CCXXXI. THE PERMEATION OF ERYTHROCYTES BY CATIONS.

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WHEN erythrocytes are suspended in salt solutions, the amount of anion permeating varies with the external p_H . If acid is added to the external phase, then hydrogen ions and anions penetrate the cell membrane freely. If the penetration of cations like sodium and potassium were also rapid, then the amount of anion penetrating erythrocytes would vary not only with the external p_H but also with the external salt concentration. It is found, however, that when human erythrocytes are suspended in salt solution at constant p_H , the amount of permeating anion changes only slightly with considerable variations in the external salt concentration [Maizels, 1934]. In view of these observations it may be concluded that human erythrocytes are not readily permeated by cations, and that the penetration of chloride is prevented by the electrostatic attraction of the non-penetrating cation.

Whilst, however, observations on the relative impermeability of erythrocytes to cations have been numerous, there has been a lack of agreement between the findings of various investigators. These disagreements arise partly from the different techniques used and partly from the fact that the cells of different animals have been employed. For this reason the permeability of erythrocytes to cations has been re-examined; in the present investigation experiments have been limited to human cells, and changes in the potassium content have been measured after the cells have been suspended in solutions of sodium chloride, potassium chloride and glucose.

Gürber [1895] originally noticed the impermeability of erythrocytes to sodium and potassium, but Hamburger and Buba [1910], using ox corpuscles, observed that cations passed across the cell membrane on changing the carbon dioxide tension of the blood, or on altering the salt concentration of the serum. Ashby [1924], using human cells, found considerable changes in the cation of human erythrocytes suspended in 10 vols. of salt solution and incubated for one or more days. The p_H of the original salt solution was 7.4, but the p_H of the fluid after mixing with blood is not given. In any case cation leakage occurring after the lapse of many hours is rather a stringent criterion of cell permeability, for the integrity of the cell is unlikely to be maintained under these circumstances; and whilst one may agree with Ashby that partial haemolysis is clear evidence of cell damage, it is equally clear that some trauma must have existed before haemolysis became apparent.

Wakeman *et al.* [1927] were unable to confirm Hamburger's observations, for they detected no passage of cation in or out of human erythrocytes on adding to the serum either salts or water. These writers limited loss of CO_2 from their preparations by saturating with CO_2 (at a pressure of 40 mm. in air). Mond [1928] found that when the erythrocytes of oxen or swine were suspended in salt solutions cation permeation only occurred at $p_H > 8$ to 8.3. Kerr [1929] found that the centrifuged erythrocytes of man, dog, sheep and ox, after 2 hours'

suspension in saline wash-fluid were all permeable to cation, but that permeability was greatly decreased when a high proportion of serum was added to the wash-fluid. Davson [1934] used a suspension of equal parts of ox corpuscles and salt solution and found a considerable and rapid permeation of cation which was unaffected by the presence of serum—indeed a considerable decrease of cell potassium occurred when the cells were left in contact with their own serum *in vitro*.

It follows from the work of Mond that in any investigation of cell permeability the p_H of the system should be known, and this is not the case with the experiments of Ashby, Kerr and Davson. Indeed it is probable that as a result of exposure to air, defibrination *etc.*, loss of CO_2 from the cells will render these definitely alkaline and that this alkalinity will be imparted to the wash-fluid, thus increasing cell permeability. Any increase in the p_H of the wash-fluid is likely to be the more marked when its volume is not great compared with the volume of the suspended cells. Under these circumstances also the composition of the wash-fluid will be materially altered should cation permeation occur. In the present investigation a small quantity of cells was suspended in 70 vols. of solution; in this way changes in the reaction and composition of the wash-fluid were limited and in addition cells remained in contact with the suspending solution without constant agitation of the system. Further, as the cells were spun down into narrow capillaries, the amount of intercellular fluid was minimum.

METHOD.

