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CATION TRANSPORT IN CHICKEN ERYTHROCYTES

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The mature human erythrocyte is non-nucleated and its consumption of oxygen is negligible. Hence, it is to be expected that in such a cell transfer of cations against gradients of concentration or potential (cation transport) will be energized by anaerobic reactions, and it has in fact been shown that these movements are based on glycolysis, being inhibited by fluoride (Harris, 1941; Maizels, 1951) and unaffected by cyanide, carbon monoxide, azide, malonate and 2:4-dinitrophenol in amounts which are known to inhibit respiration (Maizels, 1951). Like human cells, the mature erythrocytes of the chicken and tortoise are specialized for oxygen carriage, but they differ from the former in their possession of a nucleus and in their greater respiratory activity. It follows that in nucleated erythrocytes cation transport might be energized either by glycolysis or by respiration or by both activities: the present communication is concerned with these various possibilities.

In principle, the method used involves a preliminary period of several days during which the blood is stored at 4° C. This reduces metabolism to a minimum and causes cations to move with the concentration gradients so that cell sodium rises and potassium falls. At the end of cold-storage glucose, lactate, metabolic poisons or other appropriate substances are added to the blood which is then incubated: if at this stage cations move against their respective gradients, active transport is judged to have occurred.

METHODS

A sample of heparinized blood was centrifuged at 2000 *g* for 30 min in graduated tubes of 6 mm bore; this 'original sample' was analysed for Na, K and water content and also used as a standard of reference for volume changes in the cells of the treated blood samples (Maizels, 1943). 100 ml. residual blood was then mixed with 300 ml. NaCl solution (0·155 M) and 10 ml. trisodium citrate (2·5 g/100 ml.) and stored for 6 days at 4° C, chloramphenicol (10 μg/ml.) being added to check bacterial growth. After cold-storage, 10–20 ml. $Na_2HPO_4.2H_2O$ (0·1 M), 15–30 ml. KCl (0·15 M) and sometimes 1·5 ml. glucose solution (2·8 M) were added and the pH adjusted electrometrically

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to about 7·5 with NaOH. Part of this suspension was then centrifuged and kept for analysis, while the rest was divided between a number of bottles, to all except one of which suitable test reagents were added (metabolic poisons, etc.). The samples were next incubated for several hours at 20–25° C and then centrifuged, analyses being carried out on a fivefold dilution of the packed cells by methods described elsewhere (Maizels, 1943; Harris & Maizels, 1952). Certain modifications were required, however, for chicken cells haemolysed in water give a ropy unworkable jelly and so cell dilutions were made with sucrose solution (2 g/100 ml.) brought to pH 7·2 with a trace of LiOH solution. Estimation of K in ashed cells was unsatisfactory, for at 500° C a small loss of K occurs and so both Na and K were estimated on a trichloroacetic acid filtrate neutralized with excess of solid $Ca(OH)_2$: this served to neutralize the acid and to precipitate the phosphates which in chicken erythrocytes are sufficiently high to interfere with the estimation of Na by zinc uranyl acetate. In a number of experiments Na and K were also estimated using a flame photometer with an internal lithium standard. Agreement was good in the case of Na and often but not always good in the case of K. Hence, the chemical method of King, Haslewood, Delory & Beall (1942) was preferred for estimating K, because duplicates usually agreed within ± 1 m.equiv/l. cells and because it gave greater consistency in total base concentration of batches of cells suspended in large volumes of saline media of known total base concentrations.

It has been shown (Harris & Maizels, 1952) that the pH of undiluted haemolysed human erythrocytes and of a haemolysate diluted 5 times are similar and probably not far removed from the pH of the intact cell. It is probable that the effect of dilution on the pH of the heavily buffered haemolysates will also be small, but chicken cell haemolysates contain nuclei or nuclear fragments and the relation which their pH bears to that of the intact cytoplasm is uncertain, though it is probably sufficiently consistent to be used as a means of controlling pH.

