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SODIUM TRANSFER IN HUMAN AND CHICKEN ERYTHROCYTES

By EVELYN M. CLARKSON and M. MAIZELS†

From the Department of Clinical Pathology, University College Hospital, London

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Little is understood of the precise way in which cations are actively transported across cell membranes, though in the case of the mammalian erythrocyte more is known of the relations of cation transport to the Na and K concentrations within and without the cell, and of the metabolic basis of transport (for references see Solomon, 1952; Harris, 1954; Maizels, 1954*a*). Thus active transport in non-nucleated erythrocytes is energized by glycolysis, and though unaffected by respiratory poisons (Maizels, 1951), is inhibited by fluoride (Harris, 1941; Maizels, 1951). Avian erythrocytes, on the other hand, are nucleated and respire, and here cation transport is based mainly, if not entirely, on respiration (Maizels, 1954*b*). The present paper is concerned with cation transport by chicken erythrocytes, and especially with the effects of certain physical factors on transport, and with the relations of Na transport to the glycolytic and respiratory activities of the cells.

Na* (^{24}Na) has been used in the study of sodium transport, and the paper falls into two parts which follow the general section on methods. The first part deals with the technique of measuring Na and Na*, and with the calculation of transfer constants. The second is concerned with the transfer constants for Na in human and chicken erythrocytes under a variety of conditions.

METHODS

As the experiments involved the measurement of cell pH, haemoglobin, Na, Na* and lactate, the use of large amounts of radioactive material was avoided by putting up similar and parallel systems differing only in that one contained Na* while the other did not. A further advantage of this procedure arose from the fact that if radioactive cells are to be examined for pH, haemoglobin and Na content they are best left until radioactivity is dissipated: but if packed chicken erythrocytes are so treated they form an adherent mass which cannot properly be resuspended. In a few cases where Na was estimated in parallel systems, one containing Na* and the other tracer-free, good agreement was obtained between the two sets of results. The actual procedure was as follows:

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blood was centrifuged in tubes of 6 mm bore, the buffy coat removed, and the cells resuspended in original plasma. This procedure was twice repeated, and the standard cell suspension prepared consisting of 1 vol. of cells, two of plasma, and seven of a medium containing 155 m-equiv Na, 10 m-equiv K, 6 m-mole phosphate, 155 m-equiv Cl and 11 m-mole glucose/l. When oxygen consumption was measured, however, the suspension contained 1 vol. of cells and two of plasma, K being added to 10 m-equiv and glucose to 11 m-mole/l. In either case the suspension was divided, radioactive sodium carbonate neutralized with HCl being added to one portion, and a corresponding amount of NaCl to the other. About 10 min before an estimation was due, a large volume of tracer-free suspension was centrifuged, the pH of the deposit being measured, while the supernatant was used to wash the corresponding radioactive cells free of intercellular tracer. Samples for the estimation of Hb, Na, etc., in unwashed cells were centrifuged for 30 min at 2000*g* in tubes of 15 mm bore whose lower ends were drawn out to sealed graduated capillaries of about 1·7 mm bore. If these tubes had been accurately calibrated and the volume of the packed cells was read with a hand-lens, the error in this measurement did not exceed $\pm 0{\cdot}25\%$. The rest of the tube above the packed cells was carefully washed, and the cells were resuspended with a capillary pipette in 74 vol. of water. The haemolysate was homogenized by shaking vigorously, the wide end of the tube being closed by a thumb covered with a carefully cleaned rubber finger-stall. 1 ml. of the haemolysate (which became quite viscid on standing) was made up to 10 ml. with very dilute ammonia, and the absorption of light compared in a photo-electric colorimeter with that of a similar dilution prepared from original untreated cells. Where it was desired to wash tracer-free cells, the suspension was centrifuged for 5 min, supernatant replaced by KCl solution (0·165 M), the cells resuspended in the wash-fluid, and the tubes recentrifuged for 30 min; subsequent procedures were as already described.

The water content of human cells has been taken as 70% (w/v) (Maizels, 1936) and the figure for chicken cells is similar (Maizels, 1954*b*), the range being 68–72%. The figure of 70% requires correction for water in the intercellular plasma and the actual value used in the present paper was 69% (w/v). The assumption of a mean figure of 69% introduces a possible error of $\pm 3\%$ in calculating cell concentrations, but since the concentrations of Na and Na* are similarly affected, the effect on the transfer constants may be neglected.

Lactate was estimated by the method of Hullin & Noble (1953).

Chloride was estimated by the method of Claudius (1924). The error was ± 1 m-equiv: the data obtained were used as a measure of the Donnan asymmetry (Harris & Maizels, 1952).

pH was measured with a glass electrode at 20° C on a fivefold haemolysate of cells. Results differed from the pH of the corresponding whole haemolysates by only $-0{\cdot}04$ in six observations (range $+0{\cdot}02$ to $-0{\cdot}06$), but may well have differed from the pH of the intact cell. However, it afforded an adequate means of controlling cell pH during an experiment.

Sodium was measured with an EEL flame photometer. Since the proportion of K to Na in the cells investigated was very high, the effects of comparably high K contents in the Na standards was investigated: with 'Specpure' KCl the error introduced was less than 1%. It was therefore considered legitimate to use simple Na standards without the addition of complementary amounts of K. It is customary to measure the Na content of human cells on haemolysates and the propriety of this procedure was investigated. An haemolysate was dialysed for a week against repeated changes of neutral distilled water and then Na standards were prepared using as diluents the dialysed cell solution, the final dialysate, and distilled water: almost identical readings were obtained on all three (Table 1). Chicken erythrocytes present special difficulties because within a few minutes of the addition of water, the haemolysate becomes viscous and ropy and cannot be atomized. Hence, in earlier techniques it was found necessary to lyse cells in water, and then to precipitate the proteins by the rapid addition of 1 vol. of trichloroacetic acid (50%, w/v) to 10 of the 75-fold dilution of cells. The mixture was then centrifuged and the supernatant passed through a sintered glass filter. To test the adequacy of this procedure, a simple haemolysate of human cells was compared with Na standards in dialysed cell haemolysate and in water, while Na in a trichloroacetic acid filtrate was compared with standards containing comparable amounts of

acid. It was found that the Na contents measured by all three methods were similar, and differed by less than 1% for six samples whose actual Na contents ranged from 0·08 to 0·15 m-equiv/l. In the case of deproteinized samples, however, certain precautions were necessary: trichloroacetic acid depresses luminosity (Bernstein, 1952), and since its addition to cells to give an overall content of 4·5 g % results in a filtrate whose acid content by titration corresponds to only 3·75 g %, Na standards must be made in 3·75 and not in 4·5% trichloroacetic acid solution. Unless this is done, the Na standards will read too low (Table 1) and the 'unknowns' seem too high. Later it was found that if nuclear remnants and stromata were removed by centrifuging at 2000 *g* the supernatant from an haemolysate of chicken erythrocytes gave results comparable to those obtained on a trichloroacetic acid filtrate. Thus, five of six paired observations differed by less than 1·5%, and the sixth by 2·8%. If polythene containers are used wherever possible, duplicate readings usually agree within one division; differences exceeding this are probably due to contamination rather than to other technical errors, and duplicates should differ by less than 0·2 m-equiv/l. cells.

TABLE 1. Readings in instrument divisions of Na standards in various diluents. Instrument set throughout for standards in distilled water

	Na standards (m-equiv/l.)			
Diluent	0	0·067	0·133	0·267
Water	0	31	54·5	100
Dialysed haemolysate	0·5	30·5	54	100
Final dialysate from above	0	31	55	100
Trichloroacetic acid 5 g/100 ml.	1·5	25·5	47	86
Trichloroacetic acid 3·75 g/100 ml.	1·5	26	48·5	90

Radioactive sodium (Na*). The entry of Na* into erythrocytes was measured in a standard suspension of cells with a content of Na* corresponding to between 5×10^5 and 1×10^6 counts/min per ml. external phase. At suitable intervals 1 ml. cell suspension was centrifuged for 5 min, the cells resuspended in 5 ml. wash-fluid (supernatant from the corresponding tracer-free suspension) and recentrifuged for 30 min. Cell volume was now read (about 0·1 ml.), the tube carefully washed down to the level of the packed cell column, and the latter transferred to the liquid counter (20th Century Electronics, Type M6). In the case of human cells transference could be in water or in saline, and the occurrence of lysis made no difference to the counts; nor indeed were the counts of a simple cell-free medium affected by the addition of tracer-free cells. However, chicken erythrocytes which yield a viscous haemolysate were always counted unhaemolysed in NaCl solution. In the case of cells containing tracer, runs were made totalling 10,000 counts for earlier samples and upwards of 15,000 for the later ones.

It will have been noted that where cells were washed, KCl solution was used for tracer-free suspensions, and plasma for cells containing Na*. But if there was sufficient material left from the previous week, it was thought better to add KCl to dialysed plasma (to 0·155 M) and use this as a wash-fluid for the cells of all systems.

Cell respiration. In experiments involving measurements of O_2 uptake, 1 part of cells was suspended in 2 parts of diluent; measurements were made in triplicate. In earlier experiments it was found that the O_2 consumption during the first half hour was higher than it was thereafter, while in the presence of high concentrations of cyanide, arrest of respiration appeared to be considerably delayed. In spite of the fact that all suspensions in the Warburg apparatus were allowed to equilibrate for 15 min before observations were begun, it seemed likely that the method involved some error, a view which was supported by the fact that human erythrocytes similarly treated appeared to consume considerable amounts of O_2 during the first 15 or 30 min of incubation. It was surmised that after blood had been placed in the flasks, escape of CO_2 was at first quicker than absorption by KOH in the well, and that later this relation was reversed, absorption of CO_2 being interpreted as uptake of O_2. It is also possible that uptake of O_2 by cell haemoglobin not fully at equilibrium with atmospheric oxygen, simulated utilization for metabolic purposes. Such differences would be much less with tissue fragments than with 3 ml. blood rich in CO_2,

$NaHCO_3$ and haemoglobin. However this may be, a method was sought which without unduly damaging the transporting mechanism, would remove CO_2 and oxygenate cell haemoglobin before suspensions were placed in the Warburg flasks. The technique (one of many) finally adopted gave only minimal apparent uptake of O_2 when tested against human cells (less than 5 μl./ml. cells.hr). 75 ml. blood was brought to pH 7, extracted five times *in vacuo* and then rocked at room temperature in a large flask, fitted with a well containing KOH (10%) into which dipped a large fluted filter-paper. After 20 min the blood was centrifuged, supernatant plasma and buffy coat being removed, and the process of centrifuging and removal of the buffy coat repeated. Meanwhile, the removed plasma had been brought to pH 6, the CO_2 aspirated *in vacuo* (12 changes) and the pH restored to 7·5. 1 part of cells freed from leucocytes and CO_2 was then mixed with 2 parts of treated plasma and the whole brought to pH 7·5. The material was now ready for transfer to the Warburg flasks, the usual 15 min at 38° C being allowed for equilibration.