Heparinised blood is centrifuged for 30 min. in tubes drawn out into sealed graduated and calibrated capillaries. The volume of erythrocytes (about 0.1 ml.) is accurately measured, the supernatant plasma removed and the cells suspended for a given time in 7 ml. of the test fluid. The suspension is then centrifuged, the new volume of the erythrocytes measured and the p_H of the supernatant fluid estimated [Maizels, 1934]. This fluid is then discarded and the centrifuge tube carefully washed with distilled water down as far as the upper face of the erythrocyte column. The cells are laked in about 2.5 ml. of water, 0.3 ml. of sodium tungstate (10%) and 0.3 ml. of $2/3N H_2SO_4$ are added and the volume made up to 4 ml. After centrifuging, 1 ml. of the protein-free fluid is placed in a cylindrical centrifuge tube (9×1 cm.) the last centimetre of which tapers to a point. 1 ml. of sodium cobaltinitrite solution is added and after 16 hours the deposit of potassium cobaltinitrite is centrifuged down. All but 0.2 ml. of the supernatant fluid is removed and the tube almost filled with distilled water, at first added drop by drop so that the precipitate of potassium cobaltinitrite and its covering layer of sodium cobaltinitrite remain undisturbed. The latter is then removed with a capillary pipette and restored to the main bulk of wash-water in the tube (which is now pale amber in colour), after which the tube is again centrifuged. It is then left for 2 hours so that any sodium cobaltinitrite in the interstices of the packed precipitate may diffuse out. The precipitate is then twice washed after the fashion of the first washing, but after the final washing all the supernatant fluid is removed. The potassium cobaltinitrite is finally estimated by the method of Kramer and Tisdall [1921].

There are several possible objections to the above method. The composition of the potassium cobaltinitrite precipitate varies slightly, and it is therefore necessary to estimate the K content of a standard solution in the same way as that of the unknown in order to provide a factor with which the K in the unknown solution may be calculated. Again, it is conceivable that K present

in the protein precipitate might be greater or less than that in the protein-free fluid. Nevertheless potassium added to cells can be quantitatively recovered and the findings agree well with the results obtained on ashed cells within the limits of experimental error. Finally, potassium cobaltinitrite is not completely insoluble and it is important that the precipitate be not disturbed during the process of washing. The error in the method rarely exceeds $\pm 3\%$ and is usually less than $\pm 1.5\%$. Duplicate experiments agree well and results are reproducible.

RESULTS.

The findings in 53 normal cases were as follows: cell K averaged 107 m.eq. per litre of cells. In 7.5% of cases it was over 113 m.eq., in 79.5% between 96 and 113 m.eq., in 7.5% it was less than 96 m.eq., and in 5.5% less than 90 m.eq. The lowest figure was 87 m.eq., and the highest 119 m.eq.

The cation content of cells suspended in solutions of potassium chloride or sodium chloride.

The effect of external concentration. The concentration of K in the normal human erythrocyte is about 160 m.eq. per kg. of cell water. In the following experiments cells were suspended in KCl solutions varying in concentration from 100 to 514 m.eq., including 175 m.eq. which is only slightly hypertonic, and it will be seen (Table I) that though cell K increases slightly with external concentration, the total gain in K is relatively small over a wide range from 100 to 350 m.eq., and it is only when the external concentration becomes excessively high that the increase in cell K becomes more evident. Similarly, cell K is but little decreased when erythrocytes are placed in NaCl solutions of varying concentrations.

In Table I and in subsequent tables Q is the ratio of K present in unit volume of "original" cells resulting from suspension in salt solution, to that present before suspension. Thus

$$Q = K_f \times V/K_0,$$

where K_f is the K content per litre of suspended cells, K_0 is the K content per litre of original cells, and V is the ratio of the volume after suspension to that before. Results are the mean of four observations.

It should be noted that Q indicates changes in the K content of the cells, or in other words changes in the actual amount of K passing across the erythrocyte membrane. It does not indicate changes in the K concentration of cells, that is the concentration per kg. of cell water. This latter value will be considered subsequently.

Table I.

Cells 1 hour in salt solution at 31°, p_H 7.6.

External concentration m.eq.	Cells in KCl Q	Cells in NaCl Q
100	1.04	0.95
175	1.07	0.95
350	1.11	0.94
514	1.40	0.90

It might be thought that the apparent increases in cell K were due to the presence of a residue of the suspending fluid between the cells of the packed centrifuged deposit. If, however, a salt like potassium ferrocyanide (which penetrates erythrocytes extremely slowly) be added to the suspending fluid, it may be shown that not more than 1% by volume of the packed cells is occupied

by ferrocyanide, and this includes ferrocyanide in, on and between the cells. Hence intercellular salt solution can contribute but little to the changes in cell cation. In the case of the more dilute solutions, at least 70 % of the small increase observed is due to the actual penetration of potassium, whilst with the very concentrated solution the real increase of cell K is at least 80 % of the apparent increase.

Temperature coefficient. Temperature has little effect on the gain in total K of cells placed in potassium chloride solution, even when the external concentration is as great as 350 m.eq. But when the external concentration is very high indeed (514 m.eq.) the amount of K taken up by cells increases with temperature. Similarly, the loss of K from cells suspended in NaCl is practically unaffected by temperature.

