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ISOENZYMES OF MALATE DEHYDROGENASE AND THEIR REGULATION IN *EUGLENA GRACILIS* Z

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SUMMARY

The malate dehydrogenase (L-malate:NAD^+ oxidoreductase, EC 1.1.1.37) isoenzymes of *Euglena gracilis* Z occur in two groups, an anodally migrating cluster of three cytoplasmic soluble malate dehydrogenase isoenzymes, which probably occur in the cytosol, and a cathodally migrating group of two particulate isoenzymes which exist in particulate cell components, probably mitochondrial malate dehydrogenase. Comparisons of some physical and kinetic properties are described. Although the molecular and kinetic properties are similar, the soluble malate dehydrogenase is much less stable than mitochondrial malate dehydrogenase. The latter supports the conclusion that the proteins are different. Cells grown heterotrophically in the dark have approximately three times more soluble malate dehydrogenase than cells grown photoautotrophically, whereas there is no significant difference between mitochondrial malate dehydrogenase quantities. This differential regulation between isoenzymes is found when the activities are measured in terms of dry weight of cells, on a unit soluble protein basis, or per cell. Cells transferred abruptly from autotrophic growth conditions to heterotrophic conditions, or *vice versa*, showed a lag in initiation of soluble malate dehydrogenase modification which correlates with lag in growth. Modulations of the isoenzyme levels in cells changed from one nutritional mode to another were studied under a variety of conditions. In other experiments, no significant alteration of soluble malate dehydrogenase was observed to occur in the absence of growth; removal of any essential parameter for growth (*e.g.*, CO_2, light, glucose) inhibits soluble malate dehydrogenase changes, *i.e.*, greening of etiolated cells in the absence of CO_2 does not cause a significant reduction of soluble malate dehydrogenase.

Analogues of substrates did not induce the soluble malate dehydrogenase, and the effects of inhibitors upon the enzyme changes was studied with paradoxical results.

Abbreviation: PCMB, *p*-chloromercuribenzoate.

INTRODUCTION

The isoenzymes of malate dehydrogenase (L-malate:NAD^+ oxidoreductase, EC 1.1.1.37) are compartmentalized in eukaryotic biological cells. In animals, research has shown that one group, mitochondrial malate dehydrogenase, is characteristically located in the mitochondria, where it functions in the tricarboxylic acid cycle. Another group of malate dehydrogenase isoenzymes, with different properties, is located in the cytosol as a "soluble" enzyme, soluble malate dehydrogenase[1–6]. Plants possess these isoenzymes, *plus* another one located in microbodies[7–12]. The function of soluble malate dehydrogenase is enigmatic. It has been variously suggested that it may function in gluconeogenesis[13], coupled with the (NADP) malate dehydrogenase (decarboxylating) (EC 1.1.1.40) in NADPH production necessary for certain syntheses[14,15], as an anaplerotic enzyme[16,17], or as a mediator in heterotrophic CO_2 fixation[18,19].

Smillie[20] working with *Euglena*, reported that malate dehydrogenase activity for cells grown heterotrophically in the dark and for streptomycin bleached cells was about twice that for photoautotrophically grown cells. Cook[21] confirmed this, reporting a steady decrease of malate dehydrogenase during growth in the light, and increase during growth in the dark. There was no attempt to determine which isoenzymes were responsible for this difference, and no other attempts have been reported except that of Chancellor–Maddison and Noll[22] who claimed that an extra isoenzyme appeared when cells were grown autotrophically.

MATERIALS AND METHODS

Stocks

Euglena gracilis Z was obtained from Dr J. F. Preston, Microbiology Department, Yale University and has been subcultured here for 2.5 years on slants of Difco *Euglena* agar. *E. gracilis* G was obtained from Dr S. Mills, University of California, San Diego; *E. gracilis* var *bacillaris*, wild-type and W_3BUL from Dr J. A. Schiff, Brandeis University; heat and streptomycin bleached *E. gracilis* Z from Dr W. R. Evans, Charles F. Kettering Laboratory; *E. gracilis* var *saccharophila* from the Culture Collection of Algae and Protozoa, Cambridge University, England.

Culture methods

Cells were grown at 25 °C in well agitated 250 ml Erlenmeyer flasks, using Hutner's[23] mineral medium at pH 3.3. Unless otherwise stated, heterotrophic cultures were grown on 1% glucose in complete darkness. Autotrophic cultures were illuminated from the side by 700 foot-candles emitted from a bank of "cool-white" fluorescent lamps, and the atmosphere above the culture was maintained at 5% CO_2 in air, as sole carbon source, by a steady stream of moist gas.

Since malate dehydrogenase activity varies with age of culture, cells were always harvested for assay between $7.5 \cdot 10^5$ and $15 \cdot 10^5$ cells/ml, unless otherwise stated. Malate dehydrogenase activity does not vary significantly between these limits. In experiments involving successive harvesting of cells from the same culture over long periods of time, the culture was regularly diluted to a cell density calculated to grow to a density between these limits at the time of harvesting.

