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CATION CONTROL IN HUMAN ERYTHROCYTES

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It is well known that during cold-storage human erythrocytes lose K and gain Na in accordance with their respective concentration gradients. References to this work are given by Maizels (1943), who also shows that total cell base increases. If, now, these cold-stored cells are incubated cations move against the concentration gradients, their passage being activated by glycolysis, so that K enters the red cell and Na leaves (Harris, 1941). This work was extended by Maizels (1948; 1949) who has shown that loss of Na exceeds gain of K so that cell base and volume which have risen during cold-storage tend to return toward the normal during incubation and may even fall below it. Output of Na was shown to be active, and uptake of K either active or else passive and secondary to output of Na. It was further found that active cation movements in unbuffered solutions were maximal when the pH at the end of incubation was between 7·4 and 7, but owing to acid formation during incubation the former corresponds to an initial pH of about 8·2 so that the optimal pH may lie anywhere between 8·2 and 7. The present paper seeks to define these matters more clearly and also to define the effects of plasma Na and K concentrations on the active output of Na. It is therefore divided into the following sections: (*a*) The demonstration that uptake of K during incubation is not active but passive. (*b*) The investigation of the effects of external Na concentration on the active output of Na by erythrocytes. (*c*) Examination of the influence of external K on Na output. (*d*) The definition of the pH range of active cation movements in cells mixed with buffer solutions. In addition, it has seemed desirable to attempt to distinguish between real and apparent cation movements: thus, in the case of Na there is a real active output from the erythrocyte which is opposed by passive diffusion into the cells in accordance with physical factors; in so far as the former exceeds the latter so does output become apparent.

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Therefore, if the real output of Na is to be assessed, it is necessary to have a measure of passive diffusion, and this added to the apparent value would give the real value. An attempt to accomplish this is described below.

The principle of the method used is as follows: blood is cold-stored with solutions containing varying amounts of NaCl, tonicity being kept constant by the addition of LiCl. Since during cold-storage passive diffusion alone occurs, cell Na will vary with the external concentration and may be made at will either high or low. At the end of cold-storage these bloods are appropriately modified by further additions so that after incubation the effects of variations in external Na and K or of pH may be observed. All bloods are finally centrifuged: packed cells from cold-stored blood give the cold-storage levels; those from bloods cold-stored and then incubated with glucose, the active incubation levels; cells from blood incubated without glucose give the passive incubation levels. All these are compared with the findings in packed cells from the original fresh untreated blood which is used as the standard for changes in cell volume and cation contents.

METHODS

These are modifications of those used elsewhere (Maizels, 1949).

Mechanism of potassium uptake. Heparinized blood was divided into four 20 ml. lots, two lots being mixed with 40 ml. NaCl solution (0·15N) and two with 40 ml. LiCl solution (0·15N); to one of each pair glucose was added to 2%, the corresponding sample being glucose free. After 6 days' cold-storage half of each sample was centrifuged to provide the cold-storage base-line values, while the several residues were incubated with 2 ml. $Na_2HPO_4.2H_2O$ and 5 ml. KCl (0·15N); further, to the glucose-containing samples 0·38 ml. NaOH (0·45N) were added and to the glucose-free samples 0·38 ml. NaCl (0·45N), while to all samples 2 ml. water were added to restore the total cation content of the incubation plasma to the cold-storage level. After 20 hr. incubation all bloods were centrifuged and the cell deposits analysed. Lithium was taken as the difference between total base (determined electrolytically) and the sum of Na + K; it has an absolute error of ± 4 m.equiv./l. In this way two systems have been examined, one with high cell and plasma Na permitting free active and passive movements of Na and one with low Na values and restricted movements. The corresponding movements of K have been examined under these conditions.

Relation of cell and plasma sodium to active sodium output. The object here is to prepare sets of cells with varying Na concentrations, to incubate these in plasmas of varying Na concentrations and to see the effects of variations in cell and plasma Na on Na output. Citrated blood was mixed with 2 volumes of the following solutions: NaCl (0·15N), equal parts of NaCl and LiCl (0·15N) and LiCl (0·15N). Glucose was added to 2%. After 6–8 days' cold-storage the Na concentrations of the corresponding cells were respectively high, medium and low. Supernatant plasmas were now removed and the cells from each sample distributed as follows: one part was used as the cold-storage control and the rest divided and incubated for 24 hr. with 8–12 volumes of diluents whose Na contents varied but whose tonicity was maintained by the addition of appropriate amounts of LiCl. All diluents contained KCl, NaOH and phosphate as in the previous section and before the addition of cells sterile glucose was added to 0·5%. It should be noted that as a result of the technique used, cells with high Na values at the end of cold-storage have plasmas that are relatively rich in Na at the end of incubation. This arises as follows: the Na content of cold-stored cells is varied by varying that of the corresponding plasma; when the settled cells are transferred to the incubation fluid, there is a 'carry over' of 1–1·5 ml. intercellular plasma which will enrich or impoverish the incubation fluid. Further, during incubation Na-rich cells discharge much more Na into the external solution than do Na-poor cells. Hence, when cells are mixed with large volumes of

incubation solutions all of the same Na content, these solutions at the end of incubation show slight differences in their Na contents which vary directly with the contents of the added cold-stored cells.

Effects of potassium on the active movements of cations. 10 ml. lots of cold-stored citrated blood were incubated for 20 hr. with phosphate, NaOH and 20–25 ml. of a solution containing varying amounts of Na and K but with a total normality of 0·15; in some cases parallel experiments were conducted without as well as with glucose.

pH *and active cation movements.* Plasma was dialysed to remove glucose and the salt content restored by adding NaCl. Next, $Na_2HPO_4.2H_2O$ (0·1 M) was added in the proportion of 1 to 6·5 and the solution 'cleared' through paper pulp. To the heparinized filtrate KCl was added in the proportion of 1 to 7 and the product Seitz-filtered into large sterile bottles. Acid or alkali was added to give the appropriate pH. Experiments were done in parallel: to one batch 10 ml. cold-stored citrated blood (1 part of 3 % trisodium citrate to 9 of blood) containing glucose was added together with a further 2·5 ml. glucose (50 %); to the other batch of bottles glucose-free blood was added. Bottles were then incubated flat for 6 hr. after which the film of settled cells was collected, centrifuged and the packed cells analysed.

