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THE INFLUENCE OF THE CHLORIDE GRADIENT ACROSS RED CELL MEMBRANES ON SODIUM AND POTASSIUM MOVEMENTS

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SUMMARY

1. A study has been made to see whether active and passive movements of sodium and potassium in human red blood cells are influenced by changing the chloride gradient and hence the potential difference across the cell membrane.

2. Chloride distribution was measured between red cells and isotonic solutions with a range of concentrations of chloride and non-penetrating anions (EDTA, citrate, gluconate). The cell chloride concentration was greater than that outside with low external chloride, suggesting that the sign of the membrane potential was reversed. The chloride ratio (internal/external) was approximately equal to the inverse of the hydrogen ion ratio at normal and low external chloride, and inversely proportional to external pH. These results show that chloride is passively distributed, making it valid to calculate the membrane potential from the chloride ratio.

3. Ouabain-sensitive (pump) potassium influx and sodium efflux were decreased by not more than 20 and 40% respectively on reversing the chloride gradient, corresponding to a change in membrane potential from -9 to $+30$ mV. In contrast, passive (ouabain-insensitive) movements were reversibly altered – potassium influx was decreased about 60% and potassium efflux was increased some tenfold. Sodium influx was unaffected by the nature of the anion and depended only on the external sodium concentration, whereas ouabain-insensitive sodium efflux was increased about threefold. When external sodium was replaced by potassium there was a decrease in ouabain-insensitive sodium efflux with normal chloride, but an increase in low-chloride medium.

4. Net movements of sodium and potassium were roughly in accord with the unidirectional fluxes.

5. The results suggest that reversing the chloride gradient and,

therefore, the sign of the membrane potential, had little effect on the sodium pump, but caused a marked increase in passive outward movements of both sodium and potassium ions.

INTRODUCTION

Passive movements of sodium and potassium in red cells are increased when external electrolyte is replaced by non-penetrating non-electrolytes such as sucrose (Maizels, 1935; Davson, 1939). These findings have been interpreted as an effect on permeability of a change in membrane potential (p.d.) although the p.d. was not directly measured (Wilbrandt & Schatzmann, 1960; LaCelle & Rothstein, 1966). Measurements of the p.d. when cells are in plasma or Ringer solution have shown that the value (about -9 mV) is in accord with that predicted from the chloride distribution by applying the Nernst equation (Lassen & Sten-Knudsen, 1968; Jay & Burton, 1969). This relationship holds because chloride is passively distributed; the ratio of internal/external concentrations for chloride depends only on pH and agrees closely with the inverse of the same ratio for hydrogen ions (Van Slyke, Wu & McLean, 1923; Harris & Maizels, 1952).

Little is known of the effect on sodium and potassium movements of replacing external chloride with non-penetrating anions or of a change in chloride distribution across the membrane. Substitution of external sodium chloride with non-electrolytes or foreign salts, such as sodium mucate and toluene di-sulphonate, causes the chloride gradient across the membrane to be reversed (Vidaver, 1964*a*; Donlon & Rothstein, 1969). In view of the effects of non-electrolytes a study has been made of the permeability changes accompanying the replacement of chloride with foreign anions. The distribution of chloride and hydrogen ions has been measured between human red cells and media with a range of chloride concentrations, in order to see how alterations in the chloride distribution influence active and passive sodium and potassium movements. The results show a marked increase in the passive efflux of sodium and potassium with a smaller (potassium) or no (sodium) effect on passive influx. Activity of the sodium pump was somewhat decreased, but not by more than 20–40%. A preliminary account of this work has been given (Cotterrell & Whittam, 1970).

METHODS

Procedure. Human blood (supplied by the Sheffield Regional Blood Transfusion Service) was stored for 3–4 weeks in 'acid-citrate-dextrose' at 4° C. The cells were adjusted to pH 7·6 with unbuffered 1·0 M-Tris (pH 13·0) before being washed at least 3 times in ice-cold medium (150 mM sodium chloride, 5 mM Na phosphate, pH 7·6).

Chloride estimations. The chloride distribution between cells and medium was determined after incubation at 37° C in various isotonic solutions. A sample (4·0 ml.) of cell suspension (haematocrit 20–25 %) was centrifuged for 5 min, and 3·0 ml. of clear supernatant were carefully removed. The sediment of cells and small amount of medium was made up to 9 ml. with 0·2 N-H_2SO_4, 1 ml. of 20 % (w/v) sodium tungstate was added and the mixture was centrifuged. Chloride was then determined in the deproteinized mixture of cells plus medium (Sanderson, 1952). From the volume of packed cells determined by haematocrit, an allowance was made for chloride in the medium in calculating the cell content, but no allowance was made for the small amount of chloride trapped in the extracellular fluid of packed cells. The chloride distribution is expressed as the ratio of chloride concentration in cell water (assuming 65 % cell water) to the external concentration.

Hydrogen ion distribution. Cells were separated from media by centrifugation and lysed in 5 ml. distilled water for pH measurements (Harris & Maizels, 1952). The pH of the haemolysate and medium was measured at room temperature (20° C) with a glass electrode and Vibron 39A millivoltometer (E.I.L., Richmond, Surrey).

Tracer studies. ^{24}NaCl and ^{42}KCl were obtained as sterile isotonic solutions from the Radiochemical Centre, Amersham, Bucks. Both isotopes were counted without organic scintillants, either on an IDL Tritomat model 6020 liquid scintillation counter (Isotope Developments Ltd, Reading), or in later experiments, a Packard Tricarb 3020 liquid scintillation counter (Packard Instruments Ltd, Wembley, Middx.), making use of the Čerenkov phenomenon (Braunsberg & Guyver, 1965; Garrahan & Glynn, 1966).

Sodium and potassium efflux. The method and calculations were as described by Lubowitz & Whittam (1969). Washed cells were loaded with ^{24}Na for 3 hr at 37° C in a medium containing (mM): NaCl, 150, and sodium phosphate, 5 at pH 7·6. Inosine (10 mM) and adenine (5 mM) were also added to restore the ATP level to that in fresh cells (Whittam & Wiley, 1967). After at least five washes in non-radioactive medium, cells were packed, warmed to 37° C and added to 10 ml. of incubation medium to give a final haematocrit of about 5 %. They were shaken in a water-bath at 37° C. Some cell suspension was taken at 10, 30 or 40 min, rapidly cooled for ½ min in an ice-water bath and centrifuged for 2 min. Samples of supernatant (2 ml.) were added to 8 ml. of ice-cold 6 % (w/v) trichloroacetic acid and counted.

Potassium influx. This was measured as described by Glynn (1956). Samples of haemolysed cells were deproteinized and prepared for counting in a manner similar to that described above.

Sodium influx. The method was essentially the same as for potassium influx, except that, in order to avoid possible back-diffusion of tracer into the medium, the incubation time was decreased as much as possible (to 15 min). The tracer uptake by cells (haematocrit of 20 %) was linear with time up to 30 min, and it was therefore concluded that back-diffusion was negligible over this period. The incubation volume was 2·5 ml., and influx was stopped quickly by diluting the cell suspension with 7·5 ml. of ice-cold non-radioactive medium. The cells were then washed, haemolysed and counted.

Cellular sodium and potassium content. Cells were washed three times in 150 mM choline chloride, 10 mM-Tris Cl, pH 7·6, and lysed in ammonium hydroxide (6–7 mM). Samples were diluted for estimation of haemoglobin and of sodium and potassium with either a Unicam SP 90 spectrophotometer (Unicam Instruments Ltd, Cambridge) or an EEL 227 integrating flame photometer with lithium internal standard (Evans Electroselenium Ltd, Halstead, Essex) (Lubowitz & Whittam, 1969).