Table II.

Temp. °C.	Cells 1 hour in salt solution at p_H 7.6.					
	Cells in KCl.			Cells in NaCl.		
	External concentration			External concentration		
	175 m.eq. Q	350 m.eq. Q	514 m.eq. Q	175 m.eq. Q	350 m.eq. Q	514 m.eq. Q
10	1.06	1.09	1.25	0.95	—	0.93
24	1.07	1.09	1.31	0.95	0.95	0.90
31	1.07	1.11	1.41	0.95	0.94	0.89

The effect of time. In KCl solutions of moderate and high concentrations, the chief gain in K is almost immediate and during the next hour further increase is very slight. But if the external concentration is very high indeed the increase of cell K is more continuous and more marked. In NaCl solution a small loss of K occurs immediately, but thereafter cell K remains constant for at least 4 hours.

In Table III "0" minutes signifies that the cells are centrifuged immediately after suspension in the salt solution; the time is actually about 10 sec. *plus* an indeterminate period during which the cells are being centrifuged out of contact with the solution.

Table III.

Time min.	Cells in salt solution at 31°, p_H 7.6.				
	External KCl			External NaCl	
	175 m.eq. Q	350 m.eq. Q	514 m.eq. Q	350 m.eq. Q	514 m.eq. Q
"0"	1.06	1.08	1.23	0.95	0.94
5	—	1.09	1.26	0.95	—
60	1.07	1.11	1.44	0.94	0.89
240	—	1.11	—	0.92	—

The effect of p_H . Between p_H 5.8 and 8.3 cells suspended for one hour in KCl (350 m.eq.) show the same increase of K—about 11 %. At lower p_H values haemolysis occurs; the increase in cell K is more marked and the influence of temperature (insignificant between p_H 5.8 and 8.3) becomes more obvious. At $p_H > 8.3$, the increase in cell K is again more marked. The observations of Mond on the increased permeation of cation at high p_H values are thus confirmed and, whilst the increase at a very high p_H may be partly due to chemical damage of the erythrocyte membrane, the increase which is still evident between p_H 8 and 8.8 appears to depend mainly on physical causes. Thus cells placed first in NaCl solutions of p_H varying between 7.6 and 8.6 and then suspended in neutral KCl, show no more increase of K than cells suspended straight away in neutral KCl.

This shows that initial soaking in NaCl of moderate alkalinity does not so damage the erythrocyte membrane as to increase the permeation of K on subsequent suspension in KCl solution.

Cells placed in NaCl lose about 5% of K except in very acid or very alkaline solutions, when the loss of K is greater.

Table IV.

Temp. ... p_H (external)	KCl.			NaCl.		
	External concentration			External concentration		
	175 m.eq. 24°	350 m.eq. 24°	350 m.eq. 31°	175 m.eq. 24°	350 m.eq. 24°	350 m.eq. 31°
	Q	Q	Q	Q	Q	Q
5.2	—	1.61	—	—	—	—
5.6	—	1.31	1.54	—	0.56	0.12*
6.0	1.06	1.11	1.10	—	0.96	0.95
7.0	1.07	1.10	1.11	0.97	0.96	0.94
8.0	—	1.11	1.11	—	0.94	0.93
8.5	1.09	1.14	1.19	0.96	0.94	0.93
9.0	—	1.22	1.33	—	0.92	0.92
9.5	—	1.30	1.52	0.90	0.88	—

* Slight haemolysis.

Summary of changes in the cation of cells suspended in salt solutions. The changes in cell cation observed hitherto fall into two groups. (1) Cells in salt solutions of moderate or fairly high concentration show a small rise of K in KCl solution and a small loss in NaCl. These changes are almost immediate and within wide limits are unaffected by the duration of exposure, or the reaction or the temperature of the solution used. They show only slight increases with external concentration, unless the latter is very high indeed, when the increases are much greater. (2) Cells in solutions of high p_H (8.8 or over) show a considerable change of cation and the change in cell K (whether increase in KCl or decrease in NaCl) increases with time and with rise of temperature. Similarly in neutral salt solutions of very high concentration changes in cell K with time and temperature are very evident.

It is thought that the small rapid changes of cell K observed in solutions of the first group occur in the undamaged cell, whilst the larger changes observed in the second group, which are greatly affected by the duration and temperature of suspension, indicate that cell damage has occurred.

