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THE RELATION BETWEEN NET CALCIUM, ALKALI CATION AND CHLORIDE MOVEMENTS IN RED CELLS EXPOSED TO SALICYLATE

BY H. BÜRGIN AND H. J. SCHATZMANN
From the Department of Veterinary Pharmacology, University of Bern, Bern, Switzerland

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SUMMARY

1. From a 150 mM-NH_4 salicylate medium salicylate enters human red cells with a rate constant of 1·9 min^{-1} at 0 °C.

2. Salicylate increases the red cell permeability for Ca^{2+} (and Mg^{2+}). There is no saturation of the Ca^{2+} transfer with respect to salicylate up to 150 mM and with respect to external Ca^{2+} up to 30 mM.

3. Ca^{2+} entering from salicylate media activates the Ca-sensitive K channel present in human but not in adult ruminant red cells.

4. The increase in K permeability which ensues hyperpolarizes the membrane in Na salicylate media, accelerating further Ca^{2+} entry and Mg^{2+} entry and favouring Cl^- loss (see Fig. 8). The Ca^{2+} inward movement is in agreement with the constant field equation if the membrane potential is assumed to equal the K equilibrium potential and if two charges are attributed to the mobile species.

5. The effect of salicylate on Ca^{2+} permeability and hence its sequelae are reversible upon washing the cells.

6. 3-OH-benzoic acid and 4-OH-benzoic acid do not exert the effect salicylate has on Ca^{2+} permeability.

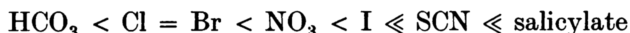
7. In 150 mM-Na salicylate media the Cl^--salicylate exchange is virtually nil at 0 °C. The exchange seen at 19 °C is obviously not across the anion exchange mechanism and proceeds at a rate comparable to that for Cl^- movement in the non-exchange-restricted mode given by Hunter (1971, 1977) for cells in a normal medium.

8. Ca^{2+} seems to increase the Cl^- permeability seen under these conditions.

9. The possibility that salicylate acts as an ionophore for Ca^{2+} is discussed.

INTRODUCTION

It is well established that monovalent anions lower the rate of Cl^- and sulphate exchange in red cells according to their position in the Hofmeister series



$$HCO_3 < Cl = Br < NO_3 < I \ll SCN \ll \text{salicylate}$$

(Passow & Schnell, 1969; Wieth, 1970*b*). The current explanation at least for the more active species is that on account of their lipophilic nature these anions are incorporated into the membrane-water interphase, imparting negative charges to regions of the membrane otherwise positive, thus blocking the access of e.g. Cl^- ions

(fixed charge hypothesis). Leu, Wilbrandt & Liechti (1942), Fischer, Willbrandt & Liechti (1944) and Wieth (1970*a*) have pointed out that thiocyanate or salicylate conversely increase K and Na permeability of the red cell membrane and that this effect has a paradoxical temperature dependence, being minimal at about 20 °C and increasing at lower (and higher) temperatures. The idea has long been fostered that altering the membrane charge should result in a mirror-image behaviour of anion and cation permeability (Mond, 1927) and seems to be successful when applied to artificial bilayers made of neutral phospholipids (McLaughlin, 1973). However, Wieth (1970) noticed that in red cells Ca^{2+} enhances the effect of SCN^- on K permeability which certainly does not fit the simple charge hypothesis. Wiley & Gill (1976) have demonstrated that salicylate also increases the permeability of red cell membranes for Ca^{2+} and hence will lead to a rise in internal Ca concentration at any external Ca^{2+} concentration above 10^{-7} M and more effectively so, of course, under conditions where the Ca pump is arrested (for reviews see Schatzmann, 1975; Sarkadi & Tosteson, 1978).

The question arose whether all these effects are independent of each other or whether some of them are primary events giving rise to the others. The present experiments were done in an effort to show that there is indeed an intricate connexion between the different changes in ionic movements brought about by the strongly lyophilic salicylate anion and that the rapid penetration of salicylate itself (Wieth, 1970*b*) and changes in the membrane potential must be taken into account. It will emerge that for the alkali cation movements in salicylate media the increase in Ca permeation is of primary importance.

METHODS

Human red cells were obtained in a small volume of blood plasma kept from clotting by acid-citrate-glucose solution from the blood donor service of the Swiss Red Cross one day after the blood was collected. Cattle red cells were from freshly drawn blood with heparin as anticoagulant and were treated as described for human cells. The red cells were washed 4 times in (mM) NaCl 145, Tris-Cl 10, pH 7·4 (4 °C) and the white cells discarded. When Ca^{2+} was present exposure to salicylate media was invariably at 0 °C in a shaking ice bath. Haematocrit values were obtained by centrifugation at 10000 rev/min for 15 min in glass capillaries. Cells were separated from the medium by ordinary centrifugation or by centrifugation through butylphthalate in a swing-out bucket rotor (Sorvall) or a fixed angle rotor of a microcentrifuge (Ecco). According to requirements the cells were washed in isotonic $MgCl_2$ (113 mM) or NaCl solution or Ca-free medium. Extracellular space in packed cells was assessed by running parallel samples without salicylate but with a known amount of $CaCl_2$ as marker for extracellular fluid. It varied from 2 to 10 vol. per 100 vol.

Active K and Ca transport was examined at 37 °C in media containing inosine (10 mM) or inosine (10 mM) + glucose (10 mM).

Chloride was determined by titration with $AgNO_3$ with a combined silver-KNO_3-calomel electrode on an automatic microtitrator (Metrohm) or by potentiometry in a concentration chain with 2 Ag-AgCl electrodes in a 100-fold dilution of sample in 0·1 N-H_2SO_4. *K and Na* were measured by emission flame photometry (EEL) after suitable dilution of media and cells with water.

Ca and Mg assays were by atomic absorption flame photometry (EEL or Il 151 instrument) in the presence of 50 mM-$LaCl_3$ in standards and diluted samples after deproteinization with 5–10% (final concentration) of trichloroacetic acid.

Salicylate was measured by the method of Stevenson (1960) in the following way: appropriately diluted cell lysate is shaken for 1 min with a tenfold volume of 0·4 g malonic acid in 100 ml. dibutyl ether. The organic phase is measured at 307 nm against a blank (haemolysate of cells

not exposed to salicylate) and compared against standards made up in the same haemolysate from salicylate free cells.

Haemoglobin was determined as cyanmethaemoglobin by photometry at 546 nm.

Tonicity of salicylate solutions was determined by freezing point depression measurement on a Knauer instrument. A mixture of Na salicylate 150 mM, Tris-Cl 20 mM and $MgCl_2$ 4 mM had 1·07 times the tonicity of NaCl 162 mM.

Membrane potential at 0 °C was calculated according to the Nernst equation

$$V_m = 54{\cdot}1 \,.\, \log \frac{[K_o]}{[K_i]} \,(mV).$$

All ionic translocations reported are net movements based on chemical determinations of concentrations in media or cells referred to original cell volume, unless otherwise stated.

