

Energy-Dependent Processes and Adenylate Metabolism in Human Erythrocytes

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Amphotericin B (1-3 mg/liter) decreases ATP content in erythrocytes by 11-26% and stimulates K^+ efflux without any effect on the adenylate pool. Adenosine added to the erythrocyte suspension increases the adenylate pool, maintains a high level of intracellular ATP during 6-8 h of incubation, and attenuates the amphotericin B-induced K^+ efflux. Incubation of erythrocytes without glucose for 4-5 h decreases the ATP concentration to 20-50% of the initial value and is accompanied by a significant reduction in the adenylate pool. Subsequent glucose addition partially restores the ATP level. In the presence of adenosine, the ATP concentration nearly reaches its initial value due to the increase in the adenylate pool.

KEY WORDS: erythrocyte, ATP, adenylate pool, energy charge, adenosine, amphotericin B, energy depletion, potassium.

The concentration of ATP in a cell is determined by the interaction between the energy metabolism, providing ATP synthesis from ADP and P_i , and adenylate metabolism, which affects the pools of ATP, ADP, and AMP. Human erythrocytes are a suitable model for the investigation of the interaction of these metabolic systems. In these cells glycolysis is the only system synthesizing ATP. Since nucleic acids are absent from mammalian erythrocytes, adenylate metabolism in human erythrocytes is not related to the synthesis and degradation of nucleic acids, but it does include AMP synthesis from adenine or adenosine and its degradation to inosine and hypoxanthine [1-5]. Interconversions between ATP, ADP, and AMP occur via the adenylate kinase reaction, which is catalyzed by a highly active adenylate kinase [6, 7]. Adenylate metabolism in erythrocytes may be mainly directed to the regulation of intracellular adenylate concentrations, especially ATP. However, its role and regulatory mechanisms in human erythrocytes are not completely clear.

Mathematic modelling suggests [8, 9] that during changes in ATP-consuming processes adenylate metabolism may stabilize the energy charge of the cell ($[ATP] +$

$0.5[ADP])/([ATP] + [ADP] + [AMP])$). When ATP-consuming processes are activated the stabilization of the energy charge can be achieved via a decrease in the adenylate pool. Theoretical predictions of modelling studies concerning the interactions between energy metabolism and adenylate metabolism in erythrocytes have not yet been experimentally tested.

In the present study we have investigated the influence of adenylate metabolism on the energy state of erythrocytes. Adenosine was used as the substrate for synthesis of adenylates. It readily penetrates into the cell [10-12] and takes part in AMP synthesis via the adenylate kinase reaction [1, 12, 13]. Activation of ATP-consuming processes and inhibition of ATP synthesis in glycolysis were used to change the energy states of the erythrocytes. Activation of ATP utilization was initiated by adding the polyenic antibiotic amphotericin B to the erythrocyte suspension. Amphotericin B in a concentration-dependent manner induces various effects in cells, including an increase in cellular membrane permeability for ions and low-molecular-weight compounds, an increase in cellular volume, and lysis of cells [14-17]. However, at low concentrations (a few mg/liter) amphotericin B-induced nonselective increase of cell membrane permeability for monovalent cations is fairly specific [16, 17]. It is accompanied by a ion imbalance followed by the subsequent activation of transport Na^+/K^+ -ATPase, decrease in ATP level, and activation of glycolysis [17-20]. The inhibition of glycolytic ATP synthesis was achieved by energy

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depletion of erythrocytes in the absence of glucose. Long-term incubation of erythrocytes without glucose decreases their ATP concentration and adenylate pool with a concomitant increase in ADP and AMP concentrations [21, 22].

MATERIALS AND METHODS

Blood from healthy volunteers was used in the experiments. A standard anticoagulant (Glugycir, 1 ml per 4 ml blood) was added to prevent coagulation. Samples were centrifuged for 10 min at 1,500g and plasma with the upper layer of cells containing leukocytes and platelets were decanted. The remaining erythrocyte mass was washed two-to-four times with three volumes of 0.9% NaCl solution by centrifuging for 10 min at 1,500g.

