Substrate-dependent utilization of the glycerol 3-phosphate or malate/aspartate redox shuttles by Ehrlich ascites cells

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The rate of transfer of reducing equivalents from cytoplasm to mitochondria has been examined in Ehrlich ascites tumour cells incubated in the presence of lactate. The flux of reducing equivalents was determined from the rate of metabolism of reduced intermediates that are oxidized within the cytosol. The magnitude of the flux of reducing equivalents was dependent on both the concentration of added lactate and the presence of carbohydrate. The rate of flux was twice as great in the presence of glucose and four times as high when glucose and lactate were added together as when lactate was the only added substrate. Fructose was less effective than glucose in stimulating reducing equivalent flux. In the presence of glucose or fructose, there was a substantial accumulation of hexose phosphates, dihydroxyacetone phosphate and glycerol 3-phosphate. Rotenone, an

inhibitor of NADH dehydrogenase, and amino-oxyacetate, which inhibits the malate/aspartate shuttle, were powerful suppressors of reducing equivalent flux from lactate as sole substrate, but were much less potent in the presence of carbohydrate. Antimycin substantially inhibited reducing equivalent flux from all combinations of added substrates, consistent with its ability to block oxidation of reducing equivalents transferred by both the malate/aspartate and glycerol 3-phosphate shuttles. The glycerol 3-phosphate shuttle represents around 80 % of the maximum total observed activity but is active only while glycolytic intermediates are present to provide the necessary substrates of the shuttle. This Ehrlich ascites cell line has an essentially similar total reducing equivalent shuttle capacity to that of isolated hepatocytes.

INTRODUCTION

The energy metabolism of a very wide range of tumours is dependent to a large extent upon glycolysis [1,2]. This contribution of glycolysis to energy metabolism is observed even in an aerobic environment and despite the presence of mitochondria. The various early attempts to explain this included Boxer and Devlin's proposal [3] that neoplastic cells are relatively deficient in reducing equivalent shuttle activity, due especially to a lack of the glycerol 3-phosphate shuttle [4]. They argued, that since the glycerol 3-phosphate and malate/aspartate shuttles [5,6] are responsible for the net transfer of reducing equivalents from cytoplasm to mitochondria, a lack of shuttle capacity would require NADH generated in the cytoplasm during glycolysis to be re-oxidized by means of the reduction of pyruvate to lactate. Thus, high rates of glycolysis would be required to compensate energetically for the depressed rate of oxidative metabolism. Subsequently, a number of workers demonstrated that in many neoplastic tissues the rate of glycolysis was not especially high, and furthermore that intercompartmental shuttle activity was present even in tissues with high glycolytic rates [7]. As a consequence of such studies the view that high glycolytic activity in tumour cells is related directly to low shuttle capacity has generally been discarded.

In our studies with isolated hepatocytes we have found that shuttle activities can have a very important regulatory function [8,9]. The study of these activities in hepatocytes is complicated, however, because substantial reducing equivalent flow occurs not only from cytoplasm to mitochondria during glycolysis but also in the opposite direction during gluconeogenesis [10]. Accordingly, we have chosen to study further the regulatory role

of intercompartmental shuttles in mouse Ehrlich ascites cells, which can be obtained readily in suspension and have a high glycolytic capacity but no gluconeogenic function. Both the malate/aspartate and glycerol 3-phosphate shuttles have been described in Ehrlich ascites cells [11–15], although this extensive literature reveals a great diversity of opinion about which of the possible mechanisms of reducing equivalent transport are functional *in vivo*.

This report describes studies of a strain of Ehrlich ascites tumour cells in which activities of both the malate/aspartate and the glycerol 3-phosphate shuttles can be demonstrated. We show that the type of shuttle used for reducing equivalent transfer is dependent on the nature of the substrates to which the cells are exposed.

MATERIALS AND METHODS

L-[U-¹⁴C]lactate was purchased from New England Nuclear, MA, U.S.A. Beckman Instruments supplied Ready Safe liquid-scintillation fluid. Boehringer Mannheim was the source of enzymes used for metabolite assay of fraction V BSA, which was defatted by the method of Chen [16], and of a calibrated protein standard containing human serum proteins. Amino-oxyacetate (AOAC), antimycin (ANTI) and rotenone (ROT) were obtained from Sigma, MO, U.S.A.; silicone oils (types 200 and 550, blended to give a specific gravity ≈ 1.03) were from Dow Corning Corporation, Midland, MI, U.S.A. Other reagents were of the highest quality commercially available.

