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FUNCTION AND REGULATION OF NADP-SPECIFIC MALATE DEHYDROGENASE IN *EUGLENA GRACILIS Z*

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SUMMARY

NADP-specific malate dehydrogenase (decarboxylating) (L-malate:NADP oxidoreductase (decarboxylating), EC 1.1.1.40) was found to predominate in *Euglena* cytosol. A single peak of enzyme activity elutes from a DEAE-cellulose column and from a Sephadex G-200 column.

The specific activity of the enzyme is low in cells grown autotrophically, *i.e.* in the light, with CO_2 as the sole carbon source, and varies little throughout the growth cycle. In cells grown heterotrophically, *i.e.* in the dark, with glucose as the sole carbon source, the specific activity of the dehydrogenase increases linearly throughout the growth cycle. The specific activity in cells grown heterotrophically is always greater than in autotrophically grown cells; the maximum difference observed is of the order of 55-fold.

Modulations of the dehydrogenase were studied under a variety of nutritional conditions. Exposure of heterotrophically grown cells to the light always causes a reduction of NADP-specific malate dehydrogenase, whatever the conditions of CO_2 and glucose. This reduction is usually exponential and can occur in the absence of growth. The specific activity of the dehydrogenase in autotrophically grown cells is not affected by removal of CO_2, or light, or both. Nor is it affected by addition of glucose, or addition of glucose and removal of CO_2, provided that the cultures are left in the light. Gassing heterotrophic cells with CO_2 in the dark has no effect upon the specific activity of the dehydrogenase.

Inhibition of growth and chlorophyll biosynthesis by chloramphenicol or DL-ethionine does not inhibit this light-induced reduction of the specific activity of malate dehydrogenase.

The possibility of a shunt bypassing the pyruvate kinase (EC 2.7.1.40) step of glycolysis, and involving phosphoenolpyruvate carboxylase, soluble malate dehydrogenase and NADP-specific malate dehydrogenase is discussed in terms of a possible function in regulation of the ratio between NADH and NADPH.

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INTRODUCTION

In a previous publication[1] we showed that the cytosol isoenzymes NAD-specific malate dehydrogenase (soluble malate dehydrogenase) are about 3.5 times more active in heterotrophically grown *Euglena*, compared with autotrophically grown *Euglena*. In this paper, we report findings upon the regulation of NADP-specific malate dehydrogenase (L-lactate:NADP oxidoreductase (decarboxylating), EC 1.1.1.40), adjacent to soluble malate dehydrogenase in the putative cytosol pathway phosphoenolpyruvate→oxaloacetate↔malate↔pyruvate, catalysed by phosphoenolpyruvate carboxylase, soluble malate dehydrogenase and NADP-specific malate dehydrogenase, respectively. The function of this pathway is uncertain. Possibilities are anaplerotic supplying of oxaloacetate to maintain the tricarboxylic acid cycle[2], which would involve the Wood–Werkman[3] reaction, gluconeogenesis, or control of NADPH production[4]. Conversion of oxaloacetate to pyruvate would involve transhydrogenation of NADH and NADP, forming NADPH, which would then be available for fatty acid biosynthesis or other NADPH requiring biosyntheses in the cytosol. This possibility was advocated by Ting and Dugger[4] for corn roots, by Lardy and co-workers[5–7] for rat liver, and by Wise and Ball[8], also using rat liver. Another possibility is that phosphoenolpyruvate carboxylase and NADP-specific malate dehydrogenase, with soluble malate dehydrogenase, are involved in cytosol malate biosynthesis. In this report we attempt to differentiate between these alternatives for NADP-specific malate dehydrogenase function.

METHODS AND MATERIALS

The organism, all media, and methods used were as described previously[1]. Since NADP-specific malate dehydrogenase activity varies continuously throughout heterotrophic growth cycles, in the experiments where cells were harvested at intervals from growing cultures (for measurement of malate dehydrogenase activity) the cultures were always diluted so that they would grow to exactly the same cell density at the next time of harvesting. Malate dehydrogenase was measured according to the method used by Mukerji and Ting[9] in the direction of malate to pyruvate and using 0.1 M *N*-Tris(hydroxymethyl)methyl-2-aminomethane sulfonic acid buffer, pH 7; phosphoenolpyruvate carboxylase according to the method used by Mukerji and Ting[10], and all other assays were as described by Peak *et al.*[1].

