Hierarchies of ATP-consuming processes: direct compared with indirect measurements, and comparative aspects

Wolfgang WIESER[1] and Gerhard KRUMSCHNABEL

Institut für Zoologie und Limnologie, Universität Innsbruck, Technikerstrasse 25, A-6020 Innsbruck, Austria

The original aim of the present study was to deal with two problems that had emerged from a study on hierarchies of ATP-consuming processes in cells [Buttgereit and Brand (1995) Biochem. J. **312**, 163–167]. Firstly, we wanted to find out whether the results of that study had been influenced by the method used for the determination of process activity and, secondly, we wondered whether and to what extent the structure of the hierarchy established for cell suspensions under energy-limiting conditions might depend on the type of cell or on the lifestyle, ecology and phylogenetic status of the species from which the cells were derived. We confined our study to the two most prominent ATP consumers of cells: protein synthesis and the Na^+/K^+-ATPase, measuring their activity directly by [^{3}H]leucine incorporation and Rb^+-flux respectively. We found large differences in the sensitivity of protein synthesis to energy limitation between hepatocytes from an anoxia-tolerant fish species and an anoxia-sensitive fish species (goldfish and rainbow trout respectively). On the other hand, Na^+/K^+-ATPase activity was hardly affected by energy limitation in the hepatocytes from both fish species. We also studied the response of a human hepatoma cell line, HepG2, to energy limitation and found both protein synthesis and Na^+/K^+-ATPase activity to be equally sensitive to energy limitation, but more sensitive than the Na^+/K^+-ATPase of the two fish species. A comparison of the indirect and direct methods for measuring protein synthesis revealed the rate of oxygen consumption to be functionally related to the concentration of cycloheximide, the inhibitor used. It was found that at 15 mM cycloheximide [three orders of magnitude higher than the concentration at which the incorporation of free amino acids (FAA) into protein is inhibited] total oxygen consumption was suppressed by 71–75 %, whereas the measured rate of [^{3}H]leucine incorporation into protein suggested that the cycloheximide-sensitive fraction should have amounted to not more than approx. 10 % of the total oxygen consumption. On the other hand, the amount of oxygen consumption suppressed with the high concentration of cycloheximide corresponded almost exactly to the increase in oxygen consumption of cells incubated in an FAA-enriched medium compared with cells incubated in a standard, FAA-free medium. Our major conclusions are, firstly, that high concentrations of cycloheximide disrupt cellular metabolism, bringing to a standstill all those processes that can be stimulated by incubating starved cells in an FAA-enriched medium, secondly, that the attempt to estimate the metabolic cost of protein synthesis by inhibiting oxygen consumption with cycloheximide leads to spurious results, and, thirdly, that the structure of a 'hierarchy' of ATP-consumers may reflect the lifestyle and physiology of the species studied.

Key words: cycloheximide, energetics, hepatocytes, Na^+/K^+-ATPase, protein synthesis.

INTRODUCTION

In the living cell at steady-state, ATP synthesis matches ATP utilization, at least when integrated over a critical time period [1,2]. If the cell is perturbed the metabolic system will pass through a transitory phase, during which the requirement for energetic equilibrium will be relaxed. Depending on the type of perturbance and on the size of the ATP pool, ATP synthesis may overshoot or undershoot ATP utilization temporarily. Following the transitory phase the metabolic system will move towards a new steady state, which is achieved either by ATP synthesis controlling ATP utilization or vice versa. However, this global description hides the fact that a given steady state of total energy metabolism can be achieved by different combinations of ATP-synthesizing and -utilizing processes. Atkinson [3] suggested that on reduction of ATP synthesis, many, or most, ATP-requiring processes will also decrease, albeit not necessarily at the same rate. He predicted the existence of a hierarchy of ATP-utilizing processes, the position in this hierarchy being determined by the sensitivity of each process to the change in energy charge. Thus pathways serving energy storage were believed to be "most sensitive to decreases in energy charge. Biosyntheses of structural macromolecules should be next, and activities that are essential for maintenance … should be able to function at lower values of charge." [3].

The first serious attempt to check Atkinson's prediction was made by Buttgereit and Brand [4]. In concavalin A-stimulated rat thymocytes these authors progressively reduced ATP synthesis in mitochondria by titration with the inhibitor of complex III, myxothiazol, and, by means of specific inhibitors, measured the activity of five ATP-utilizing processes, i.e. protein synthesis (inhibition with cycloheximide), Na^+/K^+-ATPase (inhibition with ouabain), Ca^{2+}-ATPase (inhibition with $LaCl_3$), RNA/DNA synthesis (inhibition with actinomycin) and proton leak. As predicted by Atkinson [3], protein synthesis was found to be most sensitive to the reduction of ATP synthesis, and Na^+/K^+-ATPase considerably less so. Proton leak proved to be the least sensitive to the lowering of ATP synthesis. This pioneering study, in combination with other studies on the balance of ATP synthesis and ATP utilization in different cell types, under a range of conditions, has increased our understanding of the pathways and mechanisms by which the ATP synthesized in mitochondria

Abbreviations used: FAA, free amino acids; TCA, trichloroacetic acid.

[1] To whom correspondence should be addressed (e-mail wolfgang.wieser@uibk.ac.at).

and by glycolysis is distributed to the ATP consumers of a cell. However, there are several critical aspects of this topic that need to be explored further.

