5	40
6. Sephadex G-100	22.5	4.7	4800	615	27

* μmoles NADH oxidized per min at 30 °C.

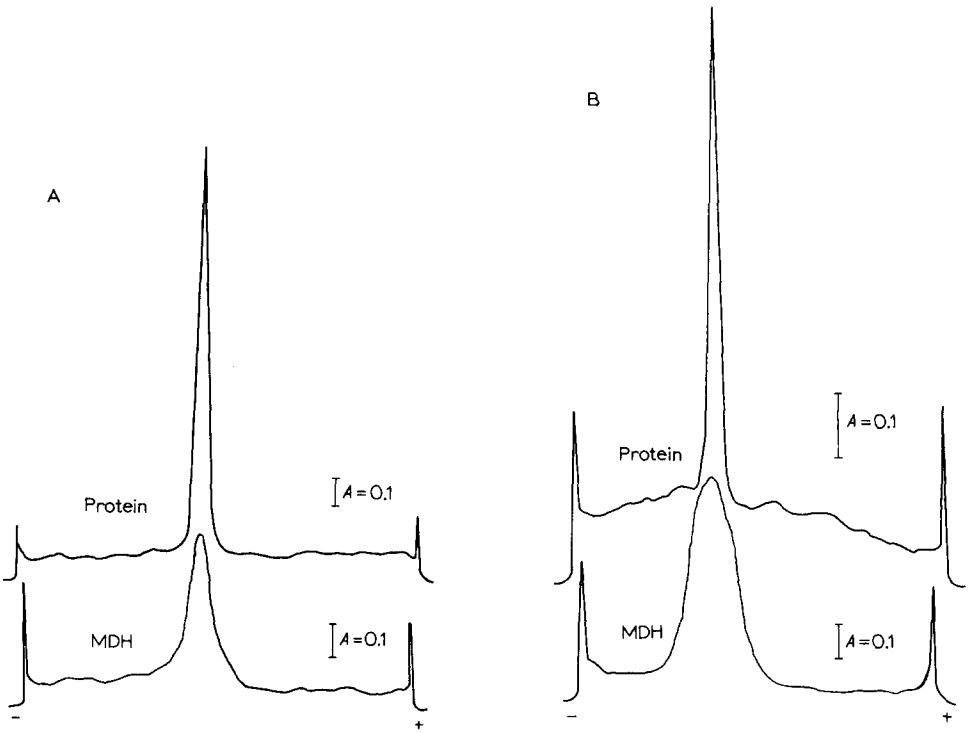

Fig. 5. Polyacrylamide gel electrophoresis of the purified isoenzymes. Electrophoresis was carried out as described in Materials and Methods. Protein was stained with coomassie brilliant blue. Activity staining was carried out according to Laycock et al. [24]. Absorbance was recorded at 576 nm (protein) and 640 nm (activity band) in a gel scanner on an Acta 111 spectrophotometer. A, isoenzyme from glucose-repressed cells; B, isoenzyme from derepressed cells. MDH, malate dehydrogenase.

derepressed cells from 7.8 to 4800. The differences in specific activity between the two isoenzymes remained constant during the purification steps. In both cases, 3 mg of pure enzyme were extracted from 1 kg of cells (wet weight) indicating similar intracellular concentrations of malate dehydrogenase present under repression and derepression, respectively (0.1–0.2 % of the extractable protein). Therefore the isoenzyme from derepressed cells is about 20-fold as active as the isoenzyme from repressed cells.

Properties of the isoenzymes

Molecular weights. A molecular weight of about 60 000 $\pm$ 4000 was estimated for both isoenzymes by gel filtration on Sephadex G-100 according to the method of Andrews [26] (Fig. 6). This value corresponds to those found for most other malate dehydrogenases from mammals [43, 44], and yeast [10]. Passing crude extracts through the G-100 column, enzyme activity was located at V_e/V_0 of 1.62 corresponding to a molecular weight of 60 000. This suggested that our purification procedure excludes any splitting yielding enzyme species of lower molecular weights.