Incubation media, in which chloride was replaced by foreign anions, were adjusted to have the same depression of freezing point as chloride medium, as determined by a

Knauer freezing-point osmometer (Knauer, Holstweg 18, Berlin). Media were prepared as follows: chloride medium, 150 mM sodium chloride, 5 mM sodium phosphate, pH 7·6; 290 m-osmole/l. Media with monovalent anion replacements for chloride, where X^- is the monovalent anion: 150 mM-NaX^-, 10 mM-Tris Cl, pH 7·6, range 280–310 m-osmole/l. Media with polyvalent anion replacements for Cl: 125 mM sodium sulphate, 10 mM-Tris Cl, pH 7·6, 300 m-osmole/l.; 100 mM sodium citrate, pH 7·6, 283 m-osmole/l.; 94 mM sodium EDTA (ethylenediamine tetra-acetic acid), 5 mM sodium phosphate, pH 7·6, 300 m-osmole/l. Potassium replacement media, 100 mM potassium citrate, pH 7·6, 288 m-osmole/l.; 91 mM potassium EDTA, 5 mM potassium phosphate, pH 7·6, 290 m-osmole/l.

Materials. All chemicals were Analar grade where possible and all solutions were made in glass-distilled water.

RESULTS

The passive distribution of chloride as an indication of membrane potential

In order to assess the validity of calculating the membrane potential from the Nernst equation, it was important to check that chloride was passively distributed between red cells and medium under a variety of conditions in which changes were made in the external chloride concentration and pH.

Dependence of chloride ratio on external chloride. Tests have been made to see whether replacement of chloride with non-penetrating anions affects the chloride distribution between cell water and medium at equilibrium. The chloride distribution (cell water/medium) was 0·71 (Table 1) with 150 mM external chloride in agreement with earlier work (Harris & Maizels, 1952), but when external chloride was reduced to 10–30 mM by replacement with citrate or EDTA, there was more chloride in the cells (90–110 μ-equiv/ml. cell water) than in the medium, giving a distribution ratio of about 3·0. The reversed concentration gradient was established during 1 hr and then stayed constant, suggesting that citrate and EDTA penetrate into the red cell very little, if at all, and that chloride is retained in the cell to act as a gegenion for internal cations. Cell chloride remained constant during incubation with 150 mM external chloride, but there was chloride loss from cells during 1 hr with low external chloride. The loss was only some 10 μ-equiv/ml. cells, which is quantitatively comparable with the net potassium loss (Table 6) and with the excess of potassium efflux over influx (Fig. 2 and Table 3), suggesting that there was a net loss of KCl from the cells. External chloride was also replaced by the monovalent anion, gluconate, which seemed to us likely not to penetrate because of its hydrophilic nature. A reversed chloride gradient of similar magnitude to that found with EDTA and citrate was maintained (Table 1), showing that the human red cell membrane is impermeable to gluconate. When sulphate was substituted for external chloride, there was a change in the chloride

TABLE 1. Chloride content of red cells

Time of incubation (min)	Medium: Cl $[Cl]_{cell}$	Cl $\frac{[Cl]_i}{[Cl]_o}$	SO_4 $[Cl]_{cell}$	SO_4 $\frac{[Cl]_i}{[Cl]_o}$	Citrate $[Cl]_{cell}$	Citrate $\frac{[Cl]_i}{[Cl]_o}$	EDTA $[Cl]_{cell}$	EDTA $\frac{[Cl]_i}{[Cl]_o}$	Gluconate $[Cl]_{cell}$	Gluconate $\frac{[Cl]_i}{[Cl]_o}$
0	85·5	0·71	64·0	4·25	67·2	3·22	60·9	3·34	65·0	5·51
5	—	—	24·0	1·50	68·8	4·10	58·8	3·26	—	—
10	—	—	14·0	0·89	60·2	3·20	53·0	2·70	—	—
15	—	—	16·0	0·92	62·0	3·30	58·1	3·06	71·0	5·65
30	—	—	17·0	1·00	57·6	2·97	54·9	2·72	71·0	5·65
60	—	—	18·0	1·10	58·6	3·00	52·4	2·54	68·0	5·30

Cells, 3–4 weeks old were prepared, incubated and analysed for chloride as described in Methods. The intracellular chloride concentrations $[Cl]_{cell}$ were converted to $[Cl]_i$ in μ-equiv Cl/ml. cell water assuming 65 % cell water. Values are means of duplicate incubations.

distribution ratio with time; starting initially at about 4·2, the ratio fell to 1·0 after 15 min and then remained at about this value. The fall was accompanied by a net loss of internal chloride from 64 to 15 μ-equiv/ml. cells, showing that chloride reached an approximately even distribution between cells and medium, consistent with entry of sulphate into the cells (Luckner, 1939). This result shows that sulphate is not suitable for use as an impermeant anion with human red cells.

Dependence of chloride distribution ratio on external pH. There is good evidence with cells under physiological conditions for the passive distribution of chloride and hydrogen ions. There was a linear relationship between the chloride ratio and external pH, and also the ratio $[H]_o/[H]_i$ agreed closely with the chloride ratio (Harris & Maizels, 1952). It seemed important to establish whether this held in cells with low external chloride.

Cell suspensions in normal chloride medium were divided into five lots and adjusted to five pH values between pH 6·0 and 8·0. Each lot of suspension was washed three times in chloride medium at the appropriate pH value and the cells were sedimented. The packed cells were resuspended in either chloride or EDTA media at the various pH values, incubated for 15 min at 37° C and the chloride distribution was determined as a function of pH with low and normal external chloride. The chloride ratio was inversely proportional to external pH in both chloride and EDTA media (Fig. 1). It fell from 1·04 to 0·56 as pH was increased from 6·0 to 8·0 with 150 mM chloride, the slope of the line, ΔCl ratio/ΔpH being 0·34, in agreement with the value of 0·35 found by Harris & Maizels (1952). For cells in EDTA medium containing 20–30 mM chloride the ratio fell from 5·50 to 2·04 between pH 6·0 and 8·0, with a slope of 0·45. These results show that the chloride distribution varied in a similar manner on altering pH whether external chloride was in high or low concentration. The findings are consistent with a passive distribution of chloride in low-chloride medium.

The interdependence of chloride and hydrogen ion distributions. A more critical test to verify that chloride is passively distributed is to see whether the chloride ratio, $[Cl]_i/[Cl]_o$, is equal to the inverse of the hydrogen ion ratio, $[H]_o/[H]_i$, under conditions of low and high external chloride and at various pH values. Two sets of five flasks were set up, at pH values over the range pH 6·0–8·0, one set for chloride estimations and the other for pH measurements. The internal pH of cells after incubation was taken to be the pH of a haemolysate prepared by lysing packed cells with 5 volumes of water (Harris & Maizels, 1952). The mean values of three experiments (Table 2) show three features. First, the ratio, $[H]_o/[H]_i$ fell from 3·08 to 2·40 in EDTA medium as external pH was increased from 6·5 to 8·0. No significant fall was seen in the same ratio for cells in chloride medium, however, probably due to errors in measuring the small pH differences

involved. Secondly, reversing the chloride gradient by suspending cells in low external chloride solutions containing an impermeant anion also reversed the hydrogen ion gradient, so that internal pH was greater than external pH (Table 2). In contrast, for cells with normal external chloride the pH inside the cells was always less than that outside. Thirdly, there was approximate agreement between the chloride and hydrogen ion ratios for pH values of 7·0, 7·5 and 8·0 with normal and low external chloride. There

