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COMPARISON OF HEAT FLUX IN WILD-TYPE AND GENETICALLY-ENGINEERED CHINESE HAMSTER OVARY CELLS

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Abstract

It is claimed, though not without dispute, that genetically engineered mammalian cells grow more slowly than their progenitor cells because the recombinant gene system causes a metabolic burden. This was found to be the case for CHO cells transfected with expression vectors for *cytochrome* b_5. The slower growth was associated with lower metabolic activity measured by heat flux and mitochondrial activity (rhodamine 123 fluorescence). The calorimetric-respirometric ratio was similar for all cell types, implying that the greater fluxes of glucose and glutamine in the recombinant cells was channelled to biosynthesis. This demand probably restricted the supply of pyruvate to the mitochondria in these cells.

Keywords: *cytochrome* b_5, genetically-engineered cells, heat flux, metabolic burden, mitochondrial activity

Introduction

In recent years, genetic engineering of mammalian cells has gained significant importance through the realization that many human therapeutic proteins cannot be produced in a biologically active form in microbial cells. Many of these macromolecules are core-glycosylated (glycoproteins) in a complex and varied manner which requires native eukaryotic post-translational machinery not available in prokaryotes and only possessed to a limited degree in yeast [1]. A second reason for using mammalian cells to obtain recombinant proteins is that folding/unfolding requires an interplay with attendant chaperones [2] in the endoplasmic reticulum and Golgi apparatus which are only found in animal cells.

In the industrial exploitation of recombinant technology in bacteria, it has been observed that specific growth rates are dramatically reduced by the presence of actively transcribing plasmids; the extent of inhibition has been correlated with plasmid numbers per cell and the level of foreign gene expression [3–5]. This is evidence for the belief that the reduced growth rate can be caused by both amplified plasmid level and recombinant gene expression [5]. It has also been reported that the same phenomena occurred for the growth kinetics of genetically-engineered

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mammalian cells. In particular, it was shown that foreign gene amplification and expression inflict a metabolic burden on the growth rate of recombinant CHO cells [6, 7]. These differences were ascribed to a redirection of host cell biosynthesis of native cellular components towards the synthesis of the cloned genes and their products. This appeared to be the case even though in most cases the foreign genes were incorporated into the host chromosomes [8]. Two of the unanswered questions in this respect are whether and how far the cells can compensate for this burden and the degree to which it is caused by the physical effects of intracellular accumulation rather than competition for the available translational and post-translational machinery.

In this paper, a novel approach to this problem has been taken in which the overall metabolic activity has been assessed by measuring the heat flow per viable cell (heat flux). For this study wild-type CHO cells were compared with cells genetically-engineered to produce a heterologous protein in a form which could not be secreted by the cells. The validity of equating heat flux to metabolic activity is well-established for microbes [9] and has recently been extended to animal cells growing in culture (in vitro) [10–12]. Briefly, it is supposed that the most adequate measure of metabolic activity is ATP turnover, the rate of which is coupled to cellular requirements in growth and maintenance, including work (mostly ion pumps). The rate of the ATP cycle (synthesis and degradation) places a demand on the catabolic pathways coupled to ATP synthesis. In exponentially growing cells, it is usually assumed that the degree of ATP-coupling does not vary, ATP phosphorylation and dephosphorylation are balanced and the ATP required for maintenance is constant. To develop this reasoning, it is important to realize that, despite the myriad of different reactions contributing to the many pathways in cell growth, a simplified growth reaction can be written for genetically-engineered cells which produce a target protein when cultured in a defined medium without serum [10–12],

$$\text{Glucose} + \text{Glutamine} + O_2 = \text{Biomass} + \text{Product} + CO_2 + NH_3 + H_2O \qquad (1)$$

