BBA 45966

# THE ROLE OF THE α-GLYCEROPHOSPHATE SHUTTLE IN THE REOXIDATION OF CYTOSOLIC NADH IN EHRLICH ASCITES TUMOUR CELLS

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(Received February 20th, 1970)

SUMMARY

1. Addition of glucose to Ehrlich ascites tumour cells is able to release partially the inhibition by rotenone of the endogenous respiration in the hyperdiploid strain, but has almost no effect on the rotenone-blocked respiration of the hyperdiploid Lettré mutant strain.

2. Measurements of redox changes of cytochromes *b*, *c* and nicotinamide-adenine nucleotides in the rotenone-inhibited cells indicate that, upon addition of glucose, (a) a translocation of glycolytic reducing equivalents to the mitochondrial cytochromes does occur in the hyperdiploid strain and (b) a reversal of mitochondrial electron transport is stimulated by the increase in the glycolytic phosphate potential in the Lettré mutant.

3. Analysis of α-glycerophosphate dehydrogenase activity in the two strains shows that the level of this enzyme is low in the hyperdiploid Lettré cells and much higher (about 20 times) in the hyperdiploid cells. Consequently, upon addition of glucose to rotenone-treated cells, α-glycerophosphate is formed in quantities adequate for the operation of the α-glycerophosphate shuttle only in the latter cells. These findings suggest that the operation of the α-glycerophosphate shuttle is mainly responsible for the aerobic oxidation of glycolytic reducing equivalents found in the wild strain. On the other hand, during glucose breakdown in the presence of rotenone only slight differences are found in the lactate accumulation between the two strains.

4. It is concluded that the high rate of aerobic glycolysis in ascites cells cannot be dependent, as suggested by other authors, on the lack of a system for the transfer of glycolytic NADH to the respiratory chain beyond the first phosphorylation site.

## INTRODUCTION

The high aerobic glycolysis in tumour cells has been ascribed to the lack of systems for the intramitochondrial oxidation of cytosolic reducing equivalents[1-3]. Among these systems the α-glycerophosphate shuttle has been extensively studied,

Abbreviations: H cells, Ehrlich hyperdiploid ascites tumour cells; H-L cells, Ehrlich hyperdiploid Lettré ascites tumour cells; TTFB, 4,5,6,7-tetrachloro-2-trifluoromethylbenzimidazole.

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and a low activity of the cytosolic enzyme involved in this shuttle, the α-glycerophosphate dehydrogenase, has been considered typical of tumours with high rates of aerobic glycolysis (see ref. 4 for review). The observations, however, that in some strains of Ehrlich ascites tumour cells α-glycerophosphate can be produced during anaerobic glycolysis[5] or in the presence of rotenone[6], and that some rat hepatomas can form this glycolytic intermediate from glucose 6-phosphate[7], have suggested a re-investigation of the problem.

In earlier studies[8–11] on Ehrlich ascites cells we observed that glucose reactivates by about 30–40 % the endogenous respiration blocked by rotenone in the hyperdiploid strain but has almost no effect in the hyperdiploid Lettré strain. The glucose-dependent respiration of the rotenone-treated hyperdiploid strain was found to be sensitive to antimycin A and sulphide.

In the present work the effect of the addition of glucose to rotenone-inhibited Ehrlich ascites cells from the two tumour strains has been further investigated. The results obtained confirm that the intramitochondrial transfer of glycolytic reducing equivalents, which can be oxidized beyond the rotenone-inhibited site, is considerable in the hyperdiploid strain but is negligible in the Lettré mutant. Further, this transfer is closely dependent on the operation of the α-glycerophosphate shuttle and does not appear to be a significant factor in the control of the rate of aerobic production of lactate in these cells.

