J. Membrane Biol. 62, 195–206 (1981)

The Journal of
Membrane Biology

Chloride Permeability in Human Red Cells: Influence of Membrane Protein Rearrangement Resulting from ATP Depletion and Calcium Accumulation

R. Motais, A. Baroin, and S. Baldy

Laboratoire de Biologie Marine, Département de Biologie du Commissariat à l'Energie Atomique, Station Marine, B.P. 68, 06230 Villefranche-sur-Mer, France

Summary. As 15% of band 3 protein, the assumed chloride channel, is associated with spectrin, the major peripheral protein of a lattice located at the red cell membrane-cytosol interface, the present study was undertaken to evaluate whether a rearrangement of the lattice modifies the functional property of band 3 protein. Such a rearrangement was modulated by depletion of cell ATP and/or by accumulation of Ca^{2+} ions within the cell.

ATP depletion induces an inhibition of the electroneutral one-for-one chloride exchanges. Neither the modification of red cell morphology due to ATP depletion (discocyte-echinocyte transformation) nor a direct effect of the decrease in internal ATP level can account for this inhibition. On the other hand, it seems reasonable to consider that inhibition is related to the changes in membrane protein organization (formation of heteropolymers) induced by the decrease in ATP level. But it does not appear that the degree of inhibition is modified when this altered assembly of membrane protein is stabilized by disulfide linkages.

Accumulation of Ca^{2+} ions in the cell at a relatively low concentration (10 μM range) inhibits chloride exchange without apparent modification of the assembly of membrane proteins. This effect of calcium on chloride exchanges is speculatively denoted as a "direct" effect of calcium.

Calcium loading of fresh red cells at higher concentrations (500 to 1000 μM) obtained by use of the ionophore A23187 induces a very strong inhibition of chloride exchanges. In this case, inhibition can be reasonably accounted for by two simultaneous effects of calcium: a "direct" effect which explains half of the inhibition and an "indirect" effect due to the formation of membrane protein complexes stabilized by covalent crosslinkages (activation by Ca^{2+} ions of a transglutaminase).

It is interesting to note that intracellular calcium, whatever the level, inhibits electroneutral exchanges of chloride but increases net chloride movements.

Key words: Red blood cell, chloride permeability, membrane protein complexes, intracellular calcium, metabolic dependence

It has recently been suggested (Bennett & Stenbuck, 1979) that about 15% of band 3 protein, an intrinsic membrane protein of the erythrocyte, is associated with spectrin, the major peripheral protein of a lattice located at the red cell membrane-cytosol interface. Such protein interactions are certainly involved in the control of intramembrane particle distribution. Since band 3 protein has been implicated in anion transport (Cabantchik & Rothstein, 1974), however, the question arises whether a rearrangement of the lattice modifies the functional property of band 3 protein.

ATP depletion and accumulation of Ca^{2+}, by altering the physical properties of the proteins of the lattice, result in modification of the red cell morphology (discocyte-echinocyte transformation). It has been suggested that in this configuration spectrin and some other polypeptides are rearranged into a more intimate contact with each other (Palek, Liu & Snyder, 1978*b*).

This discocyte-echinocyte transformation of red cells is generally reversible upon restoration of normal ATP and calcium levels. It becomes irreversible, however, after a prolonged ATP depletion or after a high calcium concentration is introduced into the cells. In these conditions it has been shown that the protein rearrangement is stabilized by spontaneous crosslinking, with formation of heteropolymers (Palek, Liu & Liu, 1978*a*). Taking advantage of these

0022-2631/81/0062-0195 $02.40

modifying effects of ATP and Ca^{2+}, the present study was undertaken to evaluate the influence of membrane protein rearrangement on chloride transport.

Materials and Methods

Preparation of Cells

Freshly collected human blood was obtained from a blood bank. It was then centrifuged for 5 min at $3,000 \times g$, and plasma and buffy coat were discarded by aspiration. The red cells were washed three times in 154 mM NaCl and used immediately, as described below.

Anaerobically and aerobically ATP-depleted cells. Red cells at 10% hematocrit were incubated in 50 mM glycylglycine, 140 mM NaCl, 5 mM KCl and 2 mM $MgCl_2$ at pH 7.4. 20 μg streptomycin and 50 U penicillin were added per ml of suspension. The pH was readjusted throughout incubation when necessary. Half of the suspension was incubated for 20 hr at 37 °C under a constant flow of prewashed humidified nitrogen gas, whereas the other half was incubated under oxygen. To restore the intracellular ATP concentration, anaerobically and aerobically depleted cells were subsequently incubated for 4 hr in the medium supplemented with 0.5 mg adenine, 12.7 mg inosine, and 2 g/liter glucose under nitrogen. After 20 min incubation, 10 mM DTT was added in the aerobically depleted cell suspension.

ATP-depleted cells in presence of $CaCl_2$. The incubations were carried out at 37 °C for 26 hr at 20% hematocrit in the same medium as above, supplemented with 2 mM $CaCl_2$. In some experiments 5 mM iodoacetic acid and 5 mM inosine were added in the medium of incubation to ensure rapid depletion.

Ionophore A23187-treated cells. The cells were suspended at 10% hematocrit in 25 mM Hepes, 140 mM NaCl, 10 mM KCl, and 2 mM $MgCl_2$ at pH 7.4 at 20 °C. 10^{-5} M ionophore A23187 (stock solution: 10^{-3} M in dimethylsulphoxide) and 1 mM $CaCl_2$ were added, and after 30 min incubation at 37 °C cells were washed twice in cold isotonic 0.5% bovine serum albumin solution. Intracellular ATP and normal intracellular Ca^{2+} levels were restored by prolonged incubation of cells (18 hr) at 37 °C with 0.5 mM adenine, 12.7 mM inosine and 2 g/liter glucose. Cells treated in the presence of histamine were incubated in medium supplemented with 40 mM dichlorhydrate histamine.

ATP and GSH Measurements

ATP was measured by the firefly bioluminescence method with a Chemglow apparatus. Typically, 50 μl of packed red cells were hemolysed in 5 ml of 10 mM Hepes buffer, pH 7.5, and placed in a boiling water bath for 2 min. The extract was stored at −18 °C. Firefly lantern extracts and ATP (adenosine 5′ triphosphate disodium salt) were purchased from Sigma or Lumac for the assay procedure.

Levels of reduced GSH were measured using methods detailed by Beutler (1975).

Cell Morphology

Cell morphology was controlled either by phase contrast or scanning electron microscopy. The red cells were suspended in a 1% glutaraldehyde cold isotonic buffer solution, pH 7.4. After incubation at 4 °C, they were either examined by use of phase-contrast microscopy or treated as described in Motais, Baroin, Motais and Baldy (1980) for examination in a Cameca MEB 07 scanning electron microscope.

Sodium Dodecylsulfate Polyacrylamide Gel Electrophoresis

In the first dimension, high molecular weight complexes were resolved by SDS-agarose-polyacrylamide gel electrophoresis (2.5% acrylamide, 3.75% Bis/mono, 0.3% agarose, and 0.2% SDS) as described by Liu, Fairbanks & Palek (1977). The discontinuous gel system described by Laemmli (1970) was employed in a gel slab for resolution in the second dimension. The stacking gel was formed of 2.67 and 8% acrylamide, 3.75% Bis/mono and 0.2% SDS. A 2-mm melted (40 °C) reducing agent band (1% agarose, 1% SDS, 40 mM DTT) was layered on top of the stacking gel and immediately covered with the first dimensional cylindrical gel.