The concentration of Na and K in cells is obtained from the estimated cell contents and the water content; the latter is calculated from the known water content of fresh cells and from the observed changes in cell volume, themselves due to movements of water (Harris & Maizels, 1952). It wil]be noted that neither the estimated content nor the concentration of a base in the cells necessarily indicates absolute changes within the cell due to movements of the base across the cell membrane, because alterations in the tonicity or pH of the suspending medium cause immediate changes in cell volume and cation content and concentration without any cation crossing the cell membrane at all; on the other hand, there may be large movements of cation in and out of cells which, if accompanied by appropriate shifts of cell water, might leave the observed cell cation content unchanged and give the impression that neither transport nor passive diffusion had occurred. Hence, in order to show whether cation has moved in or out of the cell, cation contents set out in the tables have been corrected for volume changes by reference to the original cell volume. An example may make this clear: suppose that cells as a result of cold-storage have swollen by 5% so that the volume correction, V, is 1·05 and that the Na content is 40 m.equiv/l. and that two similar samples incubated under different conditions attain the following respective values: V 1·3, Na 35 and V 0·85 and Na 45: the corrected contents are 42 m.equiv before incubation and 45 and 36 m.equiv respectively after incubation. In the first incubated sample the uncorrected Na content has fallen, but Na has actually entered the cell; in the second, Na appears to have risen but after correction for volume it is seen that Na has moved out of the cell. Further details of all the methods already mentioned are given elsewhere (Maizels, 1949; Harris & Maizels, 1952). Lactate was estimated by the method of Hullin & Noble (1953).

RESULTS

Dimensions and properties of untreated erythrocytes

The figures set out in Table 1 are not described as normal, since in the case of the chickens information is lacking about the duration of transport from farm to slaughter house and also about salt and fluid intake during that interval:

as for the tortoises their selection was guided by economic rather than aesthetic principles and some of the subjects were in pretty poor shape at the time of receipt. As the subjects were few in number and had been kept in conditions which could hardly be described as normal, the data submitted show mean values and the overall range, but not the standard deviation.

The figures presented for cations in centrifuged chicken cells are subject to correction for intercellular plasma—corrections which are specially significant in the case of cell Na. Intercellular plasma estimated by the Evans blue dye method (Maizels, 1945) appears to lie between 3 and 4% and so the true values for cell Na are about 5 m.equiv/l. less than the gross values shown in the tables. However, the latter are strictly comparable and are valid for the purposes of the present paper.

TABLE 1. Some data for fresh untreated chicken and tortoise bloods

	Chicken			Tortoise		
	No. of bloods		Range	No. of bloods		Range
Hb, g/100 ml. blood	9	9·5	(8·6–10·2)	5	8·1	(7·5–8·8)
Haematocrit	11	31	(28–34)	6	28	(23–32)
Hb, g/100 ml. cells	8	30·5	(26–34)	5	29	(28–33)
R.B.C., millions/cu. mm blood	9	2·52	(2·3–2·65)	5	0·57	(0·52–0·64)
Mean cell volume, μ^3	9	123	(111–136)	5	490	(420–540)
Water, g/100 ml. cells	8	69·5	(68–72)	5	69	(67–70)
Na, m.equiv/l. cells	15	12·6	(10–15)	5	11·1	(10·5–12·2)
K, m.equiv/l. cells	14	105	(96–112)*	5	108	(105–116)
Na, m.equiv/l. plasma	14	155	(144–167)	5	152	(145–158)
K, m.equiv/l. plasma	14	5·3	(4·8–6·6)	5	4·5	(4·0–5·4)

* Excluding one value of 84.

Note. 100 cells were measured on a dried blood film from a single chicken; the figures with standard deviation were—long diameter $10{\cdot}2 \pm 1{\cdot}8$ μ and short diameter $6{\cdot}4 \pm 0{\cdot}6$ μ. For a single tortoise the diameters were $18{\cdot}0 \pm 1{\cdot}7$ and $9{\cdot}4 \pm 1{\cdot}1$ μ.

During cold-storage Na and K move with the concentration gradients and as the increase of cell Na exceeds decrease of K, total base increases slightly and the cells swell. These changes are qualitatively similar to those observed in the human cell, but are less evident. Thus during 7 days at 4° C the erythrocytes of human blood unmixed with saline medium gained about 30 m.equiv/l. Na, while chicken cells gained only 10–15 m.equiv. Since transport of Na from the cell during incubation is best seen when the initial cell Na is high, it was found desirable to cold-store chicken blood with 3 vol. of NaCl solution (0·155 N): under these conditions about 20–30 m.equiv Na were gained in a week. During subsequent incubation at 37° C chicken erythrocytes, like human cells, transport cations against the concentration gradients, loss of Na exceeding gain of K. Because of this and possibly through loss of non-penetrating cell anion, such as occurs from dephosphorylation of phosphoric esters, total cell base is absolutely decreased and slight shrinkage