## MATERIALS AND METHODS

Two strains of Ehrlich ascites tumour cells, the hyperdiploid (H) and the hyperdiploid Lettré mutant (H-L), were grown in albino Swiss mice. The cells, withdrawn 7–8 days after the inoculation, were washed in an isotonic saline medium (154 mM NaCl, 6.2 mM KCl, 11 mM sodium phosphate buffer (pH 7.4))[12] and resuspended in the same medium for the experiments. When slightly haemorrhagic, the ascitic fluid was freed from haemoglobin by differential centrifugation. Samples with gross contamination by erythrocytes were discarded.

Oxygen uptake was measured polarographically with a Clark oxygen electrode. Redox changes of cytochromes *b* (430–410 nm) and *c* (550–540 nm) were measured at room temperature in a dual wavelength/split-beam Aminco-Chance spectrophotometer. Nicotinamide-adenine dinucleotides were measured at room temperature in an Eppendorf photometer with the fluorescence attachment. Excitation was at 313–366 nm (primary filter), and the emitted fluorescence was recorded at 430–3000 nm (secondary filter). Lactate dehydrogenase (L-lactate: NAD oxidoreductase, EC 1.1.1.27) and α-glycerophosphate dehydrogenase (L-glycerol 3-phosphate: NAD oxidoreductase, EC 1.1.1.8) activities were determined in cell-free extracts obtained by Ultra-Turrax (Janke and Kunkel K.G) disintegration at 0° (6 times for 4 sec) and centrifugation (twice at 1600 × *g* for 5 min and once at 26000 × *g* for 15 min). The enzymic activities were measured by following the rate of NADH oxidation at 366 nm, in the presence of excess pyruvate or dihydroxyacetone phosphate, using an Eppendorf photometer.

Mitochondria were prepared by the method of KOBAYASHI *et al.*[13] with slight modifications. The respiratory control ratios[14] with succinate and rotenone were 3.03 and 3.25 for mitochondria from H and H-L strains respectively (average of four

experiments). α-Glycerophosphate oxidase activity was measured by the method of KADENBACH[15] in submitochondrial particles; the particles were obtained by subjecting mitochondria to Ultra-Turrax disintegration for 8 periods of 15 sec each, at intervals of 30 sec. During the disintegration procedure mitochondria were held in tubes immersed in an ice–salt mixture (about —15°).

α-Glycerophosphate and lactate were measured in neutralized perchloric acid extracts by enzymic methods[6]. Proteins were determined by the biuret method[16].

The 4,5,6,7-tetrachloro-2-trifluoromethylbenzimidazole (TTFB) was kindly supplied by Dr. R. B. Beechey of Shell Research Ltd., Milstead Laboratory of Chemical Enzymology, Sittingbourne, Kent (Great Britain). Enzymes, coenzymes and substrates for metabolite and enzymic activity determinations were obtained from Boehringer and Soehne (Mannheim). Rotenone was purchased from K and K Lab. Inc., Plainview, New York. All other chemicals were products of Sigma (St. Louis) or E. Merck (Darmstadt).

## RESULTS AND DISCUSSION

### *The effect of glucose on rotenone-inhibited respiration*

Fig. 1 shows a comparison of the effect of 10 mM glucose on the rotenone-inhibited respiration in the two strains of ascites cells. In the H cells (A) the addition of glucose causes a release of about 30 % of the endogenous respiration, whereas in the H-L cells (B) it elicits only a slight effect (less than 5 % reactivation). Further addition of 25 mM α-glycerophosphate causes no effect on H cells and only a doubling of the rotenone-insensitive respiration in H-L cells. Results for a number of experi-

