

# HOW ALUMINIUM IS TAKEN UP BY RED BLOOD CELLS: A STUDY IN RELATION TO HYPOCHROMIC MICROCYTIC ANEMIA IN HEMODIALYZED PATIENTS

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## ABSTRACT

Investigation of aluminium uptake by human erythrocytes was the major aim of this study. Packed red blood cells were incubated in Earle's medium (pH 7.4) containing varying concentrations of aluminium (0-160  $\mu\text{g/l}$ ) as  $\text{AlK}(\text{SO}_4)_2$  and aluminium content of the cells were determined using flameless atomic absorption. There was significant increase in aluminium content of the cells. Addition of 5 mM glucose caused an elevation of red cell aluminium, whereas depletion of red cells from ATP caused a marked reduction in aluminium uptake. Both ouabain and vanadate, when added to the medium, caused a significant reduction in aluminium uptake in line with a decrease in ATPase activity.

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## INTRODUCTION

Patients with chronic renal failure who are undergoing regular hemodialysis suffer from a number of physiological disorders, including hypochromic microcytic anemia,<sup>1</sup> dialysis osteodystrophy,<sup>2</sup> and neurological disorders including dialysis dementia, and Alzheimer's disease.<sup>3</sup> The exact mechanism by which aluminium causes these diseases is still a matter of discussion. Aluminium enters blood circulation via two major routes, aluminium phosphate binder agents and dialysis fluid.<sup>4</sup> Aluminium phosphate binders including aluminium hydroxide are used for the prevention of phosphate absorption through the gastrointestinal tract by making a complex. Preparation of dialysis fluid in water supply leads to its contamination with aluminium when aluminium sulfate is added for the clarification of water.<sup>5</sup> From either route, aluminium enters the circulation and distributes into two major fractions. Approximately 82 percent of the aluminium in the circulation was found to be transferrin-bound and the remaining was ultra-filtrable.<sup>6</sup> Transferrin is a  $\beta$ -glycoprotein with a molecular weight of 80 KD.<sup>7</sup> It is the major iron carrier

protein in the plasma.<sup>8</sup> In 1983, the binding of aluminium to human transferrin was reported by G. Trapp and it was then confirmed by a number of other researchers throughout the world by using different biochemical techniques including spectrophotometric titration, gel filtration, affinity chromatography and equilibrium dialysis.<sup>9,10</sup> The occurrence of hypochromic microcytic anemia in chronic renal failure patients who are undergoing hemodialysis has been reported to be due to aluminium intoxication which might be due to the interference of aluminium with iron metabolism, particularly since these two elements bind to serum transferrin.<sup>11</sup> Aluminium-transferrin as well as iron-transferrin binds to the same receptor at the cell surface membrane,<sup>12</sup> and is internalized to the cells by endocytosis. In the cell, aluminium has been reported to interfere with heme synthesis by isolated mitochondria.<sup>13</sup> The mechanism by which aluminium is taken up by mitochondria is still a matter of speculation but the existence of transferrin and transferrin receptors in the mitochondria has been recently reported.<sup>14</sup> The accumulation of aluminium in human red blood cells might be either transferrin-mediated through transferrin receptors of the immature cells, or by direct

## Aluminium Uptake by Red Blood Cells

**Table I. Aluminium measurement in human red blood cells and plasma**

No	Plasma Al µg/l	Red blood cell Al µg/l
1	22	41
2	22	61
3	26	61
4	40	33
5	32	37
6	22	29
7	28	21
8	24	33

Each value is the mean of three observations. The blood cell Al is 5x the Al concentration of the Triton extract because of the 5-fold dilution of the packed cells.

transportation through the erythrocyte membrane by active or passive transportation. Investigation of aluminium uptake and its mechanism by human erythrocytes was the major aim of the present study.

### MATERIALS AND METHODS

#### Preparation of Packed Erythrocytes -

Human fresh whole blood samples were obtained from healthy blood donors. Cells were separated from plasma by the centrifugation at 2500 g for 10 min at 4°C. The resultant buffy coat was removed and cells were washed three times with cold 0.9% NaCl and used immediately.

#### Aluminium Uptake by Human Erythrocytes

Washed fresh erythrocytes were suspended in Earle's medium<sup>13</sup> with or without aluminium and incubated at 37°C for different periods of time. The reaction mixtures were removed and cooled on ice bath and centrifuged at 2500 g for 10 min. The supernatant was used for aluminium and protein determinations.