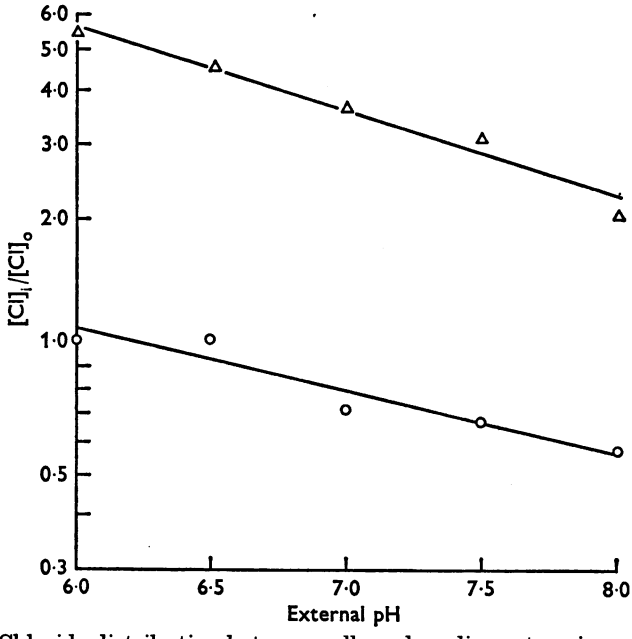

Fig. 1. Chloride distribution between cells and medium at various external pH values. Cells, 3–4 weeks old were prepared as described in Methods and adjusted to the various pH values in sodium chloride medium. Chloride distribution was measured with cells suspended in normal external chloride medium (○) or sodium-EDTA medium (△). $[Cl]_i$ and $[Cl]_o$ are the concentrations of chloride in cell water and medium respectively. Mean values of duplicate incubations of a typical experiment are shown.

was some deviation at lower pH, a possible explanation being that the glass electrode underestimated internal pH at the low internal pH values (Table 2). Gary-Bobo & Solomon (1968) found a similar discrepancy when measuring chloride and hydroxyl movements, and as with our results, the disparity increased as pH was decreased.

As a consequence of the redistribution of chloride there was an acidification of the low external chloride media. The pH of a cell suspension (initially at pH 7·6) added to EDTA medium was decreased to and maintained at pH 7·4, which is consistent with the findings of Funder & Wieth

(1966) using citrate as anion. There was a similar initial acidification of sulphate medium (to pH 7·2), but this returned to the original pH of 7·6 (after about 10 min) as sulphate exchanged with internal chloride.

Relationship between chloride ratio and membrane potential. Since chloride and hydrogen ions appear to be passively distributed under conditions of normal or low external chloride, the membrane p.d. can be calculated where p.d. = 61·5 log $[Cl]_i/[Cl]_o$ mV at 37° C. Our value for cells in 150 mM external chloride is 9 mV with the inside negative, whereas with

TABLE 2. Chloride and hydrogen ion distributions between cells and normal or low external chloride medium

Ringer pH	$[Cl]_i$	$[Cl]_o$	pH_i	pH_o	$[Cl]_i/[Cl]_o$		$[H]_o/[H]_i$
NaCl							
6·0	174	167	5·83	6·06	1·06	>	0·65
6·5	155	161	6·31	6·48	0·96	>	0·67
7·0	124	158	6·78	6·95	0·78	=	0·71
7·5	105	155	7·33	7·45	0·68	=	0·75
8·0	85	153	7·60	7·77	0·56	=	0·66
Ringer pH EDTA							
6·0	136	33	6·35	5·90	4·37	>	2·90
6·5	122	31	6·86	6·37	4·00	>	3·08
7·0	99	29	7·28	6·81	3·48	⩾	2·98
7·5	75	27	7·66	7·23	2·79	=	2·72
8·0	49	26	8·08	7·70	1·97	=	2·40

Cells, 3–4 weeks old, were incubated for 15 min at 37° C. Cl and pH estimations were performed as described in Methods. $[Cl]_i$ and $[Cl]_o$ are the chloride concentrations in cell water and medium respectively, pH_i and pH_o are cell and medium pH, respectively.

low external chloride the p.d. rose to about 30 mV, with the inside positive. Cells equilibrated in solutions with a range of chloride concentrations are effectively maintained under voltage clamp conditions, where the p.d. is a constant value according to the nature of the external anion and the chloride ratio.

Operation of the sodium pump with low external chloride. To investigate whether the nature of the predominant external anion affects the sodium pump, the ouabain-sensitive (pump) components of sodium efflux and potassium influx have been measured in Ringer with modified anion composition. Potassium chloride (10 mM) was always included to activate the sodium pump. Replacement of up to 90 % of external sodium chloride was first made with isotonic solutions of sodium EDTA, sodium citrate or sodium gluconate because these anions are non-penetrating. Similar results were obtained with each substituent and in most experiments EDTA was used.

Potassium influx. The pump potassium influx (in μ-equiv/ml. cells $\times$ hr) in EDTA medium was somewhat less than in normal-chloride medium, although there was a considerable spread of absolute values ranging from 1·79 to 3·50 with normal external chloride and from 0·90 to 3·19 in EDTA medium (Table 3). The decrease in influx was found in nine out of eleven experiments, and the mean values fell 20 % from 2·82 to 2·24. The magnitude of the decrease ($0{\cdot}58 \pm 0{\cdot}14$ S.E. of mean) was not associated with the absolute values. When citrate was substituted for chloride, the pump potassium influx was not significantly decreased; the mean difference was $0{\cdot}11 \pm 0{\cdot}13$ (S.E. of mean; four experiments).

Sodium efflux. The pump component of sodium efflux showed a considerable spread of results between five experiments, values ranging from 3·09 to 6·17 (mean 4·74) with normal external chloride and from 1·44 to 4·13 (mean 2·76) in EDTA medium (Table 4). The fall in sodium efflux with low external chloride was greater than the fall in potassium influx, the mean decrease with EDTA being $1{\cdot}97 \pm 0{\cdot}25$ and with citrate $0{\cdot}86 \pm 0{\cdot}27$ (S.E. of mean). Comparison of the results shows that the ratio of sodium efflux/potassium influx for the pump components was $1{\cdot}6 \pm 0{\cdot}3$ (S.E. of mean) for cells in normal-chloride medium, in agreement with a stoichiometry of three sodium ions extruded per two potassium taken up (Post & Jolly, 1957; see Whittam, 1964). In low chloride medium the ratios were $1{\cdot}2 \pm 0{\cdot}3$ with EDTA and $1{\cdot}4 \pm 0{\cdot}2$ with citrate; these values are not significantly different from 1·5, and are within the range found by other authors with normal external chloride. It seems that the stoichiometry of the sodium pump was not materially altered by changing the external anion so as to reverse the sign of the membrane potential.

Decrease in pump potassium influx with 10–50 mM external potassium. There would seem to be three possibilities to account for the apparent fall in pump fluxes with low external chloride. First, since activation of the sodium pump depends on external potassium, a concentration of 10 mM potassium was perhaps insufficient to activate the pump fully with low external chloride. Potassium influx was therefore measured at external potassium concentrations of 10, 20 and 50 mM. There was still a fall in the ouabain-sensitive potassium influx from 3·11 to 2·14 at 20 mM external potassium, and from 3·41 to 2·64 with 50 mM potassium (Table 3). The fall in potassium influx was thus independent of the external potassium concentration, as shown by the similar values for the ratio of potassium influx for normal chloride/EDTA medium (1·31, 1·47 and 1·29, at 10, 20 and 50 mM external K). Increasing external potassium from 10 to 50 mM did not raise the sodium pump activity in low chloride conditions to that of the control normal chloride medium, suggesting that 10 mM potassium was sufficient for activation.