Three general points common to all experiments require further comment:

(1) Na and K contents are corrected for changes in cell volume by reference to unit volume of fresh unstored and unincubated cells.

(2) Concentrations of cell and plasma cation. Previously it was assumed (Maizels, 1949) that an average value for cell water sufficed to calculate cation concentrations. The composition of normal plasma varies but little; the cation content of cells from fresh blood varies between 105 and 125 m.equiv./l. with an average of 115. Since plasma and cells are in equilibrium, it follows that the cation concentration (though not the content) of fresh untreated erythrocytes must also be fairly constant and hence that cell water varies directly with the cation content; both, in fact, are determined by the haemoglobin content of the cells (Maizels, 1936). Cell water, then, has been calculated on the basis that water in fresh cells averages 69·5 % wt./vol. and cation, 115 m.equiv./l. Hence, if a batch of fresh cells has a cation content of 110 m.equiv., water is taken as $69{\cdot}5 \times 110/115$ or 66·5 %; should the volume of these cells increase by 15 % during storage the water content will now be $(66{\cdot}5 + 15)/115$ or 70·5 %. Calculation of plasma cation is carried out as described elsewhere (Maizels, 1949). Errors in the method for cell-Na and K concentrations are ± 3 % and for plasma concentrations ± 6 %. It follows that the concentrations of cell and plasma base are mainly of use in affording a clear but approximate indication of the concentration gradients with or against which cations move.

(3) Apparent and real cation movements. Estimation of these involves the measurement of the amount of Na which would enter the erythrocyte and of K which would leave in the absence of active movements, and this in turn involves the inhibition of glycolysis. Fluoride and iodoacetate were found unsuited to the purpose for their inhibitory effect increases progressively with the dose used, as did the haemolysis which was the usual accompaniment. Inhibition of active movements was finally attained by omitting glucose from a control sample both during cold-storage and incubation; this gives the passive incubation level. The difference between the cold-storage and active incubation levels gives the apparent output of Na, while that between the passive and active incubation levels might be expected to equal the true turnover. Unfortunately, the passive incubation samples are not perfect controls because: (*a*) cold-storage in the absence of glucose may adversely affect the nutrition and hence the permeability of erythrocytes. This effect is probably slight since, although there occurs during cold-storage a leakage of Na into the cells and of K out, this leakage is practically the same in cells stored with and without added glucose—at least for 6 or 8 days. (*b*) Interference with cell nutrition in glucose-free bloods, though slight during cold-storage, may well be more marked when glucose is absent during incubation: thus haemolysis in incubated glucose-free systems is perceptibly more than in glucose-containing systems (where it is often absent), and this may well have an adverse effect on permeability. (*c*) Bloods cold-stored without added glucose might still contain a residue of their original natural glucose, and this might permit a small degree of active cation movement during incubation. In practice, such residual glucose is insignificant (Maizels, 1949). (*d*) Bloods incubated with glucose become much more acid during

incubation, and this acidity inhibits active movements. Alkali is therefore added to the glucose-containing systems just before incubation until the pH is about 8. On incubation this shifts to about 7. Glucose-free systems without added alkali usually have a pH of about 7·1 at the end of storage and of 7 after incubation. Thus, although both systems reach roughly the same pH at the end of incubation, in the absence of glucose the shift is small, while in the presence of glucose it is considerable, and so the pH conditions of the two systems are not truly comparable. (*e*) The concentration gradient: the effects of this are discussed later.

RESULTS

Mechanism of sodium output and potassium uptake

To investigate this matter, one blood was stored and incubated with Na-rich plasma and a second with plasma poor in Na but rich in Li. At the end of cold-storage, Na in the cells of the first blood was high and in the second, low. Clearly, if there is uptake of K by the cells of the first blood where output of Na is large and active and not by the cells of the second blood where by virtue of the low cold-storage level, significant output of Na during incubation is not possible, then uptake of K, when it occurs, must be passive and secondary to output of Na. Experimental details are given in the section on methods and results are shown in Table 1.

TABLE 1. Incubation and exchange of sodium and potassium in sodium-rich and sodium-poor bloods

(Fresh original cells, K content 102 and Na 15 m.equiv./l.)

	Plasma			Cells								
	Concentration at start of incubation (m.equiv./l.)			Volume (% original)	pH at 20°	Contents (m.equiv./l.)			Concentration (m.equiv./l. cell water)		
Exp.	K	Na	Li			K	Na	Li	K	Na	Li
1*a* CSL G 0	6·5	139	0	104	7·02	64	56	0	86	75	0
b CSL G +	6·5	139	0	106	6·98	65	56	0	86	74	0
c PIL G 0	26	123	0	118	6·94	58	77	0	66	87	0
d AIL G +	26	123	0	100	6·86	88	20	0	126	28	0
e AIL G +	26	64	59	102	6·82	76	18	21	105	25	29
f CSL G 0	4·1	33	110	105	6·96	78	12	30	104	16	40
g CSL G +	4·1	33	110	108	6·95	81	9·7	34	103	12	43
h PIL G 0	25	42	83	118	7·03	76	18	43	86	20	49
i AIL G +	25	42	83	108	6·90	80	6	40	102	8	51

CSL = cold-storage level; PIL = passive incubation level; AIL = active incubation level. G 0 = no glucose added; G + = glucose added. Contents are referred to original cell volume.

If the data for Na-rich systems are examined it will be seen that during cold-storage Na rises and K falls with the concentration gradients and that total base (Na + K) and volume rise above the values in the fresh cells (1 *a*, *b*, Table 1). On incubation, erythrocytes from the sample containing no glucose (1 *c*) show an increased gain of Na, a small loss of K and a further rise in Na + K and in volume. The Na-rich systems to which glucose has been added, on the other hand, show a marked decrease of Na, a relatively smaller gain of K, in each case against the gradients and total base falls, as does cell volume (1 *d*).

