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CHARACTERISTICS OF A SULPHYDRYL GROUP ESSENTIAL FOR SODIUM EXCHANGE DIFFUSION IN BEEF ERYTHROCYTES

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SUMMARY

1. In the beef red blood cell, the component of the Na efflux which is insensitive to ouabain but depends on the presence of external Na, is not affected by furosemide but is reduced by several agents: ethacrynic acid, dinitrofluorobenzene, *p*-chloromercuribenzene (PCMB), N-ethylmaleimide and *p*-chloromercuribenzene sulphonate (PCMBS). Some of these agents increased a parallel passive permeability which could mask the reduction of efflux.

2. N-ethylmaleimide and PCMBS, which in our experimental conditions (initial concentration 5×10^{-4} M and 5×10^{-6} M respectively, haematocrit 7·7 %) do not increase the leak, inhibit Na influx and efflux markedly and equally. This provides further evidence of the existence of a typical ouabain-insensitive Na exchange diffusion in beef red blood cell.

3. Inhibition of the exchange diffusion mechanism by N-ethylmaleimide or PCMBS is not total and their inhibitory effects are slightly additive. Various arguments suggest that their effects on exchange diffusion can be attributed to a reaction with sulphydryl groups.

4. These sulphydryl groups are rapidly titrable by a poorly penetrating agent such as PCMBS, and the inhibitory effect is rapidly reversible. Thus, it is assumed that the sulphydryl groups containing proteins are superficially located on the outer border of the membrane.

5. After inhibition, there is no change in half saturation constant for the complexing reaction for transfer, suggesting that the inhibited sites are no longer functioning but that the uninhibited sites are in every way normal.

6. N-ethylmaleimide and PCMBS act similarly in sheep red blood cells.

7. PCMBS does not affect sodium movement in human erythrocytes, but N-ethylmaleimide inhibits markedly the ouabain-insensitive Na efflux.

INTRODUCTION

Evidence has been presented in a previous paper (Motais, 1973) that the major part of Na movements across the cell membrane of LK beef erythrocyte corresponds to an exchange of intracellular for extracellular Na. This exchange, which is ouabain insensitive does not require energy or nucleotide and is thought not to be mediated by the Na pump.

The purpose of this paper is an attempt, by using chemical modifiers of Na^+–Na^+ exchange, to obtain information concerning the chemical nature of the membrane molecules involved in this exchange diffusion process and their location in the membrane.

METHODS

Fluxes were measured as described in the previous paper (Motais, 1973). The substances used were: dinitrofluorobenzene (Calbochiem), N-ethylmaleimide (Koch Light), ethacrynic acid, *p*-chloromercuribenzene sulphonate, *p*-chloromercuribenzoate and dithiothreitol. The last four substances were purchases from Sigma. Furosemide was a gift from Hoechst.

RESULTS

Inhibition of Na efflux by some chemical agents

The purpose of the experiments described here was to find drugs able to reduce the Na efflux to an extent similar to that caused by removal of external Na.

In preliminary experiments we used the diuretics ethacrynic acid and furosemide which were found to inhibit, in human red cells, the component of the Na efflux which is insensitive to ouabain but dependent upon the presence of external Na (Hoffman & Kregenow 1966; Dunn, 1970; Sachs, 1971; Rettori & Lenoir, 1972). As shown in Fig. 1, Na movements are unaffected by furosemide (10^{-3} M). Ethacrynic acid (10^{-3} M) causes a reduction of Na efflux. This action, however, is very slight (a decrease of 15 %) in comparison with the size of the exchange diffusion component (approximately 85 %). Of interest, however, is the fact that furosemide is not thought to have a significant effect upon membrane sulphydryl integrity while ethacrinic acid is known as a potent sulphydryl reagent. These results could indicate some relationship of sulphydryls to exchange diffusion in beef red cells. The inhibition of Na efflux following reaction with several more or less specific sulphydryl reagents was therefore investigated. In these experiments, as with those using ethacrynic acid and furosemide, 1 ml. of packed cells labelled with ^{22}Na was suspended in 12 ml. Na Ringer containing sulphydryl reagents (temperature 37° C, pH 7·4) and the measurement was started when cells were squirted in the

medium. The initial concentrations of drugs in the medium were: dinitrofluorobenzene 5×10^{-3} M, PCMBS 5×10^{-6} M, PCMB 10^{-4} M, N-ethylmaleimide: 5×10^{-4} M.