Washed erythrocytes were resuspended in incubation medium containing 135 mM NaCl, 3 mM KCl, 1 mM MgSO₄, 2 mM CaCl₂, 1.2 mM NaH₂PO₄, 2 g/liter BSA, 24 mM HEPES, pH 7.4.

Erythrocyte suspensions (hematocrit 10-20%) were incubated in teflon cells at 37°C with constant stirring for 6-8 h. For each experiment erythrocytes from one donor were used and they were incubated simultaneously in different cells.

Glucose powder was added to the erythrocytes to final concentration 5 mM at the beginning of the incubation (except in experiments on erythrocyte depletion). Adenosine and amphotericin B were added after preincubation for 30-40 min. In the experiments with energy-depleted erythrocytes, glucose and adenosine were added simultaneously after a 50% decrease in the ATP level in the erythrocytes. Adenosine deamination in erythrocytes was prevented by adding aqueous 1 mM solution of coformycin (Calbiochem, USA) to final concentration 4 μM [23, 24]. Coformycin penetrates the cell membrane slower than adenosine [25]: it was added 30-40 min before the adenosine. Adenosine (Sigma, USA) was added to the erythrocyte suspension as 10 mM solution in 0.9% NaCl. Amphotericin B (Sigma) initially dissolved in dimethylsulfoxide (0.5-2.0 g/liter) was added to the erythrocyte suspension to the final concentration of 1-3 mg/liter.

During incubation of the erythrocyte suspensions, aliquots were taken for the determination of metabolite concentrations. They were treated with four volumes of 0.5 M HClO₄ added with mixing. After 10 min the mixture was centrifuged for 10 min at 1,500g. The supernatant was neutralized with saturated solution of K₂CO₃ to pH 7 and kept frozen.

ATP concentration in these extracts was determined by a modification of the luciferin-luciferase method [26]. In some experiments ATP content was determined in aliquots immediately, without fixation with HClO₄. In

this case the erythrocyte suspension was mixed with ten volumes of distilled water, and ATP concentration was determined within 1-2 min in the lysate [26].

Adenosine, inosine, and hypoxanthine contents in the extracts were determined by HPLC on a column (4.6 × 250 mm) packed with Zorbax ODS sorbent (DuPont, USA) using isocratic elution with 0.2 M potassium phosphate pH 4 as eluent at flow rate 2 ml/min. Samples were injected in a volume of 100 μl and eluted during 30 min. The elution of nucleosides and the nitrogenous bases was detected at 254 nm. The identity of peaks and the concentration of individual components of the mixture were determined using calibration plots obtained using mixtures of nucleosides and nitrogenous bases of known concentrations.

Individual components of the adenylate pool were determined in the perchloric extracts by the HPLC method using the same type of column pretreated with the ion-pair reagent dodecyltrimethylammonium bromide (DTAB). A new column was washed with 0.05 M potassium phosphate pH 6.0 (solution A) for 30 min at flow rate 2 ml/min and then with the same solution containing 0.5 mM DTAB for 10 h at flow rate 0.2 ml/min. Such DTAB-treated columns act as ion-exchangers and can be used without loss of stability for at least two months without any additional treatment. Before the beginning of an analysis the column is equilibrated with solution A at flow rate 2 ml/min. Using an injector, 20-100 μl of the analyzed extract is applied onto the column. Nucleotides are eluted from the column at flow rate 2 ml/min using a linear gradient from solution A to solution B (0.7 M potassium phosphate, pH 6.0) during 30 min, then 100% solution B for 10 min. The column is equilibrated again with solution A for 20 min at flow rate 2 ml/min. Eluted nucleotides are monitored by the absorbance at 254 nm. The identity of peaks and the concentration of individual components of the mixture are determined using calibration plots obtained using mixtures of nucleotides of known concentrations.