PART I. MEASUREMENT OF TRANSFER CONSTANTS

Transfer constants are best calculated from the exchange of Na and Na* between cells and a suspending medium, and for the figures to approach any sort of absolute value, it is necessary that the true concentrations of cell Na and Na* be known, and hence that a suitable allowance be made for these in the intercellular plasma.

Estimation of the true concentrations of Na *and* Na* *in erythrocytes*

Corrections for Na and Na* trapped between packed cells may be effected either by washing the cells with KCl solution or by making a theoretica deduction, if the amount of intercellular plasma be known.

In the present investigation Evans Blue (T1824) was used as a 'marker' for intercellular plasma. Technical details are given by Maizels (1945), Jackson & Nutt (1951) and Chaplin & Mollison (1952). Intercellular plasma of erythrocytes centrifuged at 2000*g* for 30 min was found to be $1{\cdot}88 \pm 0{\cdot}15$% of the packed cell volume (range in seven cases, 1·6–2·22%), figures which agree with the results of workers already mentioned. It may be noted that when cells were suspended in a mixture of dye and NaCl solution instead of dye and plasma, intercellular fluid appeared to be about twice as great, either because of increased penetration, or adsorption of dye in the absence of plasma proteins; addition of as little as 10% of plasma to the suspending medium again reduced intercellular fluid to about 2% of the packed cell mass. In the case of chicken erythrocytes which haemolyse rather easily in plasma-free wash-fluids, Evans Blue trapped between the cells became discoloured on extraction with NaCl solution, and glycogen was preferred as a marker (10% in 0·165 M-NaCl). It was estimated in the saline wash by boiling with KOH, precipitating with alcohol, and estimating as glucose. Results were somewhat variable, $3{\cdot}0 \pm 0{\cdot}4$% (range in seven cases, 2·5–3·6%).

It follows that if human erythrocytes are washed with KCl solution they should lose about 3 m-equiv Na/l. cells, while if centrifuged immediately from media containing Na* they should gain an equivalent amount of tracer, and

lose the same amount on being washed in tracer-free solutions. Similarly, the average loss on washing chicken erythrocytes should be about 4·5 m-equiv Na/l. cells. These expectations are not fulfilled.

Effects of washing erythrocytes. Fresh human erythrocytes contain about 10 or 12 m-equiv Na/l. When washed in 50 vol. of KCl solution (0·155–0·165 M) and immediately centrifuged, they lose about 5 m-equiv: the mean loss and its standard deviation were $5{\cdot}12 \pm 0{\cdot}22$ m-equiv/l. cells. Resuspension and re-centrifuging result in a further loss of about 0·25 m-equiv, and if the process be repeated, the third loss is only about 0·15 m-equiv. The total loss averaged $5{\cdot}50 \pm 0{\cdot}25$ m-equiv for twelve bloods, or 2·6 m-equiv more than could be due to loss of Na in the intercellular plasma. This discrepancy cannot be explained by normal loss through diffusion, for if during washing, the cells were exposed to KCl solution for as long as 10 min (and the actual time is probably a good deal less), then with passive transfer at 0·03 hr^{-1}, the Na loss with an initial cell concentration of 10 m-equiv/l. would amount to about 0·05 m-equiv. Moreover, even if it be assumed that cells in a glucose-free medium still transport Na with a rate constant of 0·06 hr^{-1} at 18° C, the temperature of the wash-fluid, then the loss in 10 min would only be 0·1 m-equiv/l. It is in any case unlikely that the unexplained loss of 2·6 m-equiv on washing could be due to normal transfer, for most of the loss occurs with the first wash, losses with subsequent washes being trivial. It is thought that the unexplained fall of Na is due to removal by de-adsorption, or to loss of Na from the interior of the cells. If the latter be the case, it must affect a small proportion of highly permeable cells, whose initial Na concentration must be higher than the mean value of about 10 m-equiv/l. cell water. This is likely because marked loss still occurs when the wash-fluid contains 15 m-equiv/l. Na and some loss can be detected when external Na is 60 m-equiv/l. Table 2 shows these net changes in cell Na on washing with media containing varying amounts of Na. Net change equals the difference between the original cell Na corrected for Na in the intercellular plasma, and the Na of washed cells corrected for Na in the intercellular wash-fluid.

In Expts. 1, 2, 3, 4*a*, 5*a*, 6*a*, 7*a* and 8*a* the volume of the intercellular plasma has been estimated, but owing to the difficulty of using Evan's Blue as a marker for plasma-free media, the volume of the intercellular plasma-free wash-fluid was not known and has been assumed to be the same as that of intercellular plasma. In Expt. 4*b* the wash-fluid contained 10% normal plasma, while in Expts. 5*b*–8*b*, cells were washed with dialysed plasma to which NaCl and KCl were added in varying proportions: in these the volumes of intercellular plasma and of intercellular wash-fluid were both known.

Temperature had little effect on the immediate loss of Na from cells washed with KCl solution. In three experiments after single washes at 37 and 7° C, the respective losses were 5·24 and 5·01, 5·06 and 5·06, 5·18 and 5·03 m-equiv/l. cells.

Oberst (1935) found that human erythrocytes lost about 4·5 m-equiv Na on washing in an equal volume of KCl solution, and assumed that this was due to removal of intercellular plasma amounting to 3% of the packed cell volume. His figure exceeds the actual plasma contribution by about 1·5 m-equiv, while it is about 0·5 m-equiv less than the Na removed by washing cells in 50 vol. of KCl solution.

Findings in chicken erythrocytes were qualitatively similar to those in human cells: Na in the intercellular plasma averaged 4·5 m-equiv/l. and the average gross loss after one KCl wash was 7·25 m-equiv. The total loss increased to 7·6 m-equiv after two more washes (S.D. for ten experiments ± 0·3). It may be noted that on washing with KCl solution human cells swell slightly (0–3%),

TABLE 2. Net loss of sodium resulting from twice washing erythrocytes with wash-fluids of varying types and composition

(Net loss equals original cell Na content corrected for Na in the intercellular plasma minus the Na content of washed cells corrected for Na in the intercellular wash-fluid.) Temp. = 20° C.

		Net loss of Na (m-equiv/l.) from cells twice washed in fluids of the following composition (m-equiv/l.)					
Blood	Type of wash-fluid	KCl 165 NaCl 0	KCl 150 NaCl 15	KCl 135 NaCl 30	KCl 105 NaCl 60	KCl 75 NaCl 90	KCl 45 NaCl 120
Human 1	Plasma-free	2·0	—	1·3	0·6	—	0
2	Plasma-free	2·5	—	1·8	0·8	—	0·2
3	Plasma-free	2·7	—	2·1	1·1	0·4	0·1
4*a*	Plasma-free	2·6	2·5	2·1	1·4	—	—
b	Plasma 10%	—	2·6	2·2	1·4	—	0
5*a*	Plasma-free	2·7	—	—	—	—	—
b	Whole dialysed plasma	2·9	—	2·1	1·4	0·4	0·1
6*a*	Plasma-free	2·9	—	2·1	1·2	0·3	—
b	Whole dialysed plasma	2·9	—	2·1	1·4	0·2	—
7*a*	Plasma-free	2·4	2·0	—	—	—	—
b	Whole dialysed plasma	2·4	—	—	—	—	—
8*a*	Plasma-free	2·3	—	—	—	—	—
b	Whole dialysed plasma	2·4	—	—	—	—	—
Chicken 1	Plasma-free	1·9	1·4				
2	Plasma-free	2·3	1·7				
3	Plasma-free	2·9	2·1				
4	Plasma-free	2·7	2·2				

probably because of a small fall (about 0·1 unit) in the pH of the cells in an unbuffered medium; haemolysis is slight or absent. Chicken erythrocytes, on the other hand, show slight lysis, though the quite definite cell swelling (7·2 ± 1·6% in twenty observations) is itself too slight to cause rupture. It follows that washing with KCl solution is not inherently objectionable in the case of human erythrocytes, but is best avoided with chicken cells.

Immediate effects of suspending erythrocytes in media containing Na*. Suspensions of human erythrocytes were centrifuged immediately after the addition of Na* and the content of tracer estimated in the packed cells. Results (in duplicate) were 6·18, 6·12; 6·13, 6·04; 6·25, 6·12; 6·12, 6·07; 6·03, 5·96.

In another experiment, the graduated capillary containing the cells was cut across just below the upper face of the cell column, so that contamination with the supernatant Na*-rich medium was avoided without the necessity of washing the upper part of the tube (cf. Maizels, 1945; Solomon, 1952). Na* corresponded to 6·08 m-equiv/l. cells, although the intercellular plasma measured in the same way with Evans Blue as marker, was only 2·1%.

Findings in the case of chicken erythrocytes were similar to those in human cells, but the gross immediate gain of Na* was rather greater at about 8 m-equiv (7·4, 8·1, 7·8, 8·2 and 8·1 m-equiv/l. cells).

Effects of washing erythrocytes containing Na*. When human or chicken erythrocytes were centrifuged immediately after the addition of Na*, and then washed in 60 vol. of tracer-free medium (plasma or KCl solution), residual Na* was about 0·20 m-equiv/l. cells: $0·19 \pm 0·05$ for seven observations on human cells, and $0·23 \pm 0·09$ for twenty-four observations on chicken cells. After two washes residual Na* was $0·022 \pm 0·010$ for five observations on human cells and $0·027 \pm 0·011$ for ten observations on chicken cells.

When erythrocytes were suspended for varying periods in media containing Na*, so that the contents of cell tracer varied, the Na* losses after rapid washing with plasma and centrifuging were similar, irrespective of the initial levels of Na*. Thus, when the initial content of tracer was 7·38, twice washing with plasma reduced this by 7·32 m-equiv; and with contents of 9·90, 12·86 and 16·57 the corresponding reductions were 7·30, 7·56 and 7·55 m-equiv/l. cells. In another experiment Na* fell on washing from 7·98 to 0·05 and from 13·80 to 6·06 m-equiv/l. cells; a further wash reduced the latter figure to 5·97. In all the preceding experiments, Na* was added as the neutralized carbonate. Na*Cl figures were higher and more variable.