In the lithium-rich sodium-poor bloods on cold-storage, Li increases in the erythrocytes and K falls, conforming in each case with the respective concentration gradients while Na falls *against* the gradient, the net result being a small increase in total base. Fall in cell K is less than in Na-rich bloods, presumably because the larger hydrated Li ion penetrates less freely than Na^+ and the escape of K in accordance with the concentration gradient is correspondingly restricted because of the osmotic requirements of non-penetrating cell anions (haemoglobin and organic phosphate).

At the end of cold-storage in Na-poor bloods then, cell Na is low, but even so there is a relatively great though absolutely small output of Na during subsequent incubation with glucose (cf. 1 *f*, *i*), the cell distinguishing between Li and Na so that the former so far from being expelled continues to increase with the concentration gradient. Under these conditions, that rise of cell K against the concentration gradient, which is observed in Na-rich systems, does not occur (cf. 1 *f*, *i*). Further, loss of Na from the cells of Na-rich systems incubated with glucose leads to a fall in cell total base and volume (1 *b*, *d*), whereas incubation of Li-rich systems leads to no such fall whether glucose be present or not (1 *f*–*i*).

Again, in another experiment cells were cold-stored in Na-rich plasma and then incubated in a Na-poor medium. Here, although cell Na at the start of incubation was high and active output marked, maintenance of cell base was mainly effected by the substitution for Na of Li (which entered the cell with the gradient) and much less by external K entering against the gradient (1 *e*); indeed, in some cases replacement of cell Na was effected entirely by Li. This also indicates that entry of K into red cells against the concentration gradient is secondary to active output of Na.

Another point emerges: on cold-storage of cells in plasma of high Na concentration (139 m.equiv./l.) the content of Na rises with the gradient (cf. Na in fresh cells and 1 *a*, *b*), while when external Na is only 33 m.equiv. Na leaves the cells even at low temperatures, against the concentration gradient, provided that the latter be not too steep. Further, it will be noted that during the cold-storage of glucose-free systems poor in Na, a small decrease of Na still occurs (cf. original cells and 1 *f*, *g*), and this must be ascribed to activation of ionic movements by a persistent remnant of the natural glucose present in fresh blood.

Relation of cell and plasma sodium to active sodium output

In most of our experiments we have been concerned with cells alone; in the present section our concern is with the precise relations of cells to plasma. Cells were suspended during incubation by a mechanical shaker in an incubating chamber and three samples of blood were so prepared as to give after cold-storage high, medium and low cell-Na contents. These were respectively incubated in media whose Na contents were high or low and sometimes high, moderate and low (see 'methods'). Results are shown in Table 2 and Fig. 1.

TABLE 2. Relation of cell and plasma sodium to sodium output

Exp.	Incubated plasma [Na]	Cold-stored cells		Incubated cells		
		Na content (m.equiv./l.)	[Na]	Na content (m.equiv./l.)	[Na]	Cell [Na] / Plasma [Na]
1*a*	126	61	81	30	41	0·325
b	122	40	53	26	37	0·304
c	116	19	25	24·5	33	0·286
d	63	61	81	14·5	20	0·317
e	58	40	53	13	17·5	0·303
f	55	19	25	11·5	15·5	0·283
2*a*	127	65	84	24	35	0·277
b	121	44	56	21·5	30	0·248
c	118	33	42	18	27	0·228
d	63	65	84	12·5	17·5	0·278
e	59	44	56	10	14	0·238
f	55	33	42	8·5	12	0·219
3*a*	124	41	53	17	23	0·186
b	120	22	30	15·5	20	0·166
c	116	10	13	13	17·5	0·152
d	63	41	53	8·5	12·5	0·197
e	59	22	30	7	10	0·170
f	54	10	13	6·5	8·5	0·160
4*a*	130	58	74	15	20·5	0·157
b	128	27	34	12	16	0·125
c	125	13	16·5	11·5	15	0·120
d	96	58	74	12	16	0·167
e	93	27	34	8·5	11·5	0·125
f	90	13	16·5	8·0	10·5	0·117
g	60	58	74	8·0	10·5	0·174
h	58	27	34	6	8	0·136
i	54	13	16·5	5·5	7	0·128
5*a*	134	65	77	20·5	26·5	0·198
b	130	35	43	17	23	0·177
c	127	14	17	16	20	0·157
d	97	65	77	16	20·5	0·212
e	94	35	43	12	15·5	0·164
f	91	14	17	10·5	13·5	0·148
g	62	65	77	10	12	0·194
h	58	35	43	7·5	9	0·157
i	55	14	17	7	8	0·146

Changes in cell K have been discussed in the preceding section. Na changes were as follows: when cells of the *same* cold-storage contents were incubated in solutions of *varying* Na concentrations, Na output varied inversely with the external concentration and the level to which cell Na fell after incubation varied directly with the plasma level (compare records *a* with *d* and *g*; *b* with *e* and *h*, etc., Table 2). This is to be expected, for the higher the external Na, the greater will be the passive backflow and the less the apparent output. When cells of *varying* cold-storage Na contents were incubated in solutions of the *same* Na concentrations (records *a–c*; *d–f*; *g–i*) it was seen that within each experiment the higher the cold-storage value, the greater was the output of Na. This also is to be expected for the higher the initial cell Na, the less is the

contrary backflow with which active output has to contend. As a result of the very high output of Na when cells of high sodium contents are incubated and the small output when initial cell Na is low, cells of varying Na contents at the beginning of incubation tend to approach the same low values at the end. Thus in Exp. 2 (Table 2) cell Na concentration falls by 49 m.equiv. to 35 in record *a*, and by 15 m.equiv. to 27 in record *c*, and there is a suggestion in Table 2, that values for cell Na at the end of incubation are tending to approach a constant relation to the plasma concentration. This matter is discussed again later.

