

VOLUME CHANGES, ION EXCHANGES, AND FRAGILITIES OF
HUMAN RED CELLS IN SOLUTIONS OF THE CHLORIDES
OF THE ALKALINE EARTHS*

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While there is an extensive literature on monovalent and divalent ion antagonism, the possibility of the salts of divalent ions forming isotonic or isoplethochontic (Ponder and Saslow, 1931) solutions for mammalian red cells does not seem to have been considered. This investigation will show that the chlorides of the alkaline earths are, in certain concentrations and for limited times, isotonic or volume-maintaining, that decreases in red cell volume occur in hypertonic solutions and increases in volume in hypotonic solutions, and that the water exchanges which correspond to the volume changes are accompanied by ion exchanges in which K leaves the red cells while a divalent ion enters. The ion exchanges to be described here are all in the direction of the diffusion gradients, and are sometimes very complex.

Methods

The red cells of heparinized human blood are washed 3 times with 1 per cent NaCl (0.172 M) and suspended in the same medium to give a volume concentration of 0.4. Recrystallized specimens of CaCl₂, BaCl₂, MgCl₂, and SrCl₂ are dissolved in water to give concentrations of 0.172 M, and these are diluted to give a series of solutions of lower concentration, usually 1.0, 0.8, 0.7, 0.6, 0.5, and 0.4 times 0.172 M.

To 10 ml. of each of these solutions, cooled to 4°C., is added 0.2 ml. of the washed red cell suspension. Samples of each system are withdrawn at once for the measurement of the volume V occupied by the cells and of the percentage p of complete hemolysis. The volume measurements are carried out in Hamburger hematocrit tubes spun in a high speed ($2 \times 10^4 G$) centrifuge for 30 minutes, as already described (Ponder, 1950). Each volume is then expressed as V/V_0 , V_0 being the volume of the same number of cells suspended in 10 ml. of 1 per cent NaCl and measured under the same conditions. The fractional increase in volume of the average intact cell is $V/V_0(1 - p)$. To follow the changes in V/V_0 and p with time, the determinations are repeated at intervals during 24 hours, and the systems are kept at 4°C. during this period.

The values of $V/V_0(1 - p)$ are plotted against the concentration of CaCl₂, BaCl₂, etc., and one concentration is found by interpolation at which $V = V_0$ (p being zero).

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This is the isotonic concentration of the salt, or, more strictly, the concentration at which the volume is the same as it is in isotonic NaCl under the particular conditions of time and temperature at which the measurements are made. Calling the tonicity of this concentration $T = 1.0$, the tonicities of the other concentrations of the salt, some greater than tonicity 1.0 and some less, are calculated. Values of $V/V_0(1 - p)$ are now plotted against $1/T$. If water transfer alone were involved and if the van't Hoff-Mariotte law were to apply without modification, the result would be a straight line passing through the origin $T = 1.0$, $V/V_0 = 1.0$, with a slope of W , W being the fraction of the cell occupied by water.

The ion exchanges accompanying the water transfer were measured by determining the K content of the cells and of the surrounding medium with a Perkin-Elmer flame photometer as already described (Ponder, 1947). Entry of Ba, Mg, Sr, and Ca into the cells was measured with a Coleman flame photometer.

1. Tonicity-Volume Relations

If the volume of the red cells is measured as quickly as the hematocrit method allows, *i.e.* within 30 minutes of their being added to solutions of the chlorides of the alkaline earths, a concentration of each salt can be found in which, at this time, the cell volume is the same as it is in 1 per cent NaCl. This concentration will be called isotonic; in more concentrated solutions the cells shrink, and in less concentrated ones they swell. Four things can be learned from the relation between $V/V_0(1 - p)$ and $1/T$, as plotted in Figs. 1 and 2: (1) the isotonic concentration of the salt, as measured with as little delay as possible, (2) whether the relation is linear, in whole or in part, (3) the slope of the line or the slopes of its linear parts, and (4) the extent to which the tonicity-volume relation changes with time. The slopes have to be expressed as RW , because they are very rarely equal to W , the fraction of the cell volume occupied by water, as they would be if water exchange alone were involved and if the cell were a "perfect osmometer" in other respects.

