pH-DEPENDENCE OF AEROBIC GLYCOLYSIS IN EHRLICH ASCITES TUMOUR CELLS*

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1. Introduction

To obtain a greater insight into glycolytic control mechanisms, we have studied the effects of changing the pH of the incubation medium on steady-state glycolysis in Ehrlich ascites tumour cells. Similar studies have already been made with erythrocytes [1, 2], leucocytes [3], and other cells and tissues [4, 5]. These previous studies have shown distinct pH effects on the glycolytic system consisting not only in changes of input and output, i.e. glucose utilization and lactate production, but also in shifts of the quantitative inter-relationships between levels of glycolytic intermediates and adenine nucleotides. These changes indicated the operation of different control points at different pH's.

2. Materials and methods

Ehrlich ascites tumour cells were grown in 6–8 week old mice of the Agnes Bluhm strain, harvested on the 8th day after inoculation and washed [6].

All incubations were at 37° and at the pH stated. A 15 min preincubation period preceded each incubation. Glycolysis was started by addition of isotonic glucose solution to give a glucose concentration of 20 mM in the cell suspension.

Experiments with 5–7%, v/v, cell suspensions. These were done as shaking incubations in a medium containing 50 mM Tris, 20 mM phosphate, and 105 mM NaCl.

Experiments with 12–15%, v/v, cell suspensions. These were done at constant pH, maintained by a pH-stat (Radiometer, Copenhagen), in a modified Krebs-Ringer phosphate buffer containing 120 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, and 20 mM sodium phosphate. The hydrogen ions produced during glycolysis were titrated with an isotonic solution containing 100 mM NaOH, 30 mM NaCl, and 20 mM sodium phosphate. Aerobiosis was maintained by bubbling oxygen through the cell suspensions.

The concentrations of glycolytic intermediates were determined in neutralized perchloric acid extracts of the cell suspensions by spectrophotometric methods [7]. The effects of dilution (maximally 10%) in the pH-stat experiments were allowed for. Approximate intracellular pH's were calculated according to Poole, Butler and Waddell [8], who determined the relationships between extracellular and intracellular pH's in ascites tumour cells in comparable conditions to ours.

3. Results and discussion

In the steady state lactate accumulation is maximal at pH 7.4 (fig. 1A). On the other hand pyruvate accumulation increases continuously with increasing pH. The pH dependence of the [lactate]/[pyruvate] ratio at the end of incubation is shown in fig. 1B, which also shows that there are negative linear correlations between log ([lactate]/[pyruvate]) and both the measured extracellular pH and the calculated intracellular pH. Assuming that the lactate dehy-

* *Abbreviations*: FDP, fructose 1,6-diphosphate; F6P, fructose 6-phosphate; G6P, glucose 6-phosphate; LAC, lactate; PEP, phosphoenolpyruvate; PFK, phosphofructokinase; 2PG, 2-phosphoglycerate; 3PG, 3-phosphoglycerate; PK, pyruvate kinase; PYR, pyruvate; TP, triose phosphate.

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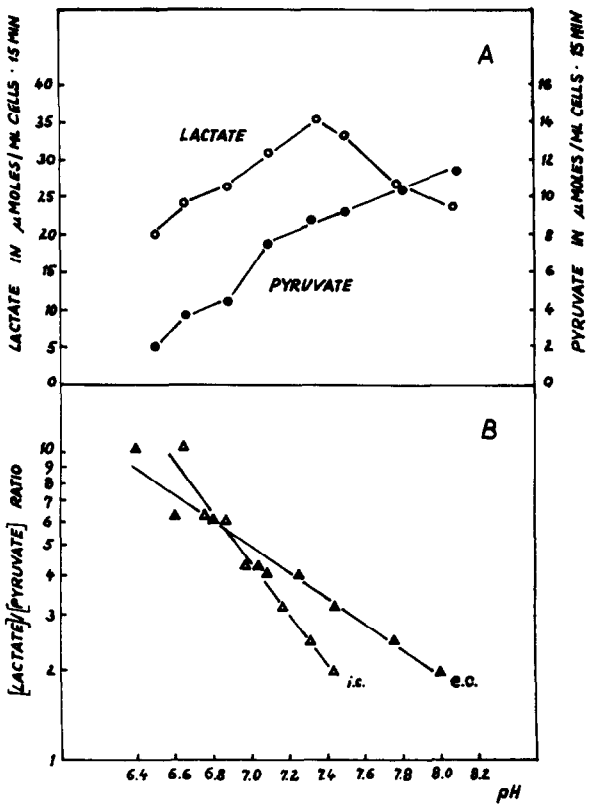