The Ehrlich ascites cell line was provided by courtesy of Dr. E. Kotlarsky (Department of Immunology and Molecular Biology, University of Adelaide, South Australia). This line was obtained

originally from Roswell Park Memorial Institute, Buffalo, NY, U.S.A. and has been preserved since its acquisition in 1955 by serial passage of the cells in C57 mice and the storage of passaged cells in liquid N₂. For each of the experiments reported here, the cells were maintained and grown by weekly intra-peritoneal transplantation in 4 or 5 female Balb/c mice aged 8–10 weeks and harvested 6–8 days after inoculation. This mouse strain is maintained as an inbred colony, being fed *ad libitum* on a standardized 'Joint Stock Ration' (Milling Industries, Australia).

The animals were killed by cervical dislocation following anaesthesia with Fluothane (ICL Pharmaceuticals) and immediately dissected, the cells being collected by flushing each peritoneal cavity with about 20 ml of a minimal salts medium [17] containing 0.25 i.u./ml heparin to suppress cell clumping. The medium and pooled cell suspensions were held on ice. After filtration of the suspension through nylon cloth (0.2 mm pore-size) the cells were gently sedimented twice by centrifugation at 140 *g* for 3 min at 4 °C in 50 ml tubes, and finally at 200 *g*, the supernatants being removed by gentle suction. The intermediate pellet fractions and the final erythrocyte-free washed cells were resuspended in the same medium (but without heparin) at a density of approximately 16 mg dry weight per ml, the actual acid-precipitable dry weight density being determined subsequently as described for isolated hepatocytes [18]. Under these conditions 1 g dry weight of cells (*n* = 8) was equivalent to 6.07 ± 0.18 g wet packed weight, 4.7 ± 0.2 × 10⁹ cells, and 0.51 ± 0.02 g of protein (determined using the Pyrogallol Red dye method [19] and a standard containing human serum proteins). Cell integrity was monitored by the ability of the cells to exclude Trypan Blue and also by leakage into the medium of lactate dehydrogenase (EC 1.1.1.27). The latter indicator was found to be more convenient, more reliable and more accurate than the former, because damaged cells fragmented completely during the course of prolonged incubations. Lactate dehydrogenase was stabilized until the time of assay by dilution of cell-free incubation mixture in 4.5% (w/v) BSA solution.

For metabolic studies at 37 °C, cells (15–19 mg dry weight) were incubated under an atmosphere of 95% O₂/5% CO₂, in 25 ml glass scintillation vials containing 2 ml total volume of bicarbonate-buffered medium [17,20,21], in which was dissolved 2.25% (w/v) defatted albumin together with substrates or inhibitors as detailed in the Results section. ANTI and ROT were added to empty vials as acetone solutions that were allowed to evaporate to dryness before the inhibitors were taken up in the medium containing albumin. When AOAC was included, cells were preincubated with the inhibitor for 10 min in the absence of any added substrate. The sealed vials were shaken in a water-bath at 120 oscillations per min with a stroke of 4.5 cm. Metabolism was terminated by the addition of an equal volume of ice-cold 1 M perchloric acid to extract intracellular contents and precipitate protein. Precipitated material was removed by centrifugation, the supernatant fractions were neutralized with a solution of 2 M KOH + 0.2 M KCl, and potassium perchlorate was removed by further centrifugation after 30 min at 0 °C. Metabolites were determined on the neutralized extract using standard enzymic assays [22], by means of a Cobas FARA automated centrifugal analyser (Roche Diagnostics, Basle, Switzerland). The uncorrected optical extinction data were transferred to a MicroVAX computer (Digital Equipment Corporation) for numerical processing. For increased precision, duplicate assays of lactate were done whenever lactate was added at concentrations greater than 10 mM. In experiments to measure the rate of cellular uptake of lactate the silicone oil cell-separation method described previously [23] was used for rapid separation of cells from incubation medium.

The calculated metabolite data are expressed either as concentrations in the total incubation mixture, as intracellular concentrations expressed per gram wet weight of pelleted cells in the case of phosphorylated intermediates, or as average rates of change per gram wet weight of pelleted cells. In the absence of added carbohydrate, the rate of reducing equivalent transfer from cytoplasm to mitochondria was calculated from the rate of lactate disappearance. When carbohydrate was a substrate, it was necessary to allow for reducing equivalents retained in intermediates and derivatives of the glycolytic pathway. The accumulation of dihydroxyacetone phosphate and lactate from glucose is not associated with intercompartmental reducing equivalent transport, whereas that of glycerol and glycerol 3-phosphate reflects a net trapping of reducing equivalents. Therefore, the rate of reducing equivalent flux can be calculated from balance studies using the rate of change of the following expression, which is an algebraic sum, where negative values represent disappearance of carbon: [2 × (glucose + fructose + hexose phosphate + glycerol phosphate + glycerol) + dihydroxyacetonephosphate + lactate]. Pilot studies showed that the level of glyceraldehyde 3-phosphate was very low and could be ignored in the calculation without introducing significant error, and that the pentose phosphate pathway had low activity. Hence, glyceraldehyde 3-phosphate- and pentose phosphate-pathway intermediates were not measured in this study.