TABLE 3. Potassium influx into red cells in normal or low external chloride conditions

External K (mM)	Replacement for Cl	Ouabain-sensitive (μ-equiv/ml. cells × hr) Expt.	Normal Cl (a)	Low Cl (b)	(b) − (a)	(b)/(a)	Ouabain-insensitive (μ-equiv/ml. cells × hr) Expt.	Normal Cl (c)	Low Cl (d)	(d)/(c)
10	EDTA	25/7	1·79	0·90	−0·89	0·50		0·85	0·52	0·61
		17/12	2·02	2·20	+0·18	1·10		0·84	0·22	0·26
		23/6	2·03	1·64	−0·39	0·82		1·06	0·30	0·28
		7/5	2·60	2·22	−0·38	0·85		0·64	0·24	0·38
		5/5	2·65	1·85	−0·80	0·69		0·54	0·24	0·44
		3/2	3·02	2·10	−0·92	0·70		0·91	0·49	0·54
		27/1	3·10	3·13	+0·03	1·01		0·87	0·34	0·39
		16/12	3·42	3·19	−0·23	0·94		0·55	0·28	0·51
		8/4	3·45	2·18	−1·27	0·63		0·72	0·47	0·65
		7/4	3·50	2·53	−0·97	0·72		0·98	0·45	0·46
		6/5	3·50	2·69	−0·81	0·77		0·84	0·39	0·46
		Mean	2·82	2·24	−0·58	0·79		0·80	0·35	0·44
		(S.E.)	±0·20	±0·20	±0·14			±0·05	±0·03	
	Citrate	23/6	2·03	1·61	−0·42	0·80		1·06	0·20	0·19
		3/6	2·25	2·06	−0·20	0·92		0·87	0·21	0·24
		27/1	3·10	3·07	−0·03	0·99		0·87	0·24	0·28
		161/12	3·42	3·60	+0·18	1·05		0·55	0·25	0·45
		Mean	2·70	2·58	−0·11	0·94		0·84	0·23	0·29
		(S.E.)	±0·17	±0·23	±0·13			±0·11	±0·01	

TABLE 3. (*cont.*)

External K (mM)	Replacement for Cl	Ouabain-sensitive (μ-equiv/ml. cells × hr) Expt.	Normal Cl (*a*)	Low Cl (*b*)	(*b*) − (*a*)	(*b*)/(*a*)	Ouabain-insensitive (μ-equiv/ml. cells × hr) Expt.	Normal Cl (*c*)	Low Cl (*d*)	(*d*)/(*c*)
20	EDTA	24/2	1·63	1·19	−0·44	0·73		1·63	0·95	0·58
		8/4	3·56	2·00	−1·56	0·57		0·98	0·83	0·84
		7/4	4·15	3·23	−0·92	0·78		1·50	0·70	0·47
							28/1	1·08	0·52	0·48
		Mean	3·11	2·14	−0·97	0·68	3/2	1·17	1·09	0·93
							2/2	1·36	1·00	0·74
							25/2	2·61	1·08	0·41
							Mean	1·32	0·88	0·63
50	EDTA	8/4	3·30	2·00	−1·30	0·61	28/1	1·61	1·06	0·66
		7/5	3·43	2·67	−0·76	0·75	3/2	3·67	2·80	0·75
		7/4	3·52	3·25	−0·27	0·92	60 2/2	3·69	1·91	0·52
							25/2	3·82	3·07	0·78
		Mean	3·41	2·64	−0·77	0·78		3·20	2·21	0·68
100	EDTA						28/1	2·20	1·67	0·76
							25/2	4·59	4·38	0·95
							3/2	5·28	2·51	0·47
							2/2	7·97	3·20	0·40
							Mean	5·01	2·94	0·65

Cells, 3–4 weeks old were pre-incubated in sodium chloride medium containing adenine (5 mM) and inosine (10 mM). Potassium influx was measured over 15 min at 37° C in the various isotonic solutions described in Methods. External potassium was not less than 10 mM, and was increased by partial replacement of the isotonic sodium salt with the potassium salt.

TABLE 4. Sodium efflux from red cells in normal or low external chloride conditions

External K (mM)	Replacement for Cl	Ouabain-sensitive (μ-equiv/ml. cells × hr) Expt.	Normal Cl (e)	Low Cl (f)	(f)–(e)	(f)/(e)	Ouabain-insensitive (μ-equiv/ml. cells × hr) Expt.	Normal Cl (g)	Low Cl (h)	(h)/(g)
10	EDTA	15/4	3·09	1·44	−1·55	0·47		1·32	2·97	2·25
		3/12	4·37	2·16	−2·21	0·49		2·97	7·64	2·57
		12/2	4·70	3·40	−1·30	0·73		1·28	4·04	3·18
		10/2	5·40	2·65	−2·75	0·49		1·45	6·75	4·65
		11/2	6·17	4·13	−2·04	0·67		1·96	6·77	3·46
							14/4	1·25	2·66	2·14
		Mean	4·75	2·76	−1·97	0·55		1·70	5·13	3·04
		(S.E.)	±0·52	±0·47	±0·25			±0·23	±0·88	
	Citrate	6/8	3·68	3·28	−0·33	0·91		2·00	5·65	2·72
		12/8	3·84	3·78	−0·06	0·98		1·77	4·10	2·32
		3/12	4·37	3·00	−1·37	0·69		2·97	4·53	1·52
		12/2	4·70	3·44	−1·26	0·73		1·28	3·44	2·70
		10/2	5·40	4·13	−1·27	0·76		1·45	4·35	3·00
		Mean	4·38	3·53	−0·86	0·80		1·89	4·41	2·45
		(S.E.)	±0·32	±0·20	±0·27			±0·30	±0·36	
50	EDTA	16/4	4·56	2·34	−1·33	0·52		1·67	3·04	1·82
		(S.E.)	±0·23	±0·12						

Conditions were as in Table 3. Sodium efflux was measured over a 30 or 40 min period at 37° C.

Influence of external sodium on potassium influx. The second possible reason for the decreased pump fluxes arises from the higher external sodium concentration which is necessary when chloride is replaced by the polyvalent anions EDTA or citrate (an increase from 150 to 350 mM). External sodium in the range 0–150 mM competitively inhibits potassium influx (Post & Jolly, 1957; Garrahan & Glynn, 1967; Priestland & Whittam, 1968), making it possible that a further increase to 350 mM might explain the decrease found with low external chloride. The magnitude of the effect can be estimated if kinetics for purely competitive inhibition are assumed. Values for half-maximal activation of potassium influx are about 0·16 mM and 0·4 mM in sodium free Ringer (K_m) and 1·4 mM and 1·5 mM in 150 mM sodium-Ringer (K_p) (Garrahan & Glynn, 1967; Priestland & Whittam, 1968, respectively). K_m and K_p are related by $K_p = K_m(1+I/K_I)$ (Dixon & Webb, 1964) where K_I is the inhibitor constant for sodium and I the concentration of inhibitor (sodium). From this equation an approximate value of K_I of between 20 and 55 mM is obtained. It can thus be predicted that raising the sodium concentration from 150 to 350 mM at 10 mM external potassium would lower potassium influx by some 12%. Although this effect would partly explain the fall which was found, it cannot be the sole explanation because raising external potassium to 50 mM did not relieve the inhibition (Table 3).

