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A Zinc-Dependent Lactate Dehydrogenase in *Euglena gracilis*

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Although deprivation of metals is known to result in striking and selective changes in the activities of certain metalloenzymes (cf. McElroy & Nason, 1954; Baumeister, 1955; Hewitt, 1959; Vallee, 1959), a number of basic questions remain unanswered: What is the role of the metal in the synthesis of the apo- or holo-enzyme? What constitutes a critical concentration of metal? What causes the activity of one metalloenzyme to decrease in metal deficiency and not another?

In order to obtain answers to some of these questions we have been studying the biochemistry and physiology of zinc metalloenzymes in the alga, *Euglena gracilis*, grown under conditions where the metal content of the cells is rigidly controlled.

In the work reported here, we have sought an enzyme in *Euglena* that might be under the control of zinc. The oxidation of lactic acid by intact cells is virtually abolished in zinc deficiency and the activity of an enzyme, tentatively identified as a D-lactate dehydrogenase, is decreased.

METHODS

Abbreviation. $-q_{O_2}$, μl. of oxygen consumed/hr./mg. of protein nitrogen.

Organism. Euglena gracilis, '*z*' strain, was kindly provided by Dr S. Hutner of the Haskins Laboratory, New York, N.Y.

Special chemicals. The iron, manganese, copper and zinc sulphates employed in the growth media were Johnson Matthey and Co. Ltd. SpecPure salts. Ammonium glutamate was prepared from L(+)-glutamic acid (Eastman Kodak). Metal-free water ammonia solution and hydrochloric acid were obtained as described by Thiers (1957). D(+)- and L(−)-Lactic acid were prepared by passage of the corresponding calcium salt (California Corp. for Biochemical Research) through a column of Dowex 50 (Brin, 1953).

Analyses. Total nitrogen was determined by acid digestion followed by nesslerization. Non-protein nitrogen was determined by treating cell samples with 4% trichloroacetic acid at 0° for 1 hr., followed by centrifuging and analysis of nitrogen in the supernatant. Protein nitrogen was estimated by difference. Trichloroacetic acid

extractions of intact cells and of homogenates of intact cells yielded identical amounts of non-protein nitrogen.

Lactate was measured by the method of Hullin & Noble (1953), with the modifications suggested by Wolf (1955).

Purification of medium components and flasks. Solutions of potassium phosphate, magnesium sulphate, calcium nitrate and ammonium glutamate at the concentrations given below were adjusted to pH 6 and shaken with successive small volumes of 0·01% diphenylthiocarbazone (dithizone) in carbon tetrachloride.

Malic acid and sucrose solutions were freed from zinc by passage through a 30 mm. × 300 mm. column of AG 50WX8 resin, processed from Dowex 50 by Bio-Rad Laboratories, Richmond, Calif.

The culture flasks were cleaned by soaking for at least 3 weeks in nitric acid–water (1:1). After each use they were returned to the nitric acid treatment until required.

Medium. This was modified from that of Hutner, Bach & Ross (1956) and contained per litre: M-potassium phosphate buffer, pH 6·0, 2 ml.; Mg, 40 mg. (as $MgSO_4$); Ca, 4 mg. (as $CaNO_3$); Fe, 2 mg. (as $FeSO_4$); Mn, 0·5 mg. (as $MnSO_4$); Cu, 0·064 mg. (as $CuSO_4$); Zn, when present, 1·0 mg. (as $ZnSO_4$); DL-malic acid, 270 mg.; ammonium glutamate, pH 6·0, 3 g.; ethanol, 3 g.; thiamine–HCl, 60 mg.; cyanocobalamin, 10 μg. The pH was adjusted to 3·5 with hydrochloric acid.

Growth conditions. Media were dispensed in 1 or 1·5 l. volumes into 2-l. Erlenmeyer flasks, autoclaved and inoculated with exponentially growing cells. The flasks were placed on a Gyrotory shaker, model G10 of the New Brunswick Scientific Co., New Brunswick, N.J., U.S.A., in the dark at a temperature of 21–25°.