RESULTS

Preliminary experiments

A mitochondrial preparation from heterotrophically grown cells showed that NADP-specific malate dehydrogenase predominates in the cytosol and traces of activity apparently associated with the mitochondria were removed by further washing.

The enzyme elutes from DEAE-cellulose as a single sharp peak at 0.04 M KCl, and elutes in the void volume of a Sephadex G-200 column, showing that its molecular weight is above 200 000*. Lardy *et al.*[11] report that the molecular weight of NADP-

* Sephadex G-200 excludes molecules above about 200 000 molecular weight (Technical Information, Pharmacia Fine Chemicals).

specific malate dehydrogenase from pigeon liver is 280 000, and one of us (I.P.T.) has found a weight of 140 000 for maize NADP-specific malate dehydrogenase. These chromatographic separations have confirmed that NAD-specific malate dehydrogenase is not being measured in error, since NADP-specific malate dehydrogenase elutes in a different position from the NAD-specific malate dehydrogenases[1].

Elution of NADP-specific malate dehydrogenase as a single sharp peak from DEAE-cellulose indicates that the enzyme may not exist in multiple forms in *Euglena* in contrast to *Opuntia*, where mitochondrial, chloroplast, and soluble organelle specific isoenzymes have been resolved (Mukerji and Ting[9]). However, since we did not resolve the three isoenzymes of soluble malate dehydrogenase or the two isoenzymes of mitochondrial malate dehydrogenase[1], the possibility that multiple forms of NADP-specific malate dehydrogenase exist in *Euglena* has not been excluded.

Comparison of specific activity of NADP-specific malate dehydrogenase in heterotrophically and autotrophically grown cells

Changes in and differences between specific activities (μmoles NADP reduced/min per mg soluble protein) of NADP-specific malate dehydrogenase in *Euglena* at various stages of either autotrophic or heterotrophic growth cycles are shown in Fig. 1. Whatever the stage of growth, there is always more NADP-specific malate dehydrogenase present in heterotrophic cells. The activity from green cells is always low, and varies little throughout the growth cycle, whereas in heterotrophic cells, it rises about 6-fold throughout the growth cycle.

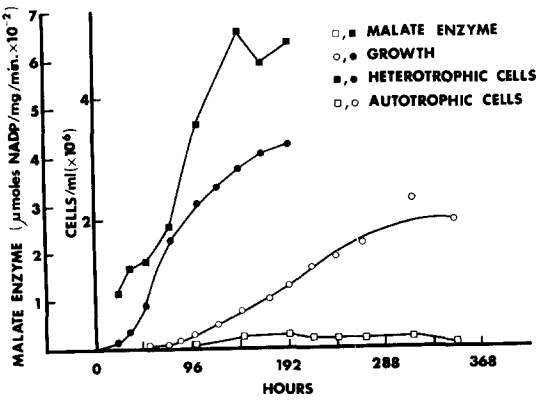

Fig. 1. Comparison of NADP-specific malate dehydrogenase (malate enzyme) activity in heterotrophic and autotrophic *Euglena* measured at various times during the growth cycles. Cells were harvested at various stages throughout the growth cycles shown by the graph, and assayed for malate dehydrogenase specific activity.

The differences in NADP-specific malate dehydrogenase and soluble malate dehydrogenase activity between heterotrophic and autotrophic cells are not quantitatively similar, since heterotrophic cells have about 3.5 times more soluble malate dehydrogenase (Peak *et al.*[1]). Thus it would seem that there is no co-ordination in the regulation of these two enzymes.

