THE EFFECTS OF CELL AGEING ON METABOLISM IN RAINBOW TROUT (*ONCORHYNCHUS MYKISS*) RED BLOOD CELLS

M. C. L. PHILLIPS, C. D. MOYES AND B. L. TUFTS*

Department of Biology, Queen's University, Kingston, Ontario, Canada K7L 3N6

*Author for correspondence (e-mail: tuftsb@biology.queensu.ca)

Summary

The effects of cell age on metabolism in the nucleated red blood cells of rainbow trout (*Oncorhynchus mykiss*) were examined. Red blood cells were separated according to age using fixed-angle centrifugation. The mean erythrocyte haemoglobin concentration in old red blood cells was found to be 120 % of that in young red blood cells. In young red blood cells, the activities of the mitochondrial enzymes citrate synthase and cytochrome oxidase were 135–200 %, respectively, of those measured in old red blood cells. The activity of the glycolytic enzyme lactate dehydrogenase in young red blood cells was 170 % of that in old red blood cells, whereas the activity of the glycolytic enzyme pyruvate kinase was not significantly affected by cell age. In addition, young red blood cells consumed over twice as much O_2 and devoted 50 % more O_2 to protein synthesis and the activity of Na^+/K^+-ATPase than old red blood cells. Red blood cell age did not significantly affect the rate of lactate production. This study shows that ageing in rainbow trout nucleated red blood cells is accompanied by a significant decline in aerobic energy production and the processes it supports, as well as a corresponding increase in the glycolytic contribution to metabolism.

Key words: rainbow trout, *Oncorhynchus mykiss*, metabolism, ageing, fish, erythrocyte, red blood cell.

Introduction

Striking differences in morphology exist between the red blood cells of mammalian and non-mammalian vertebrates. One of the most notable differences is that mammalian red blood cells lose their nuclei before entering the blood, whereas non-mammalian red blood cells remain nucleated throughout their circulatory lifespan (Sekhon and Beams, 1969; Boutilier and Ferguson, 1989). Mammalian red blood cells are also typically biconcave and extrude their organelles shortly after release into the circulation, whereas non-mammalian red blood cells are biconvex and retain a significant number of organelles and ribosomes while in the blood (Sekhon and Beams, 1969; Lane and Tharp, 1980; Lane et al., 1982). In contrast to their enucleated counterparts, the retention of organelles such as mitochondria enables nucleated red blood cells to utilize aerobic metabolism (Boutilier and Ferguson, 1989). This aerobic ATP production may be required for processes, such as protein synthesis (Speckner et al., 1989; Keen et al., 1989), that are absent from enucleated red blood cells. To date, however, the functional significance of the additional aerobic capacity of nucleated red blood cells has not been thoroughly investigated.

The effects of cell ageing on energy producing and utilizing pathways in nucleated red blood cells are also unknown (Walsh et al., 1998). The loss of mitochondria with ageing described in ultrastructural studies (Sekhon and Beams, 1969; Keen et al., 1989) suggests a significant reduction in the capacity to produce ATP aerobically. It is not known, however, whether these changes reflect an overall reduction in total (oxidative and glycolytic) ATP production or whether increased glycolytic ATP production might compensate for a decline in aerobic energy production as nucleated red blood cells age. Reductions in polyribosome content (Lane and Tharp, 1980; Keen et al., 1989) also provide evidence that energy utilization for protein synthesis declines during ageing in nucleated red blood cells. Assuming that total energy production does decline with age, changes in energy utilization pathways, such as that for protein synthesis, would be expected since energy utilization must be matched to energy production under steady-state conditions. At present, however, the effects of ageing on specific energy utilization pathways in nucleated red blood cells have not been well described.

On this background, the purpose of the present study is to examine the effects of cell age on aerobic and glycolytic metabolism, as well as the energy requirements of protein synthesis and the Na^+/K^+-ATPase, in the nucleated red blood cells of rainbow trout *Oncorhynchus mykiss*. It is hypothesized that ageing will be associated with a decline in the rate of respiration and possibly a compensatory increase in glycolysis. Moreover, any reductions in total energy production will be matched by declines in energy-consuming processes.