The validity of this equation can be tested by the enthalpy balance method [12] because the reaction is accompanied by a change in enthalpy. The equation can be reduced to the catabolic and anabolic processes, known as half reactions. The latter is usually considered to have an enthalpy change close to zero ($\Delta_{ana}H \sim 0$) [13–14]. Therefore the enthalpy change of the growth reaction is very similar to that of the catabolic half-reaction, which consists of respiration, glycolysis, the pentose phosphate pathway, glutaminolysis and β-oxidation (when fatty acids are present, e.g. in serum) [11]. Except in the latter case (which nevertheless produces CO_2 and H_2O), these pathways of catabolism are summarized in Eq. (1). Because the rate of the catabolic half-reaction is determined by ATP demand, heat flux quantitatively measures ATP turnover which, as stated above, is equivalent to metabolic activity. In thermodynamic terms, cells are grown at constant temperature and pressure, and because growth is irreversible, they perform no net work. So the exothermic enthalpy change of the growth reaction in cells is equivalent to the quantity of heat produced by that system which can be conveniently and accurately measured by a heat conduction microcalorimeter [15].

ATP is, of course, produced in the glycolytic pathway serving the need for biosynthetic precursors [16] as well as in oxidative phosphorylation. Since heat flux measurements are inclusive of all sources, it was decided to measure the activity of mitochondria using the cationic dye rhodamine 123 [17], and so distinguish between the two routes for synthesis.

In order to make a true assessment of metabolic burden, it was important to make use of definitive genetic transfections of wild-type CHO cells rather than use genetically-engineered cells of uncertain parentage. The plasmid-borne expression of the globular domain of rat liver *cytochrome* b_5 (cyt) was favoured because its behaviour in *Escherichia coli* is well-characterized in this laboratory [18] but, more importantly, the holo oxidised form can be easily quantified spectroscopically by its intense and highly characteristic Soret absorbance peak at 413 nm, thus making it a particularly convenient model protein and dispensing with the need for more arduous assays. Two constructs were used: first, a 300 bp synthetic gene encoding the 99 amino acids of cyt was cloned downstream of the Rous sarcoma virus (RSV) promotor in the expression vector pBA-RSV (CHO-pBA-cyt); and secondly, to introduce an optimised start 'scan' from the 5′ of the transcript, a synthetic Kozak sequence, ACCACC, was appended immediately upstream of the initiator codon (CHO-pAKH-cyt).

The two cell types were compared against wild-type cells in terms of growth rate, size, cyt production, heat flux, and mitochondrial activity in order to discover if the activity of the foreign genes placed a metabolic burden on them. In addition the fluxes of the two major metabolites in the growth equation (Eq. (1)) were measured as an initial stage in determining the source of any changes to catabolism as a result of transfection.

Materials and methods

Materials

All chemicals, unless otherwise stated, were purchased in the UK from (i) Fluka Chemicals, Glossop, Derbys. (ii) Sigma Chemicals, Poole, Dorset, (iii) BDH, Poole, Dorset, or (iv) Oxoid, Unipath, Hants. Media for culturing mammalian cells and geneticin sulphate (G-418) were obtained from GIBCO, Paisley, and Sigma Chemicals; tryptone and yeast extract from Lab M, Bury, Lancs, and DNA modifying enzymes from New England Biolabs, Stevenage, Herts and NEB Gene Sciences Ltd., Berwick Northumberland; Chinese Hamster Ovary Kent-1 (CHO-K1) were supplied by the European Collection of Animal Cell Culture (ECACC), Salisbury, Wiltshire, U.K.

Bacteria, culturing and plasmids

Escherichia coli TB-1 [F ara D (*lac-pro*AB) *rps* f80d *lac*ZDM 15*hsd* R17 ($r_k^+m_k^+$)] was used [19] throughout this study. The bacteria were grown on Lu-

ria Bertani (LB) medium (1% (*w/v*) Tryptone, 0.5% (*w/v*) yeast extract, 1% (*w/v*) NaCl (pH 7.5)) containing either kanamycin (50 μg cm^{-3}) or ampicillin (75 μg cm^{-3}). Agar plates were prepared by adding 1.5% (*w/v*) agar to LB medium. *E. coli* cells were rendered competent by $CaCl_2$ treatment [20, 21] and kept on ice for a period of 20 h in order to increase the state of competency [22]. *Cytochrome* b_5 was isolated from pA-cyt [18], and the vector used for mammalian cell transfections was the pBK-RSV plasmid [10] purchased from Stratagen, Cambridge.