The composition of media is given in the legends to Figures and Tables.

Water was redistilled after passage through an ion exchange column. All chemicals were reagent grade from Merck or Fluka. Inosine was from Boehringer Mannheim. Salicylate was bought as Na salicylate or free acid (Merck). K salicylate, NH_4 salicylate and Ca salicylate was made by dissolving the free acid in sufficient KOH, NH_4OH or $Ca(OH)_2$ to give neutral pH. $Ca(OH)_2$ was analytical grade of Merck which, however, contains maximally 3% carbonate (which was sedimented before use), 0·4% sulphate and 0·05% iron. 3-OH-benzoic acid and 4-OH-benzoic acid were from Fluka.

Quinidine hydrochloride was pharmacopoeia grade.

RESULTS

Salicylate effect on K and Na movements

Fig. 1 shows that in intact cells at 0 °C in 150 mM-Na salicylate and 5 mM-$CaCl_2$, buffered with Tris-Cl to pH 7·4, K and Na rapidly move down their gradients. The fluxes reach their maximal rates only after 10 min incubation. The K movement is twice as fast as the Na movement although, as we shall see, the driving force on Na is much larger than on K (the concentration difference between inside and outside is similar for Na and K but there is a considerable membrane potential inside negative; see Figs. 6, 8).

Both the rapid K movement and the more sluggish Na translocation depend on the presence of Ca^{2+} as demonstrated by the experiment of Fig. 1. Replacing Ca^{2+} by EGTA (1·0 mM) nearly completely abolishes both movements. It is clear, therefore, that Ca^{2+} ions in the medium are necessary for salicylate to induce the alkali cation leaks. The latency in onset, best seen in the K escape, strongly suggests that it is Ca^{2+} entering the cell which is responsible for the effect.

Salicylate effect on Ca and Mg movements

From Fig. 2*A* it may be seen that Ca^{2+} rapidly enters the cells from a medium with 150 mM-Na salicylate and Fig. 2*B* shows how Ca^{2+} entry depends on external Ca^{2+} concentration. Fig. 3 demonstrates that the rate of Ca^{2+} entry is a linear function of the initial external salicylate concentration between zero and 150 mM.

Fig. 4 indicates that in the Na salicylate medium Mg^{2+} also enters the cells but at a much slower rate than Ca^{2+}, not accounted for by the slightly smaller Mg^{2+} gradient (according to Flatman & Lew (1977) the free Mg^{2+} concentration may be 0·3–0·4 mM in the cell water) and that Ca^{2+} in the medium accelerates the Mg^{2+} entry.

In Fig. 5 the meta and para isomers (3-OH-benzoic acid and 4-OH-benzoic acid)

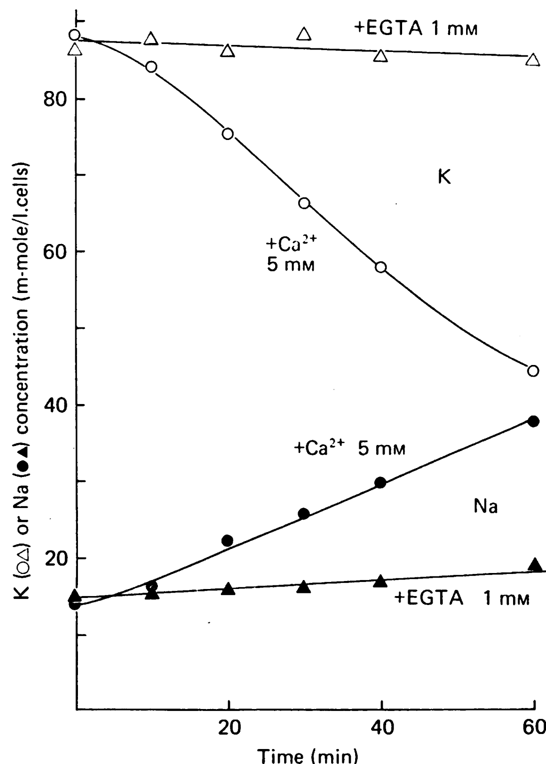


Fig. 1. K loss and Na gain of red cells in 150 mM-Na salicylate at 0 °C. Medium (mM): Na salicylate 150, Tris-Cl 10, $CaCl_2$ 5 or Tris-EGTA 1, $MgCl_2$ 1·5, pH 7·4 at 0 °C. Ordinate: cellular K or Na concentration calculated per litre initial cell volume. Haematocrit at zero time approx. 0·1. ○, ●, 5 mM-Ca^{2+}; △, ▲, no Ca^{2+} but 1 mM-EGTA. Open symbols, K; filled symbols, Na. Notice that absence of Ca from medium prevents rapid leak of alkali cations.

Fig. 2. *A*, Ca entry into red cells in 150 mM-Na salicylate at 0 °C. Medium (mM): Na salicylate 150, Tris-Cl 20, $MgCl_2$ 2, $CaCl_2$ 2 or 5, pH 7·4 at 0 °C, haematocrit at zero time approx. 0·17. Samples were washed twice with (mM) NaCl 145, KCl 5, Tris-Cl 10 (pH 7·4 at 0 °C), $MgCl_2$ 2 before analysis. Ordinate: Ca concentration calculated per litre initial cell volume. ○, 2 mM-Ca in medium; △, □, 5 mM-Ca in medium (duplicate runs). *B*, dependence of rate of Ca gain and K loss on external Ca^{2+} concentration at 0 °C. Ordinate: rate of Ca or K movement per litre original cell volume. Before experiment cells washed 4 times in ice-cold 154 mM-NaCl to reduce concn. of inorganic phosphate. Medium: salicylate 150, Tris-Cl 20 (pH 7·4), $MgCl_2$ 1·5. Incubation 25 min at 0 °C. Ca concentration in medium set by mixing Na and Ca salicylate solutions. Haematocrit approx. 0·035. P_i concn. in suspension 0·029 mM. Before analysis 0·35 ml. cells washed with 10 ml. NaCl 154 mM in the cold. Filled symbols: dependence of rate on $[Ca_o]$. Circles: dependence of rate of Ca gain on square of $[Ca_o]$. Notice that Ca entrance rate depends linearly on $[Ca_o]^2$ at low Ca concn. From the data half-saturation of the K channel by Ca_i is at approximately 100 μmole Ca/l. cells.