Control

Studies with the aim of balancing ATP-synthesizing and -utilizing processes [4–11] have so far been carried out exclusively on mammalian cells. In all cases the two components of ATP turnover would appear to have been in perfect equilibrium. That is, every change in ATP synthesis is followed by a proportional change in ATP utilization and vice versa, and, to quote Buttgereit and Brand [4] "Each ATP consumer had strong control over its own rate but very little control over the rates of the other ATP consumers." However, the generality of this principle is questionable, particularly for tissues under energy-limited conditions. Thus Mandel and Balaban [12] suggested that in the midgut of the tobacco hornworm "a sharing of energy existed between transport and other activities such that energy was shifted between them to maintain the total amount of energy utilized constant." A similar phenomenon was observed in stressed rabbit kidney tubules, which appeared to be capable of "borrowing" a "portion of basal Q_{O_2} to support pump [i.e. Na^+/K^+-ATPase] activity" (where Q_{O_2} is the rate of oxygen consumption) [13]. Finally, Krumschnabel and Wieser [14] established that under conditions of anoxia the rate of glycolytic ATP synthesis in hepatocytes of the goldfish was not altered by ouabain inhibition of Na^+/K^+-ATPase activity. This was interpreted to indicate that the ATP spared by the cessation of Na^+/K^+-ATPase activity was used to fuel other, undisclosed, metabolic processes. If this compensatory principle were found to prevail in energy limited cells, the claim [3] that the relationship between ATP synthesis and ATP utilization is controlled solely by the energy charge of the system would be difficult to uphold.

Comparative aspects

Hierarchies of ATP-consuming processes in relation to the rate of ATP synthesis have so far only been quantified in cells of homoiotherms [4,11]. We consider it unlikely that the hierarchies established in these cells represent a general model valid for all cells. Particularly in poikilothermic animals living in fluctuating and stressful environments, different priorities with respect to energy charge and rate of ATP synthesis may have become selected.

Methodological considerations

The rate of an ATP-consuming process can be determined directly or indirectly, i.e. by measuring the rate of accumulation of a reaction product or by measuring total ATP synthesis (as oxygen consumption or heat production) before and after specific inhibition of the process of interest. Buttgereit and Brand [4] used the indirect method for the determination of ATP-consuming processes. Two problems are attached to this approach: firstly, some inhibitors in common use, particularly cycloheximide, elicit side effects that may lead to under- or over-estimation of the contribution to total ATP synthesis of the particular process studied, and, secondly, if, contrary to the expectation of Buttgereit and Brand [4], a particular ATP-consuming process were to exert strong control over the rate of another consumer, the activity of this process would not be reflected in the change of ATP synthesis after inhibition of the process. Thus the validity of a given hierarchy should be checked by comparing the results of direct and indirect methods.

In the course of the present study on the comparative energetics of hepatocytes from anoxia-tolerant and anoxia-sensitive fish species we studied the responses of the two dominant ATP consumers in these cells, i.e. Na^+/K^+-ATPase activity and protein synthesis, to a gradual reduction in the rate of oxidative ATP synthesis. A comparison of our results with those of Buttgereit and Brand [4] on rat thymocytes revealed both similarities and differences between the poikilothermic and the homoiothermic systems, which we suggest might establish a baseline for future studies on 'hierarchies' in comparative cellular energetics. We also encountered a serious problem with respect to the direct and indirect methods for determining protein synthesis. Although unable to resolve this problem completely we have nevertheless included in the present study the results so far obtained, their being of general importance for any attempt to estimate the 'cost of protein synthesis' by means of inhibition with cycloheximide.

MATERIALS AND METHODS

Chemicals

[^{3}H]leucine (i.e. L-[4,5-^{3}H]leucine) was purchased from Amersham. Collagenase (type VIII), BSA, Leibovitz L-15 medium, cycloheximide and myxothiazol were obtained from Sigma. All other chemicals were of analytical grade and were purchased from local suppliers.

Preparation of cells

Hepatocytes were isolated from goldfish (*Carassius auratus*) acclimated to 20 °C, and from rainbow trout (*Oncorhynchus mykiss*) acclimated to 15 °C, using a collagenase digestion procedure as previously described [15,16]. Following isolation, cells were maintained either in amino acid-free saline [16] or in a modified cell culture medium [standard Leibovitz L-15 medium containing physiological levels of amino acids, modified by addition of 10 mM Hepes, 5 mM $NaHCO_3$ and 1 % (w/v) BSA, pH 7.6]. Incubation and experimental temperatures corresponded to the acclimation temperatures of the two fish species. HepG2 cells were seeded on to 50 ml cell culture dishes and were maintained in Dulbecco's modified Eagle's medium containing 2 mM pyruvate, 2 mM glutamine and 10 % (w/v) fetal calf albumin (pH 7.4) under an atmosphere of 5 % CO_2. When the cultures had reached confluence, cells were harvested by mild trypsin-treatment, pelleted by centrifugation (5 min at 267 ***g***), and suspended in modified Leibovitz L-15 medium (composition as above, pH 7.4) maintained at 25 °C. In all experiments cells were kept in the incubation medium for 1 h before further experimentation.

Measurements of oxygen consumption

Rates of oxygen consumption were determined using a Cyclobios Oxygraph [17]. To this end, cell suspensions were injected into an Oxygraph chamber containing medium that had been equilibrated with air and was devoid of BSA. After a stable rate of oxygen consumption had been attained inhibitors were added from concentrated stock solutions in DMSO as described below.