Materials and methods

Blood preparation

Freshwater rainbow trout *Oncorhynchus mykiss* (Walbaum) (1–4 kg) were obtained from a commercial fish hatchery (Pure Springs Trout Farm, Belleville, Ontario, Canada) and held in the Animal Care Facility at Queen's University, Kingston, Ontario. Fish were kept in aerated dechlorinated water at 10–15 °C and fed commercial fish pellets at regular intervals of 3–4 days.

Individual trout were anaesthetized in dechlorinated water containing $250\,mg\,l^{-1}$ tricaine methane sulphonate (MS-222; Sigma) and $500\,mg\,l^{-1}$ $NaHCO_3$ buffer. Blood (approximately 15 ml) was drawn into a syringe *via* caudal puncture and placed in a chilled flask containing heparinized $(40\,i.u.\,ml^{-1})$ saline (in $mmol\,l^{-1}$: 124 NaCl, 5 KCl, 0.5 $MgCl_2$, 1.1 $CaCl_2$, 5.5 glucose, 10 $NaHCO_3$). The red blood cells were washed three times with saline, and special care was taken to remove all white blood cells using an aspirator.

Separation of age fractions

Following the washing procedure, red blood cells were separated into different age fractions using fixed-angle centrifugation. This technique has been used successfully in previous studies (Murphy, 1973; Cohen et al., 1976; Speckner et al., 1989), and only minor modifications were incorporated into the present experiments. Initially, red blood cells were adjusted to a haematocrit of 80 % and filled to the top of a narrow centrifuge tube (diameter 13 mm, length 100 mm, volume 6.5 ml). The red blood cells were then centrifuged $(10\,000\,\boldsymbol{g}$, temperature 14 °C) for 15 min with a JA-20 rotor inside a centrifuge (Beckman; model J2-21M) at a fixed angle of 30 ° to the vertical. Following centrifugation, six equal 1 ml volumes of red blood cells were carefully removed from the tube using a pipette. Each 1 ml fraction was numbered according to its position in the centrifuge tube (fraction 1 for red blood cells at the top, fraction 6 for red blood cells at the bottom). Individual fractions were then washed twice in saline and kept on ice for subsequent experiments.

Experimental protocol and analyses

The protocol consisted of four separate series of *in vitro* experiments. In the first series, the mean erythrocyte haemoglobin (Hb) concentration (MEHC) was measured in fractions 1–6 for varying centrifugation speeds and times to obtain the best age separation possible. In the second series, the activities of the metabolic enzymes citrate synthase (CS), cytochrome oxidase (COX), pyruvate kinase (PK) and lactate dehydrogenase (LDH) were measured in fractions 1–6. In the third series, O_2 consumption was measured for fractions 1–6, and inhibition experiments with cycloheximide and ouabain were conducted on fractions 1 and 6 only. In the fourth series, lactate production was measured for fractions 1 and 6 only.

Haemoglobin

The Hb content of each fraction was determined using the Hb-cyanide method (Betke and Savelsberg, 1950; van Kampen and Zijlstra, 1961) and a DU 640 spectrophotometer (Beckman). The haematocrit (Hct) of each fraction was measured after centrifuging the samples for 4 min in an IEC MB microhaematocrit centrifuge (Damon/IEC Division). MEHC for each fraction was calculated as [Hb]/Hct.

Pyruvate kinase, lactate dehydrogenase and citrate synthase

Enzyme samples were prepared for each age fraction by adding 100 μl of packed red blood cells to 1 ml of cell extraction buffer $(20\,mmol\,l^{-1}$ Hepes, pH 7.0, $1\,mmol\,l^{-1}$ EDTA, 0.1 % Triton X-100) followed by vortexing and sonication for several seconds at a low setting. Samples were frozen at −80 °C until the day of the assay. PK activity was measured at 25 °C using a Spectramax Plus plate spectrophotometer (Molecular Devices) and detected at 340 nm as the rate of disappearance of NADH from 300 μl of a mixture containing $50\,mmol\,l^{-1}$ Hepes, pH 7.0, $5\,mmol\,l^{-1}$ ADP, $100\,mmol\,l^{-1}$ KCl, $10\,mmol\,l^{-1}$ $MgCl_2$, $0.15\,mmol\,l^{-1}$ NADH, $10\,\mu mol\,l^{-1}$ fructose 1,6-biphosphate, $5\,mmol\,l^{-1}$ phosphoenolpyruvate and 5 units of LDH. The activity of LDH was determined at 25 °C as the rate of removal of NADH from 300 μl of a mixture containing $20\,mmol\,l^{-1}$ Hepes, pH 7.0, $0.15\,mmol\,l^{-1}$ NADH and $0.5\,mmol\,l^{-1}$ pyruvate. CS activity was measured at 15 °C using a DU 640 spectrophotometer (Beckman) and detected at 412 nm as the rate of formation of 5′,5′-dithiobis-(2′-nitrobenzoic acid) (DTNB) in 1 ml of a mixture containing $20\,mmol\,l^{-1}$ Tris base, $0.1\,mmol\,l^{-1}$ DTNB, $0.12\,mmol\,l^{-1}$ acetyl coenzyme A and $0.2\,mmol\,l^{-1}$ oxaloacetate.