Hexokinase (EC 2.7.1.1) and lactate dehydrogenase were assayed using standard spectrophotometric methods [22]. Cell extracts for hexokinase assay were prepared by suspending 100 mg of Ehrlich cells for 30 min at 0 °C in 5 ml of 0.2 M triethanolamine buffer, pH 7.4, containing 0.5% (w/v) Triton X-100, then centrifuging at 5000 *g* for 3 min to remove major cell debris. Apparent *K_m* values of hexokinase for glucose and fructose were calculated from initial rate data obtained at 37 °C with an ATP concentration of 10 mM, using a non-weighted linear least-squares fit to a Lineweaver–Burk function.

When radioactive lactate was included in incubation mixtures, lactate, pyruvate and amino acids were separated from each other by ion-exchange chromatography. A 0.5 ml sample of each incubation mixture was quenched in 1.5 ml of ice-cold ethanol, and 1 ml of the supernatant obtained by subsequent centrifugation was passed through a sequence of columns of Dowex resins [24]. Radioisotope measurements were performed with a Beckman model LS3801 liquid-scintillation spectrometer.

Data are means of values from the stated number of independent experiments together with the corresponding values for the standard error of the mean. Data presented graphically but without error bars are representative examples of at least three similar experiments.

RESULTS

The metabolism of lactate

Lactate was consumed at a rate that was a function of concentration, when added to Ehrlich ascites cells as sole substrate over a concentration range from 1 to 30 mM (Table 1). Lactate removal continued throughout the incubation period, which was normally 60 min in duration. No significant accumulation of glycolytic intermediates or glycerol 3-phosphate was observed when lactate was the only added substrate. Thus, the rate of transfer of reducing equivalents from cytoplasm to mitochondria was taken to be equal to the rate of disappearance of lactate and dependent on the concentration of added lactate (Table 1). To eliminate the possibility that uptake into the cell could be the rate-limiting step, we measured directly the rate of entry of lactate into Ehrlich cells using rapid separation of cells from

Table 1 Metabolism of added lactate

Washed Ehrlich ascites cells were incubated as described in the Materials and methods section with initial concentrations of lactate as indicated. The average rate of lactate removal was calculated over a 30 min incubation period, during which time the rate was constant. The lactate:pyruvate ratio and pyruvate concentration are the values measured at 20 or 30 min. The data are mean values together with standard errors of the means for the number of separate experiments indicated in parentheses.

Lactate added (mM)	Average rate of lactate removal ($\mu\text{mol} \cdot \text{min}^{-1} \cdot \text{g}^{-1}$ wet weight)	Steady-state values	
		Lactate:pyruvate ratio	Pyruvate concentration (mM)
1	0.33 ± 0.01 (4)	4.3 ± 0.5 (4)	0.18 ± 0.01 (4)
2	0.38 ± 0.01 (4)	6.8 ± 0.9 (4)	0.22 ± 0.02 (4)
5	0.54 ± 0.09 (14)	13.2 ± 1.3 (13)	0.35 ± 0.03 (11)
15	0.67 ± 0.05 (45)	40.1 ± 2.1 (44)	0.40 ± 0.02 (36)
30	0.94 ± 0.12 (17)	62.6 ± 2.3 (17)	0.49 ± 0.02 (15)

Figure 1 Time course of lactate metabolism

Ehrlich ascites cells were incubated for various time periods with initial concentrations of lactate of: 1 mM (○); 2 mM (□); 5 mM (△); 10 mM (●); 15 mM (■); and 30 mM (▲). Concentrations of lactate (a), pyruvate (b) and the corresponding values of the lactate:pyruvate ratio (c) were measured as described in the Materials and methods section. The data are from a representative experiment.

Figure 2 Time course of pyruvate metabolism

Ehrlich ascites cells were incubated with initial concentrations of pyruvate of: 0.25 mM (○); 0.5 mM (□); 0.75 mM (△); 1 mM (●); and 2 mM (■), and the amount of pyruvate remaining measured with time. For the inset, the tangential rates of pyruvate removal were calculated for each point of the main figure; the reciprocal values of these rates are plotted against the reciprocal of the corresponding residual pyruvate concentrations. The data are from a representative experiment.

medium by centrifugation through silicone oil. This confirmed that lactate uptake was much faster than the rate of subsequent lactate disposal, the initial and maximal rate of entry being $8.4 \mu\text{mol} \cdot \text{min}^{-1} \cdot \text{g}^{-1}$ when cells were exposed to 15 mM exogenous lactate. Furthermore, intracellular levels of lactate had reached a steady-state within 5 min of the commencement of the incubation.

