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CATION LOADING OF RED BLOOD CELLS

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SUMMARY

1. *p*-Chloromercuribenzenesulphonate induces an increase in the permeability of red cell membranes to monovalent cations. Under suitable conditions the increase in permeability is large enough to allow cell cation concentration to approach equilibrium with that in the suspending media in 24 hr at 5° C.

2. The effect of the mercurial on permeability is fully reversed by cysteine.

3. These effects provide the basis for a simple procedure for preparing red blood cells of almost any desired sodium or potassium content.

4. If hypertonic media are used, cells can be obtained with normal volume but in osmotic equilibrium with the hypertonic solutions.

INTRODUCTION

It has been shown that the slowly penetrating (VanSteveninck, Weed & Rothstein, 1965) organic mercurial *p*-chloromercuribenzenesulphonate (PCMBS) has two major effects on monovalent alkali metal ion fluxes of red blood cells: passive permeability is increased (Sutherland, Rothstein & Weed, 1967) and active transport is inhibited (Rega, Rothstein & Weed, 1967). Both effects are associated with the combination of PCMBS with cell membrane sulphydryl groups, whereas PCMBS bound within the cell does not affect cation movements (Sutherland *et al.* 1967; Rega *et al.* 1967). The mentioned actions of PCMBS can be rapidly reversed by cysteine (Sutherland *et al.* 1967; Rega *et al.* 1967). Uncontrolled increases in membrane permeability induced by the mercurial may also produce irreversible changes in cell integrity such as haemolysis (Sutherland *et al.* 1967). Available data (Sutherland *et al.* 1967) indicate that three main parameters govern the extent of PCMBS interaction with red blood cells: (i) amount of mercurial per cell, (ii) duration of exposure, and (iii) temperature. This information suggested that under appropriate experimental

conditions large cation shifts might be obtained in a short time with little cell loss.

The detailed study of cation movements in red cells often requires that their internal ionic composition be changed. Procedures commonly in use are cumbersome and time consuming. For this reason, we thought it worth while to study the extent and reversibility of PCMBS action with the aim of developing a simple and reliable method for varying the ionic composition of red blood cells.

METHODS

Fresh human blood from haematologically normal adults was used in all experiments. The blood was centrifuged at 1750 *g* for 5 min and the plasma and buffy coat removed. The remaining red cells were washed 4 times with about ten volumes of isotonic salt solutions, packed at 1750 *g* for 30 min and submitted to the following procedure.

Step 1, *cation loading*. The washed and packed red cells were suspended in a solution containing (mM): PCMBS (p-chloromercuriphenyl sulphonic acid, Sigma Chem. Co.) 0·1 or 0·5, XCl (X stands for Na, K or their mixture) 150, $MgCl_2$ 1·0, XH_2PO_4-X_2HPO_4 (pH 7·4) 2·5, to give a final haematocrit of 5%. Phosphate buffer can be replaced by 5 mM-Tris-HCl. The suspension was stored at about 5° C for 20 hr when using 0·1 mM PCMBS and for 14 hr when using 0·5 mM PCMBS. Cells in 0·5 mM PCMBS were re-exposed under similar conditions in fresh medium for a further 10 hr. Low temperature during the exposure to mercurial helps to preserve cell integrity and diminishes the rate of transference into the cell of PCMBS bound to the membrane (Sutherland *et al.* 1967). Thus, the binding sites in the membrane can be kept almost saturated throughout the period of exposure. Renewal of suspending medium prevents accumulation of the cation leaking from the cells as well as binding of extracellular PCMBS by sulphydryl compounds (chiefly haemoglobin) released by the cells. At completion of the loading period, cells were packed by centrifuging at low temperature. At this stage, cell permeability remains altered and has to be restored.

Step 2, *sealing*. The loaded but leaky cells were suspended in a PCMBS-free solution having the salts of the medium in which the previous incubation was performed together with 11 mM glucose and 2 mM cysteine (L-cysteine hydrochloride, British Drug Houses Ltd.) for cells loaded in 0·1 mM PCMBS, or 12 mM cysteine for 0·5 mM PCMBS. The suspension was incubated for 0·5 hr for 0·1 mM PCMBS or for 4 hr for 0·5 mM PCMBS with intermittent shaking. The temperature was kept at 37° C in order to increase the rate of desorption of mercurial from the cells. At the end of the incubation, the cells were packed by centrifuging at low temperature and were washed 3 times with ten volumes of ice-cold 150 mM choline chloride, 5 mM Tris-HCl, (pH 7·4) solution.

Relative cell volume was measured by the microhaematocrit technique. Haemoglobin was estimated as oxyhaemoglobin from absorbance measurements at 541 mμ on suitable dilutions of haemolysates.

