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The effects of phosphate- and substrate-free incubation conditions on glycolysis in Ehrlich ascites tumour cells

Anthony R. Grivell *, Michael N. Berry

Department of Medical Biochemistry, School of Medicine, Flinders University, G.P.O. Box 2100, Adelaide, South Australia, 5001, Australia

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Abstract

The influence of phosphate-free medium buffered with synthetic organic buffers, and of a preliminary incubation of cells in medium lacking added substrate ('pre-incubation') was investigated with mouse-cultured Ehrlich ascites tumour cells. In comparison to phosphate-containing bicarbonate-buffered balanced-salts medium, organic-buffered medium, without a preliminary substrate-free pre-incubation, was associated with 20–30% reduction in the rate of glycolysis, the 3- to 4-fold accumulation of fructose 1,6-bisphosphate and the halving of both ATP and total adenine nucleotide levels. These perturbations were reversed by the inclusion of 5 mM sodium phosphate in the organic-buffered medium. Pre-incubation for up to 90 min, before inclusion of glucose, resulted in greater depression of the glycolytic rate and concentrations of adenine nucleotides. This occurred in both the balanced-salts medium and the organic-buffered medium. During pre-incubation cells were lysed, releasing lactate dehydrogenase, when physically agitated too vigorously. It was concluded that the use of phosphate-free medium and pre-incubation are not advisable procedures for routine metabolic investigations with this cell line.

Keywords: Ascites tumour cell; Glycolysis; Synthetic organic buffer; Substrate-free incubation

1. Introduction

It has been proposed that, for metabolic studies, Ehrlich ascites tumour cells should be incubated at 37°C in the absence of added substrate for a period of up to 90 min before addition of substrate. This substrate-free incubation is generally referred to as 'pre-incubation'. A pre-incubation regime of this nature has been advocated [1–3] as a means of obtaining cell preparations that are uniformly depleted of endogenous substrate and thus expected to exhibit reproducible metabolic rates under controlled experimental conditions, at least with respect to pathways of energy metabolism. On the other hand, other workers, including ourselves [4], have studied these cells by incubation in the presence of added substrates immediately following harvesting and washing in ice-cold buffer. As part of our study of mitochondrial redox shuttle activity in Ehrlich cells that were not pre-incubated, we observed rates of glycolysis four times as great as those reported recently by Gonzalez-Mateos et al. [3], who routinely pre-incubated cells. Such a large difference raised the possibility that a substrate-free pre-incubation period may be associated with cell damage. This report describes a comparison of the metabolic properties of cells that were either pre-incubated or incubated immediately after washing, using two different types of basic medium.

2. Materials and methods

Experimental details were as described previously [4]. 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 Cancer Institute, Buffalo, New York, USA, 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 N_2. 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.

The cells were collected with about 20 ml of a minimal salts medium [5] containing 0.25 i.u./ml heparin to sup-

* Corresponding author. Fax: +61 8 3740139.

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press cell clumping, and this suspension was filtered through nylon cloth and washed with gentle centrifugation. The medium and pooled cell suspensions were held on ice. The erythrocyte-free washed cells were resuspended in the same medium (but without heparin) at a density of approx. 16 mg dry weight per ml, the actual acid-precipitable dry weight density being determined subsequently as described for isolated hepatocytes [6]. 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) [6]. 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 stabilised 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% O_2/5% CO_2 (balanced salts medium) or 100% O_2 (organic-buffered medium) in 25 ml glass scintillation vials containing 2 ml total volume of medium. Two basic incubation media were compared in this study, the previously-described bicarbonate-buffered balanced-salts medium [4], but without albumin, and a medium buffered with organic salts (85 mM NaCl, 50 mM Pipes, 50 mM Tes and 6 mM KCl [3]). Cells incubated in the organic-buffered medium had a final cell-washing step with that medium. The sealed vials were shaken routinely on a waterbath at 120 oscillations per min with a stroke of 4.5 cm. For pre-incubation, unless otherwise indicated, bulk volumes of cells were incubated at 37°C in medium at half the cell density of the final incubation, collected (with one wash step in appropriate wash medium) by centrifugation, and resuspended at final density in the appropriate incubation medium containing 5 mM glucose. For comparative purposes the control treatment involved cells prepared and incubated, without pre-incubation, in albumin-free bicarbonate-buffered balanced-salts medium. Metabolism was terminated by the addition of an equal volume of ice-cold 1 M perchloric acid to extract intracellular contents. Precipitated material was removed by centrifugation, and the supernatant fractions were neutralised and analysed as previously described [4].