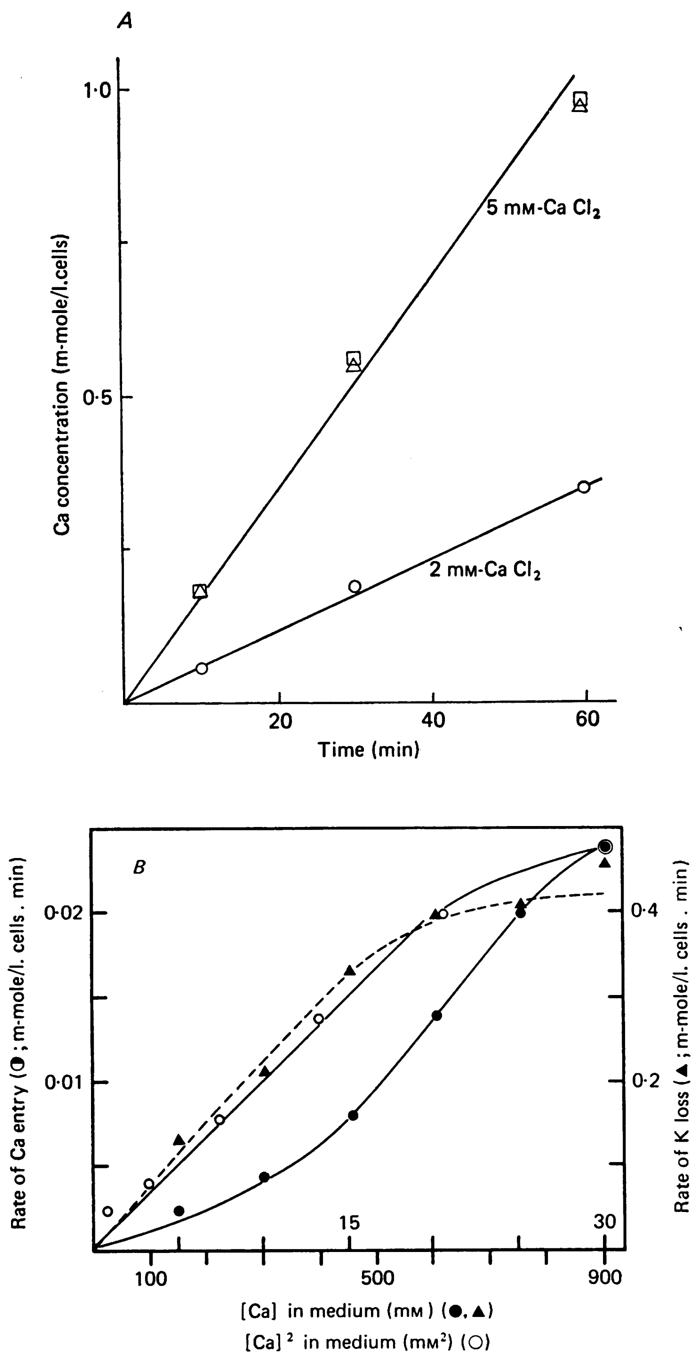


Fig. 2. For legend see facing page.

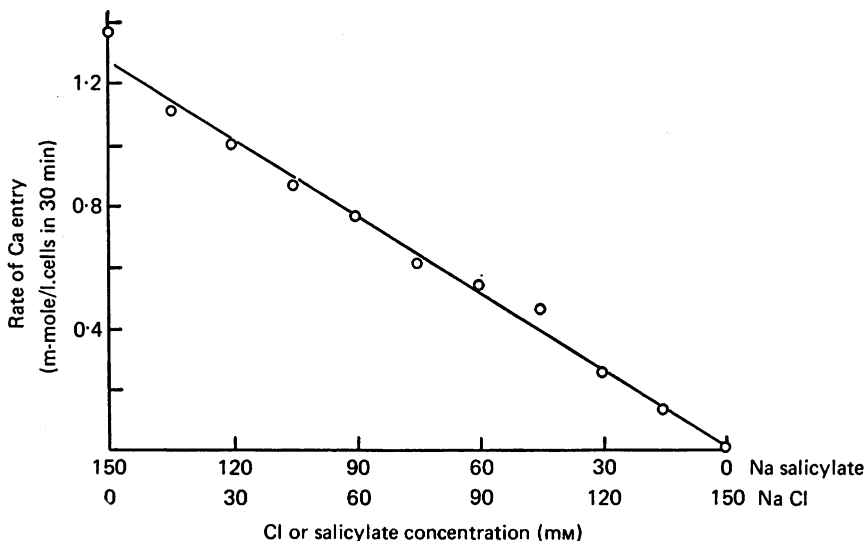


Fig. 3. Dependence of Ca entry on salicylate concentration at 0 °C. Incubation at 0 °C, 30 min. Medium (mM): NaCl + Na salicylate 150, Tris-Cl 10, $MgCl_2$ 1, $CaCl_2$ 5. Salicylate was stoichiometrically replaced by Cl. Haematocrit 0·08. Cells packed through oil without washing before analysis. Ordinate: rate of Ca movement (m-mole/l. cells.30 min, calculated for original cell volume).

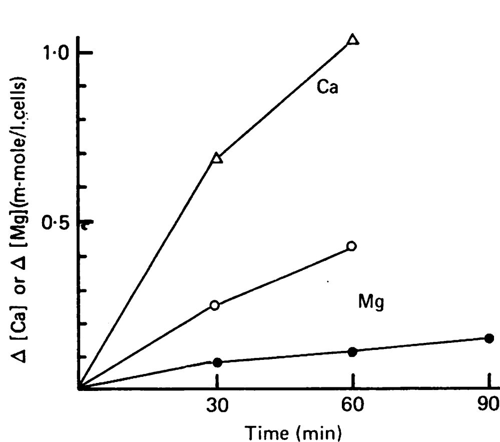


Fig. 4

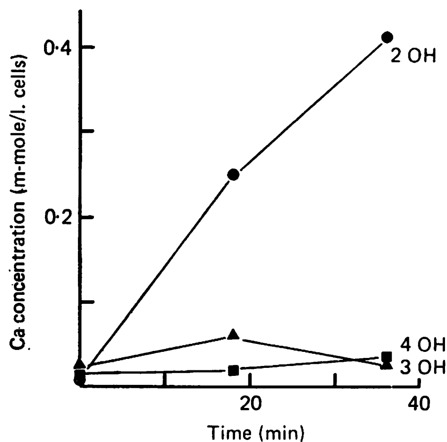


Fig. 5

Fig. 4. Mg entry into red cells in 150 mM-Na salicylate with and without Ca in medium, at 0 °C. Medium (mM): Na salicylate 150, Tris-Cl 10, $MgCl_2$ 5, $CaCl_2$ 5 or Tris-EGTA 0·5, pH 7·4 at 0 °C. Cells were washed 3 times in 20-fold volume of cold NaCl 154 mM before analysis. Ordinate: change in Ca or Mg concentration calculated per litre initial cell volume. Fresh cells contained 1·62 m-mole Mg/l. cells. △, Ca concentration in cells in Ca + Mg medium; ○, cellular Mg concentration in Ca + Mg medium; ●, cellular Mg concentration in EGTA medium.