In order to establish defined levels of inhibition of mitochondrial respiration, cellular oxygen consumption was titrated by the addition of increasing concentrations of either myxothiazol, an inhibitor of complex III of the electron transport chain (trout and HepG2 cells), or NaCN, an inhibitor of complex IV (goldfish), at 3 min intervals. NaCN was used for goldfish hepatocytes, because in these cells myxothiazol inhibition of oxygen consumption did not exceed 60 % (results not shown).

Titration experiments with cycloheximide were conducted in a similar fashion; increasing levels of cycloheximide dissolved in DMSO were added at 3 min intervals. As outlined above, from the difference in the rate of oxygen consumption before and after addition of cycloheximide an indirect estimate for the rate of protein synthesis was derived. Since at higher cycloheximide concentrations the DMSO concentration reached 1% of the respiration medium, parallel experiments were run in which only DMSO was added. The DMSO effect was taken into account in calculating cycloheximide-sensitive rates of oxygen consumption.

We want to stress that in all experiments in which the rate of oxidative phosphorylation was inhibited with myxothiazol or NaCN, the measured rate of oxygen consumption remained linear for the duration of the experiment. This indicated that neither time by itself (between 3 min, the interval chosen by Buttgereit and Brand [4], and 30 min, the interval used in the present experiments) nor time-dependent effects of the inhibitors on cellular metabolism (such as pH or redox state) were decisive factors in determining the rate of ATP turnover.

Determination of ATP-consuming processes

Protein synthesis in fish hepatocytes was measured by following the incorporation of [^{3}H]leucine into trichloroacetic acid (TCA)-precipitable cellular protein as already described in detail [18]. In brief, [^{3}H]leucine was added to cells incubated in modified L-15 medium, and duplicate samples of the suspensions were removed on to Whatman GF/A filters at various times. Filters were then briefly air-dried and placed into ice-cold 10% (w/v) TCA containing 5 mM unlabelled leucine for at least 10 min. This was followed by two 15 min washes in 5% TCA at room temperature and three consecutive 10 min washes in pure ethanol. Finally, filters were air-dried, put into 5 ml scintillation vials containing scintillation cocktail and counted for radioactivity. Samples were taken after 5, 15 and 30 min, at which times myxothiazol or NaCN was added at the desired concentration before further samples were taken at 45 and 60 min. In this way each [^{3}H]leucine incorporation curve served as its own control when comparing rates before and after the addition of inhibitor. As we have previously shown [18], [^{3}H]leucine incorporation is perfectly linear over 60 min under control conditions in the cells from goldfish and trout, so that an underestimation of protein synthetic activity due to proteolysis can be ruled out. In HepG2 cells, due to limited cell material, [^{3}H]leucine and myxothiazol were added simultaneously at time zero and samples were taken after 5, 15 and 30 min of incubation. In this case, cells treated only with DMSO were used as controls. For the conversion of [^{3}H]leucine incorporation (pmol/10^{-6} cells per min) into rates of protein synthesis (g of protein/10^{-6} cells per min) we assumed a mean leucine content in cellular proteins of 8% [19], and a mean molecular mass for amino acids of 110 Da.

Estimates for Na^+/K^+-ATPase activity were obtained by determination of cellular uptake of Rb^+. This was achieved for fish hepatocytes by preincubating the cells with various concentrations of either myxothiazol or NaCN for 15 min, followed by transfer of the cells into modified L-15 medium containing Rb^+ instead of K^+, and the same concentration of inhibitor. Rb^+ uptake was then followed by taking three consecutive samples over a time course of 15 min, as described previously [16]. This protocol ensured that rates of Na^+/K^+-ATPase activity and protein synthesis could be compared after similar times of exposure to mitochondrial inhibitors. Again, because of insufficient cell material, the protocol for HepG2 cells differed in that only one duplicate sample was taken at the end of the Rb^+ incubation period. We have repeatedly shown that in teleost hepatocytes more than 95% of Rb^+ uptake is sensitive to inhibition by ouabain [15,16,20], making Rb^+ uptake a fairly good estimate of enzyme activity. However, since we have not shown this to be true for HepG2 cells, our results may tend to overestimate Na^+/K^+-ATPase activity in these cells.

RESULTS

Cycloheximide titration

In a series of titration experiments we established the effects of the concentration of cycloheximide on the rate of both oxygen consumption and [^{3}H]leucine incorporation into protein in the three cellular systems studied. Figure 1 shows that the effects of cycloheximide concentration are unequivocal and similar in all three systems. Firstly, cycloheximide totally inhibits ^{3}H-incorporation at concentrations as low as 25 μM. Secondly, the inhibitory effect on the rate of oxygen consumption increases with cycloheximide concentration, following a nearly hyperbolic

