ATP Dependent Uptake of Zinc by Human Erythrocyte Ghosts

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The energy dependence of Zn^{2+} accumulation by human erythrocytes was studied by preparing resealed ghosts loaded with ATP or AMP. Uptake of Zn^{2+} was followed by radioactivity or atomic absorption spectroscopy measurements. Zn^{2+} taken up is not adsorbed by the membrane but transported into the ghosts. Ghosts containing ATP took up more Zn^{2+} than those containing AMP. The effect is not specific for ATP, however, GTP and CTP showing the same increased uptake. Zn^{2+} uptake by ATP ghosts can be further drastically enhanced by addition of Cu^{2+}. Therefore, in spite of the enhancement of Zn^{2+} uptake by ATP, Zn^{2+} seems to be accumulated by a passive mechanism even when the ghosts contain ATP. The accumulation is probably brought about by binding of Zn^{2+} to substances inside the ghosts.

Introduction

All living cells contain zinc. Although some of the functions of Zn^{2+}, *e. g.* as cofactor of certain enzymes, are well established, surprisingly little is known about the way Zn^{2+} enters into the cells.

Zn^{2+} is accumulated by many cells. Zn^{2+} uptake into the green alga *Chlorella* [1], yeast [2], and *Escherichia coli* [3] is energy-dependent. For mammalian cells, an energy-dependent Zn^{2+} uptake could be shown only for rat jejunum [4]. While erythrocytes also accumulate Zn^{2+} [5], Fuchswans and Springer-Lederer found that inhibition of glycolysis, and therefore of ATP synthesis, had no effect on Zn^{2+} uptake [6]. They could not, however, exclude the possibility that during their experiments a pool of ATP continued to exist that was large enough to sustain an energy-dependent mechanism.

In the present work this difficulty has been overcome by hemolysing the cells and thus depleting them of their contents. By the method of reversible hemolysis ATP or AMP were included into the resealed ghosts ("ATP ghosts" and "AMP ghosts", respectively). The ghosts were then incubated in isotonic medium containing labeled $ZnCl_2$, and the uptake of Zn^{2+} measured. It was found that uptake by ATP ghosts exceeded that by AMP ghosts. Further experiments, however, supported the view that despite this ATP dependence Zn^{2+} is taken up into ghosts by a passive mechanism.

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Material and Methods

$ZnCl_2$ labeled with ^{65}Zn and carrier free $^{22}NaCl$ were purchased from the Radiochemical Centre, Amersham. Ouabain was bought from Fluka, Buchs, Switzerland, and the nucleoside phosphates (as sodium salts) from Boehringer, Mannheim. $CuCl_2$ labeled with ^{64}Cu was prepared at the Forschungsreaktor der österreichischen Hochschulen in Wien.

Fresh blood from healthy donors, collected into citrate buffer, was used within 3 hours after collection. Erythrocytes were isolated at 4 °C by centrifugation and aspiration of the plasma and buffy coat. Resealed ghosts containing ATP were prepared according to Askari [7], except that the Na^+ of the hemolysis solution usually was not labeled. In one experiment, however, ATP-containing ghosts were labeled with ^{22}Na to make sure that they extruded Na^+ actively, as described by Hoffman [8]. Since this was found to be the case (see Table I), the ghosts were taken to be intact and not freely permeable to inorganic ions.

The hemoglobin content of the ghosts was determined by the hemiglobin cyanide method (Merck, Darmstadt).

Different kinds of ghosts were prepared by hemolysing erythrocytes in solutions of equal osmolarities (not molarities), substituting 3 mM NaAMP or 6 mM sorbitol for 2 mM Na_2ATP, as used for the ATP ghosts. Thus differences between the ghosts, which might arise from different osmolarities of the hemolysis solutions [9], were precluded.