Fig. 1. pH dependence of pyruvate and lactate accumulation in aerobically glycolysing Ehrlich ascites tumour cell suspensions. Experimental: cell contents 5 to 7% (v/v), incubation procedure as described in sect. 2. (A) Accumulations after 15 min incubation with glucose. (B) Relation of the [lactate]/[pyruvate] ratio at the end of incubation to the measured extracellular pH (e.c.) and the calculated intracellular pH (i.c.).

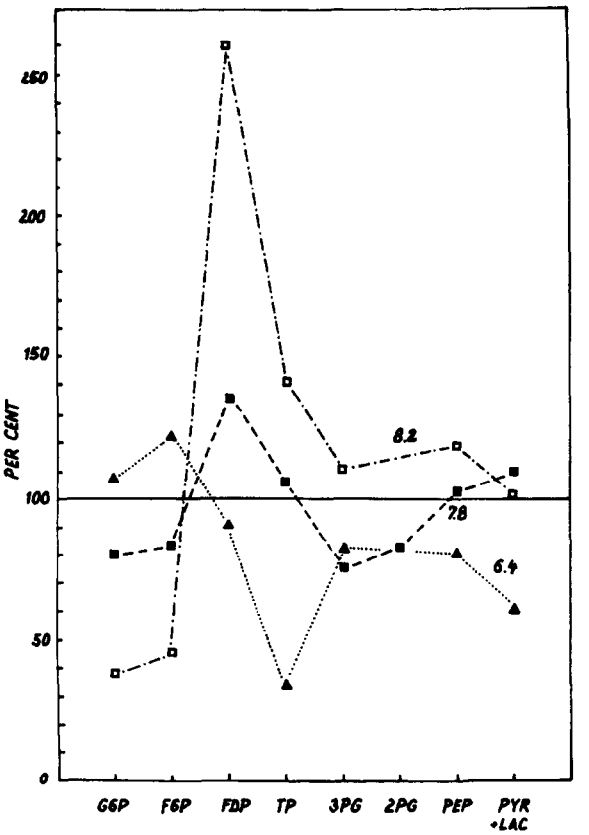

Fig. 2. Cross-over plot of glycolytic metabolites in Ehrlich ascites tumour cells under steady state conditions. Experimental: cell contents 12 to 15% (v/v), pH-state incubation as described in sect. 2. The 100% line indicates the steady state concentrations of G6P, F6P, FDP, TP, 3PG, 2PG and PEP at pH 7.4. Pyruvate and lactate are represented by their summed accumulation rates. At pH 7.4 the following steady state levels were measured: G6P 1.08, F6P 0.27, FDP 1.40, TP 0.63, 3PG 0.45, 2PG 0.06, PEP 0.82. The accumulation rates were at pH 7.4: pyruvate 0.54, lactate 2.77. Levels are given in μmoles per ml cells, accumulation rates in μmoles per ml cells per min.

drogenase reaction is at thermodynamic equilibrium [9], it is possible to compute the $[NAD^+]/[NADH]$ ratios obtaining. The negative proportionality of log ([lactate]/[pyruvate]) to pH means that there is a direct proportionality of [lactate]/[pyruvate] to hydrogen ion concentration. This implies, from the mass action equation for the lactate dehydrogenase reaction, that $[NAD^+]/[NADH]$ is independent of pH in our experimental conditions. From the results of our pH-stat experiments the calculated $[NAD^+]/[NADH]$ values were 2400–2800.

By identifying control points in glycolysis one must take into account that lactate and pyruvate are both final products of glycolysis and that their concentration ratio is a direct function of pH.

