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CATION CONTROL IN HUMAN ERYTHROCYTES

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The accumulation of potassium in animal cells against the concentration gradient has long been a matter for speculation, and it seemed to the writer that the use of a method devised by Harris (1941) might indicate some of the factors concerned in the paradoxical distribution of cations between cells and body fluids.

It is well known that in citrated human blood stored at low temperatures the erythrocytes lose potassium (Dulière, 1931; Drew, Esdall & Scudder, 1939; Downman, Oliver & Young, 1940) and gain sodium (Jeanneney, Servantie & Ringenbach, 1939; Maizels & Paterson, 1940) in accordance with the concentration gradients. Harris (1941) showed that if such cold-stored blood be kept at 37° for a few hours cation distribution tends to return toward the paradoxical levels found in the circulating blood. Repetition of Harris's experiments gave variable results, and it seemed of interest to examine possible factors concerned in the cation movements and especially pH, interdependence of cations, glycolysis and phosphorolysis.

METHODS

A sterile technique was used throughout. Forty-five parts of blood were mixed with 5 parts of trisodium citrate solution (3%) and 1 part of glucose solution (30–50%). 10 ml. of the mixture were centrifuged, supernatant plasma removed and 3 ml. packed cells kept frozen till needed; this was the 'original cell solution'. The residue of the citrated blood was kept 6 days at 4–7°, and then 10 ml. portions were placed in screw-capped bottles containing 1 ml. KCl solution (1·86%) and either acid or alkali. Sometimes the alkali was glycine (0·6 M) plus NaOH (N), and in this case the amount was made up to 1 ml. by the addition of the appropriate amount of NaCl solution (N/2). At other times Na_2CO_3 (N) was used with NaCl solution (N). All plasmas thus had the same initial amounts of Na and K, the former being six to eight times cell Na and the latter about half cell K. To one of the little bottles containing blood neither acid nor alkali was added. This was centrifuged immediately and the packed cells kept frozen; this was the 'cold-storage level' control. All other bottles were kept at 37° for 16–24 hr., after which supernatant plasma was removed, the deposit poured into calibrated haemoglobinometer comparator tubes, corked and centrifuged. Residual plasma was then pipetted off, dregs being washed away with sucrose solution (10%) and 3 ml. of the packed cells decanted with washings into a 15 ml. flask, the volume being made up with distilled water. 'Original' and 'control' cells were treated in the same way. In this cell solution Na, K, phosphate (total acid soluble and inorganic) and glucose were estimated and also the haemoglobin content relative to that of the original cell solution. Details of methods used are described elsewhere

(Maizels, 1943). It will be noted that the alkaline-saline additions to the blood samples were hypertonic, since the addition of iso-osmotic alkali to give the requisite shift of pH involves excessive dilution of the blood plasma. Hypertonic solutions, moreover, check the swelling of cells during storage and so minimize changes in cell and plasma water.

The headings in the various tables require fuller consideration:

(*a*) pH *of cells.* This was measured with a small tubular glass electrode on a fivefold dilution of cells, loss of carbon dioxide being avoided as far as possible. Owing to heavy buffering pH will differ but little from that of undiluted haemolysed cells, but may well differ somewhat from the pH of the intact cell to which, however, it probably bears a consistent relation. In any case, cell pH as set out is the reading taken at the end of a long period of incubation, during which pH is progressively falling possibly by 1 unit or more. Again, bloods mixed with NaOH and NaOH-glycine buffer may both achieve the same final pH, but the former may for some time during the first part of incubation be much more alkaline. This may alter the rate at which cation exchanges proceed during the early stages of incubation, and may also induce early and irreparable cell damage absent in the buffered system. Another factor affecting results is the speed and smoothness of mixing, a slow uneven mix exposing a proportion of erythrocytes to prolonged high alkalinity and causing cell damage which might have been avoided by quicker mixing. For these reasons, therefore, pH figures recorded in a table are merely indices of the relative reactions of the various systems and must be so understood throughout this paper.

(*b*) *Sodium and potassium.* The errors in the estimations are about $\pm 2\%$. To make values comparable these must be referred to the original cell volume. Thus if cold-stored cells have shrunk to 95% of their original volume and their K content is 65 m.equiv./l., K content per 1000 ml. original cells is 62. If after incubation the volume is 110% and K 65, K content per 1000 ml. original cells is 68 and 6 m.equiv. K have entered the cells from the plasma. Changes in cell volume are obtained by comparing haemoglobins in the various cell solutions with that of the original cell solution. The error in this estimation is $\pm 2\%$, giving an overall error of $\pm 4\%$. Concentration of K and Na in cells is obtained by multiplying the cell content (per 1000 ml. cells *final* volume) by 100 and dividing by the percentage of water in the cells; the latter, which is taken as $69{\cdot}5\%$, is subject to correction for changes in cell volume due to swelling or shrinkage. Thus, if the water content of the original cells is $69{\cdot}5\%$, and if the treated cells swell to 120%, then the water content will rise to $74{\cdot}5\%$, or allowing for errors in measuring changes in cell volume, $74{\cdot}5 \pm 0{\cdot}5\%$.