Immediately after preparation, 1 ml of the pelleted ghosts was pipetted into 30 ml of isotonic in-

cubation medium (37 °C, if not stated otherwise), which contained 154 mM NaCl, 6 mM KCl, and 10 mM Tris-HCl (pH 7.45). The hematocrit of this suspension was found to be 15 ± 1 µl packed ghosts per ml. After 20 min preincubation, 1 µM or 10 µM $^{65}ZnCl_2$ and other substances as needed (see Results) were added.

At intervals samples of the incubation suspension were centrifuged 1 min at $5200 \times g$, and 1 ml of the supernatant withdrawn. The radioactivity of this sample (A) and of 1 ml of the whole incubation suspension (B) were measured in a well-type γ-scintillation counter. The results of the Zn^{2+} uptake measurements are expressed as percentage (P_{Zn}) of the total added Zn^{2+} that was taken up by the ghosts: $P_{Zn} = 100(1 - A/B)$.

In some experiments, the Zn^{2+} added to the incubation medium was not labeled with ^{65}Zn. Samples were then taken as described above, and their zinc content determined with a Perkin-Elmer 300 S Flame Atomic Absorption Spectrometer. For these measurements the supernatant samples were used directly. The zinc contents of the ghost suspension before and after the addition of Zn^{2+} and of the intact cells could be determined only after complete solubilization of the membranes (0.3 ml 1% sodium dodecyl sulfate per ml of suspension). The ghosts were found to contain about one fifth as much zinc as intact erythrocytes, considerably more than reported by other workers [10]. (This is not surprising, because the cited work was performed with "white ghosts", *i. e.* ghosts completely free of hemoglobin. Such ghosts were not used in the present work as they will not reseal on reversal of isotonicity [11]. On the other hand resealed ghosts always contain some residual hemoglobin. The ghosts used in this work retained about 8% of their original hemoglobin content.)

Results

The time course of Zn^{2+} uptake varied considerably with ghosts from different preparations, although the ghosts always displayed the same properties qualitatively. Therefore for quantitative comparisons use was always made of ghosts from the same batch or from parallel batches prepared simultaneously.

Fig. 1 shows that during incubation for 4 – 5 h in medium containing 1 µM labeled Zn^{2+} the ATP ghosts took up more than 30% of the zinc. As the ghosts made up only 1.5% of the total suspension volume, this uptake corresponds to an accumulation factor of about 20. Fig. 1 further demonstrates that Zn^{2+} taken up by the ghosts cannot be removed by washing the ghosts once with Zn-free incubation medium. (Although this medium is isotonic, repeated washing was accompanied by rehemolysis of the ghosts, indicated by release of residual hemoglobin.)

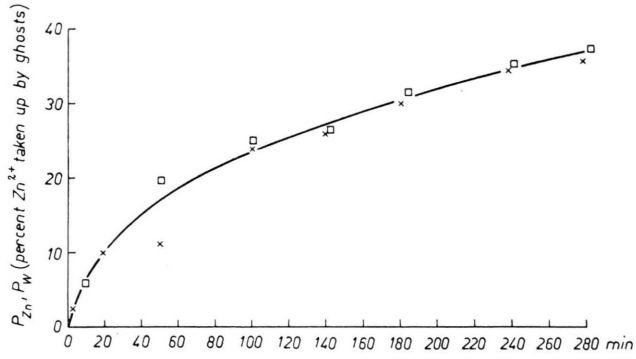

Fig. 1. Uptake of Zn^{2+} by ATP ghosts: effect of washing the ghosts. ×, P_{Zn} calculated from radioactivity in supernatant; □, P_W calculated from radioactivity of washed ghosts. Ghosts were prepared and incubated as described in Methods. Zn^{2+} concentration was 1 µM. Samples were taken as follows: 2 ml of the incubation suspension were centrifuged at $5200 \times g$ for 1 min. 1 ml of the supernatant was pipetted off, the radioactivity measured and P_{Zn} calculated as described in Methods. The rest of the supernatant was removed quantitatively and 1 ml of Zn^{2+}-free incubation medium added. The ghosts were resuspended, centrifuged, the supernatant removed, and the radioactivity of the washed ghost pellet determined. Zn^{2+} uptake was again calculated as the percentage of the total added Zn^{2+}, by $P_W = 100\ C/2\ B$. C is the radioactivity of the washed ghosts (from 2 ml suspension), B that of 1 ml of the whole incubation suspension.