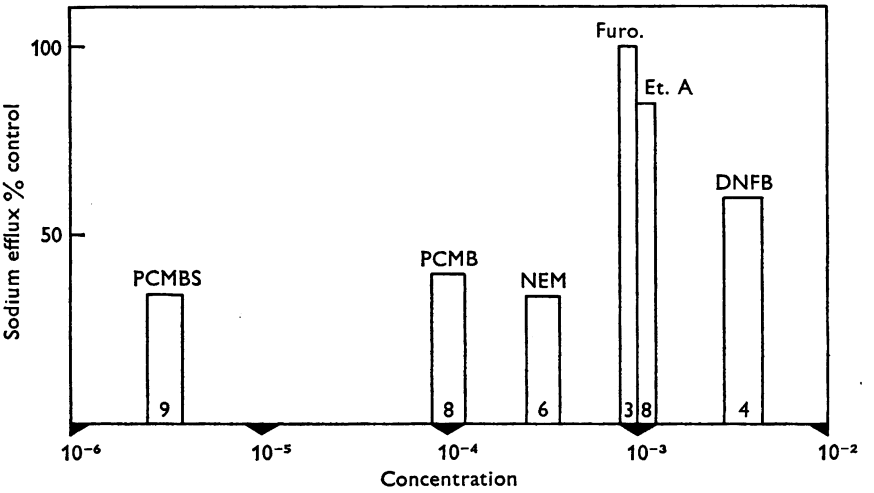

Fig. 1. Action of different sulphydryl agents on Na efflux (expressed as %control value) measured in Na Ringer. The exact values are: ethacrynic acid (Et. A): 86 % ± 1·48; dinitrofluorobenzene (DNFB): 59 % ± 1·12; PCMB: 39·9 % ± 2·12; PCMBS: 36·1 % ± 0·95; N-ethylmaleimide (NEM): 33·8 % ± 0·83. The number of experiments is recorded at the base of each column. The initial concentration of drugs is given on the abscissa. Haematocrit 7·7 %.

It can be seen (Fig. 1) that all of these sulphydryl reagents inhibit a part of the total Na efflux, but the magnitude of the effect and the doses used are different: in the presence of dinitrofluorobenzene inhibition is less important (41 %) than in presence of PCMB (60 %) PCMBS (64 %) and N-ethylmaleimide (66 %); the inhibitory potency of PCMBS is the highest since the maximal effect is obtained at 5×10^{-6} M, i.e. 3 orders and 2 orders of magnitude less than the concentration of DNFB and N-ethylmaleimide respectively. Nevertheless, it must be pointed out that the reduction of Na efflux is always, even in the best conditions, smaller than after removal of external Na (approximately 85 %), i.e. smaller than the exchange diffusion component.

Of interest is the fact that in a previous paper (Motais, 1973) and with the same biological material, PCMBS was used at a higher concentration (2×10^{-4} M) to increase passive cation permeability and thus to alter the internal Na, following the procedure described by Garrahan & Rega (1967) for human red cells. Thus, treatment with PCMBS has a dual effect:

increase of passive permeability and inhibition of exchange diffusion component (it will be demonstrated later that it is really inhibition of exchange diffusion system). Such a dual effect is found for all the drugs used here, except for N-ethylmaleimide which does not induce an increase of membrane permeability. These data are in accordance with the fact that in the human red cell also, all of these sulphydryl agents, except N-ethylmaleimide, act on two targets (N-ethylmaleimide has been claimed to have no effect on Na permeability and on Na active transport in human

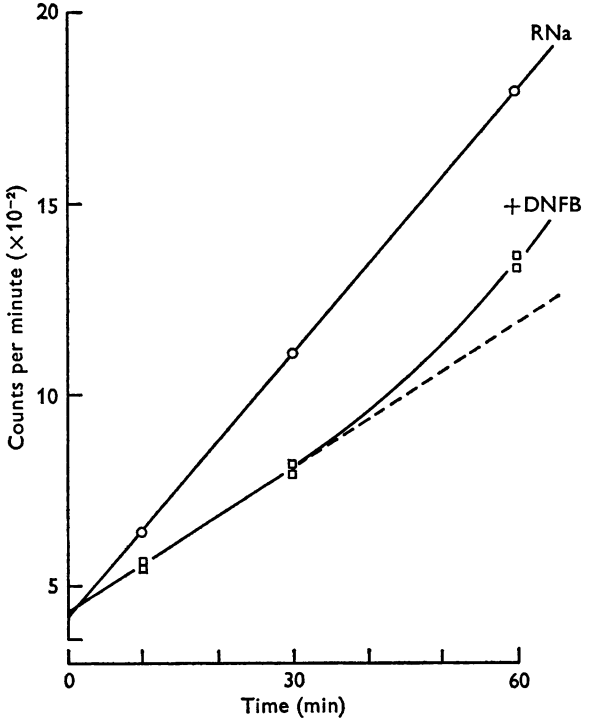

Fig. 2. Time-dependent behaviour of ^{22}Na efflux measured in Na Ringer (○) (RNa) and in Na Ringer containing 5×10^{-3} M DNFB (□). The rate of efflux is expressed in counts/min. Abbreviations as Fig. 1.

