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The Effects of Maleic Anhydride on the Ionic Permeability of Red Cells

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Summary. Maleic anhydride (MA) has been shown to react specifically and rapidly with amino groups of proteins; the maleyl amino groups are negatively charged and completely stable at neutral pH. Treatment of human red cells with this reagent results in a significant increase in K^+ permeability which is associated with a much smaller increase in Na^+ permeability. Opposite effects are observed on anion permeability, the $SO_4^{=}$ and Cl^- permeability being decreased to an approximately similar extent upon treatment with MA.

Studies on the distribution of MA between membrane lipids and proteins shows that most of the membrane-bound MA is associated with membrane proteins. These results suggest that the observed effects of MA on the ion permeability of the red cell are caused by its combination with amino groups of cell membrane proteins.

Current evidence (*see* Passow, 1969) suggests that amino groups of cell membrane proteins are involved in the mechanism responsible for the high selectivity for anions which is one of the salient features of the red cell membrane. Maleic anhydride (MA) has been shown to react rapidly and specifically with amino groups of proteins. The reaction proceeds in aqueous solution with an optimal pH of 8.5 to 9.0. The maleylamino groups are negatively charged and completely stable at neutral or alkaline pH (Butler, Harris, Hartley & Leberman, 1969). The mild conditions necessary for the reaction of MA together with its specificity for amino groups suggest that MA may be one of the choice reagents to study the effects of the blockage of cell membrane amino groups on the passive permeability to ions. The present paper reports results of experiments designed to test the effects of MA on anion and cation fluxes in human red cells, and gives an account on the mode of action of the reagent. The results are compared with previous finding by other authors who used different amino-reactive agents.

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Materials and Methods

Freshly drawn human blood from hematologically normal adults was used; coagulation was prevented with acid-citrate-dextrose solution. The blood was centrifuged at 1,750 × *g* for 10 min and the plasma and buffy coat were removed by aspiration. The remaining cells were washed three times with about 5 volumes of a 1% (w/v) NaCl solution. After the last wash, the cells were spun down for 15 min at 12,000 × *g*.

Treatment of Red Cells with Maleic Anhydride

A sample of 3 ml of washed and packed cells was suspended in 200 ml of a medium containing (mM) $KHCO_3$, 160; K_2CO_3, 8; sucrose, 150; pH 9.1. Reaction with MA was started by quickly adding finely powdered MA to the cell suspension. The reaction was allowed to proceed for 5 min at 3 °C under vigorous stirring. After this step, the cells were spun down and washed three times with at least 10 volumes of the final incubation solution. After the last wash, the cells were centrifuged for 15 min at 12,000 × *g*. In some cases, before the final wash, the cells were submitted to further treatments with MA. Control cells were treated in the same way except that MA was omitted from the solution.

K^+ Efflux

K^+ efflux was measured by continuously monitoring the build-up of extracellular K^+ using a K^+-sensitive glass electrode. For this purpose, 1 ml of washed and packed cells were squirted as quickly as possible into 100 ml of a solution containing (mM): LiCl, 120; Tris HCl, 55 (pH 7.5 at 37 °C). When MA-treated cells were used, the solution contained sucrose (150 mM). The suspension was submitted to continuous magnetic stirring. Immersed in the suspension was a K^+-sensitive glass electrode (Beckman cation electrode) connected with a calomel reference electrode through an agar bridge containing 120 mM LiCl. The potential difference between both electrodes was detected by a pH meter (Radiometer type TTT 1) and fed into a pen recorder (Heath Servo recorder model EU 208). After each run, a calibration curve was drawn by plotting the log of K^+ concentration from 0 to 1 mM against the reading in the recorder. This plot gave a straight line for K^+ concentrations above 200 μM. The calibration curve was used to draw an extracellular K^+ concentration *vs.* time curve. A sample of the cell suspension was hemolyzed in water to measure K^+ and hemoglobin concentration.

K^+ efflux was calculated from the initial slopes of the external K^+ concentration *vs.* time curve. All fluxes were related to the volume of original cells assuming that the 541-nm absorbance of packed cells was 284. This mode of expression allowed us to measure the fluxes across a constant membrane area independently of the osmotic changes in cell volume.

Rate constants for K^+ efflux were calculated by dividing the flux value by the concentrations of K^+ in cell water. The water content of cells suspended in a sucrose-free medium was assumed to be 0.7 (v/v) (Savitz, Sidel & Solomon, 1964). The water content (v/v) of cells suspended in media, to which 150 mM sucrose were added, was calculated according to the following equation:

$$\text{Cell } H_2O = 1 - 0.3\left(\frac{Hb_{suc}}{Hb}\right)$$

where Hb is the concentration of hemoglobin per liter of control cells and Hb_{suc} is the concentration of hemoglobin per liter of cells suspended in the sucrose-containing medium.