(*c*) *External concentrations of sodium and potassium.* These cannot be clearly defined in relation to the cells; the blood is shaken after cold storage and before incubation. Exchange of ions begins while erythrocytes are fully suspended and continues as the cells settle until at last it is mainly confined to the fully settled but still lightly packed cells and the intercellular plasma. Theoretically, it might seem desirable to keep the blood rocking and the cells suspended throughout incubation, but apart from technical difficulties this was thought undesirable, as constant movement might damage the cells and so alter their permeability. In any case, one is concerned less with a final 'steady state' than with initial external concentrations when ions begin to move with or against their gradients.

In some cases, K and Na contents have been estimated directly on the plasma from the cold-stored unincubated control. Conversion to plasma concentration is effected as follows: plasma from the treated but unincubated controls will vary in volume according to the amount of initial shrinkage or swelling of the cells. It has been found that this varies between $\pm 5\%$, and since plasma volume plus added reagents is 2·3 times cell volume the effect of changes in cell volume on the plasma will be $\pm 2\%$. Water in citrated plasma has been put at $94{\cdot}5 \pm 0{\cdot}7\%$, so that with the experimental errors in estimating Na and K the figures showing plasma concentrations of these ions are subject to an error of $\pm 4{\cdot}5\%$. In other instances plasma Na and K have been calculated from the original haematocrit values, from the known additions of Na and K and from known changes in cell Na and K during storage. Values for K and Na in the original plasma have been assumed to be $4{\cdot}5 \pm 0{\cdot}5$ and 145 ± 5 m.equiv./l. respectively. Figures based on this assumption are subject to an overall error of $\pm 7\%$; they are thus approximate, but afford a measure of the concentrations with or against which cations move.

(*d*) *Glucose.* In some cases blood glucose was measured, but in others only cell glucose. The latter figure does not give an absolute value for glucose metabolized, since glucose used up by the cells will be partly replenished by sugar diffusing in from the plasma, but it does afford an index of the speed of glycolysis in erythrocytes.

RESULTS

pH *and cation movements*

It will be clear from the data in Table 1 that during incubation cations are moving against their gradients, and that the process cannot be explained by any simple physical process but must be ascribed to active transport requiring energy. The actual figures set out, however, are the resultants of an active movement against the gradient and a passive diffusion with the gradient; one must presume that in the cell membrane during incubation are areas devoted to active movement and others through which simple diffusion occurs.

TABLE 1. Cation changes in erythrocytes of stored citrated blood

(Cold-stored 6 days; incubated 18 hr. Plasma concentrations at beginning of incubation: K, 4$\frac{6}{4}$±4; Na, 164±10 m.equiv./l. water.)

No.	Cell pH at 20°	K content (m.equiv./l.)*	Na content (m.equiv./l.)*	K concentration†	Na concentration†	Inorganic P (mg./100 ml.)*	Acid-soluble P (mg./100 ml.)*	Glucose (mg./100 ml.)
1 CSL	6·98	61	58	85	82	6·6	52·5	312
2	6·62	55	66	72	86	24·1	28·4	295
3	7·00	68	54	98	77	21·7	29·3	300
4	7·28	80	39	116	57	18·7	31·4	210
5	7·38	82	32	124	48	14·8	33·2	—
6	7·51	85	30	134	47	12·3	34·6	154
7	7·84	87	47	140	76	7·3	46·2	118
8	8·40	80	78	117	114	6·9	38·1	170

CSL = cold-storage level of unincubated cells.
* Values at the end of incubation corrected to original cell volume.
† Concentration, m.equiv./l. cell water at the end of incubation.

Between pH 7 and 7·8 K enters the erythrocytes and Na leaves, output of Na and uptake of K being maximal at about pH 7·3–7·4, above and below which point movements are less active. Below pH 6·7 cell Na usually rises above and K falls below the cold-storage value, and somewhere about pH 6·7 passive sodium diffusion and active sodium expulsion balance, a steady state resulting in which it appears as though Na does not penetrate the erythrocyte at all. Above pH 8 passive cation diffusion also exceeds active movement, and again somewhere between pH 7·4 and 8 there is a second point where Na in contrast to K appears to be non-penetrating.

It has been said that the cation movements are most active at pH 7·4, but it must be remembered that this figure is the value at the end of incubation, and the actual optimum may lie anywhere between the initial pH of 8·2 and 7·4. Owing to the large amount of acid liberated during incubation the use of

phosphate or plasma buffer (three parts to one of blood) has not permitted much closer definition of the optimum pH but suggests that it lies between pH 7·8 and 7·3.

Compared with the cold-storage value, total cell base content (Na + K) shows a slight fall below pH 7, up to pH 7·51 and probably up to about pH 7·7, although increased base binding by cell haemoglobin might be expected to lead to a rise in cell base; this point is discussed later. Below pH 7 cell base remains steady or rises slightly, presumably entering the cell in company with citrate which is practically non-penetrating at higher pH. So, too, above pH 7·7 cell base rises, partly because of increased base binding by haemoglobin and partly perhaps because of increased penetration by citrate.