Isotonic Concentrations.—The upper of the pairs (three in the case of CaCl_2) of curves shown in Figs. 1 and 2 is the relation between $V/V_0(1 - p)$ and $1/T$, the measurements being made within 30 minutes after the cells were added to the salts of the divalent cations. The relation is the simplest in the case of BaCl_2 and most complex in the case of CaCl_2 , the results in MgCl_2 and SrCl_2 being intermediate in complexity. In each case, it is possible to pick a concentration in which the red cells have the same volume as in 1 per cent NaCl when the measurements are made under the same conditions; these isotonic concentrations are 0.105, 0.110, 0.130, and 0.10 m for BaCl_2 , MgCl_2 , SrCl_2 , and CaCl_2 respectively. On the basis of a relation between the depression of freezing point of the substances and their isotonic concentrations, one would expect 0.123, 0.120, 0.117, and 0.121 m (the isotonic concentration of NaCl being 0.172 m); the method is not sufficiently accurate, however, to be able to detect the dif-

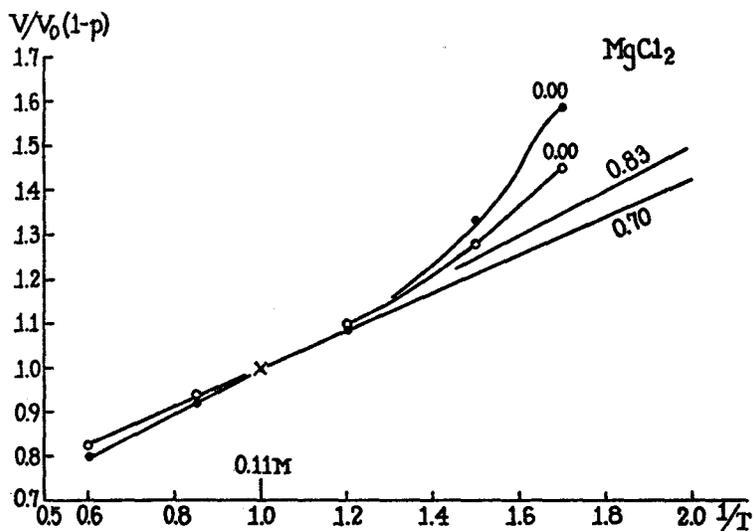
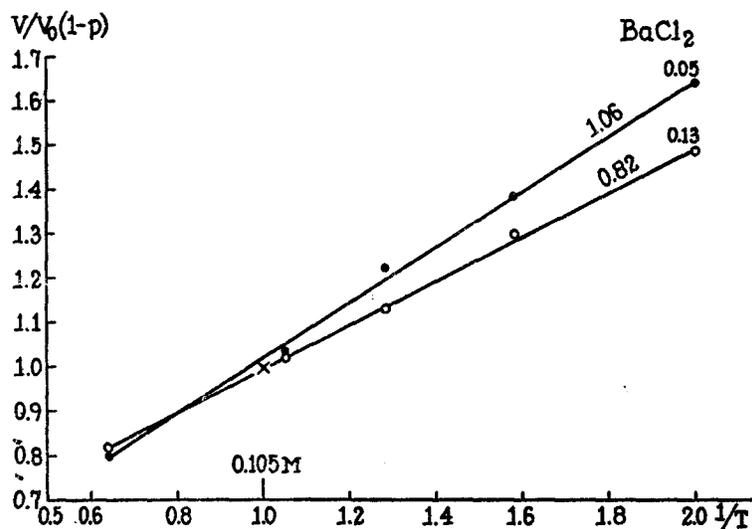


FIG. 1. Tonicity-volume relations for BaCl₂ and MgCl₂. Ordinates, red cell volume relative to volume in an isotonic concentration (marked on the abscissa); abscissae, reciprocal of tonicity. Upper curves (dots), results obtained immediately after adding the cells to the solutions; lower curves (open circles), results obtained after 24 hours at 4°C. The figures opposite the linear part of each curve are the value of R ; the figures opposite individual points give p , the fractional amount of hemolysis.