#### Preparation of Human Erythrocyte Ghosts and Determination of ATPase

Human erythrocyte ghosts were prepared as described by Dodge, et al.<sup>15</sup> Washed cells were suspended in 10 volumes of ice-cold 10 mM Tris buffer, pH 7.4 and lysed by stirring gently for 2 min. The suspension was left at 4°C. The supernatant (hemolysate) was carefully removed and the pellets were washed three to four times with cold 0.9% NaCl until the final supernatant was free of any red color. The assay of Na-K-ATPase was determined in human erythrocyte ghosts by the method of Lai, et al.<sup>16</sup>

Aluminium determinations were carried out by the method of Parkinson, et al. (1982) using a Perkin-Elmer 603 flameless atomic absorption spectrophotometer with

**Table II. Determination of aluminium in human red blood cells and media**

Time (hours)	0	1	2	3	4
Incubation medium	Aluminium content of Triton extract of cells (µg/l)				
0.9% w/v NaCl (No Al)	-	16	28	24	32
0.9% w/v NaCl 160 µg/l Al	-	36	36	40	48
0.9% w/v NaCl	-	28	20	20	18
Incubation medium	µg/l Al in Media				
0.9% w/v NaCl (No Al)	7	<1	<1	<1	<1
0.9% w/v NaCl 160 µg/l Al	165	126	136	125	119
0.9% w/v NaCl 200 mg DFO	5	6	11	10	12

Each value is the mean of three observations

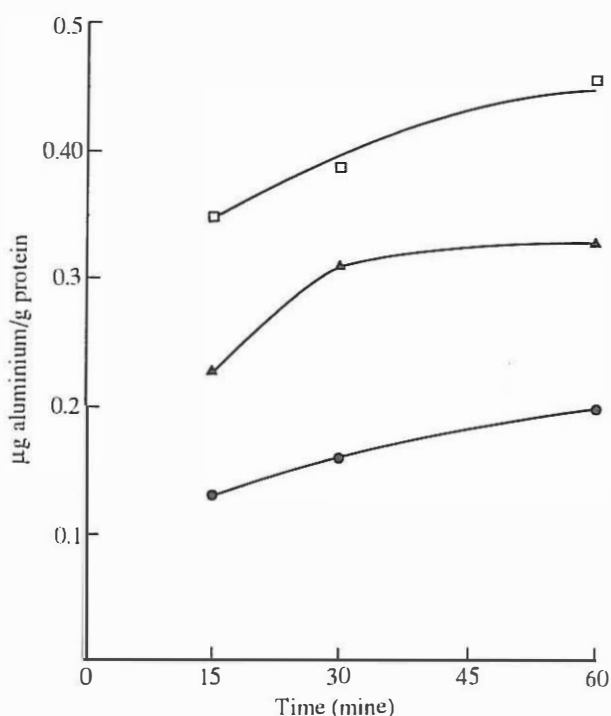
aluminium hollow cathode lamp operating at 25 mA. The wavelength used was 309.3 nm with a spectral band width of 0.7 nm. Argon gas was used as the inert gas.<sup>17</sup>

SDS-PAGE has been used according to our previous reports.<sup>18</sup> Adenosine triphosphate (ATP) concentrations in human red blood cells were determined by a bioluminescent method using a LDB kit. Protein determination was carried out by the method of Lowry.<sup>19</sup> All chemicals were reagent grade and were obtained from Sigma Chemical Company unless otherwise stated. Deionized water was used through this project.

### RESULTS

#### Aluminium Content of Red Blood Cells

Preliminary experiments were carried out to determine the aluminium content of plasma and red blood cells from eight chronic renal failure patients maintained on regular hemodialysis. Heparinised blood samples were collected and centrifuged at 1500 g for 7 min within 2 h of collection. The buffy coat was removed and the cells resuspended in 6 volumes of cold (4°C) 0.9% sodium chloride and centrifuged as before. The supernatant was discarded and the cells re-washed twice by the same procedure. 185 µL of packed cells and 740 µL 10% V/V Triton X-100 were mixed and the aluminium content of the dissolved erythrocytes determined. The results are shown in Table I and are compared with the aluminium content of the corresponding plasma. There was poor correlation between the values for plasma and erythrocytes within batch reproducibility for erythrocyte aluminium was better than 4%.



