Effect of Ouabain on the Breakdown of Adenine Nucleotides in Glucose-Depleted Nucleated Red Blood Cells. Characterization of ATPase

M Kaloyianni, O Tsikriktsi and P Tsianopoulou

Laboratory of Animal Physiology, Zoology Department Science School Aristotle University of Thessaloniki Thessaloniki 54006 Greece

Abstract. The present report confirms the presence of Na^+-K^+-Mg^{2+} ATPase in the erythrocyte membranes of the frog *Rana balcanica* (previously *Rana ridibunda*) (Schneider et al 1993, Sofianidou et al 1994) The Na^+-K^+-Mg^{2+} ATPase activity was 60% reduced by the presence of ouabain The pH optimum was 8 0 the optimum Mg^{2+} ATP concentration ratio was 2 2 1 The existence of an ATPase with a high K_m for ATP (1 48 mmol/l) was postulated

At pH 7 4 and 8 0, the adenine nucleotide pattern of glucose-depleted erythrocytes showed a characteristic reduction in ATP contents Adenine nucleotide concentrations were higher at pH 7 4 than at pH 8 0 Ouabain inhibited ATP breakdown at both pH values studied The strongest inhibition was observed at pH 7 4 The decline of the total contents of adenine nucleotides appears to be determined by the rate of AMP breakdown

Key words: *Rana balcanica* Red cells ATPase — Adenine nucleotides

Introduction

Adenine nucleotides are known to be the principal nucleotides in mature human and amphibian erythrocytes (Jacobasch et al 1974, Kaloyianni-Dimitriades and Beis 1984a), and to play an important role in the normal metabolism and functioning of the red cell (Nakao et al 1961) In contrast to the metabolic stability of hemoglobin (Shemin and Rittenberg 1946) adenine nucleotides in mammalian erythrocytes are in a dynamic state (Shemin and Rittenberg 1946) The closely connected processes of synthesis and breakdown of adenine nucleotides remains a major obstacle of a

Correspondence to Dr M Kaloyianni Laboratory of Animal Physiology, Zoology Department Science School Aristotle University of Thessaloniki Thessaloniki 54006 Greece E-mail Kaloyian@bio auth gr

comprehensive analysis of their metabolism in the red cell

Even if the importance of ATP breakdown in red cells has long been recognized, it has not yet been subject of systematic studies in nucleated erythrocytes ATPase is among the mechanisms involved in ATP consumption According to Siems et al (1983) 23% of ATP in human reticulocytes is split by Na^+-K^+ ATPase ATP concentrations can also be affected by varying concentrations of fructose-2 6-bisphosphate and triose phosphate in *Rana balcanica* erythrocytes (Kaloyianni et al 1994) On the other hand, in frog erythrocytes ATP is responsible for lowering of Hb-O_2 affinity (Bartlett 1970) Consequently, maintenance of ATP concentration is crucial for proper oxygenation of the frog tissues In addition to the possible role in ATP consumption, ATPase plays a fundamental role in ion transport and osmoregulation in aquatic organisms (Epstein et al 1967)

To the best of our knowledge however, there have not been any reports on ATPase activity in amphibian erythrocytes so far As a first step we studied ATPase in the membrane of red blood cells of the amphibia *Rana balcanica* and tried to characterize it Moreover, pH-induced (pH 7 4 and 8 0) changes of adenine nucleotides in glucose-depleted red cells of *Rana balcanica* were studied By eliminating the hexokinase-phosphofructokinase (HK-PFK) step and preventing ATP regeneration via the main pathway of glycolysis the process of ATP breakdown was expected to become more transparent Additionally, ouabain added to glucose depleted cells was hoped to help elucidate the role of ATPase in *Rana balcanica* erythrocytes

Materials and Methods

Animals

Frogs (*Rana balcanica*) weighing 50 120 g were supplied by a local dealer after having been caught in the vicinity of Larisa They were kept in containers in fresh water and used for experiments within a week

Experimental procedures

Cells were separated from heparinized frog blood by centrifugation at 3000 rpm for 10 min at 4°C Plasma and buffy coat were discarded All further preparation and centrifugation steps were carried out at 25°C

Preparation of erythrocyte membranes for ATPase activity measurements

Hemoglobin-free membranes for ATPase measurements were prepared by modification of the method of Burger et al (1968)

The red cells were washed three times in ice-cold 0 002 mol/l Tris-HCl, 0 10 mol/l NaCl pH 7 4 The buffy coat was removed after each centrifugation