An important point is whether Zn^{2+} taken up enters into the interior of the ghosts. It was found that heavy shaking of the ghosts in isotonic incubation medium leads to slow rehemolysis: the ghosts become leaky so that residual hemoglobin and other substances inside the ghosts are released into the medium. If accumulated Zn^{2+} is located inside the ghosts (in whatever form), rehemolysis should therefore cause Zn^{2+} release into the medium. If, however, Zn^{2+} was bound to the membrane, rehemolysis should have no effect on Zn^{2+} uptake. The former was found to be the case, as shown in Fig. 2.

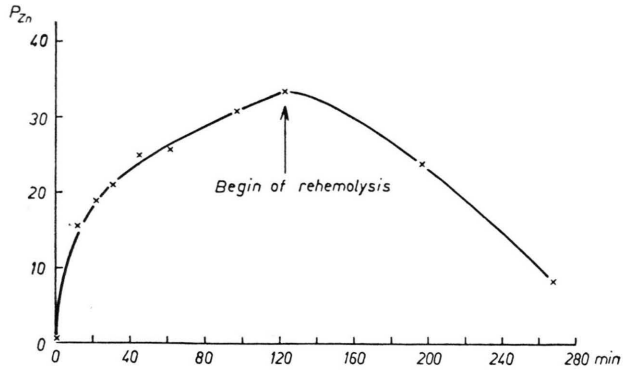

Fig. 2. Effect of rehemolysis on Zn^{2+} uptake. ATP ghosts were prepared and incubated in medium containing 10 μM Zn^{2+} as described in Methods. The ghosts took up Zn^{2+} for 2 hours, when heavy shaking of the incubation vessel began. This method of rehemolysis avoids interruption of the incubation or change of the chemical environment of the ghosts. As the rehemolysed ghosts could be easily centrifuged down, sampling was the same before and after onset of rehemolysis (see Methods).

The energy dependence of Zn^{2+} accumulation was tested by comparing ghosts containing ATP or AMP. Fig. 3 shows that at 37 °C ATP ghosts took up more Zn^{2+} from 1 μM solution than AMP ghosts. At 16 °C, however, Zn^{2+} uptake was vey low, with no difference between ATP and AMP ghosts.

As the ghosts used still contained considerable amounts of Zn^{2+} (2.8 μg/ml packed cells), it was checked whether the increased uptake of labeled Zn^{2+} by ATP ghosts was due to increased exchange for Zn^{2+} originally contained in the ghosts. For this

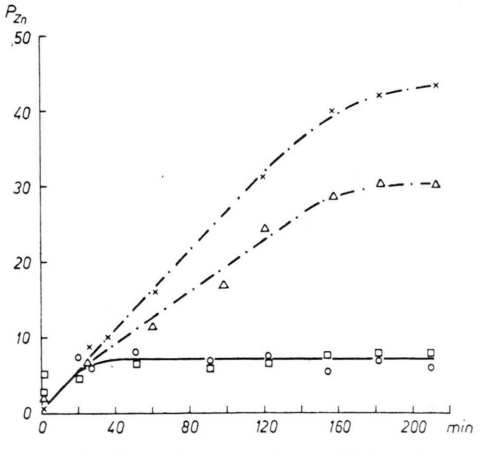

Fig. 3. Zn^{2+} uptake into ghosts filled with ATP or AMP. Radioactivity measurements ×, ghosts containing ATP, incubation at 37 °C; △, ghosts containing AMP, incubation at 37 °C; ○, ghosts containing ATP, incubation at 16 °C; □, ghosts containing AMP, incubation at 16 °C.