The rate of net pyruvate accumulation was initially equal to that of lactate removal, but decreased rapidly after the first 5–10 min of incubation as a consequence of further metabolism of the pyruvate. In contrast to the comparatively uniform rate of lactate removal (Figure 1a), the rate of pyruvate accumulation approached zero between 20 and 30 min as the pyruvate concentration neared a steady-state of approximately 0.4 mM (Figure 1b). This steady-state pyruvate concentration increased much less than proportionately with the added lactate concentration at lactate concentrations exceeding 5 mM (Table 1 and Figure 1b). Thus, by about 20 min a near steady-state [lactate]:[pyruvate] ratio was reached (Figure 1c), the magnitude

Figure 3 Time course of glucose removal

Ehrlich ascites cells were incubated for various time periods with initial concentrations of glucose of: 0 mM (○); 2 mM (●); 5 mM (■); and 10 mM (▲). The concentrations of residual glucose (a), of glucose 6-phosphate + fructose 6-phosphate (b) and of dihydroxyacetone phosphate + glycerol 3-phosphate (c) were determined as described in the Materials and methods section. The data are from a representative experiment.

of which was directly related to the lactate concentration (Table 1). Experiments in which cell incubation volume was varied, while cell mass and initial lactate concentration were both unaltered, confirmed that in the presence of lactate the pyruvate concentration was adjusted to approximately 0.4 mM, despite the change in volume (results not shown).

The metabolism of pyruvate

Pyruvate (2 mM), added as sole exogenous substrate, was removed at an initial rate of $0.71 \pm 0.01 \mu\text{mol} \cdot \text{min}^{-1} \cdot \text{g}^{-1}$, approximately one third being reduced to lactate and the remainder, it is assumed, oxidized (Figure 2). The rate of pyruvate removal decreased with time, regardless of the initial concentration added (Figure 2), and at concentrations from 2 mM to

less than 0.1 mM this removal rate was consistent with a Michaelis-Menten kinetic relationship (Figure 2 inset), from which an apparent $(S)_{0.5}$ for pyruvate removal of 1.8 mM was calculated. No steady-state value for the [lactate]:[pyruvate] ratio was achieved during a 90 min incubation period with pyruvate, the ratio remaining less than 5:1 although increasing gradually with time.

The metabolism of glucose and fructose

Glucose was removed at a constant rate, when added alone at initial concentrations of 2–10 mM, until its concentration fell to less than 0.5 mM (Figure 3a). Glucose metabolism was associated with a substantial accumulation of phosphorylated glycolytic intermediates and glycerol 3-phosphate (Table 2), as well as pyruvate and lactate (Table 3). In addition, glycerol accumulated at rates up to $0.16 \mu\text{mol} \cdot \text{min}^{-1} \cdot \text{g}^{-1}$. Measurable amounts of glycolytic intermediates were present until virtually all the added glucose was consumed (Figures 3b and 3c). During glycolysis the cells maintained a [lactate]:[pyruvate] ratio much lower than that in the presence of lactate alone (Table 3), but similar to that achieved by isolated hepatocytes metabolizing glucose [9].

We also examined the metabolism of fructose (Tables 2 and 3) and found that the rate of utilization of this ketose was slower than that of glucose, especially at concentrations less than 10 mM. The much slower rate of substrate uptake at low concentrations of fructose allowed glycolytic metabolism to be studied without substrate depletion and with maintenance of comparatively stable concentrations of phosphorylated intermediates. Hexokinase activity in cell-free extracts of Ehrlich cells was observed to have apparent K_m values of 6.07 ± 0.36 and 0.23 ± 0.02 mM ($n = 7$) for fructose and glucose respectively.

The influence of added carbohydrate on lactate oxidation

With glucose as added substrate, the calculated rate of reducing equivalent transfer was double that observed with 15 mM lactate (Table 3). This removal of reducing equivalents from the cytosol was greatly accelerated when glucose at concentrations of 2–10 mM was added in combination with lactate (Table 3), and exceeded the sum of the rates from glucose and lactate added separately. This was despite the fact that the rate of glucose removal was decreased by 20–30% when lactate also was added. Hence the rate of oxidation of lactate to pyruvate was increased in the presence of glucose. A consequence of the greater accumulation of pyruvate when carbohydrate and lactate were both present was that the steady-state [lactate]:[pyruvate] ratio attained was much lower than that observed with lactate added alone (Table 3). On consumption of the added glucose and the depletion of glycolytic intermediates, the stimulation of lactate oxidation ceased.

Dihydroxyacetone and glyceraldehyde were tested in place of glucose but had no measurable influence on concentrations of glycolytic intermediates or on the rate of lactate oxidation (results not shown). At concentrations below 10 mM, fructose brought about a lesser increase in glycolytic intermediates than did glucose, but in the presence of added lactate also stimulated the flow of reducing equivalents into the mitochondria. With increasing concentrations of lactate, the presence of glucose greatly stimulated the flux of reducing equivalents; fructose increased this flux to a lesser extent (Figure 4). Examination of the effects of a range of fructose concentrations showed that reducing equivalent flux and pyruvate accumulation were promoted when dihydroxyacetone phosphate and glycerol 3-phosphate concentrations were increased above approximately 0.1 and $0.9 \mu\text{mol} \cdot \text{g}^{-1}$ wet weight respectively (results not shown).