Harvesting. The culture flasks were first cooled in ice, then the contents transferred to 1 l. polythene centrifuge bottles and centrifuged at 1000 rev./min. for 1 min. at 0° in the International refrigerated centrifuge, model PR-2. The supernatant fluid was carefully siphoned off and the cells were washed twice in metal-free water.

Manometry. Respiration was measured by conventional manometric techniques (Umbreit, Burris & Stauffer, 1957). Standard conditions for following lactate oxidation were as follows: the side arm of Warburg vessels contained 30 μmoles of substrate or water; the main compartments contained 0·03 M-glycylglycine–0·03 M-potassium phosphate buffer, pH 3·5, cell suspension (about 500 μg. of protein nitrogen) and metal-free water to a final volume of 3·0 ml. In addition, 0·3 ml. of 10% potassium hydroxide was added to the centre wells. The vessels were shaken at 25° or 30°. After equilibration for 30 min., the contents of the side arms and main compartments were mixed. Respiration rates were calculated from the linear rates of oxygen uptake subsequently established. Oxygen uptake was calculated as q_{O_2} or as μl. of oxygen consumed/hr.

Assay of lactic dehydrogenase. For the manometric measurement of lactate dehydrogenase, the cells were suspended in 0·1 M-potassium phosphate buffer, pH 8, to a concentration of about 1 mg. of protein nitrogen/ml., frozen and thawed twice in an acetone–solid carbon dioxide bath, and stored briefly in ice–water. To obtain a homogenate (greater than 99% cell breakage), the frozen and thawed cells were passed three times through a Logeman mill (Fisher Scientific Co., Pittsburgh, Pa.). The assay conditions were: side arm, 0·3 ml. of neutralized 0·9 M-potassium lactate or water; main compartment, 0·1 M-potassium phosphate buffer, pH 8, 0·3 mM-phenazine methosulphate, 1 mM-DPN, about 1 mg. of protein nitrogen of cells, all final concentrations; the final volume was 3·0 ml. In addition 0·2 ml. of 10% potassium hydroxide was included in the centre well. The mixture was shaken at 25°; after 15 min. of equilibration the side arm and main compartment were mixed. Activity was calculated from the linear rate of oxygen uptake subsequently established.

Spectrophotometric measurement of lactate dehydrogenase was similar to the manometric assay: 0·1 M-potassium phosphate buffer, pH 8·0, 1 mM-DPN and 0·09 M-potassium lactate (when present) in a final volume of 3·0 ml. were placed in silica cuvettes. The enzyme preparations were clear supernatants after the centrifuging (20 000*g* for 30 min. at 2°) of total homogenates of *Euglena*. DPNH production was observed in a Beckman DU spectrophotometer at 340 mμ with the sample compartment maintained at 30°. Activities were obtained from the slope of the linear plot of extinction readings against time taken for 3 min.

RESULTS

Since several pyridine nucleotide-dependent dehydrogenases are zinc metalloenzymes (Vallee, 1959), in looking for an enzyme under the control

Table 1. *Effect of zinc deficiency on lactate oxidation by intact* Euglena

In Warburg vessels: 0·01 M-lactate, 0·03 M-glycylglycine buffer, pH 3·5, and cells (about 0·5 mg. of protein N). Oxygen uptake was measured by conventional manometry at 30°. Figures in parentheses refer to extra oxygen uptake found upon addition of lactate.

Expt. no.	Culture	$-q_{O_2}$ No substrate	DL-Lactate	D-Lactate	L-Lactate
1	Normal	211	688 (477)	664 (444)	758 (518)
	Exponential-phase cells	227	739 (512)	617 (401)	739 (505)
2	Normal	96	646 (550)	586 (487)	—
	Stationary-phase cells	110	579 (469)	550 (460)	—
3	Normal	71	—	888 (817)	—
	Stationary-phase cells	78	—	818 (740)	—
4	Zinc-deficient	115	156 (41)	139 (23)	152 (38)
	Stationary-phase cells	114	154 (40)	142 (25)	160 (48)
5	Zinc-deficient	93	—	125 (32)	—
	Stationary-phase cells	86	—	113 (27)	—