DNA manipulations

These procedures involving DNA separation, purification, digestion and bacterial transformation were performed essentially as described by Maniatis [23]. Oligonucleotides were synthesised by the phosphoramite method on an automated Pharmacia-LKB Gene Assembler. The deprotected oligonucleotides were purified by filtration through an NAP™-10 column. Site-directed mutageneses, performed by replacing deleted portions with synthetic oligonucleotide duplex in double-stranded plasmid DNA, were confirmed by DNA dideoxy sequencing [24]. Plasmid DNA was routinely isolated by use of Mini- and Midi-Qiagen isolation kits (Qiagen, Dorking, Surrey).

Mammalian cells culturing and transfection

CHO-K1 cells were cultured in Ham's F-12 medium supplemented with 2 mM glutamine, 10% (*v/v*) GIBCO fetal calf serum and 50 μg cm^{-3} gentamycin. The adherent-cell monolayers in cultures were detached from growth flasks by treatment with 0.05% (*w/v*) crystalline porcine trypsin and 0.02% (*w/v*) Na_2 EDTA. They were washed in trypsin inhibitor before being resuspended in the culture medium for counting by the Coulter method (Model D; Coulter Ltd., Luton, Beds). Cell viability was assessed by ethidium bromide and fluorescein diacetate differential staining [25].

Cells plated at a total of 10^4 in a 25 cm^2 T-flask were cultured for 24 h and then transfected by the standard calcium phosphate procedure [26] using 30 μg plasmid DNA suspended in 0.5 cm^3 0.25 M $CaCl_2$. Recombinant cells harbouring the *neo*^{r} gene were selected for by propagation in the presence of 500 μg cm^{-3} geneticin sulphate (a version of kanamycin for mammalian cells) which reflected their ability to express the recombinant haemoprotein [27] from the stably-integrated gene.

Cell size was measured by use of an automated laser-light Skatron Argus 100 Flow Cytometer [28] standardized with dynosphere monosized polymer beads (Dyno Particles A. S. Lillestrøm, Norway) ranging in size from 5 to 20 μm diameter. This instrument was also used to measure the intensity of mitochondrial staining by rhodamine 123. For this procedure, a stock solution was added directly to the cell suspension to a final concentration of 10 μg cm^{-3}. The cells were incubated for 20 min at 37°C, centrifuged and resuspended in Dulbecco PBS, pH 7.4 [29].

Fluorescence intensity was measured using 450–490 nm excitation filters. Peaks were statistically analysed for covariance, which is defined by

$$\mathrm{Cov} = \frac{\sum(l - l_m)(Z - Z_m)}{n} \quad (2)$$

where l and Z are the channel number and the corresponding cell counts respectively, l_m and Z_m are the means of l and Z respectively, and n is the total number of channels.

Microcalorimetry

The heat flow ($\Phi = dQ/dt$, where Q is heat) of the cells was measured in the perfusion vessel of the Thermal Activity Monitor (Thermometric AB, Järfälla, Sweden) [15]. The 4 cm^3 open stainless steel vessel was loaded with 3 cm^3 cell suspension at pH 7.2±0.02. The temperature was 37°C exactly, the stirring rate 60 rev/min and the amplification was 100 μW. Electrical calibration was performed daily and checked periodically by the chemical hydrolysis of triacetin [15].

