

Adenine Nucleotide Synthesis in Human Erythrocytes Depends on the Mode of Supplementation of Cell Suspension with Adenosine

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Svetlana V. Komarova, Eugene V. Mosharov, Victor M. Vitvitsky, and Fazoil I. Ataullakhanov

ABSTRACT: In suspensions of washed human erythrocytes, adenosine added in a single dose to concentrations of 0.1–10.0 mmol/l suspension was deaminated at rates ranging from 10 to 50 mmol/l cells h. The sum of adenosine, inosine, and hypoxanthine concentrations in the suspension, as well as the intracellular concentration of ATP, remained constant. In the presence of 25–50 mmol/l orthophosphate, addition of a single dose of adenosine into erythrocyte suspension increased the ATP concentration by up to 280% of the initial level. If the initial adenosine concentrations were greater than 5 mmol/l suspension, ATP increased independently of adenosine concentration to the level determined only by the concentration of orthophosphate. After orthophosphate was returned to its initial level, ATP in erythrocytes began to decrease. In the presence of cofomycin, erythrocytes utilised adenosine at a rate of 0.2–0.3 mmol/l cells h. Their adenylate pool increased at a rate of 0.10–0.16 mmol/l cells h for several hours, but intracellular ATP increased only slightly. The energy charge of cells decreased significantly from 0.86 ± 0.05 (control) to 0.82 ± 0.06 . Adenosine continuously pumped into erythrocyte suspensions at rates of 0.02–5.0 mmol/l cells h for several hours caused the adenylate pool of erythrocytes and intracellular ATP to increase synchronously at a rate of 0.02–0.35 mmol/l cells h. The energy charge of these erythrocytes increased significantly up to 0.91 ± 0.03 . After pumping of adenosine was stopped, the intracellular ATP and the adenylate pool began to decrease, returning sometimes to the initial level in 2–3 h. © 1999 Academic Press

Keywords: adenosine, ATP, adenylate pool, energy charge, human erythrocytes

INTRODUCTION

The study of interaction between energy and adenylate metabolism is of particular interest for understanding how the intracellular ATP level is controlled. Energy metabolism feeds ATP-ADP turnover and determines the energy charge of a cell. Adenylate metabolism involves adenylate nucleotide synthesis and degradation and determines the adenylate pool value and, hence, the absolute value of intracellular ATP concentration. The possible interrelationships between energy and adenylate metabolisms in various tissues and

cells are discussed in several reports [1–6]. Human erythrocytes, owing to their simplicity and availability, are a convenient object for studying the relationship between these two metabolisms. Regulation of energy metabolism has been extensively studied in erythrocytes. This is not the case for adenylate metabolism. Not only its regulation, but even the role in erythrocyte function is as yet obscure. In erythrocytes, adenosine is usually used as a substrate for adenylate synthesis. Within an erythrocyte, adenosine can be either phosphorylated to AMP by adenosine kinase, or deaminated to inosine by adenosine deaminase (Fig. 1) [7–10]. Due to the presence of high adenylate kinase

Research Center for Hematology of RAMS, Moscow, 125167 Russia.

Reprint requests to: Dr. Victor M. Vitvitsky, Research Center for Hematology of RAMS, Moscow, 125167 Russia. Fax: (7–095) 212–88–70. E-mail: victor@blood.ru.



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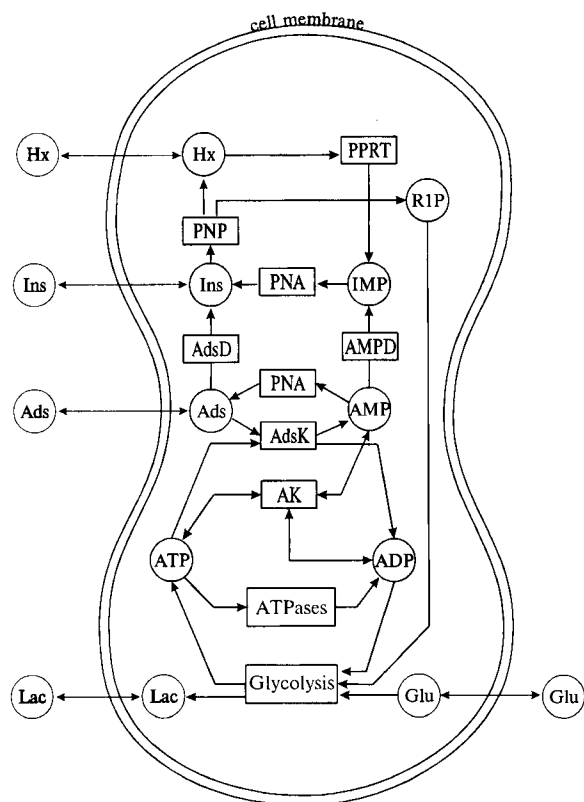