Cell membranes were prepared by lysing packed red cells in 30 volumes of cold 10 mM Tris HCl buffer, pH 7.4. Membranes were collected by centrifugation at $29,000 \times g$ for 20 min and were washed 3 times with Tris HCl. They were solubilized in a 50% sucrose, 5% SDS, 5 mM EDTA, 50 mM Tris, 0.1 mg/ml pyronin γ solution for 20 min at 37 °C.

Flux Measurements

The chloride self-exchange fluxes were measured at Donnan equilibrium at 0 °C. Labeling of cells with ^{36}Cl, isolation of labeled cells, determination of radioactivity in cell-free medium, and the automatic technique of flux measurements have previously been described (Cousin & Motais, 1976). The final hematocrit of the cell suspension was 0.5%. In all cases, the medium used was identical to the corresponding medium of incubation. The pH of the medium during flux measurements was 7.8. We did not readjust the pH of the cell suspension when it was transferred from 37 to 0 °C because pH readjustment brings about hemolysis of the depleted cells. All efflux measurements were done on triplicate samples of the labeled packed red cells.

Calculations

The kinetics for Cl^- efflux were well described by a two-compartment model with a constant volume. The equation describing the time dependence of the specific activity in a cell-free medium is:

$$Q_t = Q_\infty (1 - e^{-(K_o + K_i)t})$$

wherein Q_t and Q_∞ are the concentration of isotopes in the external medium at time t and at isotopic equilibrium, respectively, the exponents K_o and K_i are the rate coefficients for isotope efflux and influx, respectively. In our experiments K_i could be neglected because the hematocrit was low (0.5%). The rate constant was calculated from the relation between $\log\left(1 - \frac{Q_t}{Q_\infty}\right)$ and the time t by linear regression analysis. The slope of the graph was assumed to be equal to $-K_o$. Treatment of the cells may vary the intracellular volume of water and so fractional inhibition of the transport cannot be related only to the fractional decrease of the rate constant K_o. We calculated the fluxes per kg of cell solids according to Funder and Wieth (1976), using the equation:

$$J = K_o \times \frac{V}{S} \times C$$

where J is the flux (mmol/kg of cell solids · min), V (cm^3) is the volume of intracellular water, S (kg) is the dry weight of the cell and Ci (moles/cm^3/intracellular water) is the intracellular chloride concentration.

The dry weight S of the cells is easily determined as the residual weight of the cells after evaporation of cell water (50 μl of packed red cells in triplicate, 48 hr at 105 °C). The apparent cell water volume evaporated was then corrected for the extracellular trapped volume. To determine this, we added ^{14}C inuline (Centre d'Etudes Nucléaires de Saclay, France) to an aliquot of the cell suspension at the end of the incubations and spun it down simultaneously with the ^{36}Cl-labeled cells prior to flux measurements. Triplicate samples of the supernatant (50 μl) and of the pellet (50 μl) were precipitated in 1 ml of 1.5% cold perchloric acid. Radioactivity was measured by liquid scintillation counting. The ratio of counts gives the percentage of extracellular trapped water content.

The intracellular chloride concentration was similarly determined as the product of the distribution ratio of radioactive chloride and the chloride concentration in the medium. Determination of the distribution ratio was done in exactly the same way as for the ^{14}C inuline space using ^{36}Cl-labeled cell suspension.

Abbreviations

SDS, sodium dodecyl sulfate; ATP, adenosine triphosphate; GSH, glutathione; Tris, Tris-(hydroxymethyl-)aminomethane; HEPES, N-2-hydroxy-ethyl piperazine-N-2-ethanesulfonic acid; DTT, dithiothreitol; EDTA, ethylenediaminotetraacetate disodique salt; RBC, red blood cells; *W.S.*, weight solid.

Results

1. The Effects of ATP Depletion

Cell morphology and ATP content. Figure 1 shows the morphology of red cells after a 20 hr incubation period at 37 °C without glucose, under anaerobic and aerobic conditions. Incubation under nitrogen leads to an heterogeneous cell population with nearly 100% echinocytes at different stages of transformation. Aerobically treated cell population is 100% echinocytic and homogeneous. Drastic decreases of the intracellular ATP content are associated with these changes of shape (Table 1). The final ATP contents of the anaerobically and aerobically depleted cells are less than 1% of the starting level.

Incubation of these depleted cells with metabolic substrates (*see* Materials and Methods) induces an almost complete reversal of echinocytes to discocytes, as illustrated in Fig. 1. However, it must be pointed out that for aerobically ATP-depleted cells, repletion treatment was performed in the presence of the reducing DTT agent. Indeed, as discussed in the next paragraph, when red cells are ATP-depleted under aerobic conditions, a polymerization of membrane proteins occurs which is reducible by DTT. The presence of DTT in the repletion medium greatly improves the capacity of the cells to resume their native shape (Palek et al., 1978*b*). After a long and drastic ATP-depletion, the kinetics of the restoration of ATP level is slow (Feo & Leblond, 1974). In fact, in our conditions (4-hr repletion treatment) ATP levels are poorly increased (Table 1): for anaerobically ATP-depleted cells, ATP content at repletion does not exceed 10% of the initial level, while in aerobically ATP-depleted cells it does not significantly increase. If repletion treatment is performed in the presence of PO_4^{2-}, the final ATP concentration is higher but it remains far below the initial level (0.31 μM ATP/ml RBC in PO_4^{2-}-containing medium, i.e., 20% of initial concentration, against 0.11 μM/ml RBC in PO_4^{2-}-free medium). All these data suggest that the ATP concentration *per se* is not the direct factor which controls cell shape. This lack of correlation between ATP level and cell morphology is illustrated in the experiment described in Fig. 2, which confirms previous data (Feo & Mohandas, 1977): aerobic ATP-depletion performed in the presence of 1 mM iodoacetic acid, an enzymatic inhibitor of glycolysis, induces a rapid drop of intracellular ATP, and it can be observed that at the time when the ATP concentration falls to zero (about 80 min) almost all the cells are discocytes.

Membrane protein association. Figure 3 shows the SDS-polyacrylamide gel electrophoresis of solubilized membrane proteins from red cells undergoing ATP depletion during anaerobic incubation and after the subsequent ATP repletion. As reported earlier by Palek et al. (1978*b*), both gel patterns are similar to that of fresh cells.

In contrast, we observed in the polyacrylamide gel electrophoresis of membrane proteins from red cells depleted under aerobic conditions (Fig. 4*a*), the formation of a slowly migrating complex of apparent mol wt 450,000 daltons and of a high molecular weight complex (mol wt $> 10^6$ daltons) which remains at the origin of the gel. Treatment of the cells with DTT reverse the formation of these polymers. Only the reducible complex of mol wt $> 10^6$ is related to a decrease of cellular ATP (Palek et al., 1978) and it can be shown that it contains, among other membrane proteins, spectrin and band 3. This can be checked by employing two-dimensional SDS-polyacrylamide gel electrophoresis as illustrated in Fig. 4*b*: since the polymer is reducible by DTT, the first dimensional gel containing the complex is treated with DTT and the cleaved components are identified by electrophoresis in the second dimension.