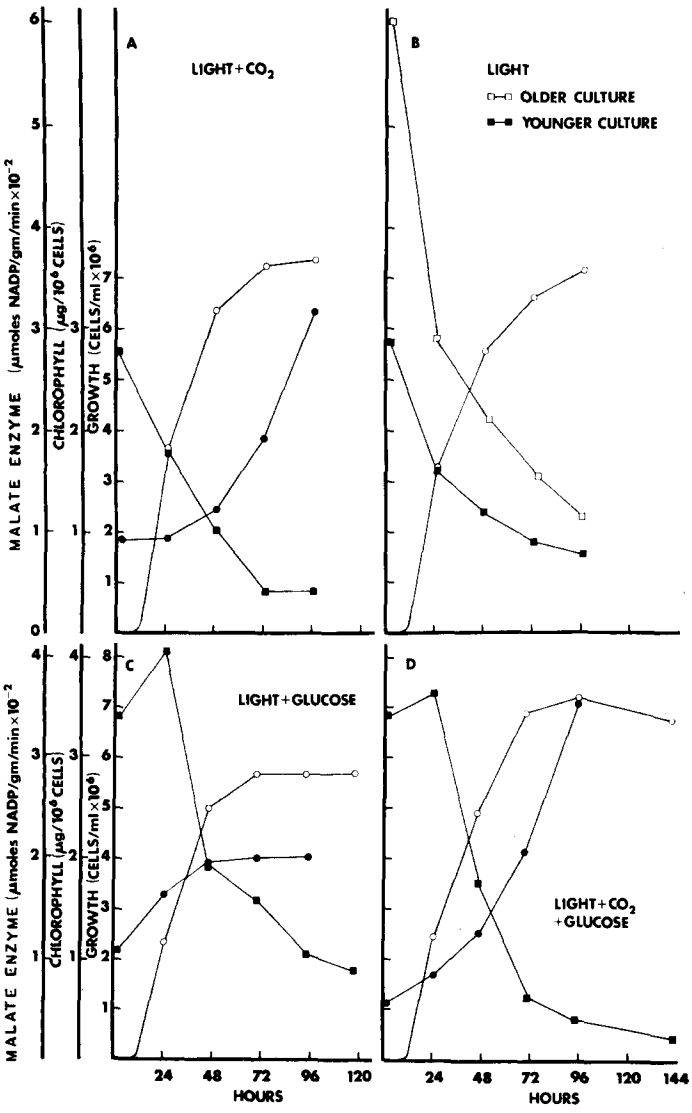

Fig. 2. Effect on NADP-specific malate dehydrogenase (malate enzyme) activity of transferring heterotrophic *Euglena* to the light, under various conditions of CO_2 and glucose. Each point represents the mean of two observations. The treatments were initiated at time zero, and aliquots were harvested for enzyme assay every 24 h. The remainder of the cultures were diluted as described in Methods and Materials. Initial NADP-specific malate dehydrogenase activities vary due to the fact that heterotrophic cultures at different stages of growth were used. (A) A complete heterotrophic to autotrophic transposition. (B) The effect of greening: heterotrophic cells were washed free of glucose and placed in the light with no CO_2 gassing. (C) Hetreotrophic cells were washed and put in 1% glucose in the light. (D) As for C, except that the cells were also gassed with CO_2. ●—●, growth; ■—■, NADP-specific malate dehydrogenase activity; ○—○, chlorophyll.

Modulations of Euglena NADP-specific malate dehydrogenase during alterations in environmental conditions

Fig. 2 shows the effects of transferring heterotrophically grown *Euglena* to the light, with, or without, glucose or CO_2, or both. Light always causes rapid reduction of NADP-specific malate dehydrogenase specific activity, whatever the conditions of glucose and CO_2, and Fig. 2B shows that the light induced reduction of NADP-specific malate dehydrogenase activity in heterotrophic *Euglena* occurs in the absence of growth, following approximately the same, but reciprocal, time course as chlorophyll biosynthesis. However, there is never an initial lag in reduction of NADP-specific malate dehydrogenase specific activity, as there is in chlorophyll biosynthesis. This light occasioned decrease in NADP-specific malate dehydrogenase activity occurs whether the cells are derived from mid-growth, or from the end of a growth cycle when the initial NADP-specific malate dehydrogenase activity is greater (Fig. 2B), and is the opposite to that observed during the greening of etiolated plants. Seeschaaf[12] showed that greening in corn is accompanied by an increase in the activity of NADP-specific malate dehydrogenase. Thus, in this respect, *Euglena* does not behave in a plant-like fashion.