It may be said that little can be learned from exposing erythrocytes to grossly hypotonic or hypertonic solutions; yet it has been seen that very slight changes in cell K occur when cells are placed in solutions whose concentrations vary between 100 and 350 m.eq. and that such changes are relatively unaffected by time, temperature or p_H . It therefore seems reasonable to conclude that no greater change of cell K would occur in solutions of physiological osmotic pressure. Further, the small change in K of cells exposed to solutions of such varying concentrations, indicates how strongly human erythrocytes in salt solutions resist gain or loss of their contained cation.

The cation content of erythrocytes suspended in solutions of glucose.

Since cell cation changes very little when erythrocytes are suspended in solutions of electrolyte, the effects of suspending cells in a solution of a non-electrolyte were investigated. It was found convenient to use a 7% solution of

glucose, for, although its osmotic pressure is slightly higher than that of normal erythrocytes and it causes some immediate shrinkage, the subsequent diffusion of glucose into the cells increases their osmotic pressure and causes them to swell, so that after one hour's suspension in 7 % glucose cell volume is 97 % of what it was before suspension. In such a solution a considerable loss of cell K occurs and this loss is greatly increased by raising the temperature or prolonging the time of suspension.

Table V.

0.1 ml. cells in 7 ml. glucose (7%). Initial p_H 6.6.						
Temp. °C.	"0" min. Glucose.		5 min. Glucose.		60 min. Glucose.	
	Final external p_H	Q	Final external p_H	Q	Final external p_H	Q
5	—	—	—	—	5.8	0.83
10	—	—	—	—	5.8	0.83
20	—	—	—	—	6.4	0.73
24	5.6	0.93	5.8	0.90	6.4	0.58
31	5.6	0.91	5.8	0.88	7.8	0.33

It will be seen that the glucose solution was slightly acid when freshly prepared, its p_H being 6.4 to 6.6. If erythrocytes are centrifuged out of such a solution after only a few moments' suspension, the supernatant glucose becomes more acid, but if the cells and fluid are left in contact for an hour, the glucose shows a marked increase of p_H (7.8–8.0). There are several possible factors concerned in these changes in reaction. Coulter [1924] remarks that this preliminary acid shift may arise from Cl^- penetrating the erythrocyte membrane more rapidly than K^+ and thus escaping from the cell more rapidly, so that in fact hydrolysis of cell KCl occurs rendering the exterior more acid and the interior more alkaline. If cell K and Cl are estimated after the erythrocyte has been in glucose for 5 min. the loss of the former is 14 m.eq. and of the latter 12.5 m.eq. These differences are hardly significant from the point of view of analysis, although it must be remembered that if 0.1 ml. of cells in 7 ml. of glucose loses an amount of chloride equivalent to only 1 m.eq. per litre of cells, this amount of anion would more than suffice to change the p_H of the glucose from 6.6 to 5.6. It is probable, however, that the following mechanism is more important. When erythrocytes are suspended in a solution of KCl, a proportion of the contained CO_2 and HCO_3^- diffuses outwards, the latter exchanging with external Cl^- , so that if the external solution were at first neutral and unbuffered, it now becomes slightly alkaline. But if cells are placed in a solution of non-electrolyte like glucose, cell CO_2 diffuses outwards, but, as there is no external anion with which cell HCO_3^- can exchange, escape of the HCO_3^- is checked by the electrostatic attraction of the more slowly permeating cell cation. Under these circumstances, the ionisation of the CO_2 diffused outwards will make the external solution more acid.

After one hour's suspension in glucose at 31°, however, practically all cell chloride, about 45 m.eq. (and presumably about 13 m.eq. of cell HCO_3^-), has diffused outwards, while some 65 m.eq. of K have been lost, and the presence of this base-bicarbonate in the external solution renders it alkaline.

These p_H changes depending on the rapid permeation of CO_2 or on the dissociated penetration of Cl^- and K^+ are best seen when the time of suspension is short, but they are also evident even when the suspension time is long, provided that the temperature be low. Thus at 10°, the glucose solution remains acid even after one hour, while at higher temperatures it becomes alkaline (Table V).

The effect of p_H on the loss of cation from cells suspended in glucose solution.

Table VI shows that erythrocytes lose cation much more rapidly when a trace of alkali is added to the glucose in which they are suspended. This effect is more evident when the period of suspension is short. The findings further confirm Mond's observations that erythrocytes are more permeable to cation when suspended in solutions of high p_H .

Table VI.

