

STABILITY OF NUCLEOSIDE TRIPHOSPHATE LEVELS IN THE RED CELLS OF THE SNAKE *THAMNOPHIS ELEGANS*

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Summary

Nucleated red cells in the nonpregnant garter snake (*Thamnophis elegans*) contain relatively high concentrations of nucleoside triphosphate (NTP), largely in the form of ATP, which is found at concentrations of approximately 10 mmol l^{-1} relative to cell volume and 15 mmol l^{-1} relative to cell water. During pregnancy, levels of NTP in adult red cells rise by approximately 50% concomitant with an increase in blood progesterone level. The stability of the NTP pool within these red cells was assessed by maintaining cells *in vitro* at 20 °C, without exogenous nutrients, and in the presence and absence of the metabolic inhibitors iodoacetate and/or cyanide. After 96 h, NTP levels in adult red cells not exposed to the inhibitors had decreased by only approximately 10%, while in the presence of both inhibitors NTP levels had fallen by less than 50%. Red cell NTP levels were not affected by acute exposure to high concentrations of progesterone either *in vivo* or *in vitro*. NTP levels were much more labile when the cells were maintained *in vitro* at either low or high pH. Maintenance of red cells at pH 6.0 for 24 h resulted in a

decrease in NTP levels of approximately 50% and at pH 10.0 the levels fell by approximately 80%, while buffers containing only ATP caused no detectable degradation. Incubation at low or high pH promoted some cell swelling; however, the magnitude of the decreases in intracellular NTP concentration prompted by these pH levels could not be mimicked by incubation of red cells in hypotonic buffer. Total nonspecific ATPase activity at pH 6.0 was approximately 55% greater than that at pH 7.4, while at pH 10.0 it was approximately 6% of that at pH 7.4. The pH-dependent decrease in intracellular NTP levels cannot, therefore, be due to stimulation of ATPase activity, at least not at high pH. Overall, the data are consistent with the hypothesis that an appreciable portion of the NTP within these cells is compartmentalized in a stable, but pH-sensitive, pool sequestered from intracellular ATP-hydrolyzing processes.

Key words: ATP, adenosine triphosphate, erythrocyte, reptile, snake, *Thamnophis elegans*, red blood cell.

Introduction

The red cells of many lower vertebrates contain high concentrations of nucleoside triphosphates (NTPs: usually in the form of adenosine triphosphate, ATP, and guanosine triphosphate, GTP) which serve, in large part, to regulate hemoglobin allosterically and therefore to modulate cell function (see, for example, reviews by Bartlett, 1980; Isaacks and Harkness, 1980). Concentrations of NTP are responsive to environmental perturbations (such as changes in oxygen availability or temperature) in numerous animals (see reviews by Wood, 1980; Weber and Jensen, 1988). In previous experiments to assess the effect of environmental oxygen tension on the oxygen-binding properties of the red cells of the garter snake (*Thamnophis elegans*), we exposed snakes to 5%, 21% and 100% oxygen. Although the red cells showed decreases in NTP content with hypoxia and increases with hyperoxia, the relatively modest maximal effect required approximately 2 weeks to develop (Herman and Ingermann,

1996). In contrast to this rather slow responsiveness of red cell NTP levels to an environmental change, we found that the low levels of NTP in the red cells of neonatal garter snakes reach adult levels within 6 h of birth (Ingermann *et al.* 1991b). Although this event suggests a more rapid turnover of NTP (6 h *versus* 2 weeks), it is, nonetheless, markedly slower than is the case in typical respiring eukaryotic cells in which ATP turns over with a half-life of several minutes (e.g. Lehninger, 1975). Koszegi *et al.* (1987) reported that the majority of ATP in two types of cultured human cells is associated with proteins and is thus compartmentalized within the cell. Such an association is likely to be ionic and sensitive to pH, and conceivably this may contribute to the relatively slow change in snake red cell NTP levels in response to environmental perturbation. To assess the stability of the NTP pool in the red cells of the garter snake, we examined the effect of *in vitro* incubation, in the presence and absence of metabolic inhibitors, on NTP content

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as a function of time. In addition, since preliminary observations suggested that extremes of extracellular pH have a profound influence on intracellular NTP levels in snake red cells, we examined in more detail the effect of pH on intracellular NTP concentrations of red cells maintained *in vitro*.

Pregnancy in *T. elegans* is associated with an approximately 50% rise in red cell NTP concentration and a concomitant decrease in red cell oxygen affinity (Ingermann *et al.* 1991a). Pregnancy in this species is associated with a rise in plasma progesterone concentrations (Highfill and Mead, 1975), and a more direct connection between red cell NTP levels and blood progesterone is indicated by the results of Ragsdale *et al.* (1993), who showed that implanting adult male *T. elegans* with progesterone-releasing capsules resulted in a rise in red cell NTP levels. The change in red cell NTP levels as pregnancy progresses or following implantation of progesterone capsules is relatively slow and requires weeks to reach maximal levels. It is possible, however, that the red cell response to progesterone is rapid, but that progesterone levels change slowly in these situations. To test this possibility, snakes were injected with progesterone and the levels of NTP in red cells were assayed 1 and 3 days later. Red cells were also incubated *in vitro* with progesterone to assess the possibility of an acute response leading to a change in the intracellular NTP level.