Oxygen consumption

An Oroboros polarographic Oxygraph (Paar KG, Graz, Austria) [30] was used to measure the oxygen consumption over a 60 min period of cultured cells maintained in 2.3 cm^3 suspension at 500 rev/min. Data was collected by Paar Oxydat software and analysed by Excel 5.0 statistical package.

Analytical procedures

Glutamine and glucose were measured using Sigma test kits after deproteinization with perchloric acid [12, 28]. The protein in carefully washed cells was estimated by the Lowry procedure (Biorad, Hercules, CA) after alkaline hydrolysis with IN NaOH [12].

Results and discussion

The growth of CHO cells in monolayers which had been transfected with the eukaryotic expression vectors (Fig. 1) encoding cyt (CHO-pBA-cyt cells) and cyt with the Kozak sequence (CHO-pAKH-cyt) were compared with their wild-type progenitor. As will be seen in Fig. 2, transfection had a marked effect on cell growth and, of the two, the vector with the consensus (Kozak) sequence [31] caused the greater burden to the cells. This point is emphasised in Table 1 which shows that the doubling time t_d, calculated from the specific growth rate μ for pAKH-cyt cells was 70% longer than for the wild-type cells. Although the vector copy numbers in the pBA- and pAKH-cyt cells are not known at present, it would be expected that, with equal numbers, the efficiency of foreign gene translation would be greater for

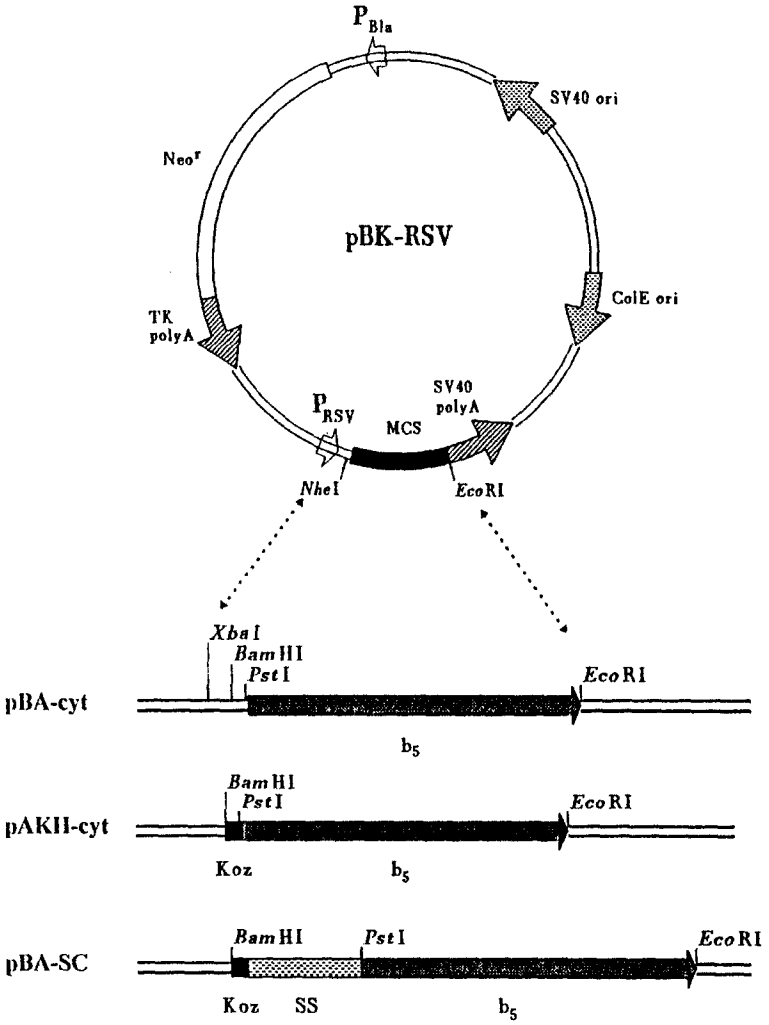

Fig. 1 Schematic depicting construction and features of the eukaryotic expression vectors encoding *cytochrome* b_5 forms addressed with Kozak and secretory signals. Abbreviations: Koz, Kozak sequence; P_{RSV}, Rous Sarcoma virus promoter; b_5, globular core of *cytochrome* b_5; *neo*^{r}, neomycin gene