Figure 1. Scheme of the relationship between energy metabolism and metabolism of adenosine in human erythrocytes. Designations: Ads, adenosine; AMP, adenosine monophosphate; ADP, adenosine diphosphate; ATP, adenosine triphosphate; Glu, glucose; Hx, hypoxanthine; IMP, inosine monophosphate; Ins, inosine; Lac, lactic acid; R1P, ribose-1-phosphate; AK, adenylate kinase; AdSK, adenosine kinase; AdSD, adenosine deaminase; AMPD, AMP deaminase; PNA, purine-5'-nucleotidase; PNP, purine nucleoside phosphorilase; PPRT, purine phosphorybpsi transferase; ATPases, sum of ATP consuming processes.

activity in erythrocytes [11, 12], AMP is rapidly phosphorylated to ATP.

Because of the low normal values of the rates of adenylate synthesis and degradation (0.05–0.1 mmol/l cells h) [7, 13–19], long-term (on the order of several hours) incubations of erythrocytes in the presence of adenosine are required to reveal its effects on ATP or other components of the adenylate pool. However, because of the rapid deamination of adenosine by adenosine deaminase, which is highly active in erythrocytes, the long-term maintenance of adenosine at the level sufficient for adenylate synthesis is a difficult problem [15, 19–21]. Therefore, numerous studies of adenylate metabolism are confined to so short time intervals

(on the order of several minutes) that the intracellular concentrations of adenylates remain unchanged and only the use of radioactive labels reveal changes in the metabolic fluxes [13–15, 20, 21]. It is possible to keep adenosine at the level sufficient for adenylate synthesis in erythrocytes by either adding specific inhibitors of adenosine deaminase [13–15, 19] or providing continuous input of adenosine [16, 22]. In [22], the quasistationary adenosine concentrations in the range from 1 to 3 mmol/l suspension were maintained by continuously delivering adenosine into erythrocyte suspensions at rates from 0.25 to 1.0 mmol/l cells h for 4 h. During this interval, the increasing ATP concentration did not reach a constant level. No data of how adenosine affects other components of the adenylate pool were presented [22].

The data accumulated so far on the adenylate metabolism in erythrocytes are still relatively incomplete in some important aspects. It remains unclear how the intracellular adenylate concentrations depend on the supply of cells with adenosine. Also unclear is the kinetics of the intracellular adenylate concentrations after cessation of the treatment stimulating the adenylate synthesis. Even the factors that determine the adenylate pool value are as yet uncertain. In this study, we tried to clarify, at least in part, these questions. Specifically, we examined the effects of adenosine on the intracellular ATP level, the adenylate pool value

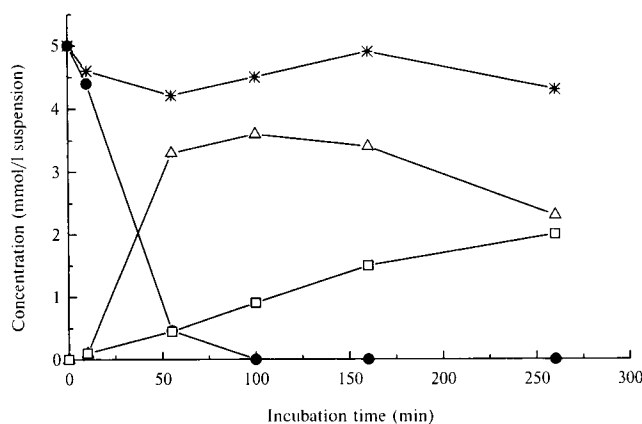


Figure 2. Kinetics of concentration changes in (circles) adenosine, (triangles) inosine, (squares) hypoxanthine, and (asterisks) their sum in a suspension of human erythrocytes (hematocrit value 23.7%) after addition of adenosine to a concentration of 5 mmol/l suspension.