Preparation of crude extracts

Harvested cells were centrifuged and resuspended in 0.1 M Tris buffer, pH 7.5 and sonicated for 60 s with a Bronwill sonicator tuned by ear to maximum noise intensity. Precautions to avoid heating the sonicate were observed. Preliminary experiments showed that 40 s sonication was sufficient to liberate all malate dehydrogenase into the solution. Extracts were clarified by centrifugation at 45 000 × *g* for 20 min. Supernatant fluids were decanted and used as a source of enzyme. All steps were kept below 4 °C.

DEAE-cellulose chromatography

DEAE-cellulose (Cellex-D from BioRad) was washed by the method of Peterson and Sober[24]. Crude supernatant fluid was first passed through a 1 cm × 10 cm Sephadex G-25 column equilibrated with 5 mM Tris, pH 8.5. Fractions containing malate dehydrogenase activity were pooled and applied to a 1.4 cm × 3 cm DEAE-cellulose column equilibrated with 5 mM Tris, pH 8.5. Proteins were eluted with a linear gradient of 100 ml of 0–0.2 M KCl in 5 mM Tris, pH 8.5. Recovery of malate dehydrogenase activity was 100%.

Assays

Protein was assayed by the method of Lowry *et al.*[25]. Chlorophyll was assayed using the method of Arnon[26]. Cell numbers were measured turbidometrically or with a Coulter cell counter. Total malate dehydrogenase activity was assayed as described by Ting[19] except that 0.1 M Tris buffer, pH 7.5, was employed.

Initially, groups of isoenzymes were assayed by measuring the areas under the individual peaks obtained from DEAE-cellulose elutions. Subsequently a shorter method was employed as follows. 5 ml of cells between $7.6 \cdot 10^5$ and $15 \cdot 10^5$ cells/ml were washed by centrifugation and resuspended in 0.05 M KCl in 5 mM Tris, pH 8.5. This was sonicated and total malate dehydrogenase measured. 50 mg of dry DEAE-cellulose (washed as described above) were added to 1 ml of the sonicate and thoroughly mixed for 30 s with a Vortex mixer. The DEAE-cellulose adsorbed the mitochondrial malate dehydrogenase under these conditions of ionic strength and was removed by centrifugation. Residual malate dehydrogenase in the supernate represents the soluble malate dehydrogenase, and the mitochondrial malate dehydrogenase activity was calculated by difference. The remainder of the sonicate was centrifuged at 45 000 × *g* for 15 min and the supernate used to assay total soluble protein. Preliminary experiments showed a close correlation between individual isoenzyme activities measured in this manner and activities obtained by integrating the areas under DEAE-cellulose curves.

Cellulose acetate paper electrophoresis

Isoenzymes were separated and located on 2.5 cm × 5 cm cellulose acetate paper strips (Schleicher and Schuell, Inc.) using the methods described by Smith[27] modified by Peak *et al.*[28], except that enzyme was applied by soaking a thin (0.25 mm) sliver of cellulose acetate paper with extract and laying it at right angles across the center of the electrophoresis strip. Stained strips were scanned with a Shimadzu scanning spectrophotometer MPS-50L.

Partial purification of malate dehydrogenase

Partially purified enzyme preparations were used to measure the properties of the isoenzymes. Steps employed, using 0.1 M Tris with 1 mM dithiothreitol throughout, were firstly, a 55–65% (v/v) cold acetone precipitation. The specific activity (μmoles NAD produced/min per mg soluble protein) increased from 17.6 to 336. Then the two peaks were separated by DEAE-cellulose chromatography. The protein from the two peaks was precipitated by 65% (v/v) cold acetone overnight and the malate dehydrogenase recovered by centrifugation. Total yield was 74% and specific activities were 144 and 123 for mitochondrial malate dehydrogenase and soluble malate dehydrogenase respectively. Several varied attempts to purify further have always resulted in radical losses of activity.

Molecular weights

Molecular weights were estimated using a calibrated Sephadex G-200 column, 1.5 cm × 65 cm. For calibration purposes, yeast alcohol dehydrogenase, molecular weight, 142 000; fraction 4 bovine serum albumin, 68 000; α-chymotrypsin, 23 000; and cytochrome *c*, 12 500, were used. For these proteins a plot of log molecular weight *versus* elution volume was linear.

Approximate sedimentation coefficients

Sedimentation coefficients were obtained by sedimentation of the enzymes through a 5-ml 5–20% sucrose gradient for 13 h at 37 000 rev./min in a Beckman Spinco SW 39 rotor. Catalase was used as a marker and S values were calculated by proportion after the method of DeMoss *et al.*[29].

RESULTS AND DISCUSSION

Electrophoresis

The inset tracing in Fig. 1 shows a paper electrophoretogram of the malate dehydrogenase isoenzymes from a crude extract derived from *E. gracilis* Z. Under these conditions of electrophoresis a group of three closely spaced isoenzymes migrate anodally (soluble malate dehydrogenase) and another group of two cathodally (mitochondrial malate dehydrogenase). The scan of an electrophoretogram shown in Fig. 1 confirms that there are five bands. We have also examined electrophoretograms of malate dehydrogenase from heterotrophic and autotrophic *E. gracilis* var *saccharophila*, *E. gracilis* var *bacillaris*, *E. gracilis* G, and the bleached mutants *E. gracilis* var *bacillaris* W_3BUL, and *E. gracilis* Z, strep and UV. In spite of repeated strip scans, we have not been able to confirm the report of Chancellor-Maddison and Noll[22]. Neither change to photoautotrophic growth nor bleached mutation causes any change in isoenzyme patterns. The scans have shown that the band in question, the most cathodal band, has about the same staining intensity whatever the mode of culture.