of zinc, it seems reasonable to examine the enzymes of respiration. Respiration of *Euglena* with a number of substrates showed the most striking respiratory response with lactate. Normal cells, both in the exponential and stationary phases of growth, oxidize lactate at extremely rapid rates (Table 1). These rates are independent of optical isomerism. On the other hand, zinc-deficient cells in the presence of lactate absorb oxygen at rates only slightly greater than controls. With both types of cells oxygen uptake was linear with time for at least 100 min. and was proportional to the cell concentration. Both types of cells were saturated at the substrate concentration used and the observed rates were reproducible (Table 1).

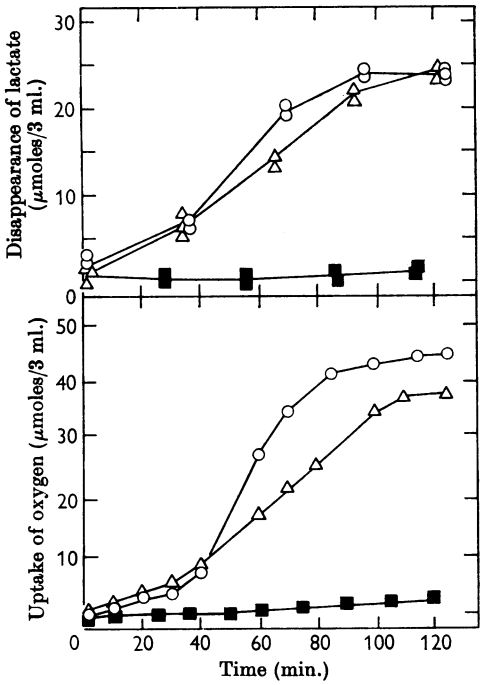

Fig. 1. Effect of zinc on lactate and oxygen consumption. Parallel flasks and Warburg vessels containing washed cells suspended in glycylglycine–phosphate, pH 3·5, were shaken at 30°; at zero time D-lactate was added at a concentration of 25 μmoles/3 ml.; samples were removed from the open flasks at various times for lactate analyses and oxygen uptake was followed in the Warburg vessels; in the upper figure the lactate disappearance and in the lower figure the oxygen uptake corrected for endogenous uptake are plotted against time; 3 ml. of reaction mixture corresponds to 0·615 mg. of protein nitrogen of normal cells in exponential phase (△), 0·701 mg. of protein nitrogen of normal cells in stationary phase (○), and 0·698 mg. of protein nitrogen of zinc-deficient cells (■). The non-linear oxygen uptake of the normal, stationary-phase cells is due to rapid depletion of the lactate in the presence of a rather high concentration of cells.

The rates of oxygen uptake reflected the rates of lactate metabolism, as shown by the measurement of lactate disappearance (Fig. 1). No lactate disappearance was detected in zinc-deficient cells.

We have thus demonstrated a lesion in lactate metabolism in zinc-deficient *Euglena*. What enzyme (or enzymes) is responsible for the failure of lactate oxidation? There is presumably a sequence of enzymes involved in the normal oxidation of lactate, but since the oxygen uptake with certain other substrates (e.g. ethanol and octanoate) is not so drastically altered by zinc deficiency, we deduce that the step under the control of zinc is specific to lactate metabolism. Our hypothesis: the step controlled by zinc is lactate dehydrogenase.

This hypothesis was tested by assay of this enzyme manometrically by coupling it to oxygen via phenazine methosulphate.