From the preceding sections, it will have been seen that human erythrocytes lose about 5·2 m-equiv Na on washing in KCl, gain about 6 m-equiv Na* immediately after suspension in Na*-containing media and lose about 5·8 m-equiv of the Na* on washing. In actual observations where loss of Na after a single wash in KCl solution, gain of Na*, and loss of Na* after one wash in KCl solution, were made on samples from the same cells, the following parallel figures were obtained: 5·16, 5·99 and 5·77; 5·23, 6·08 and 5·90. The figures are similar, but a consistent discrepancy exists in each set, for which no explanation is offered. It should also be noted that Solomon (1952) ascribed immediate increase of cell Na*, when tracer was added to suspensions, to the presence of Na* in the intercellular fluid. His average figure for trapped plasma is 3%, and although precise figures for Na* are not given, his data correspond to 4 m-equiv Na*/l. cells. It is possible, however, that Solomon's figure of 3% for trapped plasma is too high: the data of Chaplin & Mollison (1952) suggest that with cells centrifuged for 50 min at $1600g$, a figure of 2·4% or less is likely; if so, intercellular Na* would account for only 3 m-equiv,

leaving 1 m-equiv Na* derived from the cells. Even so, Solomon's total figure of about 4 m-equiv for gain of Na* is 2 m-equiv less than the values in the present paper, and 1 m-equiv less than our figures for loss of Na on washing human cells with KCl solution. The difference does not seem to lie in unsatisfactory samples of Na*, which in the present work were derived from 'Specpure' Na_2CO_3, and had a half-life of 15·1 hr. However this may be, the experiments described here indicate that in packed erythrocytes total Na is about 12 m-equiv/l., to which intercellular plasma contributes about 3 m-equiv in human cells and 4·5 in chicken cells, while there is a second fraction of about 2–2·8 m-equiv/l. which exchanges very rapidly. Solomon (1952) has produced evidence in favour of yet another fraction, inexchangeable or exchangeable only with difficulty; this in our experiments amounted to about 1 m-equiv/l. cells.

Treatment of cells for measuring transfer constants. Since net values of Na and Na* are required before transfer constants can be calculated, allowance must be made for these in the intercellular plasma, which must be measured with Evans Blue or other marker so that the appropriate deduction may be made. Even so, the basal figure for Na* at 0 hr is high (about 6 m-equiv) and detracts from the accuracy of later readings, especially when these are still quite low. Hence, although the method gives the mean transfer constants for all the several fractions of cell Na together, it is thought that some form of washing is desirable, even though this removes the easily exchanged as well as the intercellular Na.

In the attractive 'soaking-out' method of Harris & Burn (1949) cells are first charged with Na*, quickly washed, and suspended in a large volume of tracer-free medium, the transfer constants being calculated from loss of Na* by the cells. When the external phase is very large intercellular Na* may be ignored, while a deduction is made from cell Na equal to the amount of Na presumed to have been trapped in the intercellular medium. In fact, soaking out removes not only intercellular Na*, but also the easily exchanged fraction of intercellular Na*, and so the values used for Na and Na* are not quite comparable. In order that the 'soaking out' technique may be valid both intercellular and easily exchanged Na must be deducted from the total Na figure. In the present work, a 'soaking-in' technique was used, cells being washed before the estimation of Na and Na*; this gives the transfer constants for all the cells in a suspension except the highly permeable fraction. Details are given below.

In the case of human and chicken erythrocytes, some of the cell suspension is centrifuged in special tubes (see Methods) for 5 min at 2000*g*, and the packed cells (about 0·07–0·1 ml.) resuspended in 5·5 ml. KCl wash-fluid (0·16 M), centrifuged for a further 30 min, and prepared for the estimation of haemoglobin and Na as already described. For most purposes it suffices to wash one sample at the beginning of an experiment and a second at the end. Loss of Na on washing is about the same in both, and the appropriate deduction may thus be made for all the unwashed samples. In the case of Na*, however, the base-line is so high, that all samples are washed before

counting. Chicken erythrocytes present special difficulties because they swell on washing in plasma-free media, and it is essential that a volume correction be made. Thus, if the cell volume is 102% of the original before washing, and Na is 12 m-equiv/l. cells, while after washing the volume is 112% and Na is 5 m-equiv, then Na removed by washing is 6·4 m-equiv, and the Na of all the unwashed cells may be corrected by this amount. In the case of suspensions containing Na* it has been assumed that the volume changes during an experiment are the same as in the corresponding tracer-free suspensions. But in order to avoid volume corrections on washing the radioactive cells with KCl, the cells may be washed instead with plasma from the paired tracer-free system. As an alternative, where material is available, cells containing either Na or Na* may be washed with dialysed plasma to which KCl has been added to 0·16 M, and whose pH has been brought to 7·4. In this case the figures for Na and Na* must be corrected for changes in volume induced by the wash-fluid, such changes as a rule being quite small.

It will have been noted that in the present work, cell volumes are measured directly throughout, and where necessary are corrected for swelling or shrinking by measuring changes in cell haemoglobin. Some workers prefer to measure the proportion of cells in a suspension and to assume that the amount of cells in an aliquot is constant. But this may not always be so, and certainly in the case of chicken erythrocytes an amount of lysis which is imperceptible in 60 volumes of wash-fluid, may actually account for a 5% loss of cells.

Calculation of transfer constants for sodium

When tracer-free cells are suspended in a large volume of medium containing Na and Na*, the external concentration may be regarded as constant. If $[Na_i]$ (the concentration of cell Na) is also constant,

$$-k_2 t = \ln (1 - [Na_i^*]_t/[Na_i])$$

(Harris & Burn, 1949), where k_2 is the crude constant for outward transfer uncorrected for the Donnan asymmetry, and $[Na_i^*]_t$ is the concentration of cell Na* at time t. When $[Na_i]$ is not constant k_2 may be derived as follows (Harris, E. J., personal communication):

$$d[Na_i]/dt = k_1[Na_e] - k_2[Na_i],$$

where k_1 is the crude constant for inward transfer uncorrected for the Donnan asymmetry, and $[Na_e]$ the external Na concentration.

So too,

$$d[Na_i^*]/dt = k_1[Na_e^*] - k_2[Na_i^*].$$

Equating $[Na_e]$ with $[Na_e^*]$:

$$d([Na_i] - [Na_i^*])/dt = -k_2([Na_i] - [Na_i^*]),$$

and by integration

$$k_2 t = \ln \frac{[Na_i]_a - [Na_i^*]_a}{[Na_i]_b - [Na_i^*]_b},$$

where $[Na_i]_a$ and $[Na_i^*]_a$ are the cell concentrations of Na and Na* at 'a' hr and $[Na_i]_b$ and $[Na_i^*]_b$ the corresponding values at 'b' hr. Where cell volume was not constant during the experiment, graphic integration of the differential equation was preferred.

When $[Na_i]$ is constant, or when it is not constant but its plot against time permits of a close approximation to the equilibrium value (at $t = \infty$), k_1 may

be calculated from the equation $k_1[\mathrm{Na}_e] = k_2[\mathrm{Na}_i]$. If, however, extrapolation of $[\mathrm{Na}_i]$ is not possible, or if k_2 changes during the course of the experiment, the preceding equation cannot properly be applied to calculating the rate of Na influx over a selected period during the experiment. In these circumstances k_1 for the time interval between 'a' and 'b' hr was obtained by solving one or other of the equations

$$-k_2 t = \ln \frac{k_1[\mathrm{Na}_e] - k_2[\mathrm{Na}_i]_b}{k_1[\mathrm{Na}_e] - k_2[\mathrm{Na}_i]_a} \quad \text{or} \quad -k_2 t = \ln \frac{k_1[\mathrm{Na}_e] - k_2[\mathrm{Na}_i^*]_b}{k_1[\mathrm{Na}_e] - k_2[\mathrm{Na}_i^*]_a}.$$

The second equation is likely to give more accurate values, for changes in cell Na* are chiefly due to influx, while in the first equation changes in $[\mathrm{Na}_i]$ are not apparent in fresh cells and even in stored cells where $[\mathrm{Na}_i]$ alters, this is towards a decrease, and due to a large active efflux, slightly modified by a small passive influx.

However the constants are obtained, they need correction for the Donnan asymmetry (Ussing, 1949; Harris & Maizels, 1952; Harris, 1954). The simplest approach (Harris, 1954) is to put k_1' equal to k_1/f where k_1' is the true rate constant for influx, k_1 is the constant for total influx measured, and f the asymmetry factor derived from the chloride distribution ratio and taken to equal $\sqrt{([\mathrm{Cl}_e]/[\mathrm{Cl}_i])}$. Similarly, k_2 the gross outward constant also requires deduction of that moiety due to passive outward transfer in order that the true transfer constant for active efflux, k_2' may be obtained. According to Harris this passive moiety equals k_1'/f, so that $k_2' = k_2 - k_1'/f$. Harris's equation perhaps involves an oversimplification of the kinetics of passive Na transfer, but at least it allows of an approximate correction for the effects of the Donnan asymmetry. The relevant data for 'f' in chicken erythrocytes are shown in Figs. 1 and 2.

PART II. TRANSFER CONSTANTS OF CHICKEN AND HUMAN ERYTHROCYTES

In these experiments the transfer constants for influx and efflux have all been corrected for the Donnan asymmetry and are denoted by k_1' and k_2'. Chicken erythrocytes were usually examined within 3 hr of collection, but showed a small fall in cell Na during incubation, perhaps because the procedures designed to remove leucocytes tended to raise cell Na, which returned to the equilibrium value during incubation at 38° C. In the case of human cells, it was convenient to collect blood at night and examine next morning: during the preliminary period of storage at 4° C cell Na rose a few m-equiv, falling correspondingly during the subsequent incubation. Hence, when determination of $[\mathrm{Na}_i]_\infty$ was of special importance, fresh cells were used.

The constants for Na transfer in human erythrocytes at 37 or 38° C are similar to those described elsewhere (Harris & Maizels, 1951; Solomon, 1952), even though our figures have been corrected for asymmetry. This is because the

correction usually lowers k_1 by less than 15% and k_2 by less than 7%. It will be noted (Table 3) that efflux tends to decrease somewhat during incubation, the rate constant being less between 2–4 hr than at 0–2 hr. This may be due to partial failure of the energizing metabolic process or to the presence of in-exchangeable Na within the cells, a possibility suggested by Solomon (1952); the matter is discussed later. The rate constant for Na efflux from chicken erythrocytes is on the whole rather greater than in human cells, while that for

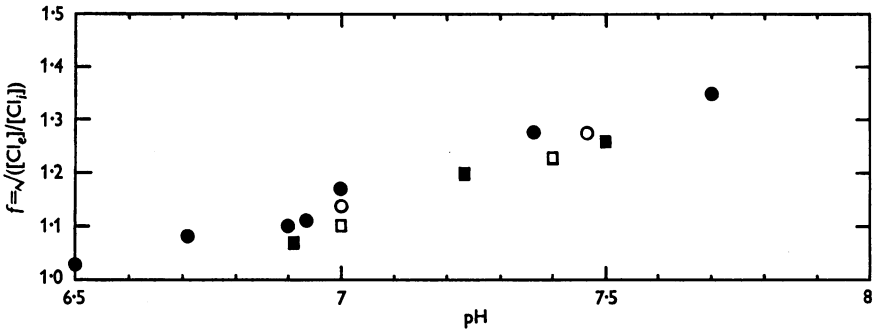

Fig. 1. pH and the asymmetry factor (f) in cell suspensions incubated at 37° C. ■, fresh cells transporting Na; □, fresh cells inactive; ●, stored cells transporting Na; ○, stored cells inactive.