Preliminary experiments demonstrated that maleylated cells have a normal osmotic response to sucrose.

LiCl was selected as the main extracellular salt because of the low sensitivity of the glass electrode to Li^+. It was found that the amount of Na^+ released from the cells had no detectable effect on the response of the electrode to K^+. In all the experiments, the degree of hemolysis was estimated from the absorbance of the supernatants at 541 nm and the loss of K^+ resulting from hemolysis was deducted from the measured losses.

Na^+ Efflux

The cells were suspended in enough ice-cold media of identical composition to that of the K^+ efflux experiments to give a final hematocrit of 10% (v/v). Ouabain (final concentration 10^{-4} M) was added to all the solutions to avoid the contribution caused by active transport in the measured fluxes. The tubes were transferred to a bath at 37 °C and individual tubes were returned to the ice-bath at measured time intervals and, after 5 min, spun down for 3 min at $1{,}500 \times g$. The sodium content of the supernatant was measured by flame photometry.

A curve of external Na^+ concentration *vs.* time was drawn and Na^+ efflux and its rate constant were calculated as described for the K^+ efflux experiments. Corrections for loss caused by hemolysis were performed as described in K^+ efflux experiments.

Cl^- Efflux

Cells treated as already described were washed three times with about 10 volumes of a solution of equal composition as that used in the K^+ and Na^+ efflux experiments and after the last wash were packed during 10 min at $12{,}000 \times g$. One ml of the packed cells was quickly squirted into 50 ml of 150 mM sucrose; 175 mM $NaCO_3H$ solution brought to pH 7.4 with gaseous CO_2. The solution was kept at 4 °C and submitted to continuous magnetic stirring. An Ag–AgCl electrode was immersed in the suspension. The potential generated by this electrode was measured against a double-junction calomel reference electrode containing KNO_3 as filling solution (Metrohm combined silver electrode Model EA 248). The potential difference was detected by a pH meter and fed into a pen recorder. Usually three runs were performed per batch of cells. After each run, a calibration curve was drawn following a procedure similar to that outlined for K^+ efflux experiments.

The calibration curves allowed us to calculate the amount of Cl^- lost from the cells at any desired time. With this data, the fraction of intracellular Cl^- that remained inside the cells at each time was calculated and plotted on a logarithmic scale against time. Straight lines were obtained with slopes equal to the rate constants for efflux.

SO_4^{--} Influx

Red cells treated as already described were suspended in a solution similar in composition to that used for Na^+ and K^+ flux experiments, except that 10 mM $NaSO_4$ replaced an equiosmolar amount of LiCl. The cell suspension was distributed into a series of tubes. To half of them enough $^{35}SO_4Na_2$ was added to give a final specific activity of 50,000 cpm/μmole. All the tubes were then transferred to a bath at 37 °C and incubated for 30 min. After this time, the tubes were returned to the ice-bath and cooled for 5 min. An identical amount of $^{35}SO_4Na_2$ was then added to the unlabelled samples. After

this step, the tubes were spun down at 12,000 × *g* for 10 min and the supernatants were discarded. Known volumes of the pellets were hemolyzed in distilled water. A sample of each hemolyzate was set aside for measuring hemoglobin and the rest was deproteinized with trichloroacetic acid (final concentration 5% w/v) and counted in a liquid scintillation counter using Bray's (1960) solution. SO_4^{--} influx was calculated from the $^{35}SO_4Na_2$ uptake by the red cells. The uptake was taken as the difference in the content of radioactivity between the pellets of the cells incubated with and without ^{35}S. This calculation assumes that ^{35}S content of cells incubated at 37 °C without ^{35}S measures the extracellular SO_4^{--} content. Under all conditions, SO_4^{--} uptakes were linear during one hour.

Rate constants for SO_4^{--} influx were calculated by dividing the flux (expressed per liter of original cells) by the SO_4^{--} concentration in the suspending media.

Measurement of Intracellular Chloride

A pellet obtained by centrifugation of red cells for 15 min at 12,000 × *g* was hemolyzed in distilled water and deproteinized following the procedure of Folin and Wu (1919). Cl^- concentration was determined in the supernatant after deproteinization according to the procedure of Schales and Schales (1941). Cl^- concentration in cell water was calculated making the assumptions already mentioned when dealing with the K^+ efflux experiments.