For the determination of extracellular K⁺, the aliquots of erythrocyte suspension were centrifuged at 1,500g for 10 min. The supernatant was either kept frozen until determination or was immediately analyzed using a flame photometer.

Hemolysis was determined spectrophotometrically by the release of hemoglobin from the erythrocytes into the medium. The relative hemoglobin concentration in the incubation medium was determined as the ratio of optical densities of the incubation medium and aqueous lysate at 415 nm under equal dilution.

The results were treated using standard statistical methods [27].

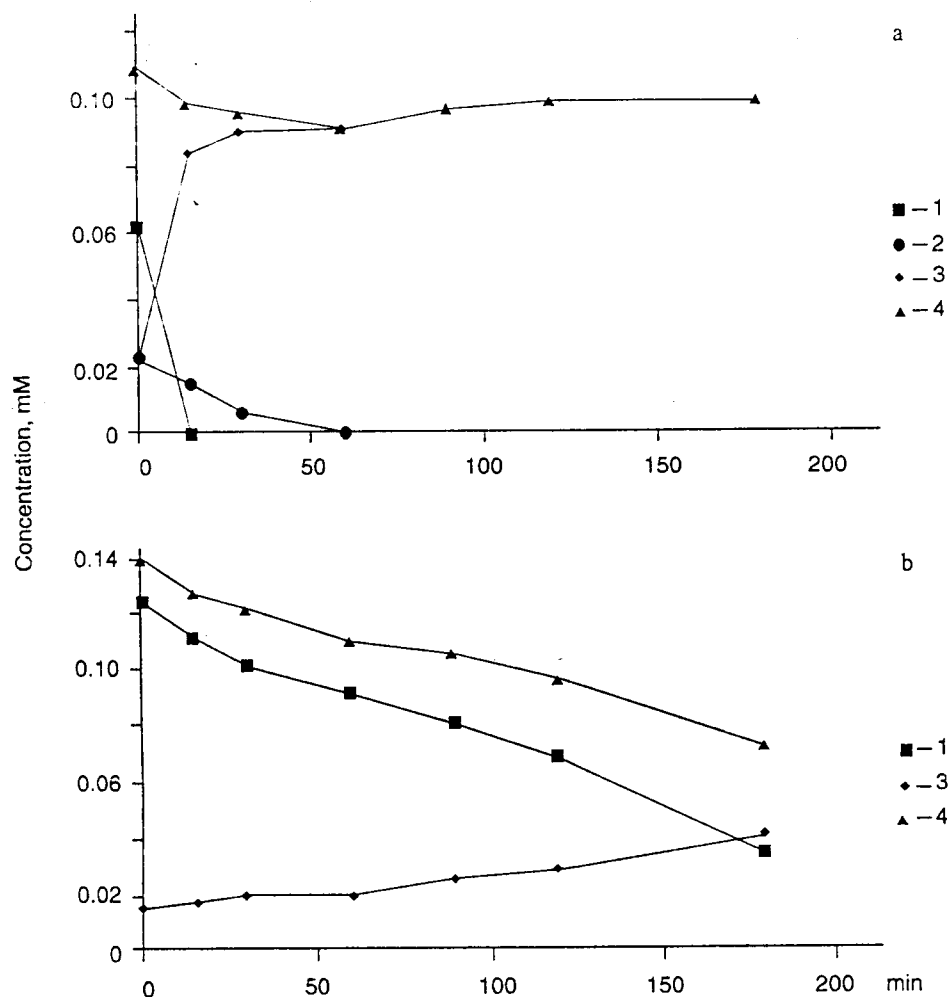


Fig. 1. Influence of adenosine (0.1 mM) added to a suspension of native erythrocytes without (a) and with 4 μ M coformycin (b) on adenosine, inosine, and hypoxanthine concentrations: 1) adenosine; 2) inosine; 3) hypoxanthine; 4) sum of adenosine, inosine, and hypoxanthine concentrations. Hematocrit of the erythrocyte suspension was 11.5%.