the latter because of the presence of the consensus sequence to initiate translation. The pBA-cyt expression vector does not have such a 'focused' section to the initiation site in the mRNA. The reduced cell growth was associated with the expression of cyt gene co-amplified with *neo*^{r} gene and selected by geneticin sulphate in the medium. As may be expected (Table 1) the pAKH-cyt produced nearly double the amount of cyt synthesized in the pBA-cyt variant. It would appear that there is a reciprocal relationship between the 'activity' of the foreign gene amplification system and the specific growth rate of the cells, to give the phenomenon of a metabolic bur-

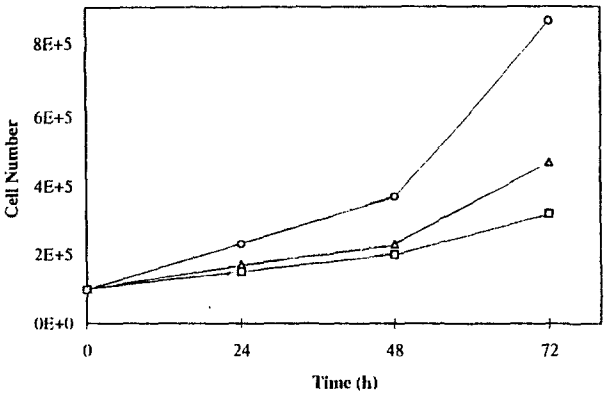

Fig. 2 A comparison of the logarithmic growth curve of wild-type CHO cells with transfected cells growing in a T-25 flask. Symbols: wild-type CHO (o); pBA-cyt (Δ); and pAKH-cyt cells (□)

den [6, 7] well known in prokaryotes but not so well characterized in mammalian cells [32]. As Gu *et al.* [33] pointed out for the *dhfr* gene system in CHO cells expressing β-galactosidase, the cause for metabolic burden could in the present case could be due to (i) the overexpression of *cytochrome* b_5, (ii) the *neo*^{r} and cyt gene co-amplification or (iii) *neo*^{r} gene expression. For the β-galactosidase expression [33], the second two factors (ii) and (iii) were more important.

Since the cyt expression vectors were not addressed with a secretory signal, the heterologous protein accumulated in the cytosol contributing to the increase in protein mass at 48 h in culture proportional to the amount of cyt produced in that time (Table 1). The cells were sized by flow cytometry and were shown to increase in volume (V) by an amount corresponding to the change in mass (m). In effect, the mass density ($\rho = m/V$) remained constant (Table 1), discounting the possibility that the increase in volume was due to an effect on osmotic pressure by the presence of greater amounts of protein.

Turning to the metabolic status of the cells (Table 2), heat flux measurements indicated that the expression of cyt caused a decrease in metabolic activity commensurate with the level of cyt production. This appeared to be caused by a decrease in oxidative processes since the oxygen consumption decreased to the same extent as the heat flow rate. This supposition is borne out by the similar values for the cal-

Table 1 Characteristic properites of wild-type and engineered cell lines

Cell type	μ/ h^{-1}	t_d/ h	ρ/ g m^{-3}	Protein/cell ($g \times 10^{-10}$)	Cell volume/ μm^3	Cyt b_5/cell ($g \times 10^{-10}$)
CHO	0.027	26	1.1×10^{-3}	8	756	0
pBA	0.020	35	1.1×10^{-3}	10	928	0.23
pAKH	0.015	40	1.1×10^{-3}	12	1150	0.45

Symbols: μ = specific cell growth rate, t_d = cell dubling time, $\rho = m/V$ the ratio of cell mass to volume where cell mass is represented by total cellular protein