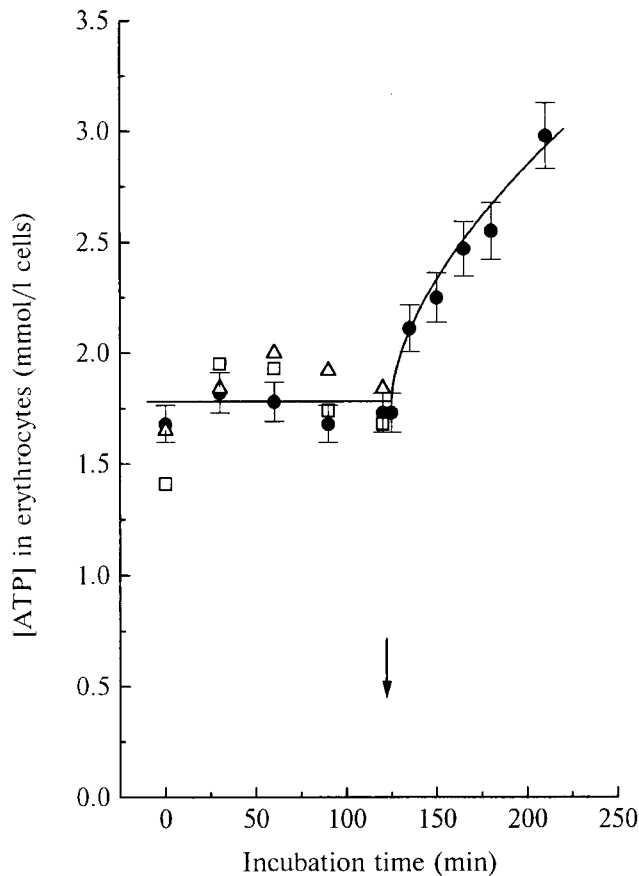


Figure 3. Effects of adenosine on ATP concentration in erythrocytes observed in the presence of orthophosphate at low and high concentrations. Initial concentrations of adenosine were (circles) 0, (triangles) 5, and (squares) 10 mmol/l suspension. The incubation medium contained orthophosphate at a concentration of 1.2 mM. The arrow indicates the moment when orthophosphate (50 mmol/l suspension) and adenosine (10 mmol/l suspension) were added to (circles) the control erythrocytes. Initial hematocrit of the suspension was 7%. The errors shown are experimental errors of the assay for ATP.

([ATP]+[ADP]+[AMP]), and the energy charge ($([ATP]+0.5[ADP])/([ATP]+[ADP]+[AMP])$) during long-term incubations of human erythrocyte suspensions using different routes of adenosine delivery. We also examined how the elevated intracellular concentrations of ATP and other adenylates behaved after the cessation of stimulation of adenylate synthesis.

MATERIALS AND METHODS

Erythrocytes were isolated from freshly collected donor blood, washed with physiological

saline, and resuspended to hematocrit of 10–20% in an incubation medium of the following composition: 135 mM NaCl, 5 mM KCl, 1 mM MgSO₄, 2 mM CaCl₂, 1.2 mM NaH₂PO₄, 2 g/l bovine serum albumin, 5 mM glucose, 24 mM HEPES (pH 7.4). When high concentrations of orthophosphate were required, phosphate buffer was used instead of HEPES-buffered medium. The suspension (from one donor in each experiment) was incubated at 37°C in 5-ml Teflon cells on a shaker. To each cell, either a single dose of adenosine (Sigma, USA) was added, or 0.1–10 mM adenosine dissolved in the incubation medium was continuously pumped with a syringe pump (Orion Res. Inc., USA) at a rate of 3 l/min. The same

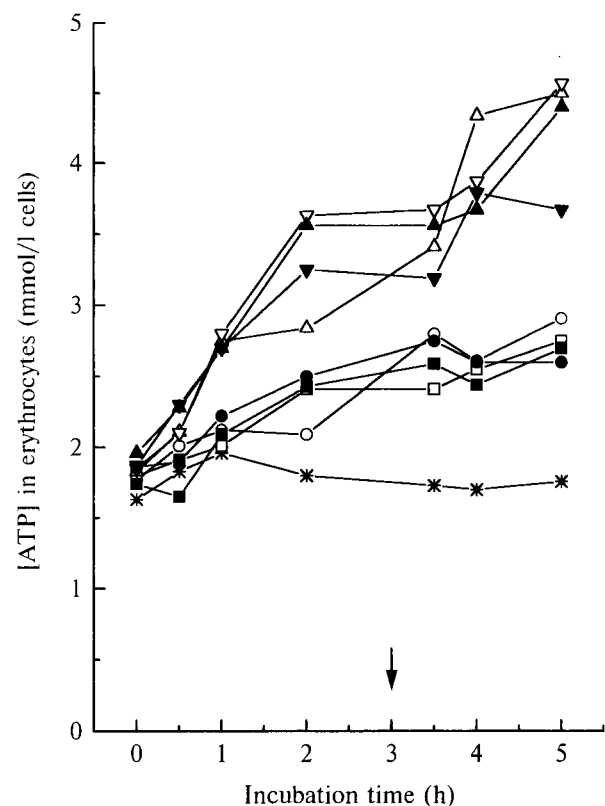


Figure 4. ATP plotted versus incubation time in (asterisks) control erythrocytes and in erythrocytes incubated with orthophosphate and adenosine at different concentrations. Circles and squares indicate data obtained at 25 mM orthophosphate. Up and down triangles indicate data obtained at 50 mM orthophosphate. The initial adenosine concentration was (open symbols) 5 mmol/l suspension and (black symbols) 10 mmol/l suspension. An arrow shows the moment of the second addition of adenosine (open squares and down triangles, 5; black squares and down triangles, 10 mmol/l suspension). The hematocrit value was 8%.