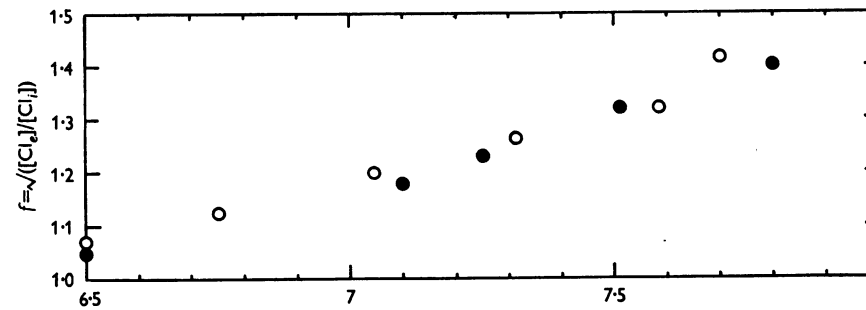

Fig. 2. pH and the asymmetry factor (f) in suspensions of stored cells incubated at 23° C. ●, cells transporting; ○, cells inactive.

influx is about the same; it follows that at equilibrium the Na concentration of chicken erythrocytes is rather less than in human cells. There is, however, the same decrease in the rate of active transport as incubation proceeds. In stored human cells the rate constant for Na efflux is less than in fresh cells, possibly due to deterioration of the energizing system (Harris & Maizels, 1951), and in spite of the fact that a high initial value for cell Na increases the passive element of efflux. In the case of chicken cells, however, the rate of active efflux (k_2') is slightly greater in stored cells than in fresh, even though allowance has been made in Tables 3 and 4 for the passive component. Thus

in fresh cells at 37 or 38° C, the value of k_2' (mean and S.D.) was $0{\cdot}44 \pm 0{\cdot}10$, while with cells stored for a week at 4° C and then incubated the value was $0{\cdot}62 \pm 0{\cdot}13$. The series in each case was small and in order to test the matter further, two samples of cells from the same blood were stored with different

TABLE 3. Transfer constants of erythrocytes: chicken blood fresh; human 2–18 hr old

Species	Temp. (° C)	Cell pH at 20° C	$[Na_i]$ at 0 hr	$[Na_i]$ at 4 hr	k_2' (hr^{-1}) 0–2 hr	k_2' (hr^{-1}) 2–4 hr	k_1' (hr^{-1}) 0–2 hr	k_1' (hr^{-1}) 2–4 hr
Chicken 1	37	7·38	8·2	7·6	0·37	0·35	0·016	0·014
2	38	7·55	8·1	6·4	0·54	0·38	0·018	0·014
3	38	7·50	8·8	7·2	0·59	0·53	0·022	0·020
4*a*	37·5	7·51	7·2	6·8	0·45	0·37	0·018	0·014
b	25	7·49	7·1	6·2	0·20	0·15	0·007	0·005
5*a*	37	7·43	8·4	7·9	0·37	0·26	0·014	0·010
b	24	7·40	8·2	7·1	0·18	0·15	0·007	0·0045
6*a*	38	7·67	8·2	7·1	0·54	0·42	0·019	0·015
b	38	7·51	7·6	6·3	0·60	0·46	0·021	0·013
c	38	7·06	7·2	7·1	0·42	0·34	0·018	0·013
7*a*	37	7·68	8·0	8·2	0·34	0·29	0·015	0·014
b	37	7·45	7·6	7·1	0·38	0·32	0·015	0·015
c	37	7·08	6·8	8·0	0·26	0·27	0·017	0·016
8*a*	39	7·52	8·1	7·7	0·35	0·28	0·014	0·011
	39	7·25	7·8	7·4	0·31	0·28	0·012	0·011
	39	7·02	7·4	8·0	0·25	0·19	0·012	0·010
Human 9	37	7·25	17·4	12·3	0·32	0·29	0·023	0·020
10	37	7·23	17·3	11·4	0·28	0·25	0·015	0·013
11	37	7·27	16·7	10·6	0·35	0·29	0·019	0·016
12*a*	30·5	7·34	17·0	12·8	0·21	0·18	0·012	0·010
b	23·8	7·34	17·0	14·5	0·12	0·09	0·0075	0·0065
13*a*	29·5	7·33	21·4	15·3	0·18	0·14	0·0095	0·008
b	23·0	7·36	21·4	18·9	0·08	0·07	0·0055	0·0055
14*a*	29·0	7·30	16·9	11·8	0·24	0·20	0·011	0·011
b	20·5	7·35	16·9	14·3	0·12	0·07	0·006	00·04
15*a*	37·0	7·49	10·4	9·3	0·38	0·34	0·019	0·018
b	27·0	7·50	10·4	9·8	0·13	0·12	0·0065	0·007
16*a*	37·2	7·36	13·0	11·2	0·35	0·30	0·025	0·020
b	28·2	7·36	13·2	12·2	0·14	0·14	0·011	0·0095
17*a*	38	7·50	10·4	10·2	0·43	0·29	0·025	0·016
b	38	7·29	9·2	8·4	0·42	0·32	0·020	0·014
c	38	7·09	8·6	8·6	0·34	0·23	0·018	0·013

$[Na_i]$ = cell sodium concentration, m-equiv/l. cell water. Bloods 9–14 kept overnight at 4° C before incubation.

k_2' and k_1' in this and later tables denote the net rate constants for Na efflux and influx, that is, the observed rate constants corrected for the Donnan asymmetry.

media so as to give a high cell Na in one and a low Na in the other: one batch was kept at 4° C for a week with 3 vol. of NaCl solution (0·16 M), glucose being omitted from the external medium so as to ensure a big rise of cell Na; the other batch was stored with a solution containing 25 or 50 mM-KCl, the complementary amounts of NaCl and also glucose, in order to limit rise of

cell Na. At the end of cold-storage both batches of cells were incubated in the standard medium, and although the experiment was weighted against the Na-rich cells because of the absence of glucose during storage, k_2' was higher during the first 2 hr of incubation than in the Na-poor cells. After 2 hr, however, $[Na_i]$ had become similar in both sets of suspensions and the k_2' values, which had fallen in both batches, were also similar (Table 5). The fact

TABLE 4. Transfer constants for Na in chicken erythrocytes stored at 4° C for 7–9 days before incubation at various pH or temperatures or with various chemical agents

No.	Special treatment	pH of cells at 20° C	Temp. ° C	Na (m-equiv/l. cell water) at 0 hr	Na (m-equiv/l. cell water) at 4 hr	k_2' (hr^{-1}) 0–2 hr	k_2' (hr^{-1}) 2–4 hr	k_1' (hr^{-1}) 0–2 hr	k_1' (hr^{-1}) 2–4 hr
1*a*	None	7·50	37	49·5	17	0·39	0·34	0·016	0·014
b	Anoxia	7·50	37	49·5	58	0·07	0	0·022	0·020
2*a*	None	7·45	38	38	12	0·63	0·35	0·024	0·017
b	NaCN 5 mM	7·45	38	38	51	−0·01	−0·05	0·017	0·021
3*a*	None	7·47	37	48·5	13	0·66	0·36	0·025	0·019
b	DNP 0·5 mM	7·44	37	48·5	55	0·02	0·03	0·028	0·019
4*a*	None	7·48	37	55	11	0·82	0·41	0·032	0·022
b	None	7·44	24	55	19	0·38	0·32	0·017	0·012
c	NaCN 5 mM	7·47	37	55	59·5	0·03	0·02	0·022	0·017
5*a*	None	7·65	37	49	12	0·62	0·52	0·027	0·022
b	None	7·36	37	47·5	12	0·63	0·43	0·024	0·018
c	None	6·96	37	46	15	0·46	0·30	0·020	0·018
6*a*	None	7·40	37·5	38	13·5	0·62	0·41	0·027	0·023
b	None	7·30	24·5	38	20	0·28	0·19	0·014	0·010
c	Compound E 0·6 mg %	7·32	24·5	38	20	0·24	0·21	0·013	0·011
7*a*	None	7·54	25	62	32	0·23	0·19	0·013	0·010
b	Compound E 1 mg %	7·54	25	60	31	0·26	0·20	0·015	0·010

DNP = 2:4-dinitrophenol. The small negative values for k_2' found here and elsewhere are probably due to experimental error.

that the rate constant for active efflux is greater in stored chicken erythrocytes with a high Na content, than in fresh cells, although the metabolic activity of the former is unlikely to be greater than that of the latter, suggests that the active component of transport works more efficiently as cell Na concentration rises.

External K *concentration and* Na *transport.* Omission of KCl from the standard diluent gave a cell suspension with a content of 1 m-equiv K/l. external phase: by the addition of KCl external [K] could be raised to any desired level. It was found that Na transport fell a little as $[K_e]$ declined from 12 to 5 m-equiv/l. and more markedly as $[K_e]$ fell below 2 or 3 m-equiv/l. (Table 6).

pH *and transfer constants.* In human erythrocytes Harris & Maizels (1951) record that k_2 and k_1 for Na is less when cell pH is 6·9 than when it is 7·3, while between 7·3 and 7·5 there is little difference in the transfer rates. These findings are confirmed by Solomon (1952), while Harris (1954) states that if

the rate constant for influx be corrected for asymmetry, the effect of pH is quite slight. Table 3, no. 17, illustrates these findings for human cells by a single experiment, where k_2' is roughly constant for cell pH 7·3–7·5, while at pH 7·09 Na transport is definitely less: k_1' decreases a little with pH. In fresh

TABLE 5. Effects of initial cell Na concentration on Na transfer

In each experiment samples of cells from the same blood were stored at 4° C with media either rich or poor in Na, to give high or low values for cell Na. After 7 days the various cell samples were all incubated at 38° C in a medium containing Na, 155 m-equiv; K, 10 m-equiv; glucose 11 m-mole/l.