Table 2 Characteristic properties of wild-type and engineered cell lines

Cell type	J_{glc}/	J_{gln}/	Heat flux/	J_{O_2}/	CR ratio/	Rhodamine 123 fluorescence	
	10^{-17} mol s^{-1} per cell		pW per cell	10^{-17} mol s^{-1} per cell	kJ mol^{-1}	Peak channel	Covariance
CHO	1.8	0.8	38	6.2	-590±66	137	14.6
pBA	3.9	1.2	30	5	-595±67	134	22.3
pAKH	3.8	1.2	21	4	-529±59	96	28.3

Symbols: J=consumption flux, and CR=respirometric-calorimetric ratio

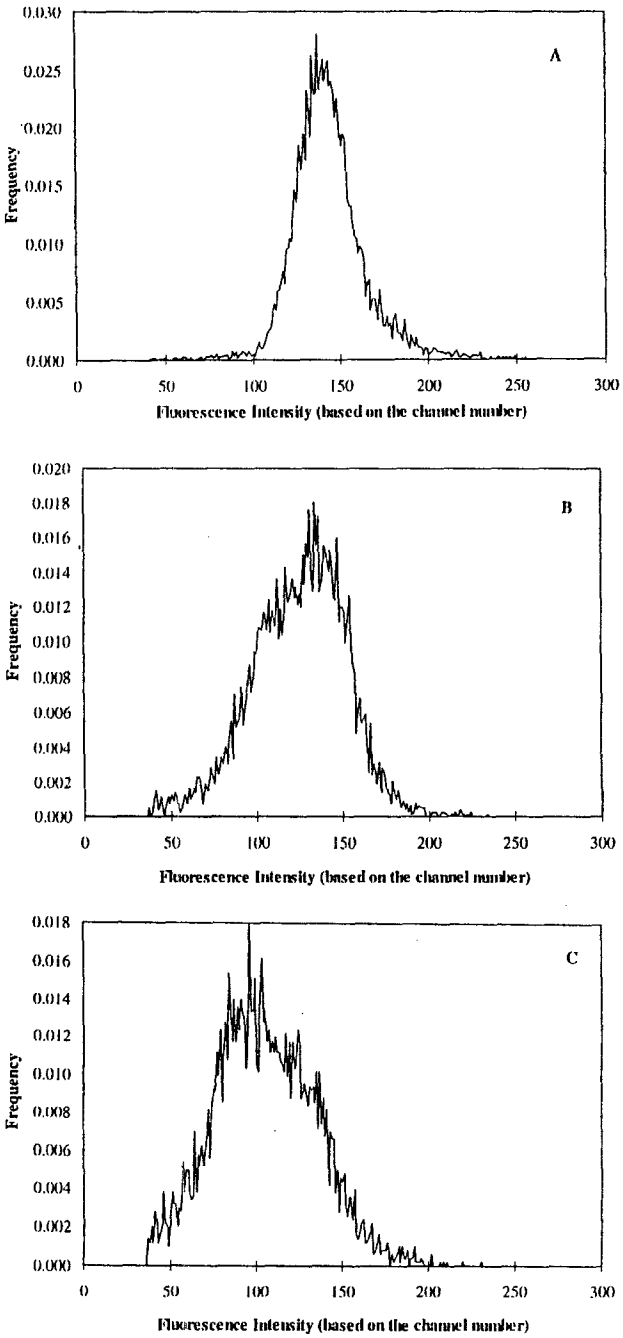

Fig. 3 Mitochondrial staining of wild-type and transfected CHO cells using the cationic dye rhodamine 123. (A) CHO cells, (B) pBA-cyt cells, (C) pAKH-cyt cells and (D) pBA-SC-cyt cells