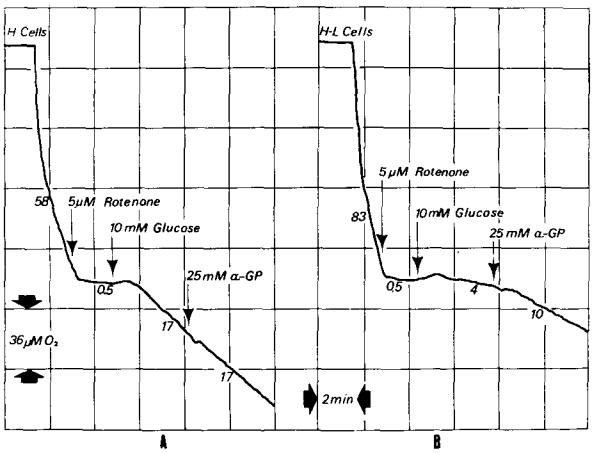



Fig. 1. Polarographic measurements of oxygen consumption in Ehrlich ascites tumour cells from normal hyperdiploid (H) and Lettré mutant (H-L) strains. Rotenone strongly inhibits endogenous respiration in both strains. The subsequent addition of glucose stimulates the respiration in H cells (A), but has almost no effect in H-L cells (B). The glucose-induced respiration is then slightly increased by α-glycerophosphate (DL-α-glycerophosphate, α-GP) only in H-L cells. 0.3 ml cell suspension, containing 18.2 mg and 17.6 mg dry weight of H and H-L cells respectively, was added to 1.7 ml of isotonic phosphate medium (pH 7.4) (for composition see MATERIALS AND METHODS). Temperature, 25°. The numbers along the traces indicate $O_2$ consumption in nmoles per min.

TABLE I

REACTIVATION BY GLUCOSE AND α-GLYCEROPHOSPHATE OF THE ENDOGENOUS RESPIRATION INHIBITED BY ROTENONE IN H AND H-L CELLS

Ascites cells (5–10 mg dry weight/ml) were treated with 10 μM rotenone. $O_2$ uptake was measured polarographically at 25°. Glucose, 10 mM; α-glycerophosphate, 25 mM. For other conditions see Fig. 1. Results are expressed as mean ± S.E. (number of observations).

| *Addition* | % *Reactivation* | |
|---|---|---|
| | *H cells* | *H-L cells* |
| Glucose | 32.4 ± 1.61 (5) | 6.17 ± 0.36 (7) |
| Glucose + α-glycerophosphate | 31.2 ± 2.15 (5) | 10.7 ± 0.08 (7) |
| α-Glycerophosphate | 8.81 ± 0.85 (10) | 6.42 ± 0.82 (11) |
| α-Glycerophosphate + glucose | 39.6 ± 4.17 (7) | 10.1 ± 1.16 (8) |

ments of this type are summarized in Table I. The purpose of adding α-glycerophosphate to intact cells was to test the possibility that an exogenous supply of this substance could compensate for its deficient endogenous production by H-L cells (*cf.* Fig. 5), and so give a release of the rotenone-inhibited respiration analogous to that obtained by glucose addition in H cells. The limited ability of added α-glycerophosphate to stimulate the respiration in the H-L cells may be due to a permeability barrier of the ascites cell membrane to this metabolite. This is suggested by two observations: (1) the stimulation by α-glycerophosphate of the rotenone-blocked respiration in H-L mitochondria ($Q_{O_2}$* at 25° = 37.7 ± 4.0 (5)); and (2) the low α-glycerophosphate accumulation inside the H-L cells after they have been incubated for different time intervals in the presence of this substance and of antimycin A to prevent its intramitochondrial oxidation (O. DIONISI, G. GELMUZZI, T. GALEOTTI AND T. TERRANOVA, unpublished results).

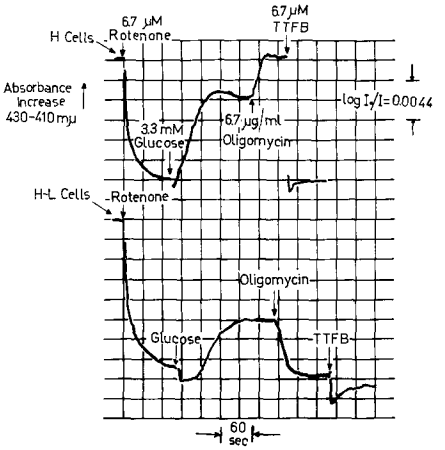
*Redox changes of cytochromes b, c and nicotinamide-adenine dinucleotides in rotenone-inhibited cells*