Fig. 5. Effect on Ca^{2+} entry of the three isomers of hydroxy-benzoic acid at 0 °C. 2 days old cells washed in (mM): NaCl 145, Tris-Cl 10 (pH 7·4). Medium (mM): Tris-Cl 20 (pH 7·4). $MgCl_2$ 1·5, $CaCl_2$ 5, Na salicylate 150 (2-OH) or Na-3-OH-benzoate 150 (3-OH) or Na-4-OH-benzoate 150 (4-OH). Haematocrit 0·073. Before analysis the cells were washed twice in cold NaCl 154 mM.

are compared to salicylate under identical conditions. The isomers definitely do not exert the action on Ca permeability characteristic for salicylate.

Relation between the effect on Ca and on alkali cations

From Fig. 1 it is obvious that Ca^{2+} is required for the K leak in a Na salicylate medium. On the other hand K in the medium inhibits the Ca entry. In Fig. 6 the result of replacing Na by K in a stepwise fashion is given. It may be seen that external K between zero and 80 mM strongly reduces uptake of Ca from a 150 mM-salicylate medium. The most obvious explanation for this behaviour is that in the presence of salicylate K has a major influence on the membrane potential and that the membrane potential largely determines the rate of Ca^{2+} influx. In the inset of Fig. 6 the rate of Ca^{2+} influx is drawn as a function of the K potential, calculated from the mean K distribution across the membrane. During the 30 min of the experiment the cellular Ca concentration rose to less than 1/5 of the extracellular Ca and the mean Ca concentration inside, therefore, was less than 1/10 of the external concentration. Thus the approximation was made that initial rate was measured such that the constant field equation for Ca^{2+} takes the form

$$\phi_{Ca} = P_{Ca}.[Ca_o].\frac{2FV_m}{RT}.\frac{\exp(2FV_m/RT)}{\exp(2FV_m/RT)-1}, \quad (1)$$

with ϕ_{Ca} = net Ca flux, P_{Ca} = Ca permeability, V_m = membrane potential, $[Ca_o]$ = Ca^{2+} concentration in the medium (5 mM).

The dashed line in the inset of Fig. 6 is fitted to the experimental points according to eqn. (1) with the linear slope through the topmost point and the origin. A better fit is obtained by a straight line through the three last points which intersects the abscissa at -15 mV. This deviation of 15 mV cannot be accounted for by a contribution of other ions to the potential or by assuming a number of charges other than two for the transported species. It must be due to experimental error or to the simplifying assumptions being incorrect (these were linearity between 0 and 30 min, perfect osmometric behaviour of the cells and a volume of 35 % solids in the cells). At any rate the experimental points are in reasonable agreement with the theoretical ratio between permeability at zero potential and the potential dependence in the steep part of the curve, i.e. the expected curvature of the function. The result is compatible with the assumption that K dominates the membrane potential and that Ca^{2+} moves across the membrane in the doubly charged form. The Ca^{2+} influx in a NaCl medium (at -10 mV inside) with 1·5 mM-$CaCl_2$ at 37 °C was found to be 30 μmole/l. cells . min . mole . l^{-1}. From the inset of Fig. 6 it may be seen that at -10 mV the influx is 400–800 μmole/l. cells . min . mole . l^{-1}, which means that 150 mM-salicylate increases the Ca permeability at 0 °C to a value 13–25 times above that valid normally at 37 °C. Since the Ca pump is virtually arrested at 0 °C the intracellular Ca^{2+} concentration will thus rise markedly even before the onset of high K permeability, i.e. the establishment of a high membrane potential.

The concentration difference across the membrane for Mg^{2+} in Fig. 4 and 6 presumably was 4·6–4·7 mM (Flatman & Lew, 1977) and at low potentials the rate of Mg and Ca entry were similar. However, Mg transfer increases less with rising potential (Fig. 6) which might mean that in the case of Mg the transported species carries only a single charge.

Fig. 7 corroborates that high K permeability is a necessary assumption in order to explain why the transmembrane K gradient enhances Ca entry. Quinidine (and quinine) is an inhibitor for the passage of K across the Ca^{2+} sensitive K channel and has been shown to have no direct effect on Ca permeability (Armando-Hardy, Ellory,

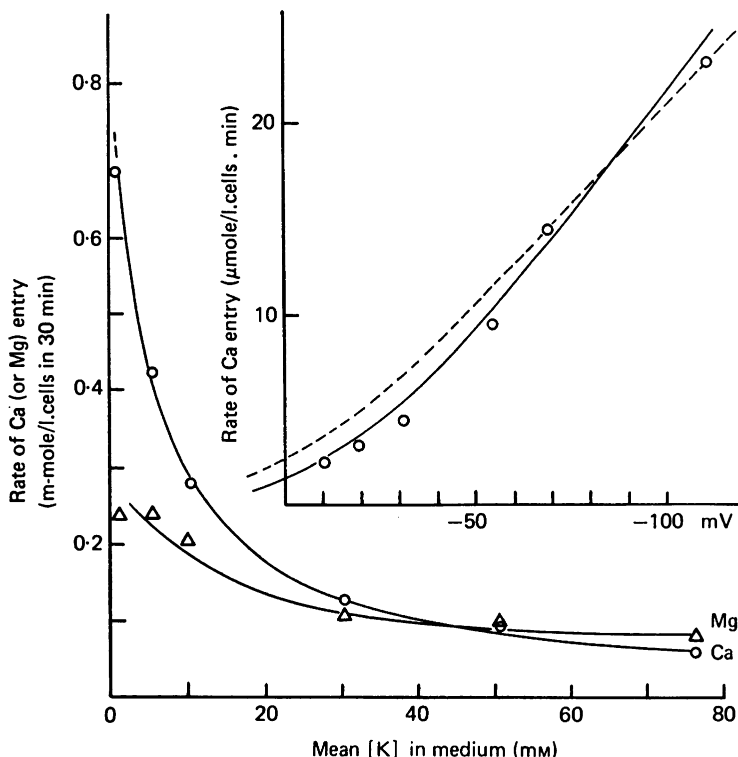


Fig. 6. Dependence of Ca and Mg entry on K content of medium at 0 °C. Medium: Na-K salicylate 150, $MgCl_2$ 5, $CaCl_2$ 5, Tris-Cl 20, pH 7·4 at 0 °C. K was stoichiometrically replaced by Na. Haematocrit approx. 0·1. Incubation at 0 °C, 30 min. Cells washed 3 times in tenfold volume of NaCl 154 mM before analysis. Ordinate: Ca uptake per litre initial cell volume in 30 min. Abscissa: mean extracellular K concentration during 30 min. Mg content of fresh cells was 1·17 m-mole/l. cells. *Inset*: mean rate of Ca entry during 30 min plotted *vs.* K potential (V_K) calculated from external mean K concn. and mean K concn. in cell water from cellular K determination corrected for haematocrit changes, assuming ideal osmometric behaviour and 35% solids in cells, and taking into account that $[K]_{cell\,H_2O}$ decreases non-linearly with $[K]_{cells}$ when the cells lose isotonic KCl solution, owing to other species contributing to total osmolarity. Dashed line: theoretical curve for a divalent cation according to eqn. (1) with $V_m = V_K$ with slope through origin and the topmost point. Continuous line: the same, with slope through 3 last points intersecting abscissa at −15 mV. Note that rate of Mg entry is similar to rate of Ca entry at low potentials but is less affected by potential increase (main figure).