Materials and methods

Garter snakes, *Thamnophis elegans* (Baird and Girard), were captured, identified and maintained as previously described (Ingermann *et al.* 1991a). Fetal snakes were at Zehr stages 35–37, where stage 37 is the last prenatal stage (Zehr, 1962). Blood was obtained from adults either by heart puncture under metofane anesthesia or by decapitation of animals rendered unconscious by a blow to the head. Blood was collected into ice-cold heparinized buffer A (in mmol l⁻¹): 143 NaCl, 3 KCl, 1.5 MgCl₂, 1.5 CaCl₂, 20 Tris, adjusted to pH 7.4 with HCl (except where indicated). Fetal snakes were excised over ice, rinsed in buffer A, sectioned in ice-cold heparinized buffer A and the cell suspension was passed through glass wool. Fetal and adult red cell suspensions were washed three times by centrifugation at 1200g for 10 min at 4 °C. The third wash was with cold buffer A containing 50 i.u. ml⁻¹ penicillin and 50 µg ml⁻¹ streptomycin, and the final red cell pellet was resuspended in this buffer with antibiotics. 10% by volume of potassium cyanide (KCN) and/or iodoacetic acid (IAC) stock solution (in buffer A containing penicillin and streptomycin) was added to samples of the resuspended red cells to give final concentrations of 0.1 mmol l⁻¹ KCN and/or 0.5 mmol l⁻¹ IAC. Owing to limited availability, fetal red cells were not exposed to IAC plus KCN. Final red cell suspensions with and without metabolic inhibitors were then placed into glass test tubes, 0.3 ml for fetal cells and 0.4 ml for adult cells. These samples were kept for varying periods of up to 96 h in a humidified chamber at 20 °C. For analyses, 1 ml of buffer A (without antibiotics) was added to each sample, these were centrifuged

in a Fisher model 235B microcentrifuge for 2 min, buffer was drawn off, and fresh buffer A was added. The cells were resuspended and the suspension was analyzed for hematocrit by centrifuging for 4 min in an IEC microhematocrit centrifuge. Tetrameric hemoglobin (Hb) concentration was determined spectrophotometrically by adding a sample of the suspension to a larger volume of Drabkin's solution and calculating the concentration using a millimolar extinction coefficient of 44.0 at 540 nm. Finally, a sample of suspension was extracted with an equal volume of ice-cold 12% (w/v) trichloroacetic acid. The nucleoside triphosphate concentration of the extract was then analyzed with an enzymatic assay kit (number 366, Sigma Chemical Co., St Louis, MO), which does not distinguish among the different NTPs. Total NTP level was expressed as a molar concentration within the red cells or as mole NTP per mole Hb. From the suspension hematocrit and Hb concentration, the mean corpuscular hemoglobin concentration (MCHC) was also calculated. Based on the measurement of free Hb in the suspension fluid, there was minimal lysis ($\leq 0.5\%$) by the end of the 96 h incubation.

For analysis of the influence of pH on red cell NTP levels, red cells from nonpregnant females were first washed three times in buffer A (without antibiotics). They were subsequently washed three additional times in the Tris-based buffer A (titrated to the desired pH within the pH range 7.2–9.0) or in modified buffer A where Tris had been replaced by BisTris or Capso: 20 mmol l⁻¹ BisTris for pH ≤ 7.2 or 20 mmol l⁻¹ Capso for pH ≥ 9.0 . Osmotic concentrations (in mosmol kg⁻¹) of the buffers were 286 \pm 1 for pH 6.0, 288 \pm 4 for pH 7.4 and 284 \pm 5 for pH 10.0 (mean \pm s.d., $N=3-5$) and were measured using a 3WII Advanced Laboratory wide-range osmometer (Advanced Instruments, Needham Heights, MA). One series of experiments at low and high pH was conducted in the presence of 1.0, 3.3 and 10.0 mmol l⁻¹ ouabain. Samples of the final suspension were incubated for 24 h at 20 °C, the pH of the suspension was determined, and the cells were then washed in buffer and analyzed for NTP and Hb concentrations. Lysis never exceeded 5%. Incubations at both low and high pH appeared to result in some cell swelling and, therefore, red cells were incubated in hypotonic buffer to establish whether swelling alone could account for the effects of low or high pH on intracellular NTP levels. For these experiments, red cells were incubated for 24 h in media which were 80%, 60% or 40% buffer A (titrated to pH 7.4) in distilled H₂O. Lysis under these conditions never exceeded 7%.