Subunits. The molecular weight of the polypeptide chain of both isoenzymes was estimated by sodium dodecylsufate electrophoresis according to Weber and Osborn [25]. Prior to electrophoresis, the proteins were denatured by heating (4 min

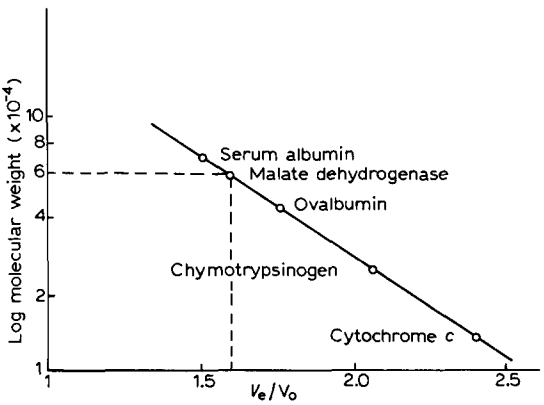

Fig. 6. Molecular weight estimation of malate dehydrogenase by gel filtration on a calibrated Sephadex G-100 column. Conditions were as described for Step 6 of purification procedure. The data were plotted according to Andrews [26]. Bovine serum albumin, ovalbumin, chymotrypsinogen and cytochrome *c* were used as reference proteins.

at 95 °C) and then dissociated for 2.5 h at 37 °C. The molecular weight of the polypeptide chain of both enzymes was determined to be 32 000 (Fig. 7). These results indicate that the enzymes are composed of two subunits identical with regard to molecular weight. Other investigators described in agreement with out findings a dimeric structure for the isoenzymes of malate dehydrogenase from some vertebrates [43, 45]. Munkres [46], however, found a tetrameric structure for the *Neurospora* enzyme.

Isoelectric points. Extracts of glucose-repressed cells contain one active malate

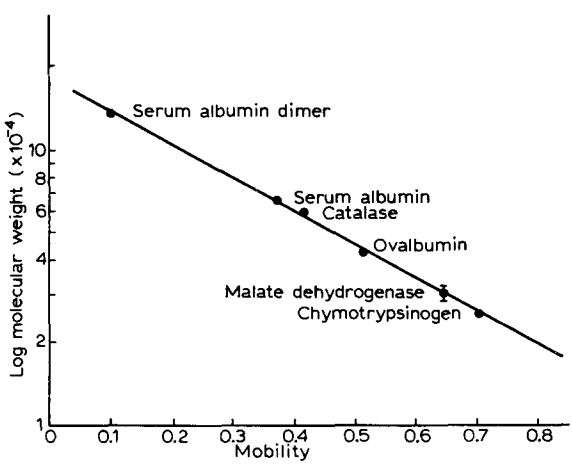

Fig. 7. Molecular weight determination of the polypeptide chain of malate dehydrogenase isoenzymes by sodium dodecylsulfate electrophoresis. The proteins were preincubated for 4 min at 95 °C and dissociated for 2.5 h at 37 °C in 1% sodium dodecylsulfate containing 1% 2-mercaptoethanol. Electrophoresis was carried out in 7% polyacrylamide gels in the presence of 0.1% sodium dodecylsulfate and 0.1% 2-mercaptoethanol. Gels were stained for proteins with coomassie brilliant blue. The mobility was calculated according to Weber and Osborn [25]. Each point represents an average of four separate experiments.