occurs. It should be noted that the figures recorded in the tables for cation in incubated cells, do not necessarily apply to suspensions at equilibrium: for though untreated cells may reach equilibrium in 4 hr, partially poisoned cells whose transport is decreased or abolished, will take very much longer. Hence, the data for cation distribution must not be regarded as an absolute measure of differences in transport, but rather as an indication of the kind of change effected by various agents.

It will be shown later that transport in chicken erythrocytes is based mainly on respiration. One sample of chicken erythrocytes cold-stored for a week and then incubated at 37° C in Ringer solution at pH 7·4 used oxygen at the rate of 120 μl./ml. per hr, the addition of cyanide (5 mM) depressing respiration by 80%. A second sample incubated in the NaCl-KCl-phosphate medium described above used 75 μl. in the first hour and 60 μl. during the second hour of incubation: if the substance oxidized were glucose, 0·4 mM should disappear from 1 l. cells in 1 hr; the observed loss, however, was 0·28 mM so that other substances besides glucose are being oxidized during respiration or else glucose metabolized is partly replaced from other stores of carbohydrate. In this system the addition of sodium cyanide (2·5 mM) depressed respiration by 78% in the first hour and by 95% in the second hour.

In eighteen experiments 1 l. chicken erythrocytes from stored blood (pH 7·2–7·4) liberated $0{\cdot}46 \pm 0{\cdot}19$ mM lactic acid per hour when respiring and $1{\cdot}62 \pm 0{\cdot}41$ mM when respiration was poisoned. Under similar conditions incubated human cells liberated $3{\cdot}1 \pm 0{\cdot}75$ mM lactic acid (fourteen observations). Further discussion of the complex metabolism of the chicken erythrocyte is outside the scope of the present paper and the preceding data merely serve as an indication of the amount of respiration and glycolysis occurring under experimental conditions.

Metabolic poisons and cation transport in chicken erythrocytes

If cation transport in these cells were based on respiration, the movements should be inhibited by agencies which prevent utilization of free oxygen. Inhibitors of glycolysis, on the other hand, should only interfere with transport when glucose is the source of energy, but not when lactate or pyruvate are available as substrates. These matters are considered below.

Inhibitors of respiration (Table 2). Cyanide and dinitrophenol cause considerable inhibition of transport in high dilution; azide also inhibits, but in higher concentration, while malonate and fluoroacetate require a still higher concentration. In the human cell, dinitrophenol up to 0·25 mM and the other agents in a concentration of 10 mM have little or no effect on transport (Maizels, 1951). The effect of oxygen lack was determined by storing blood in the cold for 6 days, evacuating air, replacing with nitrogen and returning to the cold-room overnight. Next day, the blood was incubated in the ordinary

TABLE 2. Effects of respiratory poisons on cation transport in chicken erythrocytes kept at 4° C for 7 days and then incubated at 37° C

(Initial external concentration of incubated systems, K 10 and Na 150 m.equiv/l. water; glucose 11 mM)