Table 2 Effect of lactate, glucose, fructose and metabolic inhibitors on intermediates and products of glycolysis

Ehrlich ascites cells were incubated for 30 min as described in the Materials and methods section with substrates and inhibitors added, where indicated, at the following initial concentrations: lactate, 15 mM; glucose, 10 mM; fructose, 10 mM; AOAC, 0.5 mM; ROT, 2 μ M; ANTI, 1 μ M. The values listed are the quantities per gram wet weight of cells measured at the end of the incubation period when, except for glycerol, steady-states were achieved. Hexose-6P is the sum of glucose 6-phosphate + fructose 6-phosphate. The data are mean values together with standard errors of the means for the number of independent experiments indicated in parentheses.

Addition	Intracellular concentration (μ mol $\cdot$ g ⁻¹ wet weight)				
	Hexose-6P	Fructose 1,6-diphosphate	Dihydroxy acetone-phosphate	Glycerol 3-phosphate	Glycerol
Lactate	0.02 $\pm$ 0.006 (20)	0.01 $\pm$ 0.005 (20)	0.01 $\pm$ 0.003 (20)	0.12 $\pm$ 0.02 (20)	0.51 $\pm$ 0.08 (16)
Glucose	3.50 $\pm$ 0.20 (15)	1.25 $\pm$ 0.06 (23)	0.92 $\pm$ 0.03 (18)	1.72 $\pm$ 0.08 (18)	1.88 $\pm$ 0.10 (17)
Glucose + ROT	3.03 $\pm$ 0.07 (5)	0.89 $\pm$ 0.15 (3)	0.59 $\pm$ 0.03 (5)	2.03 $\pm$ 0.10 (5)	2.12 $\pm$ 0.16 (5)
Glucose + ANTI	2.64 $\pm$ 0.09 (7)	0.38 $\pm$ 0.04 (4)	0.33 $\pm$ 0.01 (7)	5.71 $\pm$ 0.14 (7)	2.97 $\pm$ 0.32 (7)
Glucose + AOAC	3.85 $\pm$ 0.12 (3)	1.68 $\pm$ 0.10 (3)	0.91 $\pm$ 0.04 (3)	2.03 $\pm$ 0.02 (3)	2.08 $\pm$ 0.36 (3)
Lactate + glucose	4.09 $\pm$ 0.15 (15)	1.41 $\pm$ 0.14 (9)	0.93 $\pm$ 0.03 (18)	2.86 $\pm$ 0.11 (18)	2.59 $\pm$ 0.21 (18)
Lactate + glucose + ROT	3.57 $\pm$ 0.06 (3)	0.93 $\pm$ 0.06 (3)	0.70 $\pm$ 0.04 (4)	2.57 $\pm$ 0.13 (4)	3.11 $\pm$ 0.10 (4)
Lactate + glucose + ANTI	2.74 $\pm$ 0.04 (3)	0.25 $\pm$ 0.04 (3)	0.31 $\pm$ 0.04 (5)	8.15 $\pm$ 0.87 (5)	5.60 $\pm$ 0.72 (4)
Lactate + glucose + AOAC	3.88 $\pm$ 0.19 (4)	2.10 $\pm$ 0.18 (3)	1.15 $\pm$ 0.06 (5)	2.79 $\pm$ 0.23 (5)	3.48 $\pm$ 0.15 (5)
Fructose	2.56 $\pm$ 0.57 (9)	1.35 $\pm$ 0.13 (7)	0.75 $\pm$ 0.14 (8)	1.68 $\pm$ 0.24 (8)	1.67 $\pm$ 0.23 (8)
Lactate + fructose	5.32 $\pm$ 0.57 (3)	1.78 $\pm$ 0.16 (3)	1.34 $\pm$ 0.06 (3)	2.95 $\pm$ 0.63 (3)	2.27 $\pm$ 0.79 (3)
Lactate + fructose + ROT	3.36 $\pm$ 0.09 (6)	0.87 $\pm$ 0.03 (6)	0.57 $\pm$ 0.03 (6)	2.64 $\pm$ 0.28 (6)	2.24 $\pm$ 0.46 (6)
Lactate + fructose + ANTI	0.72 $\pm$ 0.08 (6)	0.49 $\pm$ 0.02 (6)	0.13 $\pm$ 0.02 (6)	6.01 $\pm$ 0.16 (6)	1.27 $\pm$ 0.26 (6)
Lactate + fructose + AOAC	3.53 $\pm$ 0.18 (6)	2.16 $\pm$ 0.11 (6)	1.32 $\pm$ 0.05 (6)	3.43 $\pm$ 0.35 (6)	3.31 $\pm$ 0.05 (6)