Determination of the Binding of Maleic Anhydride to Red Cells

Cells were treated with 30 mM MA labelled with ^{14}C following the procedure already outlined. After washing, a known volume of cells was hemolyzed in 100 volumes of 1 mM ethylenediamine tetraacetic acid; 30 mM Tris HCl (pH 7.2). An aliquot of the hemolyzate was used to measure the total incorporation of MA. The rest of the hemolyzate was spun down and, on a portion of the supernatant (cell-free hemolyzate), the incorporation of MA was determined. The pellet was used to isolate red cell ghosts according to the procedure already described (Garrahan, Pouchan & Rega, 1969). A portion of the ghosts was employed to determine the incorporation of MA into the cell membrane. The remaining ghosts were submitted to lipid extraction following the procedure of Reed, Swisher, Marinetti and Eden (1960) and the amount of MA in the lipid extract was measured. In one experiment, the lipid extract was submitted to thin-layer chromatography using chloroform/methanol, 2:1 (v/v) as the solvent, following the procedure of Bohner, Soto and Cohan (1965). For determining the distribution of MA among the different classes of phospholipids, the chromatogram was slightly stained with iodine vapors and the spots outlined; each spot was carefully scraped and its radioactivity and phospholipid content measured.

All samples were counted in a liquid scintillation counter using Bray's (1960) solution as scintillator. With the exception of the lipids, all samples were previously digested in four volumes of Hyamine X-10 (Packard Inst. Co.) for 24 hr at 80 °C and then decolored with a 30% hydrogen peroxide solution. Corrections caused by quenching were made by the internal standard method using ^{14}C urea as standard.

Sources of Materials

MA was obtained from British Drug Houses Ltd.; before use the crystals were ground into a fine powder in a mortar.

^{14}C-labelled MA was prepared as follows: 50 μC MA — 1, 4 — ^{14}C, specific activity 14.7 mC/mmole (obtained from the Radiochemical Centre, Amersham, England) in

0.5 ml of benzene, was added to a solution of 588 mg of cold MA in 5 ml of ethyl ether. The clear solution so obtained was brought to dryness by evaporation at low pressure and at room temperature. (^{35}S) SO_4^{--} was obtained from the Commissariat a L'Energie Atomique (France). Ouabain was strophantin-G octahydrate from Sigma Chemical Co. (U.S.A.). All other solvents, reagents and salts were A.R. grade. All the solutions were prepared in doubly glass-distilled water.

Results

The Effect of Maleic Anhydride on Ion Fluxes

Cation Fluxes. The effect of the treatment of red cells with MA on cation fluxes was first studied on the efflux of K^+ from red cells. Fig. 1 shows the time course of the build-up of K^+ in the external solution when cells treated with increasing amounts of MA were suspended in a K^+-free medium. The slopes of the curves give the value of the K^+ efflux. It can be seen that treatment with MA results in a dose-dependent increase in K^+ efflux. The effect of MA on the rate constant for K^+ efflux is given in Table 1. It is clear that as the intensity of the treatment is increased, the rate constant of K^+ efflux rises in an approximately linear fashion without showing any sign of saturation, reaching a value about 30 times that of the control when the cells are treated three times with 30 mM MA.

Fig. 2 shows the time course of hemolysis during an experiment similar to that in Fig. 1. It is clear that after treatment with MA, hemolysis rises with increasing dose and that, whereas in cells treated twice with 30 mM MA,

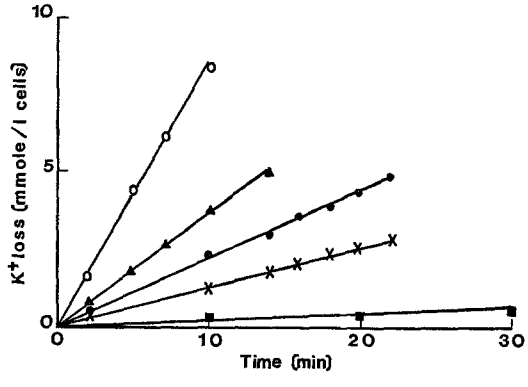

Fig. 1

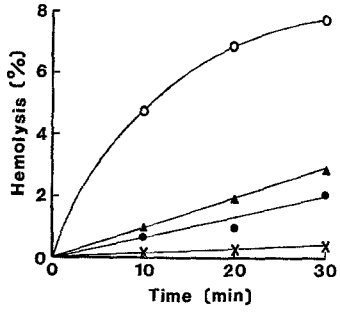

Fig. 2

Fig. 1. The time course of K^+ loss from control cells (▪), from cells treated with 15 mM MA (×) and from cells treated once (•), twice (▲) and three (○) times with 30 mM MA

Fig. 2. The time course of hemolysis of cells treated with 15 mM MA (×) and of cells treated once (•), twice (▲) and three times (○) with 30 mM MA

Table 1. The rate constant of K^+ efflux in cells treated with increasing amounts of MA

Treatment	Rate constant (hr^{-1})
None	0.009
15 mM MA	0.037
30 mM MA	0.067
30 mM MA repeated twice	0.117
30 mM MA repeated three times	0.269

The rate constants were calculated from the experiment in Fig. 1 following the procedure described in Materials and Methods.