Expt.	Hr at 37° C	Additions (mM/l. cell suspension)	Erythrocytes: pH at 20° C	Contents m.equiv/l. cells* K	Contents m.equiv/l. cells* Na	Concentrations m.equiv/l. cell water K	Concentrations m.equiv/l. cell water Na	Lactic acid liberated mM/l. cells
1 *a*	0	(Unincubated)	7·30	59	57	81	78	—
b	4	No addition	7·28	95	14	134	20	—
c	4	Cyanide 0·06	7·31	94	17	132	24	—
d	4	Cyanide 0·2	7·29	63	50	87	70	—
e	4	Cyanide 0·6	7·33	61	54	83	74	—
f	4	Cyanide 2·5	7·37	57	61	78	83	—
g	4	Cyanide 10	7·48	56	65	76	88	—
h	4	Azide 2	7·30	62	53	84	72	—
2 *a*	0	(Unincubated)	7·27	71	51	95	68	—
b	4½	No addition	7·21	91	19	132	27	—
c	4½	Dinitrophenol 0·015	7·19	89	24	124	34	—
d	4½	Dinitrophenol 0·06	7·18	73	47	100	65	—
e	4½	Dinitrophenol 0·24	7·20	63	57	85	77	—
f	4½	Malonate 1·5	7·24	86	26	122	37	—
g	4½	Malonate 3	7·24	79	36	110	50	—
h	4½	Malonate 9	7·20	68	52	93	71	—
3 *a*	0	(Unincubated)	7·26	82	36	111	49	—
b	4½	No addition	7·30	94	19	138	28	—
c	4½	Fluoroacetate 1	7·31	94	19	138	28	—
d	4½	Fluoroacetate 4	7·38	93	23	131	32	—
e	4½	Fluoroacetate 8	7·36	88	29	122	40	—
4 *a*	0	(Unincubated)	7·27	76	38	114	57	0
b	4½	No addition	7·27	88	23	130	34	0·60
c	4½	Cyanide 0·6	7·24	75	39	108	57	1·81
d	4½	Cyanide 2·5	7·30	72	43	106	63	1·96
e	4½	Dinitrophenol 0·06	7·25	76	41	108	58	1·62
f	4½	Dinitrophenol 0·25	7·30	75	43	104	60	1·96
g	4½	Malonate 9	7·31	75	39	110	57	1·53
h	4½	Fluoroacetate 10	7·28	76	37	114	55	1·48
5 *a*	0	Deoxygenated (unincubated)	7·10	71	37	99	51	—
b	4	Deoxygenated; then reoxygenated	7·11	91	16	132	23	—
c	4	Deoxygenated; not reoxygenated	6·99	64	38	95	56	—
d	4	Deoxygenated; saturated with CO	6·96	66	41	96	60	—
6 *a*	0	(Unincubated)	7·47	74	46	106	66	0
b	5	Deoxygenated; then reoxygenated	7·45	98	21	143	31	0·45
c	5	Deoxygenated; not reoxygenated	7·44	70	52	97	72	2·23

* Contents corrected for changes in volume by reference to the original cell volume (see methods).

way. Blood so treated showed active transport if oxygen were admitted at the beginning of incubation, but not otherwise. Admission of carbon monoxide leaves the inhibition of transport unaffected. The addition of any of these agents greatly increases the production of lactic acid by the cells.

Lactate and cation transport (Table 3). To demonstrate the effects of lactate on cation transport, it is first necessary to remove the natural energizing

TABLE 3. Effects of glucose, lactate, fluoride, monoiodoacetate and glutamate on washed chicken erythrocytes kept at 4° C for 7 days and then incubated at 37° C

(Erythrocytes were washed by separating from plasma and storing at 4° C with 30 vol. of NaCl solution, 0·16M. Before incubation the supernatant was replaced by 25 vol. of glucose-free NaCl-KCl suspending medium, which contained initially K 10 and Na 150 m.equiv/l.)