red cells (Sutherland, Rothstein & Weed, 1967) but is known to inhibit Na-K activated ATPase at the step of K-dependent diphosphorylation): passive cation permeability which is increased (Vansteveninck, Weed & Rothstein, 1965) and active transport (Pump I, or Pump II for ethacrynic acid) which is inhibited (Sutherland *et al.* 1967; Hoffman & Kregenow, 1966). As it has been shown that increase in passive permeability to cations is associated with the reaction of the drug within the membrane after a diffusion-limited step (Vansteveninck *et al.* 1965), the relative importance

of the two targets of sulphydryl agents will depend on the rate of penetration of the agent, its concentration and localization of the reaction site involved in exchange diffusion. Both effects can be seen simultaneously in our experimental conditions with PCMB (10^{-3} M) which penetrates the erythrocyte membrane easily (Vansteveninck *et al.* 1965), since a large inhibition is observed in Na Ringer (60%) but an increase of Na loss is observed in Na-free Mg Ringer. In the dinitrofluorobenzene (5×10^{-3} M) on the other hand, both effects do not occur simultaneously, as can be seen in Fig. 2: Na efflux is first partially inhibited (the cells are squirted in Na Ringer containing dinitrofluorobenzene at time 0) and then increases progressively with time. The explanation for the complex time-dependent behaviour is as follows: the increase in Na passive permeability, which appears later, tends to obscure the inhibitory effect of the agent on the exchange diffusion mechanism. This indicates that the sensitive sites involved in exchange diffusion could be more superficially located than the sensitive sites involved in passive permeability.

TABLE 1. Effect of PCMBS on Na efflux and Na influx

Na efflux		Na influx	
Control	With PCMBS	Control	With PCMBS
$6{\cdot}69 \pm 0{\cdot}05$ ($n = 6$)	$2{\cdot}35 \pm 0{\cdot}04$ ($n = 6$)	$6{\cdot}68 \pm 0{\cdot}08$ ($n = 6$)	$2{\cdot}36 \pm 0{\cdot}06$ ($n = 6$)

1 ml. packed cells suspended in 12 ml. Na Ringer without or with PCMBS (5×10^{-6} M).

Fluxes are expressed in m-mole/l. cells.hr.

Cells are separate samples of the same individual's blood.

Characterization of the inhibition by PCMBS and NEM

Since passive permeability is not affected by N-ethylmaleimide or by PCMBS in our experimental conditions (haematocrit 7·7%, initial concentration of PCMBS 5×10^{-6} M and of N-ethylmaleimide 5×10^{-4} M) we selected these two sulphydryl reagents for further study.

Before drawing conclusions concerning the chemical nature of ligands involved in the exchange diffusion process, it is, of course, necessary to demonstrate that the thiol reagents effectively inhibit Na–Na exchanges; in other words a symmetric inhibitory effect of the drug on Na influx and Na efflux has to be observed. It is evident that an asymmetric effect would invalidate the assumption that an exchange diffusion mechanism occurs. In experiments made with different batches of cells it was found that in the presence of N-ethylmaleimide the sodium efflux represents $33{\cdot}83 \pm 0{\cdot}83$ ($n = 6$), and the Na influx $31{\cdot}55 \pm 1{\cdot}19\%$ ($n = 6$) of the control

value. In the presence of PCMBS the data were $35{\cdot}48 \pm 0{\cdot}75$ ($n = 6$) for efflux and $36{\cdot}54 \pm 0{\cdot}51$ ($n = 6$) for influx. Table 1 presents the data obtained from separate samples of the same individual's blood. Obviously N-ethylmaleimide and PCMBS inhibit Na influx and efflux equally, and these results form additional evidence for a Na exchange diffusion mechanism in beef red cell. In assessing the equality of the inhibitory effects it should be pointed out that the amount of agent added per cell is more relevant than a consideration of concentration alone. For example, in experiments reported in Table 2, the initial concentration of N-ethylmaleimide in the Na Ringer was 5×10^{-4} M for measurements of both Na

TABLE 2. Influence of amount of NEM added per cell on Na movements

	Na efflux		Na influx	
Expt.	Control	With NEM	Control	With NEM
1	6·69	2·39	6·68	6·53
	6·63	2·27	6·61	6·33
2	5·20	2·19	5·15	4·69
	5·18	2·04	5·12	4·78
3	9·35	2·83	9·58	7·99
			9·52	7·88

Experimental conditions: the initial concentration of NEM in Na Ringer was 5×10^{-4} M for all the experiments. In efflux measurements however the haematocrit was 7·7 % (1 ml. packed cells in 12 ml. Ringer) and for influx measurements was 28·6 % (2 ml. packed cells in 5 ml. Ringer). Fluxes are expressed in m-mole/l. cells. hr. NEM would appear to have an assymetrical effect on Na fluxes.

influx and Na efflux, but the haematocrit was different: for the efflux experiments we used 1 ml. packed cells in 12 ml. Ringer and for the influx experiments 1 ml. packed cells in 5 ml. Ringer. These data give the impression that N-ethylmaleimide has an asymmetrical effect on influx and efflux. It should be noted that evidence put forward to claim that in the human red cell the ouabain-insensitive, ethacrynic acid-sensitive Na efflux is not exchange diffusion but active transport, was the asymmetrical effect of furosemide at concentrations below 10^{-4} M (Rettori & Lenoir, 1972). However, in efflux measurements 1 ml. packed cells was suspended in 25 ml. solution, but in influx experiments 1 ml. packed cells was suspended in 3 ml. solution.