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. Data are the 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. Statistically significant differences indicated in the table were identified by means of Student's *t*-test for unpaired independent samples [7].

3. Results

3.1. Comparison of two incubation media

When incubated in standard balanced-salts medium equilibrated with carbogen, Ehrlich cells consumed glucose at a uniform rate of 1.9 μmol min^{-1} g^{-1} wet weight, 80% being recovered as lactate plus pyruvate (Table 1). Cells incubated in the organic-buffered medium containing glucose alone, utilised glucose at an average rate that was about 20% lower and accumulated lactate plus pyruvate about 30% less rapidly. In this latter medium the rate of glucose uptake was not uniform with time, being most rapid during the initial 5 min of the incubation period. Moreover, after approx. 15 min of incubation the concentrations of ATP and total adenine nucleotides were more than halved, while that of fructose 1,6-bisphosphate was elevated almost 4-fold in comparison to balanced-salts medium (Table 1), although there was no statistically significant difference between the incubation media with respect to the accumulation of dihydroxyacetone phosphate and glycerol 3-phosphate. The amounts of 3-phosphoglycerate and phosphoenolpyruvate remained below 0.10 and 0.05 μmol g^{-1} respectively and were unaffected by medium composition (data not shown). The ratio of lactate/pyruvate was increased about 2-fold in organic-buffered medium.

3.2. Effect of pre-incubation

Cells were incubated in the absence of substrate for a period of 90 min, washed and resuspended in fresh medium of the same composition but with added glucose, and incubated for a further 30–60 min. The 90 min pre-incubation resulted in a greatly decreased rate of glycolysis following glucose addition, irrespective of whether balanced-salts or organic-buffered medium was used. Thus, the rate of glucose uptake was depressed by about 40% following pre-incubation in the conventional balanced-salts medium, but by almost 60% when the organic-buffered medium was used, as compared with the rate observed in cells incubated in glucose-containing balanced-salts medium without pre-incubation (Table 1). In both media concentrations of ATP and total adenine nucleotide were again much lower than in the control incubations, and fructose 1,6-bisphosphate was increased 2- to 3-fold above the concentration in the control (Table 1). The lactate/pyruvate ratio was elevated by 20–30%.

The deleterious effects of pre-incubation in each medium were apparent by 30 min pre-incubation, as revealed by the rates of glycolysis and intracellular levels of ATP (Fig. 1). Despite the variability of absolute values, ATP concentrations and glycolytic rates were consistently lower in the cells incubated in organic-buffered medium when other conditions were the same.

Table 1
Effect of pre-incubation and medium composition on glycolytic rate and products of glycolysis

