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INHIBITION OF ANION TRANSPORT ACROSS RED BLOOD CELLS WITH 1,2-CYCLOHEXANEDIONE

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SUMMARY

The effects of three different α dicarbonyl reagents on SO_4 equilibrium exchange across the red cell membrane have been studied. It was found that 1,2-cyclohexanedione, an arginine specific reagent [1], causes inhibition of transport and a reduction of the capacity of the band 3 protein to bind the specific transport inhibitor H_2DIDS. The effect on the H_2DIDS binding capacity was, however, considerably smaller than the inhibition of sulfate transport indicating that the inhibition involved a modification of sites other than the H_2DIDS binding site. Squaric acid and 1,3-cyclohexanedione produced no inhibition under conditions where 1,2-cyclohexanedione was fully inhibitory. The results suggest that besides the lysine residues [2] involved in H_2DIDS-binding guanidino groups may also play a role in anion transport across the red blood cell membrane.

INTRODUCTION

Anion transport in red blood cells can be inhibited by a large number of covalently binding amino reactive reagents [3,4,5,6]. Studies with radioactive DNFB and SITS [5,6], H_2DIDS [7] and isothiocyanoto-phenyl-sulfate [8] have led to the conclusion that the so-called band 3 protein is the protein which mediates anion transport.

In the present work, studies are done with another group-specific reagent, the 1,2 cyclohexanedione. This agent is known to react selectively with the guanidino group of arginyl residues [9]. Patthy and Thesz even demonstrated a re-

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Abbreviations used in the paper:
1,2-CHD = 1,2-cyclohexanedione.
1,3-CHD = 1,3-cyclohexanedione.
SQ = squaric acid.
H_2DIDS = 4,4'-diisothiocyano-dihydrostilbene-2,2'-disulfonate.
SITS = 4-acetamido 4'-isothiocyanostilbene-2,2'-disulfonic acid.
DNFB = 1-fluro-2,4-dinitrobenzene.

This work is dedicated to Prof. Dr. G. Pfleiderer on the occasion of his 60th birthday.

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markable selectivity of α dicarbonyl reagents for arginine residues that are involved in the anion recognizing sites of enzymes [9].

MATERIALS AND METHODS

ORh^+ blood from healthy donors was obtained from the blood bank of the Red Cross in Frankfurt/Main and stored at 4°C for no longer than 2-5 days. After removal of plasma and buffy coat, the cells were washed three times in isotonic saline. Resealed ghosts were prepared essentially as described previously [10]. They were hemolyzed at a cell:medium ratio of 1:20 in a medium containing 4 mM $MgSO_4$ and 1.45 mM acetic acid. 5 min after hemolysis, sufficient EDTA was added to obtain a final concentration of 20 mM EDTA in the hemolysate. After centrifugation, the ghosts were resuspended and resealed in a medium containing 50 mM Na borate, 5 mM Na_2 SO_4, and 77.5 mM NaCl pH 8.0 at 37°C for 45 min.

The reaction of the resealed ghosts with 1,2 CHD was performed at 37°C in a medium containing 50 mM Na borate, 5 mM Na_2SO_4 and 77.5 mM NaCl pH 8.0 for 60 min. The ghost:medium ratio was 1:20 and the concentrations of CHD were in the range of 1 - 20 mM. $^{35}SO_4$ equilibrium exchange was measured after removal of excess 1,2-CHD in the same medium at a hematocrit of 5%. The flux measurements, the isolation of white membrane, the subsequent SDS polyacrylamide gel electrophoresis and the determination of radioactivity in the gel were executed as described previously [6].

Chemicals: 1,2 Cyclohexandione ('purum') was obtained from Fluka. 1,3 Cyclohexandione ('for synthesis') from Merck-Schuchardt. Squaric acid was a gift from Professor Ried, Frankfurt/Main. $^3H_2$DIDS was synthesized by Professor H Fasold as previously described [11].