RESULTS

Influence of Adenosine on Adenylate Pool and ATP Level in Intact Erythrocytes. Adenosine added to an erythrocyte suspension is readily deaminated with the formation of inosine and hypoxanthine [1, 12]. At adenosine concentrations less than 1 mM its level in the suspension could be maintained for a long time only in the presence of the adenosine deaminase inhibitor coformycin. Under these conditions a constant decrease in adenosine concentration (~ 0.2 - 0.3 mmole/liter of cells per hour) is observed. The rate of hypoxanthine accumulation is significantly lower and inosine is not detected (Fig. 1). The decrease in adenosine concentration observed in the presence of coformycin is accompanied by an insignificant increase in ATP concentration (Table 1, Fig. 2a) and augmentation of the adenylate pool in the erythrocytes (Table 2).

Influence of Adenosine on Adenylate Pool and ATP Level in Amphotericin B-Treated Erythrocytes. Preliminary experiments revealed that amphotericin B (AMB) (1-3 mg/liter) stimulates K^+ efflux from erythrocytes and decreases the ATP concentration in them (Fig. 2, Table 1). The volume of the erythrocytes remained unchanged and hemolysis was less than 1%. Increased K^+ efflux and decreased ATP concentration were observed over several hours of incubation and the adenylate pool remained unchanged. Addition of adenosine compensates the AMB-induced decrease in ATP level in erythrocytes (Fig. 2, Table 1). This compensation was provided by the increase in the adenylate pool (Table 2). The rate of adenosine utilization in AMB-treated erythrocyte suspensions did not differ from that of intact erythrocytes (Fig. 3). This is related to the same increase in the adenylate pool in both cases (Table 2).

TABLE 1. Influence of Adenosine on ATP Content in Intact and Amphotericin B-Treated Erythrocytes^a

Experiment number	Steady-state ATP concentration, mmole/liter cells			
	Control	With adenosine	With amphotericin B	With adenosine and amphotericin B
1	1.7 ± 0.1 (5)	1.9 ± 0.2 ^c (5)	1.51 ± 0.08 ^b (4)	1.86 ± 0.08 ^b (5)
2	1.5 ± 0.2 (6)	1.7 ± 0.1 ^c (5)	1.15 ± 0.05 ^b (6)	1.7 ± 0.2 (5)
3	1.4 ± 0.1 (4)	1.47 ± 0.08 (4)	1.04 ± 0.05 ^b (4)	1.24 ± 0.07 ^b (4)
4	1.37 ± 0.07 (6)	1.5 ± 0.2 (6)	1.2 ± 0.1 ^b (6)	1.3 ± 0.1 (6)
5	1.2 ± 0.1 (7)	1.3 ± 0.1 (5)	1.0 ± 0.1 ^b (7)	1.3 ± 0.1 (7)

^aAdenosine concentration was 0.8 mM in experiment No. 5 and 0.3 mM in other experiments. Amphotericin B concentration was 1 mg/liter in experiment No. 4 and 3 mg/liter in other experiments. Data represent means and standard deviations. Number of observations are given in brackets.

^bData differ from control with probability $\alpha < 0.05$.

^cData differ from control with probability $\alpha < 0.1$. Other data do not differ from control ($\alpha > 0.1$).

TABLE 2. Influence of Adenosine on Adenylate Pool in Intact and Amphotericin B-Treated Erythrocytes^a

Experiment number	Incubation time, h	Adenylate pool, mmole/liter cells							
		Control		With adenosine		With amphotericin B		With adenosine and amphotericin B	
		initial	final	initial	final	initial	final	initial	final
1	7	2.45	2.25	2.45	3.50	2.45	2.30	2.45	3.10
2	8	1.90	2.10	2.00	3.25	1.90	2.10	1.80	2.60
3	5.5	2.00	1.70	2.20	2.75	2.10	1.60	1.85	2.45
4	5	-	-	1.85	2.40	1.85	1.85	1.85	2.35

^aData represent initial and final levels of adenylate pool obtained during incubations. Error of determinations did not exceed 10%. Numbers of experiments here correspond to those listed in the Table 1.