The cross-over plot (fig. 2) compares the steady-state concentrations of a number of glycolytic intermediates at four different pH's. Since both lactate and pyruvate are being accumulated in the steady state and their concentration ratios are pH-dependent, they are represented by their summed accumulation rates and not by their concentrations. This is necessary to show up any controlling effect of PK in the cross-over plot.

The cross-over plot shows up three control points: (1) *PFK.* The changes in the concentrations of F6P and FDP as the pH is lowered from 8.2 to 6.4 indicate an increasing control by this enzyme. This control function is well documented at pH 7.4 [10, 14] and the present data show it also to be operative at pH 6.4. As the pH is raised from 7.4 to 8.2, the rate-determining role of this enzyme apparently steadily decreases. (2) *Glyceraldehyde 3-phosphate dehydrogenase or phosphoglycerate kinase.* As the pH is increased to 7.8, one or other of these enzymes acquires a distinct control function. (3) *PK.* As the pH is increased from 7.8 to 8.2, this enzyme becomes rate-limiting.

At the lower pH's the concentrations of hexose 6-phosphates reach relatively higher levels but these intermediates are not accumulated since hexokinase is subject to an effective product inhibition by G6P [11–13], the level of this intermediate being controlled in turn by the activity of PFK. This G6P-mediated feedback between PFK and hexokinase has been described in other cells [1, 2, 11].

The *in vivo* pH-dependence of the activity of PFK conforms with results obtained with isolated enzyme from several animal cells and tissues [14–17] in at least three ways: (1) The pH optimum is between 7.8 and 8.2; (2) the increasing inhibition by ATP with decreasing pH; and (3) the increasing affinity for F6P with increasing pH. The two latter findings point to hydrogen ions as negative allosteric effectors for animal PFK's. One may suppose that the increasing control by this enzyme at lower pH's is a direct effect of this action of hydrogen ions.

Table 1

Steady state concentrations of the adenine nucleotides in aerobically glycolysing Ehrlich ascites tumour cells. The experimental conditions are the same as for fig. 2. The levels are given in μmoles per ml cells.

	pH 6.4	pH 7.4	pH 7.8	pH 8.2
AMP	0.11	0.08	0.21	0.73
ADP	0.31	0.26	0.57	0.54
ATP	2.89	2.86	2.07	0.82

Table 1 shows the steady state adenine nucleotide levels at the same four pH's. Since AMP and ADP are

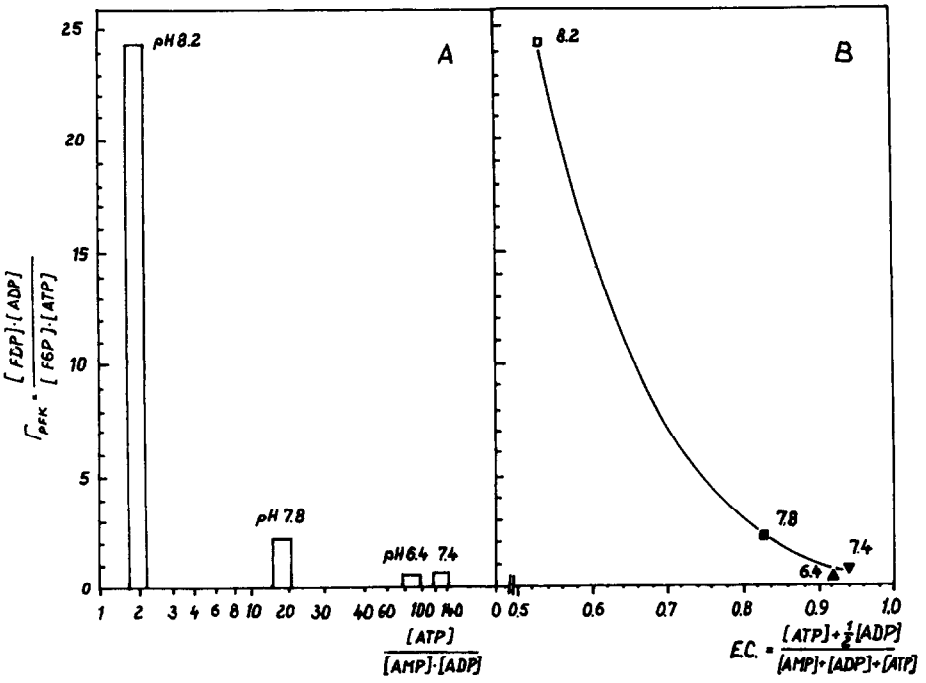

Fig. 3. Relation between the ratio [FDP][ADP]/[F6P][ATP], an indication of relative PFK activity, to the adenine nucleotide ratio (fig. 3A) and to the energy charge (fig. 3B). The steady state ratios were calculated from the data given on fig. 2 and table 1.