For analysis of ATPase activity, red cells were collected from nonpregnant females, washed three times in cold buffer A, suspended in diluted buffer A (15% buffer A, 85% water), frozen, thawed and centrifuged at 4 °C for 20 min at 20 000g. The pellet was resuspended in diluted buffer A and washed twice more at 20 000g. The final pink pellet was resuspended in a small volume of dilute buffer A and samples were taken for protein analysis by the method of Markwell *et al.* (1976) using commercial bovine serum albumin as a standard. Samples were also taken to measure nonspecific ATPase activity (assayed by a modification of the method of Zaugg and

McLain, 1970). To a volume of cell membrane suspension in diluted buffer A was added an equal volume of a buffer containing (in mmol l^{-1}): 300 sucrose, 20 EDTA, 24 sodium deoxycholate and 100 Tris, adjusted to pH 7.4 with HCl. This mixture was drawn through a 30 gauge needle six times, kept on ice, and assayed within 3 h. Samples were not centrifuged. Approximately 0.03 ml of the mixture was added to a tube in an ice bath containing (1) 0.65 ml of a buffer consisting of (in mmol l^{-1}): 23 MgCl_2 , 86 NaCl, 75 KCl and 115 Tris (for pH 7.4), 115 BisTris (for pH 6.0) or 115 Capso (for pH 10.0) and (2) 0.1 ml of 30 mmol l^{-1} ATP (titrated to a pH of approximately 7.0). The tube was placed into a water bath at 30°C for 30 min and then returned to the ice bath, where 2.25 ml of distilled water, 1.0 ml of 15.5 mmol l^{-1} ammonium molybdate in 3 mol l^{-1} HCl, 0.85 ml of 7.5% (w/v) sodium dodecyl sulfate and 0.15 ml of 0.25% (w/v) Fisk Subbarrow reducer were added. The mixture was then warmed to 20°C and the absorbance of the solution read 40 min later at 700 nm. Absorbance of the unknown samples was related to phosphate standards run simultaneously.

The stability of ATP in buffer A at pH 5.8, 7.4 and 10.2 was assessed by keeping 1 mmol l^{-1} ATP in buffer at 20°C for 24 h. Initial and final concentrations were measured using the NTP enzymatic assay kit.

To assess whether red cell NTP levels could respond acutely to a high concentration of progesterone *in vivo*, males were injected with 0.1 mg of progesterone (or a comparable molar amount of cholesterol) in 10 ml of 5% ethanol per kilogram animal mass. This dose was calculated to yield approximately 20 times the maximal progesterone levels found in *T. elegans* by Highfill and Mead (1975). Eight cholesterol-injected and eight progesterone-injected animals were maintained on a 12 h:12 h light:dark cycle with an electrically heated rock for 24 or 72 h. They were subsequently anesthetized and blood samples taken by heart puncture. In addition, to establish whether red cell NTP levels were acutely sensitive to a high progesterone concentration *in vitro*, washed red cells from three males were incubated in pH 7.4 buffer containing 5 mmol l^{-1} glucose, 5 mmol l^{-1} pyruvate and 5 mmol l^{-1} glutamine with $1 \mu\text{mol l}^{-1}$ cholesterol or progesterone (and 0.5% ethanol). After 24 h at 20°C , cells were washed once in pH 7.4 buffer and red cell [NTP]/[Hb] ratios determined.

All data are presented as mean \pm s.d. Slopes of [NTP]/[Hb] ratios *versus* time in the *in vitro* incubation studies were analyzed by one-way ANOVA followed by Scheffe's *F*-test. The influence of metabolic inhibitors on fetal [NTP]/[Hb] ratios was analyzed with an unbalanced randomized-block ANOVA with a 3×3 factorial. To test the influence of pH on ATPase activity, randomized-block ANOVAs were followed by either protected LSD or one-sample *t*-test analyses. $P < 0.05$ was considered significant.

All reagents were obtained from Sigma Chemical Co.

Results

Incubation of red cells from adult snakes *in vitro* at 20°C

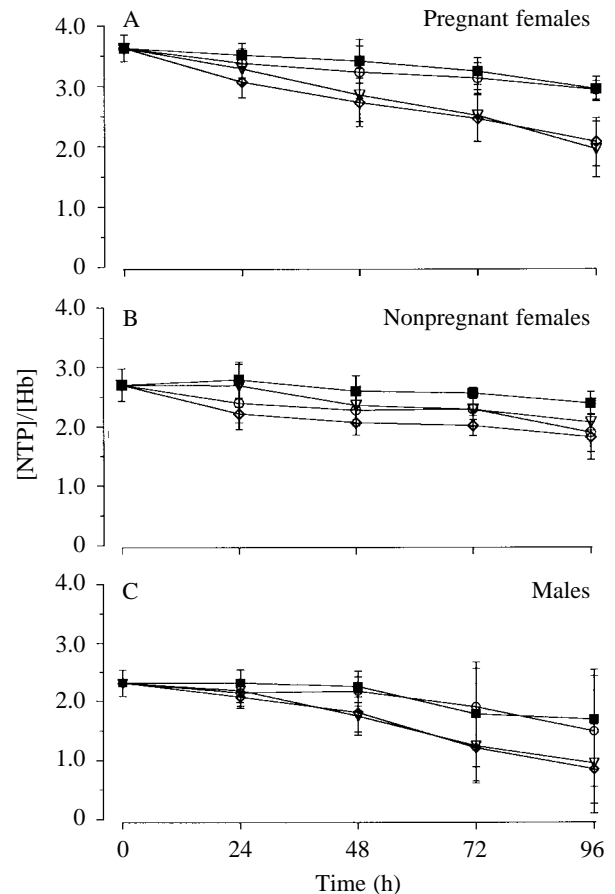
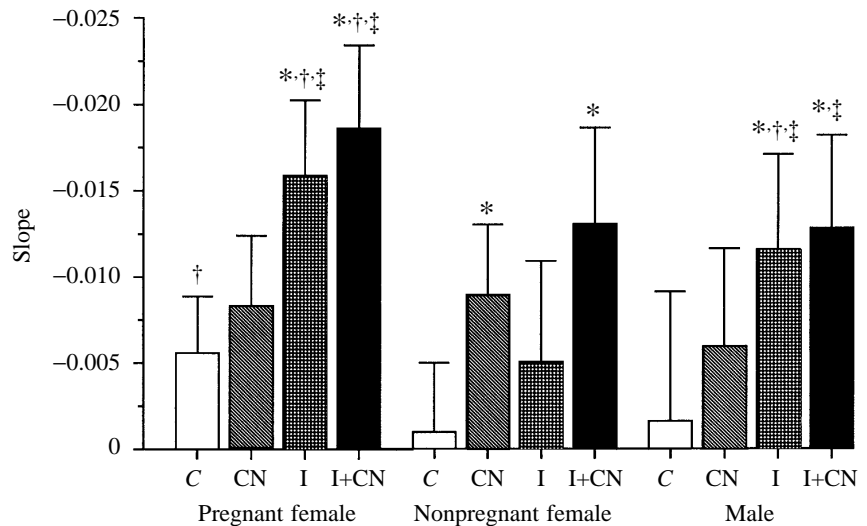