The third factor which may contribute to the reduction of active transport concerns the method of measurement. There is a net potassium loss and sodium gain when cells are in low chloride medium (Table 6). Thus, internal ^{24}Na will be diluted by ^{23}Na gained during measurement of sodium efflux, which would thus be underestimated since the initial specific activity was used in the calculation. Attempts were made to allow quantitatively for a fall in specific activity from the net changes in 3 hr because the measured net changes in 15 min were within experimental error. Calculations show that a small entry of ^{23}Na could cause a reduction of sodium efflux by some 10–20%. For comparable reasons, ^{42}K influx would be underestimated as counts gained by the cell would be lost due to the net loss of potassium into the low chloride media.

It may be concluded that although both the active potassium influx and sodium efflux are somewhat reduced with low external chloride the changes may be adequately accounted for. There would seem to be little or no effect of reversing the sign of the membrane potential by changing the chloride distribution on the activity and stoichiometry of the sodium pump.

The dependence of passive movements of sodium and potassium on chloride distribution between cells and medium

When measuring transport by the sodium pump, values were also obtained for ouabain-insensitive (passive) potassium influx and sodium efflux. Reduction of external chloride had a considerable effect which has been investigated in detail.

Potassium influx. Passive potassium influx with 10 mM external potassium was reduced (Table 3) from a mean of 0·80 (in μ-equiv/ml. cells × hr) with 150 mM external chloride (range 0·55–1·06) to a mean of 0·35 in low chloride (10 mM) EDTA solution (range 0·22–0·52), the fall being $0{\cdot}44 \pm 0{\cdot}05$ (S.E. of mean). A similar feature was found with citrate replacement when potassium influx was reduced from 0·84 to 0·23, a decrease of $0{\cdot}61 \pm 0{\cdot}11$ (S.E. of mean). These values show that passive potassium influx was decreased about 60% when external chloride was reduced to about 10 mM. The effect of higher external potassium concentrations (20, 50 and 100 mM) on ouabain-insensitive potassium influx was measured with normal and low external chloride. The results in Table 3 show that potassium influx was raised by increasing $[K]_o$ under both conditions, the increase being greater with chloride than EDTA. Thus, the values with 150 mM chloride were 0·80, 1·32, 3·2 and 5·0, compared with values with 10 mM chloride in EDTA of 0·36, 0·88, 2·2 and 2·9 when external potassium was 10, 20, 60, and 100 mM. These results show that whatever the level of external potassium the value of potassium influx was always higher with chloride as anion than with EDTA. Furthermore, the ratio of potassium influx in normal chloride/EDTA solutions was comparable for all levels of external potassium.

When external chloride was almost completely replaced by other anions to which the membrane is only slightly permeable, the passive potassium influx fell, e.g. from 0·9 with chloride to 0·3 with acetate, 0·3 with nitrate and 0·2 with lactate, pyruvate and iodide. There was no change with bromide. Partial replacement of chloride by non-penetrating anions gave progressive changes in the passive fluxes between the two extreme conditions given above. These results show that lactate, pyruvate and iodide act in a similar manner to EDTA and citrate in causing a fall in potassium influx.

Potassium efflux. Potassium efflux was also measured to see whether it was affected like potassium influx. External potassium (10 mM) was always added to the Ringer solutions. Preliminary experiments showed that the response of potassium efflux to chloride substitution was uninfluenced by ouabain, which was not routinely added. The results showed little change in potassium efflux until 40% of the chloride had been replaced, but on

further reduction (to about 50 mM chloride) potassium efflux was approximately doubled with citrate and trebled with EDTA (Fig. 2). When external chloride was 10–15 mM there was a rise in potassium efflux (in μ-equiv/ml. cells × hr) from the control of 1·4 in chloride to 15 with EDTA and to 8·0 with citrate. Potassium efflux was therefore raised in a most striking manner, the low-chloride medium producing a tenfold increase in efflux when EDTA replaced chloride.

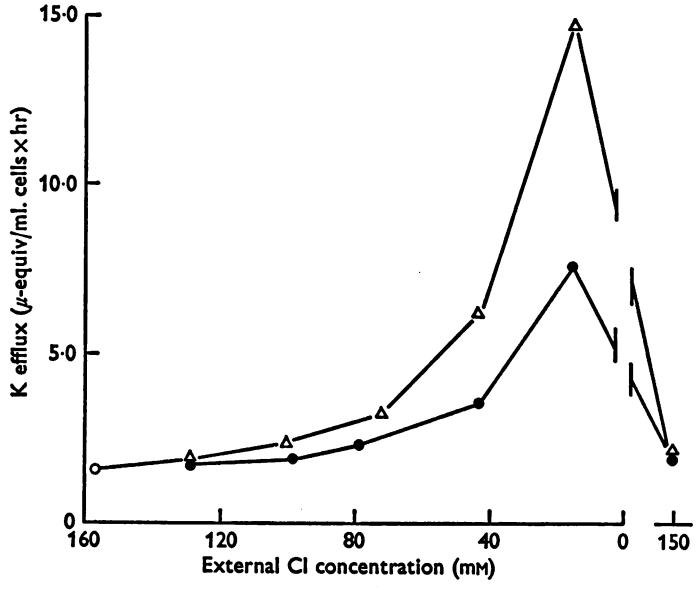

Fig. 2. Potassium efflux from cells in low external chloride media in which chloride was replaced by citrate (●) or EDTA (△) anions. Cells, 3–4 weeks old were pre-incubated in sodium chloride medium containing inosine (10 mM), adenine (5 mM) and ^{42}KCl. K efflux was measured over 30 min at 37° C and also in a parallel lot of cells which were treated with low external chloride medium and returned to normal chloride conditions. Mean values of duplicate incubations of a typical experiment are shown.

Dependence of potassium efflux on external pH. Since the chloride distribution ratio is inversely proportional to external pH in both normal and low external chloride solutions, experiments were designed to see whether potassium efflux depended on external pH in a way related to changes in the chloride ratio. Previous work has established that varying the pH of isotonic sodium chloride solutions has little effect on the loss of potassium from cells (Flynn & Maizels, 1949; Romero & Whittam, 1971). This was confirmed in the experiments shown in Fig. 3. In contrast, potassium efflux from cells in EDTA medium increased steeply from 3·2 (μ-equiv/ml. cells × hr) at pH 6·0 to 16·5 at pH 8·0. Two points arise from this result, first,

that at all pH values with low external chloride, potassium efflux was always greater (two to tenfold) than the normal-chloride control, but secondly, that the raised potassium efflux which occurred with increased external pH did not appear to be related to the chloride ratio, which must have decreased under these conditions. Although reversing the chloride gradient increases potassium efflux the permeability of the cell membrane is also independently influenced by the external pH.