Interdependence of cation movements

Expulsion of sodium during incubation. The question arises whether Na expulsion during incubation is secondary to an active accumulation of K, or K accumulation to a primary expulsion of Na, or whether both are independently active processes. With regard to the first: in normal circulating blood with an active K accumulating mechanism, expulsion of Na is not a logical outcome. Erythrocytes contain haemoglobin and organic phosphate which exert a far greater osmotic effect than all the non-penetrating anions of the plasma. It follows from the Gibbs-Donnan equilibrium that since both Na and K penetrate, these, along with accompanying anions and water, must progressively penetrate the erythrocyte, the cell swelling and finally bursting. This is, in fact, what happens when red cells are placed in solutions of urea or ammonium chloride where lysis appears to be as rapid as in distilled water. In the experiments shown in Table 1, however, two osmotically active non-penetrating external substances have been added: citrate and glycine, the latter in the pH range investigated being largely undissociated. These will oppose the osmotic effects of haemoglobin. If it could be shown that after incubation these substances had entered the erythrocyte to achieve equilibrium, the state of the experimental cells might compare with those in the circulating blood. But in fact at pH 7·4 and after 6 days' incubation, cell citrate was only 5·3 % of the external citrate. It was therefore necessary to devise a new set of experiments where heparin replaced the anti-coagulant citrate and Na_2CO_3 the buffered alkali NaOH-glycine. Neither of these replacements are quite satisfactory; omission of citrate by removing an external osmotic attraction leads to increased cell swelling during cold storage, while the unbuffered alkali Na_2CO_3 gives too high an initial pH during incubation, leading to early lysis and also to an ultimate pH of about 7 but rather unpredictable. Further, it was found difficult to achieve a pH greater than 7·1 without marked haemolysis. However, Table 2 shows that even in the absence of external non-penetrating anion K accumulates in and Na is expelled from the erythrocyte.

TABLE 2. Cation changes in erythrocytes of heparinized blood

(Cold-stored 6 days; incubated 16 hr. Plasma concentrations at beginning of incubation: K, 43±4; Na, 196±11 m.equiv./l. water.)

No.	Cell pH at 20°	Contents at end of incubation (m.equiv./l.)*		Concentrations in cell water (m.equiv./l.)	
		K	Na	K	Na
1 CSL	—	82	75	126	115
2	6·20	79	63	120	95
3	6·71	92	45	146	72
4	7·05	95	56	140	83
5	7·19	93	81	138	122

CSL = cold-storage level. Haemolysis slight in no. 4, moderate in no. 5.
* Corrected to original cell volume.

It may be noted that external Na was particularly high because in order to limit the volume of additions normal alkali was used. It was thought that a high external concentration might tend to check cell swelling at least during the cold-storage stage of the experiment. In spite of the steep contrary gradient, however, we have seen that Na passes out of the cell. It follows that expulsion of Na is an active process. Loss of Na could also result from active ejection of water, but in this case cell K would also be expelled unless it were held by some active process.

Uptake of potassium during incubation. It will, of course, be realized that while active intake of K does not imply passive output of Na (since, as we have seen, such a passive output is not compatible with a cell-plasma system freely permeable to cation), it is, on the other hand, possible that active expulsion of Na could lead to a passive uptake of K, against the gradient. It is true that electrical neutrality alone could be satisfied by the simultaneous expulsion of Cl^- along with Na^+, and this doubtless occurs, but such a process would leave unsatisfied the osmotic requirements of non-penetrating cell anion. If then it be accepted that Na expulsion is a primary activity, uptake of K might be either active or passive.

Superficial inspection of Tables 1 and 2 might suggest the former; thus in record 3 of Table 2 cell K has risen by 10 m.equiv./l. and Na fallen by 30; but in record 5, K has risen 11 m.equiv. above the control while Na has also risen 6. It thus appears as though Na and K were now entering the cell together and that K entry is independent of Na expulsion. However, there are several alternatives which do not involve the assumption of active uptake of K, one of which emerges when the exchange of cations is studied at intervals during a period of 24 hr. incubation (Table 3). It will be seen (1 *b*, *d*, *f*; 2 *d*, *e*, *g*) that after 6–8 hr. incubation cell Na is unaltered or else Na flows with the concentration gradient into the cell rising to well above the cold-storage level. This, as we have seen, is only to be expected because of the preponderating effect of non-penetrating cell anion. It is therefore likely that at about 8 hr. Na is

tending towards a simple physical equilibrium as a result of diffusion. Glycolysis is variable in this period, but between 6 and 24 hr. it is invariably active and pH falls by a whole unit or more while Na ebbs from the cell against the gradient, 39 m.equiv./l. being swept away in a few hours (2*e* and *f*). In the first stage K is little affected, but after 8 hr. it begins to enter the cell. It is possible that this entry is passive and compensates for active Na expulsion even though the expulsion is from a very high level to one which is still above the control value. In other words, it is possible that findings at high pH where both Na and K have increased in the erythrocyte at 16–24 hr. have occurred as follows: first Na enters to well above the cold-storage value tending toward a simple physical equilibrium; then Na is actively expelled and K enters passively to satisfy the osmotic requirements of cell haemoglobin and organic phosphate; there will be a stage when K has increased and Na though falling is still above the cold-storage value.

TABLE 3. Effects of pH and time on cation exchanges of incubated erythrocytes

(Cold-stored 6 days. Plasma concentrations at the beginning of incubation: Exp. 1, K 44 and Na 210; Exp. 2, K 38 and Na 198 m.equiv./l.)