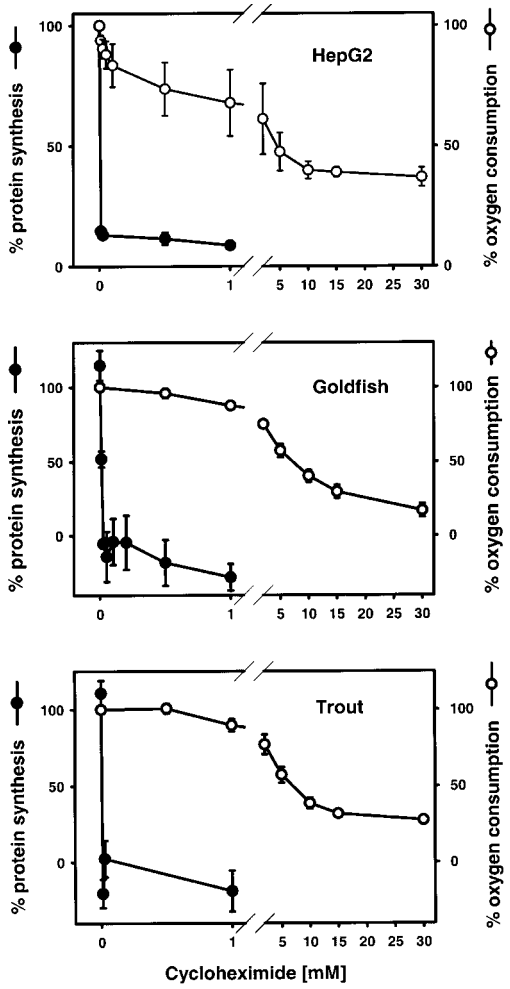

Figure 1 Titration of oxygen consumption and [^{3}H]leucine incorporation with cycloheximide

Hepatocytes from two fish species (rainbow trout and goldfish) and a human hepatoma line (HepG2), suspended in modified Leibowitz L-15 medium with FAA, were titrated with increasing concentrations of cycloheximide. In one series of experiments oxygen consumption of the cells was measured simultaneously. In another series the rate of [^{3}H]leucine incorporation into TCA-precipitable proteins was determined after addition of a specific dose of cycloheximide to the medium.

Table 1 Estimates of rates of protein synthesis in three cellular systems based on an indirect and a direct method of determination

The cellular systems studied were hepatocytes from goldfish and rainbow trout, and a human hepatoma line (HepG2). Cell suspensions were measured in medium without (standard) and with (Leibovitz) FAA. For the indirect determination of protein synthesis oxygen consumption was measured before and after treatment of the suspension with 1 mM and 15 mM cycloheximide (CH), the difference representing cycloheximide-sensitive oxygen consumption. Rates of oxygen consumption ($\dot{V}o_2$; total and CH-sensitive) are given as nmol of oxygen/10^{-6} cells per min, and the incorporation of [^{3}H]leucine is given as pmol of [^{3}H]leucine/10^{-6} cells per min. Values are given as means ± S.E.M. n.d., not determined.

		Process rate			
			CH-sensitive $\dot{V}o_2$		
Cellular system (temperature)	Medium (*n*)	Total $\dot{V}o_2$	[CH] = 1 mM	[CH] = 15 mM	[^{3}H]leucine incorporation
Goldfish (20 °C)	Standard (4)	0.209 ± 0.034	0.022 ± 0.016	0.108 ± 0.026	–
	Leibovitz (4–6)	0.738 ± 0.063	0.095 ± 0.022	0.522 ± 0.117	2.518 ± 0.246
Trout (15 °C)	Standard (6)	1.013 ± 0.072	0.052 ± 0.020	0.517 ± 0.072	–
	Leibovitz (6–8)	5.266 ± 0.596	0.952 ± 0.277	3.962 ± 0.385	5.776 ± 0.302
HepG2 (25 °C)	Leibovitz (3–4)	0.898 ± 0.095	0.150 ± 0.049	n.d.	13.077 ± 0.982

trajectory; not reaching a lower plateau until approx. 30 mM cycloheximide (note the change in scale in Figure 1). As will be explained in the Discussion section we assume the dramatic effect of high doses of cycloheximide on the rate of oxygen consumption to be due to disruption of cellular metabolism. However, even the highest doses did not impair the viability of the cells, the best proof being the constancy of the ATP pool throughout the cycloheximide-titration experiments (e.g. 15 mM cycloheximide did not significantly affect the ATP concentration in trout hepatocytes over time: $t_0 = 3.67 \pm 0.21$ nmol/10^{-6} cells; $t_{15\ min} = 3.23 \pm 0.57$ nmol/10^{-6} cells). Moreover, in the goldfish hepatocytes the activity of the Na^+/K^+-ATPase was hardly affected by incubation with 15 mM cycloheximide (results not shown).

Apparent energetic cost of protein synthesis

Since cycloheximide interferes with translation by binding to the 60 S subunit of ribosomes, the application of cycloheximide to cells has always been considered to provide information on what may be called the 'true' cost of protein synthesis, i.e. that due to peptide-bond formation. A more comprehensive approach to estimating the cost of protein synthesis is to compare the rates of oxygen consumption of cells in a standard saline medium and in a medium enriched with free amino acids (FAA). The results of such an experiment with goldfish and trout hepatocytes were striking and unexpected. As Table 1 indicates, incubating the hepatocytes in the FAA-enriched medium led to an increase in their rates of oxygen consumption by factors of 3.5 and 5.2 in goldfish and trout respectively. In absolute values, the difference in total oxygen consumption between cells incubated in an enriched (Leibovitz) medium and cells incubated in a standard, FAA-free medium turned out to be close to the cycloheximide-sensitive component of total oxygen consumption at the highest cycloheximide concentrations used. For example, with 15 mM cycloheximide, oxygen consumption of the hepatocytes was suppressed by 0.522 and 3.962 nmol/10^{-6} cells per min respectively in goldfish and trout cells, which corresponds almost exactly to the difference in oxygen consumption between enriched and standard medium (0.529 and 4.253 nmol/10^{-6} cells per min). We have not carried out the same experiment with the hepatoma cells, but we know from the literature that in cells from homoiothermic animals the addition of FAA to the medium causes standard rates of oxygen consumption to increase by not more than 30 % (see the Discussion section).