RESULTS

3.1 Effect of 1,2-CHD on sulfate equilibrium exchange in resealed ghosts

Fig. 1 shows the inhibition by 1,2-CHD of sulfate efflux from the resealed red cell ghosts. The reaction with the agent was performed prior to the flux measurement in a buffer containing 50 mM borate. The presence of borate serves both to accelerate the reaction with arginyl residues and to stabilize the product [12].

In some experiments before measuring $^{35}SO_4$ efflux, the modified ghosts were incubated for 60 min at 37°C in the absence of the modifier. After three washes, SO_4 efflux was measured. The degree of inhibition of the modified cells was the same, regardless of whether or not the exposure to 1,2-CHD was followed by incubation in the absence of the agent. This demonstrates that the

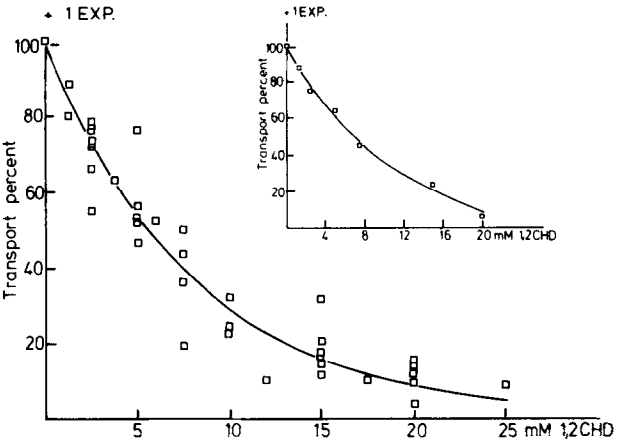

Fig. 1: Effect of 1,2-CHD on sulfate equilibrium exchange in resealed ghosts.
Ordinate = penetration rate in per cent of control value without 1,2 CHD.
Abscissa = concentration of 1,2-CHD in mM. Hematocrit 5%. Temperature 37°C, pH 8.0.
The drawn curve represents a single exponential.

effect of 1,2-CHD on the anion sites remains unaltered during the time course of the flux measurements.

3.2 Relationship between inhibition of anion transport and 1,2-CHD concentration.

Fig. 1 also shows that the relationship between anion transport as measured in the modified cells and the 1,2-CHD concentration at which the modification had been carried out can be represented by a single exponential. The plotted line represents a least square fit to the equation [13].

$$\frac{100}{{}^{o}k_{s(c=0,t)}} \times {}^{o}k_{s(c,t)} = a \times e^{-kct} \quad \text{with } a = 99 \; kt = 0.12$$

The symbols ${}^{o}k_{s}$, c, a, k and t represent, respectively, the rate constant for sulfate efflux, the 1,2-CHD concentration, empirically determined co-efficients and the time of treatment with 1,2-CHD (60 min). The inset curve shows that the data points of individual experiments agree even better with a single exponential than the composite of all data.

Table I

Conditions	Sulfate transport
	$^{o}k_{s} \times 10^{-3}$ min^{-1}
Control	7.37
2.5 mM SQ	7.837
5.0 mM SQ	7.919
7.5 mM SQ	7.763

3.3 Effect of squaric acid on anion transport

Table I shows that the cyclobutendione squaric acid has no effect on sulfate transport in resealed ghosts. The rate of SO_4 transport was indistinguishable from that in untreated ghosts.

3.4 Effect of 1,3-cyclohexandione on anion transport

Resealed ghosts were allowed to react with 1,3-CHD at the concentration indicated in Fig. 2. The reaction condition was the same as in 3.1 and 3.3. Two controls were done: one without any modifier and the other with 15 mM 1,2-CHD. From Fig. 2 and Table II one can see that 15 mM 1,2-CHD was able to inhibit