**Fig. 1.** Aluminium uptake by human erythrocytes.  $\square$  = 160 µg/L aluminium,  $\triangle$  = 80 µg/L aluminium  $\bullet$  = No aluminium. Each point is the mean of three observations.

### Aluminium Uptake by Erythrocytes

Investigations were carried out to determine how aluminium is taken up by erythrocytes. To do this, washed red cells as mentioned in Methods were incubated at 37°C for 4 h in (I) 0.9% (W/V) sodium chloride, (II) 0.9% sodium chloride containing 160 µg/L aluminium as Al K(SO<sub>4</sub>)<sub>2</sub>, and (III) 0.9% (W/V) sodium chloride containing 200 mg/L desferrioxamine (DFO). Following incubation time aluminium content of the red cells and the medium was determined. The results are shown in Table II. Except when DFO was present, the aluminium content of the medium was less after incubation. The amount of aluminium taken up by red cells incubated in aluminium-loaded medium was time-dependent.

Erythrocytes were then incubated at 37°C in Earle's medium containing different amounts of aluminium as aluminium potassium sulfate (0, 80, 160 µg/L Al). At appropriate time intervals aliquots of the mixtures were withdrawn, cooled in an ice bath and centrifuged for 8 min at 2500 rpm at 4°C.

The supernatants were discarded and the cells washed three times with 4 mL ice-cold 0.9% saline. The sedimented cells were dissolved in 4 volumes of 10% V/V Triton X-100 and their aluminium and protein contents determined. It shows that the red blood cells could take up aluminium from

**Table III.** Effect of glucose and aluminium on the ATP production by erythrocytes

Time (min)	0	60
	µmole ATP/g Protein	
Earle's medium	10	7.4
Earle's medium + 120 µg/L Al	8.8	6.8
Earle's medium + 5 mM glucose	9.97	10.8
Earle's medium + 120 µg/L Al + 5 mM glucose	8.65	7.3

**Table IV.** Effect of aluminium on ATP determination

ATP concentration	10 <sup>-11</sup>	10 <sup>-10</sup>	10 <sup>-9</sup>	10 <sup>-8</sup>	10 <sup>-7</sup>	10 <sup>-6</sup>
	ATP concentration (mVolts)					
Standard	4.7	4.5	5.6	18.5	143	1376
Standard + 270 µg/L Aluminium	4.3	4.5	5.6	17.9	141	1359

Each value is the mean of three observations

the medium (Fig. 1).

Possible aluminium binding proteins in hemolysate and red cell membranes was studied. Fresh prepared erythrocytes were incubated in Earle's medium and the same medium containing 160 µg/L aluminium for 1 h. Hemolysates and membranes were prepared and protein patterns determined by SDS-PAGE. Data are not shown. The hemolysate from cells incubated with aluminium showed an increase in one band (B) and decrease in two bands (A or C) as compared with patterns from controls.

### Effect of Glucose on Aluminium Uptake by Erythrocytes

Washed erythrocytes were suspended in Earle's medium (pH 7.4) containing varying concentrations of aluminium (0-160 µg/L) and then incubated for 3 h at 37°C. After incubation the erythrocyte suspensions were cooled in ice, 300 µL removed and added to 4 volumes of 20% trichloroacetic acid containing 4 mM EDTA.

The ATP content of the TCA extract was determined as mentioned in Methods. The remaining erythrocyte suspension was centrifuged at 2500 rpm for 5 min. The supernatants were discarded and the erythrocytes washed three times by repeated suspension in 0.9% saline and centrifuged as before. The erythrocytes were then dissolved in 4 volumes of 10% V/V Triton X-100 and the aluminium and protein content of the extracts determined.

The aluminium content of red blood cells incubated in medium containing 5 mM glucose was consistently higher than that of cells incubated in the same medium but without glucose. The obtained results were expressed as µg Al/g erythrocyte protein. It was found that the cells incubated in different concentrations of aluminium contained some 40% more aluminium than did cells

## Aluminium Uptake by Red Blood Cells

incubated in medium without added glucose (Fig. 2).