In Table 2, we summarize the information required for calculating the 'cost of protein synthesis' expressed as mmol of cycloheximide-sensitive oxygen consumption/g of protein synthesized. From the titration experiments we chose the two concentrations, 1 and 15 mM cycloheximide, as used in similar experiments reported in the literature [4,21]. In agreement with these older findings we found that the so-called 'cost of protein synthesis' based on inhibiting oxygen consumption with cyclo-

Table 2 Apparent cost of protein synthesis in three cellular systems, based on the inhibition of oxygen consumption with two concentrations of cycloheximide

The cellular systems studied were hepatocytes from goldfish and rainbow trout, and a human hepatoma cell line. CH-sensitive $\dot{V}o_2$, cycloheximide-sensitive oxygen consumption; [^{3}H]leucine, incorporation of labelled leucine; pb, peptide bonds. All the cells were incubated for 1 h in Leibovitz medium before the measurements were made. The oxygen consumption data shown are based on the set of mean values summarized in Table 1, but all of them are expressed in pmol/10^{-6} cells per min. [^{3}H]leucine incorporation was converted into rates of protein synthesis (g of protein/10^{-6} cells per min) assuming a mean leucine content of cellular proteins of 8% and a mean molecular mass of amino acids of 110 Da. The apparent cost of protein synthesis (bold) is expressed in mmol of cycloheximide-sensitive oxygen consumed/g of protein synthesized.

	Goldfish		Trout		HepG2
Variable	1 mM CH	15 mM CH	1 mM CH	15 mM CH	1 mM CH
CH-sensitive $\dot{V}o_2$	95	522	952	3962	150
Protein					
pmol of [^{3}H]leucine	2.5	2.5	5.8	5.8	13.1
pmol of pb	31	31	72.5	72.5	164
ng	3.4	3.4	8.0	8.0	18.0
Apparent cost of protein synthesis	**28**	**154**	**119**	**495**	**8.3**

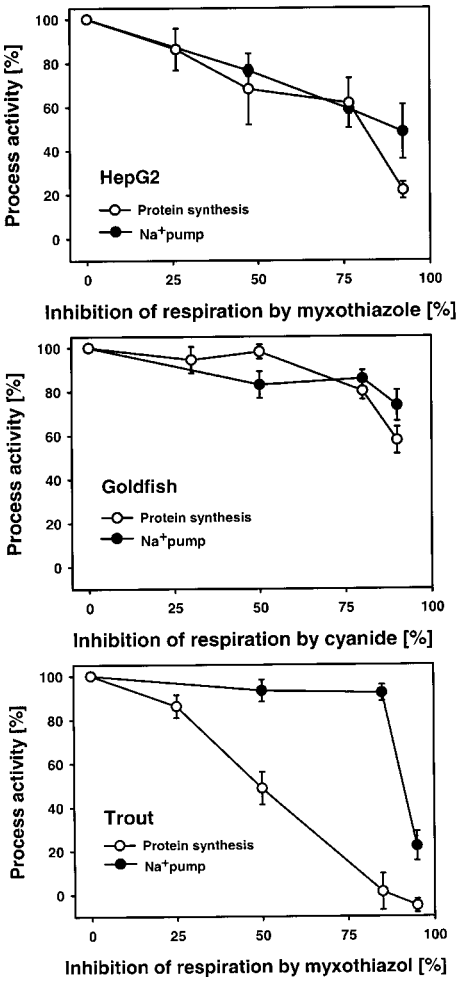

Figure 2 Effects of myxothiazol or cyanide on the rate of protein synthesis and Na^+/K^+-ATPase activity in three cellular systems

Relative process activity at different concentrations of myxothiazol or cyanide in the medium was determined directly by measuring [^{3}H]leucine incorporation (protein synthesis) or Rb^+ uptake (Na^+/K^+-ATPase activity) in the cells.

heximide covers an implausibly wide range, in our case from 8.3 to 495 mmol of oxygen/g of protein. Obviously such a range disqualifies all similarly derived data from indicating the cost of protein synthesis in terms of peptide-bond formation.

Sensitivity of two ATP consuming functions to changes in energy supply

Figure 2 summarizes the effects of the reduction in aerobic energy (ATP) supply (by titrating cytochrome oxidase activity with cyanide or complex III with myxothiazol) on protein synthesis and the activity of the Na^+/K^+-ATPase. These experiments were planned to simulate the effects of progressive hypoxia on the two most prominent ATP consumers in the cellular systems studied. The largest difference observed was that between protein synthesis in the hepatocytes from the anoxia-tolerant goldfish and those from the anoxia-sensitive rainbow trout. The responses of the two ATP-consuming processes in the hepatocytes from the poikilothermic goldfish were qualitatively similar to those of the same processes in human hepatoma cells, although the former proved to be much less sensitive to the reduction of aerobic ATP production than the latter.