Cell sodium and potassium were measured by flame photometry after the cells had been washed 3 times with ten volumes of the ice-cold choline solution.

RESULTS

Table 1 shows that alteration in membrane permeability elicited by PCMBS during the loading step is large enough to allow the intracellular composition of the red cells to approach that of the external medium in a short time. This effect can be accomplished to a large extent using 0·1 mM PCMBS. Under these conditions, a residual amount of sodium (up to

4·1 m-moles/l. original cells) or potassium (up to 13·6 m-moles/l. original cells) remained in the cells. The remaining cation was not lost even after lengthening of the exposure time by 4 hr and/or renewal of the suspending medium after 14 hr of incubation. Gentle stirring during the incubation seemed to improve the results only slightly.

TABLE 1. Effects of PCMBS-containing solutions on cation composition of red blood cells

Suspending medium (mM)			Incubation time	Cation content (m-moles/l. original cells)		Total haemolysis
NaCl	KCl	PCMBS	(hr)	Na	K	(%)
Fresh cells			—	8·4–9·6	98–104	—
150	—	0·1	20	92–103	11·5–12·6	1·4–1·7
150*	—	0·1	20	116–124	9·8–13·6	2·9–3·9
—	150	0·1	20	2·5–4·1	113–133	1·8–2·9
—	150*	0·1	20	2·8–2·8	126–141	4·2–4·5
75	75	0·1	20	65–68	71–73	4·1–4·0
150	—	0·5	24	124–126	2·4–2·5	12·8–25·6
—	150	0·5	24	1·7–1·9	128–134	18·7

Figures of cation content after loading are ranges of two experiments. All suspending media were buffered with 5 mM Tris-HCl (pH 7·4). (*) Cell suspensions were slowly rotated to provide stirring.

A fivefold increase in PCMBS concentration, together with renewal of the suspending medium, resulted in an almost complete equilibration of intracellular cations with those in the external medium.

The results obtained with 75 mM-NaCl and 75 mM-KCl (Table 1) suggest that the procedure allows almost any variation of intracellular sodium and potassium content to be obtained merely by adjusting the salt composition of the suspending medium.

Cation contents in Table 1 are referred to the haemoglobin content of 1 l. of fresh cells and are therefore over-estimated to an extent proportional to the change in volume that results from incubation. This way of expressing the data, however, allows us to estimate the fractional increase in cell water, provided we make the reasonable assumption that the cell content of osmotically active material is proportional to the sum of sodium and potassium in the cells. The fractional increase in cell water calculated on this basis ranged from 1·1 to 1·3 with 0·1 mM PCMBS and from 1·3 to 1·4 with 0·5 mM PCMBS.

Using 0·1 mM PCMBS, only 2–5% of the cells were lost through haemolysis while with 0·5 mM PCMBS the loss ranged from 15 to 25% of the original cells. This shows that even at the higher PCMBS concentration the procedure gives a recovery of at least 75% of the original cells. It has been demonstrated previously (Jacob & Jandl, 1962) that haemolysis induced by organic mercurials is 'colloid osmotic' in nature.

As mentioned before, change in cell membrane properties induced by PCMBS can be reversed by cysteine. Since all the studies in this respect (Sutherland *et al.* 1967; Rega *et al.* 1967) were carried out using lower PCMBS concentrations for shorter times, it seemed necessary to check

whether cysteine was still effective under our experimental conditions. Three criteria were used to test the effectiveness of cysteine: (i) restoration of normal passive permeability, (ii) reappearance of active transport at more or less normal rates, and (iii) ability of the cells to sustain prolonged incubations at 37° C without undue haemolysis. To test these points, Na-loaded and sealed cells were suspended at 37° C in Na-free media with and without ouabain. As some 'slowly desorbable' PCMBS presumably remains bound by the large reservoir of haemoglobin (VanSteveninck *et al.* 1965; Weed, Eber & Rothstein, 1962), cysteine was also present in the final suspension media. The use of Na-free external media and low haematocrit during experiments of cation movements allowed us to estimate the sodium efflux by measuring the changes with time in internal sodium concentration. This procedure is acceptable, since it has been shown that removal of external sodium does not affect the rate of either the sodium 'pump' or 'leak' in human red cells under these conditions (Garrahan & Glynn, 1965). The final incubation media contained either 155 or 15 mM potassium (choline chloride making up the tonicity). Only in the first case was an estimate of potassium 'leak' possible.