### ATP Content of Erythrocytes: Effect of Glucose and Aluminium

In the initial experiment designed to assess the effect of glucose on aluminium uptake by erythrocytes, aliquots of erythrocytes were removed and their ATP content determined as described in Methods. The ATP content of erythrocytes incubated for 3 h at 37°C in Earle's medium containing aluminium was significantly less than that of erythrocytes incubated in medium to which aluminium had not been added (Fig. 3). When glucose was added to the medium, the red blood cells' ATP increased (Fig. 3). In another experiment, a sample of washed erythrocytes was divided into four portions and these were incubated for 1 h at 37°C in different media (Earle's medium, Earle's medium containing 120 µg/L aluminium, Earle's medium containing 5 mM glucose and Earle's medium containing 5 mM glucose plus 120 µg/L aluminium). Once again in the absence of added glucose, there was a fall in the erythrocyte ATP content. When both aluminium and glucose were added to the medium, the erythrocyte ATP content fell out to a lesser extent than found when only aluminium but not glucose was added to the medium (Table III). The possibility of aluminium interfering with the ATP determination was examined. Using 270 µg/L aluminium as  $\text{AlK}(\text{SO}_4)_2$  and varying concentrations of ATP standard ( $10^{-11}$  -  $10^{-6}$  M), it was found that aluminium did not interfere with the ATP assay (Table IV).

### Aluminium Uptake by Erythrocytes: Effect of Ouabain

Washed erythrocytes were incubated in Earle's medium containing  $10^{-4}$  M ouabain and increasing concentration of aluminium for 2 h at 37°C. As a control, cells were also incubated in a similar medium but without added ouabain. The cells were collected by centrifugation, washed as described earlier, dissolved in 10% W/V Triton X-100 and their aluminium and protein content determined. Table IV shows that the aluminium content of the Triton extract of red blood cells incubated in aluminium-containing medium was reduced by approximately 50% when  $10^{-4}$  M ouabain was present in the medium. When the aluminium content of the extract was expressed per g erythrocyte protein, the results in Fig. 4 were obtained.

### Effect of Vanadate on Aluminium Uptake by Erythrocytes

Washed erythrocytes were incubated at 37°C for periods up to 2 h in Earle's medium containing 160 µg/L aluminium as  $\text{KAl}(\text{SO}_4)_2$ . At appropriate time intervals, aliquots were removed, cooled in ice, washed in ice-cold 0.9% W/V saline, dissolved in 10% V/V Triton X-100 and the aluminium and protein content determined. The effect of adding varying concentrations of sodium vanadate was

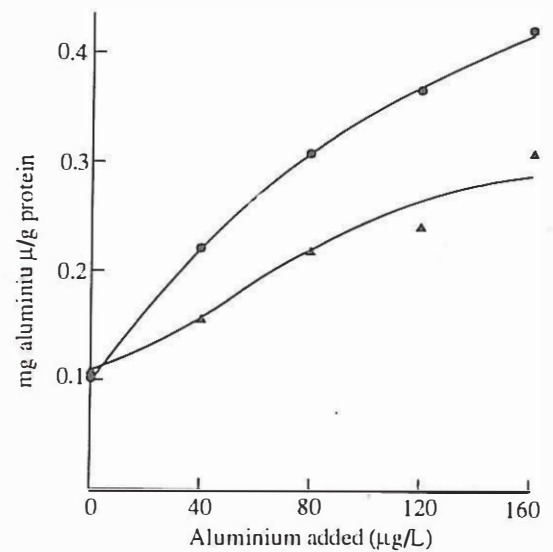


Fig. 2. Effect of glucose on aluminium uptake by human erythrocytes. (● = glucose, ▲ = No glucose) Each point is the mean of six observations.

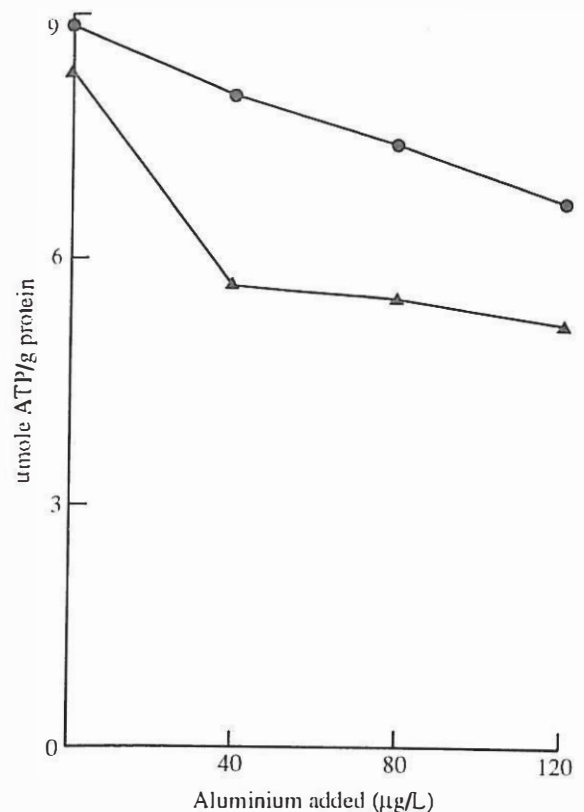


Fig. 3. ATP concentration of human red blood cells after incubation in Earle's medium containing varying concentrations of aluminium. (● = glucose, ▲ = No glucose) Each point is the mean of six observations.