It is of importance to determine whether the inhibitory effect of the sulphydryl agents can be attributed to interactions with sulphydryl groups either within the cell or within the membrane or at the outer surface of the membrane. With regard to the action of DNFB (Fig. 2) it has been discussed that sites involved in exchange diffusion may be more super-

ficially located than sites involved in passive permeability, which are known in the human red cell, to be within the membrane. Fig. 3 shows that the inhibitory effect of N-ethylmaleimide like that of dinitrofluorobenzene is very rapid. These two however enter erythrocytes easily. The only certain conclusion is that an agent acts on a membrane if it is essentially non-penetrating and reacts very rapidly. PCMBS is a good tool for

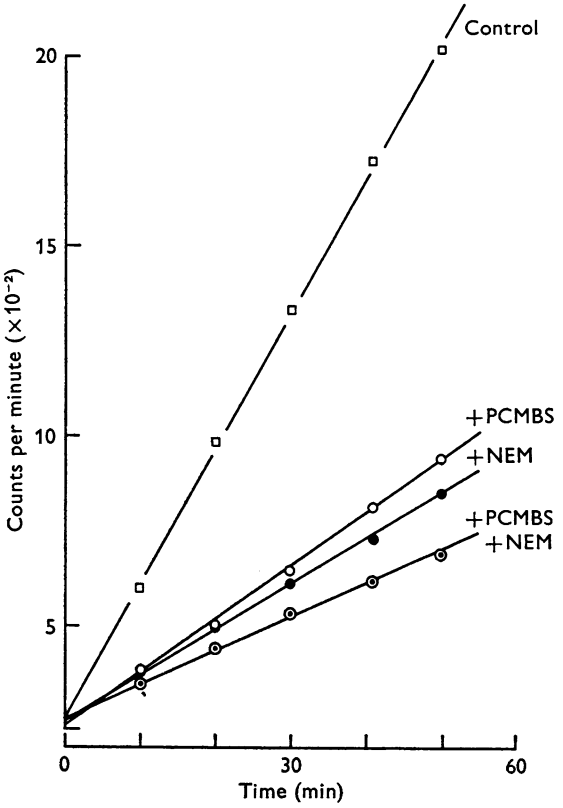

Fig. 3. The inhibitory effect of NEM (●), PCMBS (○), and PCMBS + NEM (⊙), on the efflux of ^{22}Na. The rate of efflux is expressed in counts/min. Haematocrit: 7·7%. Initial concentrations: NEM 5×10^{-4} M, PCMBS 5×10^{-6} M. Abbreviations as Fig. 1.

such a test because it penetrates very slowly (Vansteveninck, Weed & Rothstein, 1965). As shown in Fig. 3, inhibition by PCMBS also develops immediately after contact. Thus, the fact that exchange diffusion is rapidly blocked by a poorly penetrating sulphydryl agent indicates that a superficially located protein is a part of the mechanism. Another method of demonstrating an outer membrane target was to study the rapidity of reversal of the effect in the case of a sulphydryl-reagent which binds

reversibly on sulphydryl. It is known that interaction of N-ethylmaleimide with sulphydryl, resulting in addition to double bonds, is not easily reversed. Mercaptides, obtained with PCMBS, on the other hand, form easily reversible complexes so that PCMBS can be removed from the protein by adding another sulphydryl-containing agent. This property has been used in a previous paper to alter $[Na]_{in}$ (Motais, 1973). For the present study ^{22}Na loaded cells were incubated with PCMBS at a convenient concentration for 5 min, then washed 3 times with $MgCl_2$, and finally the radioactive efflux was measured in Na Ringer free of PCMBS. After 31 min a sulphydryl containing agent was added (dithiothreitol). A typical experiment is