TABLE 2. Cation movements in loaded red blood cells

Loading conditions	Sealing conditions: Time (hr)	Sealing conditions: Cysteine (mM)	Cation movements: Ouabain-sensitive (m-moles/l.orig.cells/hr)	Cation movements: Ouabain-insensitive (m-moles/l.orig.cells/hr)	% haemolysis (in 3 hr)
'Na Ringer', 0·1 mM PCMBS	0·5	2	Na efflux 3·32 K influx 2·01	1·54 3·13	1·3
'Na Ringer', 0·5 mM PCMBS	2·0	4	Na efflux 1·77 K influx 1·56	1·56 —	1·5
'Na Ringer', 0·5 mM PCMBS	4·0	4	K influx 2·46	—	1·2
'Na Ringer', 0·5 mM PCMBS	4·0	12	K influx 2·79	—	1·2
'Na-K Ringer', 0·5 mM PCMBS	4·0	12	Na efflux 4·12	1·0	0·9
3-week-old cold-stored cells*	—	—	Na efflux 4·13	0·94	—

'Na Ringer' contained (mM): NaCl 150, $MgCl_2$ 1, NaH_2PO_4-Na_2HPO_4 2·5 (pH 7·4). In 'Na-K Ringer', 100 mM-KCl replaced an equivalent quantity of NaCl. For measuring cation movements, cells were suspended in (mM): choline chloride 140, KCl 10, $MgCl_2$ 1, KH_2PO_4-K_2HPO_4 2·5, glucose 11, cysteine as in the corresponding 'sealing' media, (pH 7·4), with the exception of the first experiment in which choline chloride was replaced by KCl. For the same loading conditions initial cell composition was similar to that given in Table 1. Cells loaded in 'Na-K Ringer' contained 60 m-moles Na and 97 m-moles K/l. of original cells. Figures for fluxes are averages of two successive 1·5 hr incubations. Haemolysis during sealing incubation was always less than 4%. (*) Cold-stored cells prepared according to Post & Jolly (1957) containing 55 m-mole Na/l. original cells. Na efflux was measured with ^{24}Na. Incubation medium was (mM): NaCl 140, KCl 10, NaH_2PO_4-Na_2HPO_4 2·5, inosine 10, (pH 7·4) (P. J. Garrahan & I. M. Glynn, unpublished).

The figures for ouabain insensitive cation movements in Table 2 show that the cells regained their normal passive permeability to Na^+ and K^+ after the sealing step. The return to normal of the passive membrane properties was also reflected in the very low rates of haemolysis during the sealing and final incubations. Haemolysis was mainly an initial phenomenon associated with mixing and warming, its subsequent rate being much lower than the figures imply.

As can be seen in Table 2, after adequate incubation in cysteine, PCMBS-treated cells pumped Na^+ and K^+ at a rate that is comparable to that of cells loaded with sodium by cold storage. Active movements found after sealing with 12 mM cysteine were also similar to those obtained by Whittam & Ager (1965) with 'lactose treated' cells. The results therefore extend previous findings on reversibility by cysteine of organic mercurial effects on active transport (Rega *et al.* 1967) and on the (Na + K)-dependent ATPase activity (Skou & Hilberg, 1965). Although passive permeability in cells treated with 0·5 mM PCMBS showed complete recovery after 2 hr in 4 mM cysteine, ouabain-sensitive fluxes failed to do so. This is in agreement with previous findings demonstrating that restoration of active transport requires a longer time than restoration of passive membrane properties (Rega *et al.* 1967). Lengthening the sealing step to 4 hr resulted in an almost 2-fold enhancement of ouabain-sensitive fluxes. Further increases in time had no observable effects. Raising cysteine concentration from 4 to 12 mM slightly increased the rate of pumping as could be seen in paired experiments.

Preparation of 'hypertonic' cells. Red blood cells placed in hypertonic salt solutions lose part of their water and shrink. Owing to their low permeability to cations, shrunken cells will recover their volume only after a long time. It can be predicted that if cells are made leaky with PCMBS, volume recovery will take place in a much shorter time. If membranes are 'sealed' with cysteine after the original volume is reached, normal-sized cells in osmotic equilibrium with the hypertonic medium will be obtained. Since such cells would permit analysis of cell behaviour over a more extended range of ionic concentrations, the feasibility of their preparation was studied experimentally.

Cells were loaded by a single 20 hr incubation in a 0·5 mM PCMBS, 250 mM-NaCl solution buffered to pH 7·4 with 5 mM Tris-HCl. Cell sealing was performed as usual. After sealing, cation contents were 161 m-moles sodium and 3·3 m-moles potassium/l. of cells (referring to the actual cell volume). Assuming that the cation concentration in cell water is 250 mM, the measured values give a normal water content (about 66 %). Recovery in cell volume was also confirmed by comparing the haemoglobin content per litre of cells before and after the treatment.

'Hypertonic' cells showed normal resistance to handling. This was evident from the low rate of haemolysis after 4 hr incubation with intermittent shaking at 37° C in an isosmolar medium.