The washed red cells were then pooled hemolyzed in 30 1 vol/vol hypotonic solution (0 005 mol/l Tris-HCl, 0 005 mol/l $MgCl_2$, pH 7 65), and centrifuged at 27,000 × *g* at 4°C for 15 min The supernatant was decanted and the membranes were washed 6 to 8 times with the same volume of ice-cold hemolyzing solution until the supernatants were almost colorless The resulting fluffy membranes were stored in an equal volume of fresh hemolyzing solution All ATPase assays were performed on the same day when the membranes were prepared

Protein concentrations in erythrocyte membranes were determined in triplicate according to the method of Lowry et al (1951)

ATPase activity in the membranes of the red blood cells was measured according to the method described by Keeton and Kaneko (1972) The activity was expressed as μmols Pi/min/mg of membrane protein

Preparation of glucose-depleted cells

The procedure to prepare glucose-depleted cells was carried out according to a modification of the method described by Tu et al (1979) The erythrocytes were suspended in a 5-fold excess of an isotonic solution containing 2 mmol/l glucose 66 mmol/l triethanolamine buffer 1 mmol/l Na_2HPO_4 and 100 mmol/l NaCl, pH 7 4 After 10 min incubation with glucose the erythrocytes were centrifuged at 1500 × *g* for 10 min The glucose depletion process was started at the second washing step with the addition of a 10-fold excess of isotonic solution containing 33 mmol/l triethanolamine 1 mmol/l Na_2HPO_4 and 100 mmol/l NaCl pH 7 4

The third washing was carried out without buffer in a 10-fold excess of a solution containing 100 mmol/l NaCl, 5 mmol/l KCl 1 mmol/l $MgCl_2$ and 0 5 mmol/l Na_2HPO_4 pH 7 4 Subsequently, the erythrocytes were separated by centrifugation at 6500 × *g* for 10 min

The packed cells were suspended in the indicated incubation medium (NaCl KCl Na_2HPO_4) to make a hematocrit value of about 30% Antimycin (4 mg/100 ml) was added to prevent bacterial contamination during the incubations The pH of the red cell suspensions was adjusted rapidly by addition of 0 3 mol/l NaOH or 0 3 mol/l HCl and corrected as necessary (± 0 08 pH) with the same solutions Ouabain 10^{-4} mol/l was added to the incubation medium if indicated

Determination of adenine nucleotides

For the determination of adenine nucleotides the samples were added to one volume of chilled perchloric acid under strong stirring After centrifugation the supernatant was taken up and neutralized with 0 25 volume of a solution containing 0 5 mol/l triethanolamine hydrochloride and 1 3 mol/l K_2CO_3 The $KClO_4$-precipitate was removed by centrifugation The samples were then frozen and kept at −120°C until the assay The concentrations of ATP ADP and AMP were determined enzymatically according to Bergmeyer (1970)

Statistical evaluation

Student's *t*-test ($p < 0.005$) for unpaired grouped samples was used

Results

Table 1 shows the effects of extracellularly added Mg^{2+}, Na^+ and K^+ ions on ATPase activity. ATPase was dependent upon Na^+ or K^+ alone but it showed less activity than when Mg^{2+} was only present. A combination of sodium and potassium without magnesium produced significant activation compared to that in the absence of the ions in extracellular medium. Also a dependence of ATPase on Mg^{2+} was suggested by a rise in activity compared with that measured in the presence of sodium only. In the absence of extracellular ions ATPase activity reached half the values measured in the presence of Na^+ and K^+. ATPase activity rose significantly to 51 μmoles Pi/min/g protein when all the free ions tested were present in the incubation medium. The maximum activity of membrane ATPase was measured at 2.87 mmol/l Mg^{2+}, 120 mmol/l Na^+ and 5 mmol/l K^+.

Table 1. Effects of magnesium, sodium and potassium on ATPase activity of *Rana balcanica* red blood cell membranes

Ionic composition of the incubation medium mmol/l	μmols Pi/min/g protein
Mg^{2+} (2.87) Na^+ (120) K^+ (5)	51.9 ± 4.3
Mg^{2+} (2.87)	40.2 ± 3.7
Na^+ (120)	33.5 ± 2.1
K^+ (120)	37.3 ± 2.4
Na^+ (120) K^+ (120)	41.8 ± 3.8
Na^+ (120) K^+ (5)	41.8 ± 3.8
No ions present	19.3 ± 2.3

Each value represents mean ± S.E.M. of 4 different experiments. Three to five animals were used for each experiment the blood cells of which were pooled

When the red cell membranes were incubated at various pH, total ATPase activity grew at pH 8.0 indicating a pH maximum in this vicinity (Table 2). Fig. 1 shows the effect of ouabain on the ATPase activity of *Rana balcanica* erythrocyte membranes. A 50% inhibition was achieved at ouabain concentration of 10^{-4} mol/l.