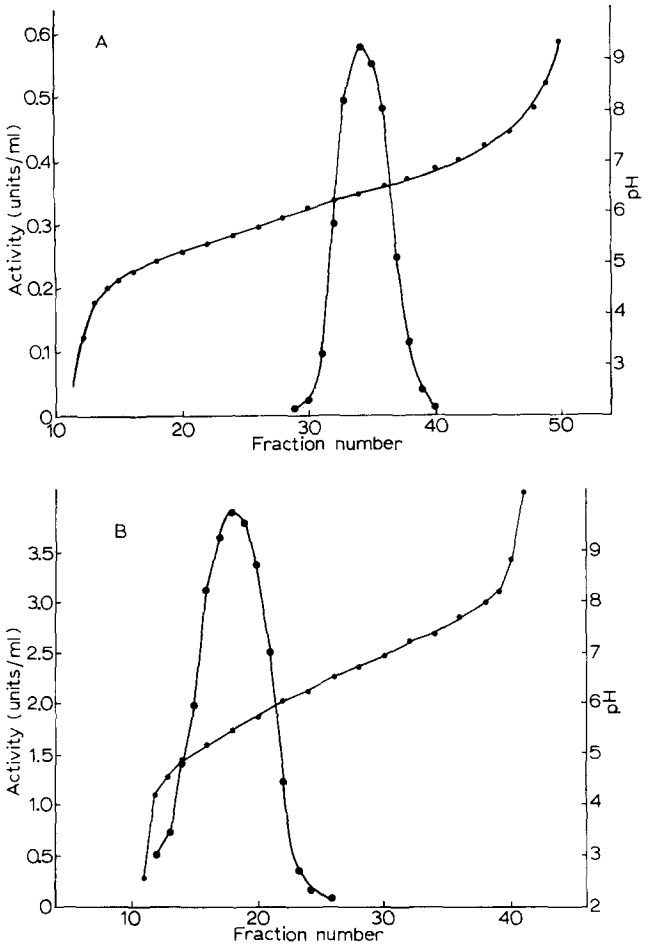

Fig. 8. Determination of isoelectric points of malate dehydrogenase ($(NH_4)_2SO_4$-fractionated extracts) in a LKB 110 ml column. Electrophoresis was carried out in 1% ampholite (pH 5–8) for 40 h at 500 V. Anode: 1.5% H_2SO_4 at column bottom, cathode: 2% ethylenediamine. Fractions of 2.5 ml were collected after electrophoresis and assayed for activity (●—●) and pH (•—•). A, isoenzyme from glucose-repressed cells; B, isoenzyme from derepressed cells.

dehydrogenase with an isoelectric point (pI) of 6.4 (Fig. 8A). On the other hand the isoenzyme present in derepressed cells is a more acidic protein with a pI of 5.5 (Fig. 8B). The difference between the pI of both isoenzymes is thus 0.9 units, which finding is in agreement with the fact that the malate dehydrogenase from derepressed cells moved faster to the anode in polyacrylamide gel electrophoresis. Both isoenzymes behaved very stably during the focusing experiment: approx. 95–100% of the activity could be recovered in the fractions, indicating that no further isoenzymic forms are present in this fission yeast which had not been detected by this method. The same isoelectric points of 6.4 and 5.5, respectively, were determined for the purified isoenzymes. Therefore it seems very unlikely that a significant proteolytic attack occurred during the purification procedures leading to changes in the charges of the isoenzymes, as has been described by Pringle [10] for the malate dehydrogenase from *S. cerevisiae*.

TABLE III

AMINO ACID COMPOSITION OF MALATE DEHYDROGENASES FROM VARIOUS ORGANISMS

m and s refer to the mitochondrial and soluble forms of the enzyme from the source indicated. The numbers refer to grams of amino acid per 100 g of total amino acids recovered from hydrolyzate. ND, not determined.

Amino acid	*S. pombe**		*Neurospora*** m-malate dehydrogenase	Bovine heart	
	Repressed	Derepressed		m-malate dehydrogenase***	s-malate dehydrogenase†
Lys	9.73	8.53	9.50	7.81	12.87
His	1.98	1.59	2.07	1.84	1.51
Arg	4.16	4.27	4.28	3.45	4.08
Asp	10.48	9.91	10.30	9.02	11.65
Thr	6.04	5.97	5.80	6.15	3.71
Ser	5.05	5.44	5.62	4.15	4.10
Glu	12.85	11.46	9.20	11.07	10.33
Pro	4.37	4.84	5.35	6.85	3.16
Gly	6.64	8.02	6.02	5.43	4.09
Ala	7.30	8.63	7.15	7.02	6.43
Cys ($\frac{1}{2}$)	ND	ND	1.09	2.87	1.42
Val	8.25	10.11	7.60	7.86	6.82
Met	0.85	0.42	1.01	2.08	2.91
Ile	5.80	5.81	4.70	6.50	6.40
Leu	8.83	10.45	8.50	9.09	10.23
Tyr	2.45	0	3.24	2.87	2.84
Phe	5.22	4.55	4.42	6.20	4.20
Trp	ND	ND	0.40	0.40	3.18