Non-specific thiolase activity for CS, determined in the absence of oxaloacetate, was subtracted from the rate in the complete mixture. The controls for both PK (absence of phosphoenolpyruvate) and LDH (absence of pyruvate) displayed no detectable activities.

Cytochrome oxidase

Packed red blood cells (200 μl) from each fraction were suspended in 800 μl of saline, sonicated (2 s, low setting) and centrifuged for 5 min at $1000\,\boldsymbol{g}$ in a MicroMax centrifuge (IEC). The resulting nuclear pellet was discarded, and the supernatant was centrifuged for an additional 10 min at $10\,000\,\boldsymbol{g}$ to isolate the mitochondrial pellet, which was then resuspended in 200 μl of cell extraction buffer. COX was assayed at 25 °C and 550 nm using a Spectramax Plus plate spectrophotometer (Molecular Devices). The specific activity of each fraction was measured by detecting the rate of reduction of cytochrome *c* in 200 μl of a mixture containing $50\,mmol\,l^{-1}$ Tris base, $50\,\mu mol\,l^{-1}$ cytochrome *c* and 0.5 % Tween 20. COX activities were expressed on the basis of the starting volumes of blood.

Rates of O_2 consumption

Blood from each of the six age fractions (2 ml at a Hct of 20 %) was equilibrated with humidified air at 15 °C in a gently rotating aerated flask for 1 h. Following the equilibration period, a 600 μl sample of blood was added to an air-tight

chamber fitted with an E5046 P_{O_2} electrode (Radiometer) and gently stirred for 12 min at 15 °C. During this time, the change in O_2 partial pressure (P_{O_2}) of each fraction was measured with an OM200 O_2 analyzer (Cameron Instrument Company) and recorded with an Omniscribe D-5000 physiograph chart recorder (Allen Datagraph). As described by Wood et al. (1990), preliminary experiments showed that Hb remained fully saturated during the measurement period and, therefore, that the change in P_{O_2} represented red blood cell O_2 consumption from the surrounding medium rather than the removal of O_2 from Hb. The relative percentage of O_2 consumption required for protein synthesis was determined by comparing O_2 consumption in the presence and absence of cycloheximide at a final concentration of 100 μg ml^{-1} (Currie and Tufts, 1997). The relative percentage of O_2 consumption required for the Na^+/K^+-ATPase was also determined by comparing O_2 consumption in the presence and absence of ouabain at a final concentration of 0.1 mmol l^{-1} (Tufts and Boutilier, 1991). An O_2 solubility constant of 1.7745 μmol l^{-1} mmHg^{-1} (1 mmHg=0.133 kPa; Boutilier et al., 1984) was used to calculate the rate of O_2 consumption from changes in P_{O_2}.