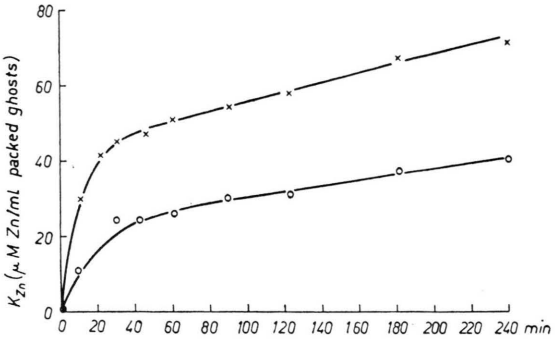

Fig. 4. Uptake of Zn^{2+} by ghosts filled with ATP or AMP. Measurements by atomic absorption spectroscopy (AAS). ×, ATP ghosts; ○, AMP ghosts. Experimental procedure as described in Methods. In experiments with AAS, non-labeled Zn^{2+} was added to a final concentration of 10 μM instead of 1 μM, in order to obtain the needed precision. (In a control experiment it was shown that the uptake curves of labeled Zn^{2+} from 10 μM solution by ATP and AMP ghosts are similar to those in Fig. 3.) The K_{Zn} values are defined by $K_{Zn}=A-B$, where B is the amount of zinc contained in the ghosts immediately after addition of Zn^{2+} to the incubation suspension, and A the amount of zinc in the ghosts after different incubation times. Both A and B are given as μM Zn/ml packed ghosts and were determined by AAS.

purpose, the net changes of Zn^{2+} concentration in the medium during the incubation of ATP ghosts and AMP ghosts were followed by atomic absorption spectroscopy (AAS). According to Fig. 4 the results of the AAS measurements match those of the radioactivity measurements (Fig. 3). Thus the amount of Zn^{2+} taken up by ATP ghosts really exceeds that taken up by AMP ghosts.

The stability constant for the complex of Zn^{2+} with ATP is much higher than with AMP ($\log K=$ 5.21 *vs* 2.23) [12]. Consequently, the additional accumulation of Zn^{2+} by ATP ghosts over AMP ghosts could be caused by complexing additional Zn^{2+} inside the ghosts. Therefore, AMP ghosts were compared with ghosts containing sorbitol instead, which does not form a complex with Zn^{2+}. No difference in Zn^{2+} uptake was found (Fig. 5).

These results suggested that ATP has a specific influence on Zn^{2+} uptake. It was therefore tested whether this was due to a linkage of Zn^{2+} transport to the active transport of Na^+ and K^+. In two experiments, the effect of Zn^{2+} on active Na^+ efflux and the effect of ouabain, a potent inhibitor of active Na^+ transport, on Zn^{2+} uptake were measured. According to Table I, Zn^{2+} had no effect on active efflux of Na^+. In the second experiment, 1 ml each of pelleted ATP ghosts was suspended in 30 ml of

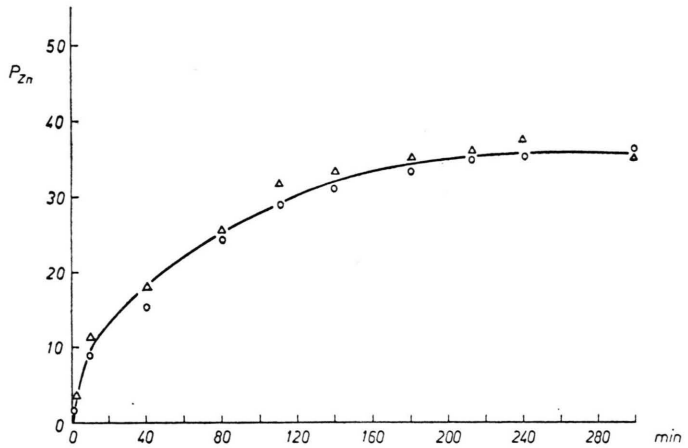

Fig. 5. Uptake of Zn^{2+} by ghosts containing AMP or sorbitol. ○, AMP ghosts; △, sorbitol ghosts. Experimental procedure see Methods. Zn^{2+} concentration was 1 μM.