As shown in Fig. 2, the total ATPase and ouabain-insensitive (Na^+-K^+-Mg^{2+}-ATPase) activities peaked at Mg^{2+} concentration of 8 mmol/l. On the other hand the ouabain-sensitive Na^+-K^+-Mg^{2+}-ATPase increment showed a maximum at

Table 2. Effect of pH on total ATPase activity

pH	Enzymatic activity (μmols Pi/min/g/protein)
6	20 9 ± 2 7
7	39 3 ± 3 8
8	18 0 ± 4 5
9	45 4 ± 4 3

Each value represents mean ± S E M of 4 different experiments Three to five animals were used for each experiment the blood cells of which were polled
The conditions of the experiment were as follows Mg^{2+} 2 87 mmol/l Na^{+} 0 12 mol/l K^{+} 0 005 mol/l

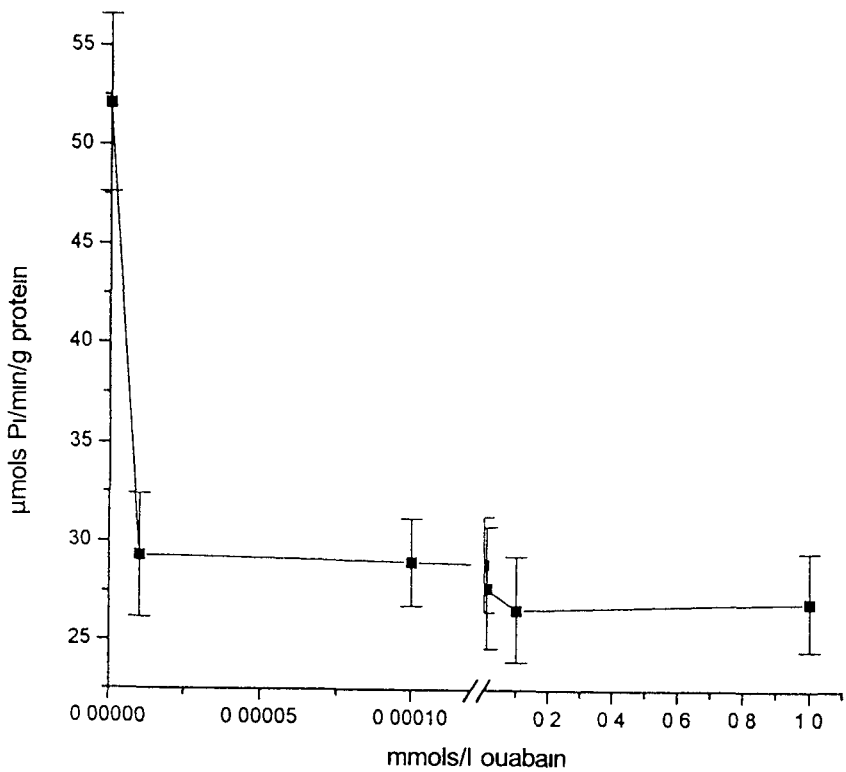


Figure 1. Effect of ouabain on the Na^{+} K^{+} Mg^{2+}-ATPase activity The following experimental conditions were employed temp 25°C pH 8 0 $MgCl_2$ 3 mmol/l, NaCl 112 mmol/ KCl 5 mmol/l time 135 min Each value represents mean ± S E M of 4 different experiments Three to five animals were used for each experiment and then blood cells were pooled