Time min.	Erythrocytes in dextrose solution, 7% at 20°.			
	Glucose initial p_H 6.6		Glucose initial p_H 9.4	
	Final p_H	Q	Final p_H	Q
5	5.6	0.94	8.6	0.67
60	6.0	0.64	8.7	0.51

Temperature coefficient of potassium loss in glucose solution (Table V). The loss of K is small at low temperatures and the values for Q at 10° and 20° are about the same. Between 20° and 30° however K loss is greatly accelerated. Thus the reciprocal of $Q_{20^\circ}/Q_{10^\circ}$ is 1.1; of $Q_{30^\circ}/Q_{20^\circ}$ 2.2. These figures are similar to the temperature coefficient for diffusion of phosphate into erythrocytes from phosphate solutions, which is 1.6 between 10° and 20° and 2.4 between 20° and 30° [Maizels, 1932].

The permeability of erythrocytes in glucose to which KCl or NaCl has been added.

The addition of quite small amounts of KCl or NaCl greatly decreases the loss of cell K. Thus the presence of only 11 m.eq. of K in the glucose solution markedly delays the diffusion outwards of cell cation, so that after one hour's suspension cell K falls from 110 m.eq. to 94 m.eq., a decrease of only 14.5%. NaCl is almost as efficient as KCl in preventing cation loss (Table VII).

Table VII.

Cells in glucose solution with and without added salts, $T=30^\circ$, time 60 min.

External glucose %	External cation species	External cation conc. m.eq.	External p_H	Cell volume %	Q
7.0	0	0	8.0	94	0.37
6.8	K	5.5	6.6	115	0.68
6.8	Na	5.5	6.6	116	0.65
6.5	K	11.0	6.6	125	0.84
6.5	Na	11.0	6.6	125	0.80
6.1	K	22.0	6.7	132	0.94
6.1	Na	22.0	6.7	135	0.88

Changes in cell volume in glucose solutions (Table VII). If cells are centrifuged immediately after suspension in pure glucose solution, their volume shrinks to 90% of the original volume in plasma, for the external solution is relatively hypertonic. If the suspension is allowed to stand for one hour, however, some of the glucose diffuses into the cells, carrying water with it, so that the cell volume increases to 95%. At this time cell glucose is about 3% and no doubt cell volume would be much larger than appears were there not a simultaneous outflow of cell K and Cl. If however this outflow is prevented by adding KCl or NaCl to the glucose, cell swelling resulting from the glucose diffused inwards is very evident.

DISCUSSION.

Hitherto, the effect of external concentration on the amount of anion gained or lost by unit volume of cells has been considered; in what follows, the external concentration is compared with the concentration of ions within the cell, that is the concentration in the cell water, the cell being at equilibrium with the external solution. The latter value may be obtained as follows: if one litre of original cells after suspension in KCl solution changes to V litres containing a total of x m.eq. K, then one litre of suspended cells contains x/V m.eq. If the proportion of water in the original cell is a , then the amount of water in one litre of suspended cells is $(V-1+a)/V$ and this water contains x/V m.eq. K. Therefore the concentration of K per kg. of cell water is $x/V \div (V-1+a)/V$ and so $[K] = x/(v-1+a)$, where $[K]$ is the concentration of cell K at equilibrium with the external solution.

In this way the concentration of the cell constituents $[S]$ has been determined where $S = [K] + [Cl] + [Hb]$. Hb has been taken as 5 m.eq. Cell water was 69.5%. Results were as follows.

External K and Cl m.eq.	V %	Internal [K] m.eq.	Internal [Cl] m.eq.	[Hb] mM	S
100	132	111	78	5	194
175	93	187	134	8	329
350	68.5	320	232	13	565
514	64	422	323	15	760

As the external p_H in this experiment was 7.3 it may be presumed that Hb is acting as an anion, and under these circumstances cell Cl^- should be less than external Cl^- , which is in fact the case, while cell K^+ should be greater than external K^+ , which is only the case with the more dilute external solutions; for when the external solution is more concentrated, cell $[K]$ is less than external $[K]$. Further the sum of cell $[K] + [Cl] + [Hb]$ should roughly equal the sum of external $[K] + [Cl]$ or twice the concentration of external $[K]$. As the cell is suspended in 70 times its volume of KCl, other cell substances like bicarbonate, urea *etc.* will diffuse outwards and their concentrations both in the cell and external solution will be so low as to be negligible. If, however, $[S]$ (fluid) and $[S]$ (cell) are compared, it will be seen that except in the lowest concentrations $[S]$ (cell) is much lower than $[S]$ (fluid), and the deficiency far exceeds any possible experimental error. Further, this deficiency becomes more evident as the external concentration increases. It may be explained in one of five ways.