Table 3 Effect of carbohydrate and metabolic inhibitors on reducing equivalent flux from lactate

Ehrlich ascites cells were incubated as described in the Materials and methods section with substrates and inhibitors added, where indicated, at the following initial concentrations: lactate, 15 mM; glucose, 10 mM; fructose, 10 mM; AOAC, 0.5 mM; ROT, 2 μ M; ANTI, 1 μ M. The average rates of carbohydrate removal, reducing equivalent flux and lactate net change (negative sign indicates net removal) were determined over a 30 min incubation period during which the rates were constant. The rate of reducing equivalent flux was calculated as described in the Materials and methods section. The lactate:pyruvate ratio values were measured at 20 or 30 min. The data are mean values together with standard errors of the means for the number of independent experiments indicated in parentheses.

Addition	Average rate (μ mol $\cdot$ min ⁻¹ $\cdot$ g ⁻¹ wet weight)			
	Carbohydrate change	Lactate net change	Reducing equivalent flux	Lactate:pyruvate ratio
Lactate	—	-0.67 $\pm$ 0.05 (45)	-0.67 $\pm$ 0.05 (45)	40.1 $\pm$ 2.1 (44)
Lactate + ROT	—	0.14 $\pm$ 0.14 (8)	0.14 $\pm$ 0.14 (8)	150 $\pm$ 22 (8)
Lactate + ANTI	—	0.12 $\pm$ 0.09 (7)	0.12 $\pm$ 0.09 (7)	192 $\pm$ 20 (7)
Lactate + AOAC	—	-0.36 $\pm$ 0.06 (6)	-0.36 $\pm$ 0.06 (6)	677 $\pm$ 78 (6)
Glucose	-2.26 $\pm$ 0.09 (19)	2.57 $\pm$ 0.12 (19)	-1.27 $\pm$ 0.11 (19)	4.7 $\pm$ 0.2 (19)
Glucose + ROT	-3.42 $\pm$ 0.06 (5)	4.93 $\pm$ 0.12 (5)	-1.17 $\pm$ 0.14 (5)	7.0 $\pm$ 0.2 (5)
Glucose + ANTI	-3.96 $\pm$ 0.20 (7)	5.86 $\pm$ 0.32 (7)	-0.24 $\pm$ 0.09 (5)	42.0 $\pm$ 6.8 (5)
Glucose + AOAC	-2.02 $\pm$ 0.02 (4)	2.28 $\pm$ 0.47 (4)	-1.53 $\pm$ 0.27 (4)	6.5 $\pm$ 1.3 (4)
Lactate + glucose	-1.71 $\pm$ 0.06 (20)	0.88 $\pm$ 0.08 (15)	-2.78 $\pm$ 0.20 (20)	10.4 $\pm$ 0.5 (20)
Lactate + glucose + ROT	-2.93 $\pm$ 0.11 (5)	3.39 $\pm$ 0.39 (5)	-2.09 $\pm$ 0.14 (5)	10.1 $\pm$ 0.4 (4)
Lactate + glucose + ANTI	-3.91 $\pm$ 0.17 (5)	5.63 $\pm$ 0.57 (5)	-1.03 $\pm$ 0.28 (4)	37.9 $\pm$ 3.9 (4)
Lactate + glucose + AOAC	-2.08 $\pm$ 0.16 (5)	1.14 $\pm$ 0.08 (5)	-2.38 $\pm$ 0.20 (5)	11.8 $\pm$ 0.8 (5)
Fructose	-1.91 $\pm$ 0.22 (9)	2.15 $\pm$ 0.22 (10)	-1.02 $\pm$ 0.12 (10)	4.9 $\pm$ 0.2 (10)
Lactate + fructose	-1.73 $\pm$ 0.27 (5)	0.93 $\pm$ 0.21 (5)	-2.17 $\pm$ 0.10 (5)	9.5 $\pm$ 0.7 (5)
Lactate + fructose + ROT	-1.50 $\pm$ 0.50 (4)	2.24 $\pm$ 0.47 (5)	-1.01 $\pm$ 0.46 (5)	44.3 $\pm$ 13.8 (5)
Lactate + fructose + ANTI	-1.70 $\pm$ 0.36 (8)	2.27 $\pm$ 0.61 (6)	-0.34 $\pm$ 0.19 (6)	113 $\pm$ 13 (6)
Lactate + fructose + AOAC	-1.75 $\pm$ 0.29 (5)	1.03 $\pm$ 0.20 (5)	-1.83 $\pm$ 0.19 (5)	11.6 $\pm$ 0.5 (5)

Experiments with L-[U-¹⁴C]-lactate confirmed that 90–97% of the pyruvate was derived from lactate, even though net pyruvate accumulation was increased 3-fold during a 60 min incubation of the cells with 15 mM lactate and fructose in combination.