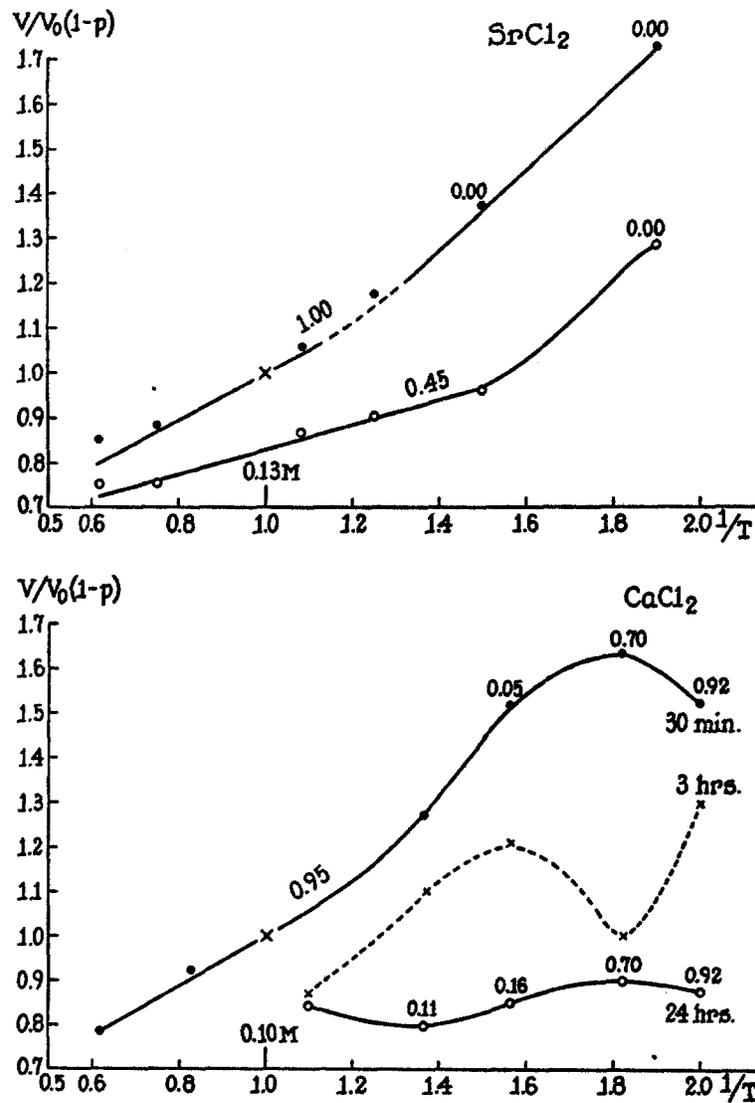


FIG. 2. Same as Fig. 1, but for SrCl_2 and CaCl_2 . A dotted curve, giving the results obtained after 3 hours at 4°C ., is included for CaCl_2 .

ference between the values "expected" and the values found experimentally, particularly as the latter are functions of time (see below).¹

¹ The solutions of the chlorides of the alkaline earths show some pH differences which, ideally, ought to be taken into consideration. In 0.1 M concentration, the pH's are: BaCl_2 , 5.0, MgCl_2 , 5.0, SrCl_2 , 5.5, and CaCl_2 , 7.8.