This formation of membrane heteropolymers through disulfide bonds in aerobically ATP-depleted cells is associated with a net decrease of the intracellular reduced GSH concentration (Table 1). GSH level reduces to about 40% of its preincu-

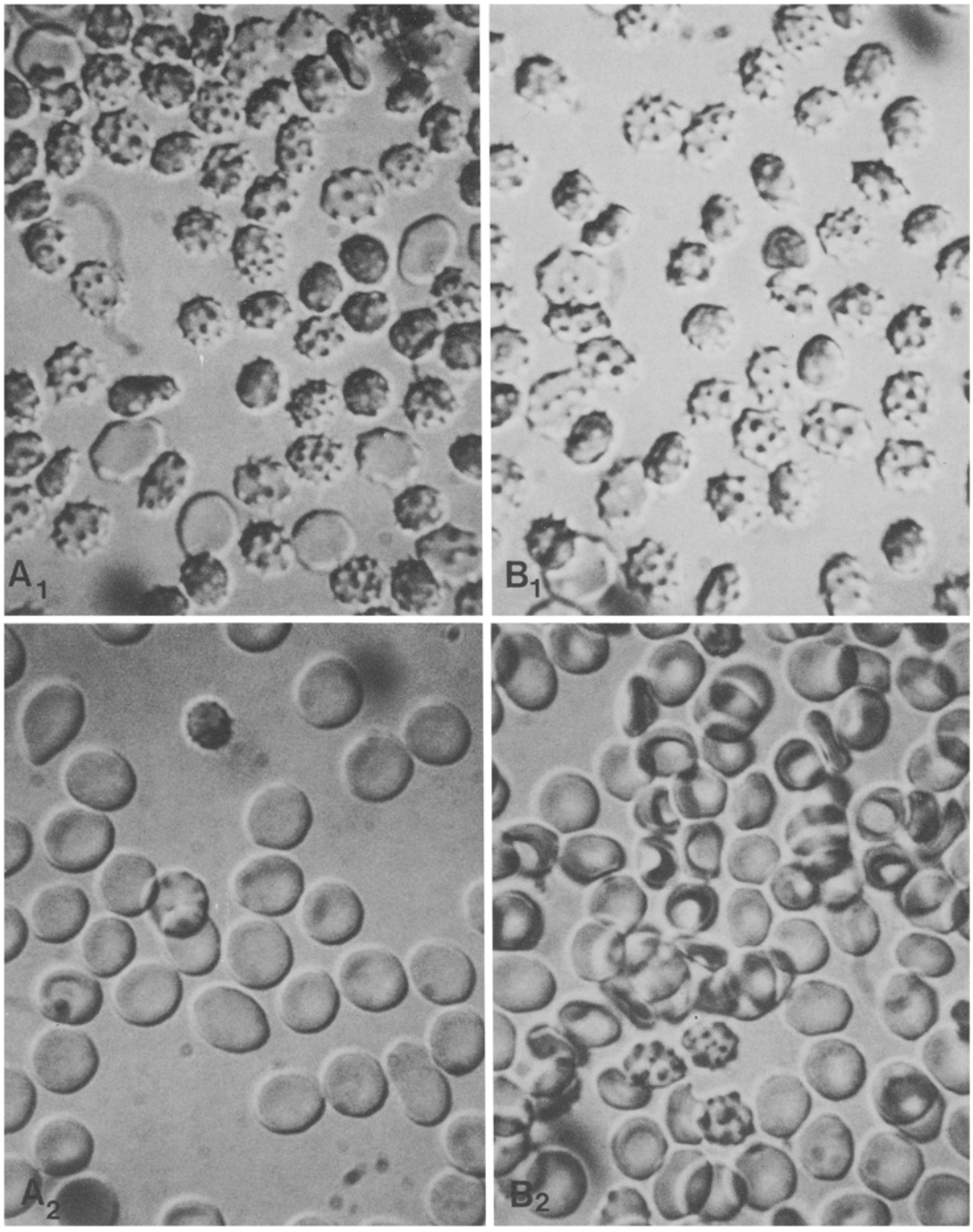

Fig. 1. Morphological aspects of red cells. (a_1): ATP-depleted cells under anaerobic conditions. (a_2): The same cells after repletion. (b_1): ATP-depleted cells under aerobic conditions. (b_2): The same cells after repletion. Repletion is carried out in the presence of 10 mM DTT

bation level. At the end of repletion treatment, GSH had practically recovered its initial value. It is worth noting that in the absence of O_2 (anaerobic ATP-depletion) the reduced GSH concentration does not vary throughout incubation apart from a slight and insignificant decrease (Table 1).

Cl^- self-exchange fluxes. As described in Materials and Methods, to take into consideration the cellular shape variations, we related the values of the Cl^- self-exchange fluxes with the weight of cell solids. Table 1 shows the values obtained for cells depleted under anaerobic and aerobic conditions, respectively.

Table 1. Evolution of ATP, GSH, and chloride fluxes under anaerobic and aerobic conditions

	ATP depletion under N_2 ($n=4$)			ATP depletion under O_2 ($n=3$)		
	ATP	GSH	Fluxes	ATP	GSH	Fluxes
A	1.35±0.21	2.41±0.37	606±52	1.620±0.086	2.50±0.10	603±60
B	0.02±0.009	2.20±0.82	414±50	0.005±0.004	1.00±0.25	389±26
C	0.11±0.075	2.03±0.53		0.008±0.001	2.02	

Incubations were performed as described in Materials and Methods. A, B, C refer respectively to fresh cells, ATP-depleted cells, and repleted cells. ATP and GSH levels are expressed in μmol per ml of RBC, and fluxes are expressed in mmol per min · per kg of weight solids. Values and standard deviations are derived from a number of experiments indicated by *n*.

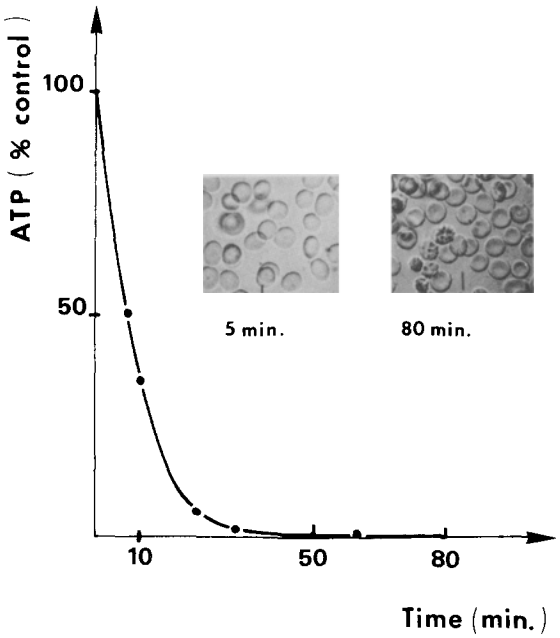

Fig. 2. ATP-depletion of red cells in 5 mM iodoacetic acid glucose-free buffer; relation to cell echinocytic transformation. *Abcissa:* incubation time in minutes. *Ordinate:* ATP content expressed as the percentage of the initial value. Incubation medium (in mM): 50, glycylglycine; 150, NaCl; 5, KCl; 2, $CaCl_2$, 2, $MgCl_2$; 5, iodoacetic acid; and 5, inosine. Micrographs show the morphological aspect of the red cells after the time of incubation indicated below