Pre-incubation period (min)	Basic medium	Incubation supplement	Rate (μmol min^{-1} g^{-1} wet weight)		Ratio lactate/pyruvate	Concentration (μmol g^{-1} wet weight)					
			Glucose	Lactate + pyruvate		ATP	Total adenine nucleotide	Hexose-6P	Fructose 1,6-bisphosphate	Dihydroxyacetone phosphate	Glycerol 3-phosphate
0	Balanced salts	–	−1.94 ± 0.08 (5)	3.12 ± 0.17 (5)	4.1 ± 0.3 (5)	2.31 ± 0.12 (5)	2.56 ± 0.13 (4)	2.93 ± 0.20 (5)	0.93 ± 0.05 (5)	0.75 ± 0.05 (5)	1.17 ± 0.19 (5)
		Glutamine	−1.94 ± 0.02 (3)	3.01 ± 0.16 (3)	4.9 ± 0.21 (3)	2.47 ± 0.11 (3)	2.74 ± 0.06 (3)	3.00 ± 0.30 (3)	0.62 ± 0.05 (3)	0.57 ± 0.03 (3)	1.15 ± 0.46 (3)
	Organic-buffered	–	−1.54 ± 0.16 (5)	2.19 ± 0.20 (5)	8.4 ± 1.5 (5)	1.03 ± 0.28 (5)	1.16 ± 0.25 (4)	1.66 ± 0.48 (5)	3.52 ± 0.86 (4)	0.91 ± 0.31 (4)	0.99 ± 0.14 (3)
		Glutamine	−1.76 ± 0.07 (3)	2.73 ± 0.14 (3)	8.1 ± 1.1 (3)	2.01 ± 0.33 (5)	2.33 ± 0.41 (3)	2.03 ± 0.31 (3)	1.02 ± 0.43 (3)	0.49 ± 0.10 (3)	0.97 ± 0.22 (3)
		Phosphate	−2.06 ± 0.08 (3)	3.02 ± 0.18 (3)	5.5 ± 0.2 (3)	2.23 ± 0.08 (3)	2.50 ± 0.09 (3)	1.99 ± 0.11 (3)	2.11 ± 0.29 (3)	1.07 ± 0.13 (3)	2.23 ± 0.19 (3)
90	Balanced salts	–	−1.23 ± 0.21 (5)	1.66 ± 0.22 (5)	4.8 ± 0.6 (5)	1.03 ± 0.15 (5)	1.11 ± 0.18 (4)	1.15 ± 0.22 (5)	2.26 ± 0.54 (5)	0.97 ± 0.28 (5)	0.52 ± 0.15 (5)
		Glutamine	−1.15 ± 0.30 (3)	1.71 ± 0.42 (3)	5.3 ± 0.5 (3)	1.11 ± 0.24 (3)	1.32 ± 0.25 (3)	1.26 ± 0.14 (3)	1.58 ± 0.67 (3)	0.60 ± 0.23 (3)	0.46 ± 0.09 (3)
	Organic-buffered	–	−0.82 ± 0.09 (5)	0.85 ± 0.05 (5)	11.4 ± 1.0 (5)	0.33 ± 0.13 (5)	0.44 ± 0.08 (4)	0.57 ± 0.25 (5)	2.81 ± 0.49 (5)	1.09 ± 0.25 (5)	0.52 ± 0.14 (5)
		Glutamine	−0.73 ± 0.08 (3)	0.96 ± 0.12 (3)	11.3 ± 0.5 (3)	0.36 ± 0.05 (3)	0.54 ± 0.14 (3)	0.28 ± 0.08 (3)	2.76 ± 0.96 (3)	0.86 ± 0.27 (3)	0.36 ± 0.08 (3)
		Phosphate	−0.94 ± 0.30 (3)	1.07 ± 0.22 (3)	9.4 ± 2.0 (3)	0.43 ± 0.07 (3)	0.62 ± 0.07 (3)	0.46 ± 0.04 (3)	1.46 ± 0.48 (3)	0.92 ± 0.51 (3)	0.93 ± 0.50 (3)

Ehrlich ascites cells, prepared as described in Section 2, were pre-incubated or not as indicated then incubated for 30 or 40 min with 5 mM glucose supplemented, where indicated, with 1 mM glutamine or 5 mM phosphate. The average rates of glucose removal and lactate + pyruvate accumulation were determined over the entire incubation period. The concentrations of intermediates were measured at 20 min, by which time steady-states were achieved. Hexose 6-P is the sum of glucose 6-phosphate + fructose 6-phosphate, 77% of this sum being glucose 6-phosphate. The data are mean values, together with standard errors of the means, for the number of independent experiments indicated in parentheses. Differences from the control treatment values were statistically significant at probability levels of 0.05 (*), 0.02 (**), 0.01 (***) and 0.001 (****).