Table I. Effect of Zn^{2+} on active efflux of Na^+ from ghosts.

Minutes from begin of incubation	Control % Na	$ZnCl_2$ (1 μM) % Na	Ouabain ($2 \cdot 10^{-4}$ M) % Na
1	9.3	8.4	8.3
60	44.6	45.3	26.1
130	61.6	64.1	33.8
200	71.8	73.2	39.1
300	79.9	80.9	47.2

ATP ghosts containing $^{22}Na^+$ were prepared as described in Methods. 1 ml of pelleted ghosts was suspended in 30 ml incubation medium (see Methods) containing 1 μM non-labeled $ZnCl_2$ or $2 \cdot 10^{-4}$ M ouabain. Control: no addition to medium. Samples were taken as described in Methods. % Na is defined by 100 A/B, where A is the radioactivity of 1 ml of supernatant, B of 1 ml of whole ghost suspension.

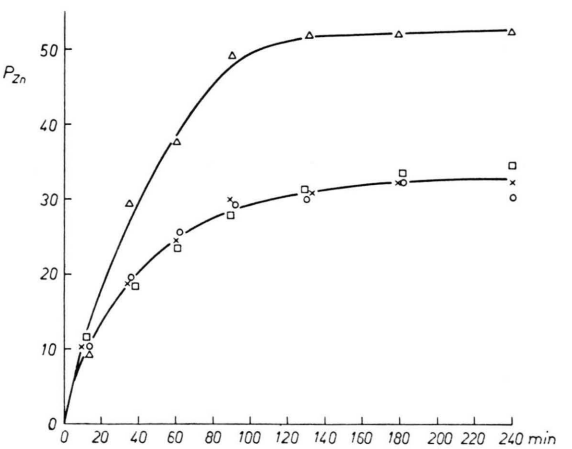

Fig. 6. Effect of other divalent metal ions on Zn^{2+} uptake by ATP ghosts. ×, no addition; ○, Cd^{2+}; □, Co^{2+}; △, Cu^{2+}. 1 ml each of pelleted ghosts were suspended in 30 ml incubation medium (see Methods). After preincubation for 20 min at 37 °C, labeled Zn^{2+} and the other ions as indicated were added as the chlorides, all to a final concentration of 10 μM.

incubation medium (see Methods) containing no ouabain and $2 \cdot 10^{-4}$ M ouabain, respectively. Both Zn^{2+} uptake curves were found to be identical, although ouabain did inhibit active transport of Na^+ as expected (Table I). These two experiments exclude an interaction between Zn^{2+} uptake and the ATP-dependent active transport of Na^+.

Therefore, the possibility of an ATP driven carrier for Zn^{2+} was considered. As such a carrier could be specific for Zn^{2+}, the effect of other divalent heavy metal ions on Zn^{2+} uptake by ATP ghosts was tested. Ions transported by the same pathway as Zn^{2+} should inhibit Zn^{2+} uptake. However, of the heavy metal ions tested, Cd^{2+} and Co^{2+} had no effect, while Cu^{2+} drastically enhanced Zn^{2+} uptake (Fig. 6). Results similar to those of Fig. 6 were obtained when Zn^{2+} accumulation into intact erythrocytes was studied (W. Fuchswans and H. Springer-Lederer, unpublished results). In search for an explanation of this unexpected result, the uptake of labeled Cu^{2+} by ghosts was studied (Fig. 7). Both ATP and AMP ghosts were found to accumulate Cu^{2+} more strongly than Zn^{2+}. As with Zn^{2+}, ATP ghosts took up still more Cu^{2+} than AMP ghosts.