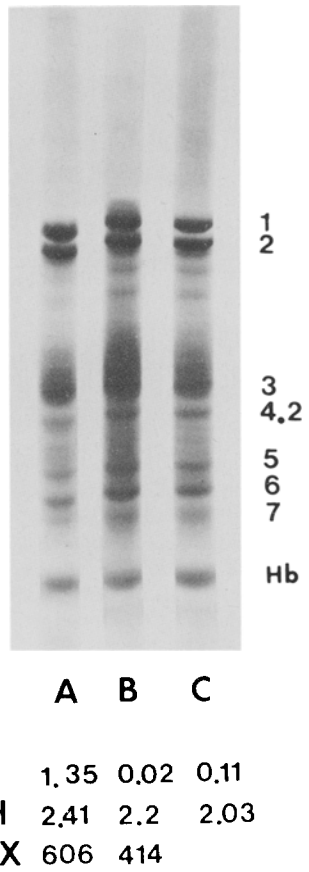

Fig. 3. Relationships between chloride self-exchange fluxes, GSH and ATP levels, and membrane protein composition of fresh cells (*A*) ATP-depleted cells under anaerobic conditions (*B*) and repleted cells (*C*). ATP and GSH levels are expressed in μmol per ml of RBC, and fluxes are expressed in mmol per min per kg of weight solids. Membrane proteins were separated by electrophoresis in SDS-agarose (0.3%), acrylamide (2.5%) without DTT reduction. The membrane polypeptides are indicated by numbers according to Fairbanks, Steck and Wallach (1971). Hb: hemoglobin

The Cl^- transport is inhibited to the same extent in the two cell populations. These inhibitions are not drastic since they are in both cases around 35%. When anaerobically and aerobically ATP-depleted cells reverse to their native shape after repletion, Cl^- transport generally does not recover its initial capacity in either population. The inhibitions of transport are reduced, but the degree of recovery varies widely from one experiment to another. We can thus only affirm that qualitatively, despite the com-

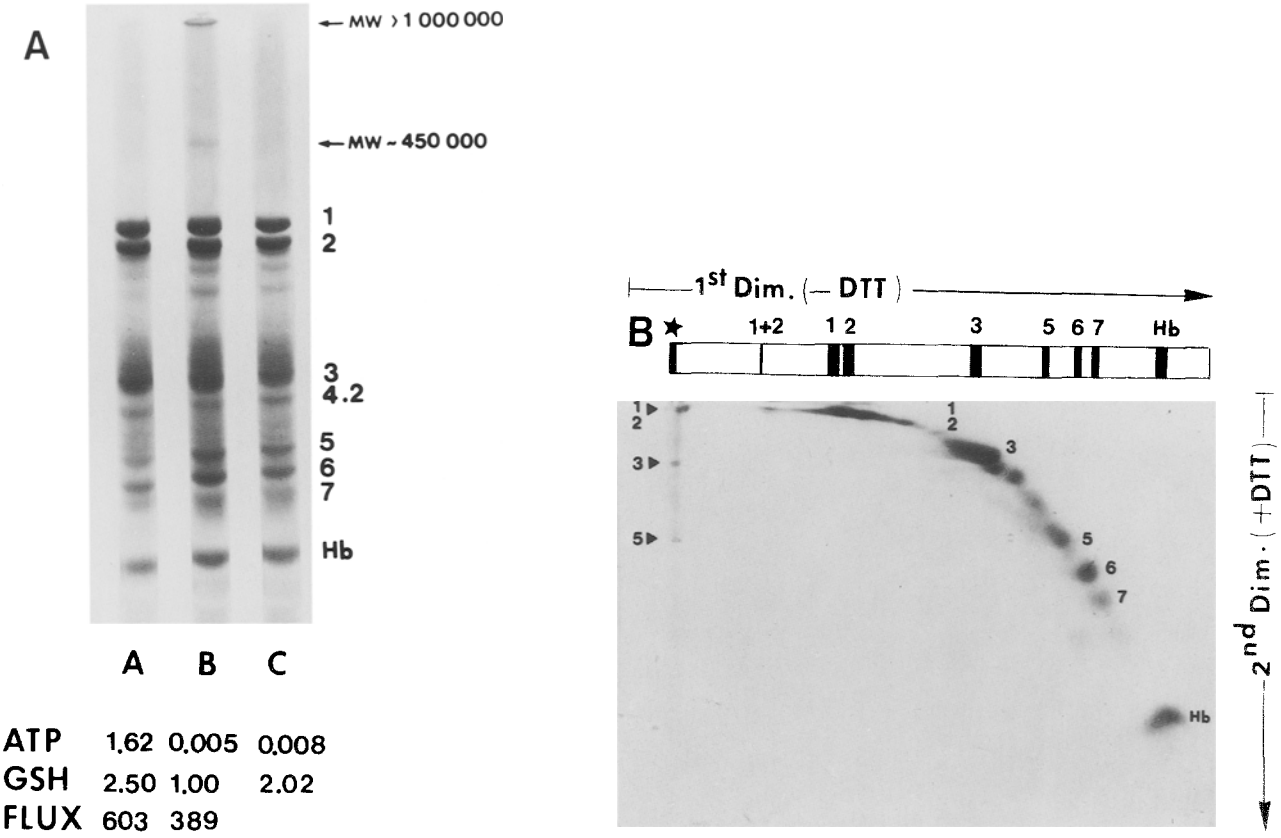

Fig. 4. (*a*): Relationships between chloride self-exchange fluxes, GSH and ATP levels, and membrane protein composition of fresh cells (*A*), ATP-depleted cells under aerobic conditions (*B*) and repleted cells in presence of 10 mM DTT (*C*). ATP and GSH levels are expressed in μmol per ml of RBC, and fluxes are expressed in mmol per min per kg of weight solids. In *A* and *B*, membrane proteins were fractionated by electrophoresis without DTT reduction. The membrane polypeptides are indicated by numbers according to Fairbanks et al. (1971). Hb: hemoglobin. (*b*): Two-dimensional gel electrophoresis of membrane proteins from ATP-depleted cells under aerobic conditions. Membrane proteins were fractionated in the first dimension without DTT reduction. Electrophoresis in the second dimension was performed in a slab incorporating a DTT zone to cleave disulfide bonds. A schematic first dimensional pattern is shown at the top of the gel. Star indicates the mol wt $> 10^6$ daltons aggregate whose protein components (▶) are released by DTT reduction

Table 2. Effect of Ca^{2+} accumulation on chloride self-exchange fluxes in ATP-depleted cells

		Chloride fluxes $\times 10^{-3}$ (moles/min/kg weight solids)	Inhibition of chloride fluxes (percent of control)
A	Control cells	485 ± 59	–
	Depleted cells	188 ± 31	61
B	Control cells	543 ± 27	–
	Depleted cells	349 ± 17	36