Fig. 1. Change in [NTP]/[Hb] ratio over time for red cells of (A) pregnant females, (B) nonpregnant females and (C) males all maintained at 20°C *in vitro* with antibiotics, but without exogenous nutrients, and with or without 0.1 mmol l^{-1} KCN and/or 0.5 mmol l^{-1} iodoacetic acid (IAC). Error bars represent \pm s.d., $N=5$. Control, ■; KCN, ○; IAC, ▽; KCN+IAC, ◇. Initial [NTP]/[Hb] levels were as follows: pregnant females, 3.63 ± 0.22 ; nonpregnant females, 2.70 ± 0.27 ; males, 2.31 ± 0.23 (means \pm s.d., $N=5$).

resulted in a decrease in the [NTP]/[Hb] ratio over a period of 4 days (Figs 1A–C, 2). However, red cells incubated *in vitro* in the absence of metabolic inhibitors showed particularly low rates of decline in intracellular [NTP]/[Hb] ratio. For example, after 96 h, [NTP]/[Hb] ratio in red cells of nonpregnant females had decreased to $89 \pm 9\%$ of initial values (mean \pm s.d., $N=5$), while in pregnant females the level had fallen to $81 \pm 8\%$ (mean \pm s.d., $N=5$). Metabolic inhibitors prompted a more pronounced reduction in [NTP]/[Hb] values over the same period so that, in the presence of IAC plus KCN, the 96 h value for nonpregnant females was $67 \pm 6\%$ and for pregnant individuals $57 \pm 12\%$ of initial values (mean \pm s.d., $N=5$ for each group). As indicated by the rates (slopes) of the [NTP]/[Hb] decline, red cells from the pregnant female and male were sensitive to IAC but not to KCN (Fig. 2). In contrast, the red cells of the nonpregnant female were more sensitive to KCN than to IAC (Fig. 2).

There were no significant differences in [NTP]/[Hb] ratios

Fig. 2. Slopes of the data shown in Fig. 1: decreases in [NTP]/[Hb] levels over time in the presence and absence of metabolic inhibitors. (IAC is shown as I and KCN is shown as CN in this figure.) Statistically significant differences ($P \leq 0.01$): *significantly different from control value (C) within a group, †significant difference from value for nonpregnant females by treatment, ‡significant difference between males and pregnant females by treatment. Data were analyzed using a two-way ANOVA. There were significant differences between groups and treatments, and a group \times treatment interaction ($P < 0.0001$ for each). In the case of significant differences, subsequent one-way ANOVAs were performed followed by Scheffe's F -tests. Values are means \pm s.d., $N=5$.



for fetal red cells incubated for 6 or 24 h in the absence of inhibitors or with KCN or IAC (Table 1). Nonetheless, there did appear to be a trend suggesting that these cells were sensitive to KCN (and less so to IAC) prior to 6 h; however, by 24 h, they appeared to be insensitive to KCN but sensitive to IAC. From 48 to 96 h (72 and 96 h data not shown), for reasons that are not clear, scatter in the data precluded any possible interpretation.

Levels of NTP were appreciably more labile when red cells of adults were maintained *in vitro* at either low or high pH (Fig. 3A,B). Maintenance of cells in buffer at pH 6.0 for 24 h resulted in a decrease in the NTP level of approximately 50% and at pH 10.0 the decrease was approximately 80%. In contrast, maintaining commercial ATP in low- and high-pH buffers for 24 h caused no detectable decrease in the ATP concentration: at 24 h, ATP kept at pH 5.8 was $101.6 \pm 2.5\%$ of the initial concentration, while at pH 7.4 it was $102.9 \pm 1.7\%$ and at pH 10.2 it was $106.6 \pm 5.8\%$ (mean \pm s.d., $N=3$ for each group). Total, nonspecific, membrane ATPase activity at pH 6.0 was approximately 55% greater than that at pH 7.4, while this activity at pH 10.0 was reduced to approximately 6% of that at pH 7.4 (Table 2).