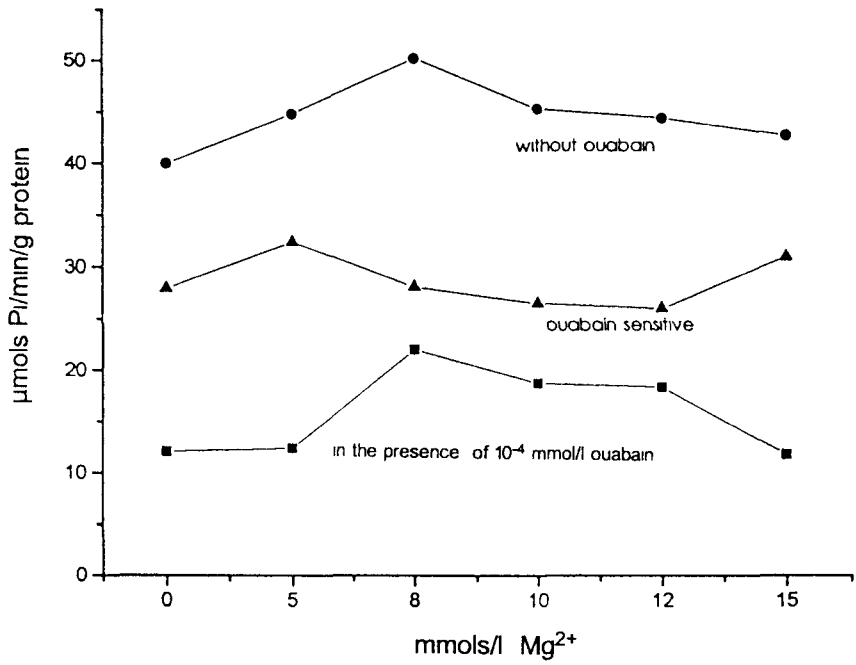


Figure 2. Effect of Mg^{2+} on total ATPase activity ouabain-sensitive ATPase and ouabain insensitive ATPase activity The concentration of NaCl was 114mmol/l that of KCl was 12 mmol/l pH 8 0 and time 120 min The activity in the absence of ouabain represents the total ATPase activity The activity in the presence of ouabain at 10^{-4} mol/l represents the ouabain-insensitive activity The difference between the two curves represents the ouabain-sensitive activity Each value represents the mean of 4 different experiments Three to five animals were used for each experiment and then blood cells were pooled

Mg^{+2} concentration of 5 mmol/l The activity peaks observed for the total ATPase and for ouabain-sensitive activities corresponded to a Mg^{2+} ATP concentration ratios of 3 2 1 and 2 1 respectively The 2 1 concentration ratio required for Mg^{2+} and ATP suggests that the actual substrate of the enzyme is Mg^{2+}-ATP complex Since the ouabain-sensitive and ouabain-insensitive activity increments both decreased as the Mg^{2+} concentration was raised above 8 mmol/l, free Mg^{2+} might have interfered by competing with Mg^{2+}-ATP complex for the enzyme or might have altered the enzyme by binding at another site independent of the active substrate site

Figs 3, 4 5 show data on the changes of ATP ADP and AMP at pH 7 4 and pH 8 0 The same blood samples were used to perform identical measurements at pH 7 4 and at pH 8 0 It may be seen that during an initial period of 30 min a decline

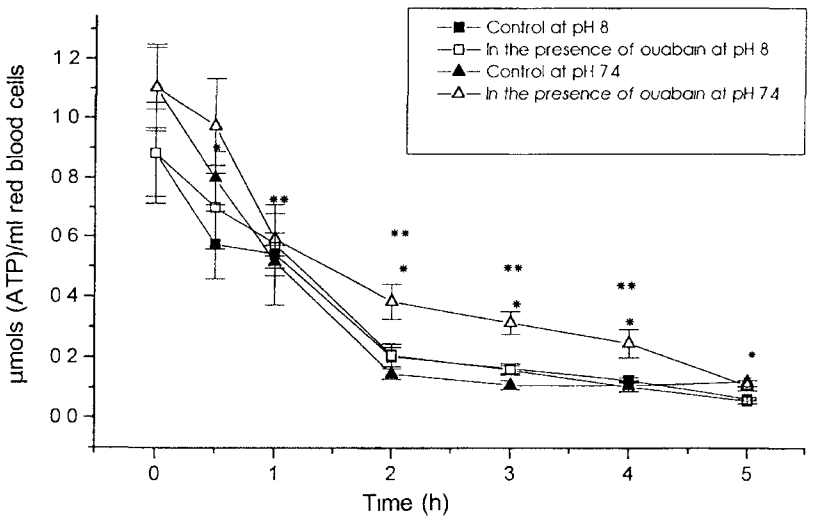


Figure 3. Time course of ATP breakdown in glucose depleted red blood cells and in glucose depleted red blood cells treated with 10^{-4} mol/l ouabain at pH 7.4 and 8.0. * Indicates statistical significance of the difference between the respective values at pH 7.4 and pH 8.0. ** Indicates statistical significance of the difference between the respective values in the absence and in the presence of ouabain at pH 7.4. All values obtained from the same experiment were statistically significant from of values obtained at time 0.