TABLE 3. Influence of Adenosine and Amphotericin B on Erythrocyte Energy Charge^a

Conditions	Energy charge	Data differ from control with probability
Control	0.86 ± 0.05 (n = 13)	
With adenosine	0.82 ± 0.06 (n = 18)	< 0.1
With amphotericin B	0.78 ± 0.04 (n = 11)	< 0.001
With adenosine and amphotericin B	0.76 ± 0.05 (n = 12)	< 0.001

^aData represent means and standard deviations of experiments listed in Table 2, including not only initial and final, but also intermediary means. Number of observations is given in brackets.

In the presence of adenosine a decrease in AMB-induced efflux from erythrocytes was observed (Fig. 2b).

Influence of Adenosine and Amphotericin B on the Energy Charge of Erythrocytes. In control erythrocytes the energy charge remains unchanged over the whole experiment. Addition of both AMP and adenosine decreases the energy charge. The combination of AMB and adenosine has an additive effect on the energy change (Table 3).

Influence of Adenosine on ATP Content and Adenylate Pool in Energy-Depleted Erythrocytes. Incubation of erythrocytes without glucose during 4-5 h decreased ATP concentration in them to 20-50% of the initial level. Energy charge decreased by 30-35%. Glucose addition partially restored ATP level during 2-3 h. In the presence of adenosine the increase in ATP concentration is more pronounced, almost reaching the initial values (Fig. 4). Coformycin addition to depleted erythrocytes did not