No.	Cell pH	$[Na_i]$ at 0 hr	$[Na_i]$ at 4 hr	k_2' (hr^{-1}) 0–2 hr	k_2' (hr^{-1}) 2–4 hr	k_1' (hr^{-1}) 0–2 hr	k_1' (hr^{-1}) 2–4 hr
Chicken 1*a*	7·20	60·5	16·5	0·56	0·42	0·023	0·025
b	7·26	44	15·5	0·48	0·41	0·022	0·024
c	7·31	22·5	12·5	0·44	0·35	0·019	0·022
2*a*	7·52	42	13·5	0·54	0·39	0·019	0·016
b	7·56	30	12	0·44	0·29	0·018	0·013
3*a*	7·42	50	15	0·72	0·33	0·034	0·022
b	7·45	43	13·5	0·69	0·38	0·030	0·022
c	7·45	32·5	12·5	0·43	0·36	0·026	0·021
4*a*	7·33	46	15	0·67	0·26	0·030	0·018
b	7·36	26·5	14·5	0·50	0·20	0·023	0·016
Human 5*a*	7·37	71·5	56	0·10	0·10	0·021	0·018
b	7·40	45	37·5	0·12	0·10	0·021	0·016
6*a*	7·32	56	37	0·22	0·16	0·028	0·023
b	7·27	29	18·5	0·25	0·18	0·021	0·018

TABLE 6. Effects of external K concentration on Na transfer in fresh chicken erythrocytes
Temp. 37° C.

No.	Cell pH	External medium [K] (m-equiv/l.)	External medium [Na] (m-equiv/l.)	k_2' (hr^{-1}) 0–2 hr	k_2' (hr^{-1}) 2–4 hr	k_1' (hr^{-1}) 0–2 hr	k_1' (hr^{-1}) 2–4 hr
1*a*	7·61	12·0	153	0·47	0·33	0·019	0·013
b	7·56	3·0	162	0·41	0·21	0·017	0·010
c	7·56	1·0	164	0·35	0·17	0·019	0·010
2*a*	7·26	12·8	154	0·34	0·20	0·018	0·011
b	7·27	4·8	160	0·30	0·21	0·017	0·013
c	7·27	1·8	163	0·24	0·14	0·016	0·011
d	7·28	0·8	164	0·21	0·13	0·017	0·015

chicken cells (Table 3, nos. 6–8) k_2' is also roughly constant between pH 7·25 and 7·67 with, perhaps, an optimal point at about pH 7·4; at pH below 7·1 active Na efflux is definitely less. Na influx, on the other hand, is little affected by change in pH between 7·02 and 7·67.

k_2' and k_1'. Except in the poisoned systems described later k_2' and k_1' vary together: thus k_1' is less during the second half of incubation than in the first half; other examples are seen in Tables 5, 6 and 10.

Temperature and Na *transfer.* Data for this are given in Tables 3 and 4 and the apparent energy of activation summarized in Table 7. The results for human cells are in qualitative agreement with those of Harris (1954) in that

the apparent energy of activation for active Na efflux (k_2') is about 20,000 cal and exceeds that for influx. Hence, as the temperature of incubation of human cells is lowered, cell Na tends to rise. Except in Expt. 3, the difference in the respective figures is small and so the presumed equilibrium value for cell Na concentration ($[Na_i]_\infty$) can only be slightly greater at the lower temperature

TABLE 7. Apparent energy of activation of Na transfer in human and chicken erythrocytes, and of lactic acid production by human cells

No.		Temperature range (° C)	Apparent activation energy (cal) for Na efflux 0–4 hr	Na influx 0–4 hr	lactate production 0–4 hr
1	Human, fresh	27 –37	19,600	18,600	16,400
2	Human, fresh	23·8–30·5	15,700	12,800	17,400
3	Human, fresh	23 –29·5	19,600	12,000	15,000
4	Human, fresh	20·5–29	17,300	15,600	19,800
5	Human, fresh	28·2–37·2	17,800	16,500	—
6	Chicken, fresh	25 –37·5	12,000	15,200	—
7	Chicken, fresh	24 –37	8,700	10,800	—
8	Chicken, stored	24 –37	8,600	8,700	—
9	Chicken, stored	24·5–37·5	10,400	10,200	—

TABLE 8. Na concentration of fresh chicken erythrocytes incubated at various temperatures

No.	Temp. (° C)	Cell Na concentration (m-equiv/l. cell water) after incubation for 0 hr	3 hr	5 hr	7 hr	9 hr
1*a*	38	5·8	6·2	5·8	—	—
b	25	5·8	5·8	5·8	—	—
2*a*	37	9·3	9·0	9·0	—	7·8
b	27	9·3	8·6	7·9	—	6·8
3*a*	38·5	9·3	9·1	8·9	9·1	—
b	24	9·3	8·6	7·9	7·3	—
4*a*	38	10·0	10·0	9·2	8·8	—
b	24	10·0	8·1	7·6	7·6	—

(23° C) than at the higher (37–38° C). With fresh chicken cells, on the other hand, the apparent energy of activation for efflux is less than that for influx: it follows that the equilibrium value, $[Na_i]_\infty$, must be less at 24 than at 38° C. The data in Tables 3 and 4 leave this uncertain, but the prolonged observations of Table 8 show that the Na concentrations of chicken cells are in fact a little lower at 24 than at 38° C. It may further be noted that the apparent energy of activation of Na influx and especially of efflux is much less in chicken erythrocytes than in human cells.

Metabolism and Na *transfer*

In fresh human blood incubated at 37° C glycolysis is rapid. Solomon's (1952) figure for the disappearance of glucose is 2·33 μmole/ml. cells. hr, while Raker, Taylor, Weller & Hastings (1950) give 1·4 μmole; for stored cells the

figure is about 1·5 μmole (Maizels, 1951). Bernstein (1953) recalls Hsu's (1935) observations that glycolysis is much more active in leucocytes than in erythrocytes, and suggests that autolysis of leucocytes may account for the lowered rate of glycolysis in the cells of stored blood. He confirmed this view by showing that consumption of glucose was between 1·2 and 2·2 μmole/ml. cells in suspensions containing leucocytes, and between 0·9 and 1·35 in cells with the leucocytes removed. He found that in human blood (including leucocytes) lactic acid formation corresponded to 70–90 % of the glucose that disappeared, while in erythrocytes freed from white cells the maximum figure of 2 mole lactate per mole of glucose was approached, the actual yield being 91–98 %. The latter observation was confirmed by estimating loss of glucose and gain of lactate in stored human cells, where it may be presumed that the leucocytes have autolysed: here, lactate formation accounts for nearly all the glucose disappearing (Table 9), this table also illustrates the marked effect of pH on glycolysis. It is worthy of note too (Table 7), that the apparent energy of activation of lactate formation is similar to that of active Na efflux.

TABLE 9. Disappearence of glucose and formation of lactate in human erythrocytes stored at 4° C for 9 days and then incubated at 37° C for 5 hr

No.	pH at 20° C	μmole glucose used by 1 ml. cells in 1 hr	μmole lactate formed by 1 ml. cells in 1 hr
1	6·83	0·49	0·81
	7·21	0·82	1·67
	7·55	1·71	3·15
2	6·87	0·50	0·81
	7·23	0·93	1·80
	7·57	1·83	3·52

Fresh chicken blood deprived of leucocytes uses about 70–80 μl. O_2 per ml. cells per hr (Tables 13 and 14) corresponding to about 0·6 μmole glucose oxidized. In this time too, about 0·05 μmole glucose is converted to lactate, perhaps because conditions *in vitro* do not favour complete oxidation. In the presence of respiratory poisons, glycolysis in chicken erythrocytes was much increased, and in view of Bernstein's results, it seemed desirable to obtain a measure of the contribution of leucocytes to glycolysis in chicken blood poisoned with cyanide. This was done as follows: two samples of heparinized chicken blood were centrifuged, the cells of one sample were simply resuspended, while the buffy coat of the second sample was removed, and the residual cells and plasma were then well shaken. The latter suspension was itself divided and centrifuged, one part being well shaken without removal of the buffy coat, which at this stage was hardly perceptible, and the second being shaken after removal of the buffy coat. After the whole process had been repeated yet a third time, it was only possible to skim the upper layer of the erythrocyte column, no white buffy coat being visible. Cyanide was added

to all the samples, and lactate production measured after incubation. After one, or at most two, 'skimmings' lactate production fell to a constant low level: this corresponded to the disappearance of leucocytes from the packed cell mass. In one experiment, liberation of lactate by fresh chicken erythrocytes together with leucocytes was 5·56 μmole/ml. cells.hr, which subsequent 'skimming' lowered successively to 1·42, 1·22 and 1·24; in another observation, the figures were 9·20 before removal of the buffy coat and 1·92, 1·61 and 1·59 after, while a third observation gave 7·90 and then 2·39, 1·84 and 1·70. With chicken blood stored at 4° C for a week, and then incubated with cyanide, initial liberation of lactate was much less at 1·46 μmole/ml. cells.hr, and successive removals of residual buffy coats had little effect, lactate production falling to 1·38 and 1·32 as a result of treatment. This doubtless correlates with the observation that the buffy coat of stored chicken blood contains few surviving leucocytes, and consists of 'smudge' cells, fibrin and the nuclei of haemolysed erythrocytes. With fresh human cells, removal of the buffy coat decreased glycolysis by 34% from 4·44 to 2·92, and after a second treatment to 2·80 μmole/ml. cells.hr. This isolated observation compares with Bernstein's (1953) mean figure of 24% reduction in glycolysis on removing leucocytes from fresh human blood (eighteen samples). It is clear that the contribution of leucocytes to glycolysis in chicken blood poisoned with cyanide is much greater than it is in human blood; whether chicken leucocytes glycolyse more actively, or whether there are more leucocytes in chicken blood has not been examined. It was evident, however, that when making quantitative measurements of glycolysis or respiration in chicken blood, the leucocytes must first be removed, even though the delay and manipulation involved, raises the base-line for lactate and Na in the erythrocytes.