of ATP and increases of ADP and AMP were observed which may accompany the accumulation of glycolytic intermediates. During the subsequent 30 min (at 60 min) AMP and ADP rose and ATP decreased to values similar as measured at pH 8.0. During this period the sum of nucleotides remained constant (Fig. 6). Thereafter ATP concentration decreased at a rapid rate which declined with time both at pH 7.4 and pH 8.0. ADP and AMP showed transient peaks after 3 h incubation to fall slowly thereafter. The sum of nucleotides decreased at a nearly constant rate of about 0.3–0.4 µmols/ml cells/h during the 2 h of incubation. The main difference between the values measured at the two pH values is a greater decrease of the sum at the lower pH during the first 2 h of incubation. Also, ATP declined stroger at pH 7.4 than at pH 8.0 (Fig. 3).

Ouabain did not affect the mass action ratios of adenylate kinase at pH 8.0 whereas at pH 7.4 the mass action ratio of adenylate kinase increased significantly after 3 h incubation of the red cells.

Figs. 3, 4, 5 and 6 also show the effect of ouabain on the changes in ATP, ADP, AMP and the sum of adenine nucleotides at pH 7.4 and pH 8.0 in glucose depleted cells. The adenine nucleotides exhibited a characteristic pattern as described earlier.

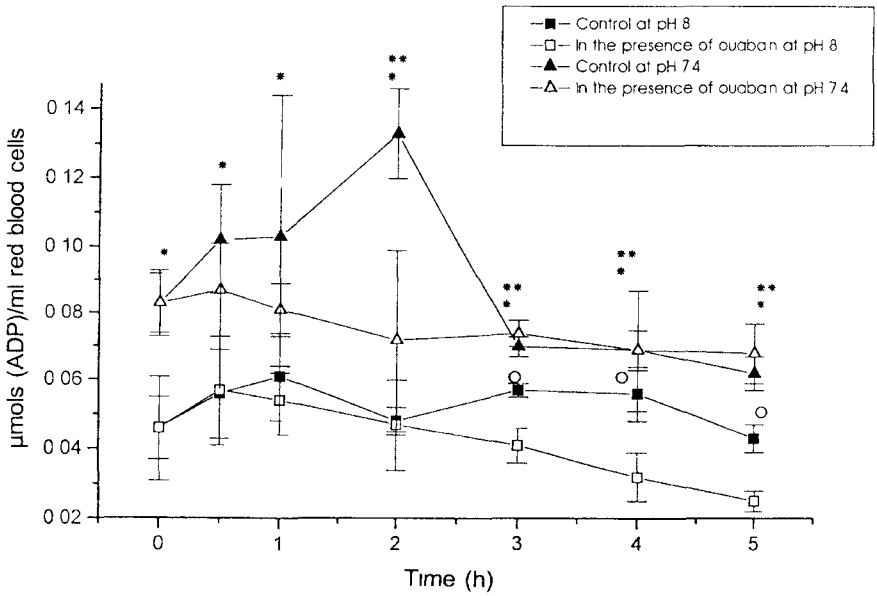


Figure 4. Time course of ADP breakdown in glucose depleted red blood cells and in glucose depleted red blood cells treated with 10^{-4} mol/l ouabain at pH 7.4 and 8.0 * Indicates statistical significance of the difference between the respective values at pH 7.4 and pH 8.0 ** Indicates statistical significance of the difference between the respective values in the absence and in the presence of ouabain at pH 7.4 o Indicates statistical significance of the difference between the respective values in the presence and in the absence of ouabain at pH 8.0 All values obtained from the same experiment were statistically significant from values obtained at time 0

with a decline of ATP whereas ADP increased to flat maximum and declined thereafter

The effects of ouabain at different pH values on the breakdown of ATP indicate a 14-46% inhibition of this process at pH 7.4 At pH 8.0 where ATPase activity is higher than at pH 7.4 (Table 2) ouabain seems to exert significant effects on ATP degradation and on the breakdown of ADP at the 3rd, 4th, and 5th hour of incubation (Fig. 4) It seems that ATPase is responsible for ATP breakdown both at pH 7.4 and pH 8.0 (Table 3)

Discussion

This is the first ATPase study in amphibian erythrocytes ATPase plays a fundamental role in ion transport and osmoregulation in aquatic organisms Therefore