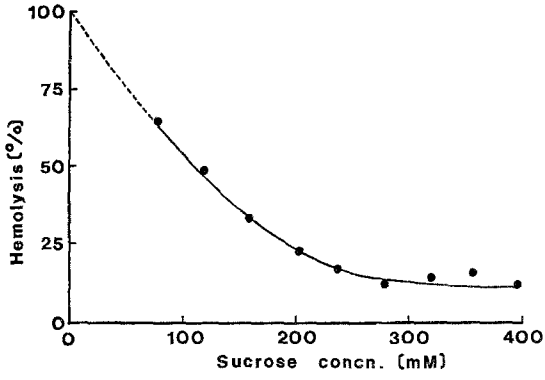

Fig. 3. The degree of hemolysis in cells treated with 30 mM MA and incubated at 37 °C for 1 hr in an isotonic salt medium to which increasing concentrations of sucrose were added

hemolysis does not exceed 3% in 30 min, there is an almost threefold increase in cell destruction when the treatment with 30 mM MA is repeated once more.

Hemolysis curves of Fig. 2 were observed in cells suspended in isotonic salt media containing 150 mM sucrose. Hence, it is unlikely that the observed hemolysis can be attributed to a colloid-osmotic effect caused by increase in cation permeability. This view is supported by the experiment shown in Fig. 3 in which the degree of hemolysis of cells, treated with 30 mM MA and incubated for 1 hr in a medium of salt composition similar to that of Fig. 1, was measured as a function of the concentration of sucrose in the incubation medium. For technical reasons, the lowest sucrose concentration used was 80 mM. Results clarify that as sucrose concentration is raised, hemolysis first decreases along a line which seems to extrapolate to 100% hemolysis at 0 mM sucrose and then, beginning at about 200 mM sucrose, levels off

Table 2. The effect of 30 mM MA on the rate constants for Na^+ and K^+ efflux

Treatment	Rate constant (hr^{-1})	
	Na^+ efflux	K^+ efflux
None	0.042	0.011
MA 30 mM	0.051	0.075

The experiment was performed at the same time and on the same batch of red cells.

reaching a steady value of about 12 % hemolysis. These results suggest that hemolysis, after treatment with MA, may be separated into two components; one prevented by sucrose and thus colloid-osmotic in nature and a second one which persists even at sucrose concentrations as high as 400 mM and which probably results from the loss of stability of the cell membrane[1].

In Table 2, the effect of treatment with 30 mM MA on K^+ efflux is compared with that on Na^+ efflux. The experiment was performed on the same batch of cells and under identical conditions. It is clear that the increase in K^+ efflux elicited by MA (about sevenfold) is accompanied by a much smaller effect on Na^+ efflux (about 1.25 times).

As the experiment in Table 2 was performed in cells whose internal content of K^+ was about six times larger than that of Na^+, it may be argued that the selective effect of MA on K^+ efflux is actually caused by competition between Na^+ and K^+ for ionic channels opened in the membrane by MA. To test this possibility, the effect of 30 mM MA was studied in cells with different internal Na^+/K^+ ratios prepared using the PCMBS technique. Results in Fig. 4 show that within the range of intracellular Na^+ and K^+ concentrations studied, the rate constants for Na^+ and K^+ efflux in cells treated with MA are independent of the intracellular cation composition. Hence, the small effect of MA on Na^+ efflux cannot be attributed to competition between Na^+ and K^+. It seems therefore that, apart from increasing cation movements across the cell membrane, MA also makes the red cell membrane more selective for K^+ than that of control cells.

1 At first look, it may seem surprising that as much as 200 mM sucrose is needed to prevent colloid-osmotic hemolysis. This probably results from the fact that, as will be shown later, treatment of red cells with MA significantly increases their intracellular electronegativity. It can be calculated (*see* Adair & Robinson, 1930) that when the value of the membrane potential is that of the cells treated with 30 mM MA (*see* Table 5) the net excess in intracellular diffusible cations when cells are in Gibbs-Donnan equilibrium with the medium is about 200 mM.