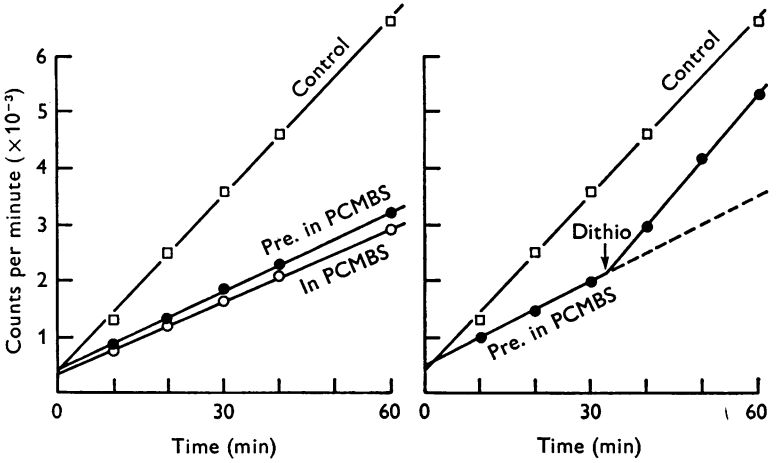

Fig. 4. Experiment showing (*a*) effect of pre-incubation with PCMBS on ^{22}Na efflux. Cells loaded with ^{22}Na were pre-incubated for 5 min (37° C) in Na Ringer with an initial PCMBS concentration of 3×10^{-5} M (haematocrit 45·8 %), then washed 3 times and finally suspended (●) as control (□) in Na Ringer without PCMBS (1 ml. cells in 12 ml. solution). The inhibitory effect can be compared with that obtained when cells not pre-incubated are suspended in Na Ringer plus PCMBS (5×10^{-6} M 1 ml. cells in 12 ml. solution) (○); (*b*) the rapid reversal of this effect by dithiothreitol (5×10^{-3} M). The rate of efflux is expressed in counts/min. Abbreviations as in Fig. 1.

reproduced in Fig. 4. First, it can be seen (Fig. 4*a*) that 5 min preincubation with PCMBS suffice to obtain a fast interaction between the slowly penetrating sulphydryl reagent and the sensitive protein. Secondly, the kinetics of recovery can be considered as instantaneous (Fig. 4*b*); thirdly the recovery is total since the slope of the straight line after addition of dithiotreitol is the same as that of the control. A similar experiment with N-ethylmaleimide showed that, as expected, the interaction of N-ethylmaleimide is practically irreversible.

It has been noted that N-ethylmaleimide and PCMBS in the conditions described above inhibit largely but not totally exchange diffusion of sodium (approximately 85 % exchange diffusion component). Fig. 5*a* shows clearly that the efflux observed in $MgCl_2$ solution, at external [Na] = 0, is N-ethylmaleimide insensitive suggesting that in a Na-free solution the Na efflux is not going through the exchange diffusion path; when the

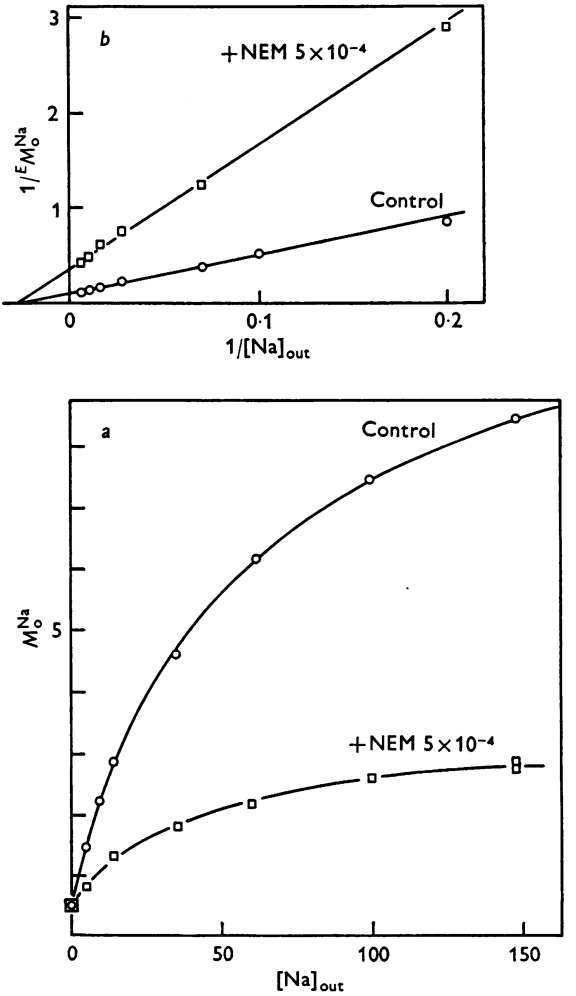

Fig. 5. Kinetics of Na inhibition by NEM. *a* Na efflux in relation to extracellular Na concentration (m-mole/l.) with (□) and without NEM (○) (5×10^{-4} M, haematocrit 7·7 %). Na was substituted by Mg. Fluxes are expressed in m-mole/l. cells.hr. *b* Lineweaver–Burk plot of the exchange diffusion component of efflux calculated from the same data. Abbreviations as in Fig. 1.

external Na is increased, however, the residual Na efflux increases; the occurrence of such an external Na-dependent efflux in the presence of N-ethylmaleimide demonstrates that the inhibition of the exchange diffusion process by the drug is in fact partial. This inhibition by N-ethylmaleimide can be described as non-competitive (Fig. 5*b*). Such an insufficient inhibition could result from a partial inhibition of the transporting sites. Some experiments were designed, by modifying our experimental conditions, to test whether a more important inhibitory effect could be obtained with larger concentrations of agent or by increasing the time of pe-incubation with the agent. As shown in Fig. 6, the inhibition is not altered either by time of incubation with N-ethylmaleimide (temperature 37° C), or by increase in its concentration.