All the *Euglenae* examined so far have shown identical mitochondrial malate dehydrogenase patterns, whereas *E. gracilis* var *bacillaris* and *E. gracilis* var *saccharophila* both reveal a single soluble malate dehydrogenase band, the most anodal one. These results are consistent with the hypothesis that random association of two polypeptides, a and b, from two gene loci, would give three soluble malate dehydrogenase

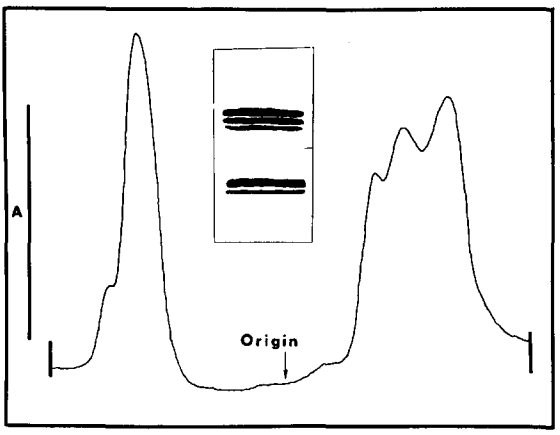

Fig. 1. Scan of cellulose acetate paper electrophoretogram showing distribution of *Euglena* malate dehydrogenase activity. The anode is to the right and the ordinate A is relative absorbance. The inset shows a tracing of a paper electrophoretogram. The sample was applied to the center of the strip and electrophoresed as described in Materials and Methods. The anode is to the top of the tracing.

isoenzymes, aa, ab, bb, and that the strains with only one soluble malate dehydrogenase lack one of the genes. This hypothesis is supported by the equal spacing between the bands and by the probability that soluble malate dehydrogenase is a dimer (Banaszak[30]). It is possible that another two gene loci control the mitochondrial malate dehydrogenase; that one of these loci directs the production of much less subunit than the other, which would account for the appearance of only two bands, the third undetected since it is beneath the sensitivity of the detecting system. Further, mitochondrial malate dehydrogenase also seems to be a dimer (Devenyi *et al.*[31], Davidson and Cortner[32]). The fact that soluble malate dehydrogenase can be modified without effect on mitochondrial malate dehydrogenase supports the hypothesis that soluble malate dehydrogenase and mitochondrial malate dehydrogenase are under the control of distinct nuclear genes.

The multiplicity of isoenzymes is not due to a population heterogeneity. Isolated individual clones reveal the same isoenzyme morphology as the parental cultures.

Intracellular location of malate dehydrogenase isoenzymes

A mitochondrial preparation was isolated, essentially by the method of Krawiec and Eisenstadt[33], and found to contain the cathodal pair of enzymes only. However, electron microscopy revealed that the mitochondrial fractions were contaminated with disrupted vesicles, in spite of many varied attempts of purification.

Gentle breakage of the cells (French pressure cell at 900 psi or shaking with glass beads) and removal of the particulate material by centrifugation revealed all five isoenzymes in the supernatant. Presumably the mitochondrial malate dehydrogenase in the supernate originates from broken mitochondria. Thus we assume, at present, that the cathodal group is mitochondrial in origin and the anodal group is derived from the cytosol. We have not yet satisfactorily excluded the possibility that one of

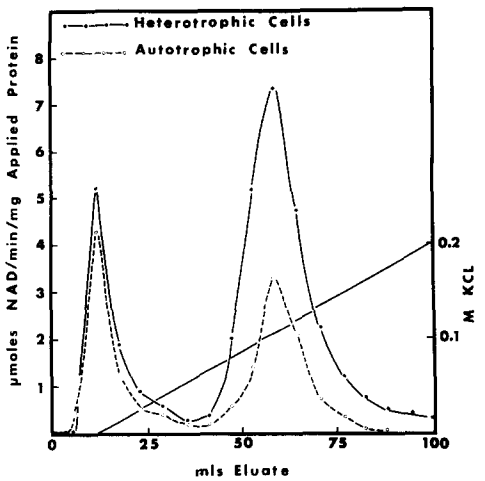

Fig. 2. Comparative DEAE-cellulose chromatography of malate dehydrogenase from heterotrophic and autotrophic *Euglena*.

the particulate isoenzymes is located in the microbodies, nor that some of this component does not occur naturally in the cytosol. However, our tentative localizations are consistent with those found in other organisms. Spinach, man, birds, trout, maize and sea urchins, for instance, all have mitochondrial malate dehydrogenase isoenzymes more positively charged compared with the soluble forms (Rocha and Ting[11], Davidson and Cortner[32], Kitto and Wilson[34], Bailey *et al.*[35], Longo and Scandalios[8], Patton *et al.*[36], respectively).