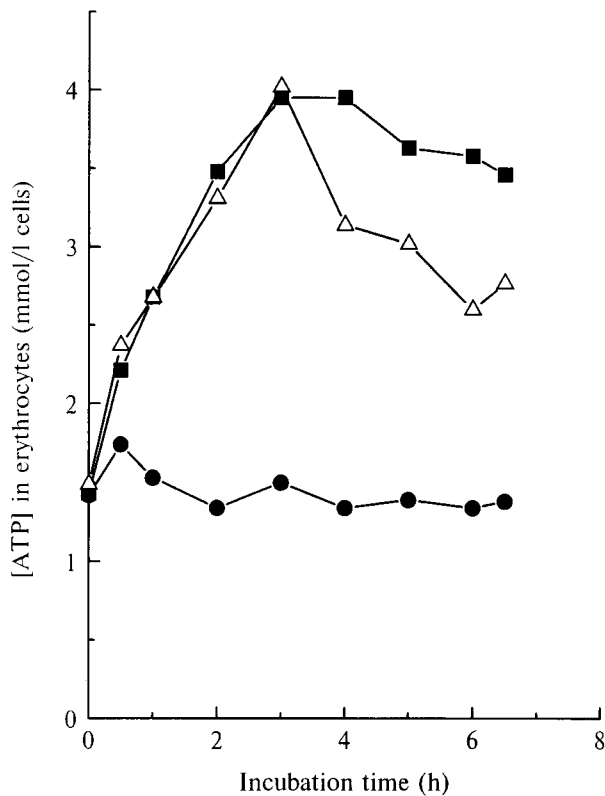


Figure 5. ATP concentration in (circles) control erythrocytes, (squares) erythrocytes that were incubated with 50 mM orthophosphate and adenosine (initial concentration of 10 mmol/l suspension), and (triangles) erythrocytes that were incubated with 50 mM orthophosphate and adenosine (initial concentration of 10 mmol/l suspension) for 3 hours and then transferred into fresh medium containing 1.2 mM orthophosphate and no adenosine. The hematocrit value was 8%.

technique was used to continuously pump inosine into the suspension of erythrocytes. Orthophosphate was added as 1M sodium phosphate buffer, pH 7.4 The inhibitor of adenosine deaminase coformycin [23] (Calbiochem, USA) was prepared as 1 mM aqueous solution and added to the erythrocyte suspension to a final concentration of 4 μ mol/l suspension at the start of incubation. All other additions were made 30 min or later after the incubation began.

During incubation, we sampled the suspension for determination of concentrations of metabolites. To each sample, 0.5 M HClO₄ was added at a 1:4 volume ratio and vigorous vortexing. Ten minutes later, the mixture was centrifuged. The supernatant, after its pH was adjusted to 7.0 with

saturating K₂CO₃, was stored frozen until determinations. ATP was determined by the luciferin-luciferase method either in the perchloric extracts of erythrocytes or their aqueous lysates prepared immediately after sampling [24]. The perchloric extracts were analyzed for nucleotides, nucleosides, and bases by HPLC [6]. The hematocrit values were determined by centrifugation of capillaries filled with erythrocyte suspension on a microhematocrit centrifuge.

RESULTS

In erythrocyte suspensions containing no effectors, adenosine added as a single dose to a final concentration of 0.1 to 10 mmol/l suspension was deaminated rapidly to inosine and hypoxanthine

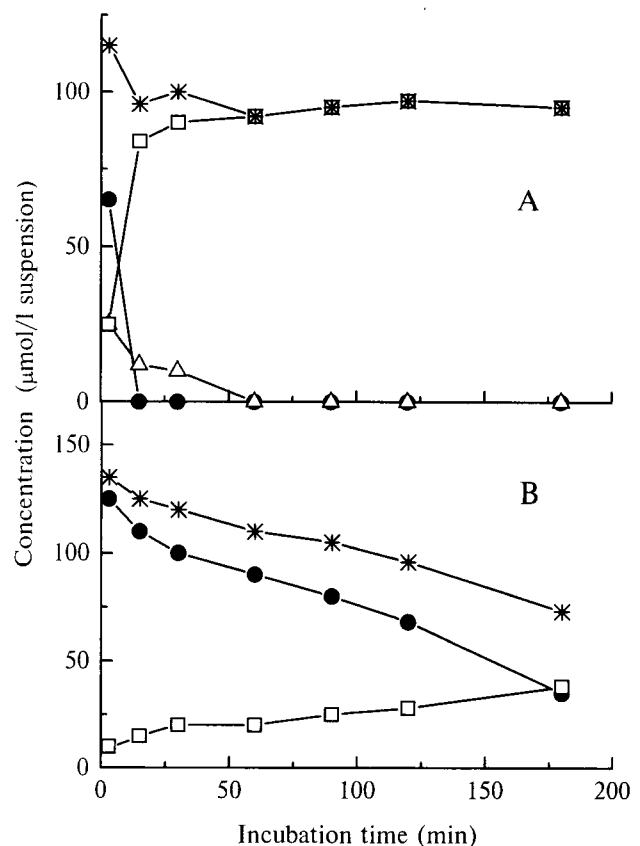


Figure 6. Kinetics of concentration changes in (circles) adenosine, (triangles) inosine, (squares) hypoxanthine, and (asterisks) their sum in a suspension of human erythrocytes after addition of (A) 0.1 mmol/l suspension adenosine and (B) 0.1 mmol/l suspension adenosine and 4 μ mol/l suspension coformycin. The hematocrit value was 11.5%.

