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URATE TRANSPORT IN HUMAN RED BLOOD CELLS

ACTIVATION BY ATP

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Summary

Urate transport in human erythrocytes were measured and compared to previous observations by other authors regarding inorganic anions, especially chloride. Conclusions were as follows:

1. Urate influx as a function of increasing concentrations showed saturation kinetics.

2. The effects of pH and of several passive anion transport inhibitors such as dinitrofluorobenzene, sodium salicylate, sodium benzoate and phenylbutazone suggest that urate and chloride are transported by different mechanisms.

3. Urate influx seems to depend on intracellular glycolysis. The results obtained on red blood cells after glycolysis inhibition agree with those obtained on ghosts where metabolism does not take place.

4. The large drop in urate influxes into erythrocytes in the presence of a glycolysis inhibitor and of a passive ion transport inhibitor seems to argue in favour of a dual urate transport mechanism, one for passive diffusion and the other connected with glycolysis.

5. The drop in the urate influx into ghosts in the absence of ATP suggests that the latter might intervene in urate transport by human red cell membranes.

Introduction

In an earlier study [1], we observed similarities between the urate anion and inorganic anions, especially as regards the variations in the coefficient of their

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in vitro distribution between the intra and extracellular media, as a function of the pH and electrolyte concentration. These results enabled us to conclude that at equilibrium, urate takes the same form on both sides of the membane and that its distribution ratio is in agreement with Donnan's equilibrium. The work done by Lassen [2] on the mechanism governing urate transport by human erythrocyte membrane suggests the intervention of a facilitated diffusion process. Other authors also observed that chloride transport by erythrocyte membranes was facilitated transport [3—6]. Other experiments again, conducted on red blood cells during the past few years, showed that both chloride and organic anions might be transported in a similar way by the same type of mechanism [7,8]. However, more recent observations seem to imply that in tubular kidney cells of rat, urate transport is an energy-dependent process [9,10]. So the aim of our present work is to compare the respective ways in which urate and inorganic anions, in particular chloride, are transported across red blood cells membranes.

Materials and Methods

1. Preparation of cells

Erythrocytes. Membrane transport of urate was studied on erythrocytes from normal subjects. Blood was drawn off extemporaneously into tubes containing heparin and immediately used for experimentation. After centrifugation, we eliminated the plasma and white leukocyte layer and treated the pellet of red blood cells, which were washed in Ringer solution at pH 7.40. After gentle shaking by constant turning over, the suspension was again centrifuged. The cells were then washed three times and used intact for some of the experiments and as ghosts for others.

Ghosts. Ghosts were prepared according to Passow's method [11], by hypotonic hemolysis in the presence of ATP and the restoration of adequate Na^+ and K^+ concentrations by adding KCl and NaCl.

It is important to note that these ghosts, which recovered their initial permeability properties, were incapable of transforming inosine, adenosine or glucose into lactic acid [11].

2. Experimental protocol

Red blood cells from the same donor were systematically divided up into four samples of the same size (tubes A—D). Tube A was used as the control, and always contained the same incubation medium: 138 mmol/l Na^+, 4.6 mmol/l K^+, 10 mmol/l Ca^{2+}, 5 mmol/l Mg^{2+}, 126 mmol/l Cl^-, 5 mmol/l PO_4^{2-}, 5.5 mmol/l glucose, 5 mmol/l Tris, and 0.3 mmol/l urate.

The other cell pellets (erythrocytes and ghosts) from tubes B—D were resuspended in the same medium. One of parameters of the media was changed, depending on the experiment planned, as described in paragraph 3 below. The hematocrit, checked in the course of each experiment, was 50%. Cell suspensions were gently rotated at 37° C for 2 h, the time required for urate to reach partition equilibrium between the erythrocytes and supernatant. At equilibrium, the addition of 100 µl [^{14}C]urate (0.125 µCi, 2.5 nM) marked time zero of the experiments. After mixing by continuous turning over, we immediately

drew off 500 µl of the suspension. 50 µl of supernatant were centrifuged and used to determine radioactivity. After the withdrawal of 500 µl at time zero, the suspension was adjusted to 37° C and gently shaken by intermittent rotation. Samples of the suspension were then taken every 5 min for 1 h, and every 20 min for a further 2 h. In this way we measured the decline in the supernatant's radioactivity as a function of time.