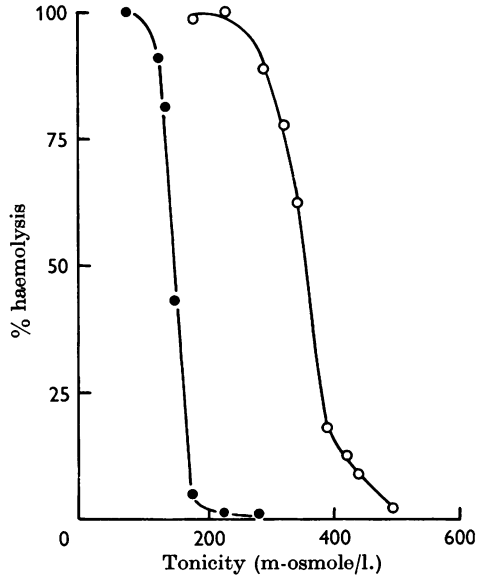

Fig. 1. Osmotic fragility of cells before (●—●) and after (○—○) 'loading' in 250 mM-NaCl solution. Haemolysis was measured after 15 min incubation at 37° C in choline chloride solutions buffered with 5 mM Tris-HCl (pH 7·4).

Osmotic fragility curves plotted in Fig. 1 show a 200 m-osmolar shift of the 50 % haemolysis point for 'hypertonic' cells. This osmotic response is to be expected from cells with normal volume but in osmotic equilibrium with a 250 mM NaCl solution. The curve for 'hypertonic' cells is symmetrical. Broadening of the curve suggests that, after treatment, the distribution of cell volume is wider than normal.

DISCUSSION

The simple procedure presented here allows controllable changes in the cation content of red blood cells even into normally hypertonic concentrations. Since the procedure involves only a short period of cold storage followed by an incubation at 37° C, it seems safe to conclude that, provided PCMBS effects are fully reversed, cells so treated cannot be very different in their metabolic state from fresh cells.

The validity of the technique rests upon the reversibility of PCMBS effects and the lack of action of cysteine on membrane properties. Both postulates have been proved. Lack of effects of up to 10 mM cysteine on (Na + K) − ATPase has been demonstrated by Skou & Hilberg (1965).

Although all organic mercurials so far tested have similar effects on membrane properties, PCMBS was selected because its low rate of penetration (VanSteveninck *et al.* 1965) allows maximal saturation of membrane sites. From previous data on PCMBS interaction with red blood cells (Sutherland *et al.* 1967; Rega *et al.* 1967), it can be calculated that at most, 1·5 m-moles Hg/l. cells are bound to the cells at the end of the loading stage using 0·5 mM PCMBS. After the 'sealing' incubation, not more than 0·35 m-moles Hg/l. cells remain within the cells. Both values are almost certainly gross over-estimates because they were calculated assuming linearity between binding and mercury concentration. Anyway, 0·35 m-moles/l. cells represents only 3% of PCMBS-titratable red blood cells sulphydryl groups (VanSteveninck *et al.* 1965).

The glycolytic system is another possible site of mercury action. Red cell glycolysis is not inhibited by *p*-chloromercuribenzoate (Jacob & Jandl, 1962) which penetrates more rapidly than PCMBS and has the same reactivity with red blood cells sulphydryl groups (VanSteveninck *et al.* 1965)

In simplicity the method compares favourably with 'lactose treatment' (McConaghey & Maizels, 1962) which induces similar cationic changes (at least in the isotonic range) in a short time. Simplicity in preparation can become a very important consideration, for instance, when effects of changes in internal cation composition have to be studied on the same batch of cells. Moreover, although it is possible in theory, no data exist about the preparation of 'hypertonic' cells by the lactose method. In yield and stability, cells prepared by the proposed procedure seem to be more satisfactory than 'lactose treated' cells: haemolysis during cation loading and final incubation is less (McConaghey & Maizels, 1962).

Volume changes and subsequent haemolysis during treatment with organic mercurials can be avoided using adequate amounts of non-penetrating solutes such as sucrose (Jacob & Jandl, 1962; P. J. Garrahan, V. L. Lew & A. F. Rega, unpublished).

Although reversible haemolysis (Hoffman, Tosteson & Whittam, 1960; Hoffman, 1962) provides a procedure for changing the cationic content of red blood cells, its usefulness in detailed studies on the kinetics of Na^+ or K^+ movements is limited by: (i) the abnormally large passive permeability to K^+, and (ii) the presence of a large number of unsealed ghosts (Hoffman, 1962).

Only some of the potentialities of the proposed procedure have been analysed. There appears to be no reason why the method should not be extended for loading cells with other monovalent cations such as rubidium (Rega *et al.* 1967), caesium, and lithium.

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