Table 1. The [NTP]/[Hb] level in fetal red cells incubated with 0.1 mmol l^{-1} KCN or 0.5 mmol l^{-1} iodoacetic acid expressed as a percentage of the initial level

Time (h)	Control (%)	KCN (%)	Iodoacetic acid (%)
0	100	100	100
6	90.4 ± 6.0	74.2 ± 8.2	80.2 ± 9.1
24	75.2 ± 13.2	74.6 ± 12.1	56.0 ± 2.7
48	53.6 ± 39.1	50.2 ± 36.1	31.8 ± 18.5

Initial [NTP]/[Hb] ratio was 2.12 ± 0.38 (mean \pm s.d., $N=5$).

Unbalanced randomized-block ANOVA with a 3×3 factorial, $P=0.38$.

Values are means \pm s.d., $N=3$.

Mean corpuscular hemoglobin concentrations (MCHC) appeared to decrease, particularly at low pH, suggesting pH-dependent cell swelling (Fig. 4). To determine to what extent cell swelling might alter the [NTP]/[Hb] value, red cells were incubated for 24 h in dilute buffer A at pH 7.4. Red cells maintained in 80% buffer A contained $99.4 \pm 1.5\%$ of the [NTP]/[Hb] value of those maintained in 100% buffer A, while at 60% buffer A this value was $90.0 \pm 10.1\%$ and at 40% buffer A it was $78.0 \pm 16.1\%$ (mean \pm s.d., $N=3$ for each group).

Cells maintained at pH 6.3 ± 0.1 in 20 mmol l^{-1} BisTris buffer showed a $51.5 \pm 3.6\%$ (mean \pm s.d., $N=4$) decline in intracellular [NTP]/[Hb] ratio by 24 h. The presence of ouabain attenuated this decline, with 1.0 mmol l^{-1} ouabain reducing the effect by $13.2 \pm 6.8\%$, 3.3 mmol l^{-1} by $14.8 \pm 7.4\%$ and 10.0 mmol l^{-1} by $19.9 \pm 8.6\%$ (mean \pm s.d., $N=3$ for each concentration). Cells maintained at pH 9.5 ± 0.1 in 20 mmol l^{-1} Capsso buffer showed a decline in intracellular [NTP]/[Hb] ratio of $49.9 \pm 9.2\%$ (mean \pm s.d., $N=4$). The presence of ouabain had no noticeable effect, 1 mmol l^{-1} ouabain altered

Table 2. Influence of pH on total, nonspecific ATPase activity of red cell membranes

pH	Activity ¹	Activity ²
	($\text{pmol P}_i \text{ mg}^{-1} \text{ protein h}^{-1}$)	(% of activity at pH 7.4)
6.0	56.0 ± 25.7^a	155 ± 14^c
7.4	36.9 ± 17.7^b	100^c
10.0	$1.9 \pm 0.5^{a,b}$	6 ± 3^c

$T=20^\circ\text{C}$; $N=4$.

¹Data were analyzed by a randomized-block ANOVA ($P < 0.003$) followed by protected LSD. Like letters indicate a statistically significant difference ($P < 0.05$).

²The analysis of the activity measured at pH 6.0 and 10.0 was based on a within-animal comparison and used a randomized-block ANOVA ($P \leq 0.0001$). One-sample t -tests were used to compare the percentage activities at pH 7.4 with the percentage activities at high ($P < 0.004$) and low ($P < 0.0001$) pH. Like letters indicate a statistically significant difference.

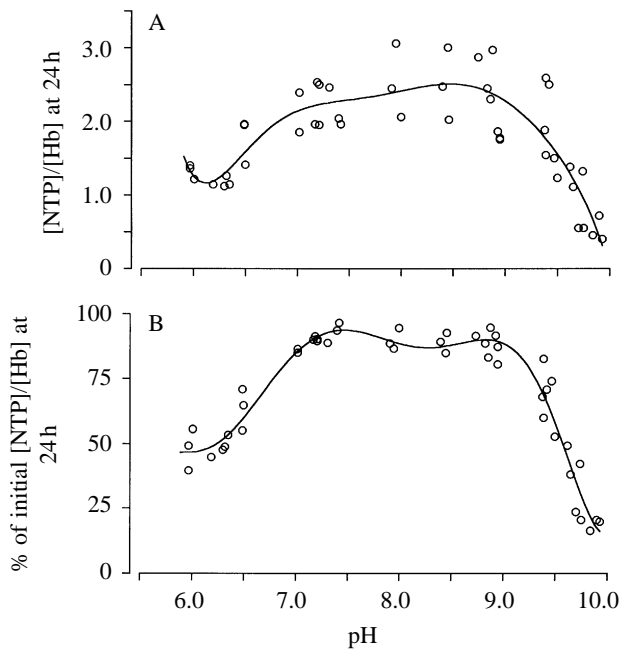


Fig. 3. The [NTP]/[Hb] ratio (A) and percentage of initial [NTP]/[Hb] ratio (B) of red cells of nonpregnant females after 24 h of incubation *in vitro* at 20 °C in buffered saline at various pH values. Buffers were 20 mmol⁻¹ BisTris for pH 6.0–7.2, 20 mmol⁻¹ Tris for pH 7.2–9.0 and 20 mmol⁻¹ Capso for pH 9.0–10.0. Initial [NTP]/[Hb] ratios were 2.69±0.58 (mean ± s.d., N=6).