Fig. 2A shows that CO_2 and growth have little effect upon the light induced decrease of NADP-specific malate dehydrogenase specific activity in heterotrophic *Euglena*. The rate of decrease of NADP-specific malate dehydrogenase activity is slightly increased. The effects of addition of glucose, with or without CO_2, upon light occasioned decrease in NADP-specific malate dehydrogenase activity are shown in Figs 2D and 2C, respectively. Neither glucose nor CO_2, either separately, or in combination, inhibit the decrease of NADP-specific malate dehydrogenase activity by light, and also, either the presence of CO_2, or the occurrence of growth, or both, accelerates the light occasioned decrease in NADP-specific malate dehydrogenase activity (compare with Fig. 2B). Also of interest is the fact that addition of 1% glucose to heterotrophic cells exposed to light always causes a transient, 24-h increase in malate dehydrogenase activity (Figs 2C and 2D), and that this transient increase is not prevented by CO_2 (Fig. 2D). This, and other "glucose" effects, are not understood, and are discussed elsewhere[15].

Irrelevant in this context, but of interest, shown by Figs 2C and 2D, is the fact that light inhibits growth on glucose, and the inhibition of growth increases as chlorophyll develops. Also, reciprocally, glucose partially inhibits chlorophyll synthesis, and less chlorophyll per cell is made. CO_2 overcomes the inhibition of growth and the partial inhibition of chlorophyll synthesis by glucose. Shugarman and Appleman[13], also Matsuka and Hase[14], showed that glucose suppresses chlorophyll synthesis in *Chlorella*. Thus it would seem that interactions between glucose and photosynthesis may be general phenomena.

The effect of a complete transposition from complete autotrophic to complete heterotrophic conditions upon NADP-specific malate dehydrogenase is shown in Fig. 3. There is a lag of about 24 h before growth commences*, however, unlike the transposition of soluble malate dehydrogenase reported previously[1], the transposition in NADP-specific malate dehydrogenase levels commences immediately, in the growth

* This lag is longer than that reported previously[1]. This is because the cultures used in the work reported here were more dense. It has consistently been observed that the lag in growth after an abrupt autotrophic to heterotrophic transposition increases with age of culture.

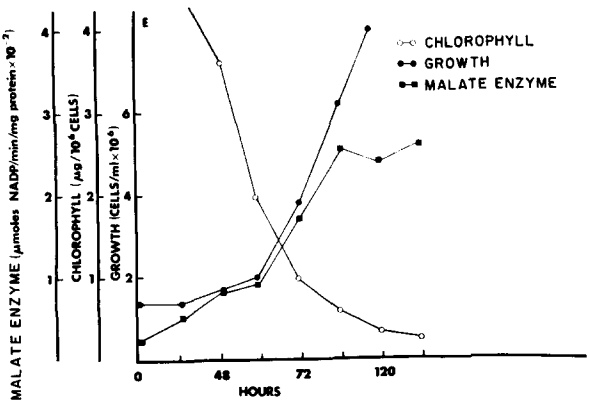

Fig. 3. Effect of an abrupt transposition from autotrophic to heterotrophic conditions upon NADP-specific malate dehydrogenase (malate enzyme) activity of *Euglena*. The transposition was inaugurated at time zero, aliquots of the culture were harvested in duplicate every 24 h, for enzyme assay. After the growth lag, the culture was diluted at the time of harvesting as described in Methods and Materials.

lag period, accelerating slightly when growth is inaugurated, and coming to completion after about 108 h. In other experiments, presented graphically elsewhere[15], the NADP-specific malate dehydrogenase levels of autotrophic cells were unaffected by cessation of CO_2 gassing, removal of light, or both. Also, gassing heterotrophic cells with CO_2 has no effect upon malate dehydrogenase specific activity. It is consistently observed that CO_2 is not of importance in the regulation of NADP-specific malate dehydrogenase. This observation is of importance since CO_2 is a malate dehydrogenase ligand. Also, in this series of experiments[15], it was shown that neither glucose alone, nor simultaneous addition of glucose *plus* cessation of CO_2 gassing, causes increase in the malate dehydrogenase specific activity of autotrophically grown cells, providing the cells are left in the light. Initiation of malate dehydrogenase synthesis occurs only when the cells are placed in the dark, following the time course shown by Fig. 3.