Table 1. Effect of Bolus Addition of Adenosine on the ATP and Adenylate Pool Levels in Intact Human Erythrocytes in the Presence of Coformycin (4 $\mu\text{mol/l}$ Suspension)

Experiment number	Incubation time (h)	ATP concentration (M \pm S.D.; mmol/l cells)		Adenylate pool (mmol/l cells)			
		Control	With adenosine	Control		With adenosine	
				Initial	Final	Initial	Final
1	7	1.2 \pm 0.1 (7)	1.3 \pm 0.1 (5)	—	—	—	—
2	7	1.7 \pm 0.1 (5)	1.9 \pm 0.2* (5)	2.45	2.25	2.45	3.50
3	8	1.5 \pm 0.2 (6)	1.7 \pm 0.1* (5)	1.90	2.10	2.00	3.25
4	5.5	1.4 \pm 0.1 (4)	1.47 \pm 0.08 (4)	2.00	1.70	2.20	2.75
5	5	1.37 \pm 0.07 (6)	1.5 \pm 0.2(6)	—	—	1.85	2.40

Note. ATP was determined several times during incubation (shown in parentheses). Adenosine concentration was 0.8 mmol/l suspension in experiment No. 1 and 0.3 mmol/l suspension in other experiments.

* — significantly different from the control ($p < 0.1$).

(Fig. 2). The sum of adenosine, inosine, and hypoxanthine concentrations remained constant. The initial rate at which adenosine decreased was 10–50 mmol/l cells h and did not depend on its concentration. The rate of hypoxanthine generation from inosine was 2–3 mmol/l cells h. ATP in erythrocytes did not change even if the initial concentrations of adenosine were high. (Fig. 3).

ATP increased significantly in response to a single addition of adenosine in the presence of high concentrations of orthophosphate (Figs. 3–5). If the initial concentration of adenosine was higher than 5 mmol/l suspension, the level to which ATP increased depended only on the concentration of orthophosphate and was not affected by adenosine concentrations (Fig. 4). After the concentration of orthophosphate was returned to its normal value, ATP in erythrocytes began to decrease (Fig. 5).

In the presence of coformycin, the rate of adenosine utilisation by erythrocytes decreased sharply to about 0.2–0.3 mmol/l cells h. Hypoxanthine accumulated at even lower rates. Inosine remained undetectable (Fig. 6). The adenylate pool and ATP in erythrocytes increased (Table 1). The mean rate at which the adenylate pool increased throughout the experiment ranged from 0.10 to 0.16 mmol/l cells h in different experi-

ments. ATP also increased, but at a lower rate. This resulted in a decrease in the energy charge of erythrocytes (Table 2).

Adenosine continuously pumped into erythrocyte suspensions at rates of 0.02–5.0 mmol/l cells h was rapidly utilised by erythrocytes. Its concentration increased to a level not higher than 5 $\mu\text{mol/l}$ suspension. Inosine and hypoxanthine accumulated in the suspension. The rate of hypoxanthine formation was proportional to the input rate of adenosine. Inosine accumulated at detectable rates only at the highest input rates of adenosine (Fig. 7). The adenylate pool and ATP in erythrocytes increased at similar rates (Fig. 8). The dependence of the rate at which the adenylate pool increased (y , mmol/l cells h) on the input rate of adenosine (x , mmol/l cells h) can be approximated closely with a hyperbolic function $y = \alpha x / (x + k)$, where $\alpha = 0.415$ mmol/l cells h and $k = 1.0$ mmol/l cells h (Fig. 9). In our experiments with constant input of adenosine, the maximum rate at which the adenylate pool increased was 0.35 mmol/l cells h (Fig. 10). This value is twice as large as the value observed in the presence of coformycin. The energy charge of erythrocytes was higher significantly in suspensions where adenosine was pumped continuously than in control suspensions (Table 2). After adenosine pumping was stopped, the

Table 2. Effects of Adenosine on the Energy Charge of Human Erythrocytes

Parameter	Control	Bolus addition of adenosine (0.1–0.8 mmol/l suspension) and coformycin (4 μ mol/l suspension)	Continuous adenosine input (1.72–5.00 mmol/l cells h)
Energy charge ($M \pm S.D.$)	0.86 \pm 0.05	0.82 \pm 0.06	0.91 \pm 0.03
Number of experiments	9	5	4
Total number of determinations	28	18	18
Significance vs. control	—	<0.02	<0.001

Note. Erythrocytes of each donor studied were incubated for several hours under the conditions indicated. The energy charge was determined at various times and averaged.

intracellular ATP and the adenylate pool began to decrease rapidly and sometimes returned to the initial level in 2–3 h.