No.	Incubation (hr.)	Cell pH at 20°	K content (m. equiv./ l.)*	Na content (m. equiv./ l.)*	K concentration†	Na concentration†	Inorganic P (mg./ 100 ml.)*	Acid-soluble P (mg./ 100 ml.)*	Glucose (mg./ 100 ml. blood)
1*a* CSL	0	—	82	75	123	112	8·4	—	460
1*b*	6	—	86	70	150	123	—	—	—
1*c*	24	6·71	92	45	146	72	—	—	—
1*d*	6	—	77	85	122	134	—	—	—
1*e*	24	7·03	95	56	140	83	—	—	—
1*f*	6	7·88	76	93	129	158	6·2	—	392
1*g*	24	7·19	93	76	140	116	15·8	—	266
2*a* CSL	0	7·10	77	63	127	104	4·8	48·0	560
2*b*	6	7·74	76	50	150	98	—	—	—
2*c*	24	6·93	92	38	150	62	—	—	—
2*d*	6	8·23	72	64	139	124	3·1	45·9	—
2*e*	9	8·14	70	73	134	140	3·7	49·0	496
2*f*	24	7·17	95	34	157	56	12·7	43·1	356
2*g*	6	8·49	77	67	152	132	—	—	—
2*h*	24	7·26	93	51	160	89	—	—	—

CSL = cold-storage level of unincubated cells.
* Values at the end of incubation corrected to original cell volume.
† Concentration, m.equiv./l. cell water, at the end of incubation.

Once it be accepted that output of Na is active, it becomes difficult to show that entry of K into erythrocytes is not passive, for so long as Na is not available to the cell any factor tending to increase cell base will call on external K. Such factors are:

(1) Active expulsion of Na leading to compensatory rise of K.

(2) Altered physical conditions in the system leading to a call for increased cell base: (*a*) Increase in cell pH. (*b*) A large unbalanced excess of non-

penetrating anion in the erythrocyte leading to a flow of water and salts into the cell. Haemoglobin and phosphate fills this role in normal and heparinized blood. In citrated blood, this osmotic force may be counteracted by adding enough non-penetrating anion (citrate) to the external phase. (*c*) In the case of systems so balanced, subsequent dilution of plasma citrate would cause base to enter the cell. (*d*) Increase of plasma base at any stage of the experiment would cause base to enter the erythrocytes.

(3) Active uptake of K. Before this can be assumed all the preceding factors must be excluded, and this has so far proved impracticable.

An example may make this clearer; blood was cold-stored with a solution containing heparin and KCl and NaCl (60 m.equiv./l. of each). At the end of cold-storage cell K was 73 and Na only 12 m.equiv./l. On incubation owing to the acidity developed cation movement of any kind was slight; little output of Na was possible from this low level, but neither did any rise occur; cell K, however, rose slightly, presumably in response to a call for increased cell base evoked by the osmotic attraction of the excess of non-penetrating anion within the red cell. To balance the latter, part of external KCl was replaced by potassium citrate at the beginning of cold storage, but on incubation this system also became so acid that little cation movement occurred. It was therefore necessary to alkalize the blood, either with strong NaOH which raised plasma base by over 12% or with dilute NaOH which decreased the external non-penetrating anion citrate, results which would in either case direct base into the cell. In one such experiment K rose from the cold-storage value of 73 to 83, while Na fell from 14 to 10 m.equiv./l. Clearly, the fall of Na may account for half the rise in K, and the change in external base or citrate at the beginning of incubation may account for the rest of the rise in cell K, in whole or in part; if the latter, then the possibility of a small active uptake of K remains, but cannot be proved in the presence of so many complicating factors all tending to increase cell base. All that can be said at present is that output of Na cannot be explained by uptake of K which, indeed, it often exceeds, and that such output against the concentration gradient must be an active process. In the case of K, on the other hand, physical processes are present which could account for its uptake against the gradient, and no evidence in favour of active transport has been found though the possibility has not been excluded.

Total base in incubated erythrocytes

The values for total base (Na + K) in cold-stored and in incubated erythrocytes are shown in Table 4. Normal erythrocytes have a total base value between 108 and 124 m.equiv./l. When heparinized and stored in the cold for several days there is a considerable rise in total base due to the fact that gain of Na exceeds loss of K, the value reaching 140 or 150 m.equiv./l. On incubation at high pH cations continue to move with the concentration gradients and

increase in base continues. But at moderate pH (moving from about 8 at the beginning to 7 at the end of 24 hr. incubation) total base shows a marked fall, so that it is less than in the cold-stored unincubated cells by 10 or 20 m.equiv./l. Since the pH of the alkali-treated incubated cells is usually greater than that of the unincubated cells an actual increase in cell base might be anticipated in the former. Thus in Exp. 5 (Table 4), with a rise in pH of 0·47, the base binding

TABLE 4. Total base in cold-stored and incubated erythrocytes

			Contents (m.equiv./l.)			
No.	Blood	pH 20°	K	Na	Na+K	Acid-soluble phosphate
1	U	—	82	75	157	—
	I	6·71	92	45	137	—
2	U	6·94	82	69	151	34
	I	7·00	88	41	129	20
3	U	6·90	82	60	142	31
	I	7·07	95	29	124	27
4	U	7·10	77	63	140	29
	I	7·17	95	34	129	26
5	U	6·91	87	53	140	33
	I	7·38	88	34	122	28
6	U	—	73	65	138	—
	I	7·30	93	36	129	—
7	U	6·85	76	67	143	31
	I	7·30	91	44	135	31
8	U	7·02	83	52	135	34
	I	6·91	97	33	130	26
9	U	6·72	67	68	135	29
	I	7·18	85	39	124	21
10	U	6·67	70	51	121	30
	I	6·82	90	27	117	27
11	U	7·23	61	58	119	26
	I	7·51	80	30	118	22
12	U	7·18	67	43	110	27
	I	7·51	92	22	114	22