3. Incubation media

Depending on the experiment planned, we varied one of several parameters in every incubation solution, in order to see how the change affected the urate influx into the erythrocytes and ghosts. Unless otherwise stated, all changes in parameters were made before 2 h incubation required to reach equilibrium.

Except where otherwise stated, the following conditions applied in all experiments: pH 7.20, $t = 37°$ C, non-radioactive urate concentration: 0.3 mmol/l and osmolarity 300 mosM.

4. Calculations

Velocity rate coefficients (k) for urate transport were calculated from the straight slope $\log[y - y_t]/[y - y_0]$ as a function of time where y, y_t, and y_0 are supernatant's radioactivity at equilibrium at times t and zero, respectively. The urate influx is expressed in mmol/cm^2 per s and calculated as follows, according to Passow's method [12], which we modified, using following symbols:

y_c, dpm in cells; y_∞, y_t, y_0, dpm in supernatant at equilibrium, at time t and zero, respectively; C_c, C_s, concentration of the non-radioactive urate in cells and supernatant (mmol/l); V_c, V_s, volumes of cells and supernatant (ml); $n_c = C_c \cdot V_c \cdot n_s = C_s \cdot V_s$, amount of non-radioactive urate in cells and supernatant (mmol); F, membrane surface (cm^2); J, urate influx (mmol/cm^2 per s); P, permeability constant (cm/s), representing the quotient flux/concentration.
We have:

$$\frac{y_\infty - y_t}{y_\infty - y_0} = e^{-kt}$$

$$B = \frac{C_s V_s + C_c V_c}{C_s V_s - C_c V_c} \text{ and } k = JBF$$

$$JF = \frac{k \cdot 1}{B} \cdot \frac{1}{B} = \frac{n_c \cdot n_s}{n_c + n_s}$$

$$PF = \frac{JF}{C_{s\infty}} = \frac{k}{c_{s\infty}} \cdot \frac{1}{B}$$

$$n_c + n_s = C_{s\infty} \cdot V_s \cdot \frac{y_0}{y_\infty}, \; n_s = C_{s\infty} \cdot V_s, \; n_c = n_c + n_s - n_s$$

Therefore

$$\frac{1}{B} = \frac{n_c \cdot n_s}{n_c + n_s} = \frac{[(C_{s\infty} \cdot V_s \cdot y_0/y_\infty) - (C_{s\infty} \cdot V_s)] \cdot C_{s\infty} \cdot V_s}{C_{s\infty} \cdot V_s \cdot \frac{y_0}{y_\infty}}$$

$$PF = \frac{k}{C_{s\infty}} \frac{[(C_{s\infty} \cdot V_s \cdot y_0/y_\infty) - (C_{s\infty} \cdot V_s)]C_{s\infty} \cdot V_s}{C_{s\infty} \cdot V_s \cdot \frac{y_0}{y_\infty}}$$

$$PF = \frac{\left\{\frac{y_0}{y_\infty} - 1\right\} C_{s\infty} \cdot V_s}{\frac{y_0}{y_\infty}} \cdot k = \frac{\frac{y_0 - y_\infty}{y_\infty}}{\frac{y_0}{y_\infty}} V_s \cdot k$$

$$PF = \frac{y_0 - y_\infty}{y_0} \cdot V_s k$$

$$P = \frac{PF}{F} = \frac{PF}{V_c \cdot r} \text{ (with } r = 15\,500 \text{ cm}^2/\text{ml)}$$

admitting according Wintrobe [13], that surface of one red blood cells is 140 μm^2 and there are $1.1 \cdot 10^{10}$ red blood cells in 1 cm^3 of pellet, i.e. a volume of 90 μm^3 for one red blood cell.

$$P(\text{cm/min}) = \frac{y_0 - y_\infty}{y_0} \cdot k \cdot \frac{V_s}{V_c} \cdot \frac{1}{15\,500}$$

$$P(\text{cm/s}) = \frac{y_0 - y_\infty}{y_0} \cdot k \cdot \frac{1 - Ht}{Ht} \cdot \frac{1}{60 \times 15\,500}$$

$$P(\text{cm/s}) = \frac{y_0 - y_\infty}{y_0} \cdot k \cdot \frac{1 - Ht}{Ht} \cdot 1.075 \cdot 10^{-6}$$