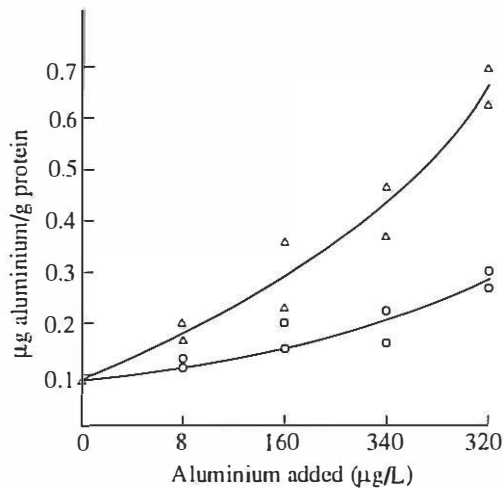


Fig. 4. Effect of 100 μM ouabain on aluminium uptake by human erythrocytes. (Δ = No ouabain, ○ = 100 μM ouabain) Each point is the mean of three observations.

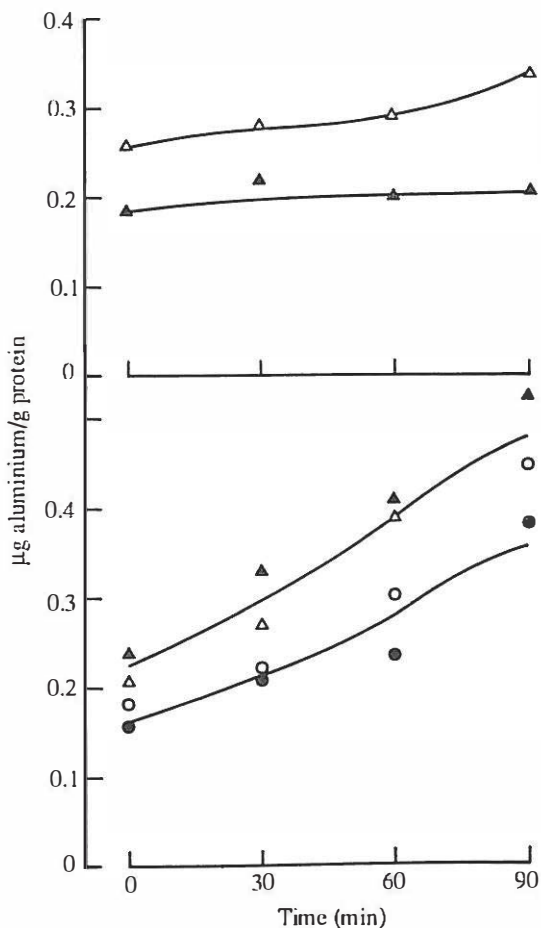


Fig. 5. Effect of vanadate on aluminium uptake by human erythrocytes. (A = No vanadate, B = 50 μM vanadate, C = 8 μM vanadate) Each point is the mean of three observations.

examined and the results obtained are shown in Fig. 5. In the absence of vanadate the aluminium content of the erythrocytes increased slightly during incubation. In the presence of 8 μM vanadate more aluminium was taken up by the erythrocytes. In the presence of 50 μM vanadate the uptake of aluminium by the cells was insignificant.

#### Effect of Aluminium on the ATPase Activity

Erythrocyte ghosts were prepared as described in Methods and the total ATPase activity determined by the method of Lai, et al.<sup>16</sup> in the presence of varying concentrations of aluminium. The results indicated that significant inhibition was found only when the aluminium concentration was in excess of 160 μg/L.