(1) *Presence of base in the erythrocyte in addition to potassium.* It is usually stated that human cells contain no base beside potassium, but according to recent work of Oberst [1935] they contain between 6 and 9 m.eq. Na with an average of 7 m.eq.

(2) *Presence of indiffusible compounds in the cell other than K, Na, Cl and Hb.* The osmotic pressure of such substances in the cell (complex organic phosphorus compounds, or proteins other than haemoglobin) is not likely to be significant and it is probable that compounds other than those of K, Na, Cl and Hb may be ignored.

(3) *Increased activity coefficient of the inorganic ions.* This is excluded by the work of Adair and Adair [1934].

(4) *Relatively high osmotic coefficient of the haemoglobin.* Except in very dilute solutions, haemoglobin exerts an osmotic pressure greater than would have been anticipated from its molar concentration [Adair, 1928; 1935]. This effect would be most evident with concentration of cell Hb resulting from

suspending the cell in hypertonic solutions. Adair's data apply to solutions of concentrations less than 5 mM and extrapolation is unsuited to the high concentrations found in the present experiments. However, as a rough approximation it may be said that when the concentrations of [Hb] are respectively 5, 8, 13 and 15 mM, the corresponding figures for [Hb]' in osmolar millimols are 9, 18, 34 and 46. Adding these values for [Hb] and Oberst's values for [Na] to cell [K] and [Cl] the value of S' is as follows:

S external	S' internal
200	205
350	350
750	605
1028	809

It will be seen that the inclusion of Na and corrected values for Hb still leaves a considerable deficiency in S (cell), while it cannot account for the observation that cell [K] instead of being more than external [K] is less.

(5) *Part of the cell water is bound by haemoglobin and only a proportion of water is free and available as a solvent.* If such were the case, the value of S' (cell) would be correspondingly increased. According to the conclusions of Hill [1930], however, about 98 % of the total erythrocyte water is free. But it is possible so to interpret Hill's data as to indicate that the ratio of free to total cell water is only 0.95 or even less. Hill estimates free water in the following manner: 1 g. of a substance (NaCl, KCl, urea, sucrose, lactic acid, succinic acid) is added to 100 g. of NaCl solution (1 %) and also to a quantity of erythrocytes known to contain 100 g. of total water. The depressions of vapour pressure of each system are measured. If the ratio

Vapour pressure depression caused by adding 1 g. solute to 100 g. water in 1 % NaCl solution
 Vapour pressure depression caused by adding 1 g. of solute to 100 g. water in corpuscles (or blood)

is unity, it is concluded that all cell water is free. If the ratio is less than unity, some water is bound; if it be greater than unity, then it is assumed that the added substance "is somehow removed from free solution by the presence of other bodies, e.g. by surface adsorption, or by 'solution' in or combination with the protein or lipins". Hill's results for corpuscles and whole blood fall into three groups: lactic acid, succinic acid and urea give ratios greater than unity; the ratios for sucrose (three experiments) are 1.02, 1.02 and 0.94; for NaCl 0.99, 0.95 and 0.955; and for KCl 0.93 and 0.97. The mean of the sucrose, NaCl and KCl results are taken, indicating that less than 3 % of cell water is bound, it being assumed that these three substances are not adsorbed. This is probably not the case with sucrose, and if the latter be excluded, the mean ratio for NaCl and KCl is 0.95, corresponding to 5 % of bound water, or less if any adsorption of NaCl or KCl occurs. Now if succinate and lactate are adsorbed, then it is certain that chloride is also adsorbed to a significant extent. Indeed, according to Katz [1933] chloride is not less strongly adsorbed than are lactate and succinate with their hydrophile groupings, and hence it follows, from the experiments with NaCl and KCl, that bound water in the erythrocyte is greater than 5 %. Hill advances another argument against the existence of bound water: "If we regard as fixed the total number of ions or molecules present in a given amount of blood, and add water to it, the depression of vapour pressure observed at any stage should be inversely proportional to the total amount of free water present at that stage. It is found that $PV = a$ constant, P being the total osmotic pressure of all the dissolved constituents and V the volume of water in the solution. By plotting the relation between $1/P$ and V and extrapolating it backwards to the

axis of V it is possible to determine the amount of 'free' water in the original blood". The results so obtained in these experiments showed that 7, 8 and 0% of total cell water was bound (average 5%). The advantage of this second method lies in the apparent exclusion of adsorption effects. The results can however be interpreted in another fashion. If only part of the cell water were free, then, on adding water to cells, the resulting osmotic pressure would be less than anticipated from the known cell water content; but if the addition of water resulted in the removal of substances normally adsorbed on the cell, then the osmotic pressure of the system would be raised. With the addition of more water, further de-adsorption would occur, and over a small range of dilution the findings might simulate those expected on the assumption that all cell water were free and that no de-adsorption occurs.