Lactate production

Blood from fractions 1 and 6 (4 ml at a Hct of 20 %) was equilibrated with humidified air in a gently rotating aerated flask for 1 h at 15 °C. Following the initial equilibration period, 2 ml of blood from each fraction was treated with humidified N_2 for 2 h to induce anoxia, while the remaining 2 ml continued to be equilibrated with humidified air for 2 h. Blood samples of 1 ml were taken for both fractions after the 1 h equilibration period and at the end of the 2 h aeration/anoxic period to be acidified in preparation for the lactate assay. Samples were acidified with 0.1 ml of 70 % perchloric acid and centrifuged for 5 min at 14 000 *g*. The supernatant was neutralized by adding 0.3 ml of saturated Tris base followed by 0.2 ml of 2 mol l^{-1} KOH and then centrifuged again for 1 min at 14 000 *g*. Supernatants were frozen at −80 °C until the day of the assay. Lactate concentrations were determined using the hydrazine sink method. Briefly, 150 μl of sample was added to 150 μl of assay mixture (400 mmol l^{-1} hydrazine, 1 mol l^{-1} glycine, 2 mmol l^{-1} EDTA, 4 mmol l^{-1} NAD$^+$, 2 units of LDH, pH 9.5), and the reaction was followed for 40 min to completion. Lactate standards of varying known concentrations were also run in parallel during this time. The lactate concentrations of both samples and standards were assayed in triplicate at 340 nm with a Spectramax Plus plate spectrophotometer (Molecular Devices). The lactate concentrations measured after the 1 h equilibration period were subtracted from those measured after the 2 h interval of aeration/anoxia to eliminate starting concentrations.

Statistical analyses

Significant differences ($P \leqslant 0.05$) between fraction means were detected using one-way analyses of variance (ANOVAs) and identified using the Student–Newman–Keuls test. In experiments involving only fractions 1 and 6, significant differences ($P \leqslant 0.05$) between fraction means were detected using paired two-tailed *t*-tests.

Results

Mean erythrocyte haemoglobin concentration

MEHC increased at a fairly constant rate with fraction age (Fig. 1); fractions 4–6 all displayed significantly higher MEHCs than fraction 1, and the MEHC of fraction 6 was also significantly greater than that of fractions 2 and 3. The fraction 6 mean MEHC of 250.9 μg μl^{-1} red blood cells was roughly 120 % of the fraction 1 mean MEHC of 211.6 μg μl^{-1} red blood cells.

Metabolic enzymes

Metabolic enzyme activity decreased with red blood cell age for CS, COX and LDH, with mean fraction 1 activities ranging from 135 to 200 % of those in fraction 6.

Mitochondrial enzymes (citrate synthase, cytochrome oxidase)

CS activity fell sharply between fractions 1 and 2, but remained fairly constant across fractions 2–6 (Fig. 2A). The mean fraction 1 CS activity of 1.75 nmol μl^{-1} red blood cells min^{-1} was higher than that of any other fraction and was 135 % of the mean fraction 6 CS activity. Unlike CS, COX activity decreased at a more constant rate across the age fractions (Fig. 2B). The mean fraction 1 COX activity of 0.06 nmol μl^{-1} red blood cells min^{-1} was greater than that in any other fraction and over twice as high as the mean fraction 6 COX activity. Thus, the decrease in COX activity between fractions 1 and 6 was larger in magnitude than the decrease in CS activity between the same two fractions.

Glycolytic enzymes (lactate dehydrogenase, pyruvate kinase)

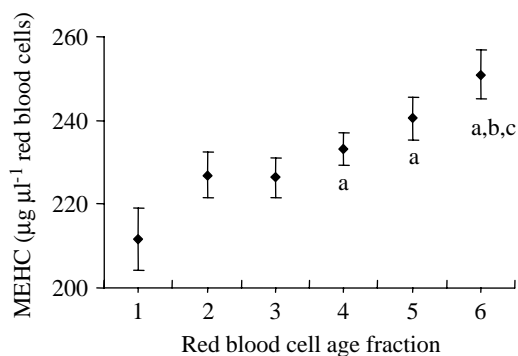LDH activity peaked at 1330 nmol μl^{-1} red blood cells min^{-1}