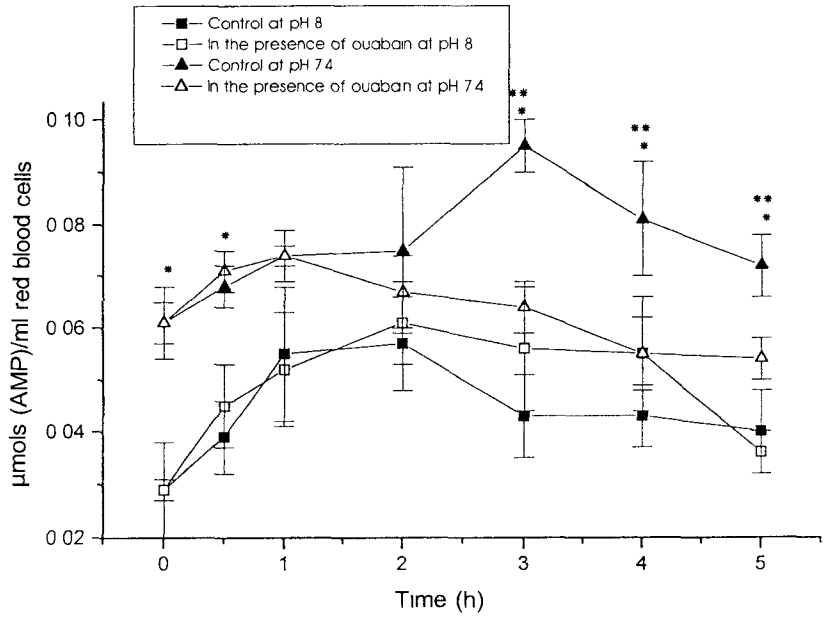


Figure 5. Time course of AMP breakdown in glucose depleted red bood cells and in glucose depleted red blood cells treated with 10^{-4} mol/l ouabain at pH 7.4 and 8.0

Indicates statistical significance of the difference between the respective values at pH 7.4 and pH 8.0 ** Indicates statistical significance of the difference between the respective values in the absence and in the presence of ouabain at pH 7.4 All values obtained from the same experiment were statistically significant from values obtained at time 0

this study is important for the elucidation of metabolic processes within the red cell The experiments presented herein yield interesting insights into the dynamics of the adenine nucleotides in *Rana balcanica* erythrocytes The data on the transitory periods during glucose depletion show clearly that the decline of ATP and the corresponding rise of ADP and AMP at pH 7.4 and 8.0 are probably due to a relative excess of the HK-PFK system activity as compared with pyruvate kinase in human red cells (Rapoport et al 1979)

Scheme 1 illustrates the glycolytic pathway and adenylate kinase that yield ATP and the two pathways of AMP breakdown, one via adenylate deaminase and the other one phosphatase-related followed by adenosine deaminase and nucleoside phosphorylase Under conditions of glucose depletion the breakdown of total nucleotides proceeds at a slowly decreasing rate which is less rapid at the alkaline pH It amounted to 0.3 µmol/ml/h at pH 8 and to about 0.4 µmol/ml red cells/h at pH 7.4 (Fig 6) The rate does not appear correlated to AMP concentration which