Effects of poisons on Na *transfer and metabolism in chicken erythrocytes.* Adequate amounts of such respiratory poisons as cyanide, azide, dinitrophenol and malonate all have similar effects on Na transport in fresh chicken erythrocytes: k_2' calculated for the first 2 hr falls to one-half or one-quarter of that in the unpoisoned cell; thereafter, active transport practically ceases (Tables 4 and 10). In some cases small negative values of k_2' occur, which are doubtless due to experimental errors. It may be wondered if after 2 or 3 hr incubation with these poisons the erythrocyte is virtually dead. But this is not so, for if the cells be twice washed with normal plasma, Na transport again becomes active (Tables 11 and 12). When measuring respiration, duplicate or triplicate observations were made on each cell suspension, and earlier experiments with cyanide (1–5 m-mole/l. suspension) suggested that inhibition of respiration was marked, but that complete inhibition was not immediate. It seemed likely that these observations arose from a failure to equilibrate the suspension fully with atmospheric oxygen, or to remove its CO_2 completely before manometric readings were begun. When special precautions were taken

to achieve removal of CO_2 and full equilibration with air (see Methods), cyanide appeared to cause immediate inhibition of O_2 uptake. The treatment, which also involved centrifuging, does not leave the cells unharmed, for while the unpoisoned cells transport Na normally, the treated cells poisoned with cyanide transport Na much less efficiently than untreated cells similarly

TABLE 10. Effects of inhibitors of respiration, and of compound F on the transfer constants of fresh chicken erythrocytes

No.	Special treatment	Cell pH at 20° C	Temp. (° C)	k_2' (hr^{-1}) 0–2 hr	k_2' (hr^{-1}) 2–4 hr	k_1' (hr^{-1}) 0–2 hr	k_1' (hr^{-1}) 2–4 hr
1*a*	None	7·30	37	0·37	0·35	0·016	0·014
b	Cyanide 5 mM	7·40	37	0·22	0·04	0·013	0·013
2*a*	None	7·52	38·5	0·59	0·37	0·021	0·017
b	Cyanide 5 mM	7·50	38·5	0·16	0	0·017	0·015
3*a*	Cyanide 5 mM	7·58	38·8	0·10	−0·04	0·017	0·014
b	Cyanide 10 mM	7·58	38·8	0·08	0·02	0·016	0·015
4	Cyanide 5 mM	7·44	37	0·05	−0·02	0·015	0·015
5	Cyanide 5 mM	7·32	37	0·16	0·02	0·018	0·016
6*a*	None	7·52	38	0·43	0·35	0·027	0·020
b	DNP 0·045 mM	7·48	38	0·07	0·04	0·022	0·014
c	DNP 0·090 mM	7·47	38	0·05	0·01	0·022	0·015
7*a*	None	7·38	37	0·37	0·35	0·016	0·014
b	Compound F 0·1 mg %	7·38	37	0·37	0·31	0·016	0·015

DNP = 2:4-dinitrophenol.

TABLE 11. Recovery of Na transport by poisoned chicken erythrocytes after washing in normal plasma

Cells stored for 1 week at 4° C before the addition of poison and subsequent incubation.

Poison added at 0 hr (m-mole/l. cell suspension)	Treatment at 2 hr	Na (m-equiv/l. cells) at 0 hr	2 hr	5 hr
None	None	44·5	19	16
NaCN 5	None	44·5	51	64
NaCN 5	Twice washed	44·5	51	24·5
Dinitrophenol 0·5	None	44·5	51·5	61
Dinitrophenol 0·5	Twice washed	44·5	51·5	21

TABLE 12. Recovery of Na transport by poisoned chicken erythrocytes after washing in normal plasma

Fresh cells suspended in a medium containing 5 mM-NaCN for 3 hr and then washed.

No.	Treatment of cells 3 hr after the addition of cyanide	k_2' (hr^{-1}) at 0–1 hr	1–2 hr	2–3 hr	3–4 hr	4–5 hr
1*a*	None	0·12	0·06	0	0·02	−0·02
b	Twice washed with plasma at 3 hr	0·12	0·06	0	0·52	0·34
2*a*	None	0·18	0·03	−0·01	0·03	0
b	Twice washed with plasma at 3 hr	0·18	0·03	−0·01	0·31	0·27
3*a*	None	0·09	0·02	−0·02	−0·03	0
b	Twice washed with plasma at 3 hr	0·09	0·02	−0·02	0·32	0·23

poisoned. Thus the rate constant for efflux of untreated poisoned cells is about 0·15 hr^{-1} for the first 2 hr of incubation (Table 10), while after full aeration and extraction of CO_2 it is only about one-quarter of this (Table 14); in short, the response of treated fresh cells to respiratory poisons differs from that of fresh cells, and resembles that of untreated stored cells similarly exposed (Table 4).

Dinitrophenol inhibited Na transport in chicken blood, but only appeared to act in relatively high concentration (0·5 m-mole/l.). Dr J. D. Judah (personal communication) suggested that this might be due to inhibition by plasma, and certainly, when the erythrocytes were suspended in saline, DNP became effective at a concentration of 0·045 mM or less. According to Dodds & Greville (1934) DNP increases the respiration of tumour tissue and a similar effect on chicken erythrocytes is shown in Table 13; it also inhibits oxidative phosphorylation (Judah, 1951) and this is doubtless the cause of its inhibitory action on Na transport (Table 13).

TABLE 13. Oxygen consumption and outward transfer constants for Na (k_2') of fresh chicken erythrocytes treated with respiratory poisons

No.	Additions (m-mole/l. cell suspension)	k_2' (hr^{-1}) 0–2 hr	k_2' (hr^{-1}) 2–4 hr	Rate of oxygen consumption (μl./ml. cells.hr) at 0–1 hr	1–2 hr	2–3 hr	3–4 hr
1	None	0·43	0·35	70	64	63	63
	DNP 0·045	0·07	0·04	91	87	80	71
	DNP 0·09	0·05	0·01	110	96	91	83
2	None	0·34	0·26	127	122	114	114
	Azide 1	0·25	0·06	133	119	102	102
	Azide 3	0·11	0	130	106	80	75
	Azide 10	0·08	−0·03	123	83	57	48

Note. These experiments differ from previous ones showing values of k_2', because CO_2 was extracted from cell suspensions, which were also aerated before incubation.

The metabolic effects of azide are complex: it inhibits the cytochrome oxidase system, and in addition, as Judah (1951) has shown, it acts like DNP and inhibits oxidative phosphorylation. Azide acts less powerfully than cyanide and even in the highest concentration used (10 m-mole/l. cell suspension) only caused partial inhibition of respiration at 2 hr (Table 13). Nevertheless, the reduction in Na transport was marked, which suggests that the action of azide on chicken erythrocytes is directed chiefly against oxidative phosphorylation. Fluoroacetate was rather ineffective (Table 14), while malonate inhibited both respiration and Na transport—but only when present in fairly high concentrations (about 8 m-mole/l. cell suspension). Hence, it seemed desirable to rely mainly on cyanide experiments, when attempting to correlate transport with respiration, although this involved the use of KCN–KOH mixtures in the central well of the Warburg flasks (Krebs, 1935). It has already been noted that high concentrations of cyanide cause an

immediate inhibition of respiration, but Na transport, though reduced, persists for ½ hr or perhaps a little longer (Table 14); after 1 hr active efflux has ceased. With small amounts of cyanide, the range of concentration is quite critical, so that 0·07 mM may give only slight inhibition of respiration and transport, while with 0·14 mM the effects are marked. On the whole, there is a rough correlation between failure of transport and inhibition of respiration.

TABLE 14. Effects of respiratory poisons on the oxygen consumption and rate constant for Na efflux (k'_2) from chicken erythrocytes

No.	Additions (m-mole/l. cell suspension)		k'_2 (hr^{-1}) 0–½ hr	½–1 hr	0–1 hr	1–2 hr	O_2 consumption (µl./ml. cells.hr) 0–½ hr	½–1 hr	0–1 hr	1–2 hr
1*a*	Fluoroacetate	0	—	—	0·47	0·42	—	—	84	71
b	Fluoroacetate	16	—	—	0·45	0·34	—	—	74	57
2*a*	Fluoroacetate	0	—	—	0·38	0·34	—	—	79	76
b	Fluoroacetate	16	—	—	0·33	0·26	—	—	75	64
3*a*	Malonate	0	—	—	0·44	0·30	—	—	80	77
b	Malonate	2	—	—	0·33	0·21	—	—	71	68
c	Malonate	8	—	—	0·19	0·14	—	—	52	28
4*a*	Cyanide	0	—	—	0·31	0·25	—	—	71	68
b	Cyanide	0·07	—	—	0·26	0·20	—	—	69	40
c	Cyanide	0·14	—	—	0·21	0·07	—	—	52	28
5*a*	Cyanide	0	—	—	0·40	0·29	—	—	78	81
b	Cyanide	0·07	—	—	0·38	0·24	—	—	69	69
c	Cyanide	0·14	—	—	0·27	0·18	—	—	58	53
6*a*	Cyanide	0	—	—	0·44	0·37	—	—	58	60
b	Cyanide	5	0·26	0·04	0·15	−0·06	8	0	4	0
7*a*	Cyanide	0	—	—	0·38	0·30	—	—	66	61
b	Cyanide	5	0·14	−0·02	0·06	0·03	0	0	0	0
8*a*	Cyanide	0	—	—	0·37	0·35	—	—	71	70
b	Cyanide	5	0·28	0·08	0·18	−0·07	4	0	2	0
9*a*	Cyanide	0	—	—	0·38	0·30	—	—	—	—
b	Cyanide	5	—	—	0·14	0·03	—	—	—	—
10*a*	Cyanide	0	—	—	0·38	0·30	—	—	76	70
b	Cyanide	5	0·11	0·02	0·07	0·02	12	0	6	0
11*a*	Cyanide	0	—	—	0·36	0·28	—	—	72	67
b	Cyanide	5	0·10	−0·02	0·04	0·02	8	0	4	0
c	Cyanide	5	—	—	0·07	0·03	—	—	—	—

Note. These experiments, like those of Table 13, differ from previous ones showing values of k'_2, because CO_2 was extracted from cell suspensions, which were also aerated before incubation.

It has already been noted that while stored chicken erythrocytes transport Na as efficiently as the fresh cell, the addition of cyanide causes a more complete and rapid inhibition of transport in the stored cell than it does in the fresh.

Effects of compounds E *and* F. The effects of compound E on stored cells (Table 4) and of compound F on fresh cells (Table 10) were examined: no action was detected.

Relation of transport to glycolysis in human erythrocytes. Chicken erythrocytes using 80 µl. O_2/ml. hr could oxidize 0·6 µmole glucose/ml. cells, or if only 80 % O_2 were so used about 0·45 µmole glucose/hr. Human cells at comparable

pH glycolyse about 1·2–1·5 μmole glucose (Table 9), so that the energy available from this source is about five times greater in chicken than in human erythrocytes (assuming that 1 m-mole glucose glycolysed liberates 54 cal, and completely oxidized 775 cal). On the other hand, the energy available solely from glycolysis in the human cell is about twice as great as that available from glycolysis in the chicken cell poisoned with cyanide, and it may be wondered if the human cell would still transport Na were its power to glycolyse reduced to the level found in poisoned chicken cells. This was investigated by measuring the transfer constants of human erythrocytes at temperatures below 38° C.