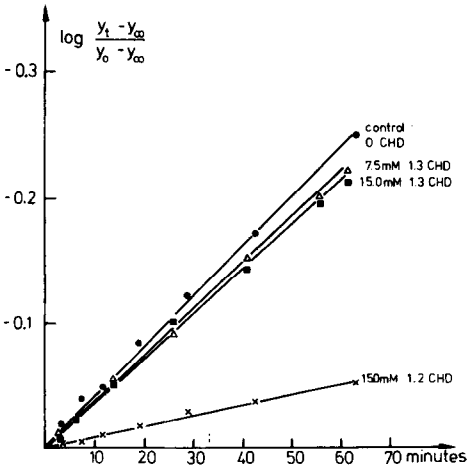

Fig. 2: Semi-log plot of the time course of release of radioactivity from $^{35}SO_4$ loaded ghosts. Before the initiation of the efflux of the labelled sulfate, the ghosts were incubated with either 1,2-CHD or 1,3-CHD at the concentration indicated in the figure. For details of the experimental conditions, see Materials and Methods.

Y_t, Y_o, Y_∞ represent, respectively, the radioactivity in the supernatant at time t, $t = o$, $t = \infty$

Table II

Conditions	Sulfate transport
	$^{0}k_{s} \times 10^{-3}$ min^{-1}
Control	7.297
7.5 mM 1,3-CHD	7.329
15 mM 1,3-CHD	7.264
15 mM 1,2-CHD	0.875

$^{35}SO_4$ efflux to about 90%, while 1,3-CHD had almost no effect. These results support the assumption that the reacting group in the membrane is most probably a guanidino residue.

3.5 Effect of 1,2 CHD on $^{3}H_2$DIDS binding to the 95 000 Dalton protein (band 3)

After modification of the resealed ghosts at various 1,2-CHD concentration and removal of the unreacted 1,2-CHD by washing, each ghost suspension was subdivided into two portions. One was used for flux measurements, and the other for the determination of the capacity of the protein in band 3 to bind H_2DIDS. The incubation with $^{3}H_2$DIDS was performed at a $^{3}H_2$DIDS concentration of 10 µM, for 45 min at 37°C in the same medium in which the reaction with 1,2-CHD was done. In some experiments, 1,2-CHD was also present during flux measurement and incubation with H_2DIDS. The membranes of the $^{3}H_2$DIDS-treated ghosts were isolated, dissolved in SDS, subjected to polyacrylamide gel electrophoresis as described in [6] and the capacity of band 3 for covalent $^{3}H_2$DIDS binding was determined. (Radioactivity on the gels was determined according to [6].)

The results represented in Figs. 3 and 4 show that even when the transport system is completely inhibited, the band 3 protein can still bind $^{3}H_2$DIDS. However, the binding capacity is reduced by about 50%. This behaviour differs from that of previous studies on the effects of covalently and non-covalently