In crude enzyme preparations activity was linear with time (Fig. 2) and with enzyme concentration; DPN promoted activity, but only slightly (Table 2); phenazine methosulphate was essential; activities in completely broken cells and in cells merely

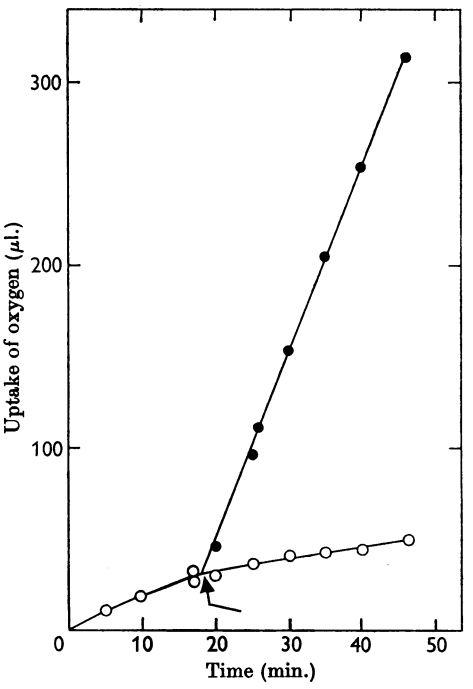

Fig. 2. Time course of oxygen uptake in manometric assay for lactate dehydrogenase. Frozen and thawed normal *Euglena* (2·25 mg. of protein nitrogen) in 0·1 M-phosphate buffer, pH 8, were shaken in Warburg vessels with 3 mM-DPN and 0·3 mM-phenazine methosulphate; at the time indicated by the arrow 0·27 m-mole of D-lactate was tipped from side arm; final volume, 3·0 ml. ○, Control; ●, plus lactate.

subjected to freezing and thawing were virtually identical; and the D(−)-isomer was strongly favoured (Table 4).

A comparison of lactate-dehydrogenase activities in normal and zinc-deficient cells provides a test of the hypothesis that lactate dehydrogenase is the site of zinc control of lactate metabolism. The results are shown in Table 3. The lactate dehydrogenase of zinc-deficient cells was one-fourth to one-third of the values found with zinc-sufficient controls.

The hypothesis is thus supported qualitatively but not quantitatively; although activity observed in the normal cell extracts is nearly sufficient to account for the observed high rates of respiration with lactate, the activity of zinc-deficient extracts is substantially greater than that of the corresponding intact cells.

One possible explanation is that the zinc-deficient cells fail to absorb or accumulate lactate. This possibility has not been tested.

A second possibility is that the lactate-dehydrogenase assay may have included the activities of several species of the enzyme, only one of which was sensitive to zinc and responsible for the oxygen uptake observed with intact cells.

Preliminary support for this hypothesis is provided by two observations:

(1) Activity towards the D- and L-enantiomorphs is highly differentiated in the normal preparations, whereas the zinc-deficient preparations are indifferent to optical activity (Table 4).

(2) When the optically clear supernatant obtained by centrifuging broken-cell preparations is examined spectrophotometrically for DPN reduction, substantial lactate-dehydrogenase activity is observed. This reaction is also linear with enzyme concentration and time and is specific for the D-enantiomorph (Fig. 3). The spectrophotometric assay, employed on normal and zinc-deficient preparations, shows activities independent of zinc concentration (Table 5).

Thus it appears highly probable that zinc-dependent and zinc-independent lactate-dehydrogenase activities are included in the manometric assay.

Table 2. *Requirements for assay of lactate dehydrogenase*

Standard conditions: in main compartment of Warburg vessels, 0·1 M-potassium phosphate buffer, pH 8, 0·3 mM-phenazine methosulphate, 1 mM-DPN and 1·34 mg. of protein nitrogen of frozen and thawed normal cells; in side arm, 0·3 ml. of 0·9 M-D(−)-lactate; final volume, 3·0 ml.; temp., 25°. Activity is expressed as μl. of oxygen/hr. and is an average of two flasks.

Conditions	Lactate-dehydrogenase activity
Standard	728
Without substrate	58
Without DPN	642
Without phenazine methosulphate	44

DISCUSSION

Effects of zinc on respiration have been only rarely noted. Probably the first such observation was that of Kosinski (1901). He found that the carbon dioxide production of *Aspergillus niger* rose from 7·4 to 23·7 mg./hr. within 1 day after the addition of 0·003 % of zinc sulphate. This observation was considered in the context of 'chemische Reizung' (cf. review by Chesters & Rolinson, 1951). The effect on respiration was considered to be a non-specific response to zinc and not a functional role of zinc in respiration.