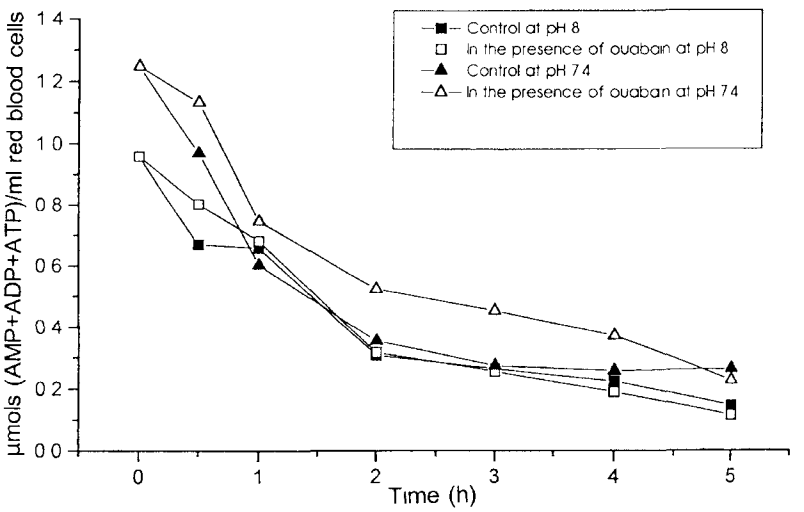


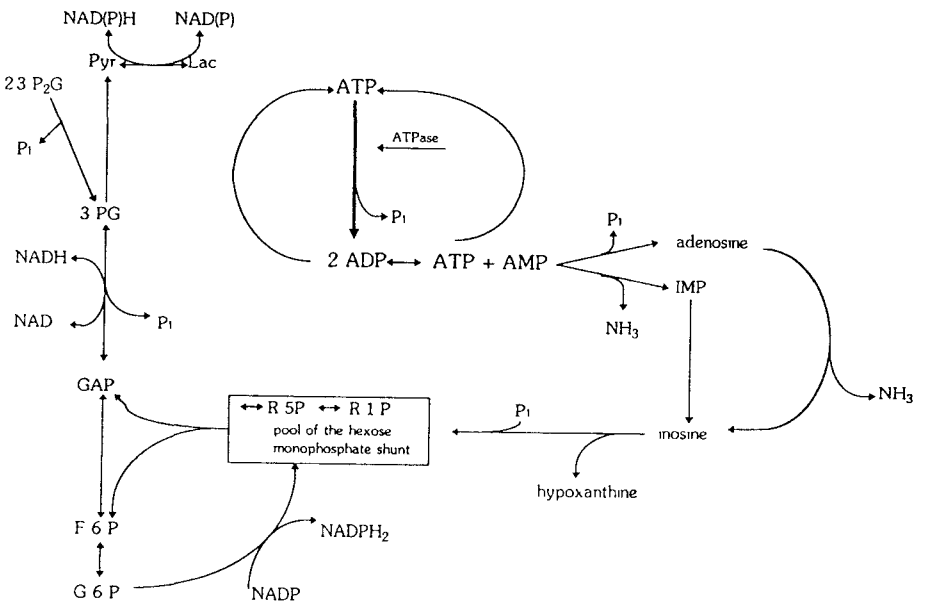
Figure 6. Changes in total levels of adenine nucleotides during glucose depletion of *Rana balcanica* red blood cells at pH 7 4 and 8 0

Table 3. Inhibition of ATP-degradation by ouabain

pH	Inhibition (%)
7 4	11 46
8 8	< 1

varies over the pH range studied This would indicate that the enzyme systems involved in the AMP breakdown are saturated with respect to their substrates

However when frog erythrocytes were incubated in the presence of glucose total nucleotides seemed to increase (Kaloyianni-Dimitriades and Beis 1984a) Two explanations may be considered for this fact For one some unknown mechanism may be postulated by which degrading enzymes are inhibited Such an explanation appears most unlikely as it has not been supported by research evidences It is much more likely that the increase of total adenine nucleotides is a result of their continuous resynthesis which proceeds as long as glucose is available For such a resynthesis in frog erythrocytes an intact adenine moiety is a necessary precondition (Miyazaki et al 1975) AMP may then be produced either by adenosine kinase from adenosine or via a specific ribosyl phosphate transferase from adenine (Scheme 1) It has been reported for *Rana balcanica* erythrocytes that the high



Scheme 1. Sources of ATP and breakdown of adenine nucleotides

concentrations of adenosine may promote deamination of adenosine rather than phosphorylation upon its entry into the erythrocytes (Kaloyianni et al 1993) At low concentrations of adenosine, frog erythrocytes incorporated adenosine both via adenosine kinase and via its conversion to the hypoxanthine pathway (Miyazaki et al 1975) Experiments on the utilization of adenosine in human red cells indicate that the metabolic fate of adenosine also depends on its concentration in the medium At low adenosine concentrations it is converted to ATP whereas at extracellular concentrations higher than 10 μmol/l all adenosine transported into the cells is deaminated (Plagemann et al 1985) Furthermore it has been suggested that since AMP deaminase is strongly inhibited in the cell mostly by 2 3 diphosphoglycerate or by unknown factors the main AMP breakdown pathway proceeds via phosphatase (Rapoport et al 1990)

AMP is an activator of *Rana balcanica* erythrocyte PFK (Kaloyianni et al 1994) Therefore, the lowering of the AMP concentration at pH 8 0 compared to pH 7 4 caused an inhibition of phosphofructokinase (PFK) This pH dependence of PFK activity (weaker activity at pH 8 0 than at pH 7 4) has been reported earlier for *Rana balcanica* erythrocytes (Kaloyianni et al 1994) Thus, the glycolytic rate of *Rana balcanica* erythrocytes is affected both by pH (Kaloyianni-Dimitriades