Ferreira, Fleminger & Lew, 1975). It may be seen that quinidine (0·25 mM) nearly completely suppresses the K leakage and at the same time quenches the rapid Ca inward movement. Low salicylate concentrations were used in this experiment to

avoid precipitation with quinidine and it was ascertained that quinidine does not affect the rapid entry of salicylate into the cells (not shown).

Finally comparison of Fig. 2*A* and *B* indicates that the K channel shows low affinity for internal Ca^{2+} as observed by Lew & Ferreira (1976) (half saturation at approximately 100 μmole Ca/l. cells).

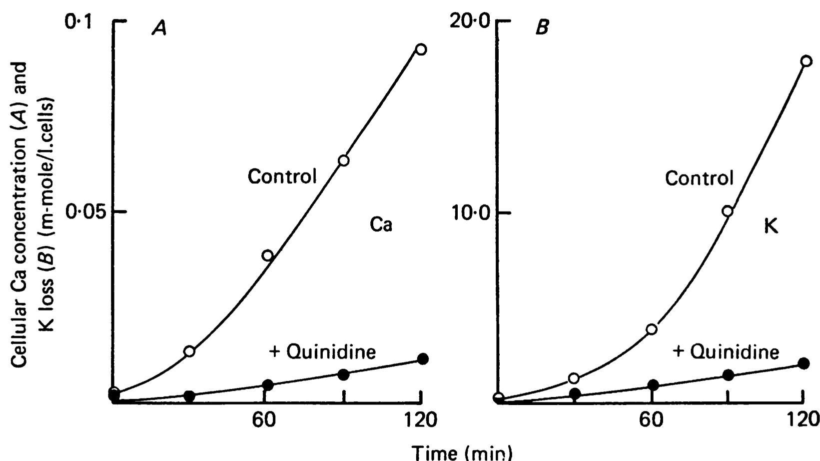


Fig. 7. Inhibition of salicylate-induced K loss and Ca entry by quinidine sulphate 0·25 mM. Medium (mM): Na salicylate 35, NaCl 115, Tris-Cl 10, $MgCl_2$ 1, $CaCl_2$ 5, pH 7·4 at 0 °C, haematocrit approx. 0·16. Ca measured in cells, calculated per litre initial cell volume. K measured in medium and calculated as change in cellular K per litre initial cell volume. Ordinate: *A*, cellular Ca concentration, *B* cellular K loss per litre cells. Notice that Ca entry is nearly completely abolished by quinidine, which acts primarily on Ca-sensitive K channel.

Salicylate effect on Cl^- movement

Figs. 8, 9 and 10 present some results on Cl^--salicylate exchange in high salicylate media. At 0 °C there is an initial rapid exchange which was probably due to inadequate cooling when the cells were added, because it was not seen when carefully chilled cells were added at a lower haematocrit value. Thereafter, in Ca^{2+}-free medium the exchange was nearly checked (Fig. 8*A*) obviously far from equilibrium. At 60 min the Cl^- distribution corresponds to +35·1 mV (positive inside) whereas the salicylate distribution corresponds to −24·85 mV (negative inside). This can only mean that either Cl^- or salicylate or both are not free to move across the membrane. Fig. 9 clearly shows that salicylate under these conditions moves exceedingly rapidly, provided that the penetrating cation NH^+_4 replaces Na^+ (Motais, 1977) in the medium. Thus it is clear that Cl^- permeability is extremely low. When Ca^{2+} was present in the medium, Cl^- moved slowly but perceptibly towards equilibrium, obviously together with K^+ (Fig. 8*B*). It may be mentioned that the rate of Cl^- movement in the absence of Ca^{2+} was the same as in an isotonic (120 mM) Na_2SO_4 medium without salicylate.

At 19 °C the rate of Cl^- movement can be measured more accurately (Fig. 10). The rate in salicylate medium is again comparable to that in a sulphate medium,

whereas in a medium with 150 mM-Na-3-hydroxybenzoate it is slightly, and in one with 150 mM-Na-4-hydroxybenzoate markedly faster. The cellular Cl^- concentration does not fall logarithmically with time: the rate constant is low but consistently increases from beginning to end of the experiment (0·015 min^{-1}–0·036 min^{-1}). The rate constant for salicylate entry calculated from Fig. 9 is 1·9 min^{-1} at 0 °C. The Cl^- movement thus is very much slower than the salicylate movement.

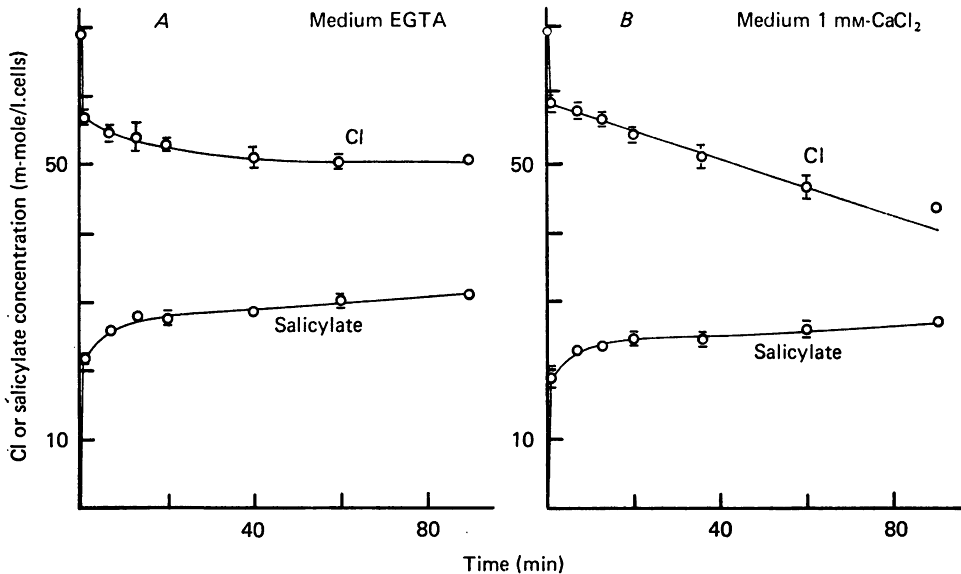


Fig. 8. Salicylate entry and Cl^- loss in 150 mM-Na salicylate at 0 °C with and without Ca in medium. Three experiments, except at 90 min (one expt.), vertical bars ± 1 S.E. of mean. Medium (mM): Na salicylate 150, Tris-Cl 10, $MgCl_2$ 1, $CaCl_2$ 1 or Tris-EGTA 1, pH 7·4 at 0 °C. Haematocrit 0·2. Cl determined in medium and calculated per litre initial cell volume. Salicylate measured in cells and calculated per litre initial cell volume. Notice rapid salicylate-Cl exchange during first min. Thereafter a non-equilibrium distribution is established in EGTA medium. Salicylate distribution at 60 min corresponds to −24·85 mV (inside negative), Cl^- distribution to +35·1 mV (*A*). In Ca medium Cl^- moves steadily into medium, obviously together with K (*B*). Difference in slope for Cl curve in *A* and *B* is statistically significant (between 1 and 60 min $P = 0{\cdot}001$).