* Mean of analyses of 24-h hydrolyzates of three preparations.
** Munkres and Richards [47].
*** Siegel and Englard [48].
† Thorne [2] and Pfleiderer et al. [49].

Amino acid composition. A summary of the amino acid compositions of both isoenzymes of malate dehydrogenase is presented in Table III. The amino acid composition of both isoenzymes differ slightly from each other in seven amino acids, namely lysine, glutamic acid (glutamine), glycine, alanine, valine, leucine and tyrosine. No tyrosine has been found in the isoenzyme from derepressed cells. The differences are not so marked as between the mitochondrial and cytoplasmic forms of bovine and porcine malate dehydrogenase [2, 3, 7, 50]. In fact the results obtained by amino acid analyses give only an indication but do not unequivocally prove that the two malate dehydrogenases isolated have different primary structures. It is possible that the absence of tyrosine in the enzyme from derepressed cells may be caused by a modification of the residue of this amino acid in vivo (during respiratory derepression). Such a modification may lead to an activated form of the enzyme. However some experimental data make such an in vivo activation (e.g. by an enzyme-catalysed chemical modification) very unlikely. First, the malate dehydrogenase present in glucose-repressed cells disappears rapidly after the glucose concentration in the medium has been dropped to zero [39]. On the other hand the second enzymatic form appears in the cells at a glucose concentration of about 1 g/l; its activity increases very

slowly and reaches its highest level approximately 10 h after the exhaustion of glucose. Thus it is very unlikely that this second enzymatic form arises from the enzyme present in glucose-repressed cells by an enzyme-catalysed chemical modification of the tyrosine residue (or another amino acid). Second, the increase in specific activity during respiratory derepression is a very slow process. This fact may rule out a simple modification. Third, the marked charge differences between the two enzyme forms may disfavour such a modification.

Although the results presented give some indications that the primary structures of both malate dehydrogenases are different, further detailed experiments are necessary to prove this matter.

Both isoenzymes from *S. pombe* are significantly different in amino acid composition from either the soluble and mitochondrial malate dehydrogenase from mammalian sources, but show a greater similarity (although they are not identical) with the mitochondrial form from *N. crassa*. Unfortunately the amino acid composition of the cytoplasmic isoenzyme from *N. crassa* and the three isoenzymes from *S. cerevisiae* are not yet known. If the malate dehydrogenase from *S. pombe* and mammals arose from a common evolutionary ancestor, it is apparent that they have undergone extensive divergence in amino acid composition during evolution.

pH optima of the reaction rates. The optimal pH for the oxaloacetate reduction was 7.5 for both isoenzymes in 0.1 M potassium phosphate. Munkres and Richards [51] have described two pH optima (6.75 and 7.65) for the mitochondrial isoenzyme from *N. crassa* under identical conditions. For the forward reaction (oxidation of malate) a pH optimum of 9.5 was found in 0.1 M glycine–NaOH buffer at 30 °C.

Influence of temperature. The temperature optimum for the oxaloacetate reduction at pH 7.5 in 0.1 M potassium phosphate buffer was 40–42 °C. The energy of activation in the temperature range of 18–35 °C was estimated to be 10 kcal/mole for the isoenzyme from glucose-repressed cells and 8 kcal/mole for the isoenzyme from derepressed cells. The Arrhenius plot was linear in this temperature range. Munkres and Richards [51] described for the mitochondrial isoenzyme from *N. crassa* a temperature optimum of about 40 °C at pH 6.7 and an activation energy of 6 kcal/mole.