Interestingly, continuous input of adenosine did not cause any significant increase in ATP if the suspension contained coformycin (Fig. 11). However, if adenosine was pumped along with inosine into the coformycin-containing suspension, ATP increased to the level observed in the absence of coformycin in the suspension with the equivalent input of adenosine (Fig. 11). When pumped into the suspension continuously, inosine alone did not change intracellular ATP significantly.

DISCUSSION

The results of this study demonstrated that the response of the adenylate pool and ATP concentration in erythrocytes to addition of exogenous adenosine depended on the way of its delivery.

Under physiological conditions, adenosine added as a single dose into erythrocyte suspension is rapidly deaminated. The rate of its degradation coincides with the literature data on adenosine deaminase activity of human erythrocytes [12, 25–27]. Therefore, even when the initial concentrations of adenosine in suspensions were high, it diminished to the undetectable level within 1–2 h. This interval seems to be insufficient for synthesis of appreciable amounts of ATP from adenosine, especially because substrate inhibition of adenosine kinase could decrease significantly the rate of adenosine incorporation into adenylates at high adenosine concentrations [13, 15, 28].

For erythrocytes, the stimulation of ATP synthesis from adenosine in the presence of orthophosphate is a well-known phenomenon [14, 15, 22, 29, 30]. Adenosine deamination in erythrocytes is insensitive to orthophosphate [14, 15, 20]; therefore, it is likely that orthophosphate plays an

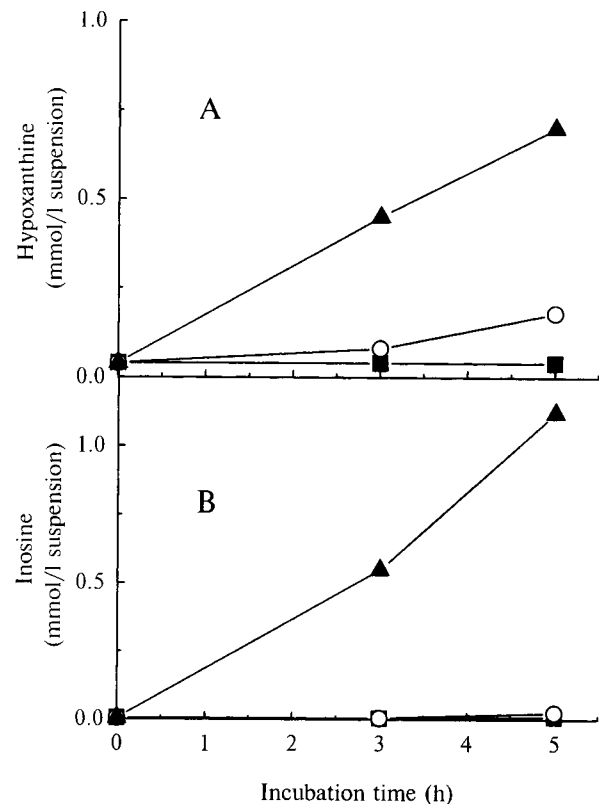


Figure 7. Changes in (A) hypoxanthine and (B) inosine in erythrocyte suspension supplemented with adenosine at a constant input rate of (squares) 0.02, (circles) 0.23, and (triangles) 2.3 mmol/l cells h. The hematocrit value was 13%.

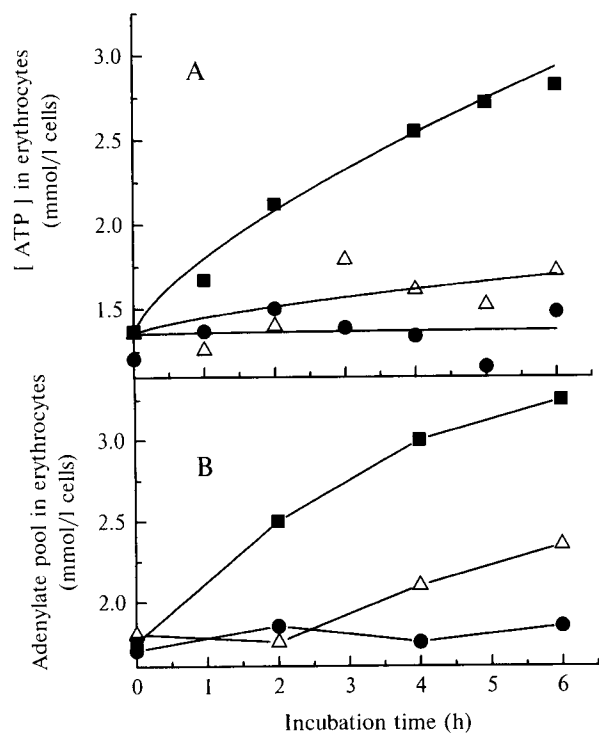


Figure 8. Changes in (A) intracellular ATP and (B) the adenylate pool (circles) in control erythrocytes and in erythrocytes supplemented with adenosine at a constant input rate of (triangles) 0.26 and (squares) 2.6 mmol/l cell h.