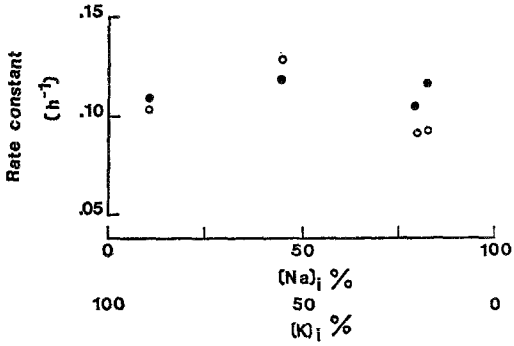

Fig. 4. The effect of intracellular cation content on the rate constants for Na^+ (○) and K^+ (●) efflux in red cells treated with 30 mM MA. The intracellular content of Na^+ and K^+ is expressed as per cent of the total cation content. The intracellular content of Na^+ and K^+ was adjusted, prior to maleylation of the cells, using the PCMBS method (Garrahan & Rega, 1967)

Anion Fluxes. Studies on the effects of amino reagents on Cl^- or Co_3H^- permeability has been hampered by the very high rate of exchange of these anions across the red cell membrane. Taking advantage of the finding of Dalmark and Wieth (1970) on the large temperature dependence of Cl^- exchange in red cells, a technique was developed which allowed us to quantify the rate of Cl^- efflux in exchange for CO_3H^- in red cells at 3 °C. The technique is based on the continuous recording of the signal from an Ag–AgCl electrode immersed in a well-stirred suspension of red cells in an initially Cl^--free isotonic CO_3H^- solution (*see* Materials and Methods). In all experiments, hematocrit was kept low enough to insure that no appreciable back-flow of Cl^- into the cells would take place during the course of the experiment.

Fig. 5 summarizes the results of three experiments in which the Cl^--efflux in exchange for CO_3H^- was measured in control cells and in cells treated with 15 mM and 30 mM MA.

The results show that: (1) In untreated cells, the Cl^- efflux follows first-order kinetics with a half time of 13 sec. This result is in good agreement with those of isotopic Cl^- exchange measured at the same temperature by Dalmark and Wieth (1970), using the rapid filtration technique. (2) Exposure of red cells to MA results in a significant reduction of Cl^- efflux. (3) Like the control cells, the cells treated with MA approach equilibrium with the external media with first-order kinetics suggesting that the effect of MA is uniform on the cell population.

The study of the effect of MA on Cl^- efflux was extended to the penetration of SO_4^{--} into red cells. Table 3 summarizes the result of an experi-

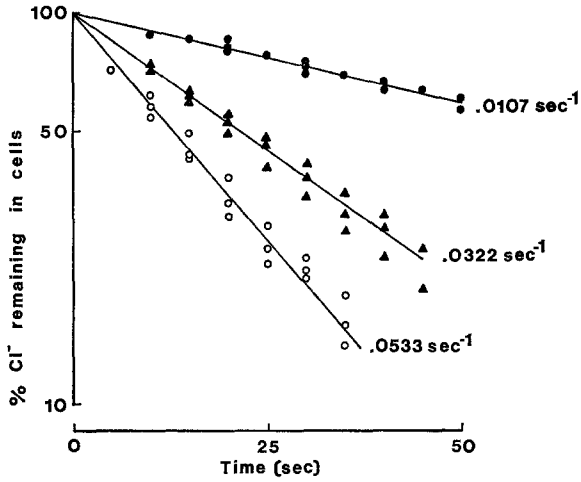

Fig. 5. The efflux of chloride from control cells (○) and cells treated with 15 mM (▲) and 30 mM (●) MA. The experiment was performed at 4 °C. Each curve was drawn pooling the results of three separate experiments. The figures on the curves represent rate constants for efflux

Table 3. The effect of treatment with MA on sulfate influx into red cells

Concentration of MA during treatment (mM)	Rate constant for sulfate influx (hr^{-1})
0	0.275
3.8	0.138
7.5	0.091
15.0	0.071
30.0	0.033

The experiment was performed on the same batch of cells and under identical conditions.

ment in which the effect of increasing concentrations of MA on the influx of SO_4^{--} into red cells was tested.

It can be seen that as the concentration of MA during pretreatment is increased, there is a progressive decline in the rate of sulfate penetration. The effect does not show saturation within the range of MA concentrations tested.

The Mode of Action of Maleic Anhydride

Table 4 shows the result of an experiment in which the distribution of MA among different subcellular fractions was studied in red cells treated with 30 mM MA labelled with ^{14}C.