Fig. 2 shows steady state changes of cytochrome *b* (430–410 mμ) in intact Ehrlich ascites tumour cells from the two strains. Addition of 6.7 μM rotenone causes oxidation of the pigment in both types of cell, as indicated by the downward deflection of the trace. The extent of the oxidation corresponds to 10.2 and 13.7 nmoles/g dry weight for H and H-L cells respectively. Subsequent addition of 3.3 mM glucose reduces in H cells 75% and in H-L cells 40% of the cytochrome *b* oxidized by rotenone. The reduction of cytochrome *b* by glucose in H-L cells is preceded by a small oxidation, as observed previously[11,12]. The further addition of oligomycin (6.7 μg/ml) causes an opposite effect in the two strains: it potentiates the effect of glucose in H cells, but completely reverses such effect in H-L cells.

This observation suggests that a different mechanism should be invoked for explaining the glucose-induced reduction of cytochrome *b* in the two kinds of cell. Oligomycin at the concentration used here inhibits the endogenous respiration by about 80% (81.0% ± 1.7 (11)) and the rotenone-insensitive, glucose-dependent,

* μl $O_2$/mg protein per h.

respiration by about 20 % (20.8 % ± 1.3 (12)) in H cells indicating that the latter is loosely coupled. Thus the oligomycin effect in H cells is probably due to reduced electron flow through phosphorylation sites between cytochrome *b* and $O_2$. On the other hand, as reported previously[11], the oligomycin sensitivity of the cytochrome *b* reduction by glucose in the Lettré cells may be attributed to the inhibition of the reversed electron transport induced by the cytosolic phosphate potential ($ATP/ADP \cdot P_i$) generated during the glycolysis. The final addition of the uncoupler TTFB at 6.7 $\mu$M concentration oxidizes completely cytochrome *b* in H cells and has obviously almost no effect on H-L cells.



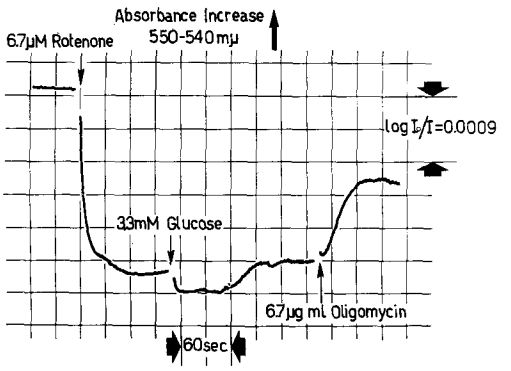




Fig. 2. Spectrophotometric measurements of cytochrome *b* in H and H-L ascites cells. The final concentrations of cell suspension were 7.15 and 6.84 mg dry weight/ml for H and H-L cells, respectively.

Fig. 3. Spectrophotometric measurements of cytochrome *c* in H ascites cells. The final cell suspension concentration was 6.05 mg dry weight/ml.

Table II summarizes the effects of oligomycin and TTFB, as well as that of other inhibitors of oxidative phosphorylation and glycolysis, upon the reduction of cytochrome *b* induced by glucose in rotenone-inhibited hyperdiploid ascites cells. Results with pyruvate are also included in this table. The glucose-induced reduction is abolished by TTFB, iodoacetate, fluoride and cyanide, and inhibited nearly 80 % by pyruvate and 70 % by antimycin A. In the presence of oligomycin the inhibitory effects of fluoride and pyruvate[18] are less evident, whereas that of iodoacetate is unchanged, indicating that the former two agents are unable to block completely the availability to the respiratory chain of glycolytic reducing equivalents, as iodoacetate does.