TABLE 15. Na transport and lactate production in erythrocytes incubated with cyanide (5 m-mole/l. cell suspension)

No.		Temp. (° C)	pH of cells at 20° C	k_2' (hr^{-1}) at 0–2 hr	k_2' (hr^{-1}) at 2–4 hr	Lactate formed (μmole/ml. cells.hr) 0–2 hr	Lactate formed (μmole/ml. cells.hr) 2–4 hr	Lactate formed (μmole/ml. cells.hr) 0–4 hr
1	Chicken	37	7·55	0·11	−0·01	—	—	1·24
2	Chicken	38·5	7·50	0·16	0	—	—	1·59
3	Chicken	38·8	7·58	0·10	−0·04	1·66	1·74	1·70
4	Chicken	37	7·50	0·12	−0·03	1·94	1·82	1·88
5*a*	Human	37	7·49	0·38	0·34	—	—	2·93
b	Human	27	7·50	0·13	0·12	—	—	1·21
6*a*	Human	30·5	7·34	0·21	0·18	—	—	1·80
b	Human	23·8	7·34	0·12	0·09	—	—	0·95
7*a*	Human	29·5	7·33	0·18	0·14	—	—	1·65
b	Human	23	7·36	0·08	0·07	—	—	0·96
8*a*	Human	29	7·30	0·24	0·20	—	—	1·30
b	Human	20·5	7·35	0·12	0·07	—	—	0·50
9*a*	Human	38	7·50	0·33	0·29	—	—	3·36
b	Human	38	7·29	0·42	0·32	—	—	2·10
c	Human	38	7·09	0·34	0·23	—	—	1·23

As in the case of chicken cells, cyanide was added to the suspensions, though transport in non-nucleated erythrocytes is virtually unaffected by respiratory poisons (Maizels, 1951). The effects of temperature on the transfer constants of human cells are shown in Table 3, while in Table 15 the constants may be compared with the corresponding productions of lactic acid. Similar data are also shown for chicken cells, and it will be seen that under appropriate conditions lactate production in human and chicken cells becomes similar, yet in the former transport occurs, and in the latter it remains absent. It will also be noted that for human cells at constant pH, the rate constant for active efflux (k_2') varies directly with lactate production (Table 15, nos. 5–8). But at constant temperature and variable pH this relation does not hold, for glycolysis is much more affected by pH changes than is the transfer constant for Na efflux (Table 15, no. 9): this confirms previous observations (Maizels, 1951; Solomon, 1952).

DISCUSSION

Na *and* K *in erythrocytes.* In human blood the ratios $[Cl]_{plasma}/[Cl]_{cell}$, $[Na]_{cell}/[Na]_{plasma}$ and $[K]_{cell}/[K]_{plasma}$ respectively approximate to 1·4, 0·11 and 30; in chicken blood the corresponding ratios are about 1·3, 0·09 and 30. If the chloride distribution is regarded as a measure of the Donnan asymmetry, then it follows that in the respiring chicken erythrocytes as in the non-respiring human cell, both Na and K are actively transported across the cell membrane.

In an earlier section of this paper it has been shown that if erythrocytes, which usually contain about 12 m-equiv Na/l. cells, are quickly washed in KCl solution, there is an almost immediate loss of about 5·2 m-equiv Na from human cells and 7·5 from chicken cells; additional washes cause little further loss. To these losses, Na in the intercellular plasma contributes about 3 m-equiv in human cells and 4·5 in chicken cells, leaving 2 or 3 m-equiv Na easily exchanged, at a rate and in an amount which is practically unaffected by changes in temperature between 4 and 38° C. This may suggest that the easily exchanged fraction is derived from the cell surface rather than from the interior. The fraction, however, increases greatly under certain conditions: thus with cells stored at 4° C it may double in 2 weeks and increase fourfold in 5 weeks (Clarkson, unpublished); so too, in sickle-cell anaemia easily exchanged Na is about 7 m-equiv in oxygenated blood, increases to about 25 m-equiv/l. cells when reduction is complete, and reverts to 7 or 8 m-equiv on re-oxygenation (Clarkson & Maizels, 1955). It is unlikely that in these various circumstances the adsorbing power of the cell increases proportionally, and it is more likely that easily exchanged Na increases because the cells become more permeable. If this applies to abnormal erythrocytes or to cells altered by cold-storage, it may well apply also to fresh normal cells, in which case it must be assumed that easily exchanged Na is derived from a small proportion of very permeable cells with a high Na content, rather than from each and every erythrocyte. This seems probable, because while the mean Na concentration of erythrocytes is about 12 m-equiv/l. cell water (excluding intercellular Na), easily exchanged Na is lost almost as quickly from cells washed in a KCl medium containing 15 m-equiv Na/l. as it is when the wash-fluid is pure KCl solution; indeed, immediate loss of Na from the erythrocytes continues, though in decreasing amounts as external Na is raised from 15 to 60 m-equiv (Table 2). The evidence that easily exchanged Na is derived from the interior rather than from the surface of the normal erythrocyte is not conclusive, though the possibility is supported by the observations of Davies (1954) that kidney tissue within 2 or 3 min of slicing loses about 30μmole K/g and gains about 40 μmole Na. However this may be, it seems probable that human and chicken erythrocytes contain two Na fractions, a large

fraction whose rate constant for passive transfer is about 0·02 hr^{-1}, and a small fraction (derived from the surface or interior of the cell, or from both sites) which exchanges very rapidly. In addition, Solomon (1952) has described a third Na fraction, inexchangeable, or exchanged only with difficulty. He emphasizes that when human erythrocytes are incubated with Na*, the concentration of Na and Na* at first converge, but show no tendency to ultimate meeting. His data suggest that about 2 m-equiv Na/l. cells are inexchangeable; methods in the present paper indicate a figure of about 1 m-equiv. Solomon & Gold (1955) have also produced evidence for two potassium fractions in human erythrocytes, the smaller exchanging twice as rapidly as the larger.

Cell Na *concentration* ($[Na_i]$) *and* Na *efflux.* As a result of storage at 4° C with different media, the concentration of cell Na may be varied. When several samples of such erythrocytes are incubated in identical media, it is found that in the case of human erythrocytes the initial value of $[Na_i]$ has little effect on k_2' (Table 5); this confirms the observations of Harris & Maizels (1951). In the case of chicken erythrocytes, k_2' is greater when the initial value of $[Na_i]$ is high. Since the Na efflux has been corrected for the passive component, and since it is improbable that metabolism is more active in Na-rich erythrocytes than in Na-poor cells, it must be presumed that Na is transported more efficiently when cell Na is high than when it is low. Thus, Na transport in human cells appears to be a first order process, while with chicken cells the reaction is of a higher order. Since $[Na_i]$ of stored cells falls during incubation, a progressive decrease in k_2' may be expected, and this accounts in part for the fall seen in Table 4 at 2–4 compared with 0–2 hr, though not for the similar but slighter falls of k_2' in fresh cells (Table 3).

External K *concentration and* Na *transport.* With human erythrocytes k_2' for Na is unaffected by changes in external K concentration ($[K_e]$) as long as this exceeds 2 m-equiv/l. (Harris & Maizels, 1951), though when $[K_e]$ falls to about 1 m-equiv a marked decrease in k_2' occurs. With chicken erythrocytes (Table 6) Na transport decreases slightly as $[K_e]$ falls from 12 to 5 m-equiv, and more markedly when $[K_e]$ is reduced below 3 m-equiv/l. suspension.

pH. The effects of pH on Na transfer in human erythrocytes have been described elsewhere (Flynn & Maizels, 1949; Harris & Maizels, 1951; Solomon, 1952): between pH 7·7 and 7·25 k_2' alters rather little, but below pH 7·25 transport decreases. The rate constant for Na influx, corrected for asymmetry, decreases slightly with fall of pH; typical findings are shown in Table 3, no. 17. With chicken erythrocytes the pH relations of efflux are similar, but influx is practically unaffected (Table 3). These findings were contrary to expectations, because it was thought that while human erythrocytes were relatively indifferent to pH, in the more actively metabolizing chicken cells the pH range might be more critical.

Temperature coefficient of influx and efflux. Between 23 and 38° C the

temperature coefficient for the rate constant of active Na efflux (k_2') from fresh chicken erythrocyte is less than that for the rate constant of influx (k_1') (Table 7). Hence, if the temperature of a suspension be lowered the equilibrium value for cell Na must also fall and this is in fact the case (Table 8). At some point between 4 and 23° C however, this relation of temperature coefficients must be reversed, since it has been seen that the Na concentration of cold-stored cells rises. This interesting observation corresponds with those of Solomon (1952) who used human cells and found for the range 30–37° C that the apparent energy of activation for influx was about 20,000 cal and for efflux about 15,000. However, in the present series of experiments with human cells, the apparent energies of activation averaged 15,000 cal for k_1' and 18,000 cal for k_2', while for the *uncorrected* constants k_1 and k_2 the figures were 15,000 and 17,700 cal. There is thus a discrepancy between Solomon's results and our own.

If the apparent energies of activation of influx and efflux be known, and also $[Na_i]$ at any given temperature, then $[Na_i]$ at any other temperature within the range reviewed may be calculated from the equation

$$A_{out} - A_{in} = \frac{RT_xT_y}{T_x - T_y} \times \ln \frac{[Na_i]_{\infty y}}{[Na_i]_{\infty x}},$$

where A_{out} is the apparent activation energy for efflux, A_{in} for influx, R is the gas constant, T_x and T_y the absolute temperatures corresponding to $x°$ and $y°$ C, $[Na_i]_{\infty x}$ the known equilibrium concentration for cell Na at $x°$ C (t = infinity) and $[Na_i]_{\infty y}$ the equilibrium concentration for cell Na at $y°$ C, which is to be calculated. The equation for $(A_{out} - A_{in})$ is based on the fact that at equilibrium $k_1[Na_e] = k_2[Na_i]$, k_1 and k_2 being the observed transfer constants uncorrected for asymmetry; it is implicit that the absolute value of the efflux at any given temperature is proportional to $[Na_i]$ and to a rate constant which is dependent on temperature. It is very uncertain whether the equation is strictly applicable to a complex system consisting of numerous reactions, each with its own temperature coefficient, but its application should have some qualitative value, and for two sets of similar data should at least yield results worth comparing. From the equation it follows that if, as in Solomon's experiments with human cells, $A_{out} - A_{in}$ is about -5000 cal, x and y respectively 37 and 27° C, and if $[Na_i]$ is 10 m-equiv/l. cell water at 37° C, then cell Na concentration at 27° C should fall to about 7·5 m-equiv. On the other hand, if as in the present paper $A_{out} - A_{in}$ is taken as $+2700$ cal, and the equilibrium value for $[Na_i]$ as 10 m-equiv at 37° C, then at 27° C $[Na_i]_\infty$ would be 11·7 m-equiv. That the equilibrium value for $[Na_i]$ is in fact higher at 27° C than at 37° C is supported by data in Table 3, which may be considered independently of, and quite apart from, any additional data on Na* concentration, or on k_1 or k_2. In these experiments, human blood had been

kept for several hours at 4° C and cell Na had risen, falling again on subsequent warming. It will be seen that after 4 hr incubation, when the systems were more nearly at equilibrium, [Na_i] was higher in the cooler suspensions: the average increase was +1·9 m-equiv/l. cell water for a mean temperature difference of −8·7° C.