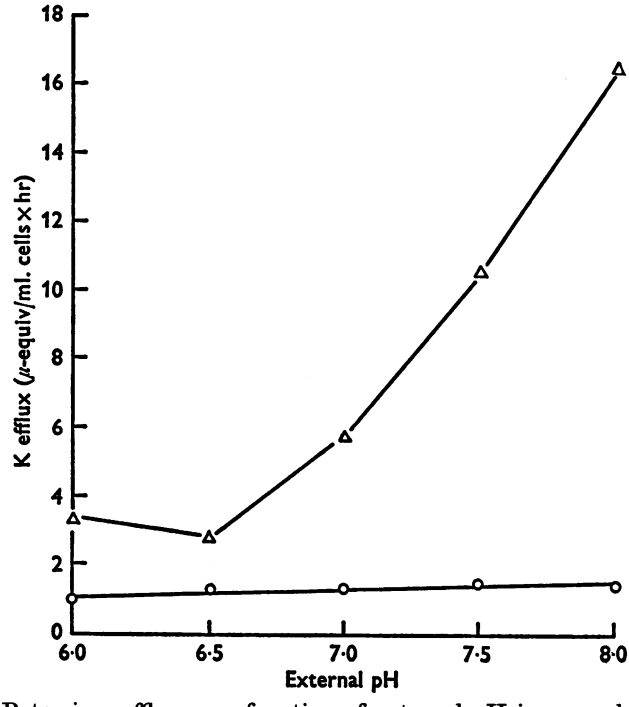

Fig. 3. Potassium efflux as a function of external pH in normal and low external chloride media. Cells, 3–4 weeks old were prepared as described in Fig. 2. After pH adjustment, cells were incubated in normal chloride (○) or EDTA medium (△) at the pH values shown. Mean values are shown from triplicate incubations in a typical experiment.

Reversibility of potassium efflux on altering the external chloride concentration. The increase in potassium efflux might arise from irreparable damage to the cell membrane, in which case it would not be reversible, or it may be associated with the change in membrane p.d. associated with the low external chloride. Two sets of cell suspensions were loaded with ^{42}K. In the first lot of cells, potassium efflux was measured with normal external chloride and in the modified media in which chloride had been largely replaced by EDTA or citrate anions. The results were in line with the experiments described above, in showing that large increases in potassium

efflux occurred when cells were suspended in low-chloride solutions (Fig. 2). The other lot of cells was treated in exactly the same way except that at the end the cells were washed briefly, and suspended in chloride medium. Efflux was then measured. In each instance, whatever the anion in the Ringer during the pre-treatment, the efflux was the same low value of about 2·0 in spite of the increase which must have occurred during exposure to the low-chloride medium. The increase in potassium efflux was therefore reversible, not due to damage of the cell membranes, and apparently related to the chloride distribution and hence to p.d.

TABLE 5. Sodium influx with 150 mM external sodium in normal or low chloride media

mM-$[Na]_o$	mM-$[Cl]_o$	Na influx (μ-equiv/ml. cells × hr)
150	150	2·81
150	100	2·37
150	50	2·77
150	10	2·78

Sodium influx was measured as described in Fig. 4, with media in which EDTA was substituted for chloride and potassium for sodium to keep external sodium at 150 mM. Mean values shown are from five incubations in a typical experiment.

Sodium influx. Sodium influx is passive, proportional to external sodium and uninfluenced by ouabain in the presence of 10 mM external potassium (Glynn, Lew & Lüthi, 1970; Lant, Priestland & Whittam, 1970). Sodium influx was therefore measured to see if it was like potassium efflux in being affected by chloride replacement with impermeant anions. Such chloride substitution inevitably requires a higher external sodium concentration and a higher influx is to be expected. The specific activity of external sodium was calculated by dividing the radioactivity by the sodium concentration, and on this basis, the sodium influx (in μ-equiv/ml. cells × hr) was increased from $2·1 \pm 0·2$ (S.E. of mean; six experiments) in 150 mM chloride to $10·2 \pm 1·1$ (S.E. of mean; five experiments) in EDTA (350 mM sodium). The influx was raised to this maximum value as chloride was progressively decreased to about 10 mM by the polyvalent anions. Because sodium influx depends on external sodium it was necessary to change the anion whilst keeping external sodium constant. This was done by mixing isotonic sodium and potassium salts of EDTA so that the sodium concentration was 150 mM (Table 5). Sodium influx was 2·8 at an external chloride concentration of 150 mM and 2·7 when chloride was reduced to 10 mM by EDTA replacement, showing that influx was not affected by substituting EDTA for chloride. When external sodium was varied, by

replacement with potassium, sodium influx was increased linearly with external sodium whether the anion was chloride or EDTA (Fig. 4). Both sets of points fell on the same line.

In other experiments almost complete replacement of chloride by monovalent ions (bromide, nitrate, acetate, lactate, pyruvate) had little effect on sodium influx. It can be concluded that sodium influx was independent of the nature of the anion and was a linear function of external sodium.

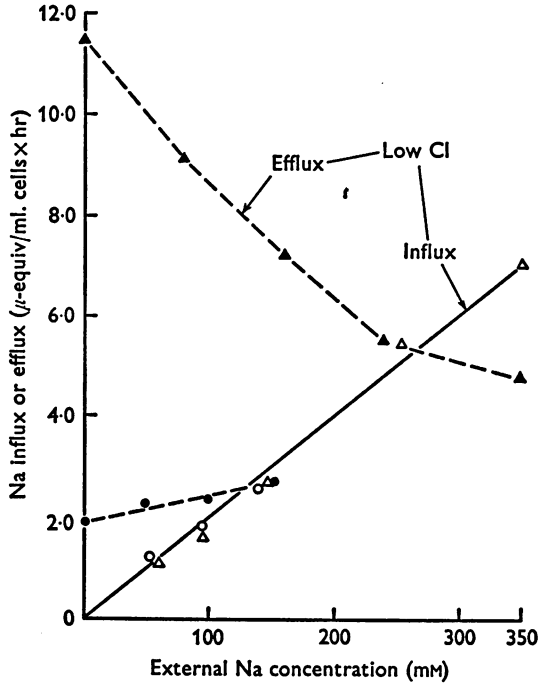

Fig. 4. Sodium influx and ouabain-insensitive sodium efflux as a function of external sodium concentration in normal chloride or EDTA medium. To give a range of external sodium concentrations the sodium salt was partly replaced by the potassium salt. Cells, 3–4 weeks old, were pre-incubated in sodium chloride medium containing inosine (10 mM) plus adenine (5 mM). Sodium influx and efflux were measured over 15 and 30 min respectively at 37° C. Mean values of triplicate incubations are shown; ○ = sodium influx, ● = sodium efflux with normal external chloride; △ = sodium influx, ▲ = sodium efflux with EDTA substitution for chloride. Sodium efflux was measured in the presence of 0·1 mM ouabain.

Sodium efflux. Passive sodium efflux (ouabain-insensitive) was increased in low-chloride conditions from a mean of 1·70 (range 1·25–2·97) with normal external chloride to 5·13 (range 2·66 to 7·75) with EDTA substitution and to 4·4 with citrate substitution for chloride (Table 4). This

effect represents a trebling of sodium efflux when external chloride was reduced to about 10 mM.

Dependence of sodium efflux on external sodium. The first point to consider is whether sodium efflux is dependent on external sodium concentration, as in the isotonic solutions of polyvalent anions the sodium concentration was higher (about 350 mM) than with normal external chloride. Since sodium efflux is stimulated by external sodium in the presence of ouabain (Hoffman, 1966; Lubowitz & Whittam, 1969), it seemed that the raised external sodium might account for the increase in sodium efflux. To test this point ouabain-insensitive sodium efflux was measured as a function of external sodium (range 0–150 mM) in both chloride and EDTA media with potassium as substitute for sodium. Sodium efflux was decreased with normal external chloride (Fig. 4) from 2·7 with 150 mM sodium to 1·9 in sodium-free medium, as found previously with magnesium as sodium replacement (Hoffman, 1966; Lubowitz & Whittam, 1969), showing that external sodium stimulated sodium efflux. In EDTA medium, in contrast, sodium efflux was decreased from 11·6 with zero external sodium to 5·5 with 150 mM sodium and to 4·8 with 350 mM sodium. The higher sodium efflux in sodium-free EDTA medium was unexpected, and is considered later in connexion with the p.d. but it is obvious that a stimulation of ouabain-insensitive sodium efflux by raising external sodium above 150 mM does not account for the increased sodium efflux caused by chloride replacement.