Measurements of redox changes of cytochrome *c* (550–540 nm) in H cells are shown in Fig. 3. After oxidation by rotenone, the glucose addition causes a small reduction which is, even for this pigment, potentiated by oligomycin. An oxidation of cytochrome *c* was, instead, observed upon glucose addition to rotenone- or amytal-treated H-L cells[11,19].

Fig. 4A shows the response of nicotinamide-adenine dinucleotides to the addition of rotenone and glucose to H and H-L cells. In both kinds of cell rotenone causes reduction of the coenzyme, which is presumably mainly mitochondrial. The lower

TABLE II

EFFECT OF DIFFERENT AGENTS UPON THE REDUCTION OF CYTOCHROME *b* INDUCED BY GLUCOSE IN ROTENONE-INHIBITED HYPERDIPLOID CELLS

6.7 $\mu$M rotenone was present in all the experiments. Substrates and inhibitors were added before (iodoacetate, NaF) or after rotenone at the following final concentrations: 3.3 mM glucose, 6.7 $\mu$g/ml oligomycin, 6.7 $\mu$M TTFB, 1.7 mM iodoacetate, 16.5 mM NaF, 3.3 mM sodium pyruvate, 6.7 $\mu$g/ml antimycin A and 1.3 mM KCN. The final dry weight for 1 ml of cell suspension (H strain) in the cuvette was 4–7 mg. The cytochrome *b* concentration was calculated by using $\Delta\varepsilon$ (430–410 nm) = 180 $mM^{-1} \cdot cm^{-1}$ (ref. 17).

| *Expt. No.* | *Substrate of inhibitor* | *Cytochrome b reduction (nmoles/g dry weight)* |
|---|---|---|
| 1 | Glucose | 7.9 |
| | + oligomycin | 11.3 |
| | + TTFB | 0.0 |
| | + oligomycin + TTFB | 0.0 |
| 2 | Glucose | 8.5 |
| | + oligomycin | 11.9 |
| | + iodoacetate | 0.0 |
| | + NaF | 0.0 |
| | + iodoacetate + oligomycin | 0.0 |
| | + NaF + oligomycin | 5.8 |
| | + NaF + oligomycin + TTFB | 0.0 |
| 3 | Glucose | 5.3 |
| | + oligomycin | 10.9 |
| | + pyruvate | 1.1 |
| | + pyruvate + oligomycin | 6.4 |
| 4 | Antimycin A | 18.2 |
| | Glucose | 8.6 |
| | + antimycin A | 21.1 |
| 5 | $CN^-$ | 23.7 |
| | Glucose | 7.1 |
| | + $CN^-$ | 23.7 |

extent of reduction in H-L than in H cells may be due to the initial, more highly reduced steady state of respiratory carriers, under the conditions of endogenous metabolism, in the former cells (*cf.* Fig. 2, for cytochrome *b*). On addition of glucose a further reduction is observed, due to the formation of NADH at the glyceraldehyde-3-phosphate dehydrogenase level, as indicated by the sensitivity to iodoacetate (not shown). The kinetics of the glucose-induced $NAD^+$ reduction are different in the two strains. In H cells a more reduced steady state of the coenzyme is reached immediately after the addition of glucose, whereas in H-L cells a large initial reduction is followed by a partial reoxidation of NADH. As shown in Fig. 4B, the phase of reoxidation in H-L cells is abolished by 50 mM sodium oxamate. This substance inhibits lactate formation by competing with pyruvate for lactate dehydrogenase[20]. The biphasic response of NADH in H-L cells may, therefore, be due to its accumulation in the cytosol and subsequent partial reoxidation only at the lactate dehydrogenase level by pyruvate. On the other hand this type of response would be absent in H cells, where reducing equivalents can also be quickly reoxidized by an active shuttle mechanism operating between cytosol and mitochondria.