As none of the ions tested inhibited Zn^{2+} uptake, it was tested whether the uptake mechanism by ATP ghosts was specific for ATP. Fig. 8 shows Zn^{2+} uptake into ghosts containing equimolar amounts of ATP, CTP, or GTP, respectively. All three uptake curves are identical within experimental error.

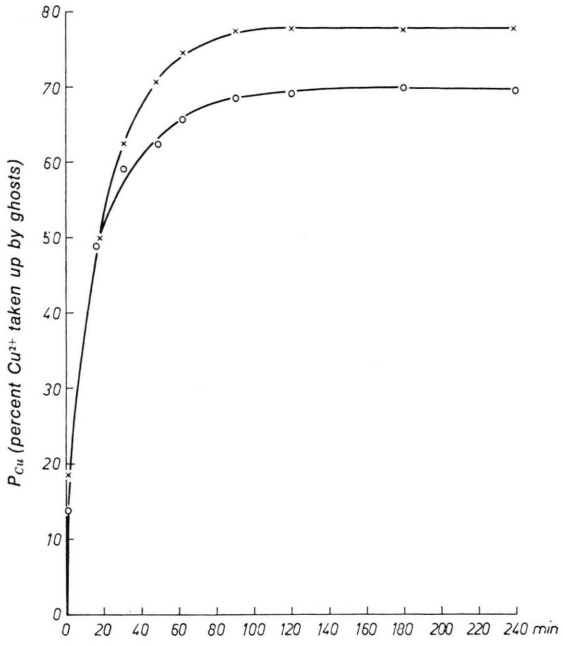

Fig. 7. Uptake of Cu^{2+} by ghosts containing ATP or AMP. ×, ATP ghosts; ○, AMP ghosts. Experimental procedure as for Zn^{2+} uptake (see Methods), except that instead of Zn^{2+} $CuCl_2$ labeled with ^{64}Cu was added to a final concentration of 10 μM Cu^{2+}. P_{Cu} is defined analogous to P_{Zn}.

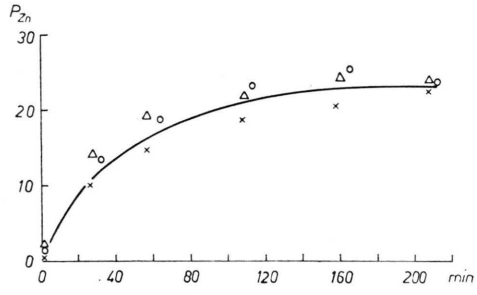

Fig. 8. Uptake of Zn^{2+} by ghosts containing different nucleoside triphosphates. ×, ATP ghosts; ○, GTP ghosts; △, CTP ghosts. GTP ghosts and CTP ghosts were prepared by substituting in the hemolysis solution 2 mM GTP or 2 mM CTP for 2 mM ATP. Otherwise procedure as described in Methods.

Discussion

Although it has long been known that erythrocytes accumulate Zn^{2+} [13], no mechanism has sofar been known. The possibility that this accumulation is energy-dependent has now been investigated by studying Zn^{2+} uptake into resealed ghosts with different contents.

All available evidence is consistent with the conception that Zn^{2+} enters into the ghosts. The fact that Zn^{2+} taken up is not readily removed by washing (Fig. 1), does not exclude, however, the possibility that Zn^{2+} is tightly bound to the outside of the membrane. At 16 °C ghosts are apparently impermeable to Zn^{2+}, the small residual uptake being due to adsorption to the outside of the membrane. Indeed it is known that erythrocyte membranes undergo a phase transition at 18 – 19 °C, at which temperature the kinetics of glucose uptake also has a discontinuity [14]. As no active transport mechanism is possible in AMP ghosts at any temperature, the difference in Zn^{2+} uptake at 16 °C and at 37 °C (Fig. 3) could be the amount of Zn^{2+} entering the ghosts. A direct proof for the entrance of Zn^{2+} into the cells is furnished by the rehemolysis experiment (Fig. 2).