While the evidence against bound cell water is not conclusive there is certain evidence in its favour. Adair [1931] has shown that haemoglobin binds water: equilibration of dried haemoglobin with the vapour of saturated ammonium sulphate gives 0.2 g. of bound water per g. Hb; the distribution of chloride between haemoglobin in a collodion sac and an external solution of sodium chloride indicates 0.21 g. of bound water, while the figure obtained from determinations of crystal density is 0.18 [Adair and Adair, 1934]. Again, according to Brooks [1934] 1 g. of muscle protein binds 0.3 g. of water. 100 ml. of normal erythrocytes contain 30 g. of haemoglobin and this should bind 6 g. of water. In addition there are other substances in cells (proteins, lipins) which might also bind a proportion of water. In Table VIII the concentrations of $[K]$, $[Na]$, $[Cl]$ and $[S']$ have been calculated per kg. of free cell water, it being assumed that bound water constitutes respectively 5 and 10% of the total water, corresponding to 0, 3.5 and 7 g. of bound water per 100 ml. of cells. Cell water is 69.5%.

$S' = [K] + [Na] + [Cl] + [Hb]'$, where $[Hb]'$ is the roughly corrected value for $[Hb]$: it has not been further corrected for the slight increase in Hb concentration implied in the assumption that part of the cell water is bound. $[Na]$ is derived from the values given by Oberst [1935].

Table VIII.

g. H_2O /100 ml. cells		External concentration m.eq.											
		100			175			350			514		
		$[K]$	$[Cl]$	$[S']$	$[K]$	$[Cl]$	$[S']$	$[K]$	$[Cl]$	$[S']$	$[K]$	$[Cl]$	$[S']$
Free	Bound	m.eq.	m.eq.	o.m.	m.eq.	m.eq.	o.m.	m.eq.	m.eq.	o.m.	m.eq.	m.eq.	o.m.
69.5	0	111	78	205	187	134	350	320	232	604	422	323	809
66.0	3.5	115	81	212	198	142	372	352	255	662	471	360	900
62.5	7.0	119	83	217	210	151	392	390	284	736	530	407	1023

o.m. = osmolar millimols.

When discussing Hill's experiments, it was suggested that the presence of bound water was concealed by the simultaneous absorption of salt, and that Hill was really measuring the vapour pressure of a solution of unadsorbed ions and molecules in bound cell water. In the present experiments cell ions and molecules adsorbed and unadsorbed have been measured and also the total cell water. If the suggestion that adsorption concealed the presence of bound water were correct, then

$$\frac{\text{unadsorbed ions and molecules}}{\text{free cell water}} \text{ should equal } \frac{\text{total cell ions and molecules}}{\text{total cell water}},$$

and each should equal the external concentration. Reference to Table VIII, line 1, shows that the ratio of the total cell content to the total cell water (S') is roughly equivalent to the sum of external $[K]$ and $[Cl]$ in certain cases, being 205 when external $[K]$ and $[Cl]$ are each 100 m.eq., and being 350 when external $[K]$ and $[Cl]$ are each 175 m.eq. But for higher concentrations the value for S' (cell) is, as has been seen, much less than S (external).

It appears that in solutions of moderate concentration the adsorption of water and salts by Hb is approximately equal, but that with unlaked cells in more concentrated solutions water is more strongly adsorbed than salts. Further, as the apparent deficiency in S' (cell) becomes more marked with increase of external concentration it is also necessary to conclude that the amount of bound cell water increases. This is probable: with increase of the external concentration the erythrocyte shrinks and with increasing concentration of the contained haemoglobin more water will be bound. Thus Kunitz *et al.* [1933-34] have shown that 1 g. of Hb in 6.3% solution binds 0.14 g. of water, whilst in 10.45% solution 1 g. binds 0.22 g.

If then it is assumed that 6 or 7 g. of bound water per 100 ml. are present in cells suspended in concentrated solutions, then the values of S within and without the cell would be roughly equal, whilst the concentration of cell $[K]$ is greater than the external concentration, which should be the case under the conditions of experiment.