Since the passage of NH_4^+ across the membrane occurs in the form of NH_3, coupled with an exchange of the concomitant anion with OH^- ions (Motais, 1977) the experiment of Fig. 9 in addition shows that the passage of OH^-, unlike that of Cl^-, is rapid at 0 °C in a salicylate medium.

Reversibility of the salicylate effects

Fig. 11 shows that the effect of salicylate on Ca and K is perfectly reversible upon simple washing. From metabolically intact cells Ca is rapidly pumped out against a gradient and K is taken up actively at a normal rate but only after a latency (Fig. 11) which presumably indicates the time required for cellular Ca^{2+} concentration to fall below a critical value. The K uptake becomes fast (3 m-mole/l. cells.hr)

when the cellular Ca concentration has reached about 0·15 m-mole/l. cells, corresponding to 30–50 μmole free Ca^{2+} per l. cells. This seems to indicate that the Ca sensitive K channel closes at these Ca^{2+} concentrations and thus displays a rather low apparent affinity for Ca^{2+} (Lew & Ferreira, 1976).

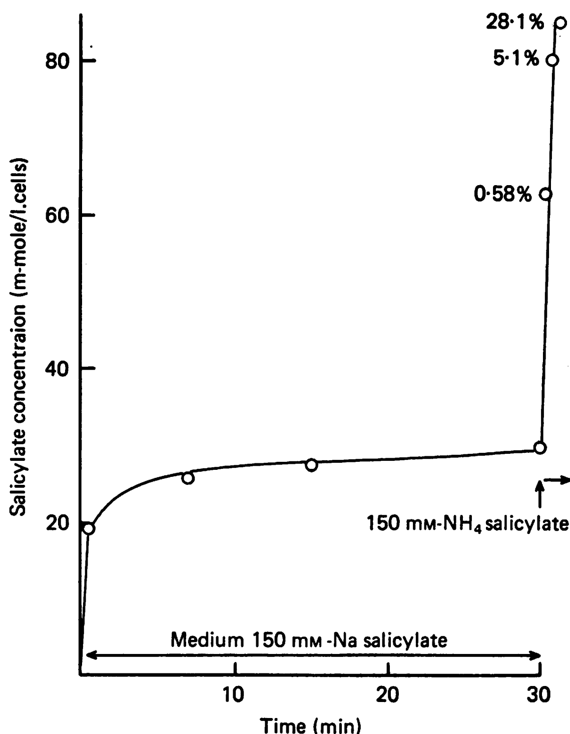


Fig. 9. Same experiment as Fig. 8. After 30 min in Na salicylate medium Na was replaced by NH_4 (at arrow cells were centrifuged and resuspended in NH_4 salicylate medium). With the easily permeating cation NH_4^+ salicylate rapidly moves into the cells which shows that permeability for salicylate remains extremely high (rate constant = 1·9 min^{-1}). Thus the odd distribution in Fig. 8 is due to a vanishingly low Cl^- permeability. The cells of course swell; figures near curve indicate % haemolysis.

In ATP depleted cells Ca concentration after removal of salicylate remains high and K continues to leak out (Fig. 11) which again shows that the effect on K permeability is not a direct action of salicylate.

Salicylate effect on Ca in ruminant red cells

Interestingly red cells from adult cows do not take up much Ca in salicylate media, whereas cells from calves do (Table 1). One known difference between red cells from adult and young ruminants is that the former lack the Ca^{2+} sensitive K channel whereas the latter do have it (Brown, Ellory, Young & Lew, 1978). This difference might easily explain the different behaviour towards Ca in salicylate media if the proposed role of K is accepted.

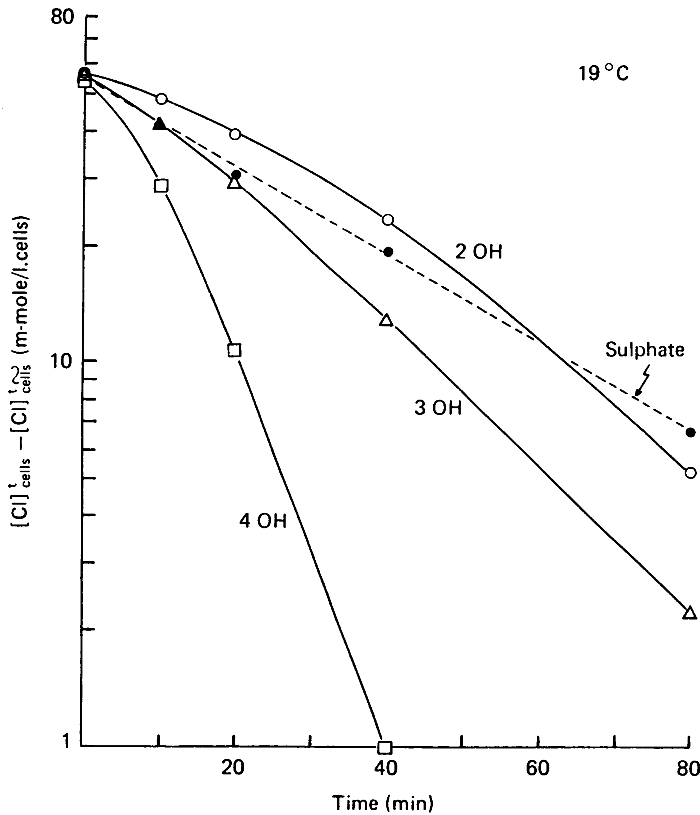


Fig. 10. Cl^- exit at 19 °C from intact cells into media with 150 mM-Na salicylate (2-OH) or its meta (3-OH) or para (4-OH) isomer. Cells washed in NaCl 145 mM, Tris-Cl 10 mM, pH 7·4. Incubation at 0 °C for 10 min at a haematocrit of approx. 0·1 in medium, zero time sampling, centrifugation in the cold, resuspension in prewarmed medium (19 °C) at haematocrit 0·08. Medium (mM): Tris-Cl 10 (pH 7·4), $MgCl_2$ 2, Tris-EGTA 1, Na salicylate or isomers 150 or for comparison Na_2SO_4 120. Cell samples centrifuged through oil. Cl^- determination potentiometrically in cells. Ordinate: log scale; difference of cellular $[Cl^-]$ at time t and infinite time.