Tsui (1949) showed that zinc-deficient tomato leaves respired at slightly higher rates than did normal leaves. The reduced rate of lactate meta-

Table 3. *Lactate-dehydrogenase activity in normal and zinc-deficient cells*

Lactate-dehydrogenase activities of frozen and thawed cells were determined by the manometric method; activities are expressed as $-q_{O_2}$; D-lactate, 0·01 M final concentration; rates marked with (*) were above linear range of enzyme concentrations and are lower than true rates.

	$-q_{O_2}$					
	Normal			Zinc-deficient		
Expt. no.	Endogenous	Plus lactate	Difference	Endogenous	Plus lactate	Difference
1	29	552	518	—	—	—
	42	586	552	—	—	—
2	58	456	398	76	172	96
	39	431	392	60	182	122
3	13	269*	256*	0	114	114
	14	302*	288*	6	108	102
4	—	—	—	17	82	65
	—	—	—	22	104	82
Average	—	401	—	—	—	97

Table 4. *Effect of optical activity on lactate-dehydrogenase activity*

Standard conditions of manometric assay; activity is expressed as μl. of oxygen/hr.; normal cells were grown in 10 μM-zinc medium; zinc-deficient cells were grown with no added zinc.

Enzyme	Addition	Lactate-dehydrogenase activity
Normal cells	None	26
	0·09 M-D-Lactate	372
	0·09 M-L-Lactate	90
		85
Zinc-deficient cells	None	21
		27
	0·09 M-D-Lactate	99
		127
	0·09 M-L-Lactate	96
		92

Table 5. *Diphosphopyridine nucleotide-dependent lactate dehydrogenase in the non-particulate fraction*

DPN reduction by high-speed supernatants was followed spectrophotometrically at 340 mμ at 30°; activities were reported as actual rates observed (ΔE/min.) and as μmoles of DPNH formed/min./mg. of protein nitrogen of total homogenate; additions, except for phenazine methosulphate, were identical with manometric assay.

Expt. no.	Zinc added to medium	Lactate-dehydrogenase activity with D(−)-lactate (ΔE_{340}/min.)	Lactate-dehydrogenase activity with D(−)-lactate (μmoles of DPNH/min./mg. of protein nitrogen)
1	0·01 mM	0·041	0·038
		0·047	0·043
2	None	0·020	0·038
		0·020	0·038

bolism in zinc-deficient *Euglena* appears therefore to be without precedent, which is perhaps not surprising considering the paucity of respiratory studies on zinc-deficient organisms. Indeed, among the organisms whose zinc nutrition has been examined (cf. review of Baumeister, 1955), relatively little is known of lactate metabolism. Nason, Oldewurtel & Probst (1952) found that lactate oxidation by tomato-leaf homogenates was unaffected by zinc deficiency, but the activity of even control homogenates was vanishingly small. In *Euglena* lactate serves as a substrate for respiration (Danforth, 1953). The question whether lactate metabolism is affected in other zinc-deficient organisms cannot be answered, nor is it known whether this phenomenon is specific to zinc.

The DPN-dependent lactate dehydrogenase of rabbit muscle is a zinc-metalloenzyme (Vallee & Wacker, 1956). Initially we interpreted our results