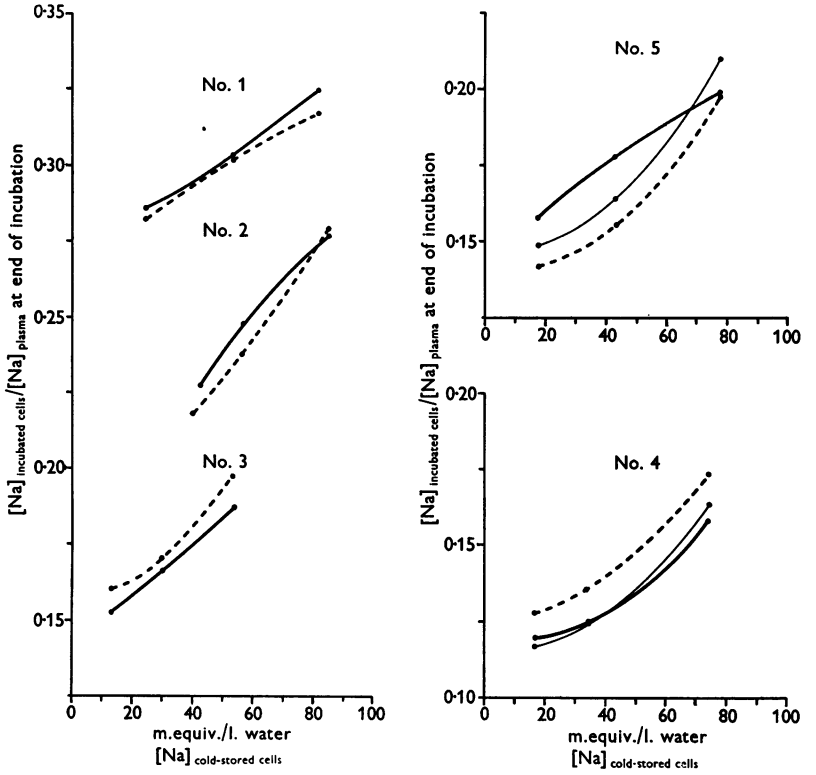

Fig. 1. Relation of $[Na]_{cells}/[Na]_{plasma}$ at end of incubation to $[Na]_{cells}$ at end of cold storage. ——, cells in plasma of high Na concentration; ——, cells in plasma of medium Na concentration; - - - -, cells in plasma of low Na concentration.

Effects of plasma potassium on the active movements of cations

The technique by which these were studied is described in the section on methods. In some cases cells were washed with saline before incubation to remove intercellular K. Results are shown in Table 3. The following observations may be made:

(1) During incubation in the presence of glucose K enters the erythrocytes and Na leaves in each case against the concentration gradient. Uptake of

K does not balance but is less than the output of Na. Thus, with a plasma K of 24 m.equiv./l., cell K rises by 14 m.equiv., from 71 to 85 m.equiv.; and 23 m.equiv. Na are discharged, while with an external K of 3·4 m.equiv. only

TABLE 3. External potassium and cation movements

(Cold-storage 6 days; incubated 20 hr. in Exps. 1 and 2; 6½ hr. in Exp. 3.)

Exp.	Final plasma concentrations (m.equiv./l.) K	Na	Volume (% original)	pH at 20°	Cells: Contents (m.equiv./l.†) K	Na	Cells: Concentrations (m.equiv./l. cell water) K	Na
1*a* CSL	20	151	102	7·14	71	48	97	66
b PIL K+	26	145	111	7·20	69	63	84	77
*c** PIL K−	1·5	172	104	7·13	57	67	76	89
d AIL K+	24	148	98	7·12	85	25	124	36
e AIL K+	9·8	165	92	7·03	83	25	132	40
f AIL K+	5·3	172	93	7·02	79	30	124	47
g AIL K+	3·4	174	91	7·00	74	32	120	52
*h** AIL K−	0·5	174	95	7·13	67	48	102	73
2*a* CSL	25	141	105	7·22	82	43	106	56
*b** PIL K+	27	128	120	7·15	82	61	89	67
*c** PIL K−	0·8	155	114	7·05	72	66	84	77
*d** AIL K+	25	132	102	7·10	97	21	132	29
*e** AIL K+	5	152	99	7·01	92	26	130	36
*f** AIL K+	2	153	97	7·01	81	34	118	50
*g** AIL K−	0·6	156	97	7·07	75	43	108	62
3*a* CSL	22	150	104	7·18	72	51	99	70
b PIL K+	3·8	162	109	7·36	74	54	95	70
c PIL K+	1·8	164	110	7·38	72	54	91	68
d PIL K+	1·2	165	108	7·38	70	56	91	73
e PIL K−	1·0	165	109	7·37	68	57	87	76
f AIL K+	3·5	162	103	7·30	86	34	119	47
g AIL K+	1·6	164	99	7·28	82	36	120	53
h AIL K+	1·1	165	99	7·31	78	39	114	57
i AIL K−	0·8	165	99	7·29	73	43	107	63

CSL = cold-storage level. PIL = passive incubation level; no glucose added. AIL = active incubation level; glucose added. K+ = K added before incubation. K− = no K added.

* = cells washed with saline before incubation.

† = contents referred to original cell volume.

In Exps. 1 and 3 phosphate was present in the plasma as a buffer. Plasma in Exp. 3 was dialysed and reconstituted.

3 m.equiv. K enter the cell and 16 m.equiv. Na leave (contents column, Table 3, records 1 *a*, *d*; 1 *a*, *g*). Further, when plasma K falls to a very low level apparent output of Na is much reduced and may cease altogether (1 *a*, *h*; 2 *a*, *g*; 3 *a*, *i*). It will be shown later that though apparent output of Na ceases, real output probably persists.

When active movements are suppressed by the omission of glucose, cell Na rises, but there is little loss of K during 20 hr. incubation (Table 3, 1 *a*, *b*; 2 *a*, *b*; 3 *a*, *b*) except in those systems designed to contain practically no external

K and even here loss of K is quite small—14 m.equiv./l., in Exp. 1 (*a*, *c*); 10 in no. 2 (*a*, *c*) and 4 in no. 3 (*a*, *e*).