1983) as well as by the activatory action of AMP on PFK Nevertheless according to Rapoport et al (1981) about one half of the usual glycolysis increase is due to the effects of adenine nucleotides

Rana balcanica erythrocytes are nucleated and show high activity of Na^+-K^+-ATPase in relation to non-nucleated mammalian erythrocytes (Palma et al 1994 Rybakowski and Lehmann 1994) At pH 8 0 the ATP concentration significantly diminished from 0 9 to 0 06 μmol/ml red cells after 5 h incubation (Fig 3) which may have been related to the enhanced activity of ATPase at this pH (Table 2) The exponential decline of the ATP concentrations in the absence of glucose indicates a strong concentration dependence of the ATP degrading processes Rapoport et al (1979) reported that when human red cells were incubated under the same experimental conditions, in the absence of glucose for 3 h the concentration of a reducing substance glutathione increased and that of pyruvate decreased

The high intracellular K^+ concentration as well the high K^+/Na^+ ratio in *Rana balcanica* erythrocytes (7 34) (Kaloyianni et al 1997) show a positive correlation with the high ATP contents of the erythrocytes (Kaloyianni-Dimitriades and Beis 1984a) the same also has been suggested for sheep erythrocytes (Miseta et al 1993) It is possible therefore that the high intracellular K^+ concentrations increase the activity of the K^+-sensitive regulatory enzyme pyruvate kinase in *Rana balcanica* erythrocytes (Kaloyianni-Dimitriades and Beis 1984b) In *Rana balcanica* erythrocytes the Embden-Meyerhof pathway is the main source of ATP (Kaloyianni-Dimitriades and Beis 1984a) Therefore K^+ transported by ATPase may be rate limiting with regard to the generation of ATP molecules via the Embden-Meyerhof pathway in *Rana balcanica* erythrocytes

Ouabain inhibits the breakdown of ATP in *Rana balcanica* red cells (Fig 3) Ouabain also inhibits Na^+-K^+-Mg^{2+}-ATPase isolated from *Rana balcanica* red cell membrane (Fig 1) The ouabain-sensitive ATPase makes up 50% of the total ATPase activity It has been postulated that lactate production in human erythrocytes may be controlled by the membrane adenosine triphosphatase activity (Whittam et al 1964) In *Rana balcanica* red cells ouabain inhibited lactate production by erythrocytes suspended in adenosine suggesting that lactate production in *Rana balcanica* red cells is related to active cation transport and possibly to Na^+/K^+-ATPase (Kaloyianni et al 1993)

The fact that ouabain is stronger in glucose depleted cells incubated for 30 min at alkaline pH supports the notion that the reduction of ATP concentration at pH 8 0 might be due to enhanced activity of the Na^+-K^+-Mg^{2+}-ATPase at this pH (Fig 3) Accordingly the element of the control matrix (S_{ij}) which gives the relative dependence of the metabolite concentration of the enzyme activity (Heinrich and Rapoport 1974) indicates that activation of frog ATPase at pH 8 0 results in a decrease of ATP ($S_{ij} = -0\ 03$) Thus, changes of ATPase activity would explain the pattern of adenine nucleotide levels at alkaline pH values On the other hand, it

seems conceivable that the high K_m value for the *Rana balcanica* Na^+-K^+-ATPase of 1.48 ± 0.84 mmol/l may not account for the breakdown of ATP observed after 2 h incubation of glucose depleted cells. According to Schrier (1966) ATP may be supplied to ATPase in the microenvironment of the membrane. Hence intracellular ATP might not play a direct role in regulating the ATPase activity.

The present report confirms the presence of Na^+-K^+-Mg^{2+}-ATPase in *Rana balcanica* erythrocytes. The observations concerning the membrane ATPase suggested that the enzyme is involved in the active transport of electrolytes across biological membranes, and that the red blood cell maintains its cation composition and volume by the action of a K^+-Na^+ exchange pump. The preponderance of ATPase activity in relation to ATP production may be responsible for changes in adenine nucleotides occurring in glucose depleted red blood cells of *Rana balcanica*. Both pH and ouabain are effective modulators of adenine nucleotide breakdown. Nevertheless, the exact physiological roles of the enzymes involved in adenine nucleotide breakdown will have to be revealed by future studies.

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