DEAE-cellulose chromatography

Fig. 2 shows that two peaks of malate dehydrogenase activity can be eluted from DEAE-cellulose, the first peak barely adsorbs onto the cellulose and the second peak elutes at 0.10 M KCl (mean of five determinations). Electrophoresis shows that the first peak to emerge from DEAE-cellulose contains only mitochondrial malate dehydrogenase, and that the second peak contains only soluble malate dehydrogenase. In these chromatograms the malate dehydrogenase recovery is 100%.

Since the ordinates of Fig. 2 are malate dehydrogenase activity per mg applied protein it is possible to compare the quantities of mitochondrial malate dehydrogenase and soluble malate dehydrogenase present in autotrophic and heterotrophic cells. Seeing that 100% of applied activity is recovered, any differences are not due to differential losses of activity. These data indicate little mitochondrial malate dehydrogenase difference between autotrophic and heterotrophic cells, whereas there is approximately 2.5–3.0 times more soluble malate dehydrogenase in heterotrophic cells. The evidence obtained from electrophoretograms, that malate dehydrogenase isoenzymes from autotrophic cells are the same proteins as those from heterotrophic cells, is supported by the conformational similarities of the DEAE-cellulose profiles. However, since DEAE-cellulose chromatography does not separate the isoenzymes comprising soluble malate dehydrogenase or mitochondrial malate dehydrogenase, it

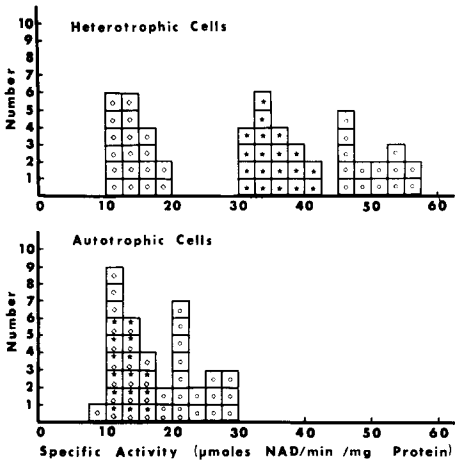

Fig. 3. Histograms showing a comparison of the distribution of soluble malate dehydrogenase, mitochondrial malate dehydrogenase and total malate dehydrogenase activities from heterotrophic or autotrophic *Euglena*. Each block represents the mean of four measurements of a sample taken from a different culture. ○, total malate dehydrogenase; ★, soluble malate dehydrogenase; ◊, mitochondrial malate dehydrogenase.

is possible that there could be changes in concentrations of individual isoenzymes that have not been detected by electrophoresis.

The apparent differences between the soluble malate dehydrogenase activities from the two types of cells were tested statistically. Fig. 3 and Table I show that the difference between heterotrophic and autotrophic cells with regard to soluble malate dehydrogenase activity is highly significant, heterotrophic cells having more than

TABLE I

COMPARISON OF ACTIVITIES OF MALATE DEHYDROGENASE ISOENZYMES DERIVED FROM AUTOTROPHICALLY OR HETEROTROPHICALLY GROWN *Euglena*

The ratios are autotrophic to heterotrophic activities and probability (P) was computed using Student's "t" test. A shows specific activity (μmoles NADH oxidized/min per mg soluble protein), B shows μmoles NADH oxidized/min per mg dry weight of cells and C shows μmoles NADH oxidized/min per 10^6 cells. Means ± standard deviations are quoted and the quantities in parentheses refer to the number of assays (each the mean of four measurements of one sample of cells taken from one culture). The results in B and C were calculated from the mean figures quoted in A, and from careful measurements of dry weights of cells and amount of soluble protein/10^6 cells.

Expt	*Malate dehydrogenase*	*Autotrophic cells*	*Heterotrophic cells*	*Ratio*	*P*
A	Mitochondrial	1.14 ± 0.27	1.42 ± 0.19	1:1.25	0.10
	Soluble	1.04 ± 0.29	3.55 ± 0.31	1:3.42	0.01
	Total	2.18 ± 0.41 (21)	4.97 ± 0.34 (18)	1:2.27	0.01
B	Mitochondrial	0.22	0.38	1:1.73	
	Soluble	0.20	0.92	1:4.60	
	Total	0.42	1.30	1:3.10	
C	Mitochondrial	0.28	0.27	1:0.96	
	Soluble	0.26	0.66	1:2.54	
	Total	0.54	0.93	1:1.73	

three times the activity, while the difference with regard to mitochondrial malate dehydrogenase activity is not significant. Further, these differences are observed whether the activities are assayed per cell, per mg protein, or per mg dry weight of cells. Thus, the elevation of total malate dehydrogenase in heterotrophically grown *E. gracilis*, reported earlier by Smillie[20] and by Cook[21], and also here, is due solely to the soluble malate dehydrogenase isoenzymes. The results were confirmed qualitatively in one experiment using strain G. The autotrophic to heterotrophic ratios in this experiment were 1:1.84 for soluble malate dehydrogenase and 1:1.32 for mitochondrial malate dehydrogenase. Thus it seems that there may be quantitative differences between strains in the degree of soluble malate dehydrogenase variation in metabolic shifts.