Form of the Tonicity-Volume Relation.—When the volumes are measured as soon as possible after the addition of the cells to the solutions of the chlorides of the divalent cations, the tonicity-volume relation between the tonicities 1.6 and 0.75 can be reasonably well represented by a straight line through the point $V/V_0 = 1$, $1/T = 1$. The slope RW of this line varies, and if W is taken as 0.6, R varies between 0.83 and 1.06; *i.e.*, it is sufficiently close to 1.0 to justify the statement that the red cell behaves, in these solutions and under these conditions, as an osmometer in which water transfer alone is involved. In tonicities lower than about 0.75, however, the tonicity-volume relation shows an upward departure from the linear relation; this departure is least marked in BaCl_2 , quite appreciable in MgCl_2 , and very marked in SrCl_2 and in CaCl_2 .

A similar upward departure from linearity has been described (Ponder, 1950) in NaCl systems sufficiently hypotonic to produce lysis varying between just commencing hemolysis and about 50 per cent hemolysis ($p = >0.00$ to 0.50). Attempts have been made to account for this upward departure on the grounds that the ghosts of the hemolyzed red cells have some rigidity and therefore occupy volume in the hematocrit column of packed red cells. In the case of systems containing hypotonic NaCl , the comparison of volume measurements made by diffraction with those made with the hematocrit supports this idea (Ponder, 1951 *b*), but the upward departure occurs *in the absence of hemolysis* in hypotonic systems containing MgCl_2 and SrCl_2 . In the case of systems containing these two salts, the properties of the ghosts cannot be used to explain the upward departure from linearity, and there is no escaping the conclusion that the volume of the intact red cells can become greater than that predicted by the van't Hoff-Mariotte law.

Change in the Tonicity-Volume Relation with Time.—The volume changes described above are largely, if not wholly, due to the rapid movement of water. They are not maintained, however, when the cells are allowed to stand at 4°C. in the solutions of the chlorides of the divalent cations. The initial entry or exit of water is followed by a slower movement of ions, intracellular K (and a much smaller amount of Na) being lost from the cells while the cation from the surrounding fluid enters. The rate of exchange varies with the divalent cation and with its concentration, so that the tonicity-volume relations may change with time in a very complex way; *e.g.*, in the case of CaCl_2 , for which three tonicity-volume relations are shown in Fig. 2, one immediately after the cells are added to CaCl_2 , one 3 hours later, and one 24 hours later, measurements made at other time intervals giving curves intermediate in form.

In all cases, the change in the tonicity-volume relation with time results in R becoming smaller. In BaCl_2 , R decreases from a little more than 1.0 to about 0.8, and in MgCl_2 the decrease is from 0.8 to about 0.7. The relation is substantially linear in the case of both these salts, and there is no hemolysis on standing for 24 hours at 4°C. In solutions of SrCl_2 the tonicity-volume is not good

to begin with, and after 24 hours R has fallen from about 1.0 to about 0.5; the upward departure from linearity is as pronounced after 24 hours as it was initially, and cannot be accounted for by hemolysis, which does not occur. Finally, in CaCl_2 the value of R after 24 hours is virtually zero; *i.e.*, the volume of the intact cells is substantially the same in all tonicities. This final state is reached in a complex way, as is shown by the shape of the curve for observations made 3 hours after the addition of the cells to the CaCl_2 ; Schreinemakers (1938) has discussed cases of this kind.