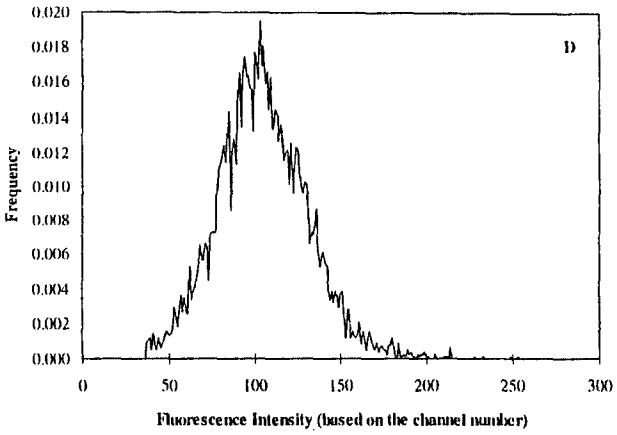

Fig. 3 Continued

orimetric-respirometric (CR) ratio [34]. Oxidation of substrates primarily occurs in mitochondria and the intensity of the protonmotive force was assessed by staining with rhodamine 123. The results are depicted in Fig. 3A–C and gave the same quantitative trend as observed with the calorimetric estimations. The fluorescence intensity was less in the pBA-cyt cells than the wild-type cells but the pAKH-cyt cells showed the least mitochondrial activity. There are several ways to analyse the data of fluorescence intensity (Table 2). Channel numbers of peak fluorescence showed very little difference between wild-type and pBA-cyt cells but it was noticeable that the normalized highest frequency was less in the recombinant cells, indicating a greater heterogeneity of mitochondrial activity. Analysis of covariance (Table 2) highlighted the fact that the decrease in metabolic activity coincident with the level of expression by the cyt gene system, was related to the level of mitochondrial activity.

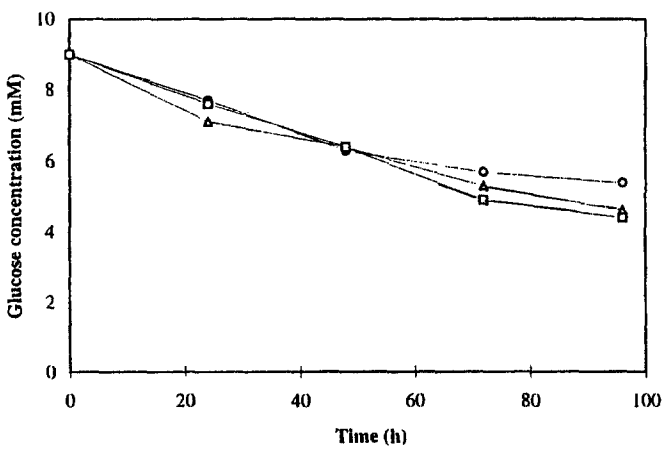

Fig. 4 Comparison of glucose consumption by wild-type and transfected CHO cells. Symbols: wild-type CHO (o); pBA-cyt (Δ) and pAKH-cyt cells (□)

The CR ratio, as previously mentioned, was not significantly different between the three cell types. Gnaiger and Kemp [34] showed that the CR ratio cannot be more exothermic than $-450\pm5\%$ kJ mol^{-1} O_2 unless anaerobic process occur at the same time. This does not mean that the environment is less than fully aerobic because it is now realized that growing cells in normoxic conditions require biosynthetic processes from glucose and glutamine [16] with ATP being produced and with lactate conversion from pyruvate as a by-product. The pathways leading to lactate accumulation produce heat which is additional to that dissipated in oxidative processes. Therefore, one of the probable contributors to the highly exothermic CR ratios could be lactate production. However, the level of glucose and glutamine consumption is insufficient to explain totally the observed CR ratios and further biochemical analysis will be required to find other sources.