Effects of inhibitors on metabolism

The effects of AOAC, an inhibitor of aminotransferases and

hence of malate/aspartate shuttle activity [25], were examined. When lactate was added alone, AOAC greatly decreased its removal and the flux of reducing equivalents into the mitochondria, as well as pyruvate accumulation (Table 3), thereby causing a 17-fold increase in the [lactate]:[pyruvate] ratio. The small quantity of pyruvate which did accumulate in the presence of AOAC was almost all derived from the added lactate, since L-[U-¹⁴C]lactate yielded pyruvate retaining over 90% of its initial

Figure 4 Relationship between flux of reducing equivalents and lactate concentration in the presence and absence of glucose and fructose

Ehrlich ascites cells were incubated as described in the Materials and methods section with various initial concentrations of lactate. Lactate was added alone (○), or together with 10 mM glucose (▲) or 10 mM fructose (△). The data are mean values with standard error bars for 4–45 separate determinations. The unit of reducing equivalent flux is $\mu\text{mol} \cdot \text{min}^{-1} \cdot \text{g}^{-1}$ wet weight.

specific activity (results not shown).

When glucose was added by itself, AOAC raised the [lactate]/[pyruvate] ratio only moderately and did not inhibit reducing equivalent flux (Table 3). Moreover, this inhibitor was much less effective in decreasing the reducing equivalent flux when glucose and lactate were combined than when lactate was present alone. Fructose also alleviated the inhibitory effects of AOAC on reducing equivalent flux and pyruvate accumulation from lactate.

The inhibitor of NADH dehydrogenase, ROT, stimulated glycolysis from glucose, added alone or in combination with lactate, but completely eliminated removal of lactate added by itself (Table 3). ROT did not increase greatly the [lactate]:[pyruvate] ratio in cells incubated with glucose but raised it almost 4-fold in cells incubated with lactate alone. Reducing equivalent flux and pyruvate accumulation from lactate were eliminated by ROT, but these effects of ROT were in large measure countered if glucose or fructose was also present. On the other hand the inhibitory effects of ANTI on reducing equivalent movement and pyruvate accumulation from lactate were relieved to only a small extent by glucose or fructose, and ANTI was much more effective than ROT in raising the [lactate]:[pyruvate] ratio, both in the absence and presence of added glucose or fructose (Table 3).

DISCUSSION

When incubated *in vitro* with lactate alone, Ehrlich ascites cells accumulate pyruvate without the concomitant accumulation of reduced products. This indicates that reducing equivalents must have been transferred to the mitochondria, since the lactate dehydrogenase of Ehrlich ascites cells is presumed to be located within the cytoplasmic compartment, as it is in other cell types. Only over very brief initial time intervals does measurement of pyruvate accumulation by itself approximate the rates of lactate removal and reducing equivalent transfer. Beyond about 5 min, pyruvate accumulation greatly underestimates this rate of transfer because much of the pyruvate produced is further metabolized, to an extent that can be determined from balance studies. Taking this into account, the rate of reducing equivalent transfer from the cytoplasm was $0.67 \mu\text{mol} \cdot \text{min}^{-1} \cdot \text{g}^{-1}$ with 15 mM lactate, and was a function of lactate concentration.

We also explored the less physiological circumstance when pyruvate alone was added to the cell suspension. The accumulation of lactate under these circumstances gives a measure of the rate of transfer of reducing equivalents from mitochondria to cytoplasm. This rate was only 8% of the rate measured in the opposite direction in the presence of glucose plus 15 mM lactate. Flow from the mitochondria is probably mediated via a malate shuttle [10], but this was not explored in the present study.

At initial lactate concentrations exceeding 5 mM, the magnitude of the steady-state [lactate]:[pyruvate] ratio was approximately proportional to the lactate concentration, in contrast to data for isolated hepatocytes [26]. This was because the steady-state pyruvate concentration was relatively constant at around 0.4 mM. The concentration of pyruvate at any time reflects the dynamic balance between the rate of formation from lactate, a function of lactate concentration (Table 1), and the rate of pyruvate removal via the mitochondria, a rate that is very strongly concentration dependent (Figure 2), especially below 1.0 mM. Thus, the steady-state concentration of pyruvate appears to be 'buffered' against extremes of change although still somewhat dependent on lactate concentration. The concentration dependence of pyruvate metabolism is most likely a property of the mitochondrial pyruvate transport mechanism, which has a reported K_m of about 0.6 mM for mitochondria isolated from Ehrlich cells [27], compared with 0.25 mM for that in rat liver [28]. The apparent ($S_{0.5}$) of 1.8 mM for removal of exogenous pyruvate by intact Ehrlich cells, in the current experiments, might additionally reflect the properties of either uptake across the plasma membrane or the reactions necessary for mitochondrial pyruvate disposal.