Long depletion under air was performed as described in Materials and Methods in medium containing 2 mM $CaCl_2$ (A) and 2 mM EDTA free of $CaCl_2$ (B). The values and the standard deviations are derived from three (A) and two (B) experiments. Control cells refer to cells incubated in Ringer supplemented with glucose 2 g/liter, inosine 12.7 mM and adenine 0.5 mM. Depleted cells refer to cells incubated in the same Ringer without glucose, inosine, and adenine.

plete restoration of the cellular morphology, the Cl^- self exchange fluxes only partially recover. Repletion experiments performed in the presence of PO_4^{2-} do not show an improved recuperation of the Cl^- transport despite the higher final intracellular ATP concentration. The following values are an example of an experiment: anaerobically ATP-depleted cells (0.01 μmol ATP/ml RBC) with a lower chloride permeability than control (392×10^{-3} mmol/mn/kg weight solid and 617×10^{-3} mmol/mn/kg weight solid, respectively) were separated into two batches for repletion treatment with or without 2 mM PO_4^{2-}. At the end of the repletion period (4 hr) we obtained a similar partial recuperation of the transport in the two batches (445×10^{-3} and 453×10^{-3} mmol/mn/kg weight solid) despite the fact that the ATP concentrations were different (0.11 μmol ATP/ml RBC and 0.31 μmol ATP/ml RBC).

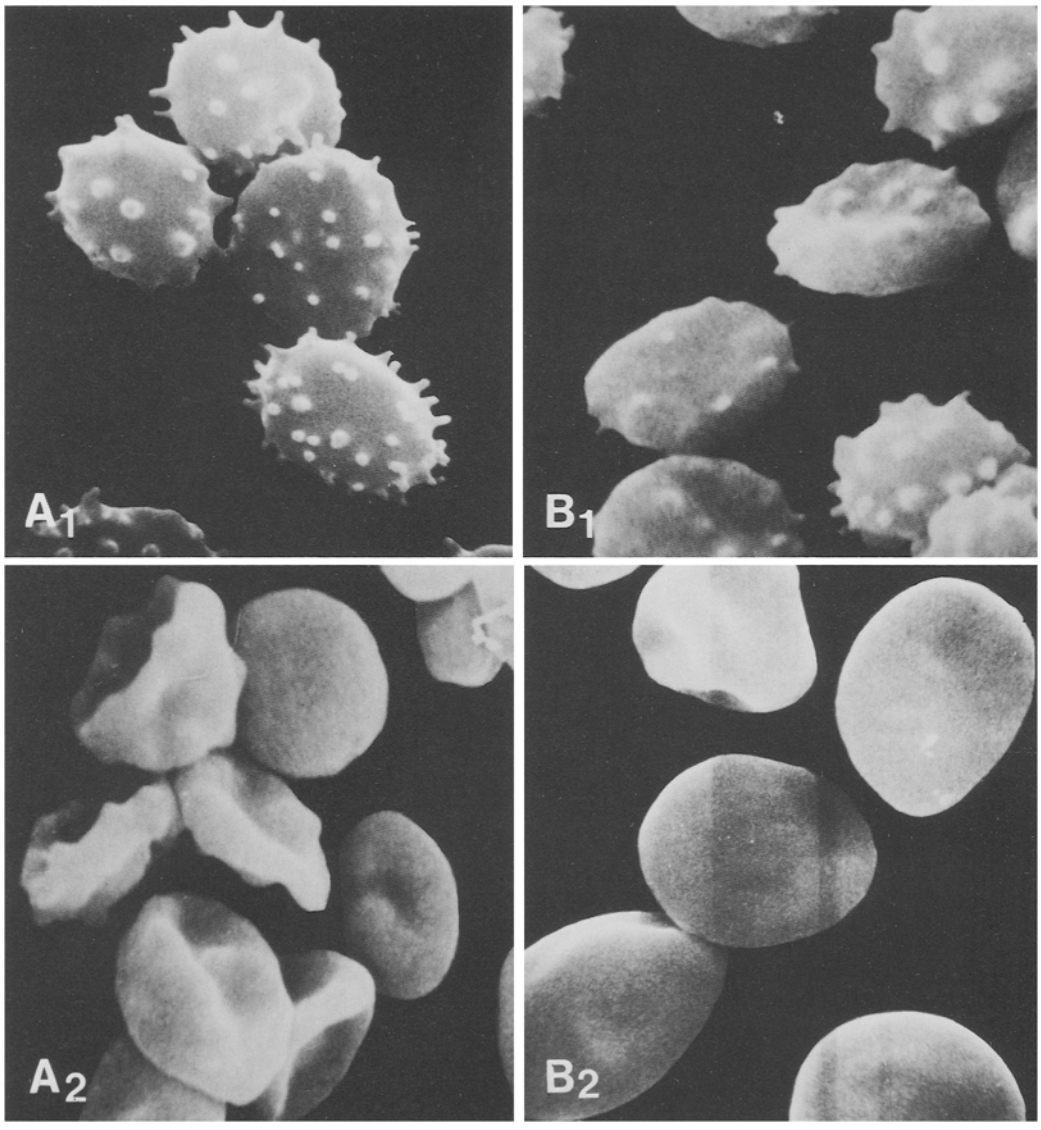

Fig. 5. Scanning electron micrographs of Ca^{2+}-loaded cells with 1 mM Ca^{2+} and 10^{-5} M ionophore A23187 (×6000). (a_1): Ca^{2+}-loaded cells in the absence of histamine. (a_2): The same cells after repletion by long incubation (18 hr) in medium supplemented with metabolic substrates. (b_1): Ca^{2+}-loaded cells in the presence of 40 mM histamine. (b_2): The same cells after repletion by long incubation (18 hr) in medium supplemented with metabolic substrates

Effect of Ca^{2+} accumulation during ATP depletion. In the above experiment, incubations were performed without calcium in the external medium. When ATP-depletion of red cells is carried out in the presence of $CaCl_2$, Ca^{2+} uptake by cells occurs. Table 2 gives the values of chloride self-exchange fluxes measured after aerobic ATP depletion in Ca^{2+}-containing (2 mM Ca^{2+}) and Ca^{2+}-free (2 mM EDTA) media. It can be seen that chloride movements are strongly reduced when Ca^{2+} accumulates. The degree of inhibition in ATP-depleted cells containing Ca^{2+} is twice that in ATP-depleted cells not loaded with Ca^{2+}. These data suggest a direct inhibitory effect of Ca^{2+} ions on chloride permeability.

2. *The Effects of Ca^{2+} Loading*

Cells loaded with Ca^{2+} by means of ionophore A23187 are useful for the study of Ca^{2+} effects inside the cells. The intracellular Ca^{2+} loading was performed (*see* Materials and Methods) in the presence and absence of histamine in the external medium for a reason which will be explained below. Briefly, histamine prevents the stabilization of membrane protein crosslinking occurring during the intracellular Ca^{2+} accumulation.