(2) During incubation in the presence of glucose, cell volume and the content of base fall, although the external base concentration remains practically unaltered, at least in Exp. 1. Further, even when external K is so low that apparent output of Na falls, the cell content of Na + K is still below the cold-storage level (1 *a*, *g*; 2 *a*, *g*; 3 *a*, *i*).

In glucose-free systems cell base and volume rise during incubation, provided, as is usually the case, that non-penetrating anion within the cells exceeds that in the plasma (Table 3, Exps. 1 and 2). But in Exp. 3, where slowly penetrating inorganic phosphate was added to the external phase, rise in total base during incubation without glucose is slight. When increase of base does occur, it is most marked when external K is plentiful (see Table 3; passive incubation levels, records 1 *b*, *c*; 2 *b*, *c*; 3 *b*, *e*).

pH *and cation movements*

This investigation presents two difficulties. First, the apparent movement of Na or K is the resultant of a true active movement against the gradient and of passive movement with the gradient, and these may each be affected differently by pH; thus, the pH of maximum apparent movement is not necessarily the same as that of true active movement. In order to attempt the definition of both points it is necessary to conduct experiments with and without glucose at various pH. Curves obtained from glucose-containing systems show active movements and the differences between these and the cold-storage base-line values for Na and K give the apparent changes in cation distribution on incubation. Curves obtained with glucose-free bloods give the incubation values for passive diffusion. The second difficulty arises from the liberation of acid during incubation with glucose. Unbuffered systems may shift from pH 8 to 7, and it is not possible to say at which point active cation movements are maximal. Theoretically this might be achieved by means of a 'time curve', but in practice this is unsatisfactory because active movements persist over a wide range and the increments are too small and the experimental errors too great to permit of precise identification. Nor was the use of buffers wholely satisfactory. Thus, on incubating 1 volume of cells with 50 of plasma, the shift in pH was considerable after 24 hr. When plasma was replaced by phosphate (0·2 M) pH was more stable, the shift being 0·15 at pH 7·4 and 0·3 at pH 8. This method gave results in good agreement with the more physiological technique finally used of mixing 1 part of phosphate buffer with 6 parts of plasma and incubating 50 parts of this with 1 of cells for only 6 hr., at which time active movements were well marked. The pH shift under these conditions was quite small. Details of procedure are given in the section on methods.

The effects of incubating blood with plasma-phosphate buffer are shown in Fig. 2. The simple diffusion data are as follows:

(*a*) Acid shift in the absence of glucose is slight: less than 0·05 pH. The shift is probably due to the level of slowly penetrating inorganic phosphate in the plasma being greater at the beginning than at the end of incubation, and perhaps to the persistence of traces of glucose and of glycolysis in the so-called glucose-free systems.

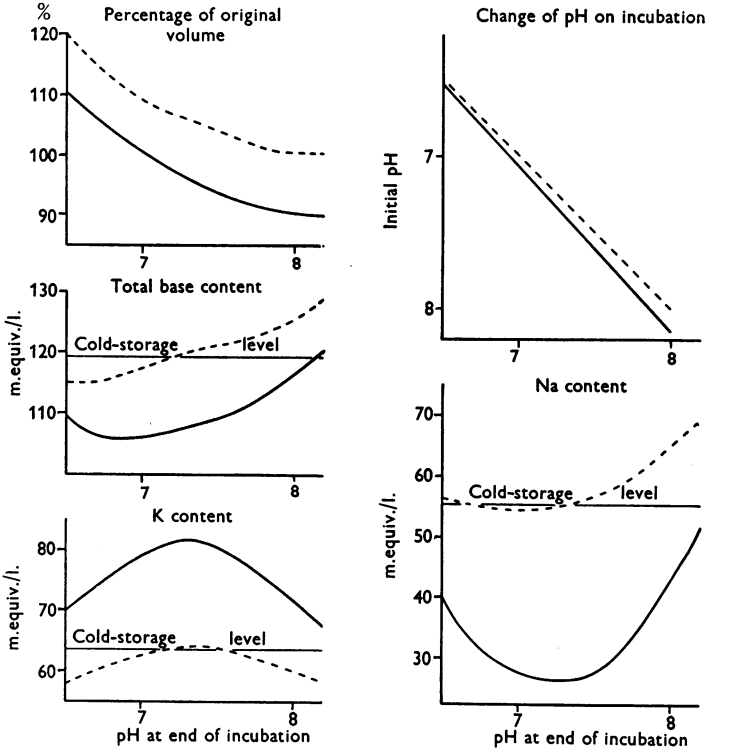

Fig. 2. Effects of incubation on pH, K uptake, Na output, total base and volume of erythrocytes. Cells in phosphate-plasma solution, 6 hr. Concentrations: K = 15, Na = 146 m.equiv./l. ————, glucose added (active incubation); – – – –, glucose not added (passive incubation).

(*b*) Little Na diffuses into the erythrocytes and little K escapes during the short period of incubation, except at high pH where Na rises and K falls, suggesting increased permeability to cation at high pH. Similar changes, probably due to cell damage, are seen below pH 6·5.

(*c*) Cell volume increases with fall of pH because less cell base is combined with haemoglobin and more with osmotically active anions.

(*d*) Total base, Na + K, increases with rise of pH owing to increase of base bound by cell Hb. Intra- and extracellular base concentrations are about the

same at pH 7·4 when cell Hb^- and $phosphate^-$ are presumably balanced by the slowly diffusing $phosphate^-$ of the plasma.

In glucose-containing systems the following observations may be made at 6 hr.:

(*a*) Acid shift is small particularly below pH 7·5.

(*b*) There is a considerable apparent output of Na over a wide range between 6·5 and 8·2 (difference between cold-storage and active incubation levels). Between pH 7 and 7·6 the base of the curve is almost flat with a probable minimum at about 7·3.

(*c*) There is a considerable uptake of K against the concentration gradient over a wide range with a maximum at about pH 7·3.