Table 4. The distribution of MA among different red cell components

Fraction	Bound MA (mmole/liter cells)
Whole hemolyzate	77.5
Cell-free hemolyzate	76.0
Hemoglobin-free membranes	0.55

Cells were treated with 30 mM ^{14}C-MA. The results represent the mean of two experiments.

Table 5. Effects of treatment with MA on the distribution of chloride between intra- and extracellular media of red cells

Treatment	$\frac{(Cl^-)_{in}}{(Cl^-)_{out}}$	Calculated membrane potential (mV)
None	0.70	−9.45
15 mM MA	0.54	−16.32
30 mM MA	0.32	−30.19

Before measuring intracellular chloride, the cells were incubated at 37 °C for 10 min in a solution similar in composition to that used for the cation flux experiments. Membrane potential was calculated applying Nernst's equation to the chloride ratio.

The data clearly show that most of the MA taken up by the cells can be recovered in the cell-free hemolyzate. This result shows that MA quickly crosses the red cell membrane. The intracellular accumulation probably is a consequence of the reaction of MA with the large intracellular pool of amino groups provided mainly by hemoglobin. A small fraction (about 0.7%) of the MA taken up by the cells is recovered with the cell membranes.

Since, as mentioned before, reaction of amino groups with MA leads to reversal of their charge, it may be expected that at a given pH the net charge of hemoglobin and hence the membrane potential in MA-treated cells will be more negative than in untreated cells. To measure this effect, the ratio of intra- to extracellular chloride concentration was estimated in control cells and in cells treated with MA. Results in Table 5 show that as the intensity of treatment with the amino reagent progresses, the intracellular Cl^- concentration decreases from 0.7 to 0.3 times the extracellular Cl^- concentration, which corresponds to a drop from −9 to −30 mV in membrane potential.

In view of these results and since electrical potential is one of the components of the driving force for ion movements, to quantify the effect of MA on membrane permeability it is necessary to cancel the effect caused by changes in membrane potential from the measured fluxes. To do this, the following assumptions were made: (1) all the measured fluxes result from the diffusion of ions across the cell membrane, and (2) the electrical field across the membrane is constant.

If these assumptions are taken for granted, the following equation (Katz, 1966) can be used:

$$J=PCf \tag{1}$$

where J is the unidirectional flux, P is a permeability coefficient, C is the concentration of the ion in the water phase of the compartment from which the flux is measured and f represents the effect of the electrical field on the rate of ion permeation. The value of f can be calculated according to the following equation:

$$f=(zEF/RT)/1-\exp(-zEF/RT) \tag{2}$$

where E is the potential difference across the membrane, its sign being taken as positive when the movement of the ion is assisted, z is the valence and F, R and T have their usual meaning. Eq. (1) can be rearranged to give

$$P=J/(Cf). \tag{3}$$

The value of P is equal to the flux at zero membrane potential and at unit concentration. In all the experiments, the fluxes were calculated per liter of original cells and hence, the area of cell membrane involved was always the same. This made it unnecessary to express the fluxes per unit membrane area to apply Eq. (3). Hence, the value of P differs only by a constant factor from the true permeability coefficient.

Since, under the assay conditions employed, Cl^- efflux is coupled in a one-to-one fashion to CO_3H^- entry, the rate of chloride efflux will be limited by the rate of permeation of the slowest anion and hence the effect of changes in membrane potential in Cl^- efflux will be opposite according to whether Cl^- or CO_3H^- are rate limiting in the exchange. Since no definite information concerning this point is available, the Cl^- fluxes at zero membrane potential were calculated considering both possibilities.

Table 6 summarizes the effect of 30 mM MA on the rate constants for the fluxes of all the ionic species studied after correction to zero membrane potential. The data show that: (1) When the effects caused by changes

Table 6. The effect of 30 mM MA on the rate constants for ion fluxes corrected to zero membrane potential

Ion	Treatment	Rate constant (hr^{-1})		Treated / Control	Control / Treated
		Experimental	Corrected		
K^+	None	0.009	0.010	–	–
	MA 30 mM	0.061	0.110	11.0	–
Na^+	None	0.042	0.052	–	–
	MA 30 mM	0.051	0.088	1.7	–
Cl^-	None	178	146	–	–
	MA 30 mM	42	22	–	6.6
Cl^- (bicarbonate-limiting)	None	178	216	–	–
	MA 30 mM	42	81	–	2.7
SO_4^{--}	None	0.275	0.410	–	–
	MA 30 mM	0.033	0.137	–	3.0

The ratios of treated to control and vice versa were calculated using the corrected rate constants.

