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The Effect of External ADP on Red Cell Nucleotide Levels[1] (40392)

G. PANGALIS,* C. TEGOS,† AND E. BEUTLER†

* *University of Athens School of Medicine, First Department of Internal Medicine, Athens, Greece, and* †*Department of Hematology, City of Hope National Medical Center, Duarte, California 91010*

When de Gruchy *et al.* (1) modified the autohemolysis test by the addition of adenosine triphosphate, they recognized that the red cell membrane was impermeable to this nucleoside triphosphate. However, Kashket and Denstedt (2) had suggested that adenosine diphosphate (ADP) added to the exterior of red cells was dismutated by adenylate kinase to adenosine monophosphate (AMP) and ATP which, they claimed, appeared in the interior of the erythrocyte. de Gruchy *et al.* (1) suggested that this mechanism might account for the apparent effect of ATP in reducing autohemolysis. However, the question of whether significant adenylate kinase activity exists in the red cell membrane and whether nucleoside triphosphates can, in point of fact, enter the erythrocyte has never been reinvestigated using modern techniques. The ability of nucleotides to penetrate the erythrocyte would be a phenomenon which might be of considerable practical importance; however, Kashket and Denstedt's studies consisted merely of qualitative estimation of nucleotide levels after paper electrophoresis.

Materials and methods. All reagents or enzymes used in this study were purchased from Sigma Chemical Co. unless otherwise indicated. Commercially available ADP was approximately 5% contaminated with AMP and ATP; it was repurified on an AG 1 × 8 (Bio-Rad Laboratories) resin column. The commercially available ADP was applied to the column which was washed with distilled water and subsequently the nucleotides were eluted from the column using 0.01 *N*, 0.02 *N*, and 0.04 *N* HCl. The ADP was eluted with 0.04 N HCl and was immediately neutralized with K_2CO_3 and placed on ice. [^{14}C]-ADP was obtained from New England Nuclear, Boston, Massachusetts. The determination of the AMP, ADP, ATP and adenylate kinase activities were carried out as described elsewhere (3). Radioactivity was counted in a Packard-Tricarb Automatic Scintillation Counter using Handifluor scintillation fluid.

Human blood was collected in a heparinized syringe. The red cells were washed three times with isotonic (0.154 *M*) KCl and the buffy coat was removed after each washing. All procedures were performed on ice except when otherwise indicated. The same experimental conditions reported by Kashket and Denstedt (2) were used for the incubation of the erythrocytes with ADP or [^{14}C]ADP. Determination of AMP, ADP, ATP and adenylate kinase activities was performed in both the erythrocytes and supernatant before incubation (zero time) and after sixty minutes incubation of the erythrocyte-ADP mixture at 37°. Radioactivity was counted on aliquots obtained from washed erythrocytes and the supernatant before incubation and after sixty minutes incubation of the erythrocyte-[^{14}C]ADP mixture at 37°.

Results. A. *The effect of incubation with ADP on nucleoside concentration and adenylate kinase activity in the red cell and in the surrounding medium.* The effect of incubation of red cells with ADP on adenine nucleotide concentrations is summarized in Table I. Although ADP which was free of contaminating AMP and ATP was used, even the supernatant separated immediately after addition of ADP to the cell suspension contained significant amounts of AMP and ATP. This was explained by the fact that a substantial amount of adenylate kinase activity was present in the supernatant, amounting to .037 U/ml before and .135 U/ml after 60 min incubation. Estimation of the quantity of hemoglobin which had been released into the medium by measuring its optical density in the Soret region indicated that the activity could be accounted for, in full, by lysis of red cells.

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TABLE I. THE EFFECT OF INCUBATION OF HUMAN ERYTHROCYTES ON ADENYLATE KINASE (AK) AND ADENINE NUCLEOTIDES.

	AK	AMP	ADP	ATP	TOTAL
Red cells					
0′	80 U/ml RBC	14.3 μM	220.6 μM	1298.6 μM	1533.5 μM
60′	77 U/ml RBC	<5 μM	336.3 μM	744.9 μM	1086.2 μM
Supernatant					
0′	0.037 U/ml	164 μM	521 μM	157 μM	842 μM
60′	0.135 U/ml	271 μM	328 μM	235 μM	834 μM

There was a rise of ADP levels in the red cells after incubation with exogenous ADP, but this occurred at the expense of ATP and AMP, so that the size of the total intracellular nucleotide pool diminished.

B. *The penetration of radioactive ADP into red cells.* Less than 0.1% of radioactivity was found in washed erythrocytes from the erythrocyte-[^{14}C]ADP mixture before incubation, and after incubation for sixty minutes at 37° even less radioactivity was associated with the erythrocytes.

Discussion. Twenty years ago Kashket and Denstedt (2) claimed that ADP added to washed red cells produced an increase in glucose utilization and an increase in the intracellular ATP levels. We have not further investigated the putative effect of ADP on red cell glucose utilization; the conditions of incubation were not defined, and it seems likely that the alterations which were observed were due to changes in intracellular red cell pH. The purpose of our study has been to determine whether ADP can, in point of fact, enter the erythrocyte. When red cells were incubated with ADP rapid formation of ATP and AMP in the external medium could be detected. This does not indicate, however, that the exterior surface of the membrane has adenylate kinase activity as suggested by Kashket and Denstedt (2). Red cells are a very rich source of adenylate kinase: each ml of normal red cells contains sufficient enzyme to convert about 90 μmoles of ADP to ATP and AMP per minute. Thus, hemolysis of even a very small fraction of the cells in the incubating mixture could result in rapid equilibration of ATP, ADP, and AMP in the external medium. In our investigations, the formation of ATP and AMP did not exceed the amount which could have been formed by enzyme released from the few red cells which had been hemolyzed in the course of the experiment. Nor was there evidence that ADP entered the red cells. The level of red cell adenine nucleotides was actually decreased after incubating the cells in a medium containing ADP, presumably because of deamination of AMP to inosine monophosphate or dephosphorylation to adenosine (4). Moreover, no detectable amounts of radioactive ADP entered the red cells upon incubation.

Our studies indicate that, contrary to the earlier studies of Kashket and Denstedt (2), no penetration of ADP into red cells occurs during incubation, and that conversion of ADP into ATP and AMP in the external medium can readily be accounted for on the basis of hemolysis of red cells, which contain copious amounts of this enzyme. Accordingly, the effect of ATP in the autohemolysis test cannot be explained on the basis originally proposed by de Gruchy *et al.* (1). It now seems likely that the effect of ATP may have been related, in large part, to the fact that a high concentration of osmotically active, polyelectrolyte was added and that the ATP solution had not been neutralized so that it exerted a profound effect on the pH of the final system.

Summary. It has previously been suggested that ADP added to human erythrocyte suspensions may enter the cells and perform a metabolic function. Incubation of human red cells with radioactive and nonradioactive ADP failed to provide any evidence of the entry of this nucleotide into the erythrocytes.

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