effector role towards enzymes involved in synthesis and degradation of adenylates. As known from the literature, orthophosphate inhibits purine-5'-nucleotidase and AMP deaminase [31–33]. The activation of adenosine kinase in the presence of orthophosphate is also possible [15]. Interestingly, orthophosphate concentration has to be high not only in order to increase ATP, but also to maintain it at the high level, because the ATP concentration returned to its normal value after orthophosphate in the medium was decreased to its basal level (Fig. 5).

It is of interest that continuous adenosine input into cell suspension or its bolus injection to the suspension containing cofomycin (an inhibitor of adenosine deaminase), exerted different effects on the ATP concentration and adenylate pool in erythrocytes. In the presence of cofomycin, a slight increase in ATP and a slow increase in the adenylate pool were observed. The rate at which the adenylate pool increased in our experiments is

in a good agreement with the values of rates of adenosine incorporation into synthesis of adenylates observed in short-term experiments with cofomycin [14, 15]. A decrease in the energy charge of cells observed in the presence of cofomycin suggests that the synthesis of adenylates from adenosine presents a significant burden for energy metabolism of erythrocytes. Under conditions of continuous input of adenosine, the rates at which the adenylate pool and intracellular ATP increased and the levels that they reached were much higher than those observed in the presence of cofomycin, and the energy charge of cells increased. It is conceivable that an increase in the energy charge is accounted for by the formation of ribose-1-phosphate from inosine in adenosine degradation to hypoxanthine (Fig. 1). In turn, ribose-1-phosphate can be incorporated into glycolysis through reactions of the pentose phosphate pathway and thereby increase the rate of glycolytic

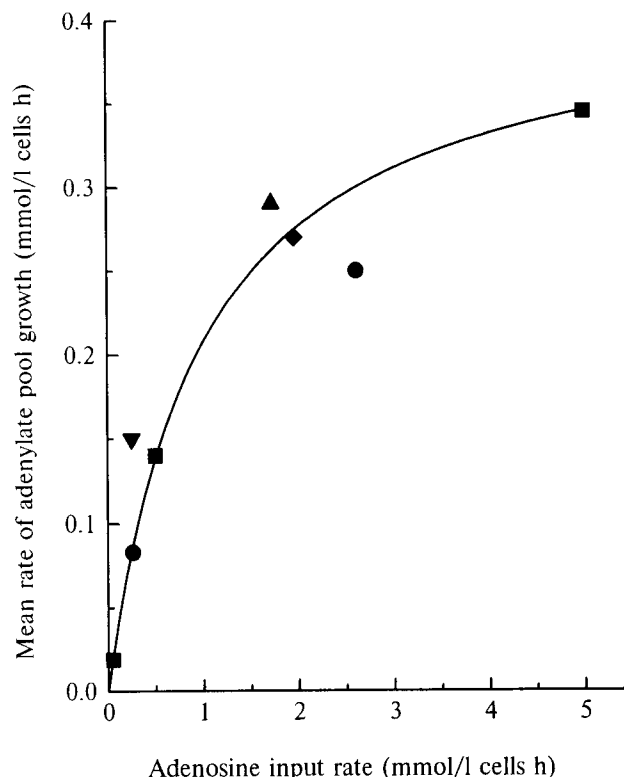


Figure 9. Mean rate of the adenylate pool growth (y) in erythrocytes as a function of the adenosine input rate (x). Different symbols indicate different donors. The best fit to these data is $y = \alpha x / (x + k)$, where $\alpha = 0.415$ (mmol/l cells h) and $k = 1.0$ (mmol/l cells h).