Nos. 1–6, heparinized; 6–12, citrated. U = unincubated; I = incubated.

capacity of cell haemoglobin increases by about 20 m.equiv./l., but base actually falls by 18 m.equiv./l. Hence, the fall of base cannot be attributed to any pH change. It could be brought about by a redistribution of some cell anion which is normally non-penetrating, the chief of these being phosphate. Actually, a small loss of total phosphate does occur which is usually negligible when compared with the base loss. This is shown in Table 4, where phosphate is given in milli-equivalents; the figures are approximate and based on data for the equivalence of cell phosphate given by Maizels & Farmer (1939). If, now, it be recalled that at the onset of incubation NaCl and Na_2CO_3 are added to the blood and increase the concentration of plasma base by about 25% and that, in spite of this accession of external base, cell base actually falls, it will be

realized that the output of base on incubation, like the output of Na on which it depends, must be active.

In order to satisfy electrical neutrality cation escaping from the erythrocytes must be accompanied by OH^- or other diffusible anions. Since the cells are not more acid after incubation than at the end of cold storage, it seems that the cations must leave the cells in company with Cl^- and HCO_3^-. Thus, in Exp. 7 of Table 4 18 m.equiv. diffusible anion must leave the cell in company with base actively discharged and about 20 m.equiv. more in association with hydrogen ions in order to free sufficient cell base to permit the observed shift in cell pH from 6·91 to 7·38; it being assumed that 320 g. haemoglobin present in a litre of erythrocytes binds about 40 m.equiv. base for an increase of 1 pH unit (Maizels & Farmer, 1939).

In citrated as in heparinized blood, K and Na move with the gradient during cold storage but the rise in total base is less, possibly because the osmotic attraction of non-penetrating cell anion is opposed by plasma citrate; similarly, during incubation, cell base falls below the cold-storage level, but the decrease is less marked than in heparinized bloods.

The figures in Table 4 are too few to permit of statistical analysis, but they suggest that the higher the rise of base during cold storage, the greater is the fall during subsequent incubation. Thus in Table 4, nos. 1, 2 and 3 have a cold-storage base level averaging 150 m.equiv./l., falling by 20 m.equiv. during incubation; 4, 5 and 6 average 139 m.equiv. with a fall of 12; 7, 8 and 9 average 138 m.equiv. with a fall of 9 m.equiv.; and 10, 11 and 12 have an average cold-storage base of 117 m.equiv., falling only 1 m.equiv. during incubation.

On the basis of the preceding, it is suggested that cells take up base during cold storage, and during subsequent incubation tend to return to a 'setting' of about 125 m.equiv./l., the exact figure depending on experimental conditions. We have little knowledge of how this is achieved. It may be accepted that the further active movements proceed, the greater will be the disturbance of the pre-existing physical equilibrium and the greater the forces opposing further active movements; thus the concentration gradients against which cations have to move grow ever steeper, while the escape of Na from the cells in company with diffusible anion and a corresponding amount of water will concentrate non-penetrating anion (haemoglobin and organic phosphate), and this in accordance with the Gibbs-Donnan equation will increase the physical tendency for base to diffuse back into the cells. Hence, it may be that the so-called setting of the base level is the resultant of the opposed physical and biological forces. However this may be and in the absence of fuller data, it would seem fair to summarize the findings by saying that the higher the rise in cell base during cold storage, the greater the fall during incubation. It is suggested that this adjustment has a physiological value in that it tends to check in the erythrocyte the natural tendency for base to rise as a result of the electrostatic and osmotic

attraction of the contained haemoglobin and organic phosphate—a tendency which is presumably present in all animal cells by virtue of the excess of non-penetrating anion they contain.

Change in cell volume during incubation

Since cold-stored erythrocytes lose base to plasma when they are incubated it follows that loss of water and cell shrinkage must occur if osmotic equilibrium is to be maintained. In the previous experiments plasma base during cold storage was about 165 and 185 m.equiv./l., which as a result of subsequent additions reached a value of 200–240 at the beginning of incubation. It follows that the relevant changes in cell volume were obscured. In Table 5 plasma-base content during cold storage and incubation were kept practically the same—at 175 m.equiv./l. in nos. 1 and 2 and at 164 in nos. 3 and 4, and it will be seen that cell-base content and volume decrease during incubation. The figures may be contrasted with the cold-storage levels (records 1 *a* and *c*; 2 *a* and *b*; 3 *a* and *b*; 4 *a* and *c*) and also with the figures obtained in glucose-free bloods incubated in otherwise identical solutions (records 3 *b* and *c*; 4 *c* and *d*). Here active cation movements are absent; cell K falls and Na rises with the concentration gradients, total base increases and the cells swell. It is possible that the rise of base is due to increased permeability arising from impaired nutrition of the cell in the absence of glucose, and certainly the cells of glucose-free bloods were prone to show slight haemolysis. On the other hand, cells of glucose-free bloods showed no increased permeability during 6 days' cold-storage (records 1 *a* and *b*; 4 *a* and *b*; 5 *a* and *b*), while incubation with glucose-free buffered plasma for only 6 hr. was associated with increase in cell base and volume without haemolysis; further, the cells of some glucose-containing bloods with definite haemolysis still showed very active cation movements with decrease in cell base and volume. It is therefore suggested that increase in cell base and volume in the absence of glucose may be due, at least in part, to the absence of active cation movements.