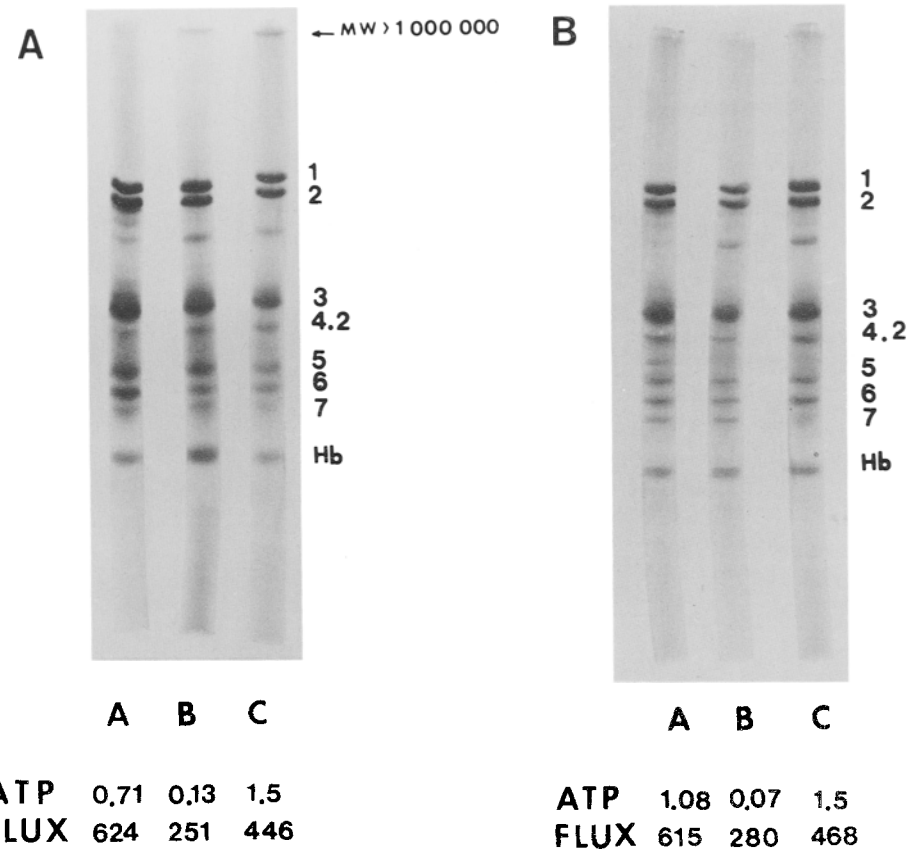

Fig. 6. Relationships between chloride self-exchange fluxes, ATP level and membrane protein composition of control cells (*A*), Ca^{2+}-loaded cells (*B*), and Ca^{2+}-loaded cells after a subsequent repletion treatment (*C*). (*a*): Incubation performed in histamine-free media. Ca^{2+} loading was obtained by incubating red cells at 37 °C in a glucose-free medium containing 1 mM Ca^{2+} and 10^{-5} M A23187. Arrow indicates the high molecular weight membrane protein aggregate. (*b*): Incubations performed in 40 mM histamine enriched media. Same legends as in *a* except that all the different incubation media are supplemented with 40 mM histamine and that control cells refer to fresh cells incubated in medium supplemented with metabolic substrates

Cell morphology and ATP content. Figure 5 shows the morphology of Ca^{2+}-loaded cells in the presence and absence of histamine. Cells of the two batches are echinocytic with an important fraction of the populations spheroechinocytic. As expected, intracellular ATP concentration decreases (*see* Fig. 6). Washing ionophore-treated cells with bovine serum albumin in free Ca^{2+} medium and incubating them with metabolic substrates leads to the reversal of the cellular shape (Fig. 5). For histamine-free medium treated cells, the morphological recovery is largely incomplete (35% echinocytes are still present), while cells always treated in the presence of 40 mM histamine recover their biconcave shape but show numerous distortions (Fig. 5). No echinocytes remained. The loss of ATP during Ca^{2+} accumulation is reversible, since in repleted cells the ATP content is largely restored to its initial value (Fig. 6).

Membrane protein association. Lorand, Weissmann, Epel and Lorand (1976) have shown that in Ca^{2+}-enriched cells, permanent membrane protein cross-linking can occur, catalyzed by a Ca^{2+} activated red cell glutaminase. Figure 6*a* shows the SDS-polyacrylamide gel electrophoresis of solubilized Ca^{2+}-loaded cell membrane proteins. Indeed, a large molecular weight protein remains at the origin of the gel. This polymer cannot be cleaved with reducing agents and the electrophoresis performed on the repleted cell membrane proteins after Ca^{2+} extrusion exhibits the same gel pattern. This permanent heteropolymer can be prevented from forming if cells are loaded with Ca^{2+} in the presence of histamine, which is a substrate for the cytoplasmic transglutaminase. This is shown in Fig. 6*b* in which gel patterns from intact cells, Ca^{2+} loaded cells, and repleted cells are identical. No membrane protein aggregate can be detected.

Cl^- transport. Our data are reported in Fig. 6. In ionophore-treated cells, either with or without histamine, the Cl^- self-exchange fluxes are strongly in-

hibited. In each case, the extent of inhibition is around 60%. After repletion Cl^- transport is not fully recuperated in the two cell populations (with and without histamine). In both, a residual inhibition around 30% of the Cl^- transport remains.

Discussion

The present study demonstrates that, in human red cells, ATP depletion induces an inhibition of the electroneutral one-for-one chloride exchange, which is considered as a nonenergy-dependent transport process. In addition it is shown that this inhibition is largely increased when red cells are depleted in the presence of calcium in the external medium, i.e., when Ca^{2+} ions can accumulate in the cells. Thus the respective roles of ATP depletion and intracellular Ca^{2+} must be analyzed.

The following possibilities can be considered to explain the observed transport inhibition occurring after ATP depletion.

1) The modification in cell morphology, associated with ATP depletion, causes an alteration in the capacity of the transport system to carry chloride ions.

2) The decrease in the internal ATP level directly modifies the transport system efficiency.

3) During ATP depletion, spectrin is rearranged to establish closer contacts with different membrane proteins allowing the formation of large molecular weight complexes. Since band 3, the assumed chloride channel, is involved in one of these complexes (Palek et al., 1978*b*), such a rearrangement could explain inhibition of chloride transport.

The first possibility appears unlikely because even when the discocyte-echinocyte transformation of red cells undergoing ATP depletion is reversed after ATP repletion ,the rate of chloride exchanged does not recover its initial value. It must be pointed out that complete recovery of the discocyte shape is obtained whereas intracellular ATP level is poorly restored, suggesting - and it is formulated as the second possibility - that ATP level could directly influence the transport system activity. This possibility also seems unlikely because the rate of chloride exchanges is the same when, after ATP repletion, the level of ATP stores obtained is 0.11 μM/ml cells (incubation in normal medium containing inosine adenosine and glucose) or 0.31 μM/ml cells (incubation in the same medium supplemented with inorganic phosphate. Furthermore we demonstrated (Fig. 2) than when ATP depletion is induced by incubation of the cells with iodoacetic acid, an enzyme-inhibitor blocking glycolysis, within 1 hr ATP fell to zero but 95% of the cells were discocytes, however, and the rate of chloride self-exchange was not significantly modified (not shown). Incidentally these data confirm the interesting finding previously obtained in similar experimental conditions by Feo and Mohandas (1977), that ATP is not necessary for maintaining the discocyte shape.