Dependence of the reaction rate on the concentrations of substrates. Apparent Michaelis constants and maximum reaction velocities of both isoenzymes were determined for oxaloacetate, NADH, L-malate and NAD^+ and are shown in Table IV. Both isoenzymes exhibit about the same apparent Michaelis constants, but the values for maximum reaction velocities (expressed as μmoles substrates oxidized or reduced per min per mg of pure enzyme) are significantly higher for the isoenzyme from derepressed cells. Higher concentrations of oxaloacetate inhibit the activity of both isoenzymes. The extent of this substrate inhibition was the same for both isoenzymes comparable to that described for the cytoplasmic forms from animals [4] and yeast [8, 9, 12, 40] and did not change during purification. In the case of our isoenzymes, therefore, it is not possible to differentiate between a mitochondrial and a cytoplasmic form, as reported by other authors [4, 40].

Intracellular localisation of the isoenzymes. The intracellular localisation of the two isoenzymes are not completely known at present; this problem is under further investigation. It can be postulated that the isoenzyme present in glucose-repressed cells is likely to be implied in the citric acid cycle and therefore localized in the mitochondria. Indeed, as has been shown by other authors, the isoenzyme which is not

TABLE IV

APPARENT MICHAELIS CONSTANTS AND MAXIMAL REACTION VELOCITIES OF MALATE DEHYDROGENASE ISOENZYMES

Reaction velocities are expressed as μmoles NAD^+ reduced (NADH oxidized) per min per mg of pure enzyme. OAA, oxaloacetate. Conditions of determinations: (1) Malate oxidation: 0.1 M glycine –NaOH, pH 9.5; $1.27 \cdot 10^{-3}$ M NAD^+; $3.3 \cdot 10^{-2}$ M malate. (2) Oxaloacetate reduction: 0.1 M potassium phosphate, pH 7.5; $3.75 \cdot 10^{-4}$ M NADH; $1.0 \cdot 10^{-3}$ M oxaloacetate.

	Isoenzyme from repressed cells	Isoenzyme from derepressed cells
Malate oxidation		
K_{malate}	2.74 mM	2.5 mM
K_{NAD+}	0.56 mM	0.26 mM
V_{malate}	117	606
V_{NAD+}	156	626
Oxaloacetate reduction		
K_{OAA}	0.3 mM	0.16 mM
K_{NADH}	0.8 mM	0.8 mM
V_{OAA}	848	6250
V_{NADH}	634	8000

repressed by glucose is localized in the mitochondria [9, 12]. Preliminary experiments have shown that in derepressed cells of *S. pombe* about 80–90% of malate dehydrogenase activity is localized in the cytoplasm (Zimmerli, unpublished results). It may be possible that this cytoplasmic activity is an evolutionary relic of the glyoxylate cycle (which is not functioning in this fission yeast). In fact, Atzpodien et al. [9] and Benveniste and Munkres [12] have reported that in *S. cerevisiae* and *N. crassa* the glucose-repressible isoenzyme is localized in the cytoplasm and it is suggested that this form is involved in the glyoxylate cycle (gluconeogenesis).

On the other hand, as suggested by Krebs [52] for animal tissues, a cytoplasmic form of malate dehydrogenase could be involved in a cycle playing a special role in hydrogen transport between the cytoplasm and mitochondria. If such a mechanism exists also in *S. pombe*, then one would expect that malate dehydrogenase activity is present in the cytoplasm. As only one isoenzymic form of malate dehydrogenase has been found in derepressed cells of this fission yeast, the mitochondria have to contain two different malate dehydrogenases in the two different physiological states. In opposition to this, mitochondria from other yeast strains contain the same isoenzymic form in the glucose-repressed as well as in the derepressed state.

The results presented here indicate that *S. pombe* appears to be a suitable organism for further detailed studies on regulatory properties, metabolic significance and subunit structure of malate dehydrogenase isoenzymes.

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