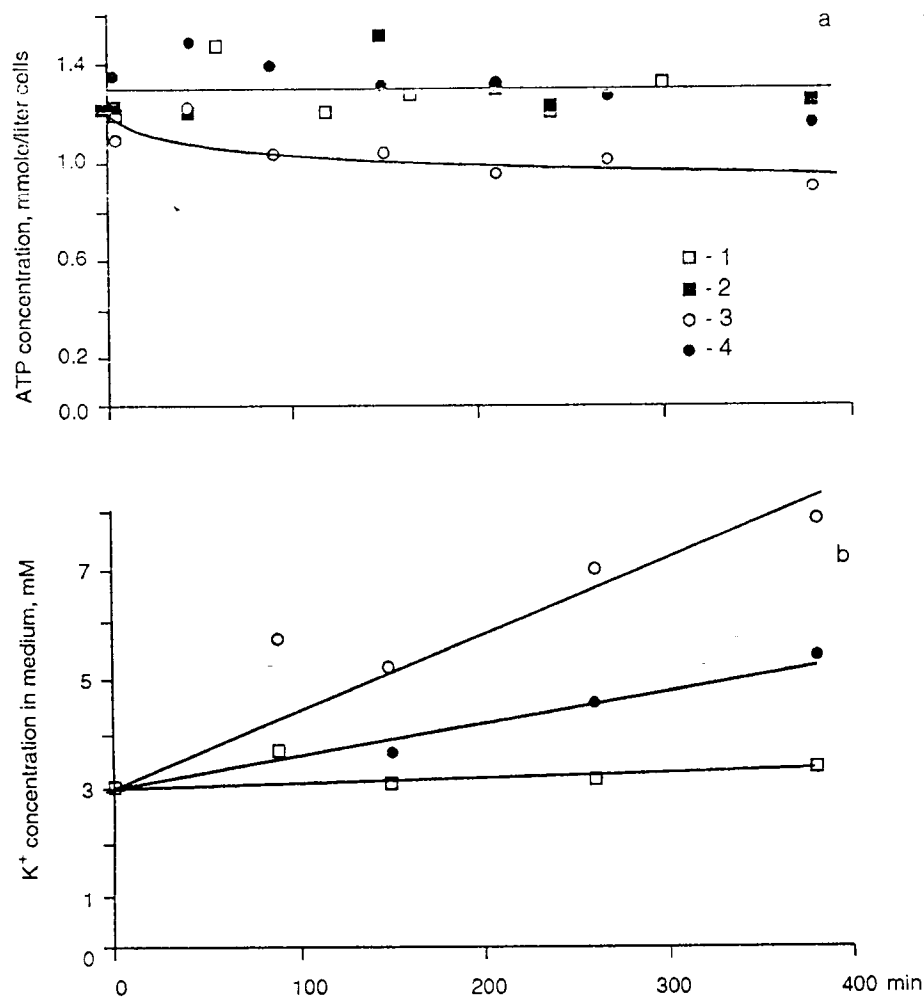


Fig. 2. Influence of adenosine on ATP level (a) and K⁺ efflux from erythrocytes (b) in the presence of amphotericin B: 1) control; 2) adenosine (0.8 mM); 3) amphotericin B (3 mg/liter); 4) amphotericin B (3 mg/liter) and adenosine (0.8 mM). Hematocrit of the suspension was 15.9%.

TABLE 4. Influence of Adenosine on Adenylate Pool (a) and Energy Charge (φ) after Glucose Addition to Depleted Erythrocytes^a

	Control	Initial level at time of glucose addition	Final level after glucose addition without adenosine	Final level after glucose addition with adenosine
a	2.35	1.85	1.63	1.97
φ	0.90	0.61	0.88	0.88
a	2.45	1.75	1.75	2.10
φ	0.78	0.58	0.80	0.84
a	1.90	1.23	1.70	1.83
φ	0.89	0.59	0.89	0.90

^aError of determination did not exceed 10%.

influence the restoration of ATP level in them. After glucose addition the ATP level in depleted erythrocytes with and without cofornycin reached the same mean, which is lower than that obtained in the presence of

adenosine. Results of experiments on the influence of adenosine on the adenylate pool in depleted erythrocytes are shown in the Table 4. In the presence of adenosine, the adenylate pool increases as in intact and AMB-treated

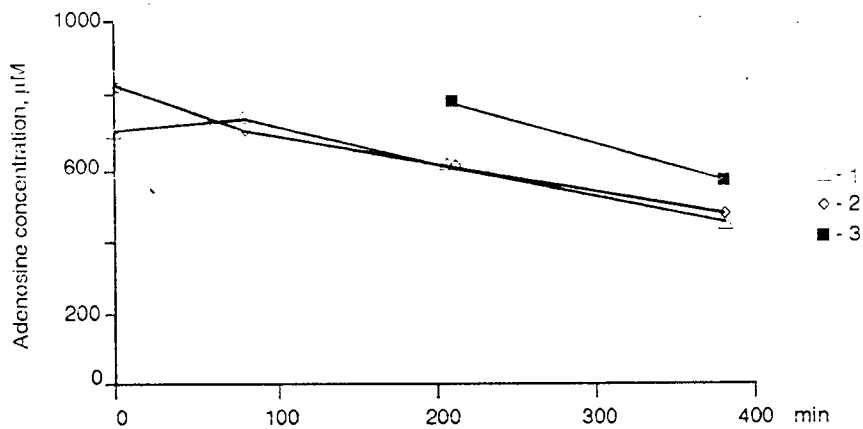


Fig. 3. Influence of coformycin on adenosine concentration in intact (1), amphotericin B-treated (2), and energy-depleted (3) erythrocytes. Initial adenosine concentration in all experiments was 0.8 mM. Concentration of amphotericin B was 3 mg/liter.