(*d*) The curve for the active incubation levels of total cation falls with pH to a flat base between 7·4 and 6·6. The cause for the general low level lies in active cation movements, with output of Na exceeding uptake of K. As a result of this, total base in samples incubated with glucose is less than in glucose-free systems or in the cold-stored controls. A further factor in the fall of total base at low pH is decreased base binding by haemoglobin. Increase of total base at very low pH (below 6·6) must be ascribed to failure of the Na output mechanism in this range. As a result minimum values for cell base are observed at about pH 7. Cell-base *concentration* rises progressively with pH.

(*e*) In accordance with the change in total base, cell volume on incubation with glucose is considerably less than when glucose is absent and at a corresponding pH 3–5% less than the volume of cold-stored cells.

It would, therefore, seem that active cation movements are most effective between pH 7·2 and 7·4 or, allowing for acid shift during incubation, between 7·2 and 7·45. They are, however, almost as effective over a much wider range—between 6·85 and 7·65.

DISCUSSION

It is now well known that the erythrocyte membrane is freely permeated by cations, yet during life the cation content of the cells is kept constant. This constancy is dynamic for there are some forces leading to passive inflow of cations into the red cells and others leading to active discharge. It is assumed that the sites of these movements in opposite directions are distinct; that there are areas for passive diffusion and others for active output. The concentration gradient tends to send Na into the cells and K out, while all ions are attracted by the non-penetrating anions within—haemoglobin and organic phosphate. Since, apart from these two, all other cations and anions are, with unimportant exceptions, permeant, the tendency to achieve an equilibrium of the kind described by Donnan will, unchecked, lead to a progressive entry of salts and water into the cells until ultimate rupture occurs. This process is opposed under physiological conditions by active output of Na, and also *in vitro* when blood is incubated with glucose.

If glucose be absent from the system, however, the ion-attracting properties of cell non-penetrating anion are clearly shown by a tendency for cell base and volume to increase during cold-storage and especially during incubation (see Table 1, 1 *a–c*; and Table 3, 1 *a, b*; 2 *a, b*). The tendency is slight in Exp. 3 (Table 3) because of opposition by the slowly penetrating inorganic phosphate added to the external phase. Attraction by cell non-penetrating anion is further shown by the slowness with which K escaped from cells even when cell K is high and plasma K low (Table 3, 1 *a–c*; 2 *a–c*; 3 *a–e*). Again, increase of cell base in glucose-free systems must always occur at the expense of plasma base. Thus, if batches of red cells are incubated with plasmas of decreasing K concentrations in the absence of glucose, fall of external K leads to increased loss of K from the cells, though rise in total base still occurs. If this rise were the same in all cases, increased passive entry of Na would be needed in low plasma K systems, but in fact though increase of cell base occurs in all cases, it is less in the low plasma K systems, presumably owing to the relatively slow penetration of Na^+. If, however, for any reason the cell is damaged, as by iodoacetate, permeability is much increased: fall in cell K is marked, but rise in cell Na is very marked and so cell base rises considerably (Table 4).

TABLE 4. Effects of iodoacetate on cation exchange

	Plasma Concentrations (m.equiv./l. water)			Cell				
							Contents (m.equiv./l.)	
	K	Na	Iodoacetate	Volume (% original)	pH at 20°	Lysis	K	Na
Cold-storage level	20	145	0	100	6·75	0	64	47
Active incubation level	26	132	0	97	6·64	0	79	24
Active incubation level	26	132	2·5	103	7·53	Trace	44	82
Active incubation level	26	132	5	111	7·40	Much	40	96

(Glucose added to all samples.)

That this increase of cell base so evident in glucose-free systems is opposed by active cation movements when systems are incubated in the presence of glucose is shown by the fall in total cell base which occurs (see especially, Table 1 and Table 3, Exp. 1, where plasma-base concentration was about the same during cold-storage and incubation). But since the fall in cell base is due to output of Na, this output must be active; indeed it could in no way be secondary to active uptake of K, for with such uptake against the gradient through active areas of the cell membrane, Na should still diffuse *into* the cell through passive areas of the cell membrane in response to the attraction of cell non-penetrating anion. Actually, Na is expelled even in the absence of K uptake (Table 3, 2 *a, f*; 3 *a, i*).

Uptake of K could still be either active or passive, and direct evidence in favour of the latter is given in Table 1, where cells are cold-stored with plasma poor in Na and rich in Li. Here the cells contain so little Na at the end of

incubation that output of Na, though relatively great, is absolutely small, only 3 or 4 m.equiv./l. (*g* and *i*); under these conditions there is no output of Li and no uptake of K. This shows that human erythrocytes distinguish between Na and Li, actively excreting only the former. In the absence of output of Na there is no call for reciprocal passive uptake of K, though if uptake of K were indeed active, some would still enter the cells. In fact, Li enters the erythrocytes and K leaves in conformity with their respective concentration gradients. It is of interest to note that active output of Na on incubation in the presence of glucose is much more rapid than passive input when glucose is absent. Thus in Table 3, Exp. 1, passive diffusion equals 15 m.equiv. in 20 hr., and active output is 23 (*a*, *b* and *d*) while in Exp. 3 the figures are 3 and 17 (*a*, *b* and *f*). It is this powerful outward drive of Na which causes the fall in cell base and volume during incubation with glucose and which is the *in vitro* expression of that mechanism which *in vivo* keeps cell volume and base constant. Cell base during *in vitro* experiments falls because Na output exceeds passive K uptake. It follows that electrical neutrality is maintained only in part by an exchange of cations and that some of the Na excreted from the cell must be accompanied to the plasma by diffusible cell anion.

It would thus appear that output of Na is dominant in maintaining the level of cell base, and the effects of other influences on this dominant factor were therefore studied—level of plasma and cell Na, plasma K and cell pH.