Fig. 1. Mean erythrocyte haemoglobin concentration (MEHC) in six age fractions of rainbow trout red blood cells. The youngest red blood cells are in fraction 1, and the oldest red blood cells are in fraction 6. The letters a, b and c denote values that are significantly different from those for fraction 1, 2 or 3 ($P \leqslant 0.05$, $N=6$), respectively. All values are expressed as means ± S.E.M.
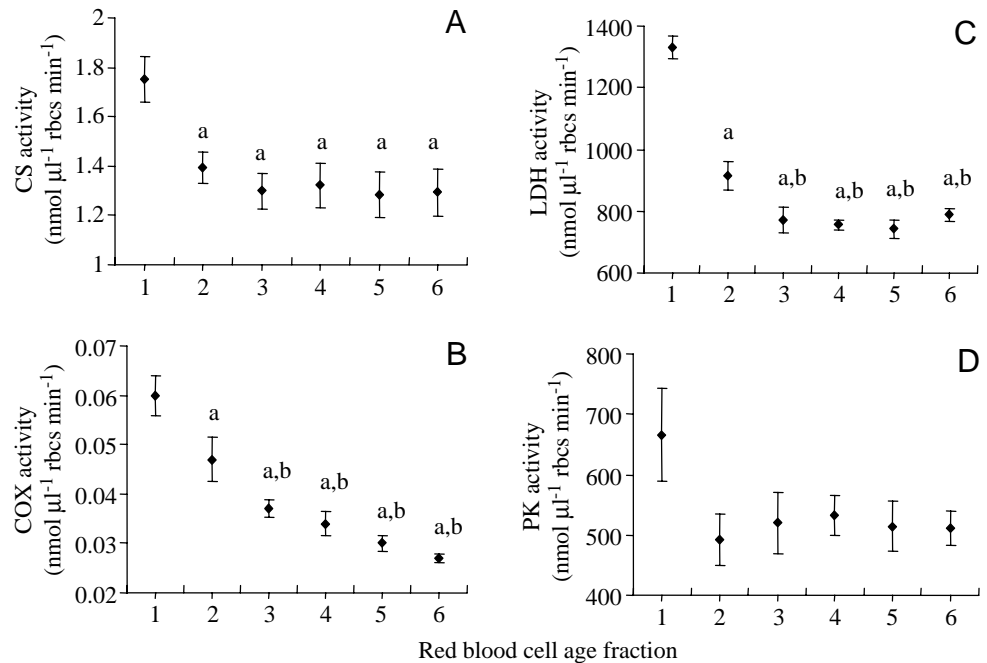
Fig. 2. Activities of the metabolic enzymes citrate synthase (CS) (A), cytochrome oxidase (COX) (B), lactate dehydrogenase (LDH) (C) and pyruvate kinase (PK) (D) in six age fractions of rainbow trout red blood cells (rbcs). The youngest red blood cells are in fraction 1, and the oldest red blood cells are in fraction 6. The letters a and b denote values that are significantly different from those for fraction 1 and 2 ($P \leq 0.05$, $N=6$), respectively. All values are expressed as means ± S.E.M.

in fraction 1 and decreased sharply in the younger fractions, but levelled off in the older fractions (Fig. 2C). The mean fraction 1 LDH activity was 170 % of the mean fraction 6 LDH activity. PK activity was not significantly affected by fraction age (Fig. 2D). However, the mean fraction 1 PK activity of 666.2 nmol μl^{-1} red blood cells min^{-1} was roughly 130 % of the mean fraction 6 PK activity.

Rates of O_2 consumption

The rate of O_2 consumption decreased significantly with fraction age (Fig. 3). The mean fraction 1 O_2 rate of consumption was 0.193 nmol $O_2 \mu l^{-1}$ red blood cells min^{-1}, higher than that in any other fraction and over twice as high as the mean fraction 6 rate of O_2 consumption. The difference in
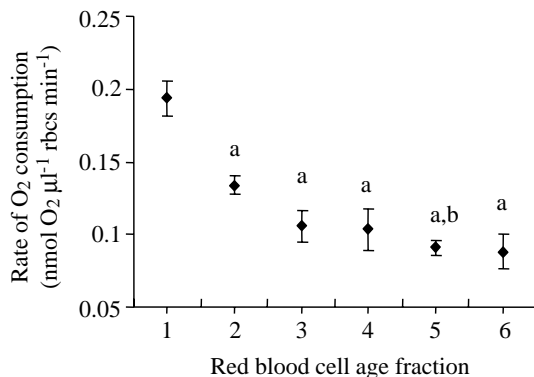

Fig. 3. Rates of O_2 consumption in six age fractions of rainbow trout red blood cells (rbcs). The youngest red blood cells are in fraction 1, and the oldest red blood cells are in fraction 6. The letter a denotes values that are significantly different from that for fraction 1 ($P \leq 0.05$, $N=6$). All values are expressed as means ± S.E.M.

rates of O_2 consumption between fractions 1 and 6 was similar in magnitude to the decrease in COX activity observed for the same two age fractions.