The low intensity of mitochondrial activity in the genetically-engineered cells (Table 2) should be reflected in glucose flux if the primary reason for utilization of it were energy production. In fact, Fig. 4 shows that glucose uptake was not inhibited and, when adjusted for cell numbers, the flux of it was greater in both the transfected cell-types than in the controls. Since the studies with rhodamine 123 indicated that the amount of glucose oxidation was low in the recombinant cells, the increased uptake of it must reflect a greater demand for glucose in glycolysis, probably to satisfy the needs of the cyt gene system incorporated into the host chromosomes [8]. Glutamine can be oxidized in mitochondria as an energy source [35] and, in some cell types, it can be the major supply [36]. Mostly, though, it is indispensable to cell growth by (i) providing amino acids through transamination; (ii) being the amino donor to form amino sugars; and (iii) being incorporated into purines and pyrimidines in nucleic acid synthesis. It can be seen from Fig. 5 that much of it is consumed in 96 h. When consumption of it is expressed as a flux (Table 2), it can be seen that slightly more is consumed by the recombinant cells. Since both cell growth and oxidative phosphorylation were less in genetically-engineered cells than the control, it must be assumed that the glutamine is required by the cyt gene system for transcriptional and translational processes.

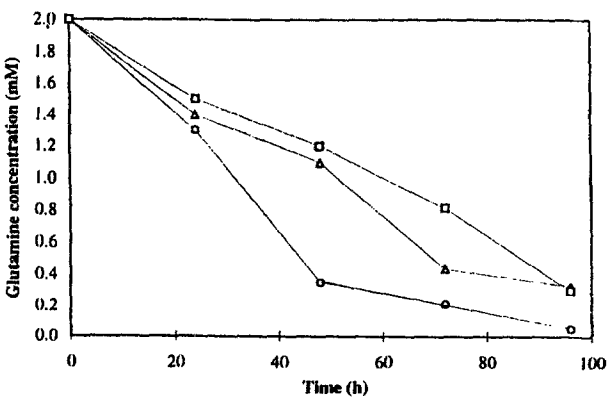

Fig. 5 Comparison of glutamine consumption by wild-type and transfected CHO cells. Symbols: wild-type CHO (o); pBA-cyt (Δ) and pAKH-cyt cells (□)

These results support the claim [6, 7, 37] that recombinant cells have a metabolic burden, but it is intriguing as to why the process of producing heterologous proteins should cause less oxidative processes in the mitochondria. The rates of consumption of glucose and glutamine (Table 2) were not high enough (Figs 4 and 5) to limit mitochondrial activity by exhaustion of the exogeneous supply (Fig. 3) and there was certainly no evidence of reciprocal regulation between them [38]. A possibility is that the demand for both substances to form biosynthetic precursors was so high that their uptake channels in the plasma membrane acted to limit the catabolic rates. The fact that the greater flux was not manifested as increased heat flux is suggestive of greater anabolic activity, the enthalpy change of which is close to zero [13, 14]. Since glucose breakdown by glycolysis only produces 2 mol ATP per mol compared to 36 mol for the oxidation of it, diversion of the glycolytic flux to producing more biosynthetic precursors, would decrease ATP supply and result in decreased growth.

It remains possible that the accumulation of cyt in the cytosol of recombinant cells causes mitochondrial damage by physical force or by physically disrupting the glycolytic pathway and cytosolic gradients. One of the aims of constructing an expression vector with the secretory sequence of alkaline phosphatase after the Kozak sequence (CHO-pBA-SC – see Fig. 1) was to test this hypothesis. Preliminary results with these cells indicate they produce as much cyt as the pAKH-cyt cells, most of which (78%) is secreted into the medium [39]. The specific growth rate is the same as for wild-type cells (Table 1) but the mitochondrial activity (Rh 123, Fig. 3D) is similar to that of pAKH-cyt cells. Having shown that the cyt gene system imposes a metabolic burden on transfected CHO cells, it is hoped that thermobiochemical studies with pBA-SC-cyt CHO cells will provide further elucidation of the mechanism of this burden.

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