Apart from the difficulty in accounting for them on the basis of the van't Hoff-Mariotte law, the tonicity-volume relations in solutions of BaCl_2 , MgCl_2 , SrCl_2 , and CaCl_2 of varying tonicities provide new objections to the "dual mechanism" (Davson, 1936; Davson and Danielli, 1938; Davson and Ponder, 1938, 1940) or "colloid-osmotic" (Wilbrandt, 1941) theory of hemolysis, either in its original or in its restated form (Ponder, 1951 *a*). This theory states that permeability of the red cell to cations must result in the movement of water into the cell and in an increase in volume (with eventual hemolysis when the volume reaches the "critical" volume) because of the colloidal osmotic pressure of the material within the cell. The volume changes in systems which contain lysins, and in which the ion exchange of K for Na is rapid (Ponder, 1948), are not such as would be suggested by this theory unless subsidiary assumptions, involving arbitrary constants, are introduced, nor are the volume changes which accompany the slower K-Na exchange which occurs in isotonic systems containing no lytic material (Ponder, 1951 *a*); in these however, the change is at least in the direction of an increase. In systems containing the salts of the alkaline earths in various tonicities, the changes in volume with time are almost all in the direction of volume decreases, and there is no evidence of effects of colloidal osmotic pressure although the cells are sufficiently permeable to cations to allow large exchanges to occur. On the contrary, the SrCl_2 and CaCl_2 data suggest that, as the cation exchange becomes more and more complete, the final state is one of equilibrium with the volume of the cell about the same as it was initially.

2. Ion Exchanges

Table I shows the amount of exchange of internal K (lost) for various external cations (gained) at the end of 24 hours at 4°C., the external solutions being isotonic in all cases. The losses are expressed as a percentage of the initial concentration of cell K, and the gains as a percentage of the isotonic concentration of the external salt.

The exchanges in isotonic solutions of the salts of the divalent cations are greater than the K-Na exchange which occurs when the cells are suspended in isotonic NaCl, but, as in the K-Na exchange, the amount of K lost and the amount of the external cation gained are approximately equal. The exchange

is most rapid in CaCl_2 , and, as has already been remarked, the volume of the intact cells is slightly less at the end of 24 hours than the initial volume (see Fig. 2). These exchanges are all in the direction of the concentration gradients, but an interesting point is raised by the observations (Solomon, 1952) which suggest that carrier systems are involved when K leaves the cell to exchange with Na, even although both ions are moving with their concentration gradients. If this is so, carrier systems may be involved in the entrance of Ba, Mg, Sr, and Ca.

3. Effects on Fragility

Even when human red cells have been exposed to isotonic solutions of the chlorides of the alkaline earths for times long enough to allow of at least 50 per cent of the intracellular K to exchange for the external cation, most of the effects on osmotic, heat, and mechanical fragilities are small. Only two are

TABLE I

External cation	Loss of K	Gain of external cation
	<i>per cent</i>	<i>per cent</i>
Ba	62	55
Mg	55	40
Sr	50	46
Ca	95	85
Na	32	33

remarkable, the effect of BaCl_2 on heat fragility, and the effect of CaCl_2 on osmotic and mechanical fragilities.

Osmotic Fragility.—Washed cells were allowed to stand in about 20 times their volume of isotonic solutions of NaCl, BaCl_2 , MgCl_2 , SrCl_2 , and CaCl_2 at 4°C. for 16 hours. At the end of this time, only the system containing CaCl_2 showed hemolysis (16 per cent). The cells were then thrown down and resuspended in isotonic NaCl to make suspensions with a volume concentration of about 0.3. Of these suspensions, 0.1 ml. of each was added to 1 ml. of various tonicities of NaCl, and the amount of lysis at the end of 1 hour was measured as a percentage of complete hemolysis. Table II shows the results.

The osmotic fragility apparently decreases in the order $\text{NaCl} > \text{BaCl}_2 > \text{MgCl}_2 > \text{SrCl}_2$, but the differences are difficult to interpret because the "isotonic" concentration of the salts changes with time. The important point is that the osmotic fragility is not greatly different under conditions in which at least 50 per cent of the intracellular K has exchanged for Ba, Mg, or Sr; this is an extension of the observation (Ponder, 1947) that the osmotic fragility is not much affected by a K-Na exchange. In systems containing 0.1 M CaCl_2

there is a considerable amount of lysis on standing for 16 hours at 4°C. As in the case of the other salts (and particularly SrCl₂), it is doubtful what the "isotonic" concentration is, as this changes with time. Red cells in which there is a substantial K-Ca exchange are, however, quite unstable osmotically, 57 per cent lysis occurring in a concentration of NaCl (0.5 per cent) which does not produce any hemolysis of cells in which the other ion exchanges (K-Ba, K-Mg, etc.) have occurred.