TABLE 1. Intracellular Ca concentration in cattle cells after loading in Na salicylate. Fresh cells incubated for 3 hr at 0 °C in (mM): Na salicylate 150, Tris-Cl 10, $MgCl_2$ 2, $CaCl_2$ 5, pH 7·4. Cells were washed 4 times in a Na-K Cl medium with 1 mM-$CaCl_2$ and analysed directly or after two washes in Ca-free medium. Where necessary results are corrected for extracellular Ca in packed cells

Age of animals (days)	Number of animals	Cellular Ca concn. (m-mole/l. cells)
6–43	4	1·027 ± 0·123 (S.E. of mean)*
60	1	0·50
Adult	3	0·226 ± 0·002 (S.E. of mean)*

* $P < 0·01$.

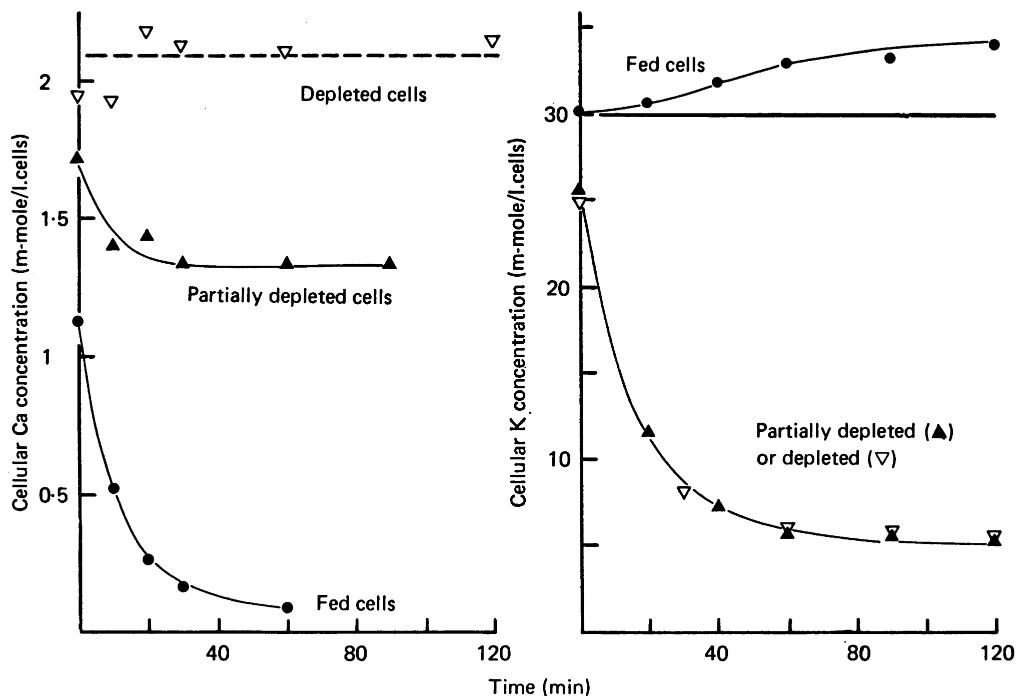


Fig. 11. Reversal of the salicylate effect upon washing. Filled symbols: complete experiment with one blood sample. Cells washed 5 times with 154 mM-NaCl solution. To either feed or starve the cells incubation at 37 °C for 2 hr in (mM) NaCl 145, KCl 5, Tris-Cl 10 (pH 7·4), $MgCl_2$ 2, inosine 10 (solution a_1) without or with iodoacetamide 6 (solution a_2). Subsequently (without washing) exposure at 0 °C for 1 hr to (mM) Na salicylate 150, Tris-Cl 5 (pH 7·4), $MgCl_2$ 2, $CaCl_2$ 5. After four washings in the cold with solution a_1 without inosine: incubation in solution a_1 and a_2 respectively at 37 °C, 1 mM $CaCl_2$. For Ca determination cells were centrifuged through butylphthalate; correction for extracellular Ca was done with a separate sample undergoing the same procedure except for absence of Ca in the salicylate medium. For K determination the cells were washed twice in 113 mM-$MgCl_2$. By mistake the starved cells were exposed for a few minutes to warm solution a_1 before solution a_2. They formed some ATP sufficient to support the Ca pump for a short while (partially depleted cells). Open triangles: data from a similar experiment with completely starved cells. Notice that ATP rich cells extrude Ca and accumulate K against a gradient whereas partially or completely ATP depleted cells retain Ca and continue to lose K until equilibrium is reached.

DISCUSSION

Salicylate, in spite of its low pK_a of about 3, penetrates the red cell membrane at pH 7·4 extremely rapidly (Wieth, 1970*b*). From Fig. 9, which shows how salicylate penetrates accompanied by NH_4^+ at 0 °C, a rate constant of 1·9 min^{-1} can be derived. Fortes & Hoffman (1971) have shown with fluorescent probes that salicylate interacts with red cell membrane constituents. It ranges very high in the so-called lyotropic series of anions. These anions share a number of actions on red cells and salicylate is the most potent among them. From the work of Leu *et al.* (1942), Fischer *et al.* (1944) and Wieth (1970*a*, *b*) it is clear that strongly lyophilic anions such as SCN^- or salicylate very effectively block the anion exchange mechanism of

red cells and markedly increase the alkali cation permeability and Wiley & Gill (1976) have pointed out that they also increase red cell permeability for Ca^{2+}. It is currently assumed that lyotropic anions accumulate at the membrane-water interphase (which they probably do) and – by imparting negative surface charges to the membrane – lower anion and increase cation permeability. This corresponds to the original notion of Michaelis about charged membranes, demonstrated to be valid for collodium membranes. At a closer look the matter seems to be more complicated, however.

Salicylate effect on cations

From Fig. 1 it is clear that salicylate has hardly any effect on K and Na permeability in the absence of Ca^{2+}. From Figs. 2 and 4 a direct effect on permeability of divalent cations becomes apparent. The increase of Ca^{2+} permeability will lead to a rise in internal Ca^{2+} concentration particularly at 0 °C where the Ca pump activity is negligible. There can hardly be any doubt that the K leak seen in Fig. 1 in the presence of Ca^{2+} is due to internal Ca activating the well known Ca sensitive K channel (Gardos, 1958; Blum & Hoffman, 1971, 1972; Simons, 1976*a*, *b*; Lew & Ferreira, 1976, 1977, 1978; Knauf, Riordan, Schuhmann, Wood-Guth & Passow, 1975). It is generally held that internal Ca^{2+} exclusively acts on K permeability whereas experiments of the type shown in Fig. 1 consistently also gave an effect on Na transfer. There may be an intrinsic difference between intact and salicylate treated cells. However, the small effect on Na might simply be due to the fact that in salicylate the membrane potential approaches the K equilibrium potential (Fig. 6). Similarly the dependence of the rate of Ca (and Mg) entry on the external K concentration (Figs. 6 and 4) is easily explained by assuming that K dominates the membrane potential once some Ca has entered the cells. The parallelism between K and Ca movements under the influence of quinidine (Fig. 7) and the fact that red cells from adult cattle, lacking the Ca^{2+} sensitive K channel (Brown *et al.* 1978), are rather insensitive to salicylate (Table 1) strongly supports this interpretation. A similar view was proposed by Sarkadi, Szasz & Gardos (1976) for the action of a combination of a Ca and K ionophore.