Net movements of sodium and potassium as a function of external chloride

From the preceding unidirectional flux results it can be predicted that cells incubated in low chloride solutions should lose potassium as well as gaining or losing sodium according to the external sodium concentration. Changes in cell sodium and potassium content have been measured in the presence of 0·1 mM ouabain when they are determined solely by the passive fluxes and are not complicated by operation of the sodium pump. The potassium loss (ΔK) is given after 1 hr by the difference in influx (M_{in}) and efflux (M_{out}), as

$$\Delta K = M^{K}_{out} - M^{K}_{in}.$$

It follows from the flux values given above that, with 150 mM external chloride cells should lose (1·5–1·0) = 0·5, whereas with low external chloride they should lose (15 – 0·5) = 14·5 with EDTA and (8·0 – 0·5) = 7·5 with citrate. The mean potassium losses (in μ-equiv/ml. cells × hr) from net movements after 1 or 3 hr were 1·4 with chloride, 4·5 with citrate and 5·7 with EDTA media. The net movements with citrate and EDTA are smaller than would be expected from the tracer fluxes, but they,

nevertheless, vary in the expected direction and clearly show a much greater potassium loss with citrate or EDTA as predominant external anion than with chloride.

External sodium was varied between 0 and 350 mM by replacement with potassium using EDTA as major anion. Cells gained or lost sodium according to the external sodium concentration. With 350 mM sodium, the gain (in μ-equiv/ml. cells $\times$ hr) was 2·6 whereas with no external sodium there was a loss of 3·2 (Table 6). Cells in 150 mM sodium gluconate gained roughly the same amount of sodium (0·3) as cells in sodium chloride medium which is to be expected for similar external sodium concentrations (Table 6). There was a potassium loss, however, of comparable magnitude (4·8) to that seen with citrate or EDTA as replacement for chloride, suggesting that the loss was related to the reversed membrane p.d. found under those conditions.

Relationships between membrane potential difference and ion movements

The chloride ratio (internal/external) was determined in cells from the same blood used for the flux measurements, and the membrane potential was calculated from the Nernst equation, when various proportions of the external chloride were replaced by EDTA (Fig. 5). The graph shows the passive sodium and potassium fluxes as a function of p.d. There was a steep increase in potassium efflux as the inside of the cell became positive, but only a relatively small fall in passive potassium influx. When the passive potassium flux ratio, M_{out}/M_{in} (right-hand ordinate), is plotted against p.d., the results do not show a linear relationship but a steep increase as the inside of the cell became positive. The steep rise was caused by the increased efflux because influx shows a slight fall. If the unidirectional potassium fluxes in red cells were dependent only on the electrochemical gradient as the driving force, and if potassium movements across the membrane were independent of each other, then a straight line relationship between flux ratio and p.d. should be obtained (indicated in Fig. 5), a tenfold change in chloride ratio equivalent to a potential change of 61·5 mV, causing a tenfold change in flux ratio (Ussing, 1949). A steep rise in flux ratio with decrease in p.d. would be expected if the ion movements were not independent, but again the flux ratio would still vary linearly with p.d. as found in nerve (Hodgkin & Keynes, 1955). The results in Fig. 5 show that this explanation does not apply to red cells, in which there appears to be a preferential increase in efflux over influx, suggesting that rectification of the membrane to potassium occurs as the inside of the cell becomes positive.

Sodium movements are influenced to a lesser extent than potassium movements when the membrane potential is reversed (Fig. 5). The

TABLE 6. Changes in sodium and potassium content of cells with normal or low external chloride, in the presence of ouabain

Expt.		Incubation (hr)		(μ-equiv/ml. cells × hr) media NaCl	KCl	Na EDTA	Na citrate	Na gluconate
			$[Na]_o$	150	0	350	390	150
Na	1	3		+0·5	+0·2	+1·9	—	+0·5
	2	1		+2·2	—	+1·7	—	+0·4
	3	1		+2·6	0	+4·2	—	0
	4	3		+0·5	—	+0·7	+1·3	—
	Mean			+1·1	+0·1	+2·1	+1·3	+0·3
K	1	3		−1·4	+1·3	−3·5	—	−3·6
	2	1		−0·2	—	−3·4	—	−3·4
	3	1		−3·5	−1·0	−8·9	—	−7·5
	4	3		−0·7	—	−7·1	−4·5	—
	Mean			−1·4	+0·1	−5·7	−4·5	−4·8

Cells, 3–4 weeks old were incubated at 37° C for 1 hr or 3 hr in the various media, as described in Methods, all of which contained inosine (10 mM) and adenine (5 mM), as substrate. Ouabain was present at a final concentration of 0·1 mM and potassium not less than 10 mM. Net changes in cell sodium and potassium are shown as + = cell gain and − = cell loss. Each value in the Table represents the mean of triplicate incubations.

increase in passive sodium efflux was only two- to threefold in contrast with the tenfold increase in potassium efflux under similar low chloride conditions. On the other hand, with low external sodium, there was an increase in sodium efflux to a value comparable to that for potassium

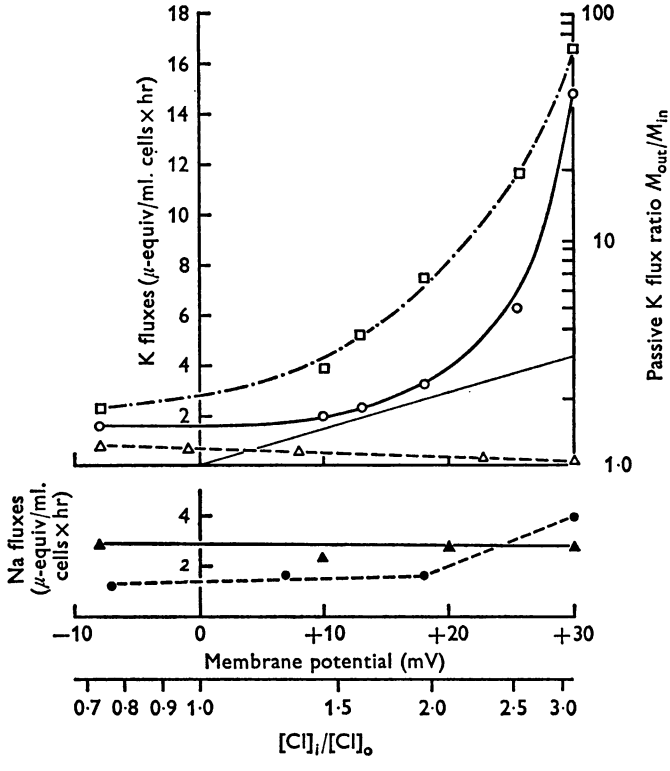

Fig. 5. Dependence of sodium and potassium fluxes on membrane potential calculated from the chloride distribution ratio when EDTA was substituted for chloride. Open triangles (△) potassium efflux; open circles (○) potassium influx in the presence of ouabain; filled circles (●) sodium influx at an external sodium concentration of 150 mM, achieved by partial replacement of sodium EDTA with potassium EDTA; filled triangles (▲) ouabain-insensitive sodium efflux; open squares (□) and right hand ordinate show passive K flux ratio (efflux/influx). Continuous straight line shows slope of a tenfold change in flux ratio per tenfold change in chloride ratio (upper graph).

efflux. As regards sodium influx, neither the sign nor magnitude of the p.d. over the range -10 to $+30$ mV apparently had any effect. The results suggest that sodium and potassium movements independent of the sodium pump depend on the sign and magnitude of the membrane potential.