In order to explore the third possibility, we investigated membrane protein composition of red cells depleted in ATP under different experimental conditions and simultaneously measured the chloride self exchanges. As previously described by Palek et al. (1978*b*) we found that membranes of human erythrocytes depleted in ATP under aerobic conditions contain several high molecular weight spectrin-rich complexes, namely a heterodimer of spectrin (1+2) of apparent molecular weight of 450,000 daltons and a large complex (mol wt $>10^6$ daltons) with a high spectrin to band 3 ratio (Fig. 4). In these conditions we observed that the rate of chloride exchanges was decreased by about 35%. Taken together these data could suggest that the formation of complexes is responsible for the observed transport inhibition. However, when ATP depletion was induced in anaerobic conditions, we observed the same degree of chloride transport inhibition (Table 2 and Fig. 3) though the membranes of these cells did not exhibit such complexes and had the same membrane protein electropherogram as the fresh, non-ATP-depleted cells (Fig. 3). This discrepancy between aerobic and anaerobic data, in fact, is only apparent. Indeed, the formation of the high molecular weight complex observed in cell depleted in ATP under aerobic conditions can be visualized as the consequence of two processes: the first, a rearrangement of spectrin and other membrane proteins into more intimate contact with each other, this rearrangement being induced by ATP depletion; and the second, the appearance of spontaneous intermolecular crosslinkings by disulfide bridges with subsequent formation of a heteropolymer which the rearranged proteins undergo under aerobic conditions. In anaerobic conditions membrane proteins rearrange to close contact but spontaneous oxidation does not, of course, occur. Consequently the rearrangement of protein which is not stabilized by disulfide covalent bridges is dissociated during solubilization of the membrane in SDS and cannot be observed on gel electrophoresis. Such an interpretation is strongly supported by the data of Palek and Liu (1979) and Liu and Palek (1979), showing that glutaraldehyde crosslinking or catalytic oxidation of ghosts from anaerobic ATP-depleted cells, but not from fresh cells, contain a large molecular weight (mol wt $>10^6$ daltons) complex, which is enriched in spectrin. Therefore it seems reasonable to consider that the inhibition of chloride transport observed after ATP depletion is related to the changes in membrane protein organization induced by the decrease

in ATP level. But it does not appear that the degree of inhibition is modified when this altered assembly of membrane proteins is stabilized by the disulfide linkage.

As a consequence of this interpretation one would expect that the dissociation of complexes would allow the normal rate of chloride exchanges to be resumed. When red cells have been ATP depleted under anaerobic conditions, it can be presumed that ATP repletion will restore the normal arrangement of membrane proteins (as it restores normal shape), since the neighboring proteins were not crosslinked through disulfide coupling. Similarly for aerobically ATP-depleted cells, the restoration of normal protein organization should be obtained if ATP repletion is carried out in the presence of dithiothreitol (DTT), which reduces disulfide bonds and completely dissociates the high molecular weight polymer (Palek et al., 1978*b*, and Fig. 4).

Surprisingly enough, we found in these two different experimental conditions that the recovery of chloride permeability was only partial and essentially variable from one experiment to another. This nontotal reversibility of inhibition can be attributed to the following possibilities:

1) Human erythrocytes incubated without glucose could release spectrin-free vesicles containing lipids, hemoglobin, and some integral proteins such as band 3 (Lutz, Liu & Palek, 1977). Thus irreversibility could correspond in our experimental conditions to a loss of band 3, the assumed chloride channel.

2) The changes in membrane protein arrangement are not completely reversible as repletion progresses.

The first possibility surely cannot entirely explain the failure of the transport inhibition to reverse because, from the data of Lutz et al. (1977), it appears that after 20 hr incubation at 37 °C (our experimental conditions) only about 2% of the membrane proteins are lost, i.e., 1% of the band 3 protein. Thus we favor the second explanation because it has been recently demonstrated (Liu & Palek, 1979), using catalytic oxidation, that the propensity of ghosts from anaerobically depleted cells to form a high molecular weight complex was not completely reversed by restoration of ATP level: this suggests that spectrin and other membrane proteins undergo an irreversible aggregation during ATP depletion.

As mentioned above, inhibition of chloride self-exchanges is strongly increased when ATP depletion is induced in the presence of calcium in the external medium (a 60% inhibition is observed when red cells are depleted in a medium containing 2 mM $CaCl_2$ against 35% in a Ca^{2+}-free medium containing 2 mM EDTA). It is well known that, in these experimental conditions, calcium accumulates in the cells. As external calcium has no effect on chloride transport in fresh cells incubated with metabolic substrate, it can be concluded that it is intracellular calcium which is involved in this additive inhibitory effect. Cellular calcium concentration in erythrocytes metabolically depleted in plasma (Lichtman & Weed, 1973; Palek & Liu, 1979) may rise to approximately 50 μM after 24 hr incubation. Such a concentration of calcium could conceivably induce, by activating transglutaminase, covalent crosslinkings between γ glutamyl-ε-lysine residues of spectrin, band 3, and other membrane protein neighbors (Lorand et al., 1976; Anderson, Davis & Carraway, 1977). Then aggregate formation due to ATP depletion plus aggragate formation due to Ca^{2+}-activated transglutaminase could account for the total inhibition observed in erythrocytes metabolically depleted in the presence of calcium. In fact, it has been shown that considerable increases in erythrocyte calcium are required to initiate transglutaminase-dependent membrane protein crosslinking. Such concentrations (about 0.5 mM) are approximately 7–8 times higher than that found in metabolically depleted erythrocytes (Lorand et al., 1976; Anderson et al., 1977; Coetzer & Zail, 1979). Thus, from our experiments performed with ATP-depleted cells, it appears that at a relatively low concentration (10 μM range) intracellular calcium inhibits chloride self-exchanges in a manner which does not involve membrane protein crosslinking. The question is evidently open whether such inhibition is related to a direct Ca^{2+} effect on the chloride channel. Recently, by controlling cellular concentration with ionophore A23187 and divalent cation buffers, Low (1978) also demonstrated an inhibitory effect of a relatively low level of Ca^{2+} on sulfate exchanges in erythrocytes. The apparent inhibition constant (K_i) was calculated to be 6 μM.

When we used ionophore to load fresh cells (10 μM ionophore, 1 mM external Ca, 1/2 hr incubation) the intracellular calcium attained a sufficiently high concentration ($C > 500$ μM) to initiate transglutaminase-dependent membrane protein complexes (*see* Fig. 6) and discocyte-echinocyte transformation (Fig. 5). In these conditions we found a very large inhibition of chloride exchanges (60% of the control). This degree of inhibition is identical to that observed when ATP depletion is induced in the presence of calcium in the incubation medium, i.e., when the additive effects of ATP depletion and low intracellular calcium level occur. We found that after restoration of normal ATP and calcium levels, the complex remains unchanged (Fig. 6) but the inhi-

bition is diminished by half: the degree of residual inhibition (30%) is similar to that observed when a complex is formed by ATP depletion in Ca^{2+}-free medium.