Lactate oxidation was strongly depressed by AOAC, which has frequently been employed to inhibit the malate/aspartate shuttle [25]. Lactate removal was also very sensitive to both ROT and ANTI. From these observations it can be concluded that the transfer to the mitochondria of reducing equivalents derived from oxidation of lactate as the only added substrate was mediated by the NAD^+ -linked malate/aspartate shuttle. This observation has been reported previously for Ehrlich cells [12,14] and also for isolated hepatocytes [29].

In contrast to the metabolic picture seen with added lactate, a consistent [lactate]:[pyruvate] ratio of about 5:1 was achieved rapidly in cells incubated with glucose or fructose. Balance studies allowed the determination of the rate of reducing equivalent transfer to the mitochondria, although with these substrates a correction was needed for reducing equivalents retained in hexose-phosphates, dihydroxyacetone phosphate, glycerol 3-phosphate and glycerol because substantial levels of these intermediates were maintained during glucose metabolism [30]. These calculations showed that, with glucose as the sole added substrate, the rate of reducing equivalent transfer was almost 2-fold that observed with 15 mM lactate added alone. Further, when glucose was the substrate, reducing equivalent transfer was not depressed by AOAC and was only slightly decreased by ROT. On the other hand ANTI had a much more inhibitory effect, but despite this both respiratory inhibitors stimulated glycolysis to similar extents. These findings imply that reducing equivalents arising in glucose metabolism, at the reaction catalysed by glyceraldehyde-3-phosphate dehydrogenase (EC 1.2.1.12), pass to the mitochondria predominantly via the flavin-linked and ROT-insensitive glycerol 3-phosphate shuttle.

When lactate and glucose were added simultaneously, the flux of reducing equivalents was more than double that when glucose was present alone. Therefore, it appears that only if adequate levels of carrier intermediates of the glycerol 3-phosphate shuttle are present, can reducing equivalents arising in lactate oxidation

also utilize this shuttle. The shuttle intermediates would probably be present normally within these cells since glucose would be expected to be available to generate them under *in vivo* conditions.

An unambiguous demonstration of the carrier role of glycerol 3-phosphate and dihydroxyacetone phosphate was provided by experiments in which cells were incubated with low concentrations of fructose in combination with lactate. Stimulation of lactate oxidation in the presence of fructose was evident when concentrations of glycerol 3-phosphate and dihydroxyacetone phosphate were increased above 0.9 and 0.1 $\mu\text{mol} \cdot \text{g}^{-1}$ wet weight respectively, although maximum rates of reducing equivalent flow were observed only when glucose was the source of carrier intermediates. In keeping with these findings, in the presence of glucose or fructose, the high [lactate]:[pyruvate] ratio observed with lactate alone was very significantly decreased. These results show that in the absence of glucose, lactate oxidation can be limited by its dependence on reducing equivalent transfer via the malate/aspartate shuttle, which, it was calculated, contributed only 12–25% of maximal shuttle activity observed in these experiments.

The many reports concerning shuttle activity in Ehrlich cells provide an incomplete picture of which of these are functional *in vivo* [13,31,32]. The variability of strains available [15], in part a consequence of the acknowledged genetic instability of such cell lines [33], and the use of differing experimental conditions, especially exposure to different substrate combinations, undoubtedly have contributed greatly to this diversity of conclusions. Thus, some researchers have concluded that the glycerol 3-phosphate shuttle is absent from some strains of these cells [11,12,14,30,34]; others that the malate/aspartate shuttle is ineffective [31,32,35,36]. In particular, proposals that tumour cells generally lack reducing equivalent shuttle activity altogether [3] have been shown to be an over-simplification and inconsistent with experimental observation in at least some of these non-solid tumours, within which shuttle enzyme activities have been measured and redox shuttle function has been demonstrated [11,13,15]. It seems clear that both the nature and the rate of reducing equivalent shuttle activity are dependent, at least in part, on which substrates are available for oxidation. The different metabolic patterns observed in this study help to explain the divergence of conclusions in the published literature [11,12,14,15,30–32,34–36].

In summary, our findings indicate that the activity of shuttle systems transferring reducing equivalents into the mitochondria of Ehrlich ascites cells do not seem to be qualitatively or quantitatively greatly different from such non-neoplastic cells as isolated hepatocytes. For example, the observed reducing equivalent transfer rate of 2.78 (Table 3) corresponds closely to that measured with isolated hepatocytes, 2.61 [9], although the proportion contributed by each shuttle system is likely to be different between different cell types. In view of the high glycolytic activity of these cells, they continue to be a useful preparation for the comparative study of the interactions between glycolysis, intercompartmental reducing equivalent transfer and mitochondrial oxidations.

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