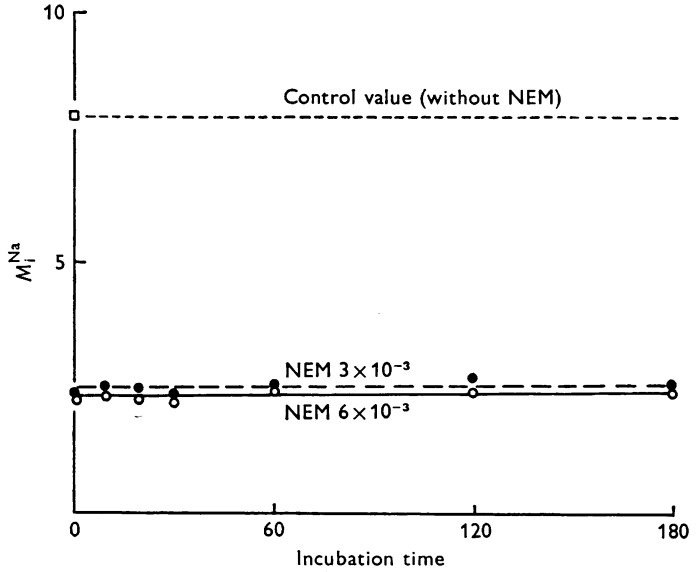

Fig. 6. No modification of the Na influx (expressed in m-mole/l. cells.hr) with time of incubation with NEM at 37° C pH 7·4. Two concentrations of NEM were used: 3×10^{-3} M and 6×10^{-3} M (haematocrit 33 %). Abbreviations as in Fig. 1.

The effect of N-ethylmaleimide (and presumably PCMBS) being assumed maximal it was therefore of interest to see whether their effects could be additive. The efflux of radioactivity was measured in sodium Ringer containing PCMBS or N-ethylmaleimide or both. The result of a typical experiment (Fig. 3) suggests that N-ethylmaleimide does not act on all the same groups as does PCMBS, since the inhibitory effects are slightly additive.

Effects of N-ethylmaleimide and PCMBS on Na efflux in sheep and human erythrocytes

The preceding results raised the question of whether or not N-ethylmaleimide and PCMBS inhibition of Na exchange diffusion is a feature peculiar to the beef red cell. As previously discussed (Motais, 1973) there are at least two forms of Na exchange diffusion: a ouabain-insensitive one, clearly demonstrated in beef red cells and which probably also occurs in

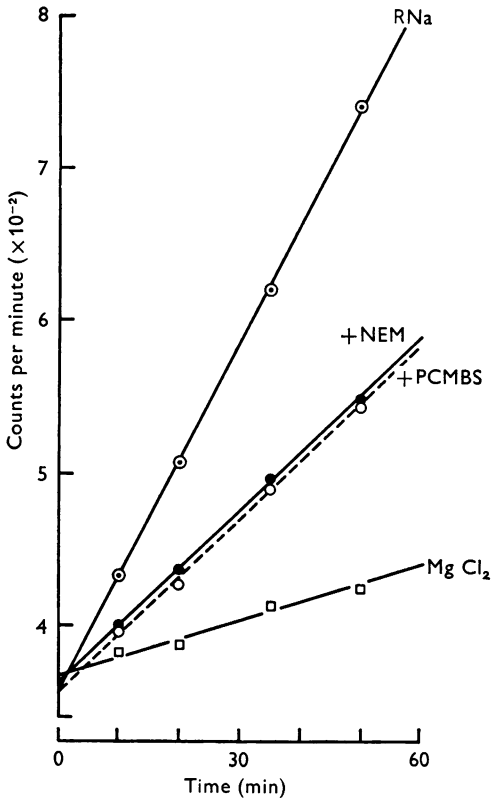

Fig. 7. The inhibitory effect of NEM (●) and PCMBS (○) on the efflux of ^{22}Na in sheep erythrocytes. The rate of efflux is expressed in counts/min. Haematocrit 7·7%. Initial concentrations: NEM 5×10^{-4} M, PCMBS 5×10^{-6} M. Abbreviations as in Fig. 1.

LK sheep erythrocytes as indicated by the occurrence of a large Na-free effect (Tosteson & Hoffman, 1960), and a ouabain-sensitive one described by Garrahan & Glynn (1967) in the human red cell. Some experiments were therefore carried out in these erythrocytes to test the effect of N-ethylmaleimide and PCMBS on exchange diffusion.