				Erythrocytes				
					Contents m.equiv/l. cells*		Concentrations m.equiv/l. cell water	
Expt.	Hr at 37° C	Additions mM/l. cell suspension (note: cells partly depleted of glucose by washing)	Plasma added	pH at 20° C	K	Na	K	Na
1 *a*	0	(Unincubated)	0	7·32	74	51	93	64
b	4	No addition	0	7·30	80	43	106	57
c	4	Glucose 9	0	7·35	93	27	124	36
d	4	Lactate 20	0	7·33	94	25	129	34
e	4	NaF 10	0	7·40	72	52	93	67
f	4	NaF 10, glucose 9	0	7·34	76	47	100	62
g	4	NaF 10, lactate 20	0	7·36	88	31	120	42
h	4	Glutamate 5, glucose 9	0	7·37	92	27	123	36
2 *a*	0	(Unincubated)	0	7·36	85	40	112	53
b	4½	No addition	0	7·36	89	37	118	50
c	4½	Glucose 9	0	7·30	95	25	129	34
d	4½	Lactate 30	0	7·34	97	23	130	31
e	4½	NaF 3	0	7·34	88	39	112	50
f	4½	NaF 3, glucose 9	0	7·31	89	32	118	42
g	4½	NaF 3, lactate 30	0	7·31	96	24	125	31
h	4½	NaF 10	0	7·40	81	43	104	55
i	4½	NaF 10, glucose 9	0	7·35	81	42	104	54
j	4½	NaF 10, lactate 30	0	7·34	86	37	112	48
3 *a*	0	(Unincubated)	0	7·40	66	51	95	73
b	4	No addition	0	7·40	73	42	104	60
c	4	Glucose 9	0	7·40	83	31	119	44
d	4	Lactate 20	0	7·32	86	26	120	36
e	4	NaF 10, glucose 9	0	7·37	72	44	102	62
f	4	NaF 10, lactate 20	0	7·36	83	31	119	44
4 *a*	0	(Unincubated)	0	7·37	78	37	106	50
b	4½	No addition	0	7·39	80	32	111	44
c	4½	Glucose 10	0	7·42	87	20	128	29
d	4½	Lactate 10	0	7·40	92	15	136	22
e	4½	Lactate 20	0	7·40	93	16	136	23
f	4½	NaF 5, glucose 10	0	7·37	80	29	118	42
g	4½	NaF 10, glucose 10	0	7·28	75	36	104	50
h	4½	Glucose 10	+	7·48	98	14	140	20
i	4½	NaF 5, glucose 10	+	7·46	91	19	130	27
j	4½	NaF 10, glucose 10	+	7·44	86	24	124	35
5 *a*	0	(Unincubated)	0	7·32	64	57	86	77
b	4	No addition	0	7·31	72	46	100	64
c	4	Glucose 9	0	7·31	85	28	120	40
d	4	Lactate 30	0	7·29	87	26	122	37
e	4	MIA 0·2	0	7·30	65	53	88	72
f	4	MIA 0·2, glucose 9	0	7·31	65	54	88	73
g	4	MIA 0·2, lactate 30	0	7·31	83	30	117	43
h	4	MIA 0·8	0	7·29	64	57	86	77
i	4	MIA 0·8, glucose 9	0	7·30	63	57	85	77
j	4	MIA 0·8, lactate 30	0	7·24	78	34	110	48

* Contents corrected for changes in volume by reference to the original cell volume (see Methods). MIA = monoiodoacetate. 4 *i* and *j* incubated with undiluted plasma.

substrate, glucose. In human erythrocytes some of the glucose naturally present disappears during the preliminary cold-storage period and the rest during the early stages of incubation by diffusion into the added diluent and especially by glycolysis. In cells so treated, transport is abolished and cation movements are directed by simple physical laws (Maizels, 1951). But when chicken cells are treated in this way, transport continues: thus in one instance storage at 4° C caused cell Na to rise from 13 to 51 m.equiv/l. During the subsequent incubation Na fell to 21 m.equiv. when the system was fortified with extra glucose and to 25 m.equiv when no glucose at all was added. This may arise from the fact that the respiring chicken erythrocyte can derive more energy from a given amount of glucose than can the glycolysing non-nucleated human cell: moreover, avian erythrocytes contain much more phosphoric esters than mammalian cells (Rapoport & Guest, 1941) and it may be presumed that the reserves of energy are correspondingly great. Whatever the cause, the effects may be minimized by the partial removal of soluble substrates from the cells. This was accomplished as follows: supernatant plasma was removed from gently centrifuged fresh chicken blood and the cells stored at 4° C without the addition of glucose in 30 vol. of NaCl solution (0·16 M). After a week the supernatant solution was removed and replaced by 25 vol. of the NaCl-KCl-phosphate solution (see Methods). In the present paper erythrocytes so treated are called 'washed cells': suspensions of these cells were incubated as such or with appropriate additions. Table 3 shows that even after such drastic treatment and in the absence of added glucose, some degree of cation transport persists (see records *a* and *b* of the various experiments) since cell Na, which may be expected to rise with the concentration gradient, actually falls slightly. It is clear, however, that the addition of either glucose or lactate to such a depleted system greatly increases the cation transport, and it may be concluded that lactate which is unable to energize transport in human erythrocytes is able to accomplish this in chicken cells. Indeed, transport effected by lactate is always slightly greater than with glucose, not only when lactate added exceeds glucose (Table 3, Expts. 2 and 5), but when the amounts are roughly equal (Expts. 1, 3 and 4), and even when the weight of lactate added is only half that of glucose in a corresponding suspension (Expt. 4, *a*, *c* and *d*).