DISCUSSION

Cycloheximide and protein synthesis

Our data corroborate the literature [6] on this topic insofar as the incorporation of labelled amino acids into proteins was found to be blocked by 25 μM cycloheximide. The rate of cycloheximide-sensitive oxygen consumption, however, turned out to be strongly dependent on the concentration of the inhibitor used. As Figure 1 demonstrates, the amount of oxygen consumption depressed is a quasi-hyperbolic function of cycloheximide concentration, reaching a lower plateau at not less than 15 mM, which is about three orders of magnitude higher than the concentration required for blocking the incorporation of FAA into proteins. In our cellular systems, cycloheximide-sensitive oxygen consumption with 30 mM cycloheximide ranged from 60 to 90% of the control oxygen consumption. The same effect has already been noted, Houlihan and co-workers [21,22] reporting a reduction of oxygen consumption in hepatocytes and other cells from the rainbow trout of the order of 40–90% using 0.7 to 7.0 mM cycloheximide and 70–90% using 30 mM cycloheximide. On the basis of these figures the authors concluded that in rainbow trout hepatocytes protein synthesis absorbed from 40 to 90% of aerobic ATP production, yielding an estimated cost of protein synthesis from 11 to 217 mmol of oxygen/g of protein. In our experiments with 15 mM cycloheximide the highest apparent cost of protein synthesis amounted to 495 mmol of oxygen/g of protein (Table 2). From a strictly biochemical point of view, assuming an average of five ATP molecules per peptide bond, the metabolic cost of peptide-bond formation corresponds to approx. 6 mmol of oxygen/g of protein [23,24], uncertainty in this respect being mainly due to the poorly defined caloric equivalent of ATP in the living cell. Obviously the extreme values reported by us and others are far beyond anything that can be accounted for by realistic estimates of the cost of peptide-bond formation. However, physiologists have long known that the actual cost of protein 'synthesis' in whole animals by far exceeds the theoretical value of peptide-bond formation. For whole pigs, a cost of 21.5 kJ/g of protein was calculated [25], and Fuller et al. [26] obtained values ranging from 7.4 to 31.6 kJ/g deposited, according to the nature of the diet, with a mean of approx. 20 kJ/g. According to Waterlow and Millward [27] there is on average a 5-fold difference between the energy cost of protein synthesis estimated directly from the stoichiometry of bond formation, and the indirect estimate based on the measurement of heat production or carcass analysis on whole animals. This difference between biochemical and physiological measurements is usually attributed to the energy costs of protein turnover and deposition, RNA turnover, amino acid transport and metabolic regulation [27]. Our study provides a new perspective insofar as, firstly, the apparent cost of protein synthesis based on the use of high concentrations of cycloheximide by far exceeds the hypothetical 5-fold difference between biochemical and physiological costs (see Table 2), and, secondly, that this difference applies even to short-lived cellular systems in which, for example, the cost of protein deposition would not appear to be a realistic assumption.

Building proteins from scratch, and the consequences

In a medium devoid of FAA the rate of protein synthesis depends entirely on protein turnover, settling to the lowest level sufficient to support essential maintenance functions. If FAA are

added to such an impoverished medium the machinery of protein synthesis will be thrown into high gear, stimulating an array of ATP-consuming functions that had been repressed during FAA-starvation. Thus by measuring the rate of oxygen consumption of cells in media with and without FAA a minimum and a maximum level of metabolic performance can be defined. The difference between these levels depends, firstly, on the types and range of physiological functions the cell is able to perform, and, secondly, on the degree to which these functions can be turned off during periods of starvation. Such experiments have been carried out, for example, on mice Ehrlich ascites tumour cells [8], and on rabbit reticulocytes [6]. In these types of cells the addition of FAA to the medium resulted in an increase in total oxygen consumption by 38 and 23 % respectively. In contrast, the addition of FAA to a standard medium with hepatocytes from goldfish and rainbow trout caused the rate of oxygen consumption to increase by 253 and 420 % respectively (the present study, see Table 1). The validity of such a dramatic increase is supported by a previous investigation [20] on two groups of rainbow trout, one kept on a maintenance ration and the other fed *ad libitum*. At 15 °C hepatocytes from the trout fed *ad libitum* displayed metabolic rates 132 % higher than the comparable rates from maintenance-fed trout. These findings seem to suggest that the addition of FAA to starved, resting or maintenance-fed cells leads to an increase in metabolic activity, the upper level of which cannot be accounted for by the metabolic cost of peptide-bond formation, and that this response is much more pronounced in cells from poikilothermic than homoiothermic organisms. We feel that the problem raised by this finding is somehow connected to a problem that was extensively studied more than 20 years ago by Todorov and Ch'ih and their respective co-workers [28–30]. They found that sublethal concentrations of cycloheximide reversibly disrupted cellular metabolism and that after removal of cycloheximide a multitude of processes were activated in order to restore the previous steady-state, including an increased uptake of nutrients, acceleration of proteolysis and protein turnover for constructing new polysomes, acceleration of protein transport from cytoplasm to nucleus and vice versa, synthesis of mRNA and more. We speculate that the dramatic increase in the rate of oxygen consumption in fish cells in an FAA-enriched medium was due to the mobilization of an equivalent range of processes depending on accelerated protein synthesis and ATP production. The large difference in this respect between the responses of poikilothermic and homoiothermic cells can be explained by assuming that in the absence of FAA, basal metabolism is more drastically reduced in cells from poikilothermic species than in those from homoiothermic species. Consequently, the scope for metabolic activity in the presence of FAA turns out to be much larger in the former than in the latter. A corollary to these assumptions is that the metabolism of fish cells suspended in a saline medium without FAA is greatly depressed and can be compared with the state of metabolism of the rat hepatocytes studied by Todorov [29] after treatment with high doses of cycloheximide.