Exposure of fractions 1 and 6 to the respiratory inhibitors cycloheximide or ouabain resulted in decreases in rates of O_2 consumption for both fractions in comparison with uninhibited rates (Fig. 4). Proportionally, the decrease in the rate of O_2 consumption from the uninhibited rates did not differ between fractions 1 and 6 for either cycloheximide (fraction 1 decreased by 26.4 % compared with 27.0 % for fraction 6) or ouabain (fraction 1 decreased by 36.6 % compared with 40.1 % for fraction 6). However, differences in the total O_2 requirements of both protein synthesis and the Na^+/K^+-ATPase were observed (Fig. 5). Inhibition of protein synthesis with cycloheximide caused the mean fraction 1 rate of O_2 consumption to decrease by 0.060 nmol $O_2 \mu l^{-1}$ red blood cells min^{-1} compared with a reduction of only 0.037 nmol $O_2 \mu l^{-1}$ red blood cells min^{-1} for fraction 6. Inhibition of the Na^+/K^+-ATPase with ouabain caused the mean fraction 1 rate of O_2 consumption to fall by 0.115 nmol $O_2 \mu l^{-1}$ red blood cells min^{-1}, while the rate for fraction 6 fell by only 0.067 nmol $O_2 \mu l^{-1}$ red blood cells $min^{-1.}$ Thus, fraction 1 red blood cells devoted approximately 50 % more O_2 consumption towards protein synthesis and the Na^+/K^+-ATPase than fraction 6 red blood cells.

Lactate production

The red blood cells in fractions 1 and 6 both significantly increased their rate of lactate production under anoxic conditions compared with those under aerated conditions (Fig. 6). However, no significant differences were observed between fractions 1 and 6 with respect to either aerated or anoxic rates of lactate production.
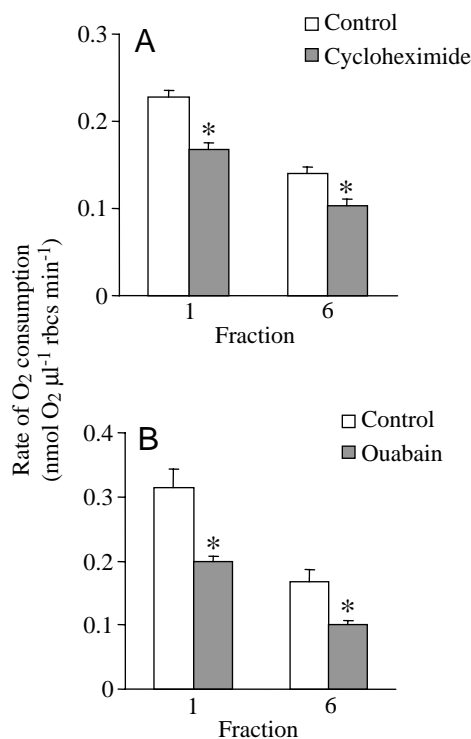
Fig. 4. Effects of the respiratory inhibitors cycloheximide (A) and ouabain (B) on the rate of O_2 consumption of young (fraction 1) and old (fraction 6) rainbow trout red blood cells (rbcs). Asterisks denote a significant difference from the value for uninhibited controls for both fractions 1 and 6 ($P \leq 0.05$, $N=4$). No significant differences in the rate of O_2 consumption exist between age groups as a proportion of control rates. All values are expressed as means + S.E.M.