TABLE II

COMPARISON OF PROPERTIES OF SOLUBLE MALATE DEHYDROGENASE AND MITOCHONDRIAL MALATE DEHYDROGENASE FROM *Euglena*

% remaining activities after exposure to urea, PCMB and 56 °C were derived from the best line drawn through the points obtained from a complete inactivation curve. K_m values, and urea and PCMB inactivations were determined at 25 °C.

	Malate dehydrogenase	
	Soluble	*Mitochondrial*
Molecular weight by gel diffusion on a calibrated Sephadex G-200 column	71 000	71 000
Sedimentation coefficient by sedimentation through a sucrose gradient, using catalase as a marker	4.3	4.3
K_m value for oxaloacetate (0.1 M Tris, pH 7.5, 0.1 mM NADH)	0.050 mM	0.050 mM
K_m value for NADH (0.1 M Tris, pH 7.5, 0.5 mM oxaloacetate)	0.12 mM	0.11 mM
Remaining activity after exposure to 8 M urea for 1 min	7.8%	98.4%
Remaining activity after exposure to 7.7 mM PCMB for 15 min	16.5%	100%
Remaining activity after 5 months storage at −22 °C in 0.1 M Tris, pH 7.5, *plus* 1 mM dithiothreitol	0.025%	100%
Remaining activity after exposure to 56 °C for 2 min (0.1 M Tris, pH 7.5)	41.0%	90%

Comparison of properties of soluble malate dehydrogenase and mitochondrial malate dehydrogenase

Table II summarizes some of the properties of soluble malate dehydrogenase and mitochondrial malate dehydrogenase using enzymes partially purified as described in Methods and Materials. The physical properties (molecular weight and sedimentation coefficient) of the isoenzymes are the same.

Recovery of malate dehydrogenase from G-200 columns, and after sedimentation through a sucrose gradient, was about 100%, even when crude extracts were used. These observations, taken together with the 100% recovery after DEAE-cellulose chromatography indicate that it is highly unlikely that there is a malate dehydrogenase inhibitor or activator present in the crude extracts.

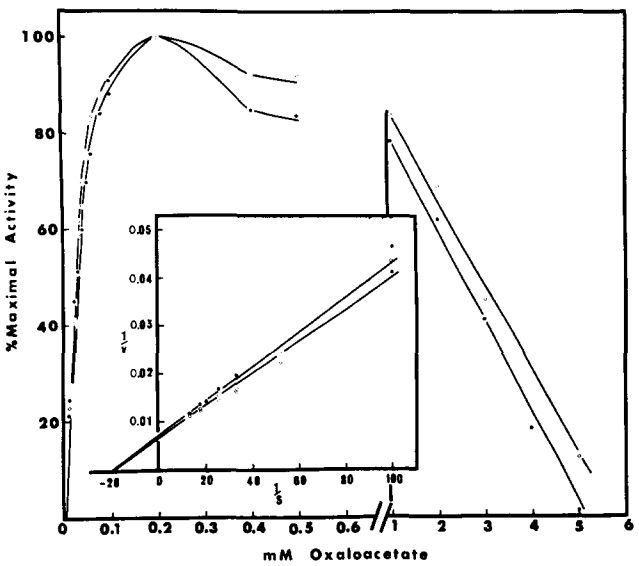

Fig. 4. Hyperbolic rate curves and double reciprocal plots of kinetic data for *Euglena* malate dehydrogenase isoenzymes. Initial rates of NADH oxidation were measured at a range of concentrations of oxaloacetate. Other details are given in Table II. The abscissa scale is increased tenfold above 0.6 mM oxaloacetate. The ordinate of the double reciprocal plot shown in the inset is the reciprocal of the % maximal activity and the slope of the regression was calculated by the method of least squares. ○—○, soluble malate dehydrogenase; ●—●, mitochondrial malate dehydrogenase.

The kinetic properties of the enzymes are quite similar. The effects of different pH values (8.5, 6.5) upon the kinetics were small. Fig. 4 shows that the degree of substrate inhibition is slightly less for soluble malate dehydrogenase than for mitochondrial malate dehydrogenase. This difference seems to be smaller for *Euglena* malate dehydrogenase than for malate dehydrogenase isolated from other organisms, such as corn root tips (Ting[19]), spinach (Rocha and Ting[12]), chick (Kitto and Kaplan[6]), tuna and chick (Kitto[37]) *inter alia*. An explanation for this difference is not known, however. The similarity in kinetics may mean that the enzymes are catalyzing the same reaction, but in different regions of the cell.

Further evidence that the soluble malate dehydrogenase and mitochondrial malate dehydrogenase are not the same protein is obtained from their differences in stability, since soluble malate dehydrogenase is rapidly inactivated by urea, heat, *p*-chloromercuribenzoate (PCMB), and cold storage whereas mitochondrial malate dehydrogenase is insensitive to PCMB and storage and, by comparison is slowly inactivated by urea and heat. The fact that these inactivations approach first order kinetics shows that the individual sub-bands within any group have similar sensitivities.