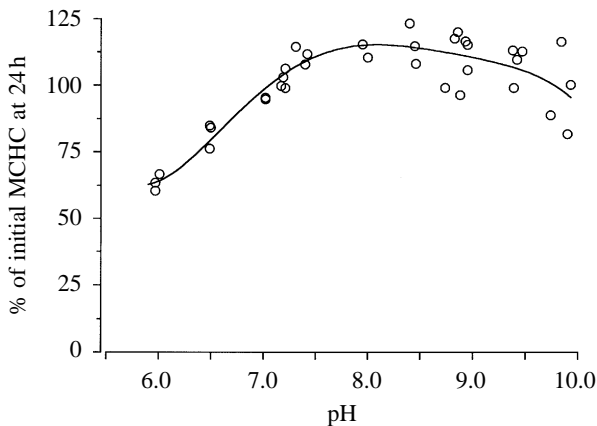


Fig. 4. The mean corpuscular hemoglobin concentration (MCHC) as a percentage of the initial value following incubation as described in Fig. 3. Initial MCHC value was 4.16±0.21 mmol⁻¹ (mean ± s.d., N=6).

the decline by $-2.6\pm 5.5\%$, 3.3 mmol⁻¹ by $4.5\pm 8.7\%$ and 10.0 mmol⁻¹ by $3.0\pm 10.1\%$ (mean ± s.d., N=4 for each concentration). Ouabain at 10 mmol⁻¹ had no significant effect on the red cell [NTP]/[Hb] ratios of cells maintained for 24 h at pH 7.4 (data not shown).

Injection of *T. elegans* with progesterone did not result in a rise in red cell NTP concentration at either 24 or 72 h. The red cell [NTP]/[Hb] value at 24 h for animals injected with 0.1 mg kg⁻¹ of animal progesterone was 2.25±0.18, and for

animals injected with the same concentration of cholesterol it was 2.26±0.13. At 72 h, these values were 2.28±0.17 for progesterone-treated snakes and 2.52±0.23 for cholesterol-treated snakes (mean ± s.d., N=4 for each group). Similarly, incubation of red cells *in vitro* with exogenous nutrients and progesterone for 24 h did not result in an increase in red cell [NTP]/[Hb] ratio relative to cells incubated with nutrients and cholesterol, as the [NTP]/[Hb] ratio of progesterone-treated cells was 101.6±3.7% (N=3) of that of cholesterol-treated cells. Cells incubated for 24 h at pH 7.4 without or with 5 mmol⁻¹ glucose plus 5 mmol⁻¹ pyruvate and 5 mmol⁻¹ glutamine showed decreases in the [NTP]/[Hb] ratio of 8.2±5.2% in the absence of additional energy sources and 5.1±7.3% when these were present (mean ± s.d., N=3 for each group), respectively. These values were not significantly different as assessed by a Student's *t*-test.

Discussion

The red cells of the killifish *Fundulus heteroclitus*, when maintained *in vitro* without exogenous nutrients at room temperature, show no decline in red cell ATP concentration over 4–8 h (Greaney and Powers, 1978). Similarly, red cells of *Squalus acanthias* show no decline in ATP concentration over 18 h when maintained at 10 °C without nutrients (Wells and Weber, 1983). Consistent with these findings is the very slight (if any) decrease in cellular NTP levels noted in the present study with red cells from the garter snake (Fig. 1A–C). In the presence of KCN or IAC, the level of ATP in *F. heteroclitus* red cells shows a 40% decline over 4 h and, under anoxia, red cells of *S. acanthias* show a 30–35% decrease in ATP levels over 18 h. In contrast to these findings, the use of a metabolic block resulted in a slower decline in NTP levels in red cells from adult *T. elegans*; total NTP levels in these cells appeared particularly stable. For unknown reasons, NTP levels in red cells from fetal snakes appeared less stable than those in red cells from adult snakes. Despite the red cells of snakes being nucleated, having mitochondria and generating ATP by oxidative phosphorylation (Ogo *et al.* 1993), 0.1 mmol⁻¹ KCN had little or no effect on the red cells from the adult male and pregnant female. It did, however, have a demonstrable effect on the cells from the nonpregnant female. In contrast, 0.5 mmol⁻¹ IAC did appear to be an effective inhibitor of ATP synthesis, at least in the red cells of the males and pregnant females, suggesting that normal ATP turnover in these cells is primarily glycolysis-based.

Since pregnancy in *T. elegans* is associated with a rise in NTP levels of the adult red cell (Ingermann *et al.* 1991a), we looked at the possibility that the turnover of the additional NTP associated with pregnancy is different from that of the basal levels in the red cells of the male and nonpregnant female. NTP levels of the red cells of the pregnant animal did indeed decrease more rapidly in the absence, as well as in the presence, of KCN+IAC than did those of the cells of the nonpregnant female. This suggests that the pregnancy-

associated NTP distributes into intracellular pools differently from the smaller amount of NTP in the nonpregnant female.