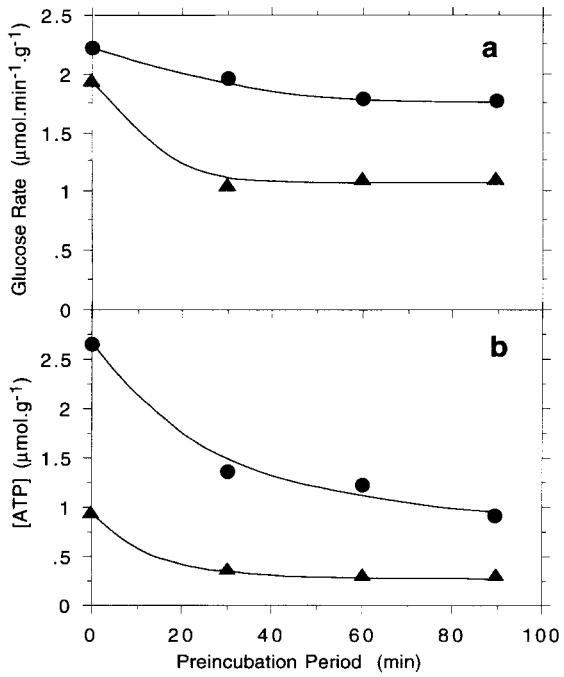

Fig. 1. Effect of pre-incubation on glycolytic activity. Ehrlich ascites cells were pre-incubated for the indicated time periods in two media: balanced-salts medium (●), and organic-buffered medium (▲). Following an additional 30 min incubation in the same media supplemented with 5 mM glucose, the rates of glucose removal (a) and concentrations of ATP (b) were determined as described in Section 2. The data are from a representative experiment.

3.3. Protective effects of glutamine and inorganic phosphate

In cells that were not pre-incubated, but incubated in organic-buffered medium containing glucose supplemented with 1 mM glutamine, there was a partial restoration of the glycolytic rate and adenine nucleotide levels towards values obtained with control-treatment cells (Table 1). This effect of glutamine was not observed with cells that had been pre-incubated previously for 90 min. In contrast, with control cells incubated in balanced-salts, glutamine had no discernible effect on either glycolytic rate or ATP concentration. Nor did it increase the glycolytic rate or raise adenine nucleotide levels in cells previously pre-incubated for 90 min, an effect similar to that observed with cells pre-incubated in organic-buffered medium.

When organic-buffered medium was supplemented with 5 mM sodium phosphate (pH 7.0) and cells incubated immediately in the presence of glucose, the rate of glycolysis was increased, and the concentrations of ATP and total adenine nucleotide were raised to the values observed in balanced-salts medium, although the lactate/pyruvate ratio remained slightly elevated (Table 1). On the other hand, the addition of phosphate together with glucose to cells that had been pre-incubated for 90 min had no effect on the observed metabolic performance (Table 1).

3.4. Effect of suspension volume and oscillation rate during pre-incubation

Under routine incubation conditions (that is, 0.1 g wet weight of cells in a total incubation volume of 2 ml), less than 5% of total lactate dehydrogenase, a reliable indicator of cell damage [6], was released into the medium during a 30-min incubation in organic-buffered medium not containing glucose (data not shown). This confirmed that the depression of ATP and total adenine nucleotide were apparently not simply a consequence of gross cell damage occurring during pre-incubation.

However, when freshly-harvested cells were pre-incubated similarly, but in the larger volumes used by other workers [3] (for example, 48–56 ml total volume in a 250-ml conical flask), they were substantially damaged when agitated at the standard oscillation rate, since about 60% of total lactate dehydrogenase activity was released by 90 min incubation under these conditions (data not shown). This apparent fragility was observed with cells suspended in either of the incubation media.