These results strongly support the earlier conclusions on the different effect exerted by the glycolytic reactions on the rotenone-inhibited respiratory chain in

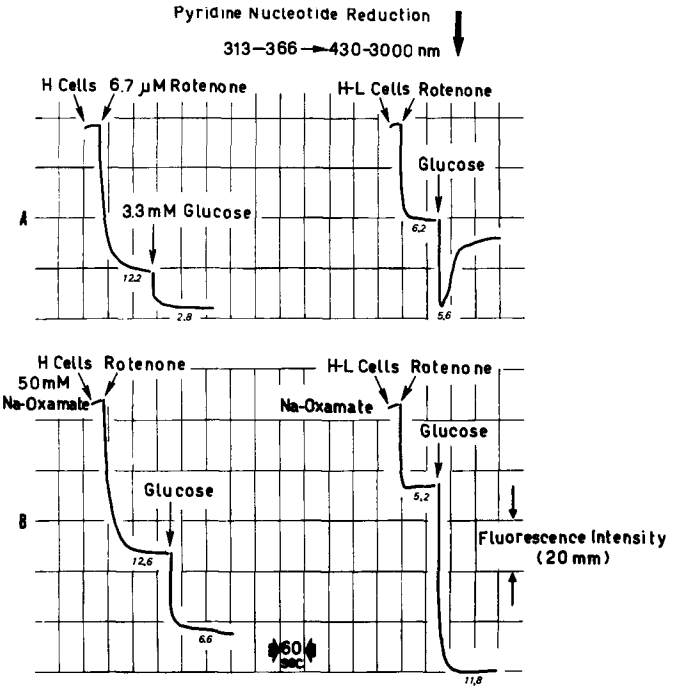



Fig. 4. Fluorimetric recordings of the redox changes of nicotinamide-adenine dinucleotides induced by rotenone and glucose in H and H-L ascites cells, in the absence (A) or presence (B) of sodium oxamate. The final cell-suspension concentrations were 4.73 (H) and 6.02 (H-L) mg dry weight/ml. The numbers along the traces represent arbitrary units (mm/mg dry weight) and refer to the increment of fluorescence intensity produced by each treatment.

the two strains of ascites cells[9–11]. In H-L cells the low efficiency of a system for the intramitochondrial translocation of glycolytic reducing equivalents makes it possible for the glycolytic phosphate potential to exert a direct control on the oxidation–reduction state of the mitochondrial electron transport chain. On the other hand, in the H cells this control is exerted by the glycolytic redox potential ($NAD^+/NADH$ ratio) through an active shuttle mechanism for the aerobic oxidation of cytosolic NADH.

*Activities of lactate dehydrogenase, α-glycerophosphate dehydrogenase and α-glycerophosphate oxidase*

Analysis of lactate dehydrogenase and α-glycerophosphate dehydrogenase activities (Table III) in cell-free extracts from the two types of cell shows that, whereas for the lactate dehydrogenase enzyme the activity is only 1.5 times higher in H cells than in H-L cells, for α-glycerophosphate dehydrogenase it is more than 20 times higher in the former cells*. Consequently, the lactate dehydrogenase/α-glycerophosphate dehydrogenase ratio appears to be 15 times higher in the H-L

* The activities of lactate dehydrogenase in the two strains and that of α-glycerophosphate dehydrogenase in H cells are comparable to those found in normal rat liver: lactate dehydrogenase, 280.2 ± 29.8 (4); α-glycerophosphate dehydrogenase, 34.5 ± 4.2 (4); lactate dehydrogenase/α-glycerophosphate dehydrogenase ratio, 8.2 ± 0.7 (4).