In Exp. 5, cold-storage and incubation plasmas were both hypertonic, and there was no change in cell volume on incubation with glucose, but neither was there any change in base. However, the experiment shows that on incubation in the absence of glucose increase in cell base and volume were marked (records 5 *d* and *e*).

One other point may be noted: in the earlier series of experiments cell base tended to fall to between 130 and 120 at the end of incubation; in these plasma base lay between 200 and 240 m.equiv./l. In Table 5, the base level after incubation was about 115, and this may be associated with the lower plasma concentration of about 170 m.equiv./l.

Glycolysis and the movement of cations

An example of the association of glycolysis with active cation movements is shown in Exp. 5 of Table 5.

It should be noted that the pH records of Exp. 5 of Table 5 differs from those in Table 3. In the latter, each experiment comprises a series of observations; in each series the *same* amounts of alkali are added to samples of blood which are then incubated for different periods of time, the pH falling with the length of incubation. In Exp. 5 of Table 5, as with the experiments of Tables 1 and 2, each experiment consists of an unincubated control blood and several other samples to which *different* amounts of acid or alkali are added before they are incubated, usually for 18 hr.

TABLE 5. Effects of incubation and glucose on cell volume and cation (Cold-stored 6 days; incubated 18 hr.)

No.	Glucose	Percentage original volume	pH at 20°	K content (m.equiv./l.)*	Na content (m.equiv./l.)*	Inorganic P (mg./100 ml.)*	Acid-soluble P (mg./100 ml.)*	Glucose (mg./100 ml. blood)
1 *a* CSL	+	100	7·14	72	54	—	—	—
b CSL	0	101	7·26	72	55	—	—	—
c I	+	94	7·03	91	24	—	—	—
2 *a* CSL	+	102	7·18	58	65	—	—	—
b I	+	94	7·01	80	32	—	—	—
3 *a* CSL	+	104	7·27	71	48	—	—	—
b I	+	101	7·12	85	26	—	—	—
c I	0	111	7·28	69	63	—	—	—
4 *a* CSL	+	105	7·22	81	42	—	—	—
b CSL	0	105	7·31	80	45	—	—	—
c I	+	100	7·01	94	23	—	—	—
d I	0	114	7·15	82	54	—	—	—
5 *a* CSL	+	89	—	74	42	4·4	48·0	306
b CSL	0	89	—	74	40	10·7	48·0	17
c I	+	96	6·92	80	44	20·0	31·4	179
d I	+	89	7·28	86	29	17·2	38·6	70
e I	0	103	7·22	66	64	21·9	30·8	12
f I	0	97	7·76	55	87	16·6	24·2	15

CSL = cold-storage level; I = incubated.
* Values at the end of incubation corrected to original cell volume.

Harris (1941) is of the opinion that the metabolism of glucose is an important factor in energizing the passage of cations across the red-cell membrane, and this view is supported by the present data, where systems incubated without glucose show little or no accumulation of K and often a definite loss, while expulsion of Na is not seen. Again, even in systems with added glucose, gain of K and loss of Na is least marked at low pH where glycolysis is less active (Table 1 and Exp. 5 of Table 5), most evident at moderately high pH where glycolysis is more active and again less marked where, in the presence of slight haemolysis indicating damage to the cell membrane, glycolysis shows some decrease (Table 1, no. 8). It would therefore seem clear that active transport of cations is closely bound up with glycolysis.

Harris (1941) has attempted to correlate sugar disappearing by glycolysis with the amount of active movement of K but finds the figures very variable; this applies also to the present data, and is not surprising when all the factors

involved are considered. Thus the actual figures for cell K are dependent on the passive movements of Na and K and on the active transport of Na. These will vary considerably with the individual peculiarities of the cells of different bloods, with the speed and smoothness with which alkali is mixed with blood before incubation, and especially with small differences in the temperatures of cold storage and incubation; such differences are unavoidable with small refrigerators or incubators in constant general use, and they will greatly influence the rate of glycolysis, the speed of active movements of Na and of the passive movements of Na and K. Hence it is unprofitable to try to equate glycolysis with K movements unless temperature can be accurately controlled and numerous other variables eliminated.