Malate dehydrogenase variations during metabolic transpositions

The results of abrupt transpositions of cells from heterotrophic to autotrophic conditions and *vice versa* are shown in Fig. 5. These results strongly confirm the conclusion that changes in malate dehydrogenase activity are attributable solely to so-

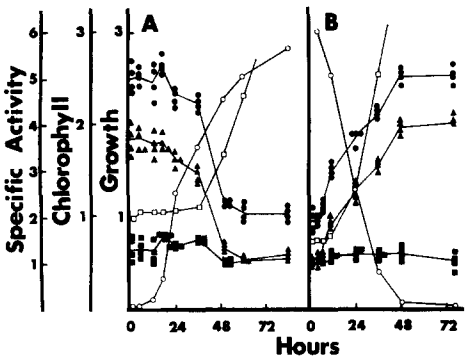

Fig. 5. Alterations in levels of malate dehydrogenase isoenzymes during transitions between autotrophy and heterotrophy. The transitions were inaugurated at time zero. A shows a transition from heterotrophy to autotrophy and B shows a transition from autotrophy to heterotrophy. Each point represents one determination and the lines are drawn through the means of four determinations. Where points superimpose completely they are drawn to one side. For clarity the 60 and 84 h mitochondrial malate dehydrogenase points (A) are omitted as they superimpose on the soluble malate dehydrogenase points; for the same reason the initial three sets of mitochondrial malate dehydrogenase points are omitted in B. Specific activity units are μmoles NADH oxidized/min per mg soluble protein, chlorophyll is in μg/10^6 cells, and growth is cells/ml ($\times 10^6$). ●—●, total malate dehydrogenase; ■—■, mitochondrial malate dehydrogenase; ▲—▲, soluble malate dehydrogenase; ○—○, chlorophyll; □—□, growth.

luble malate dehydrogenase and not to mitochondrial malate dehydrogenase. Regulation of soluble malate dehydrogenase, independently of mitochondrial malate dehydrogenase has been reported before in eukaryote cells, by Witt *et al.*[38], using yeast, and by Benveniste and Munkres[39], using *Neurospora*, and we are now able to confirm this observation using *Euglena*. These observations all support the possibility that soluble malate dehydrogenase and mitochondrial malate dehydrogenase are under different genetic control, as also supported by Davidson and Cortner[31,39], Longo and Scandalios[8], and by Kitto and Wilson[34] for human, maize and bird malate dehydrogenase respectively. It seems unlikely that this regulation of soluble malate dehydrogenase is due to the inactivation or stimulation by small ligands since these latter would have been removed by the initial G-25 sieving of the crude extract.

During transposition* from autotrophic to heterotrophic growth, activity of soluble malate dehydrogenase does not change during the lag phase of growth, but rapidly increases with the onset of logarithmic phase of growth (Fig. 5). The transposition of soluble malate dehydrogenase is complete by 48 h. Similarly, during the transposition from heterotrophic to autotrophic growth, disappearance of soluble malate dehydrogenase is correlated with the growth phase, and is complete by 48 h. It thus appears possible that control of soluble malate dehydrogenase is exerted *via* the metabolic situation that occurs as a result of autotrophic or heterotrophic growth. To test for this hypothesis, we have examined some incomplete transpositions, such that all factors except one were present, thus growth did not occur. The results of some of these experiments are shown in Fig. 6. Fig. 6A shows that elaboration of the photosynthetic apparatus, *i.e.*, greening, is not accompanied by a soluble malate de-

* In this paper, a transposition is defined as a complete switch over from the heterotrophic enzyme activity levels to autotrophic levels, or *vice versa*.

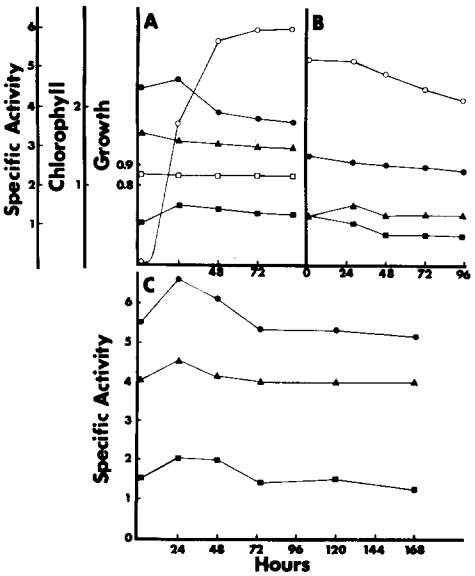

Fig. 6. The effects of various treatments upon the malate dehydrogenase isoenzymes of *Euglena*. A shows the effects of greening upon the malate dehydrogenase isoenzymes. Heterotrophic cells were washed once by centrifugation and transferred to the light, in autotrophic medium less glucose, at time zero. CO_2 was not gassed. In B autotrophic cells were placed in the dark, and CO_2 removed at time zero. In C, the glucose was removed from heterotrophic cells, at time zero, by centrifugation. In none of these experiments did any growth occur after the change in environment. Symbols and units are as shown in Fig. 5 and the zero time point for soluble malate dehydrogenase is omitted in B as it superimposes upon the mitochondrial malate dehydrogenase value. Each specific activity is the mean of four determinations. (In Fig. 5 these four determinations are shown in full.)

hydrogenase transposition. Thus the depression of soluble malate dehydrogenase in autotrophic cells is not due to the presence or synthesis of chlorophyll. The slow decline in both malate dehydrogenase species is always observed whenever the carbon source is removed. This may be some function of the cells entering the palmelloid stage. Fig. 6B demonstrates that light and CO_2 are not direct agents repressing soluble malate dehydrogenase. At time zero, autotrophic cells were placed in the dark and CO_2 removed, and no further growth occurred. Fig. 6C demonstrates that glucose *per se* is not inducing soluble malate dehydrogenase, since removal of glucose at time zero is not followed by a soluble malate dehydrogenase transposition, but by the normal slow decline observed in the absence of carbon source. The initial transient increase is inconsistently observed when the cells' environment is abruptly changed (see Figs 6A, 7B). The explanation for this is not known.