Conversely, pre-incubation either in a bulk volume but at half the standard oscillation rate of 120 oscillations per

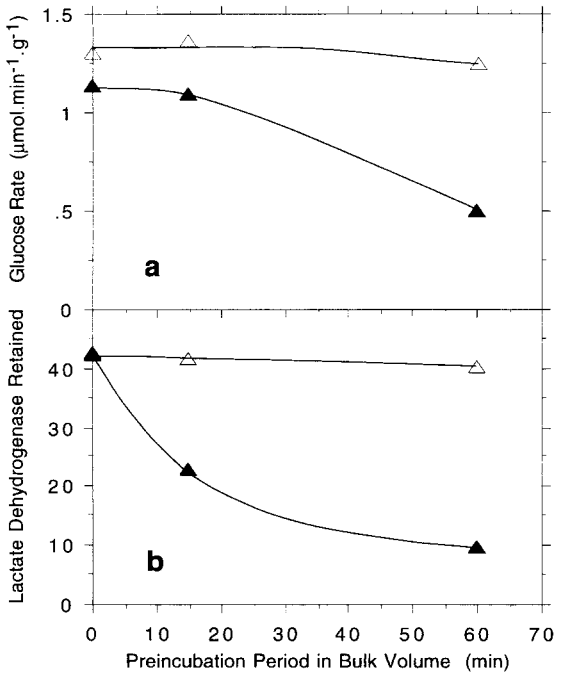

Fig. 2. Effect of agitation rate on cell integrity. Ehrlich cells were pre-incubated in 48 ml volumes of organic-buffered medium, in 250 ml conical flasks that were agitated at rates of 60 oscillation per min (△) and 120 oscillation per min (▲), for the times indicated, after which 2-ml volumes of cell suspension were transferred to standard incubation vessels, gassed and incubated at the same oscillation rate for a total pre-incubation period of 60 min. Glucose (5 mM) was added and the vessels incubated for a further 30 min at which time the rate of glucose removal (a) and the intracellular retention of lactate dehydrogenase (b) were determined as described in Section 2. The data is from a representative experiment.

min (Fig. 2), or in small volumes (2 ml) under the standard oscillation conditions (data not presented), was not associated with significant leakage of lactate dehydrogenase, although the metabolic perturbations previously described were still apparent (Fig. 2).

4. Discussion

The rate of glycolysis described in the report of Gonzalez-Mateos et al. [3] was only about 25% of the average rate measured under control conditions within this series of experiments, raising the issue of which cell incubation protocol is more appropriate for examining the normal state. Our control rates are the same as published previously on the basis of a more extensive experimental set [4], but are about 1.5-fold greater than analogous rates reported by other investigators for this type of cell line [1,8–10]. There are two factors that are clearly different between the two incubation protocols, the composition of the medium and the preliminary incubation period without exogenous substrate; hence the influence of each of these was examined independently.

Cells incubated with glucose in organic-buffered medium had a relatively reduced rate of glucose utilisation and significantly perturbed levels of phosphorylated compounds. The decreased level of ATP suggests that the accumulation of phosphorylated intermediates of glycolysis resulted in the 'trapping' of phosphate moiety, thereby depressing the residual level of adenine nucleotide. The data of Table 1 are consistent with the phosphate-balance that is implied by such a mechanism, since the total phosphate content of the measured phosphorylated intermediates was approximately equal in treatments that did not include pre-incubation. The observation of such apparent perturbation of the glycolytic pathway in cells incubated in organic-buffered medium, particularly the accumulation of fructose 1,6-bisphosphate, strongly suggests an inhibition of activity at some point beyond the phosphofructokinase reaction. It is significant that the addition of 5 mM phosphate to the organic-buffered medium caused the cells incubated immediately in this medium to behave much more like control-treatment cells in terms of glycolytic rate, levels of adenine nucleotides and levels of glycolytic intermediates. This strongly implies that this major effect of organic-buffered medium simply may be related to the deficiency of inorganic phosphate, a basic component of balanced-salts medium. In addition, it points to the reaction catalysed by glyceraldehyde 3-phosphate dehydrogenase as the possible point of blockade of the pathway. There is ample evidence that deficiency of phosphate can limit the rate of glycolysis, at least in some cell lines [11,12].