In spite, however, of the poor correlation between glycolysis and movements of cations it is probable that the two are intimately connected although Harris (1941) considers that some intermediate product of sugar metabolism is responsible for energizing cations rather than glucose itself. Thus, he finds that the cells of blood stored without glucose still show some accumulation of potassium. It is not clear, however, from Harris's experiments that all glucose had in fact been used up before incubation. Moreover, bloods incubated with little glucose, in vessels closed merely with a cotton-wool plug tend to be fairly alkaline, a reaction which favours any active movement of cation that may be possible. In my own experiments with cold storage at 4–7°, glucose indeed largely disappeared and here little or no accumulation of K occurred. Still, it is not impossible that some derivative rather than glucose itself is responsible for activating cations and some of this derivative may persist in erythrocytes after glucose has disappeared. Support for this view might seem to be gained from the observation that although glycolysis proceeds throughout incubation active cation movements are not seen for 6 or 9 hr. (Table 3) at which time it might be presumed that sufficient of the appropriate intermediate substance had been formed. It is possible, however, that the very high pH prevailing during the first half of incubation favours passive diffusion of cations and so conceals any active movements. Certainly, as the pH falls during incubation, the passive factor wanes and the active becomes more evident.

Phosphate and cation movements

In general, the data suggest that active movements of cations vary inversely with the inorganic phosphate, but numerous apparent exceptions will be found. Thus at pH 7·1 where active movements are rapid, inorganic phosphate may appear to be little lower than at pH 6·7 where movements are less, but this is because much of the inorganic phosphate formed in the erythrocytes has leaked out at the lower pH as shown by the low total phosphate (Table 1, nos. 2, 3 and 4). Hence it would probably be more correct to say that active cation movements vary directly with the acid-soluble organic phosphorus, that is, the

difference between total acid-soluble P and inorganic P. Exceptions are met at fairly high pH where inhibition of phosphorolysis is marked while expulsion of Na and uptake of K are less evident (Table 1, nos. 7 and 8). These findings are usually associated with some haemolysis and may be due to increased permeability of erythrocytes at high pH. Again, when cells are incubated at moderately high pH actual synthesis of organic phosphate occurs during the first 7–9 hr. so that inorganic phosphate may be lower than in the original unincubated samples (Table 3, nos. 1 *a* and *f*; 2 *a*, *d* and *e*), yet Na moves with the gradient and no accumulation of K occurs. Hence, the mere occurrence of synthesis is not associated with the active transport of cations. It seems likely that movements of cations require that phosphorylated compounds break down actively, yet not so rapidly as to overtake resynthesis. This would explain the findings at low pH, where with rapid conversion of organic to inorganic phosphate, glycolysis ceases and with it the active transport of cations.

Hydrolysable phosphate in cold-stored blood is best preserved at moderately low pH, disappearing rather more quickly at high or very low pH (Maizels, 1943), but in incubated blood it survives best between pH 7 and 7·5 and disappears very quickly below pH 7. On the whole, correlation between cation movements and hydrolysable phosphate is not good, though it would be correct to say that when hydrolysable phosphate falls below 1·5 mg./100 ml. active transport of cations falls.

If the site at which active movements of cations originate be at the same place in the cell as the sites of the associated phenomena, glycolysis and the synthesis of phosphoric esters, then it must be located in or on the red-cell membrane, for it has been shown that in the erythrocyte with its membrane intact glycolysis is rapid and phosphorolysis slow, while after haemolysis, glycolysis is inhibited and phosphorolysis accelerated (Rona & Arnheim, 1913; Guest, 1932). It may further be shown that the outer face of the red cell is not the site of activity in the following way: intact cells are suspended in a watery haemolysate of the same cells made isotonic with sodium chloride; on incubation it is found that glycolysis is active and phosphorolysis slow in the intact cells, while glycolysis is slow and phosphorolysis active in the cell haemolysate which is in contact with the external surfaces of the intact cells. It may therefore be assumed that glycolysis, ester synthesis and the activation of cation movements are located in the substance or possibly on the inner face of the corpuscle membrane. Support for this view is gained from the work of Brooks (1939), who found that radioactive K before concentrating in the sap of *Valonia* showed intense accumulation in the protein envelope of the cell.

DISCUSSION

It may be said at once that there is no clear knowledge of the way in which human erythrocytes maintain a high K and low Na concentration though suspended in a plasma which is rich in Na and poor in K. The earlier conceptions were purely physical; it was first thought that the distribution arose in some inexplicable manner during the active stage of cell development and was perpetuated in maturity by the cell becoming impermeable to all cations. That this view gained acceptance probably arose from the fact that some permeability experiments were of such short duration that cation penetration was not detected. In others it is possible that a steady state existed; thus at low temperatures cations diffuse passively, at higher, actively, so that at suitable 'room temperatures' these might well balance. pH and time relations might provide further opportunities for steady states. However this may be, there can be no doubt that the theory of erythrocyte impermeability is wrong. This follows from the work of Jeanneney *et al.* (1939) and Maizels & Paterson (1940) on passive diffusion of cations into cold-stored erythrocytes, from the passive diffusion of radioactive isotopes (Cohn & Cohn, 1939; Hahn & Hevesy, 1941), from the active passage of Na out of and K into cold-stored erythrocytes after transfusion (Maizels, 1943), and from the observations of Steinbach and Harris. Steinbach showed (1940) that excised frog's muscle placed in K-free Ringer solution lost K and gained Na, but that if after some hours K in the Ringer fluid was raised to only 10 m.equiv./l., K in the muscle fibre rose to 70 m.equiv., while Na was simultaneously expelled. Then in 1941, Harris demonstrated that active cation movements in and out of human erythrocytes occurred at 37°, and that the processes were activated by glycolysis, either directly or indirectly. Support for these observations is given in the present paper, where it is shown that no active cation movements occur in the absence of glycolysis and not even in the presence of glucose unless the pH is appropriate. Thus if stored blood is brought to pH 8 and incubated Na rises and K falls in the cells in accordance with the concentration gradient and in spite of glycolysis; but later, as the pH falls cations move actively against the gradient. The actual findings are the resultant of active transport and passive diffusion, and it would seem reasonable to assume that certain areas of the cell surface are devoted to the former and others to the latter. Active expulsion of Na is inhibited at low pH and accelerated by alkalis up to pH 7·3 (approx.). It follows that passive uptake of K against the gradient is also hastened by rise of pH, and this finding is comparable to that of Fenn & Cobb (1933–4), who showed that the amount of external K required to prevent loss of K from muscle is less at high than at low pH and to that of Jacques & Osterhout (1933–4), who found a similar pH relation to the entry of K into *Valonia*.