and

$$J(\text{mmol/cm}^2 \text{ per s}) = \frac{P}{C_{s\infty}}$$

Results

1. Experiments on erythrocytes

Effect of increasing urate concentrations. Fig. 1 shows saturation kinetics in agreement with Lassen's results [2]. Fig. 2 indicates the reciprocal flux as a function of the urate concentration, according to the Lineweaver and Burk plot. The straight line obtained enabled us to evaluate the Michaelis constant (K_m) at 3.8 mmol and the maximum velocity (V) at $17 \cdot 10^{-11}$ mmol/s per cm^2. As uric acid has poor solubility in water, we had to use 13 mmol/l lithium carbonate for our experiments, lithium urate solubility being satisfactory. We checked that lithium carbonate had no specific effects on the urate influx into the red blood cells. Thus, for a urate concentration of 0.3 mmol/l the influx was identical, whether lithium carbonate was present or not. The same checking process could not be applied to the highest concentrations on account of the insolubility of urate in the absence of lithium carbonate.

Effects of extracellular chloride. Fig. 3A makes clear that for identical pH values, the urate influx is larger when chloride is replaced by citrate or

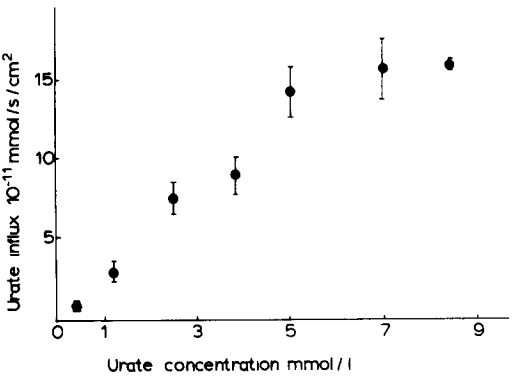

Fig. 1. Relation between urate extracellular concentration (in mmol/l supernatant) and urate influx in human erythrocytes ($X10^{-11}$ mmol/cm^2 per s). Each point: mean ± S.D. of at least eight experiments.

saccharose. The presence of chloride therefore appears to reduce urate transport.

Effects of anion transport inhibitors. Some substances, especially sodium salicylate, phenylbutazone, dinitrofluorobenzene and sodium benzoate, are known for their inhibitory effect on the passive cellular influx of many mineral and organic anions [14—16]. We studied their effect on the urate influx into the erythrocyte by adding them to the incubation medium in successive experiments. In each case, we compared the results to a control without any inhibitor. Fig. 4 shows that dinitrofluorobenzene (4 mmol/l), salicylate (30 mmol/l) and benzoate (20 mmol/l), inhibit urate transport. The rate coefficients obtained in the presence of inhibitors were as follows (mean ± S.D.):

k (control) = 0.028 ± 0.003; k dinitrofluorobenzene = 0.015 ± 0.003;

k (benzoate) = 0.016 ± 0.002, and k (salicylate) = 0.018 ± 0.001.

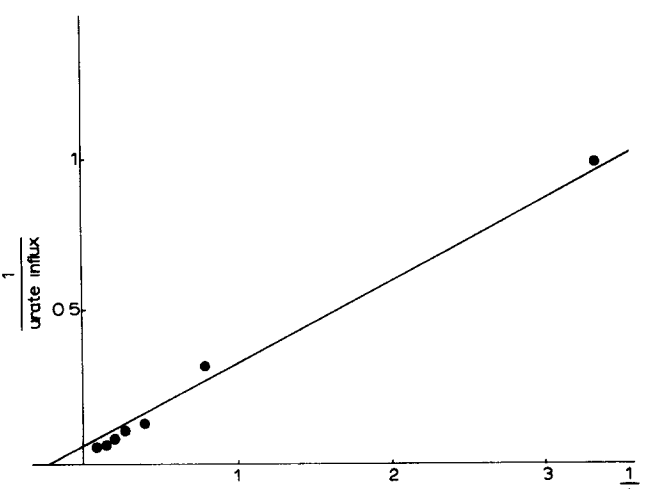

Fig. 2. The urate influx is plotted against extracellular urate concentration in a Lineweaver-Burk plot ($1/J$ and $1/C$). The K_m and V determined from this graph were 3.8 mmol and $17 \cdot 10^{-11}$ mmol/s per cm^2, respectively.