Fig. 5 shows that for the Ca^{2+} movement there is specificity: the m and p isomers of salicylate do not show the action of salicylate itself.

The question immediately arises whether salicylate may be looked upon as an ionophore for Ca^{2+} (and Mg^{2+}). Salicylic acid is known to form very stable complexes with divalent cations such as Fe^{2+} and Cu^{2+} but the formation constant for the Ca^{2+} complex is very low ($10^{0.15}$) (Sillen & Martell, 1964). This is no obstacle because high formation constants are rather unfavourable for effective action as an ionophore and Figs. 2*B* and 3 show that affinity both for Ca^{2+} and salicylate in the Ca translocation is indeed low. The main point is whether the Ca^{2+}-complex is liposoluble. Table 2 shows that salicylate is able to partition some Ca into a somewhat polar organic solvent but not into a very apolar one. The efficacy of salicylate in facilitating Ca passage across the red cell membrane is orders of magnitude less than what renowned Ca ionophores achieve: A 23187 leads to equilibration of Ca^{2+} across the red cell membrane in minutes if present in 10 μM concentration (Ferreira & Lew, 1976). The fact that the potential dependence of the Ca^{2+} transport can best

be fitted to theory by assuming two positive charges on the mobile species creates a difficulty. The 1:1 complex would carry only one charge but a dimer or the product of an interaction between two Ca-salicylate molecules with a membrane constituent might comply with this requirement. One might be inclined to support the idea of a mobile complex involving two Ca^{2+} ions by the linear dependence of Ca movement on the square of external Ca concentration at non-saturating Ca^{2+} concentrations shown in Fig. 2*B*. However, this seems risky since it is not known whether in this experiment the membrane potential was equal at all Ca^{2+} concentrations.

TABLE 2. $CaCl_2$ in 12 mM-Tris-Cl buffer pH 7·4 (at 25 °C) was shaken with a twofold volume of ethylacetate for 3 min with or without 100 mM-Na salicylate. After phase separation Ca was determined in aqueous and organic phase by atomic absorption flame photometry

	a Ca concn. in H_2O (μM)	*b* Ca concn. in ethylacetate (μM)	*b/a*
With salicylate	764 ± 13	29·8 ± 0·67	0·039 ± 0·0011
Control	796 ± 18	0	0

The same experiment with chloroform + 1% ethanol gave unmeasurable Ca or Mg concentration ($b/a < 0·01$) in organic phase.

The alternative to an ionophoric action is the formation of salicylate lined channels for Ca^{2+} and here again the complexing configuration of salicylic acid might be advantageous.

All the experiments with Ca were done at 0 °C in order to keep interference from the Ca pump low. Leu *et al.* (1942), Fischer *et al.* (1944) and Wieth (1970*a*) have explained the paradoxical temperature dependence of the K leakage (rising salicylate effect with falling temperature below 20 °C) by assuming that salicylate binding to the membrane is exergonic. This may well be so. However, Ca^{2+} contamination of reagents in these experiments must have been sufficient to cause K leakage and the apparent negative temperature coefficient may have been due rather to the Ca pump opposing the salicylate induced Ca entry near 20 but not near 0 °C.

The salicylate effect on cations is easily reversible upon washing the cells. Fig. 11 shows that the actions of the cation pumps are necessary to reverse the cation leaks. Since the Na-K pump cannot reverse the Ca leak but the Ca pump can remedy the K leak this is further proof for the primary role of Ca. It may be mentioned that the Ca pump in these salicylate treated cells shows the low-affinity behaviour towards Ca^{2+} described by Sarkadi, Szasz, Gerloczy & Gardos (1977), which is seen when no EGTA is incorporated into the cells (Fig. 5 in Schatzmann, 1973). Similarly, the K channel shows low affinity for Ca^{2+} (see Results) as observed in intact cells (Lew & Ferreira, 1976).

Salicylate effect on Cl^-

It is certain that in red cells there is a saturable, very fast anion exchange mechanism (Gunn, Dalmark, Tosteson & Wieth, 1973) and a net anion transporting mechanism, which is orders of magnitude slower (Hunter, 1971, 1977; Lassen, 1978). Whether the latter is due to a slip in the exchange mechanism or is an

independent pathway, has been discussed (Knauf, Fuhrmann, Rothstein & Rothstein, 1977). From Wieth's investigations (1970*b*) it is sufficiently clear that salicylate (or SCN^-) drastically reduce Cl^--Cl^- exchange. The experiments of Figs. 8 and 10 show that salicylate cannot substitute for the normal partner in the exchange mechanism (HCO_3^- or Cl^-) but enters exceedingly rapidly by another pathway when accompanied by a penetrating cation (rate constant 1·9 min^{-1} at 0 °C). Cl^- movement is quite slow and in fact the rate constant found (0·015–0·036 min^{-1} at 19 °C) is in agreement with the figure of 0·036 min^{-1} at 37 °C given by Hunter (1971, 1977) for the non-exchange restricted Cl^- permeability (Fig. 9), and is very similar to that for SO_4^{2-}-Cl^- exchange (Fig. 10).

Thus the Cl^--salicylate 'exchange' of Fig. 10 is not through the exchange mechanism and since its rate is of the same order as the Cl^- movement in Hunter's experiments with K ionophore it may safely be stated that the non-exchange-restricted Cl^- flux is by far not as much inhibited by salicylate as the exchange mechanism and indeed is perhaps not inhibited at all at 19 °C. This does not exclude the possibility that net Cl^- permeability can be increased by Ca^{2+} as suggested by Fig. 8. In the absence of Ca Cl^- equilibrates exceedingly slowly although the high salicylate permeability (Fig. 9) would allow its movement. In the presence of Ca Cl^- moves *g* times faster. The potential increase accounts for only one third of this, which suggests that Ca^{2+} not only activates the K channel but in addition and independently increases Cl^- permeability (Lassen *et al.* 1978) and that Cl^- permeability might be rate-limiting for the K efflux.

Fig. 8*B* shows that the Cl^- movement induced by Ca^{2+} is not compensated by an extra salicylate uptake. In a K medium this extra Cl^- efflux was prevented or even reversed (not shown). This means that this Cl^- movement is potential sensitive, which again rules out the possibility that it is across the electroneutral exchange mechanism.

The curvature seen in Fig. 10 seems to indicate that the system involved, whether inhibited or not, shows saturation kinetics with respect to internal Cl^- concentration. Since the sulphate-chloride exchange in the same experiment is perfectly logarithmic with time this provides a further argument for the claim that the exchange system is not involved in the Cl^- movement in salicylate media. This may be different with the para isomer, with which Cl^- exchanges considerably more rapidly (Fig. 10).

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