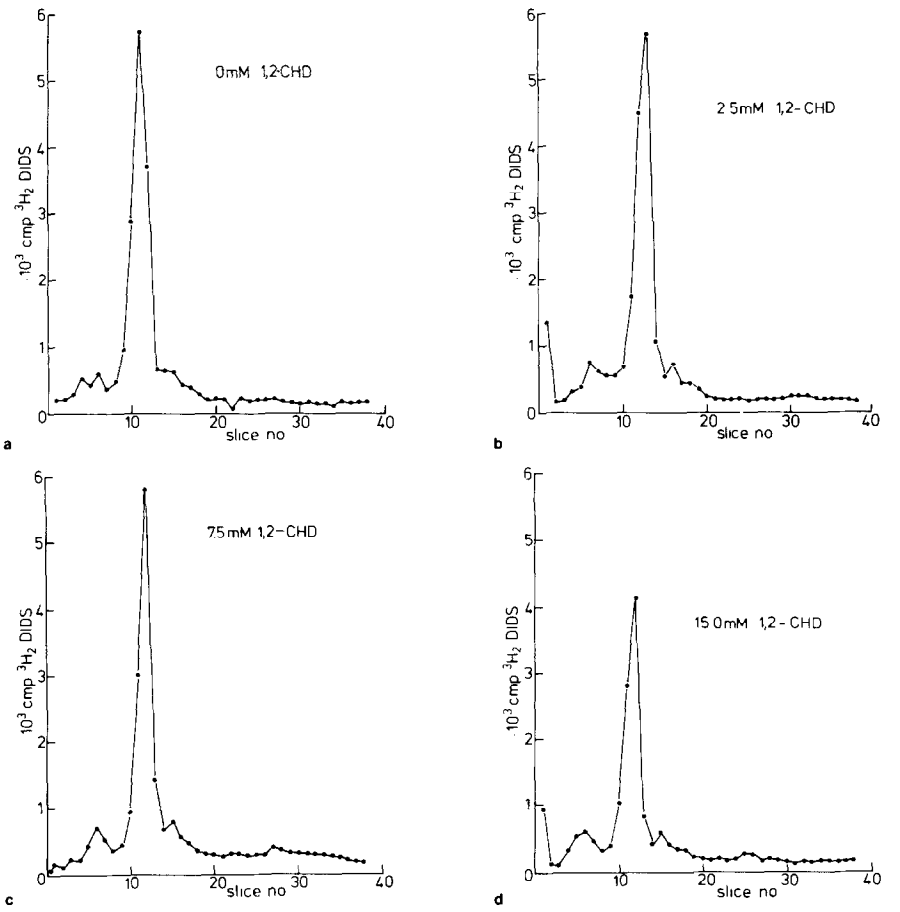

Fig. 3: $^3H_2$DIDS binding patterns to red cell ghosts. Prior to the exposure to $^3H_2$DIDS, the ghosts had been treated with 1,2-CHD. 1,2-CHD concentration is indicated in the figure.

binding anion transport inhibitors on the H_2DIDS binding site in the band 3 protein [5,6,7,2,12,14].

DISCUSSION

1,2 CHD, which is known to react selectively with arginine residues, is found to be a potent inhibitor of anion equilibrium exchange across the red blood cells.

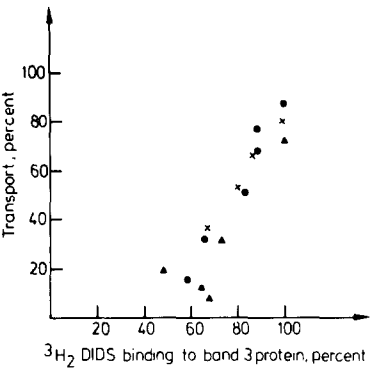

Fig. 4: Effect of 1,2-CHD on anion transport and $^3H_2$DIDS binding site on band 3.

Transport and $^3H_2$DIDS binding are expressed as per cent of corresponding values in resealed ghosts that had not been exposed to 1,2-CHD.

It has also been found that the inhibitory effect caused by this reagent exceeds the reduction of the capacity of the anion transport protein to bind the anion transport inhibitor $^3H_2$DIDS. This indicates that the 1,2-CHD binding site is not identical with the amino acid residue that is involved in the covalent binding of $^3H_2$DIDS (presumably a lysine residue, Passow et al [2]).

This result demonstrates the existence of another functional amino acid residue - most probably an arginine residue which seems to participate in anion transport.

The role of arginine can be supported by experiments of Funder et al. and Brahm [15, 16] on the effect of pH and temperature on chloride exchange flux. Their results showed that monovalent anion transport is independent of pH in the range pH 7.2 to at least 11.0 at 0°C and that the pK of the transporting groups decreases at least by two pH units by a temperature increase to 38°C, suggesting a group with a very high ionization enthalpy; for example, the guanidino group of an arginine residue.

Legrum et al [17] also found after dansylation of the ghosts at pH 6.6 that by increasing the pH, the rate of divalent anion equilibrium exchange increases

until a plateau is reached extending from pH 6.0 to at least 8.5. This shows that under this condition, the transport process is carried out by groups which do not change their degree of protonation over this pH range. One may conclude, therefore, that the effect of 1,2-CHD on anion transport in red blood cells is in agreement with the general rule that arginine residues are located at the anion recognizing sites in many functional proteins [3].

It is still premature to speculate about the location of the 1,2-CHD binding site in relation to the $^{3}H_{2}$DIDS binding site. The fact that SQ has no effect on transport supports the general notion that the anion transporting site is embedded in a hydrophobic region. Experiments are now being done to identify the position and kinetic function of these sites in more detail.

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