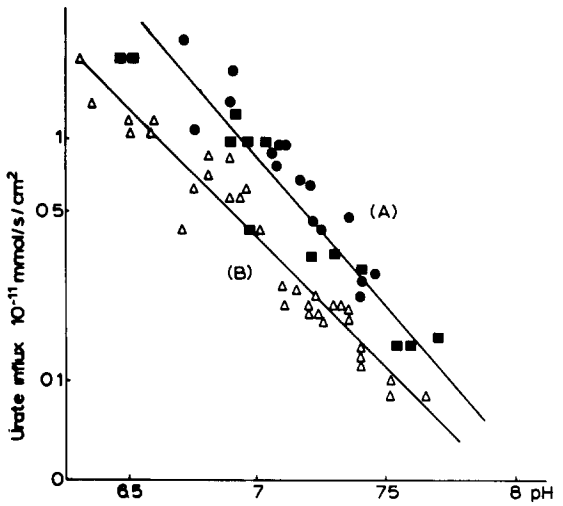

Fig. 3. Urate influx as a function of pH. (A) Much of the chloride in the incubation medium was replaced by citrate (68 mmol/l) (●) or by saccharose (210 mmol/l) (■). (B) Control (△) with chloride (126 mmol/l). The pH of the incubation solutions was changed by using HCl or NaOH.

Higher concentrations of these inhibitors did not increase inhibition (for 4—16 mmol/l dinitrofluorobenzene, $k = 0.017 \pm 0.01$, and for 30—120 mmol/l salicylate, $k = 0.019 \pm 0.002$). However, these results are difficult to interpret because these large concentrations of inhibitor can bring about other changes.

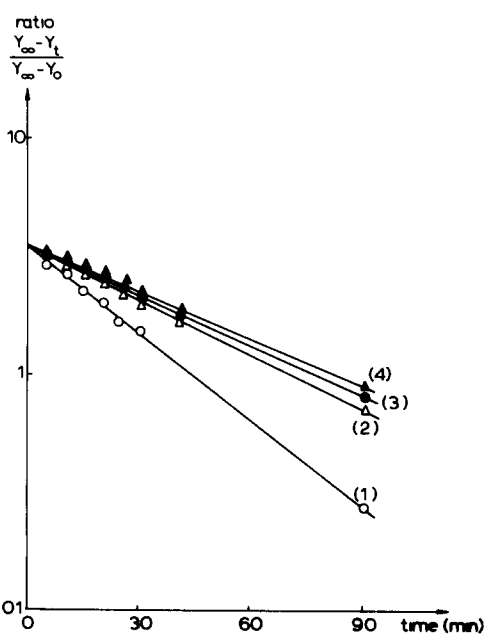

Fig. 4. Inhibitory effect of three drugs, inhibitors of passive anionic transport on urate transport measured under steady-state conditions. (1) control cells, (2) dinitrofluorobenzene (4 mmol/l); (3) benzoate (20 mmol/l); (4) salicylate (30 mmol/l). Addition of inhibitor in incubation medium is made 2 h before adding labeled urate.

Effect of pH. The relationship between urate influx into erythrocytes and the extracellular pH (Fig. 3B) is linear. There is thus a drop in the urate influx when the pH is raised from 6.30 to 7.70.

Metabolic effects. We compared the urate influx into normal red blood cells with the influx into the cells after inhibition of glycolysis. Intraerythrocyte glycolysis was eliminated by preincubating the erythrocytes for 1 h at 37° C in a control solution containing no glucose. (Control erythrocytes were incubated in the control solution containing glucose at the same time.) This preincubation was carried out in the absence of urate. Throughout the remaining part of the experiment, the cells were treated with a glucose-free solution and compared to control cells in which active glycolysis occur.

In a different series of experiments, we inhibited the intracellular metabolism by adding 10 mmol/l sodium fluoride and comparing the urate influx with controls containing no sodium fluoride.

Table I shows that either the absence of glucose or the presence of sodium fluoride cause a reduction of about 50% in urate transport. The latter therefore appears to be glycolysis dependent.

Sodium fluoride inhibits enolase, which transforms 3-phosphoglycerate into phospho*enol*pyruvate. Enolase inactivation lowers the concentration of the compounds formed from 3-phosphoglycerate. It is clear from Table I that the addition of these different metabolites to the incubation medium does not restore an urate influx comparable to the control.

Possible intervention of two mechanisms in urate transport. We compared

TABLE I

PEP, phospho*enol*pyruvate.