Relation of sodium output to cell sodium and plasma sodium

It may be assumed that in a system containing a large excess of plasma, if active output proceeded to equilibrium, the Na concentration, of the cells would be related purely to the plasma concentration, and there is indeed a suggestion in Table 2 (more especially, perhaps, in Exps. 1, 4 and 5) that under ideal conditions the value $[Na]_{cell}/[Na]_{plasma}$ might be constant. However, it is clear that under experimental conditions the cold-storage value of cell Na has an influence on the incubation concentration, a large change in the former being associated with a small change in the same direction, of the latter. This is not surprising. The cell has to excrete not only Na constantly diffusing into it from the plasma through passive areas of the cell membrane in amounts depending on the plasma level, but also Na already present in the cell at the beginning of incubation, and the concentration of the latter which has been made to vary between 13 and 84 m.equiv. is by no means negligible. In order to evaluate the influence of cell Na at the beginning of incubation on the value at the end, the ratios of cell- and plasma-Na concentrations, $[I]/[P]$, have been plotted against the Na concentrations of the corresponding cold-stored cells, $[C]$. In Fig. 1, the relation $[I]/[P]$ to $[C]$ is shown for plasmas of high, medium and low Na concentrations. It must be noted that a high degree of accuracy was not possible in fitting curves, partly because it was not practicable to

investigate more than three cold-storage levels, partly through difficulties in reproducing exact pH conditions in each blood sample incubated, and partly through increasing error in the Na method when low contents were estimated. However, the following conclusions seem justified: if one accepts, what is probable, that at equilibrium in an ideal system $[I]/[P]$ is constant, then the degree of obliquity of the curves to the X-axis is an index of the functional activity of the cells, for the less the obliquity, the less will be the influence of cold-storage Na on the value at the end of incubation. Further, if at equilibrium $[I]/[P]$ were constant, then the distance of the corresponding horizontal line from the X-axis would be an index of the balance between cell permeability and speed of passive diffusion on the one hand and activity of output on the other. This indication will also be gained by extrapolating the curves of systems not in equilibrium in Fig. 2, to $[C]=0$, and the values of $[I]/[P]$ so obtained will mainly be an index of cell permeability, for when cell Na falls from a high level against the concentration gradient, the chief factor is active output; when it rises from a low level with the gradient, passive diffusion predominates. In support of this view, it will be noted that in Exps. 1 and 2, where incubated cells were not kept suspended by rocking and where active output of Na produced a local rise of Na round the cells, thus increasing back diffusion, the heights of the curves above the X-axis were also much increased.

But although it is likely that at true equilibrium $[I]/[P]$ would be constant, under experimental conditions this constancy is not attained. Preliminary investigations indicate that cell Na falls sharply for 9–15 hr. and then for the rest of the 24 hr. incubation, alters very little, $[I]$ remaining constant for each individual system, while the various values of $[I]/[P]$ for the constituent systems of each experiment are not quite identical, being still somewhat dependent on $[C]$. It is suggested that several days would be required for $[I]/[P]$ to become constant and that long before this the activating mechanism has begun to fail, so that although after 12 hr. incubation it still suffices to keep cell Na at a constant low level, it is unable to lower the level still further to that point at which $[I]/[P]$ is constant. Such weakening of output activity might be due to decay in the phosphorylation cycle, since after 24 hr. incubation, although glucose is still present in the cell, organic phosphate has fallen from 95 to 30% of the total phosphate.

If, however, at equilibrium $[I]/[P]$ is in fact constant, then $([P]-[I])/[P]$ is also constant, and in Table 2 this relation roughly holds though the experiments there are not presumed to have reached equilibrium. This probably arises because the systems are approaching equilibrium and partly because in the fraction, $[P]$ is relatively great, while $[I]$ is small with variations which, though relatively moderate, are absolutely small. It is probable that fuller study of the dynamics of output (with special reference to Na permeability as measured by trace and to the effects of variation of lithium in the external phase)

would clarify the problem; at present it can be said that $[Na]_{cell}$ after incubation is mainly determined by $[Na]_{plasma}$, but owing to failure of the system to reach equilibrium, is affected to some extent also by $[Na]_{cell}$ at the start of incubation. *In vivo*, however, absence of metabolic failure ensures a constant relation of cell Na to plasma Na, the balance struck between active output and passive diffusion determining individual and species variations in cell Na and cell K.

Effects of plasma-potassium concentration on active sodium output

It has been seen that when erythrocytes are incubated in a medium containing glucose but poor in K, apparent output of Na falls; this is because when external K is not available as a compensating cation, cell anion attracts Na which diffuses back passively into the cells as fast as it is actively excreted. It may be questioned if real output also fails or whether sufficient output persists to overcome in whole or part the tendency for Na to diffuse into the cells, and since in Exp. 2 (Table 3) cell Na rises by 23 m.equiv./l. in the absence of glucose (contents column *a* and *c*), while in the presence of glucose no Na enters (*a* and *g*), it seems likely that even when plasma K is very low and no apparent output of Na is seen, some real output still occurs. However, it was said when considering 'methods' that the glucose-free control is not always valid for various reasons, of which the chief was the divergence of its pH during incubation from that of bloods containing glucose. This objection in the present context is not serious, since the real outputs compared are in each case the difference between values in a glucose-free system whose pH shifts from 7·1 to 7 and in a glucose-containing system with a shift from 8 to 7, so that although each real output may not have an absolute meaning, one has a significance relative to the other. In any case in Exp. 3, pH was similar in all systems owing to the use of buffered plasma. But one difficulty still remains: during the incubation of glucose-free blood the concentration gradient for Na falls throughout incubation, while with glucose present it rises, so that passive back-diffusion in the latter, though not manifest, might well be greater than in the former. On the other hand, it may be that passive return of Na in glucose-containing systems is restricted by the simultaneous inward diffusion of K through the same areas of the cell surface, and that concealed back-diffusion in the presence of glucose does not differ appreciably from simple manifest diffusion in the absence of glucose. If this were so, then in Exp. 3 of Table 3, in addition to the obvious fall in apparent output of Na as external K falls, there would also be a fall in real output, which with high external K was 20 m.equiv. (*b* minus *f*) and with low external K was between 14 and 18 m.equiv. (*e* minus *h* or *i*). So too in Exp. 2, with high and low external K, the respective real outputs were 40 (*b* minus *d*) and 23 m.equiv. (*c* minus *g*). The question arises if in the complete absence of external K, real output would fail altogether. If so, the glucose-free