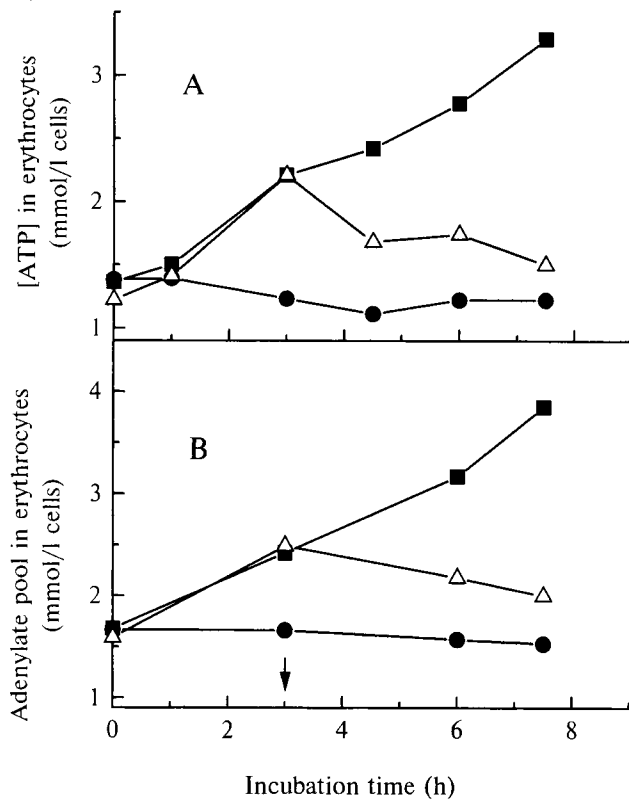


Figure 10. Changes in (A) intracellular ATP and (B) the adenylate pool (circles) in control erythrocytes, (squares) erythrocytes supplemented with adenosine at a constant input rate of 1.72 mmol/l cells h throughout the experiment, and (triangles) erythrocytes supplemented with adenosine at a constant input rate of 1.72 mmol/l cells h up to the moment indicated by an arrow.

reproduction of ATP from ADP in cells. The relationship between the rates of the increase in the adenylate pool and adenosine input (Fig. 9) seems to reflect the dependence of the rate of adenosine kinase reaction on the adenosine concentration (which is proportional to its input rate [22]). Under conditions of continuous input of adenosine into erythrocyte suspensions, coformycin inhibited both adenosine deaminase and synthesis of ATP from adenosine. Synthesis or degradation of nucleotides can hardly be the target of coformycin, because if adenosine was pumped along with inosine into coformycin-containing suspensions, ATP increased to the level observed in suspensions without coformycin where only adenosine was pumped (Fig. 11). It is more plausible that the metabolites formed upon adeno-

sine deamination (inosine, hypoxanthine, and/or inosine-5'-monophosphate) either stimulate the incorporation of adenosine into adenylates or inhibit the degradation of adenylates in erythrocytes.

Our data demonstrate that after cessation of stimulation of adenylate synthesis, both adenylate pool and ATP concentration returned to their initial levels. This fact suggests that normal erythrocytes keep the adenylate pool at a fixed level. This regulation seems to be disrupted in the presence of orthophosphate at high concentrations

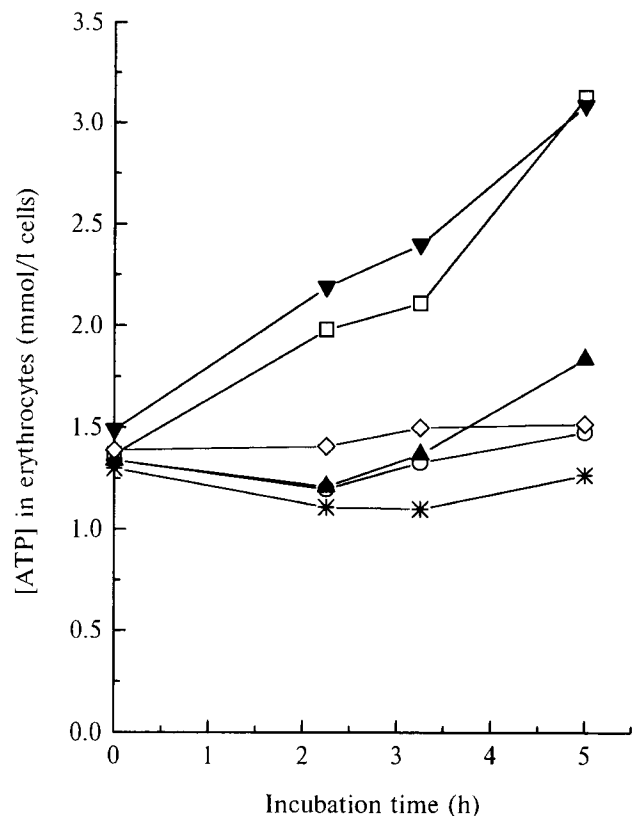


Figure 11. Effect of coformycin on the synthesis of ATP from adenosine in human erythrocytes: (asterisks) control, (circles) 0.3 mmol/l suspension adenosine added at zero time to the suspension containing 4 μmol/l suspension coformycin, (squares) adenosine input at a rate of 2.6 mmol/l cells h, (up triangles) adenosine input at a rate of 2.6 mmol/l cells h to the suspension containing 4 μmol/l suspension coformycin, (down triangles) simultaneous adenosine and inosine inputs, each at a rate of 2.6 mmol/l cells h, to the suspension containing 4 μmol/l suspension coformycin, (diamonds) inosine input at a rate of 2.6 mmol/l cells h. The hematocrit value was 17.3%.

and/or added adenosine and tends to recover after removing of the challenging factors.

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