In summary, the regulation of NADP-specific malate dehydrogenase is complex. Light appears to repress the enzyme in heterotrophic cells, but glucose must be present for the apparent induction of the enzyme by the removal of light.

Effect of inhibitors upon the repression of NADP-specific malate dehydrogenase by light

It is possible that the repression of NADP-specific malate dehydrogenase activity is related to the synthesis of chlorophyll. Alternatively, synthesis of chlorophyll and repression of NADP-specific malate dehydrogenase may be unrelated processes both regulated by light. These alternatives were tested by inhibiting chlorophyll development using two inhibitors, and the results are shown in Fig. 4. DL-Ethionine prevented growth completely, and there was minor synthesis of chlorophyll compared with controls. Chloramphenicol stopped growth, and inhibited chlorophyll synthesis by about 50%. However, the decrease in NADP-specific malate dehydrogenase activity was not inhibited in either case. The small differences between control and treated curves are probably insignificant. Thus it seems probable that the action of light in repression of NADP-specific malate dehydrogenase is independent of chlorophyll biosynthesis.

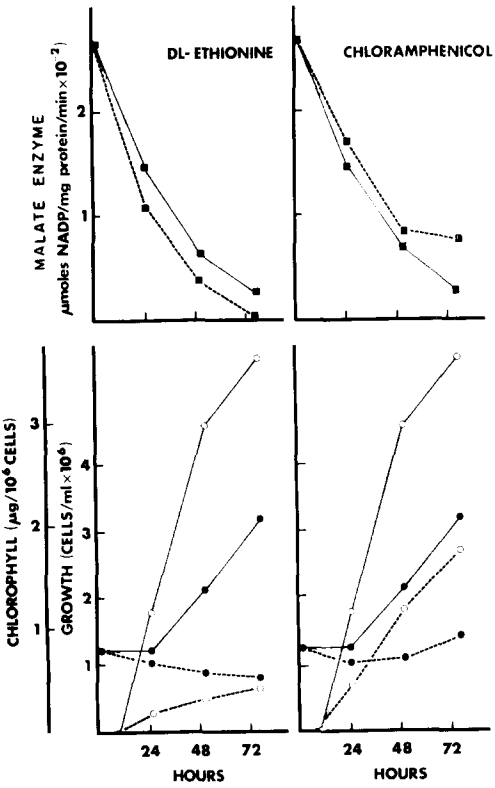

Fig. 4. Effect of inhibitors on NADP-specific malate dehydrogenase (malate enzyme) reduction in heterotrophic to autotrophic transposition of *Euglena*. Each point represents the mean of two determinations. The treatments were initiated at time zero. Heterotrophic cells were washed and placed in autotrophic medium, and aliquots were harvested for enzyme assay every 24 h, and the remainder of the cultures were diluted as described for Fig. 2. The culture was split into three; 500 μg/ml chloramphenicol was added to one, 10 μg/ml DL-ethionine to the second, and the third culture acted as control for both inhibitors. Left hand graphs show the effects of DL-ethionine, right hand graphs the effects of chloramphenicol. Top graphs show the effects on NADP-specific malate dehydrogenase activity and bottom graphs the effects on growth and chlorophyll biosynthesis. Broken lines represent the actions of the inhibitors and continuous lines are the control results. Symbols are as in Fig. 2.

DISCUSSION

We have demonstrated here, and previously[1], that two cytosol enzymes, soluble malate dehydrogenase and NADP-specific malate dehydrogenase, which catalyze adjacent steps in a possible glycolytic bypass (see Fig. 5, Reactions 2, 3 and 4)* rerouting metabolites between pyruvate and phosphoenolpyruvate, are elevated in heterotrophically grown, compared with autotrophically grown *Euglena*. Further, both enzymes are elevated in heterotrophic cells as the cultures age, but not in autotrophic cells. We have also shown that these two enzymes are not regulated in the same fashion. Most significantly, NADP-specific malate dehydrogenase is regulated in

* In this discussion, this bypass will be referred to as the dicarboxylic acid bypass, since both metabolites involved are dicarboxylic acids.