The addition of 1 mM glutamine resulted in a similar effect to that of inorganic phosphate, in that it restored partially the rate of glycolysis and the levels of adenine nucleotides for cells in organic-buffered medium, but only when there was no pre-incubation step, although it did not stimulate glycolysis in cells incubated in balanced-salts medium. On the other hand, it did not inhibit glycolysis with any of the cell treatments, in contrast to the observations of Gonzalez-Mateos et al. [3]. We have no explanation for this difference in the effect of glutamine between these two reports. On the basis of separate studies using balanced-salts medium (unpublished results), in which the presence of glutamine stimulated respiration of these cells by around 5–10% in the presence of glucose and by 20–30% endogenously or when palmitate, lactate or pyruvate was added, we suggest that the glutamine response in the current experiments was due to supplementation of energy metabolism as a consequence of the mitochondrial oxidation of glutamine, a preferred mitochondrial substrate for Ehrlich ascites cells [13].

When cells were pre-incubated for 90 min in organic-buffered medium, before incubation with added glucose, the average rate of glycolysis was depressed even further. Significantly, glycolytic rates were decreased similarly even with the standard balanced-salts medium, if the cells were first pre-incubated. This loss of glycolytic capacity was a function of the period of pre-incubation, being significant even when this was as brief as 15 min. In both media, levels of adenine nucleotide and glycolytic intermediates were greatly perturbed following pre-incubation. However, in contrast to the situation with non-pre-incubated cells in organic-buffered medium, neither phosphate nor glutamine supplementation resulted in a protective effect, implying a different cause for the cell impairment. Prolonged incubation of Ehrlich cells in the absence of metabolisable hexose leads to severely depressed ATP levels and even cell death [14,15]. Perhaps more directly relevant, Glaser et al. [2] demonstrated that depletion of ATP and the corresponding accumulation of fructose 1,6-bisphosphate following pre-incubation is most likely caused by a pyruvate deficiency and a resultant inability to regenerate oxidised NAD, thus potentially forcing the accumulation of fructose 1,6-bisphosphate.

Our experimental observations suggested therefore that the two factors examined, the use of the organic-buffered medium and the extended pre-incubation period, independently contributed to metabolic impairment, at least as measured by glycolytic activity. When both these factors were present in the same treatment, cell glycolytic rate was depressed maximally.

The extreme loss of adenine nucleotides that was observed with some treatments in these experiments led to the preliminary expectation that a large proportion of the cells had been killed. However, direct measurement of lactate dehydrogenase loss to the medium (verified in several experiments with parallel measurements using trypan blue) demonstrated that neither the medium used, nor the pre-incubation period, were associated per se with significant cell death. On the other hand, reasonably severe

agitation of the cells in either medium, as occurred typically during pre-incubation of larger batches of cells, did result in release of most of the cellular lactate dehydrogenase. It may be that a proportion of the cells are only partially damaged after 60 min pre-incubation and thus beginning to leak lactate dehydrogenase, while retaining a relatively greater proportion of glycolytic activity. It is possible that the enhanced shear forces developed in a pre-incubation volume that was less than 25% the capacity of a relatively large flask, under the standard agitation conditions, were strong enough to damage cell structure. This possibility is consistent with the observation that reduction of the severity of agitation, either by using much smaller containers or by halving the shaking rate, prevented this cell destruction.

As well as differences in experimental technique, there is the likelihood of a further fundamental difference between experiments performed in different laboratories, namely the genealogy of the cell lines used. It is to be expected that there will be differences between various strains with respect to physical fragility and also in relation to metabolic responses to changing substrate availability. The acknowledged existence [16] of cell variability requires that due allowance should be made for it in comparing data from separate laboratories.

The rate of the energy-yielding reactions, including glycolysis, will respond to any processes, whether normal or abnormal, that increase ATP turnover [11,17]. Thus there is a genuine element of arbitrariness in any claim that a given cell preparation possessing a particular metabolic rate capacity is more 'metabolically competent' or 'normal' than another. However, the observation of dramatic and largely irreversible depression in the levels of total adenine nucleotide, and of ATP in particular, is difficult to dismiss as being normal or a minor perturbation, even in the absence of significant leakage of intracellular enzyme content. Therefore, at least with the cell strain used (and the experimental protocols followed) in this laboratory, pre-incubation of cells and the use of organic-buffered incubation media appear not to be advisable for routine metabolic studies.

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