The presence of a suitable transglutaminase substrate, like histamine, inhibits crosslinkings of membrane proteins (i.e., the formation of complex) in intact red cells exposed to Ca^{2+} and ionophore (Fig. 6) but, of course, does not impair the altered assembly of spectrin and other membrane proteins. In this experimental condition a high degree of inhibition (55%; *see* Fig. 6) is also observed, and restoration of normal ATP and calcium levels, which restores the normal biconcave shape, only permits a partial recovery of chloride permeability (residual inhibition 24%).

A reasonable interpretation of these two sets of experiments is that the introduction of a large amount of Ca^{2+} into fresh cells by treatment with ionophore produces a strong inhibition chloride self-exchanges in two simultaneous ways:

1) A "direct" effect of Ca^{2+} on chloride permeability which accounts for about half of the observed inhibition and which is released after ATP repletion and Ca^{2+} extrusion. This action could be similar to the effect observed with a low intracellular Ca^{2+} concentration.

2) An "indirect" effect through a rearrangement of spectrin and other membrane proteins and which accounts for the second half of the inhibiting action. This altered assembly of proteins being stabilized by permanent protein crosslinkings by a Ca^{2+}-activated transglutaminase, the indirect inhibitory effect is not released after ATP repletion and Ca^{2+} extrusion. In the presence of histamine, covalent stabilization cannot occur, but it is possible, as in the case of anaerobic ATP depletion, that irreversible aggregation occurs, preventing a total release of chloride inhibition after Ca^{2+} extrusion.

The possibility that this irreversible inhibition could be due to the release into the external medium of microvesicles containing band 3, as we discussed above for ATP depletion, is unlikely. This phenomenon has indeed been described (Allan, Billak, Finean & Michell, 1976) when red cells are incubated in the presence of ionophore A23187 plus Ca^{2+}, but in conditions more drastic than those we used. Furthermore, the microvesicles have a very low content of band 3 when freshly collected blood is used.

Concerning the action of Ca^{2+} on chloride movements, it is interesting to note the converse effect of Ca^{2+} on the fast anion exchange mechanism and on the net anion transporting mechanism: on the first process, an inhibitory effect according to the present results on chloride and the data of Low (1978) on sulfate; on the second one, a stimulatory effect according to Bürgin and Schatzmann (1979). It is actually questioned whether the net anion transporting mechanism is an independent pathway or a slip in the exchange mechanism. One could speculate that Ca^{2+} induces the working of the mechanism in the direction of the "slipping" way to the prejudice of the "normal" way.

This study was supported by C.N.R.S. (ERA 495) and D.G.R.S.T. (n° 57.7.0052). We are particularly grateful to Dr. Donat and Dr. Cortegiani (Centre de Transfusion Sanguine) for blood supply. It is a special pleasure to acknowledge the expert assistance of Dr. F. Sola.

References

Allan, D., Billak, M.M., Finean, J.B., Michell, R.H. 1976. Release of diacylglycerol-enriched vesicles from erythrocytes with increased intracellular Ca^{2+}. *Nature (London)* **261**:58–60

Anderson, D.R., Davis, J.L., Carraway, K.L. 1977. Calcium-promoted changes of the human erythrocyte membrane. *J. Biol. Chem.* **252**:6617–6623

Bennett, V., Stenbuck, P.J. 1979. The membrane attachment protein for spectrin is associated with band 3 in human erythrocyte membranes. *Nature (London)* **280**:468–473

Beutler, E. 1975. Red cell metabolism. *In:* A Manual of Biochemical Methods. (2nd Ed.) Gune & Stratton, New York

Bürgin, H., Schatzmann, H.J. 1979. The relation between net calcium, alkali cation and chloride movements in red cells exposed to salicylate. *J. Physiol. (London)* **287**:15–32

Cabantchik, Z.I., Rothstein, A. 1974. Membrane proteins related to anion permeability of human red blood cells. I. Localization of disulfonic stilbene binding sites in proteins involved in permeation. *J. Membrane Biol.* **15**:207–226

Coetzer, T.L., Zail, S.S. 1979. Cross-linking of membrane proteins of metabolically-depleted and calcium loaded erythrocytes. *Br. J. Haematol.* **43**:375–390

Cousin, J.L., Motais, R. 1976. The role of carbonic anhydrase inhibitors on anion permeability into ox red blood cells. *J. Physiol. (London)* **256**:61–79

Fairbanks, G., Steck, T.L., Wallach, D.F.H. 1971 Electrophoretic analysis of the major polypeptides of the hyman erythrocyte membranes. *Biochemistry* **10**:2606–2617

Feo, C.J., Leblond, F.P. 1974. The discocyte-echinocyte transformation: Comparison of normal and ATP-enriched human erythrocytes. *Blood* **44**:639–646

Feo, C.J., Mohandas, N. 1977. Role of ATP depletion on red cell shape and deformability. *Blood Cells* **3**:153–161

Funder, J., Wieth, J.O. 1976. Chloride transport in human erythrocytes and ghosts: A quantitative comparison. *J. Physiol. (London)* **262**:679–698

Laemmli, U.K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature (London)* **227**:680–685

Lichtman, M.A., Weed, R.I. 1973. Divalent cation content of normal and ATP-depleted erythrocytes and erythrocytes membranes. *In:* Red Cell Shape M. Bessis, R.I. Weed and P.F. Leblond, editors. p. 79. Springer, New York

Liu, S.C., Fairbanks, G., Palek, J. 1977. Spontaneous, reversible protein cross-linking in the human erythrocyte membranes. Temperature and pH dependence. *Biochemistry* **16**:4066–4074

Liu, S.C., Palek, J. 1979. Metabolic dependence of protein arrangement in human erythrocyte membranes. II. Cross-linking

of major proteins in ghosts from fresh and ATP-depleted red cells. *Blood* **54**:1117–1130

Lorand, L., Weissmann, L.B., Epel, D.L., Lorand, B.J. 1976. Rule of the intrinsic transglutaminase in the Ca^{2+} mediated cross-linking of erythrocyte proteins. *Proc. Natl. Acad. Sci. USA* **73**:4479–4481

Low, P.S. 1978. Specific cation modulation of anion transport across the human erythrocyte membrane. *Biochim. Biophys. Acta* **514**:264–273

Lutz, H.U., Liu, S.C., Palek, J. 1977. Release of spectrin-free vesicles for human erythrocytes during ATP-depletion. *J. Cell Biol.* **73**:548–560

Motais, R., Baroin, A., Motais, A., Baldy, S. 1980. Inhibition of anion and glucose permeabilities by "Anesthetics" in erythrocytes: The mechanisms of action of positively and negatively charged drugs. *Biochim. Biophys. Acta* **599**:673–688

Palek, J., Liu, P.A., Liu, S.C. 1978*a*. Polymerisation of red cell membrane protein contributes to spheroechinocyte shape irreversibility. *Nature (London)* **274**:505–507

Palek, J., Liu, S.C. 1979. Membrane protein organization in ATP depleted and irreversibly sickled red cells. *J. Supramolec. Struct.* **10**:79–96

Palek, J., Liu, S.C., Snyder, L.M. 1978*b*. Metabolic dependence of protein arrangement in human erythrocytes membranes. Analysis of spectrin-rich complexes in ATP-depleted red cells. *Blood* **51**:385–395

Received 23 October 1980; revised 2 January 1981