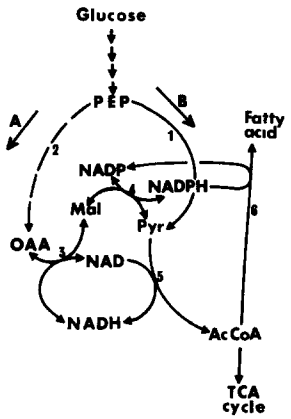

Fig. 5. Hypothesis for regulation of NADPH/NADH ratio in fatty acid biosynthesis. A, dicarboxylic acid shunt; B, glycolysis; 1, pyruvate kinase; 2, phosphoenolpyruvate carboxylase; 3, soluble malate dehydrogenase; 4, NADP-specific malate dehydrogenase; 5, pyruvate dehydrogenase; 6, fatty acid synthetase; PEP, phosphoenolpyruvate; OAA, oxaloacetate; Mal, malate; Pyr, pyruvate; AcCoa, acetyl coenzyme A; TCA cycle, tricarboxylic acid cycle.

the absence of cell growth, unlike soluble malate dehydrogenase, and light alone reduces the activity of NADP-specific malate dehydrogenase in heterotrophic cells, but not the activity of soluble malate dehydrogenase. One interpretation of these differences in regulation of the two enzymes is that regulation of the dicarboxylic acid bypass is biphasic. In the first phase, NADP-specific malate dehydrogenase is regulated by events which occur as a result of the presence or absence of light. In the second phase, soluble malate dehydrogenase is regulated, when growth commences by metabolic pool sizes, which have been changed by the different levels of NADP-specific malate dehydrogenase.

Regardless of the mechanisms of regulation, this new knowledge of the essential similarities between changes in the activities of the two enzymes, their common localization in the same cell compartment and the fact that they catalyze adjacent steps in the same sequence, provides evidence that the dicarboxylic acid bypass is more prominent in the cytosol of heterotrophic cells than that of autotrophic cells, and that the older the heterotrophic cells are, the more prominent the pathway becomes.

The function of the dicarboxylic acid bypass most strongly supported, by this information, is that it is concerned with supplying NADPH for cytosol fatty acid biosynthesis. The essentials of the reaction sequence are shown in Fig. 5. Phosphoenolpyruvate, from glucose, is the first metabolite in a branched pathway. If direction B is followed, the phosphoenolpyruvate is converted to pyruvate, catalysed by pyruvate kinase, producing one molecule of ATP from ADP. Alternatively, phosphoenolpyruvate may be converted to pyruvate *via* oxaloacetate and malate, catalysed by phosphoenolpyruvate carboxylase, soluble malate dehydrogenase and NADP-specific malate dehydrogenase, respectively. We have found substantial amounts of phosphoenolpyruvate carboxylase (about $4.2 \cdot 10^{-2}$ μmoles NADP reduced/min per mg soluble protein) in supernates of sonicates of both heterotrophic and autotrophic cells. If route B is followed, a transhydrogenation between pyridine nucleotides is effected, whereby, for each phosphoenolpyruvate converted to pyruvate, one NADPH

is produced at the expense of one NADP, essentially at the expense of one ATP. The acetyl-coenzyme A produced can be used in the synthesis of lipid, which is catalysed by the cytosol complex, fatty acid synthetase (Wakil *et al.*[16]) which requires NADPH for reductive syntheses. Thus NADP is regenerated; as long as there is a constant supply of phosphoenolpyruvate, there is a constant re-oxidation and re-reduction of the same coenzyme molecules in a pair of self-regenerating cycles.