Heat Fragility.—This was measured as already described (Ponder, 1952). Washed human red cells were allowed to remain at 4°C. in contact with isotonic solutions of NaCl, BaCl₂, MgCl₂, SrCl₂, and CaCl₂ for 6 hours; 0.5 ml. volumes of the suspensions, each with a volume concentration of 0.08, were then heated for 3 minutes at 53°C. The initial number of cells, N , the final number of cells

TABLE II

Tonicity	NaCl	BaCl ₂	MgCl ₂	SrCl ₂	CaCl ₂
0.5	0	0	0	0	57
0.4	16	0	0	0	69
0.35	62	52	38	14	80
0.30	87	83	76	19	87

TABLE III

	NaCl	BaCl ₂	MgCl ₂	SrCl ₂	CaCl ₂
f	2.6	6.4	2.5	2.8	2.7
p	0.10	0.00	0.02	0.00	0.08
P	3.9	9.1	7.0	9.9	19.6

plus fragments N_f , and the fractional hemolysis p give the amount of fragmentation $f = N_f/N(1 - p)$. The fragments are almost invariably spherical. The values of f and of p are shown in the first two rows of Table III.

Exposure to isotonic BaCl₂ results in an increase in the extent to which the cells are fragmented by heat. The number of fragments is about twice that obtained by heating, under the same conditions, cells which have been exposed to isotonic solutions of the other salts. The fragments, moreover, are very much smaller than those in systems containing NaCl or the other salts, and large numbers of still smaller fragments can be seen by phase contrast. They look like tiny myelin forms.

Mechanical Fragility.—This was measured as already described (Ponder, 1952). Washed human red cells were allowed to stand in contact with isotonic solutions of NaCl and of the chlorides of the alkaline earths for 6 hours at 4°C.; the suspensions were then centrifuged and sufficient supernatant fluid removed to leave suspensions with a volume concentration of 0.4. The cells of 0.5 ml of these suspensions were rotated, with 3 glass beads, for 45 minutes at 30

R.P.M. and the percentage hemolysis P at the end of this time was measured. The results are shown in the last row of Table III.

The mechanical fragility of the cells exposed to isotonic solutions of the salts of the alkaline earths is increased, in the cases of BaCl_2 , MgCl_2 , and SrCl_2 to about double, but in the case of CaCl_2 to about 5 times, that found in isotonic NaCl . Since the meaning of osmotic, heat, and mechanical fragilities, in terms of red cell structure, is still obscure, no clear interpretation can be given of the effect of Ca on osmotic and mechanical fragilities or of the effect of Ba on heat fragility. For the present, the negative aspect of the observations is probably the most interesting. Since divalent ions can be introduced in large amounts into the red cell by ion exchange without greatly changing its fragility, it should be possible to introduce these ions in their radioactive form, for example, and yet leave the cell in many respects uninjured.

SUMMARY

1. Concentrations of BaCl_2 , MgCl_2 , SrCl_2 , and CaCl_2 can be found in which the volume of washed human red cells remains almost unchanged for short periods of time; in more concentrated solutions the cells shrink, and in less concentrated ones they swell. Between tonicities of about 1.5 and 0.75, the van't Hoff-Mariotte law applies roughly, but at lower tonicities the red cell volume is anomalously great, sometimes in the absence of hemolysis.

2. If the cells are allowed to stand at 4°C . in the media of different tonicities, the volume changes are not maintained. The volumes decrease in a complex way, and the decreases are accompanied by a loss of K from the cells and an entry of the external cation into them.

3. With two exceptions, these ion exchanges are not accompanied by any important changes in the osmotic, mechanical, or heat fragility of the red cells. The exceptions are a marked effect of BaCl_2 on heat fragmentation, and of CaCl_2 on osmotic and mechanical fragilities.

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