At least part of the Zn^{2+} accumulation by ghosts must be energy-independent, because AMP ghosts also accumulate Zn^{2+}, though only by a factor of about 10 – 15 as compared with 20 – 30 for ATP ghosts (Figs 3 and 4). Energy-independent Zn^{2+} accumulation (as measured with AMP ghosts) is only possible if Zn^{2+} taken up is bound within the ghosts. The concentration of free Zn^{2+} can thus remain lower inside than outside the ghosts and Zn^{2+} can be transported into the ghosts by passive diffusion. An important complexing agent inside the ghosts is undoubtedly the residual hemoglobin, which is known to bind Zn^{2+} tightly [13, 15]. For the additional accumulation by ATP ghosts three mechanisms are possible: 1. As ATP forms stronger complexes with Zn^{2+} than AMP, the additional uptake could be caused by additional binding of Zn^{2+} inside the ghosts. 2. ATP causes binding of additional Zn^{2+} to other substances (*e. g.* by phosphorylating them). 3. Active transport of Zn^{2+} by ATP ghosts.

Table I shows that Zn^{2+} accumulation is independent of the Na^+ and K^+ pump. The effect of Ca^{2+}, which is actively extruded by ghosts [16], on Zn^{2+} uptake was also studied, but the results (not shown) were inconclusive. No indication of an active transport of Zn^{2+} by means of the Ca^{2+} pump was obtained, however. If Zn^{2+} were transported actively, there would have to be a carrier of its own. Although the existence of such a carrier cannot be ruled out, it appears likely that even in ATP ghosts no energy-dependent mechanism is involved, because GTP or CTP have the same effect as ATP (Fig. 8). In contrast, energy-dependent carriers

such as the Na^+ and K^+ dependent ATPase of erythrocyte membranes have different affinities to different nucleotides [17].

Cu^{2+} is known to increase the permeability of the erythrocyte membrane for K^+, causing passive efflux of accumulated K^+ from whole cells [18]. Therefore the drastic increase by Cu^{2+} of Zn^{2+} uptake into ATP ghosts (Fig. 6) also points to a passive mechanism. For if Zn^{2+} were accumulated by active transport, the addition of Cu^{2+} would be expected to cause leak, *i. e.* efflux of Zn^{2+}, thus lowering net Zn^{2+} uptake. (Similar results have been obtained with the green alga Chlorella fusca, where on addition of Cu^{2+} the plasma membrane becomes more permeable to K^+ and Zn^{2+} uptake is increased [19].) Although the reason for the enhancement of Zn^{2+} uptake by Cu^{2+} is unclear, it is interesting to note that Cu^{2+} is accumulated by ghosts to a higher degree and more rapidly than Zn^{2+}, but also ATP-dependent (Fig. 7). Uptake of Cu^{2+} by intact erythrocytes (which contain, of course, ATP), however, is already known to proceed by a passive mechanism [20]. This also shows that enhancement of Zn^{2+} uptake by ATP need not be an active process.

Although active transport of Zn^{2+} can thus be ruled out, the mechanism of the increase of uptake by ATP is not quite clear. Fig. 8 suggests that the additional Zn^{2+} is bound by the nucleoside triphosphates. (The binding constants for GTP and CTP are not known, but can be assumed to be very similar to that of ATP, because the phosphate moiety is the same in all three molecules.) On the other hand, ghosts containing AMP or sorbitol, which have very different binding constants for Zn^{2+}, also take up equal amounts of Zn^{2+}. There are reports that ghosts containing ATP differ morphologically [21, 22] and chemically (*e. g.* [23, 24]) from those without ATP. No comparable data are available for the other nucleoside triphosphates. It might, however, be speculated that the triphosphates alter some substance inside the ghosts so that more Zn^{2+} can be bound.

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