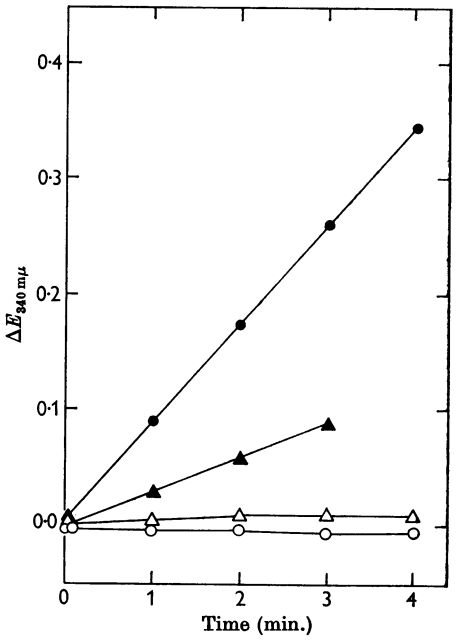

Fig. 3. DPN reduction in the presence of lactate by non-particulate cell fraction. Normal *Euglena* were homogenized in 0·1 M-potassium phosphate and then centrifuged at 25 000*g* for 30 min.; the clear supernatant was decanted and employed as enzyme; enzyme, 3 mM-DPN, plus 0·09 M-D-lactate (●), L-lactate (△), DL-lactate (▲) or water (○) were incubated in the cuvette and DPNH formation was observed at 340 mμ. The low activity with DL-lactate is probably due to the low proportion of D-lactate in the racemate.

with *Euglena* in terms of a rabbit-muscle-like enzyme. The *Euglena* activity, however, is largely D-specific, whereas the rabbit enzyme is L-specific. However, D-specific enzymes have been identified and partially purified from animal mitochondria (Tubbs & Greville, 1959; Tubbs, 1960), yeast (Boeri, Cremona & Singer, 1960; Lebeyrie, Slonimski & Naslin, 1959), *Leuconostoc mesenteroides* (DeMoss, Bard & Gunsalus, 1951) and *Lactobacillus plantarum* (Dennis & Kaplan, 1960). The bacterial enzymes are pyridine nucleotide-dependent. The first two of these enzymes are pyridine nucleotide-independent and are active with D-hydroxy acids generally. They are sensitive to chelating agents, and, for the yeast enzyme, Curdel, Naslin & Lebeyrie (1959) and Boeri *et al.* (1960) have suggested that the enzyme contains zinc as an essential component.

From the data of Fig. 4 and Table 4, it is clear that *Euglena* contains a pyridine nucleotide-dependent lactate dehydrogenase; the DPN–lactate-dehydrogenase activity, however, is unaffected by the zinc concentration of the cells. The

next step is to separate the zinc-sensitive enzyme. This may be an enzyme similar to the mitochondrial lactate dehydrogenase described by Tubbs (1960) or the D-hydroxy acid dehydrogenase of yeast.

SUMMARY

1. Intact cells of *Euglena gracilis* grown in a medium containing sufficient zinc oxidize lactate at high rates (400–800 μl. of oxygen consumed/hr./mg. of protein nitrogen). Zinc-deficient *Euglena* oxidizes lactate at a rate of about 40 μl. of oxygen/hr.

2. The failure of lactate oxidation in zinc deficiency is due in part to decreased activities of D-lactate dehydrogenase.

3. A manometric method is described for measuring lactate-dehydrogenase activity in *Euglena* homogenates or frozen and thawed cells.

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The Enzymic Synthesis of Aryl Sulphamates

3. THE SPECIFICITY AND MECHANISM OF THE ACTIVATION OF RAT-LIVER ARYLAMINE SULPHOKINASE BY 17-OXO STEROIDS*

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In the previous papers of this series it was shown that the arylamine sulphokinase of rat liver was activated by 17-oxo steroids (Roy, 1960) and that the kinetics of the activation by 3β-methoxyandrost-5-en-17-one (Roy, 1961) were consistent with a reaction mechanism formally similar to that found in partial competitive inhibition (Dixon & Webb, 1958). In this type of inhibition the competitive effect is only partial in the sense that an enzyme–inhibitor–substrate complex can be formed and the velocity constant for the breakdown of this complex into the reaction products is the same as that for the breakdown of the normal enzyme–substrate complex. As in true competitive inhibition the net effect of the addition of the inhibitor is a decrease in the apparent affinity of the enzyme for the substrate. Activation can occur by a mechanism which is formally similar if the net effect of the modifier is to increase the apparent affinity of the enzyme for the substrate.

* Part 2: Roy (1961).