Fig. 7 shows attempts to induce the soluble malate dehydrogenase using analogues or stereoisomers of normally utilized substrates, *viz.*, 3-*O*-methylglucose, L-glucose, and D-glutamine. None of the analogues used supported growth and no increase in soluble malate dehydrogenase was observed. Also, Table III shows that glucose concentration does not markedly affect soluble malate dehydrogenase levels, as might be expected were the substrate acting as an inducer. In other experiments, heterotrophic cultures gassed with CO_2 do not show reduced soluble malate dehydro-

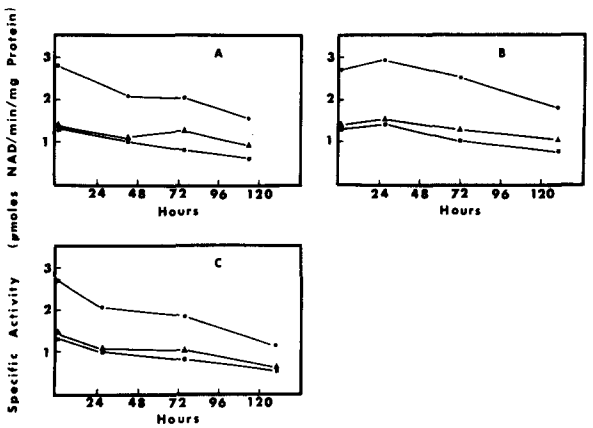

Fig. 7. Effects of analogues or stereoisomers of substrates upon malate dehydrogenase isoenzymes of *Euglena*. (A) 3-*O*-methylglucose. (B) D-glutamine. (C) L-glucose. Autotrophic cells were washed and placed in the dark and 1% analogue or isomer added at time zero. No growth occurred. Each point represents the mean of four determinations. Symbols are as shown in Fig. 5.

genase nor did removal of CO_2 from autotrophic cultures cause any elevation of the isoenzyme.

These experiments have all shown that transposition of soluble malate dehydrogenase does not occur unless the cells are growing. Incomplete autotrophic or heterotrophic conditions such that growth does not occur are not accompanied by malate dehydrogenase transposition. Thus it seems that soluble malate dehydrogenase levels are not controlled directly by external parameters, but by internal factors, such as different metabolic pool sizes associated with type of growth.

TABLE III

EFFECT OF GROWTH OF *Euglena* UPON VARIOUS SUBSTRATES UPON MALATE DEHYDROGENASE ISOENZYMES

In this case, cells were always harvested for assay when the cultures reached $1.1 \cdot 10^6$ cells/ml. Quantities are malate dehydrogenase specific activities (μmoles NADH oxidized/min per mg soluble protein) $\pm$ one standard deviation. Quantities in parentheses are numbers of cultures assayed.

Substrate	*Malate dehydrogenase*		
	Total	*Mitochondrial*	*Soluble*
Fructose (4)	3.99 ± 0.24	0.91 ± 0.14	3.08 ± 0.11
Malic acid (4)	4.29 ± 0.20	1.32 ± 0.07	2.97 ± 0.21
Glucose (18)	4.97 ± 0.34	1.42 ± 0.19	3.55 ± 0.31
Pyruvic acid (4)	5.87 ± 0.11	1.81 ± 0.90	4.06 ± 0.14
Succinic acid (4)	6.42 ± 0.33	2.16 ± 0.18	4.26 ± 0.39
Acetic acid (8)	6.47 ± 0.46	1.87 ± 0.16	4.60 ± 0.42
Ethanol (8)	6.63 ± 0.69	2.13 ± 0.30	4.50 ± 0.26
Glutamic acid (8)	8.00 ± 0.24	3.21 ± 0.18	4.78 ± 0.13
0.5% glucose (4)	4.16 ± 0.12	1.13 ± 0.15	3.03 ± 0.21
1.0% glucose (18)	4.97 ± 0.34	1.42 ± 0.19	3.55 ± 0.31
5.0% glucose (5)	5.34 ± 0.19	1.71 ± 0.90	3.63 ± 0.13
10.0% glucose (4)	5.37 ± 0.29	1.88 ± 0.11	3.49 ± 0.22