Previous findings have suggested that the pregnancy-associated rise in red cell NTP levels was due to, or at least correlated with, a chronic rise in plasma progesterone levels (Ragsdale *et al.* 1993). To test whether the red cells themselves could respond quickly to elevated progesterone levels, animals were injected with progesterone or red cells were incubated *in vitro* with progesterone. In neither case was an acute change noted in red cell NTP values. Therefore, it appears that these cells do not respond to progesterone directly and/or that they cannot respond quickly to this potential stimulus.

The pronounced stability of total NTP levels in the red cells of the snake, in the absence or presence of metabolic inhibition, appears inconsistent with ATP having a half-life of minutes, as in other eukaryotic cells (e.g. Lehninger, 1975). However, ATP has been reported to bind to various proteins; it binds to lamin C (Schwartz and Clawson, 1991), serum albumin (Bauer *et al.* 1992) and α -crystallin (Reddy *et al.* 1992), and Koszegi *et al.* (1987) have suggested that in several cell types the bulk of cellular ATP is associated with proteins rather than being in a soluble form that is uniformly distributed in the cytoplasm. Conceivably, this may represent a possible resolution to the paradox of the stability of NTP levels in the snake red cell. The apparently low rate of NTP turnover in the snake red cell may be due to a compartmentalization of NTP with cellular proteins or membranes. Compartmentalization of ATP appears to account for the high ATP content of mammalian platelets (Ugurbil *et al.* 1979) and has been reported in the human red cell (Gupta *et al.* 1978; Petersen *et al.* 1989, 1990). Perhaps such an association in the snake red cell renders NTP relatively unavailable for hydrolysis by nucleoside-triphosphate-consuming processes.

Since NTP is highly charged at physiological pH, any association with intracellular proteins (or membranes) would probably be ionic. Extremes of pH should therefore disrupt these putative interactions and possibly make ATP accessible to intracellular ATPase activity. As indicated in Fig. 3A,B, 24 h of incubation at low or high pH (in the absence of metabolic inhibition) resulted in a pronounced and relatively rapid decrease in red cell NTP levels. That incubation of commercial ATP at low and high pH did not result in any detectable change in concentration suggests that ATP was not itself unusually labile under these conditions. This is consistent with the reported inherent stability of the nucleoside triphosphates (Miller and Westheimer, 1966; Westheimer, 1987).

The decrease in the level of NTP in red cells noted at extreme pH could have been due to osmotic challenges to the cell; however, incubation in hypo-osmotic buffer at pH 7.4 resulted in no comparable decrease in red cell NTP levels after 24 h. The decrease in NTP concentration could have been due to activation of ATPase activity. Indeed, ATPase activity at low pH was greater than that at pH 7.4. Thus, the decrease in red cell NTP levels after 24 h at low pH could have been due to direct stimulation of ATPase activity under these incubation

conditions. In contrast, increased ATPase activity could not have accounted for the marked reduction of intracellular NTP levels after incubation at high pH, as nonspecific red cell ATPase activity at high pH was only approximately 6% of that at pH 7.4. Therefore, the decrease in red cell NTP levels during incubation at high pH does not appear to have been due to an inherent instability in ATP at this pH, to osmotic perturbation or to an enhanced total nonspecific ATPase activity. A net reduction in total NTP at the pH extremes could have resulted from an increased availability of NTP to a variety of intracellular consuming processes and/or to cellular export (Forrester, 1990; Bergfeld and Forrester, 1992).

Overall, it appears that total NTP levels of the red cells of *T. elegans* are very stable *in vitro*, relatively insensitive to metabolic inhibition and completely insensitive to short-term progesterone exposure, but are labile under extremes of pH. These results are consistent with the hypothesis that intracellular NTP is compartmentalized into at least two pools within these snake red cells: a labile, cytoplasmic pool and a more stable, protein- or membrane-associated pool.

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References

- BARTLETT, G. R. (1980). Phosphate compounds in vertebrate red blood cells. *Am. Zool.* **20**, 103–114.
- BAUER, M., BAUMANN, J. AND TROMMER, W. E. (1992). ATP binding to bovine serum albumin. *FEBS Lett.* **313**, 288–290.
- BERGFELD, G. R. AND FORRESTER, T. (1992). Release of ATP from human erythrocytes in response to a brief period of hypoxia and hypercapnia. *Cardiovascular Res.* **26**, 40–47.
- FORRESTER, T. (1990). Release of ATP from heart. Presentation of a release model using human erythrocyte. *Ann. N.Y. Acad. Sci.* **603**, 335–352.
- GREANEY, G. S. AND POWERS, D. A. (1978). Allosteric modifiers of fish hemoglobins: *in vitro* and *in vivo* studies of the effect of ambient oxygen and pH on erythrocyte ATP concentrations. *J. exp. Zool.* **203**, 339–350.
- GUPTA, R. K., BENOVIC, J. L. AND ROSE, Z. B. (1978). Magnetic resonance studies of the binding of ATP and cations to human hemoglobin. *J. biol. Chem.* **253**, 6165–6171.
- HERMAN, J. K. AND INGERMANN, R. L. (1996). Effects of hypoxia and hyperoxia on oxygen-transfer properties of the blood of a viviparous snake. *J. exp. Biol.* **199**, 2061–2070.
- HIGHFILL, D. R. AND MEAD, R. A. (1975). Sources and levels of progesterone during pregnancy in the garter snake, *Thamnophis elegans*. *Gen. comp. Endocr.* **27**, 389–400.
- INGERMANN, R. L., BERNER, N. J. AND RAGSDALE, F. R. (1991a). Effect of pregnancy and temperature on red cell oxygen affinity in the viviparous snake *Thamnophis elegans*. *J. exp. Biol.* **156**, 399–406.
- INGERMANN, R. L., BERNER, N. J. AND RAGSDALE, F. R. (1991b). Changes in red cell ATP concentration and oxygen-affinity following birth in the neonatal garter snake *Thamnophis elegans*. *J. exp. Biol.* **157**, 579–584.
- ISAACKS, R. E. AND HARKNESS, D. R. (1980). Erythrocyte organic