## DISCUSSION

In blood, aluminium is present in both plasma and erythrocytes. Hewitt, et al.<sup>20</sup> has reported that the concentration of aluminium in serum is 60% of that in erythrocytes. Molitoris, et al.<sup>21</sup> have also shown that the concentration of aluminium in erythrocytes and plasma are very similar. How does this aluminium get into the cells? Reticulocytes, the precursors of mature erythrocytes contain transferrin receptors and thus one would expect that reticulocytes could readily take up aluminium from extracellular fluid in the form of transferrin. However, other mechanisms for aluminium uptake may well be present, particularly as erythrocytes in blood will be circulating in a plasma medium which contains significant amounts of non-protein blood aluminium. With regard to the distribution of aluminium between plasma and erythrocytes, the aluminium content of the packed cells was determined. The aluminium content of the packed cells was consistently higher than that of plasma but there was a poor correlation between the two. To address the question of how aluminium gets into red cells, the general principles of how ions enter red cells and how they are retained by red cells needed to be examined.

In relation to sodium and potassium, under physiological conditions, internal Na<sup>+</sup> is exchanged for external K<sup>+</sup> at the expense of energy derived from the hydrolysis of ATP at the inner surface of the cell membrane. Sodium and potassium interchange across cell membranes is controlled by an adenosine triphosphate and (Na<sup>+</sup>-K-ATPase) commonly referred to as Na<sup>+</sup> pump.<sup>22</sup> Waisman, et al.<sup>23</sup> showed that in the presence of ATP, calcium accumulation by human erythrocytes was linear for 30 min. The transport of Ca<sup>2+</sup> across the plasma membrane by red cells is influenced by the anion composition of the incubation medium. In order to investigate aluminium uptake by erythrocytes, washed red cells were first incubated for 0-4 h in Earle's medium containing 160 μg/L of aluminium as AlK(SO<sub>4</sub>)<sub>2</sub>. The results showed that aluminium taken up by erythro-

## Aluminium Uptake by Red Blood Cells

**Table V. Effect of 100  $\mu$ M Ouabain on aluminium uptake by erythrocytes**

Aluminium concentration of medium ( $\mu$ g/L)		Aluminium content of Triton extract of cell ( $\mu$ g/L)				
		0	80	160	240	320
Sample 1	10 <sup>-4</sup> M Ouabain	16	20	22	24	41
	No Ouabain	16	30	63	71	94
Sample 2	10 <sup>-4</sup> M Ouabain	-	17	23	43	56
	No Ouabain	-	32	40	66	90

cytes was a time-dependent process. When erythrocytes were incubated in medium containing 160  $\mu$ g/L aluminium compared with control erythrocytes and the hemolysate and the ghosts separated, it was found that the aluminium content of the cytosol was increased by 67% where as that of the ghosts fell by 47%.

Having established that aluminium enters red cells, experiments were carried out to investigate this process. When red blood cells were incubated in medium containing varying concentrations (0-160

AlK(SO<sub>4</sub> added to the medium, the aluminium content of the cells was significantly increased. This is analogous to the observation of Long and Mount,<sup>24</sup> who showed that washed human red cells were able to take up 336  $\mu$ g/L calcium when they were incubated in a medium containing glucose.

In relation to the present work, if glucose increases aluminium uptake by providing energy, this may be ATP-mediated. When red blood cells are incubated in a medium containing 5 mM glucose, there was an increase in the ATP content whereas with red blood cells incubated without glucose, the ATP content fell (Fig. 2). At the same time, the aluminium content of cells incubated with glucose showed a 2-fold increase compared to those incubated without glucose in the medium. If ATP hydrolysis was involved in aluminium uptake, one might expect to see altered ATPase activity in red cells incubated with aluminium. However, when the ATPase activity of erythrocyte ghosts was measured in the presence of different concentrations of aluminium, no effect was seen when the aluminium concentration was of the order found in blood of patients with aluminium overload (0-160 aluminium concentrations (22 activity was reduced by 40%. Lai, et al.<sup>19</sup> who also investigated the effect of aluminium on a variety of ATPase activities, found reduced ATPase activities by 50% in the presence of 224 mg/L aluminium.

The effect of ATPase inhibitor on aluminium uptake by erythrocytes was also examined. Ouabain (100

reduces aluminium uptake by 50% and ATPase activity by 42%, respectively. A marked decrease in aluminium uptake by erythrocytes and ATPase activity may suggest a probable energy-dependent mechanism for aluminium uptake by erythrocytes.

However, more investigation is needed to elucidate the exact mechanism by which aluminium is taken up by red cells.

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## A.A. Moshtaghi

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