TABLE III

COMPARISON OF CYTOSOLIC AND MITOCHONDRIAL ENZYMIC ACTIVITIES IN H AND H-L CELLS

Enzymic activities, expressed in μmoles/h per mg protein, were determined as described in MATERIALS AND METHODS. The values represent the means ± standard error of mean; number of observations in parentheses.

| *Ehrlich ascites cells (strain)* | *Lactate dehydrogenase* | *α-Glycerophosphate dehydrogenase* | *Lactate dehydrogenase/ α-glycerophosphate dehydrogenase* | *α-Glycerophosphate oxidase* |
|---|---|---|---|---|
| H | 301.2 ± 29.8 (15) | 31.3 ± 3.3 (15) | 11.2 ± 1.8 (15) | 0.251 ± 0.033 (5) |
| H-L | 200.4 ± 14.7 (15) | 1.36 ± 0.13 (15) | 167.1 ± 21.8 (15) | 0.477 ± 0.051 (5) |

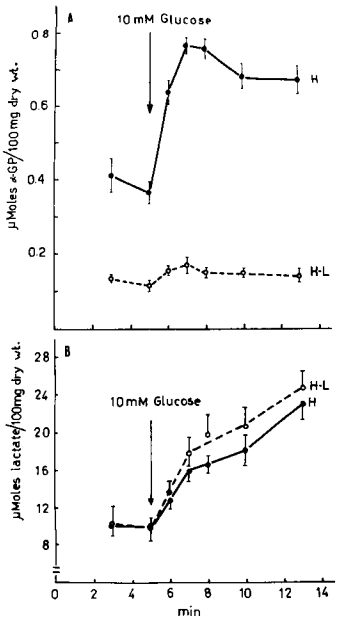



Fig. 5. α-Glycerophosphate (α-GP) and lactate production upon addition of glucose to H and H-L ascites cells inhibited by rotenone. At time = 0 the cells were treated with 10 μM rotenone. The glucose addition is indicated by the arrow. Erlenmeyer flasks containing 20–25 ml of cell suspension were incubated at 25° under air tension in a Dubnoff shaker. Samples (1 ml containing 25–30 mg dry weight cells) were withdrawn at intervals. The samples were added to the same volume (1 ml) of 6% (w/v) ice-cold perchloric acid and centrifuged. The neutralized extracts were assayed enzymically as described in ref. 6. The vertical lines represent twice the standard error of the mean of 6–7 experiments.

cells than in H cells. From these results it is possible to infer that the lack of the glucose-dependent respiration in rotenone-inhibited H-L cells is largely due to the low activity of α-glycerophosphate dehydrogenase. That the rate-limiting factor in the operation of the α-glycerophosphate shuttle in H-L cells is represented by the low activity of the 'soluble' enzyme is also supported by measurements of the α-glycerophosphate oxidase (L-glycerol-3-phosphate: cytochrome *c* oxidoreductase, EC 1.1.2.1) activity in the mitochondria from the two strains. As shown in Table III, this flavoenzyme is even more active in H-L than in H cells.

*α-Glycerophosphate and lactate formation by glucose in rotenone-inhibited cells*

Fig. 5 shows the steady state changes of α-glycerophosphate (A) and the lactate accumulation (B) in the two strains upon addition of glucose to rotenone-treated cells (at time zero). The production of α-glycerophosphate, as expected, is very small in the H-L cells, whereas in H cells it is appreciably higher, a value equal to twice the initial, endogenous level being attained within 2 min of the addition of glucose. However, as shown in Fig. 5B, the greater efficiency of the α-glycerophosphate shuttle in H cells has only a slight influence on the rate of lactate formation in these cells. Indeed this product accumulates in almost equal amounts in the two strains.

From the results obtained in this work it is possible to conclude that the high rate of aerobic lactate production in Ehrlich ascites tumour cells is largely independent of the ability that these cells have to reoxidize glycolytic NADH in the mitochondria beyond the first phosphorylation site.

AKNOWLEDGEMENTS

We are grateful to Dr. Britton Chance for his encouragement and helpful criticism of the manuscript. We thank Messrs. Marco Cagossi and David Oscier for their help during the first phase of this work. The Ehrlich-Lettré hyperdiploid strain of ascites tumour cells was kindly provided by Dr. E. Patterson of the Cancer Research Institute, Fox Chase, Pa. (U.S.A.). This work was in part supported by a grant from Consiglio Nazionale delle Ricerche, Italy.

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