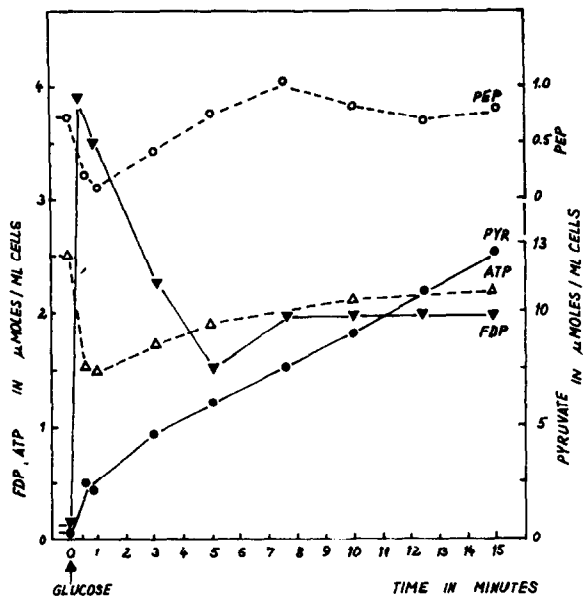

Fig. 4. Variations with time of the FDP, ATP, PEP and pyruvate levels after glucose addition to Ehrlich ascites tumour cells. Experimental: pH-stat incubation. The extracellular pH was 7.8 throughout the incubation.

activators and ATP is an inhibitor of PFK [14], the adenine nucleotide ratio, [ATP]/[AMP] [ADP], is a measure of the inhibition of PFK by adenine nucleotides (fig. 3A). The ATP-regenerating kinase of the later reactions of glycolysis tend to increase the ratio [ATP]/[AMP] [ADP] and hence reinforce the inhibition of PFK produced by hydrogen ions. At higher pH's PFK control is diminished, the FDP and TP levels increase and, at pH 8.2, there is a small but significant accumulation of these intermediates. This accumulation, together with the lowered [ATP]/[AMP] [ADP] ratio and the cross-over points in the later reactions of glycolysis, indicate a dynamic disproportionality between the ATP-consuming and the ATP-regenerating processes inside the cells at pH 8.2 [18].

The relation between PFK activity and Atkinson's energy charge [19] is shown in fig. 3B. Although PFK, being an enzyme involved in the regulation of an ATP-regenerating sequence, should show a type R curve, our results differ from Atkinson's theoretical predictions [19]. The difference may be assumed to occur because ATP and ADP are both allosteric effectors of PFK and substrate and product, respectively. Further, the very steep slope of the curve may in part be due to the effects of pH changes on PFK activity. Fig. 3B does, however, demonstrate the great sensitivity of PFK to the energy charge.

Fig. 4 shows the variations with time of the levels of some intermediates involved in the PK reaction in a glucose-induced transient state. The initial steep rise in pyruvate and fall in PEP concentrations indicate an activation of PK upon glucose addition, this activation coincides with an increase in FDP and a fall in ATP levels, the latter substance being a well-known inhibitor of PK [20]. At all pH's studied there is a quantitative relationship between increased FDP and decreased ATP levels and PK activation. One PK type (liver, yeast) is activated by FDP while another type (muscle) is not [21, 22]. Ascites-tumour-cell PK has not as yet been assigned to one of these types, nor do the present results allow such an assignation to be made, since one cannot decide whether the activation shown in fig. 4 is due to activation by FDP or removal of ATP inhibition. Further work on the characterization of ascites-tumour PK is being undertaken.

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