- phosphates and hemoglobin function in birds, reptiles and fishes. *Am. Zool.* **20**, 115–129.
- KOSZEGI, T., KELLERMAYER, M., BERENYI, E., JOBST, K. AND HAZLEWOOD, C. F. (1987). The bulk of ATP is associated to proteins in the living cell: a release kinetics study. *Physiol. Chem. Phys. med. NMR* **19**, 143–146.
- LEHNINGER, A. L. (1975). *Biochemistry*. New York: Worth Publishers, Inc.
- MARKWELL, M. A. K., HASS, S. M., BIEBER, L. L. AND TOLBERT, N. E. (1976). A modification of the Lowry procedure to simplify protein determination in membrane and lipoprotein samples. *Analyt. Biochem.* **87**, 206–210.
- MILLER, D. L. AND WESTHEIMER, F. H. (1966). The hydrolysis of phenylpropyl di- and triphosphates. *J. Am. chem. Soc.* **88**, 1507–1511.
- OGO, S. H., BERNARDES, C. F., GLASS, M. L., TORSONI, M. A. AND VERCESI, A. E. (1993). Functional mitochondria in snake *Bothrops alternatus* erythrocytes and modulation of HbO₂ affinity by mitochondrial ATP. *J. comp. Physiol. B* **163**, 614–619.
- PETERSEN, A., KRISTENSEN, S. R., JACOBSEN, J. P. AND HØRDER, M. (1990). ³¹P-NMR measurements of ATP, ADP, 2,3-diphosphoglycerate and Mg²⁺ in human erythrocytes. *Biochim. biophys. Acta* **1035**, 169–174.
- PETERSEN, A., PEDERSEN, E. J. AND QUISTORFF, B. (1989). The Na⁺/K⁺-ATPase reaction of human erythrocytes is not near equilibrium. A ³¹P-NMR study. *Biochim. biophys. Acta* **1012**, 267–271.
- RAGSDALE, F. R., IMEL, K. M., NILSSON, E. E. AND INGERMANN, R. L. (1993). Pregnancy-associated factors affecting organic phosphate levels and oxygen affinity of garter snake red cells. *Gen. comp. Endocr.* **91**, 181–188.
- REDDY, M. C., PALMISANO, D. V., GROTH-VASSELLI, B. AND FARNSWORTH, P. N. (1992). ³¹P NMR studies of the ATP/α-crystallin complex: functional implications. *Biochem. biophys. Res. Commun.* **189**, 1578–1584.
- SCHWARTZ, A. M. AND CLAWSON, G. A. (1991). Cooperative ATP binding by cloned lamin C. *Exp. Cell Res.* **193**, 432–434.
- UGURBIL, K., HOLMSEN, H. AND SHULMAN, R. G. (1979). Adenine nucleotide storage and secretion in platelets as studied by ³¹P nuclear magnetic resonance. *Proc. natn. Acad. Sci. U.S.A.* **76**, 2227–2231.
- WEBER, R. E. AND JENSEN, F. B. (1988). Functional adaptations in hemoglobins from ectothermic vertebrates. *A. Rev. Physiol.* **50**, 161–179.
- WELLS, R. M. G. AND WEBER, R. E. (1983). Oxygenation properties and phosphorylated metabolic intermediates in blood and erythrocytes of the dogfish, *Squalus acanthias*. *J. exp. Biol.* **103**, 95–108.
- WESTHEIMER, F. H. (1987). Why nature chose phosphates. *Science* **235**, 1173–1178.
- WOOD, S. C. (1980). Adaptation of red blood cell function to hypoxia and temperature in ectothermic vertebrates. *Am. Zool.* **20**, 163–172.
- ZAUGG, W. S. AND MCLAIN, L. R. (1970). Adenosinetriphosphatase activity in gills of salmonids: seasonal variations and salt water influence in coho salmon, *Oncorhynchus kisutch*. *Comp. Biochem. Physiol.* **35**, 587–596.
- ZEHR, D. R. (1962). Stages in the normal development of the common garter snake, *Thamnophis sirtalis sirtalis*. *Copeia* **1962**, 322–329.