Incubation	Urate influx (10^{-11} mmol/cm^2 per s)	S.D.	*n*
Red blood cells			
Control	0.49	± 0.03	10
Without glucose	0.20	± 0.02	10
With NaF	0.19	± 0.01	10
With NaF	0.26	± 0.079	6
With NaF and PEP (30 mmol/l)	0.29	± 0.047	6
With NaF	0.29	± 0.086	5
With NaF and pyruvate (36 mmol/l)	0.27	± 0.04	5
With NaF	0.38	± 0.039	6
With NaF and lactate (38 mmol/l)	0.36	± 0.022	6
With NaF	0.35	± 0.027	10
With NaF and NAD (2 mmol/l)	0.35	± 0.027	10
Red blood cells			
Control	0.55	± 0.03	10
Without glucose	0.33	± 0.01	10
With glucose and salicylate (30 mmol/l)	0.35	± 0.03	10
Without glucose and with salicylate (30 mmol/l)	0.17	± 0.01	10
Red blood cells			
Control	0.45	± 0.006	6
With NaF	0.28	± 0.02	6
Ghosts			
With ATP	0.28	± 0.018	8
Without ATP	0.16	± 0.012	8

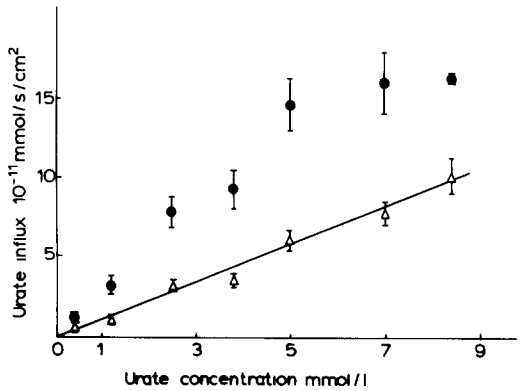

Fig. 5. Effect of the addition of NaF (10 mmol) in the incubation medium. Ordinate, urate influx (10^{-11} mmol/cm^2 per s); abscissa, urate concentration (mmol/l). •, control cells; △, incubation medium supplemented with NaF. Each symbol: mean ± S.D. of at least eight experiments.

urate influx in four different cases (Table I): with glucose (control), without glucose (glycolysis inhibition), with salicylate (inhibition of passive anion transport) and with salicylate but without glucose. These two last conditions combined to produce a cumulative inhibitory effect. In addition, we compared the effect of increasing urate concentrations on a medium without sodium fluoride (control) with the effect on a medium containing 10 mmol/l sodium fluoride (Fig. 5). We did not observe any saturation process in the presence of sodium fluoride but a linear relationship between the urate influx and the concentration levels. In the absence of glycolysis, urate transport seems to be a simple diffusion mechanism. But it is important to note that an increased K_m could explain the data equally well.

2. Experiments on ghosts

Effect of the pH. Experimental protocol was identical to that for erythrocytes. Fig. 6 shows that between pH 6.10 and 6.40, the urate influx increases and reaches its maximum at pH 6.40. After that, it decreases up to pH 7.50. A similar curve was reported earlier for sulfate [16,17] and chloride [3,6] in the case of erythrocytes. However, the optimum pH for urate (6.40) is different from the optimum for chloride (7.80). Between pH 6.10 and 6.40, there are comparable increases of urate transport in ghosts and of chloride transport in erythrocytes. Thus, the urate influx increases four fold and the chloride influx, five fold. Above all it is important to note that, for the same pH, the urate influx into red blood cells is higher than the urate influx into ghosts, especially when pH is less than 7.

Effect of ATP. We compared the urate influx into ghosts prepared with hemolyzing solutions with and without ATP. The results are given in Table I. We obtained a reduction in the urate influx in the absence of ATP. The same table also shows that urate transport is comparable in erythrocytes without glycolysis, and in the ghosts. On the other hand, such transport increased in red blood cells in which active glycolysis occurred.

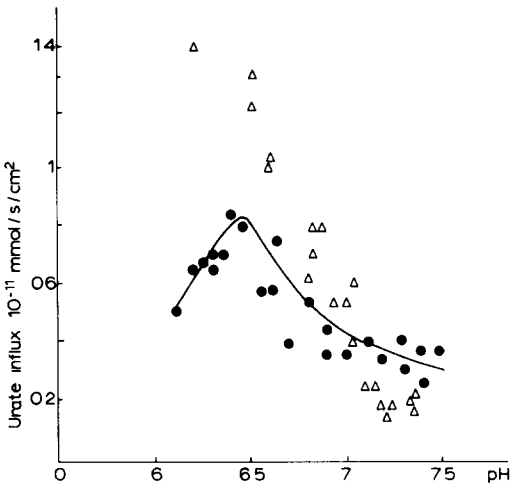

Fig. 6. The influence of pH on urate influx in ghosts (•) and in erythrocytes (△). Ordinate, urate influx (10^{-11} mmol/cm^2 per s); abscissa, pH of incubation medium. In ghosts, the graph shows an optimum influx for pH 6.40. At pH <7, urate influx in erythrocytes in greater than in ghosts. Each point represents one experiment.