Fig. 7 shows that the pattern of inhibition by N-ethylmaleimide and PCMBS is very similar in sheep and beef erythrocytes: the inhibitory effects are rapid; the drugs inhibit largely but not totally the exchange diffusion component; the same initial concentrations of drugs are effective. These data suggest that great similarities may exist between the Na exchange diffusion mechanisms of the erythrocytes of these two species. In the human red blood cell, we have tested the sulphydryl reagents in two experimental conditions: firstly in K-free Na Ringer, i.e. under conditions in which the Na–K pump exchanges extracellular Na for intracellular Na

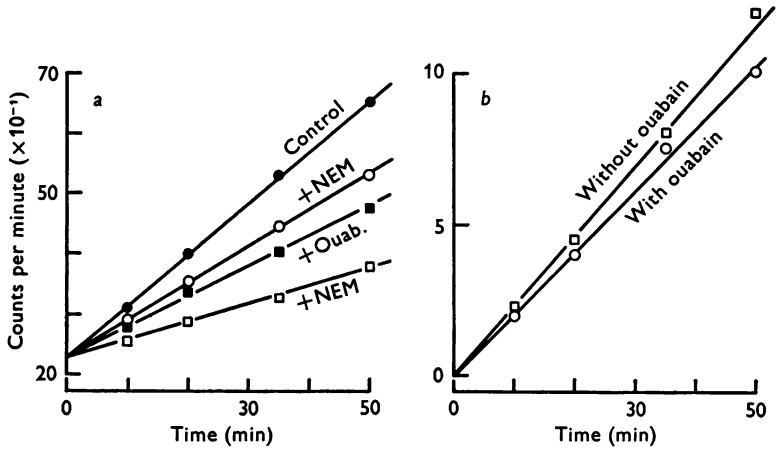

Fig. 8. Effect of NEM on the human red blood cell. *a* Time-dependent behaviour of ^{22}Na efflux measured in K-free solution (●) and in K-free solution containing ouabain (■), NEM (○) and NEM + ouabain (□). The rate of efflux is expressed in counts/min. Solutions in which the measurements were made contained (mM): NaCl 150, Tris 20, glucose 10 pH: 7·4. 1 ml. packed cells was suspended in 12 ml. solution. The initial concentrations of inhibitors were: ouabain 10^{-4} M, NEM 5×10^{-4} M. *b* Inhibitory effect of NEM in presence (○) or in absence (□) of ouabain. Data calculated from Fig. 8*a*, by subtracting the Na efflux in the presence of NEM from the control, and in the presence of NEM + ouabain from the control + ouabain. Abbreviations as in Fig. 1.

(these Na–Na exchanges, mediated by the pump, are ouabain sensitive) (Garrahan & Glynn, 1967); secondly, in the same solution containing ouabain. It is known that the Na efflux occurring in the presence of ouabain does not require external K, but depends on the presence of external Na (Hoffman & Kregenow, 1966). This ouabain-insensitive Na efflux has been claimed either to represent an active transport mediated by the so called Pump II (Hoffman & Kregenow, 1966; Sachs, 1971; Rettori & Lenoir, 1972) or to be an ouabain-insensitive exchange diffusion

(Lubowitz & Whittam, 1969; Dunn, 1970; Lubowitz, 1972). It has been found that PCMBS, in our experimental conditions (initial concentration 5×10^{-6} M, haematocrit 7·7 %) shows a very slight inhibitory effect in K-free Na Ringer and in the same solution containing ouabain. Conversely, as it is evident in Fig. 8*a*, N-ethylmaleimide markedly inhibits Na efflux in K-free Na Ringer and also in the same solution containing ouabain. The inhibitory effects are roughly equivalent in these two conditions (Fig. 8*b*). It appears therefore that N-ethylmaleimide does not inhibit the ouabain-sensitive exchange diffusion but does inhibit the ouabain insensitive sodium efflux. It would be of interest to use N-ethylmaleimide, which does not cause haemolysis, as a tool to study this ouabain-insensitive Na efflux in order to choose between the two previously discussed hypothesis (Pump II or exchange diffusion). If it is an ouabain-insensitive diffusion it is in some respects different from this process as described in beef red cells since it is not inhibited by PCMBS but is inhibited by furosemide (Dunn, 1970; Sachs, 1971) which has no effect in beef erythrocytes.

DISCUSSION

In this paper it has been demonstrated clearly that two sulphydryl agents, N-ethylmaleimide and PCMBS, which strongly inhibit Na efflux, inhibit equally Na influx. It affords further evidence, additional to all that already advanced (Motais, 1973) of the presence of a Na exchange diffusion, insensitive to ouabain, in the beef erythrocyte.