in membrane potential are cancelled, both the increase in cation fluxes and the reduction in anion fluxes elicited by MA are still present. After correction to zero membrane potential, the effects of MA on ion fluxes have to be attributed to changes in the permeability properties of the red cell membrane induced by the amino reagent. (2) The almost complete lack of effect of MA on the experimental rate constant for Na^+ efflux seems to be due to the fact that, in this particular case, the opposite effects of the reagent on the driving force and on the cell membrane permeability for cation efflux cancel each other. When the effect of MA on membrane potential is taken into account, a small but significant increase in Na^+ permeability after treatment with the reagent becomes apparent. (3) The larger depression of the experimental rate of SO_4^{--} permeation as compared with that of Cl^- can be fully accounted for by the fact that for SO_4^{--}, being a divalent anion, the dependence of its flux on electrical potential is larger than that of monovalent ions like Cl^- or CO_3H^-. When corrected to zero membrane potential, the effect of 30 mM MA on Cl^- permeability is equal to or larger than that on SO_4^{--} permeability, depending on whether CO_3H^- or Cl^- is taken as rate limiting in the Cl^- efflux in exchange for CO_3H^-.

Distribution of Maleic Anhydride Among Membrane Components. As already shown in Table 4, when red cells are treated with 30 mM MA,

Table 7. The distribution of MA among different cell membrane components in cells treated with 30 mM MA

Membrane component	Maleic anhydride (mmole/liter cells)	Maleic anhydride (mole/mole amino group) × 100
Protein	0.46	21
Total Lipids	0.09	6
Lecithin + lysolecithin	0.013	–
Sphingomyelin	0.035	–
Phosphatidylserine	0.008	1.4
Phosphatidylethanolamine	0.016	1.8
Solvent front	0.018	–

Lipid-bound MA is the amount of MA which is extracted together with the membrane lipids. Protein-bound MA was calculated as the difference between the total membrane-bound MA and the lipid-bound MA. The distribution of MA among the different phospholipid classes was calculated from the radioactivity recovered on each spot of the chromatogram. 97.9% of the radioactivity present in the lipid extract was recovered after chromatography. The amino content of the membrane protein fraction was assumed to be 2.3 mmole/liter cells and of the membrane lipid fraction was assumed to be 1.46 mmole/liter cells (Knauf & Rothstein, 1971).

No quantitative determination of the phospholipids after chromatography was made. The distribution of amino groups among the different classes of phospholipids on a molar basis was assumed to be equal to that published by Reed *et al.* (1960) for lipid phosphorus.

0.55 mmole of the reagent is incorporated into the membrane fraction of a liter of cells. Since both the major components of the cell membrane, namely proteins and lipids, contain amino groups potentially able to react with MA, the distribution of the reagent between these two fractions was studied.

Results in Table 7 show that: (1) 84% of the membrane-bound MA is not extracted with the membrane lipids and thus presumably is bound to membrane proteins. The amount of protein-bound MA calculated on this basis is sufficient to react with approximately 20% of the amino groups present in cell membrane proteins. (2) 16% of the membrane-bound MA can be recovered with the lipids after solvent extraction of the membrane. If all the lipid-bound MA were distributed between phosphatidyl serine and phosphatidyl ethanolamine, about 6% of the amino lipids would have reacted with MA. (3) Thin-layer chromatography of the lipid extract showed that only 27% of the lipid-bound MA migrated with amino lipids, which reduces to 1.6% the amount of amino lipids which reacted with MA.

Discussion

The main conclusion to be drawn from the results presented in this paper is that, as a consequence of the reaction of MA with the red cell membrane, cation permeability is increased while anion permeability is depressed. Furthermore, since the major part of the MA incorporated into the red cell membrane is associated with the protein fraction, it seems likely that reaction of MA with membrane proteins is responsible for the observed changes in permeability. It is unlikely that the small amount of reagent that can be recovered with the lipid fraction is of any physiological significance.

As already mentioned, studies of the interactions of MA with proteins in solution show that, under conditions similar to those employed by us, the compound reacts specifically with amino groups. It seems, therefore, reasonable to think that a reaction of this sort with cell membrane proteins is the primary event in eliciting the observed changes in cell membrane permeability.

Since the environment within the cell membrane may differ from that necessary for MA to react specifically with amino groups, the possibility that, apart from amino groups, cell membrane sulfhydril groups may also be involved in the reaction (*see* Butler *et al.*, 1969) has to be considered. It is known that specific blockage of sulfhydril groups increases Na^+ and K^+ permeability to a similar extent (Sutherland, Rothstein & Weed, 1967) without observable changes on anion permeability (Knauf & Rothstein, 1971). These effects contrast with those observed after treatment with MA and makes it unlikely that blockage of sulfhydril groups play any important role in eliciting the observed effects of MA.