This hypothesis for the function of NADP-specific malate dehydrogenase and soluble malate dehydrogenase is supported by the fact that there may be more than 3-fold greater requirement for NADPH for lipid biosynthesis in heterotrophically growing *Euglena* extraplastid space compared with autotrophically grown *Euglena*. It is possible to calculate the NADPH requirements for the cytosol of autotrophically and heterotrophically grown cells, knowing the total lipid content (Rosenberg[17], Hulanicka *et al.*[18]), distribution of chloroplastic and extrachloroplastic lipid types (Von Debuch[19], Zill and Harmon[20], Erwin and Block[21], Rosenberg *et al.*[22]) and different growth rates (Fig. 1). The amount of NADPH required for each fatty acid was calculated by summing two NADPH molecules for each 2-carbon fragment of the aliphatic chain, and deducting one NADPH molecule for each double carbon to carbon bond. Knowing that the total lipid is the same in both types of cell, (Rosenberg[17]), we calculated NADPH requirements by summing the product of the number of NADPH molecules required to synthesize one molecule of fatty acid and the percentage of the fatty acid present as quoted in Table II, of Hulanicka *et al.*[18]. The computation is presented completely elsewhere (Peak[15]), but shows that the cytosol of heterotrophic cells requires more than three times NADPH for cytosol fatty acid biosynthesis compared with autotrophic cells, assuming lipid turnover to be similar and that no chloroplastic lipid is synthesized outside the chloroplast. Further, autotrophic cells may have another source of NADPH, from photosynthesis. More evidence that the dicarboxylic acid shunt goes in the direction of malate decarboxylation is that equilibrium constants for phosphoenolpyruvate carboxylase and NADP-specific malate dehydrogenase support this direction. Also, under physiological conditions of malate concentration, the equilibrium of the step catalyzed by malate is in favor of malate decarboxylation[24].

Whatever the function of these enzymes, there is little doubt that heterotrophic cells have a greater requirement for an extrachloroplastic NADPH generating system, and that two adjacent enzymes which could supply that NADPH are found to have elevated activities in heterotrophic cells.

Possible alternate functions of the NADP-specific malate dehydrogenase were discussed elsewhere[15].

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REFERENCES

1 Peak, M. J., Peak, J. G. and Ting, I. P. (1972) *Biochim. Biophys. Acta* 284, 1–15
2 Ohman, E. and Plhak, F. (1969) *Eur. J. Biochem.* 10, 43–55
3 Werkman, C. H. and Wood, H. G. (1952) *Adv. Enzymol.* 2, 135–182
4 Ting, I. P. and Dugger, W. M. (1965) *Science* 150, 1727–1728
5 Shargo, E., Lardy, H. A., Nordlie, R. C. and Foster, D. O. (1963) *J. Biol. Chem.* 238, 3188–3192
6 Lardy, H. A., Foster, D. O., Shargo, E. and Ray, P. D. (1964) *Adv. Enzyme Regul.* 2, 39–47
7 Young, J. W., Shargo, E. and Lardy, H. A. (1964) *Biochemistry* 3, 1687–1692
8 Wise, E. M. and Ball, E. G. (1964) *Proc. Natl. Acad. Sci. U.S.*, 52, 1255–1263
9 Mukerji, S. K. and Ting, I. P. (1968) *Biochim. Biophys. Acta* 167, 239–249
10 Mukerji, S. K. and Ting, I. P. (1971) *Arch. Biochem. Biophys.* 143, 297–317
11 Lardy, H. A., Paetkau, V. and Walter, P. (1965) *Proc. Natl. Acad. Sci. U.S.* 53, 1410–1415
12 Seeschaaf, K. W. (1971) *Angew. Bot.* 45, 65–81
13 Shugarman, P. M. and Appleman, D. (1966) *Plant Physiol.* 41, 1701–1708
14 Matsuka, M. and Hase, E. (1969) *Plant Cell Physiol.* 10, 277–282
15 Peak, M. J. (1972) Ph. D. Thesis, University of California, Riverside
16 Wakil, S. H., Tichener, E. B. and Gibson, D. M. (1959) *Biochim. Biophys. Acta* 34, 227–233
17 Rosenberg, A. (1963) *Biochemistry* 2, 1148–1154
18 Hulanicka, D., Erwin, J., and Block, K. (1964) *J. Biol. Chem.* 239, 2779–2787
19 Von Debuch, H. (1961) *Z. Naturforsch.* 16b, 246–248
20 Zill, L. P. and Harmon, E. A. (1962) *Biochim. Biophys. Acta* 53, 579–581
21 Erwin, J. and Block, K. (1963) *Biochem. Z.* 338, 496–511
22 Rosenberg, A., Pecker, M. and Moschides, E. (1965) *Biochemistry* 4, 680–685
23 Ochoa, S., Mehler, A. H. and Kornberg, A. (1948) *J. Biol. Chem.* 174, 979–1000
24 Harary, I., Korey, S. A. and Ochoa, S. (1953) *J. Biol. Chem.* 203, 595–604