Discussion

As indicated by Lassen's results [2], urate transport appears to take place, at least in part, by a passive facilitated diffusion process. At the same time it should be born in mind that we were unable to determine the effect of lithium carbonate itself at high urate concentration. Indeed Li^+, as Cl^- can apparently be transported by the anion transport mechanism [18]: according to the ion pair hypothesis, negatively charged in pairs, $LiCO_3^-$ could be transported through the cation-tight membrane by the specific anion.-exchange system. Therefore it is not impossible that, at high concentration of urate, $LiCO_3^-$ compete with urate for some membrane sites. So, slight doubts persist regarding the saturation obtained at such concentrations.

The fact that the presence of chloride in the extracellular medium reduces urate transport suggests that this transport might not depend on ionic strength but could be connected with the presence of chloride itself. This may mean there is competition between chloride and urate anions for identical membrane sites. However, it is important to note that the replacing of extracellular chloride by sucrose or citrate also changes the cell membrane potential, and it is not impossible that the latter might interfere with urate transport.

Moreover, our other results do not argue in favour of the existence of an identical mechanism for the transport of inorganic anions and urate, since urate transport is much less sensitive to passive anion transport inhibitors than sulfate for instance: the inhibitory effect of salicylate (30 mmol/l) on urate transport, for which we obtained a significant reduction in the influx (30%) is much smaller than it is for sulfate transport, for which Schnell [19] obtained a 93% reduction with a salicylate concentration of $2 \cdot 10^{-2}$ mmol/l. This concentration salicylate has no inhibitory effect on urate transport. Dinitrofluorobenzene

is known to cause outflux of cell potassium [16]. We checked that this outflux was not responsible for reducing the urate influx, by adding ouabain, which inhibits active sodium and potassium transport, and let persist only potassium outflux, to an incubation medium containing no dinitrofluorobenzene. There was no reduction in the urate influx compared to the control.

The effects of pH on urate and chloride transport are not comparable: for chloride transport Gunn et al. [3] observed a reduction with increasing pH, however over the same pH range, whereas their chloride flux increased 1.3 fold, urate influx increased 14 fold. In addition, urate transport, contrary to that of chloride and sulfate, appears to be glycolysis dependent. The cumulative inhibitory effect obtained in the presence of a glycolysis inhibitor shows that there might be two different urate transport mechanisms, a passive diffusion mechanism and a mechanism connected with glycolysis. The linear relationship obtained between the urate influx and increasing concentrations seems to bear out this hypothesis.

As glycolysis appears to alter the urate influx, we set out to verify this hypothesis by studying the urate influx into ghosts prepared according to Passow's method [11] which renders them incapable of converting glucose into lactic acid but enables them to recover their initial permeability properties.

The fact that the urate influx is smaller in the ghosts and erythrocytes, where there is no glycolysis, than in control erythrocytes might suggest that glycolysis intervenes in urate transport. The drop in the concentration of the glycolysis-mediating metabolites formed from 3-phosphoglycerate does not appear to be the cause of the reduced urate influx. On the other hand, as the results obtained on ghosts in the absence of ATP seem to show, the latter appears able to modify urate transport. In addition, the fact that the urate flux into the red blood cells is larger than in the ghosts in the presence of ATP (Table I) seems consistent with this: thus ATP is regenerated by glycolysis in the red cells but not in the ghosts. In the latter, the initial ATP is used to activate the sodium/potassium pump, and its concentration therefore tends to decline. Moreover, the urate influx into the ghosts in the presence of ATP is comparable to the urate influx into the erythrocytes in the presence of sodium fluoride. This may be explained by the fact conditions are comparable in both cases, i.e. there is no glycolysis and the sodium/potassium pump functions thanks to the ATP present initially. All this seems to argue in favour of a possible relationship between the presence of ATP and urate transport. The part played by ATP is not known, but it is not impossible that the mechanism concerned involves a protein. In this connection we recently observed a great affinity between urate and a red cell membrane protein (unpublished data). However, further experimentation is necessary to study this interaction and the part it might play in urate transport.

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