'Hierarchies' of ATP-utilizing processes

As mentioned in the Introduction section, a 'hierarchy' of ATP-utilizing processes with respect to the rate of ATP production has been reported in concavalin A-stimulated rat thymocytes [4]. The order of sensitivity was as follows: protein synthesis > RNA/DNA synthesis > Na^+ cycling > Ca^{2+} cycling > proton leak. In hamster fibroblasts, protein synthesis was reported to be much more sensitive to a decrease in ATP content than protein breakdown, falling by 50 % when ATP was reduced by only 15 % [31]. In the case of rat thymocytes protein synthesis was shown to fall by 50 % when the reduction of ATP synthesis had reached approx. 25 % [4]. In this case the ATP requirement for protein synthesis and other ATP-utilizing processes was defined by measuring oxygen consumption before and after the application of specific inhibitors, which in the case of protein synthesis was cycloheximide. In view of what has been said above concerning problems connected with this inhibitor we used direct methods for determining process activity, i.e. incorporation of [^{3}H]leucine for protein synthesis, and Rb^+-flux for the Na^+/K^+-ATPase [15,16]. The data presented in Figure 2 allow us to grade the three cellular systems studied by their sensitivity towards a reduction of ATP production. The following two series indicate the average relative process activities when ATP production was reduced by 50 %: (1) protein synthesis, goldfish hepatocytes (99 %) > human hepatoma cells (68 %) > trout hepatocytes (50 %); (2) Na^+/K^+-ATPase, trout (93 %) > goldfish (82 %) > human (78 %).

Unexpectedly, the most striking difference in sensitivity to energy limitation was not found between the two poikilothermic fish species and human hepatoma cells, but between the two fish species. Hepatocytes from the anoxia-sensitive trout responded to the reduction of ATP production by massive down-regulation of protein synthesis, a characteristic of animals capable of 'metabolic depression' [32,33]. Why the goldfish differed in its response to the same stress scenario is unclear, but it may indicate that the capacity for protein synthesis was maintained in this anoxia-tolerant species so as to allow the selective production of proteins adapted to periods of energy limitation.

The activity of the Na^+/K^+-ATPase was considerably less sensitive to energy limitation in the two fish species than in human hepatoma cells. The data presented in Figure 2 indicate that the sensitivities of protein synthesis and Na^+/K^+-ATPase activity as determined by direct methods were practically identical in the hepatoma cells and in the goldfish hepatocytes. This is in contrast with Atkinson's expectation [3], and the findings of Buttgereit and Brand [4] in rat thymocytes based on indirect methods of determination. The similarity of mammalian hepatoma cells and hepatocytes from an anoxia-tolerant fish species in this respect may be due to the higher dependence of the energy metabolism of these two cellular systems on glycolytic activity compared with the metabolism of the anoxia-sensitive trout hepatocytes. We have shown this to be the case for the hepatocytes from goldfish [15].

This work was supported by the Fonds zur Förderung der wissenschaftlichen Forschung in Österreich, project number 13464-BIO. G.K. is the recipient of an APART grant from the Austrian Academy of Sciences. Our experiments with the human hepatoma line were made possible through a gift from Professor G. Utermann (Institut für Medizinische Biologie und Humangenetik der Universitat, Innsbruck, Austria) and the expert technical assistance of Ms Linda Fineder.

REFERENCES

1 Arthur, P. G., Hogan, M. C., Bebout, D. E., Wagner, P. D. and Hochachka, P. W. (1992) Modeling the effects of hypoxia on ATP turnover in exercising muscle. J. Appl. Physiol. **73**, 737–742
2 Hochachka, P. W. (1994) Solving the common problem: matching ATP synthesis to ATP demand during exercise. Adv. Vet. Sci. Comp. Med. **38A**, 41–56
3 Atkinson, D. E. (1977) In Cellular Energy Metabolism and its Regulation, p. 218, Academic Press, New York
4 Buttgereit, F. and Brand, M. D. (1995) A hierarchy of ATP-consuming processes in mammalian cells. Biochem. J. **312**, 163–167
5 Siems, W., Dubiel, W., Dumdey, R., Muller, M. and Rapoport, S. M. (1984) Accounting for the ATP-consuming processes in rabbit reticulocytes. Eur. J. Biochem. **139**, 101–107

6 Siems, W., Muller, M., Dubiel, W., Dumdey, R. and Rapoport, S. (1986) ATP production and consumption of rabbit reticulocytes increase in an amino-acid-enriched medium. Biomed. Biochim. Acta **45**, 585–591

7 Siems, W. G., Schmidt, H., Gruner, S. and Jakstadt, M. (1992) Balancing of energy-consuming processes of K 562 cells. Cell Biochem. Funct. **10**, 61–66

8 Muller, M., Siems, W., Buttgereit, F., Dumdey, R. and Rapoport, S. M. (1986) Quantification of ATP-producing and consuming processes of Ehrlich ascites tumour cells. Eur. J. Biochem. **161**, 701–705

9 Schmidt, H., Siems, W., Muller, M., Dumdey, R. and Rapoport, S. M. (1991) ATP-producing and consuming processes of Ehrlich mouse ascites tumor cells in proliferating and resting phases. Exp. Cell Res. **194**, 122–127

10 Culic, O., Gruwel, M. L. and Schrader, J. (1997) Energy turnover of vascular endothelial cells. Am. J. Physiol. **273**, C205–C213