DISCUSSION

The distribution of chloride

The first point to consider is the fact that a reversed chloride gradient can be maintained between red cells and an external medium containing impermeant anions instead of chloride. The chloride distribution ratio, $[Cl]_i/[Cl]_o$, could be varied from the normal value of 0·7 to between 3·0 and 4·0 as external chloride was replaced with anions such as EDTA, citrate or gluconate. Previous work has shown a reversed chloride gradient under conditions in which external chloride was replaced by non-penetrating non-electrolytes (LaCelle & Rothstein, 1966; Donlon & Rothstein, 1969), or impermeant electrolytes such as mucate or toluene-disulphonate (Vidaver, 1964*a*). Under low chloride conditions the chloride distribution depended on external pH as found previously (Harris & Maizels, 1952; Funder & Wieth, 1966), and the hydrogen ion distribution changed as the inverse of the chloride distribution so that the cell interior was at higher pH than under the normal chloride conditions (Table 2).

Since the Results show that chloride is passively distributed under a variety of conditions, it seems justified to calculate the membrane p.d. from the chloride distribution ratio and values were in the range of -9 to $+30$ mV. The direct measurement of membrane p.d. in red cells is technically difficult (Lassen & Sten-Knudsen, 1968; Jay & Burton, 1969) but the values are, nevertheless, in agreement with those predicted from the chloride distribution for cells under physiological conditions.

Sodium and potassium movements in cells with a reversed chloride gradient

The main aspect of the results relates to the effect of a reversed chloride gradient across the membrane on sodium and potassium fluxes, both active and passive. When account is taken of the technical considerations mentioned in the Results section, there seems to be no appreciable effect of a change in chloride gradient, corresponding to an alteration in membrane p.d. from -9 to $+30$ mV, on ion movements of the sodium pump or on the ratio of sodium transported/potassium transported. This is consistent with the finding that the ratio remains constant when the sodium pump is made to function at different rates by changing internal sodium and external potassium concentrations, and therefore the electrochemical gradient (Whittam & Ager, 1965). Another point is that the energy requirement for sodium and potassium transport by the sodium pump is decreased from 8·4 kcal in chloride to 3·6 kcal in EDTA, so that energetically, conditions are more favourable for the sodium pump. Attempts were not made to make the chloride gradient even bigger because this would have been

accompanied by high internal pH, which in itself would have led to decreased activity of the sodium pump. There have been few other studies on the relation between sodium pump activity and membrane potential and the conclusion from the present experiments would not appear to apply to frog striated muscle in which pump activity was decreased as the inside of the fibre became positive (Horowicz, Gage & Eisenberg, 1968).

Ion movements independent of the sodium pump were asymmetrically affected on reversing the chloride gradient, for there was an increase in efflux but not influx. Thus, sodium influx depended only on the external sodium concentration and was independent of the nature of the external anion. In contrast, sodium efflux was markedly increased in two noteworthy ways. In the first place, with constant external sodium concentration the efflux was greater with EDTA than with chloride as the predominant external anion. The energy requirement for the outward movement of 1 mole of sodium is decreased from approximately +1·7 kcal with chloride to −1·6 kcal/with EDTA according to the following equation:

$$\Delta G = RT \ln \frac{[Na]_i}{[Na]_o} + RT \ln \frac{[Cl]_o}{[Cl]_i}.$$

These values show that conditions are favourable for efflux being greater than influx, so that internal sodium can approach its equilibrium level, when

$$\frac{[Na]_i}{[Na]_o} = \frac{[Cl]_o}{[Cl]_i}.$$

The other feature is that a decrease of external sodium on replacement with potassium caused an increase in efflux with EDTA, but a decrease with chloride. A similar decrease has been previously found when magnesium was substituted for sodium (Hoffman, 1966; Lubowitz & Whittam, 1969). It follows that the reason for the large increase with EDTA does not arise because of replacement of sodium with potassium, but because of replacement of chloride with EDTA and the consequential reversal of membrane potential. This means that sodium efflux is raised on lowering external sodium for two reasons: first, reversing the membrane potential and, secondly, lowering and reversing the sodium concentration gradients. These two factors make up the driving force (or electrochemical potential gradient) which thus appears to determine the outward movement of sodium. In agreement with the unidirectional fluxes a change has been demonstrated in the net sodium movement according to the external anion. It can be concluded therefore that with the same external sodium concentration reversal of the chloride gradient brings about an increase in sodium efflux.

The potassium permeability was also changed in an asymmetrical manner, and as with sodium, the most conspicuous feature was an increase in efflux. The independence relation of Ussing (1949) does not seem to apply, as previously concluded by Funder & Wieth (1967) from a consideration of potassium fluxes in cells in normal Ringer. Moreover, it is clear from Fig. 5 that the fluxes were not altered only because of changing the electrical gradient across the cell membrane. The non-linear dependence of flux ratio on p.d. is perhaps most simply interpreted in terms of a preferential increase in outward permeability. A somewhat similar phenomenon, but in the opposite direction, is the rectification of potassium conductance in depolarized frog muscle fibres (Katz, 1949; Adrian, 1969) when inward current is raised more than outward.

Normal cell permeability could be restored by returning cells to chloride medium, showing that the foreign anions used to alter the chloride gradient did not irreparably damage the cells. Similar permeability changes were induced by both gluconate and the calcium-chelating anions (EDTA, citrate), and appreciable changes in sodium and potassium fluxes on partial replacement of chloride with citrate or EDTA did not occur until the concentrations of these ions were at least 40 mM. Calcium chelating effects of these two anions are presumably more than saturated at this high concentration.

It is evident that the membrane potential is one factor which determines membrane permeability. Another factor is pH, which affects permeability independently of changes which would arise from chloride redistribution. Thus, if potassium efflux had simply depended on chloride distribution then it would have decreased with increasing external pH, whereas it was, in fact, raised. Passow (1969) has considered the mechanism for the control of permeability by pH in terms of a lattice of fixed charges in a membrane. It is not clear, however, how a lattice model of a simple kind can account for spatially asymmetrical changes in permeability of the kind described above.

The net potassium loss and small sodium gain found in the present work is in line with past work on the relation between potassium loss in non-electrolyte media and membrane p.d. (Wilbrandt & Schatzmann, 1960; LaCelle & Rothstein, 1966; Donlon & Rothstein, 1969), although the effects with impermeant polyvalent anions are not as dramatic as those in very low ionic strength sucrose media. Another aspect of the influence of the potential is Vidaver's (1964*b*) demonstration that a reversed chloride gradient between pigeon red cell ghosts and Ringer was able to induce sodium-linked glycine movement when the sodium concentrations were equal on both sides of the membrane. When external chloride is replaced by foreign monovalent anions to which the membrane is permeable, cells

also lose potassium, but in contrast, gain more sodium and consequently swell (Funder & Wieth, 1967; Wieth, 1970).

The changes in membrane permeability induced by reversing the membrane potential are in contrast with those which accompany a raised internal calcium (Romero & Whittam, 1971), because calcium acts predominantly on potassium permeability by increasing potassium movements both inwards and outwards, such that a net potassium loss could be converted into a net potassium gain by raising the external potassium concentration. The general conclusion is that membrane potential in human red cells controls passive sodium and potassium efflux with only little or no effects on influx.

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