control would be fully valid because passive diffusion alone would be occurring both in glucose-free and glucose-containing systems. Unfortunately media cannot be made K free, for if cells are washed free of intercellular K and suspended in K-free media, a little K soon enters the medium either by lysis of cells or simple leakage, Thus in Exps. 1 *c*, *h*, 2 *c*, *g* and 3 *e*, *i*, external media were originally K free but acquired a little K by the end of incubation. A solution might be attempted by plotting so-called real output against external K and extrapolating to K = 0, but points on the curve are too few and the lowest values for K too high for this to be possible. However, it seems likely that real output of Na decreases as external K falls, and that though output does not depend on uptake of K by the cells it is in some way potentiated by the presence of K in the plasma; but a conclusive statement cannot be made until passive movement of Na can be measured, while active output is proceeding and this may require the use of radioactive Na.

It does, however, seem quite definite that although apparent output of Na fails when external K is low, total base and volume after incubation are still below the cold-storage levels because there is no uptake or actual loss of K. Thus, in Exp. 1 of Table 3, the cold-storage level of total base is 119 m.equiv./l., while the incubation levels are 110 with high plasma K and 115 with low (1 *a*, *b* and *h*). In Exps. 2 and 3 the findings are similar. Thus the volume and base control mechanism still works effectively even when output of Na seems relatively inactive, so that the ratio of Na/K in the cell does not fall as low as usual after incubation.

pH *and cell cation*

It has been seen earlier that apparent active cation movements are most marked between pH 7·2 and 7·4. It is probable that passive movements in the presence of glucose are qualitatively, if not quantitatively, similar to diffusion in the glucose-free controls. Since the gap between the two curves is widest between pH 7·2 and 7·4, it follows that real output is most active in this range (Fig. 2). But when considering the maintenance of the constancy of cell base *in vivo*, it is the apparent and not the real cation exchange which is effective. It has been seen that this is maximal, or nearly so, over a wide range between 6·85 and 7·65. Normal pH measured by similar methods is 7·2–7·4 at 20°, and it is unlikely that in acidosis or alkalosis cell pH will transgress the range of maximum cation activity. Nevertheless, Butler and his co-workers (1947), investigating diabetes, and Darrow and his associates (1948), in infantile diarrhoea, have found that with severe acidosis, there is a loss of K and water from the body. It must be presumed that the very actively metabolizing tissue cells are more adversely affected by acidosis than are the erythrocytes, and that they lose K in circumstances which leave the red cell almost unaffected.

Control of cation in erythrocytes

It has been seen that major factors in fixing cell base are the concentration gradients sending Na into and K out of the erythrocytes, non-penetrating cell anion attracting all ions and also water, and active output of Na and water removing base from the cell, but also tending to raise the concentration of cell non-penetrating anion and so increase backflow of penetrating ions. Subsidiary factors are the pH of the cells and the levels of plasma Na and K. In man the ratio *in vivo* of $[Na]_{red\ cells}/[Na]_{plasma}$ is about 0·13, and in successful experiments similar ratios may be obtained on incubation *in vitro*. But constancy of cell base and volume may also be found in systems with low plasma K, where the cell-plasma [Na] ratio is high because of increased back-diffusion of Na and cell K low. Such a system would give incubation curves of the type shown in Fig. 1, but placed higher above the base-line, even with rapidly glycolysing and respiring cells. It is possible that the erythrocytes of certain species (ox, dog, cat, etc.) with a high cell-plasma [Na] ratio and low cell K are similar to these experimentally modified human cells in having a powerful active output largely neutralized by an increased passive backflow of Na; the latter due to a low level of plasma K or to high cell permeability. On the other hand, one may conceive of systems in which the cell-plasma [Na] ratio is high not because of raised permeability, but because output is low, in which case constancy of cell base would be achieved with a real saving in work done. Incubation curves from such cells would be more oblique than those in Fig. 1 and cell glycolysis would be small and respiration slight.

However this may be, without some such mechanism for controlling the entry of salts and water into cells incapable of resisting an internal pressure, rupture is inevitable. The mode of activation is still unknown; its dependence on glycolysis is clear and work in hand shows that it is inhibited by fluoride and iodoacetate, but not by carbon-monoxide or cyanide.

SUMMARY

1. When human blood is cold-stored cell Na rises and cell K falls in accordance with the respective concentration gradients. Total base increases.

2. On incubating such cold-stored cells in the presence of glucose, Na leaves the erythrocyte and K enters against the concentration gradients, the value for Na + K falling. The result of these actions in the intact circulation is to prevent that accumulation of salts and water which would otherwise occur because of the excess of cell over plasma non-penetrating anion. Exit of Na is active and uptake of K passive.

3. When red cells take up lithium from an appropriate diluent during cold-storage, this is not actively excreted during incubation.

4. Output of Na is very active between pH 6·85 and 7·65, the actual maximum lying between 7·2 and 7·4. It is unlikely that pathological variations in the

pH of erythrocytes suffice to cause significant changes in the balance of cell Na and cell K, but this does not exclude the possibility of similar pH changes in more actively metabolizing tissue cells causing serious cation disturbance.

5. The level to which cell Na falls on incubation depends chiefly on the plasma-Na concentration and to a less extent on the value of cell Na at the beginning of incubation. Active output and passive backflow balance in man when the red cell-Na concentration is about one-eighth of the plasma concentration.

6. When erythrocytes are incubated in plasma poor in K, output of Na falls.

7. A distinction has been drawn between apparent and real output of Na. The former is the effective output and equals the difference between cell Na at the beginning and end of incubation. Real output includes apparent output together with that output of Na which opposes and overcomes the tendency for Na to enter the erythrocytes by simple diffusion.

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