The rate of permeation of cations into erythrocytes. Reference has already been made to the small and rapid changes in cation observed in cells suspended in solutions of KCl and NaCl. It has been noted that these changes are almost immediate and are stable for periods up to 4 hours; they are somewhat affected by the external concentration but only slightly by changes of temperature between 8 and 33°. These cation changes of the first group are contrasted with the cation changes found in solutions of extreme hypertonicity or of marked alkalinity, where cell cation increases with time and also with temperature. The findings in the first group also contrast with the findings in the case of cells suspended in glucose solutions: for whilst erythrocytes suspended in a solution of potassium-free electrolyte like NaCl retain most of their potassium, potassium is lost fairly rapidly when the cells are placed in a potassium-free non-electrolyte like glucose, and this loss increases with time and with rise of temperature. In short, there are certain small changes of cell cation which are stable and other larger changes which are influenced by time and temperature factors. It is suggested that whilst a part of the small increase of cell $[K]$ in KCl solution may be due to the presence of intercellular solution, and to cell penetration, some at least is due to combination of KCl with the materials of the cell membrane or to adsorption at the cell surface, whilst the decrease in NaCl solution may be due to displacement of pre-existing potassium from the cell membrane and its replacement by Na. Davson [1934] rejects this possibility in the case of ox corpuscles, because the assumption implies a difference of osmotic pressure between cells and the fluid in which they are suspended; but if one accepts the presence of bound water, this discrepancy will disappear. The only alternative to the previous suggestion is as follows: only a small part of the erythrocyte interior is freely permeable to cations, while the greater part is impermeable except in the presence of grossly hypertonic external solutions. The simplicity of structure of the erythrocyte makes this alternative improbable.

If one assumes then that 5% of K in the normal cell is present on the surface, and a further 5% is adsorbed by cells in KCl solution, then in NaCl solution all the surface K will be replaced by Na, with an actual loss of 5% of K. Apart

however from this surface cation, no other cation will pass in or out of the cell. In non-electrolyte solutions on the other hand surface cation will be removed and not replaced by any other cation. Under these circumstances all the contained cation will gradually leak out of the cell.

It has been noted that the presence of only 11 m.eq. of K or Na in the external glucose will delay the escape of cation and will actually maintain a concentration of 94 m.eq. of K per g. of cell water for over one hour, whilst sodium is almost as effective as potassium. It is suggested that as long as the cation (whether Na or K) in or on the cell membrane is preserved, so long will cation within the cell remain more or less intact, and as the amount of a substance adsorbed from a solution decreases much less rapidly than the external concentration, a marked decrease of external salt might occur, without any significant decrease in adsorption. Alternatively, impermeability to cation might depend on the electrical charge on the cell, which is altered in pure glucose solution, but maintained if a small quantity of electrolyte be added.

In the case of other species which lose cation more readily than human cells, this surface action may be less important. Thus the erythrocytes of dog and ox lose potassium in sodium-free electrolyte solution, whilst human cells retain their cation even in solutions consisting mainly of non-electrolytes, so long as a trace of electrolyte is present.

SUMMARY.

1. Human erythrocytes are more resistant than the cells of other species to changes in their cation content. In NaCl solutions a slight loss of K occurs and in KCl a slight gain (5–10%). The gain in KCl solutions increases slightly with external concentration, but is practically unaffected by temperature, moderate changes of p_H or by duration of exposure up to 4 hours. It is suggested that these small changes in cell cation may be due to adsorption.

2. In solutions of moderate alkalinity (p_H 8.3–8.8) increased permeation of cation occurs, which is probably dependent on physical causes.

3. In solutions of extreme concentration (514 m.eq.), or of marked alkalinity (p_H 9 or more) or marked acidity (p_H 5.6 or less), increased permeation of cation occurs as a result of cell damage. These changes in cell cation increase with temperature and with duration of exposure.

4. In glucose solutions, CO_2 at first diffuses out of the cell more rapidly than base-bicarbonate and renders the external solution acid. Thereafter, potassium and HCO_3^- diffuse out of the cell and the suspending fluid becomes neutral or slightly alkaline. The rate of loss of K from cells in glucose solution increases with time and temperature. If small quantities of KCl or NaCl are added to the external glucose, loss of cell K is greatly delayed and even after one hour's suspension the concentration of K in the erythrocyte will be eight times as great as the external concentration of K or Na. It is thought that the presence of small quantities of electrolyte in the external fluid delay de-adsorption or maintain the charge on the cell surface and so prevent loss of cation.

5. When cells are suspended in concentrated solutions of KCl, the internal concentration is apparently less than the external and it is necessary to assume that only 90% of cell water is free.

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Note added to proof. The analyses on p. 1977 have been confirmed by the estimation of base according to the electrolytic method of Adair and Keys [1934].