Reaction of MA with proteins in solution has two main observable effects: there is an increase in the negative charge of the protein and, as a consequence of this, the protein changes its native conformation (Butler *et al.*, 1969). There seems to be no reason for excluding the possibility that similar conformational changes also occur in membrane-bound proteins upon treatment with MA. As judged by the appearance of hemolysis which is not prevented by sucrose, the membrane of cells treated with MA appears to be less stable than that of control cells. It may well be that the cause of this phenomenon is a change in conformation of cell membrane proteins after reaction with MA.

If partial disorganization of the cell membrane structure is one of the effects of the treatment of the red cell with MA, it becomes necessary to analyze to what extent the observed changes in ion permeability are

caused by this phenomenon rather than being the direct result of the blockage of amino groups. If disorganization of the membrane were the sole result of the maleylation of membrane proteins, a generalized and non-specific increase in the permeability to all small solutes would have been expected. In sharp contrast to this prediction, the experimental results show that MA is able to elicit specific, and in some cases opposite, effects in the permeability of the red cell membrane to ions of similar size. It seems, therefore, reasonable to conclude that apart from and superimposed to the partial disorganization of membrane structure, maleylation of membrane amino groups damages a mechanism which is concerned with the control of the ionic selectivity in red cells. In this mechanism amino groups as such may be directly involved.

The very low cation permeability of the red cell membrane may be caused by fixed positive charges provided to the cell membrane by protein amino groups (for an assessment of this hypothesis *see* Passow, 1969). This view is mainly supported by the finding that reaction of a number of amino reagents with red cells, as well as high pH values which discharge amino groups, lead to an increase in cation fluxes together with a decrease in anion fluxes. The increase in K^+ permeability observed in MA-treated cells is in agreement with the above-mentioned hypothesis. However, in its simplest form, the positive fixed-charge hypothesis cannot account for the significantly larger increase in K^+ permeability as compared to Na^+ permeability upon treatment of red cells MA. Furthermore, if this hypothesis is taken for granted, the selective effect of MA on K^+ permeability would seem to suggest that separate pathways for Na^+ and K^+ permeability, with different sensitivities to MA, are present within the red cell membrane. Some indirect evidence for different pathways for Na^+ and K^+ movements is available; *viz*, the different responses of Na^+ and K^+ fluxes to changes in pH (Pfleger, Rummel & Seifen, 1967) and the fact that K^+ permeability may be increased independently to Na^+ permeability by Ca^{++} (Whittam, 1968; Lew, 1970).

As mentioned before, it has been reported that blockage of cell membrane amino groups with reagents different from MA depresses anion permeability (Passow, 1969; Passow & Schnell, 1969; Knauf & Rothstein, 1971; Poensgen & Passow, 1971). This conclusion is mainly based on the observation that amino agents reduce SO_4^{--} permeability. Under physiological conditions the rate of SO_4^{--} exchange across the red cell membrane is several orders of magnitude lower than that of halides and CO_3H^-. It may therefore be risky to draw conclusions concerning the overall properties of the anion permeability of red cells from studies on SO_4^{--} permeability.

The use of the Ag–AgCl electrode allowed us to extend to rapidly diffusing anions the studies on the effects of an amino-reactive agent like MA on anion permeability. Results show that in red cells treated with enough MA to elicit large increases in K^+ permeability, the rate of $Cl^- - CO_3H^-$ exchange is significantly less than that of control cells. The reduction in the permeability to monovalent anions due to MA is paralleled by a similar decrease in the rate of SO_4^{--} permeation.

The effect of MA on anion permeability, suggests that amino groups are involved in the mechanism responsible for the high anionic selectivity which is a characteristic feature of the red cell membrane.

The mechanism through which amino groups intervene in mantaining the high anionic permeability of the red cell membrane is still an open question, which probably will not be answered by the use of chemical probes. One of the currently held views (for references *see* Passow, 1969) mantains that amino groups act as the main source of fixed positive charges within the membrane structure. If this were the case, the observed effects of MA would have been caused by the cancellation or reversion of such charges, with a concomitant decrease in the intramembrane concentration of mobile anions. However, present knowledge does not allow us to reject the possibility that most of the rapid halide exchanges observed in red cells are caused by a neutral one-to-one carrier-mediated process (Hunter, 1971). If this were the case, our results would suggest that the integrity of amino groups is required for the process to take place.

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