Parallelism of rate constants for influx and efflux. Apart from changes in k_2' induced by respiratory poisons or by low external K concentration, any change in the constant for efflux is paralleled by a corresponding change in the constant for influx. Thus, both k_2' and k_1' decline after several hours' incubation of any given sample of chicken or human blood (Tables 3–5 and 10), while when a number of different samples are all incubated for the same time, cells with the higher k_2' also have a higher value for k_1'. The reason for this correlation is obscure, and Harris & Maizels (1951) suggest that it may be due to 'a single mechanism for transport in either direction'.

Effects of respiratory inhibitors on transport. Agents which inhibit respiration in chicken erythrocytes also inhibit Na transport, while inhibitors of glycolysis have little effect, provided some respirable substrate like lactate be present (Maizels, 1954*b*). An attempt has been made to correlate oxygen consumption with transport, and the data are shown in Tables 14 and 15. Some inhibitors were unsatisfactory, for while the effects on transport were marked, the metabolic effects were confusing. Thus dinitrophenol inhibits oxidative phosphorylation (Judah, 1951), but increases O_2 consumption (Table 13). Azide has a similar action (Judah, 1951) besides inhibiting cytochrome oxidase, and this agent appeared to inhibit transport with only a moderate decrease in the oxygen consumed. Fluoroacetate (Table 14) acted feebly, perhaps because chicken erythrocytes lack the specific activator, which according to Buffa & Peters (1949) fluoroacetate requires. Malonate was more satisfactory, though its action was also rather weak (Table 14). Cyanide was very active even in low concentrations, provided that equilibrium mixtures of KOH and KCN were present in the flask wells. With fluoroacetate, malonate and low concentrations of cyanide, there was rough correlation between reduction of respiration and decrease in the rate constant for efflux (Table 14). When, however, the concentration of cyanide was high, transport remained fairly active during the first half-hour of incubation, though respiration was almost completely inhibited (Table 14, nos. 7–11), and it must be presumed that Na efflux was energized by metabolic reserves accumulated during respiration, and still available after respiration had ceased. After incubation for 1 hr both respiration and transport had ceased: indeed, a number of negative values for k_2' were observed, some of which are shown in Tables 4, 10, 14 and 15. The values were small, averaging $-0{\cdot}02$ hr^{-1} in ten experiments carried out by the method used for obtaining the data of Table 14. However, it is thought that special weight should be given to Expts. 9–11 of Table 14 because in these the

technique was most exacting: Na was estimated in quadruplicate and Na* in triplicate, and while in earlier experiments tracer-free cells were washed with KCl, and cells containing Na* with normal chicken plasma, in these three experiments both sets of cells were washed with plasma that had been dialysed, and then reconstituted with KCl. The values for k_2' thus obtained were about $+0{\cdot}02$ hr^{-1} between 1 and 2 hr, and it is thought that such small negative values as are found in other experiments, are due to experimental error.

Finally, the relation of energy liberated to transport effected may be considered. Chicken erythrocytes respiring at 38° C liberate about 400 cal/l. cells.hr; after partial reduction of respiration with cyanide, the value may fall to 140 cal, while when fully poisoned with cyanide, glycolysis is the only source of energy, corresponding to about 45 cal: the values of k_2' corresponding to these various conditions are respectively about 0·4, 0·1 and 0 hr^{-1}. In human erythrocytes, glycolysis is the only source of energy, corresponding to 80 cal/l. hr at 37° C, to about 45 cal at 30° C and to about 25 cal at 23° C: the corresponding values of k_2' are approximately 0·3, 0·17 and 0·1 hr^{-1} (Table 15). It thus seems that the respiring erythrocyte requires a greater amount of total energy for transporting Na, than does the glycolysing human cell, possibly because its general activities are so numerous and complex as to leave only a small fraction of the total energy available for transport. The possibility also remains, though no supporting evidence has been found, that in the respiring erythrocyte glycolysis cannot be linked to transport.

SUMMARY

1. In the nucleated chicken erythrocyte, as in the non-nucleated human cell, potassium as well as sodium is transported actively.

2. Sodium trapped in the intercellular plasma of centrifuged erythrocytes contributes about 3 m-equiv/l. to the gross content of human cells and about 4·5 m-equiv to that of chicken cells. Net cell Na left after intercellular Na is deducted includes an inexchangeable fraction, a large fraction whose rate constant for passive penetration is about 0·02 hr^{-1}, and also a Na fraction which exchanges very rapidly with the external medium and which accounts for 2–3 m-equiv Na/l. cells.

3. When fresh human erythrocytes are incubated for 2 hr at 37° C, the rate constants for Na efflux and influx corrected for the Donnan asymmetry (i.e. k_2' and k_1') are respectively about 0·35 and 0·02 hr^{-1}; for chicken cells the values are about 0·45 and 0·02 hr^{-1}. The rate of efflux declines with time: in part at least, this may only be apparent, and due to a failure to allow for inexchangeable Na within the cells.

4. In human cells k_2' is largely independent of the initial level of cell Na, but with chicken erythrocytes k_2' increases with the concentration of cell Na.

5. In chicken erythrocytes Na efflux decreases when external potassium

($[K_e]$) is very low (less than 1 m-equiv/l. external medium) and tends to become maximal as $[K_e]$ rises above 3 m-equiv.

6. In both human and chicken erythrocytes k_2' is roughly constant for a pH range of 7·25–7·65; below pH 7·1 Na efflux decreases.

7. Between 24 and 37° C the apparent activation energy of Na efflux is about 18,000 cal and for influx about 15,000; hence fresh human erythrocytes gain Na on cooling to 24° C. For chicken erythrocytes the activation energies for efflux and influx are about 10,000 and 11,000 cal and hence with cells at equilibrium with the external medium, cell Na concentration is lower at 24° C than at 37° C. However, the activation energy for efflux must come to exceed that of influx somewhere between 4 and 24° C, for when chicken cells are cooled to 4° C, cell Na rises. In human erythrocytes there is close correlation between k_2', glycolysis and temperature. But at constant temperature, there is much less correlation between k_2' on the one hand, and glycolysis and pH on the other.

8. There is rough correlation between the action of respiratory poisons on respiration and on Na transport, though with high concentrations of cyanide inhibition of respiration is almost immediate, while between $\frac{1}{2}$ and 1 hr elapses before inhibition of Na transport is complete.

REFERENCES

BERNSTEIN, R. E. (1952). The determination of potassium and sodium in biological fluids by flame photometry. *S. Afr. J. med. Sci.* **17**, 101–116.

BERNSTEIN, R. E. (1953). Rates of glycolysis in human red cells in relation to energy requirements for cation transport. *Nature, Lond.*, **172**, 911–912.

BUFFA, P. & PETERS, R. A. (1949). The *in vivo* formation of citrate induced by fluoroacetate and its significance. *J. Physiol.* **110**, 488–500.

CHAPLIN, H. & MOLLISON, P. (1952). Correction for plasma trapped in the red cell column of the haematocrit. *Blood*, **7**, 1227–1238.

CLARKSON, E. M. & MAIZELS, M. (1955). Sodium transfer in the erythrocytes of sickle-cell anaemia. *J. Physiol.* **129**, 504–512.

CLAUDIUS, M. (1924). A new method for the microdetermination of chlorine in blood. *Acta med. scand.* **61**, 4–7.

DAVIES, R. E. (1954). Relations between active transport and metabolism in some isolated tissues and mitochondria. *Symp. Soc. exp. Biol.* **8**, 453–475.

DODDS, E. C. & GREVILLE, G. D. (1934). Effect of dinitrophenol on tumour metabolism. *Lancet*, i, 398–399.

FLYNN, F. & MAIZELS, M. (1949). Cation control in human erythrocytes. *J. Physiol.* **110**, 301–318.

HARRIS, E. J. (1954). Linkage of sodium- and potassium-active transport in human erythrocytes. *Symp. Soc. exp. Biol.* **8**, 228–241.

HARRIS, E. J. & BURN, G. P. (1949). The transfer of sodium and potassium ions between muscle and the surrounding medium. *Trans. Faraday Soc.* **45**, 508–528.

HARRIS, E. J. & MAIZELS, M. (1951). The permeability of human erythrocytes to sodium. *J. Physiol.* **113**, 506–524.

HARRIS, E. J. & MAIZELS, M. (1952). Distribution of ions in suspensions of human erythrocytes. *J. Physiol.* **118**, 40–53.

HARRIS, J. E. (1941). The influence of the metabolism of human erythrocytes on their potassium content. *J. biol. Chem.* **141**, 579–595.

HSU, F. Y. (1935). Factors affecting blood glycolysis *in vitro* and *in vivo*. *J. Physiol.* **84**, 173–186.

HULLIN, R. P. & NOBLE, R. L. (1953). The determination of lactic acid in microgram quantities. *Biochem. J.* **55**, 289–291.

JACKSON, M. D. & NUTT, M. E. (1951). Intercellular plasma and its effect on absolute red cell volume determination. *J. Physiol.* **115**, 196–205.

JUDAH, J. D. (1951). The action of 2:4-dinitrophenol on oxidative phosphorylation. *Biochem. J.* **49**, 271–285.

KREBS, H. A. (1935). The synthesis of glutamine from glutamic acid and ammonia, and the enzymic hydrolysis of glutamine in animal tissues. *Biochem. J.* **29**, 1951–1969.

MAIZELS, M. (1936). The anion and cation content of normal and anaemic bloods. *Biochem. J.* **30**, 821–830.

MAIZELS, M. (1945). Intercellular plasma in the centrifuged erythrocytes of normal blood. *Quart. J. exp. Physiol.* **33**, 129–136.

MAIZELS, M. (1951). Factors in the active transport of cations. *J. Physiol.* **112**, 59–83.

MAIZELS, M. (1954*a*). Active cation transport in erythrocytes. *Symp. Soc. exp. Biol.* **8**, 202–227.

MAIZELS, M. (1954*b*). Cation transport in chicken erythrocytes. *J. Physiol.* **125**, 263–277.

OBERST, F. W. (1935). The determination of sodium in human red blood cells. *J. biol. Chem.* **108**, 153–160.

RAKER, J. W., TAYLOR, I. M., WELLER, J. M. & HASTINGS, A. B. (1950). Rate of potassium exchange of the human erythrocyte. *J. gen. Physiol.* **33**, 691–702.

SOLOMON, A. K. (1952). The permeability of the human erythrocyte to sodium and potassium. *J. gen. Physiol.* **36**, 57–110.

SOLOMON, A. K. & GOLD, G. L. (1955). Potassium transport in human erythrocytes: evidence for a three compartment system. *J. gen. Physiol.* **38**, 371–388.

USSING, H. H. (1949). Transport of ions across cellular membranes. *Physiol. Rev.* **29**, 127–155.