The failure of physical processes to explain the observed cation distribution

in frog's muscle led Dean (1941) to accept the occurrence of active exchange and to propose a 'pump' theory. He suggests that either K is pumped into the cells with a complementary output of Na, or Na is pumped out of the cells with a complementary uptake of K or that both processes occur. It is implicit in his writings that any of these processes acting alone would explain the findings, but in the present paper it is emphasized that the first of Dean's suggestions is not correct, since the presence of a large amount of protein and organic phosphate acting as a non-penetrating anion within cells, while permitting active uptake of K would oppose the passive output of Na.

Indeed, this aspect of the matter seems largely to have been overlooked. Since, as we have seen, the erythrocytes contain a large excess of non-penetrating anion and are permeable to cations, these should, together with anions and water, progressively diffuse into the cells in accordance with the thermodynamic requirements until the cell bursts, and this should be true of all body cells and of any cell without an internal or external structure capable of withstanding osmotic swelling. Since in the case of the body cells Na would tend to enter by diffusion from the plasma, and in the case of single or small groups of cells in sea water Na is again likely to be the chief invading cation, there exists a primary need for cells to develop a mechanism for excreting Na if they are to keep their total base constant and survive. It is therefore interesting to recall that such a mechanism exists even in the non-nucleated human erythrocyte, where during cold storage cell base and volume increase and where during incubation the excess base and water are expelled, the mechanism being so adjusted that the greater the previous rise during cold storage, the greater is the active fall during incubation.

Active excretion of Na, however, does not of necessity imply active uptake of K, and it is difficult to see what primary function such an uptake might subserve; in the absence of any evidence in favour of such active uptake, it is suggested that passage of K into cells is passive and secondary to active output of Na.

If this view be accepted, it becomes possible to explain the various Na/K ratios of the different species, for erythrocytes with an active metabolism will expel a large amount of Na and have a large compensatory uptake of K with a low Na/K ratio, while cells with a low metabolic rate will have a small output of Na and a small uptake of K, with a high Na/K ratio; further, those species with a high Na/K ratio and a low metabolic rate might have a low ratio in muscle and other more actively metabolizing body cells. There is, however, an alternative explanation; the cells of different animal species may all have similar metabolic rates but differ in their permeability to cations. Low permeability would then be associated with a standard active output of Na, a small passive leakage back of Na with a preferential passive inflow of the smaller hydrated-K ion, and a low Na/K ratio; high permeability would have the reverse

effect. But whichever mechanism exists, its end must be the maintenance of a constant level of cell base and water.

But while the assumption of a 'pump' for the active expulsion of Na may explain many of the known findings in cell-base distribution, there is (as Dean remarks) no explanation of how the pump works, and though it is clear that glucose supplies the energy and that Na must adsorb on the inner face of the erythrocyte membrane, be rotated to the outer face and ejected there, further knowledge is completely lacking. The practical outcome of these activities is less obscure; they permit cells to survive in a Na-rich fluid medium without swelling or bursting. Hence, phylogenetically, the mechanisms must be very old, and it is possible that passive uptake of K and possibly Mg, due to active output of Na, may have acquired an extra and later significance by facilitating certain enzymatic processes.

The pathological implications of the foregoing may also be of interest; in prolonged severe acidosis a rise in the Na/K ratio might be expected in the erythrocytes and presumably in all the body cells, while in alkalosis cell Na/K might fall. On the other hand, any shift in cell pH compatible with life might be too small to affect the distribution of cations.

SUMMARY

When blood is stored at 37°, there is an active expulsion of Na from the erythrocytes with uptake of K, the processes being energized by glycolysis. It is thought that uptake of K is passive, though the possibility of an active factor has not been excluded.

When blood is stored in the cold there is progressive loss of K and a faster gain of Na with a consequent increase in the amount of cell base. Incubation in the presence of glucose reverses these cation movements, Na being actively expelled and K regained, in each case against the concentration gradient. Since expulsion of Na exceeds uptake of K, cell base which had increased during cold storage tends to return toward the normal level during incubation. These processes are accelerated by the maintenance of a slightly alkaline reaction during incubation; their result is to restore the physiological Na/K ratio in erythrocytes and plasma and to keep cell base and water constant. In their absence, the erythrocytes and, indeed, any cell unprotected by some external or internal resistant structure would burst because of the osmotic and electrical conditions imposed by its own protein.

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