Discussion

Vertebrate red blood cells can be separated into fractions of different ages using fixed-angle centrifugation (Murphy, 1973; Cohen et al., 1976; Speckner et al., 1989). This technique relies on density differences between young and old cells which result largely from an increase in MEHC during maturation (Tooze and Davies, 1963; Härdig, 1978; Keen et al., 1989). The magnitude of the change in MEHC observed across the six cell fractions in the present study (Fig. 1) was similar to that in previous studies (Speckner et al., 1989). These results therefore confirm that the methodology used in this study was effective in separating trout red blood cells into fractions with different mean ages. Several other aspects of this methodology also warrant discussion. First, previous studies have shown that some degree of contamination between age fractions should be expected when cells are separated using this approach (Piomelli et al., 1967; Beutler, 1985). Thus, the true magnitude of the differences between age fractions will probably be even greater than that measured. Moreover, the magnitude of the differences between young and old cells will also be influenced by the number of fractions chosen for examination. Six fractions were chosen for examination in the present study. This represented the maximum number of fractions that still provided an adequate volume of cells for the analyses.
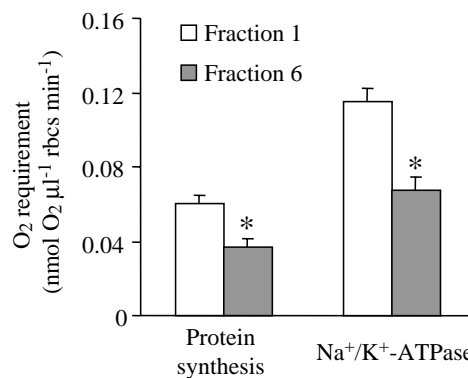

Fig. 5. The O_2 consumption requirements for protein synthesis and Na^+/K^+-ATPase in young (fraction 1) and old (fraction 6) rainbow trout red blood cells (rbcs). Asterisks denote a significant difference from the value for fraction 1 ($P \leq 0.05$, $N=4$). All values are expressed as means + S.E.M.

Nonetheless, it would be expected that the magnitude of any observed differences between the youngest and oldest cell fractions might have been even larger had the cells been separated into a greater number of fractions. Lastly, it is noteworthy that we have shown that the concentration of total DNA does not vary between age fractions (S. G. Lund, M. C. L. Phillips, C. D. Moyes and B. L. Tufts, in preparation). Thus, the differences observed between the age fractions do not reflect significant differences in the number of cells per unit volume between fractions. As documented in numerous previous studies using this approach, the differences observed between fractions in the present study therefore represent the impact of ageing on red blood cell variables.

It is well known that loss of organelles accompanies nucleated red blood cell ageing (Sekhon and Beams, 1969; Lane et al., 1982; Keen et al., 1989). However, the metabolic
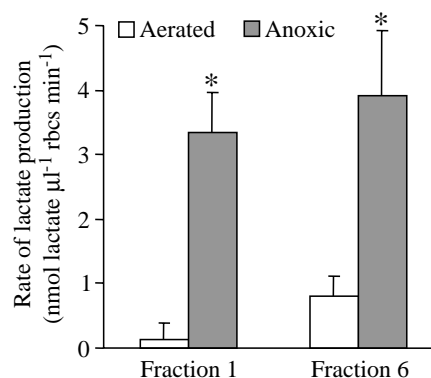

Fig. 6. Rates of lactate production in young (fraction 1) and old (fraction 6) rainbow trout red blood cells (rbcs) under conditions of aeration and anoxia. Asterisks denote a significant difference between anoxic and aerated rates of lactate production for both fractions 1 and 6 ($P \leq 0.05$, $N=4$). No significant differences exist between fraction 1 and 6 aerated or anoxic rates of lactate production. All values are expressed as means + S.E.M.

impact of degradation in organelles such as mitochondria is unknown. In the present study, the rate of O_2 consumption was found to decline by at least 50 % in ageing rainbow trout red blood cells (Fig. 3). The underlying basis for this change appears to be due to coincident changes in both mitochondrial properties and the cellular demands for energy.

From a mitochondrial perspective, CS and COX activity measurements show that ageing in rainbow trout red blood cells is associated with a significant reduction in aerobic metabolic capacity. The CS activity in fraction 1 is 135 % of that in any other fraction (Fig. 2A), suggesting that only the youngest cells have an elevated capacity for tricarboxylic acid cycle activity. Unlike CS, the activity of COX decreases at a more gradual rate in ageing red blood cells (Fig. 2B), and the decline in activity (50 %) between fractions 1 and 6 is larger. The different trends observed between CS and COX activity are interesting for a number of reasons. First, these results suggest that cell ageing may be accompanied by changes in mitochondrial structure as well as decreased mitochondrial abundance. Specifically, CS is localized in the mitochondrial matrix (i.e. a marker for mitochondrial volume) and COX is localized in the mitochondrial inner membrane (i.e. a marker for cristae surface area). These observations are also supported by studies which show that mitochondrial degradation in ageing red blood cells is characterized by symptoms such as swelling and cristae disorganization (Sekhon and Beams, 1969; Keen et al., 1989). Second, the magnitude of the decline in COX activity is very similar to that observed for O_2 consumption, which suggests that COX activity may be a more accurate indicator of actual aerobic flux than is CS activity.