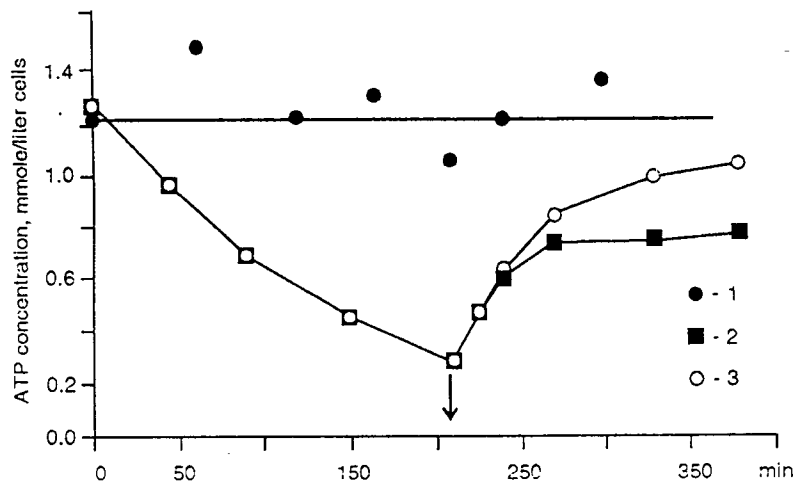


Fig. 4. Influence of glucose and adenosine on ATP level in energy-depleted erythrocytes: 1) control; 2, 3) erythrocyte preincubation without glucose. Arrow indicates addition of glucose or glucose with adenosine (0.8 mM), respectively. Hematocrit of the erythrocyte suspension was 15.9%.

erythrocytes. The restoration of ATP level in depleted erythrocytes is coupled to the rapid restoration of the energy charge to the control mean (Table 4).

DISCUSSION

The results of the present report demonstrate that intact erythrocytes maintain constant ATP concentration and adenylate pool over several hours of incubation *in vivo*. Decrease in the energy charge and ATP level in erythrocytes after treatment with AMB can be explained by the activation of transport ATPases as a consequence of the AMB-induced ion imbalance observed in cells [17-20]. However, the adenylate pool remains unchanged. Decrease in ATP concentration and energy charge is more

pronounced in energy-depleted erythrocytes and is coupled to significant decrease in the adenylate pool. This suggests that a significant decrease in ATP level is required for a decrease in the adenylate pool. Other possibility is that the rate of decrease in the adenylate pool is regulated by some factors which are differently changed under activation of ATP-consuming processes and in the energy depleted erythrocytes.

Adenosine addition increases the adenylate pool both in intact erythrocytes and in erythrocytes with activated ATP consumption and also in energy-depleted cells. The involvement of adenosine in the adenylate kinase reaction may account for the increase in the adenylate pool. The latter is increased with a same rate in all cases under study. However, a significant increase in ATP concentration in the presence of adenosine is observed only in the

erythrocytes with altered energy metabolism. Nevertheless, even in these erythrocytes the increase in ATP concentration does not exceed the control value.

The data obtained do not support predictions of mathematical models of the energy metabolism and metabolism of adenylates [8, 9] that suggest a decrease in the adenylate pool during stimulation of ATP utilization. In these experimental conditions the adenylate pool is increased, and this requires reevaluation of model considerations of the system of adenylate metabolism.

The increase in the adenylate pool during stimulation of transport ATPases provides the maintenance of high ATP levels, close to normal values. We suggest that the increase in ATP concentration in the presence of adenosine leads to additional activation of ion transport by Na^+/K^+ -ATPase which results in a decrease in K^+ efflux from erythrocytes induced by AMB. Thus, adenylate metabolism may have an important role in the regulation of ATP-dependent processes, in particular for ion pumps. Our results cannot exclude the possibility of a direct influence of adenosine on ion transport; however, such an influence seems unlikely.

The energy charge of erythrocytes is markedly decreased in the presence of adenosine. Apparently, ATP consumption required for the synthesis of adenine nucleotides from adenosine represents a significant influence on the energy metabolism of erythrocytes. This suggests that relatively low rates of adenylate metabolism in normal erythrocytes [21, 22, 28, 29] are stipulated by the possible influence which adenylate metabolism may exert on energy metabolism.

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