11 Buttgereit, F., Burmester, G. R. and Brand, M. D. (2000) Therapeutically targeting lymphocyte energy metabolism by high-dose glucocorticoids. Biochem. Pharmacol. **59**, 597–603

12 Mandel, L. J. and Balaban, R. S. (1981) Stoichiometry and coupling of active transport to oxidative metabolism in epithelial tissues. Am. J. Physiol. **240**, F357–F371

13 Soltoff, S. P. and Mandel, L. J. (1984) Active ion transport in the renal proximal tubule. III. The ATP dependence of the Na pump. J. Gen. Physiol. **84**, 643–662

14 Krumschnabel, G. and Wieser, W. (1994) Inhibition of the sodium pump does not cause a stoichiometric decrease of ATP-production in energy limited fish hepatocytes. Experientia **50**, 483–485

15 Krumschnabel, G., Schwarzbaum, P. J. and Wieser, W. (1994) Coupling of energy supply and energy demand in isolated goldfish hepatocytes. Physiol. Zool. **67**, 438–448

16 Krumschnabel, G., Biasi, C., Schwarzbaum, P. J. and Wieser, W. (1996) Membrane-metabolic coupling and ion homeostasis in anoxia-tolerant and anoxia-intolerant hepatocytes. Am. J. Physiol. **270**, R614–R620

17 Haller, T., Ortner, M. and Gnaiger, E. (1994) A respirometer for investigating oxidative cell metabolism: toward optimization of respiratory studies. Anal. Biochem. **218**, 338–342

18 Krumschnabel, G., Biasi, C. and Wieser, W. (2000) Action of adenosine on energetics, protein synthesis and K^+ homeostasis in teleost hepatocytes. J. Exp. Biol. **203**, 2657–6526

19 Carter, C. G., He, Z.-Y., Houlihan, D. F., McCarthy, I. D. and Davidson, I. (1995) Effect of feeding on the tissue free amino acid concentration in rainbow trout (*Oncorhynchus mykiss* Walbaum). Fish Physiol. Biochem. **14**, 153–164

20 Krumschnabel, G., Biasi, C., Schwarzbaum, P. J. and Wieser, W. (1997) Acute and chronic effects of temperature, and of nutritional state, on ion homeostasis and energy metabolism in teleost hepatocytes. J. Comp. Physiol., B **167**, 280–286

21 Pannevis, M. C. and Houlihan, D. F. (1992) The energetic cost of protein synthesis in isolated hepatocytes of rainbow trout (*Oncorhynchus mykiss*). J. Comp. Physiol., B **162**, 393–400

22 Smith, R. W. and Houlihan, D. F. (1995) Protein synthesis and oxygen consumption in fish cells. J. Comp. Physiol., B **165**, 93–101

23 Land, S. C., Buck, L. T. and Hochachka, P. W. (1993) Response of protein synthesis to anoxia and recovery in anoxia-tolerant hepatocytes. Am. J. Physiol. **265**, R41–R48

24 Fuery, C. J., Withers, P. C. and Guppy, M. (1998) Protein synthesis in the liver of *Bufo marinus*: cost and contribution to oxygen consumption. Comp. Biochem. Physiol., Part A: Mol. Integr. Physiol. **119**, 459–467

25 Reeds, P. J., Cadenhead, A., Fuller, M. F., Lobley, G. E. and McDonald, J. D. (1980) Protein turnover in growing pigs. Effect of age and food intake. Br. J. Nutr. **43**, 445–455

26 Fuller, M. F., Cadenhead, A., Mollison, G. and Seve, B. (1987) Effects of the amount and quality of dietary protein on nitrogen metabolism and heat production in growing pigs. Br. J. Nutr. **58**, 277–285

27 Waterlow, J. C. and Millward, D. J. (1989) Energy cost of turnover of protein and other cellular constituents. In Energy Transformations in Cells and Organisms (Wieser, W. and Gnaiger, E., eds.), pp. 277–282, G. Thieme Verlag, Stuttgart

28 Todorov, I. N., Smal'ko, P. Ya. and Galkin, A. P. (1978) State of the polyribosomes as a reflection of the functional interaction of the systems of translation and transcription during the restoration of protein biosynthesis inhibited by cycloheximide. Biochemistry **42**, 1693–1700

29 Todorov, I. N. (1990) How cells maintain stability. Sci. Am. **263**, 32–39

30 Ch'ih, J. J., Duhl, D. M., Faulkner, L. S. and Devlin, T. M. (1979) Regulation of mammalian protein synthesis *in vivo*. Simulated transport of nuclear ribonucleoprotein complexes to the cytoplasm after cycloheximide treatment. Biochem. J. **178**, 643–649

31 Gronostajski, R. M., Pardee, A. B. and Goldberg, A. L. (1985) The ATP dependence of the degradation of short- and long-lived proteins in growing fibroblasts. J. Biol. Chem. **260**, 3344–3349

32 Guppy, M. and Withers, P. (1999) Metabolic depression in animals: physiological perspectives and biochemical generalizations. Biol. Rev. Cambridge Philos. Soc. **74**, 1–40

33 Hand, S. C. and Hardewig, I. (1996) Downregulation of cellular metabolism during environmental stress: mechanisms and implications. Annu. Rev. Physiol. **58**, 539–563

Received 25 October 2000/2 January 2001; accepted 5 February 2001