From a functional perspective, young and old red blood cells devote roughly the same proportion of the O_2 they consume to protein synthesis (Fig. 4A). Protein synthesis is energetically expensive and may account for up to 40 % of whole-animal oxygen consumption in fish (Lyndon et al., 1992). In the present study, protein synthesis accounts for 26 % of the oxygen consumption of young red blood cells and 27 % of the oxygen consumption of old cells. However, the absolute O_2 consumption devoted to protein synthesis in young red blood cells is approximately 50 % greater than that in old red blood cells (Fig. 5). Younger red blood cells contain more polyribosomes than old red blood cells (Lane and Tharp, 1980) and have a higher rate of oxygen consumption than older red blood cells, so it is not surprising that they devote more total O_2 to protein synthesis. Similar trends were observed for the energy requirements of the Na^+/K^+-ATPase. Previous studies on Na^+/K^+-ATPase inhibition show that it accounts for 20–25 % of the total red blood cell O_2 consumption in rainbow trout red blood cells (Tufts and Boutilier, 1991; Wang et al., 1994). In our experiments, the Na^+/K^+-ATPase accounted for a greater proportion of the total oxygen consumption; 37 % and 40 % in young and old cells respectively (Fig. 4B). The reason that our current values are somewhat higher than those of previous studies is not entirely clear, although it is noteworthy that varying degrees of adrenergic stimulation may contribute to these differences (Tufts and Boutilier, 1991). As observed for

protein synthesis, the absolute O_2 consumption devoted to the Na^+/K^+-ATPase in young red blood cells is approximately 50 % greater than that in old red blood cells (Fig. 5). As a result of the accumulation of Ca^{2+}, ageing red blood cells experience a disruption in membrane asymmetry (Schwartz et al., 1985). Since Na^+/K^+-ATPase activity is dependent upon the asymmetry of the phospholipid environment (Roelofsen, 1981; Deuticke and Haest, 1987), this may explain why the O_2 requirements of the Na^+/K^+-ATPase also decrease with cell age.

The alternative route of ATP production, glycolysis, becomes increasingly important as nucleated red blood cells age. Since lactate production is unaffected by red blood cell age (Fig. 6), whereas the rate of O_2 consumption decreases as red blood cells age, a higher proportion of the total ATP produced in old red blood cells is derived through glycolysis. Shifts in the relative importance of aerobic and anaerobic metabolism are known to occur in other instances, such as during myogenesis in mouse C2C12 myoblasts (Leary et al., 1998). In addition, previous studies on rainbow trout red blood cells have shown that anaerobic metabolism plays a significant role in red blood cell energy production and can account for up to 28 % of cell metabolism even when O_2 is present (Walsh et al., 1990). It is interesting that the decline in LDH activity (Fig. 2C) does not appear to have a negative effect on glycolytic flux. The functional significance of an apparent surplus of LDH activity in young compared with old cells is therefore unclear. By maintaining a higher concentration of LDH, young red blood cells may be able to respond more quickly to conditions of hypoxia or anoxia. Alternatively, difference in LDH activity between young and old cells may reflect different abilities to use lactate as an oxidative fuel.

In conclusion, this study shows that ageing in rainbow trout nucleated red blood cells is accompanied by significant changes in metabolism that are at least as large as those recorded and are probably even greater. Young red blood cells consume at least twice as much O_2 as old red blood cells. The underlying reasons for this difference appear to be mitochondrial degradation, as indicated by reduced CS and COX activity, and lower cellular energy demands, as indicated by a decline in the proportion of O_2 consumption devoted to protein synthesis and Na^+/K^+-ATPase ionoregulation. Since the rate of O_2 consumption in the ageing red blood cell decreases, whereas lactate production remains constant, the role of glycolysis becomes increasingly important with cell age.

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