Before claiming that the inhibitory effects obtained with N-ethylmaleimide, PCMBS and other drugs strongly suggest the involvement of thiol groups in the exchange diffusion system, the degree of specificity of these reagents for sulphydryl groups must be discussed. None of the agents is absolutely specific for sulphydryl. N-ethylmaleimide and PCMBS are highly specific for sulphydryl groups but are not completely so under all conditions. When these compounds react with proteins the sulphydryl binding sites are titrated first; any excess of reagent however may begin to react with other functional groups such as carboxylic or amino groups. The ready reversibility by dithiotreitol of the effect of PCMBS and the low concentrations used (5×10^{-6} M) are consistent with the assumption that its primary effect is on sulphydryl groups. One procedure often used to check further that sulphydryl groups are involved is to use agents which react differently on sulphydryl groups: two such agents are PCMBS which forms easily reversible mercaptide bonds, and N-ethylmaleimide which forms covalent bonds. It therefore seems reasonable to conclude that the exchange diffusion system in the beef red cell contains a protein with an sulphydryl group and that the reaction of this group with sulphydryl

agents results in inhibition of the system. It is interesting to note that if sulphydryl groups containing proteins are inhibited there seems to be little or no change in the half saturation constant for the complexing reaction for transfer (Fig. 5). The simplest explanation is that the inhibited sites are no longer functioning but that uninhibited sites are in every way normal.

Another problem to be discussed is the localization of the sulphydryl group involved in the Na^+–Na^+ exchanges. It is obvious that when a chemical agent penetrates a cell, the first part of the cell to be exposed is the membrane. In the case of a sulphydryl reagent, the primary targets are the superficial sulphydryl groups, some of which could be involved in the system studied and others not. Other populations of sulphydryl groups are located within the membrane structure: they are reached later by the agent; if it is a slowly penetrating agent it would act on these inner groups after a relatively long period of time. So, by appropriate selection of agents and conditions it is possible to obtain information concerning the 'topographic' location of sensitive sites in the membrane.

Such information is available in human red cells for several transporting systems (Vansteveninck *et al.* 1965; Rega, Rothstein & Weed, 1967; Sutherland *et al.* 1967). A brief résumé of these data will provide a useful background for a discussion of the characteristics of the SH group involved in Na^+–Na^+ exchange in the beef red cell.

Sugar transfer is facilitated diffusion involving binding of the sugar by a membrane 'carrier'. It is blocked rapidly by poorly penetrating sulphydryl agents such as PCMBS, indicating that a superficially located protein is a part of the mechanism (Vansteveninck *et al.* 1965). Sugar transfer is also inhibited by the penetrating agent N-ethylmaleimide but in this case inhibition develops slowly and progressively (Dawson & Widdas, 1963); thus, it probably does not act on the same group as does PCMBS but must inhibit at internal sites within the membrane. Sugar transfer would therefore seem to be inhibitable at two locations in the membrane.

Active transport of Na and K and passive permeability to Na and K are controlled by internal sulphydryl groups which are considered as not titrable by N-ethylmaleimide (Sutherland *et al.* 1967) but titrable by organic mercurials (PCMBS) and $HgCl_2$; passive permeability is increased by these agents: the leakage is initiated without delay in the case of inorganic mercury which penetrates rapidly (Weed, Eber & Rothstein, 1962) but only after a delay of several hours in the case of PCMBS (Sutherland *et al.* 1967). Active transport of Na and K is inhibited by PCMBS and because sulphydryl groups are located within the membrane the effect is only initiated after a delay (Rega *et al.* 1967). The mechanism of the effect

of sulphydryl agents on active transport of Na and K is related to their inhibitory effect on Na-K ATPase.

We have shown that in the beef red cell, exchange diffusion of Na is inhibited by a slowly penetrating agent (PCMBS) and by a penetrating agent N-ethylmaleimide. The reaction always occurs rapidly and, for PCMBS, can be reversed rapidly by the addition of a sulphydryl-containing compound. From the foregoing it may be concluded that while in the human erythrocyte the active and passive cation movements are controlled by sulphydryl groups which are non-titrable by N-ethylmaleimide and situated within the membrane, in the beef red cell the sulphydryl groups involved in the Na exchange diffusion are superficial and titrable by N-ethylmaleimide. The behavioural characteristics of these sites are thus analogous to those of the sulphydryl groups involved in sugar transport. It is important to note in any case that the action of both N-ethylmaleimide and PCMBS is very rapid and does not increase progressively with increasing incubation time (Fig. 6). The exchange diffusion therefore does not appear to be inhibitable at two locations in the membrane.

The experiments with N-ethylmaleimide and PCMBS carried out on sheep erythrocytes provide some evidence in support of the occurrence in these cells of an exchange diffusion process showing great similarities with the mechanisms described in beef red cells.

In human red cells it has been shown that the Na-dependent component of the Na efflux, which is considered either as active extrusion (Pump II) or as an exchange diffusion flux, can be markedly inhibited by N-ethylmaleimide without reducing the ouabain-sensitive component of the efflux. It should be pointed out that N-ethylmaleimide has previously been claimed to have no effect on Na movement in human red cells (Sutherland *et al.* 1967). Conversely, PCMBS in our experimental conditions has been found to have no significant inhibitory effect.

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