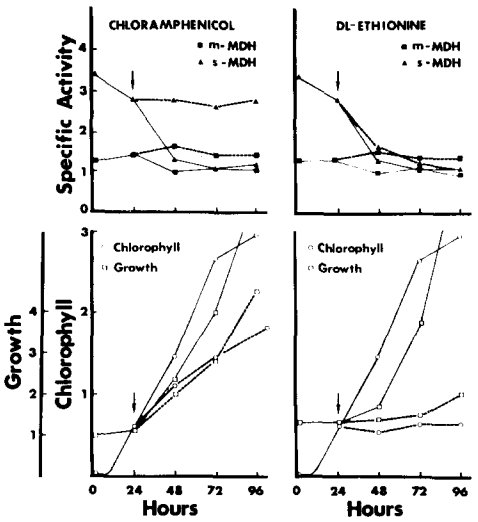

Fig. 8. Effects of chloramphenicol and DL-ethionine on malate dehydrogenase. Heterotrophic cells were washed and resuspended in autotrophic medium at time zero. After 24 h illumination the cultures were split into two and inhibitor added to one, shown on the graphs by arrows. Samples were harvested periodically from control cultures and inhibitor treated cultures, and malate dehydrogenase activities determined. Left hand graphs show the effects of 500 μg/ml chloramphenicol on the cultures; right hand graphs show the effects of 10 μg/ml DL-ethionine; upper graphs show the effects of both inhibitors upon malate dehydrogenase; lower graphs show the effects of both inhibitors upon growth and chlorophyll biosynthesis. Control curves are shown by continuous lines and inhibitor treatment curves are shown by broken lines. Symbols and units are as in Fig. 5 and each specific activity point is the mean of four determinations. m-MDH, mitochondrial malate dehydrogenase; s-MDH, soluble malate dehydrogenase.

Effect of chloramphenicol and ethionine

If soluble malate dehydrogenase is regulated by factors associated with chlorophyll synthesis and growth we would anticipate a diminution of the rate of soluble malate dehydrogenase transposition if these factors are inhibited. Fig. 8 shows the effects of adding 500 μg/ml chloramphenicol or 10 μg/ml DL-ethionine to heterotrophic cultures just after the commencement of a transposition to autotrophy. Chloramphenicol inhibits chloroplast DNA replication (Richards *et al.*[40]) and DL-ethionine is an inhibitor of some parameters of chloroplast synthesis including chlorophyll (J. M. Bernstein and Harvard Lyman, personal communication). Fig. 8 shows that chloramphenicol slows growth and chlorophyll synthesis, and, as anticipated, stopped the soluble malate dehydrogenase transposition whereas DL-ethionine causes immediate cessation of chlorophyll synthesis, a marked reduction in growth but, paradoxically, only a small reduction in rate of soluble malate dehydrogenase diminution. The effect of chloramphenicol on growth and chlorophyll synthesis is in agreement with the results of Richards *et al.*[40]; however, we cannot compare our results with those of Bishop and Smillie[41], who found no inhibition of growth by 500 μg/ml of chloramphenicol, since their conditions were different, *viz.* glucose was present and the light intensity was twofold greater.

The failure of DL-ethionine to prevent a soluble malate dehydrogenase transposition may be explained by either of two hypotheses. Either chlorophyll synthesis

and growth are not the factors governing the reduction of soluble malate dehydrogenase during transposition or else DL-ethionine is altering regulatory components of the metabolic pools in such a way that they achieve an autotrophic composition, despite inhibition of chlorophyll synthesis and growth. M. R. Atkinson and Polya[42] have shown that ethionine may act as an ATP trap and Raven[43] presents evidence that it may alter the energy charge. D. E. Atkinson[44] has shown that the energy charge is an important regulatory parameter, and such phenomena may coincidentally cause a soluble malate dehydrogenase transposition.

Effects of various substrates

Table III shows the levels of the malate dehydrogenase species when *Euglena* is grown for long periods (over 14 generations) on various substrates in the dark. Cells would not grow on glycine, citrate, sorbitol, sucrose, galactose, lactose, glycerol, maltose, ribose or aspartate. In spite of the fact that we have not measured the effect of culture age upon enzyme levels for each of these substrates, certain generalizations may be made about these results. Except for malic acid, fructose and glucose have the lowest levels of soluble malate dehydrogenase. The possibility of increased gluconeogenesis with the tricarboxcylic acids entering directly into the tricarboxylic acid cycle, as opposed to the sugars, should be investigated, since this may be associated with increased soluble malate dehydrogenase.

These measurements show that mitochondrial malate dehydrogenase activities are altered by different substrates. For instance, glutamate doubles the mitochondrial malate dehydrogenase activity and ethanol and succinate cause about a 50% increase compared with glucose. These results are not understood; it is possible that these two tricarboxylic acid cycle intermediates are regulator molecules for mitochondrial malate dehydrogenase.

The difference between glucose-grown and acetate-grown cells is too small for us to claim an involvement of *Euglena* soluble malate dehydrogenase in glyoxylate metabolism as asserted by Witt *et al.*[37] for yeast, or by Benveniste and Munkres[38] for *Neurospora*.

Final summary

The important findings that we have presented in this paper are that the soluble malate dehydrogenase of *Euglena* is two- to threefold more active in cells grown heterotrophically compared with those grown autotrophically and that there is no such change in mitochondrial malate dehydrogenase. Chloramphenicol inhibits the reduction in soluble malate dehydrogenase during change to autotrophy. We present evidence that regulation of the soluble malate dehydrogenase occurs as a result of the metabolic state of the cells. Other considerations, concerning the metabolic functions of soluble malate dehydrogenase will be published later.

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