Fluoride and cation transport (Table 3). Since cation transport in chicken erythrocytes depends especially on respiratory activities, fluoride should inhibit transport in the presence of glucose, but not when lactate is added, and this may be expected whether the cells be depleted of glucose or not. In fact, cells suspended in diluent from which plasma has not been excluded are much more resistant to the action of fluoride than are suspensions of washed cells. This is shown in Expt. 4 where cold-stored suspensions of washed chicken erythrocytes are incubated without and with the addition of plasma (Expt. 4, *e*, *f*, *g*; *c*, *i*, *j*). It may be noted that suspensions of chicken cells containing plasma

are also much more resistant to the action of fluoride than are the corresponding suspensions of human cells, for the former shows a decreased but still definite output of Na in the presence of 10 mM fluoride, while when dilutions of whole human blood are incubated with only 4 mM-NaF, manifest transport of cation may cease (Maizels, 1951). It is probable that plasma protects transport in chicken erythrocytes because under experimental conditions it contains even when relatively fresh an amount of lactate equivalent to about 1·9 mM/l. cells; an amount which increases to 2·4 mM after a week at 4° C (mean of seven observations): suspensions of cold-stored washed cells, on the other hand, contain the equivalent of only 0·4 mM lactate/l. cells (mean of thirteen observations). It follows that the high content of lactate in suspensions of unwashed cells affords a source of energy for cation transport which is independent of glycolysis—a source which is slight in washed cell suspensions. Even washed cell suspensions, however, are fairly resistant to fluoride, for in the presence of 4 mM fluoride incubated human erythrocytes (unwashed) gain 4 or 5 m.equiv Na and as much as 10 m.equiv Na when the fluoride content is 10 mM; washed chicken cells, on the other hand, show practically no increase of Na in the presence of 10 mM-NaF, while with 5 mM-NaF some Na is still transported from the cells at 37° C (Table 3). It is possible that this resistance to fluoride poisoning may lie in the high magnesium content of chicken erythrocytes, which in three cases amounted to 5·2, 5·4 and 4·9 mM/l. cells, or about twice as great as the value in human cells.

Since the effects of fluoride on unwashed cells are slight unless a relatively high proportion of anion in the suspending medium is replaced by fluoride, it has been thought more satisfactory to examine the effects of fluoride on washed cells. If the suspensions of such cells contain no added glucose, manifest output of Na and uptake of K are virtually inhibited (Table 3, Expts. 1 and 2), but in the presence of added glucose a small amount of cation transport is observed. If, however, lactate is added to washed cells incubated with fluoride, cation transport is much increased (Table 3, Expts. 1–3). Nevertheless, fluoride has some inhibitory action on the oxidation of lactate, for in Expt. 1 of Table 3, incubation with lactate causes cell Na to fall by 26 m.equiv in the absence of fluoride and by 20 m.equiv in its presence; the findings in Expts. 2 and 3 are similar. However, in spite of this partial inhibition, a considerable amount of cation transport can be energized by lactate even in the presence of fluoride.

Monoiodoacetate (MIA) and cation transport (Tables 3 and 4). The findings here conform to those of the previous experiments: transport is inhibited, the effect being more apparent in washed cells, presumably because of their low content of natural lactate. Even this, however, may suffice to energize a small amount of transport, for when incubated with 0·2 mM MIA chicken erythrocytes show a decrease of 2 or 3 m.equiv Na (Table 3), while human cells even

when unwashed show a passive increase of about 10 m.equiv (Maizels, 1951). Lactate, but not glucose, energizes transport in the presence of MIA, but the transport is not as great as in the absence of MIA: presumably, the latter has some inhibitory action of the respiratory cycle.

Glutamate and cation transport. Terner, Eggleston & Krebs (1950) have shown that glutamate is essential to cation transport in the brain and retina of certain mammalia. The natural glutamate of these tissues is considerable—about 6 mM/kg tissue: in the chicken, glutamate in the erythrocytes of three bloods kept 7 days at 4° C and then incubated for 4 hr, was estimated by paper chromatography (Fowden, 1951) and found to be only 0·8, 0·3 and 0·3 mM/l. respectively; yet such erythrocytes transported cation very freely on incubation, half the total Na output and K uptake occurring in the first hour. Addition of glutamate to a level of 10 mM/l. cell suspension had no effect on the amount of transport, possibly because little or no glutamate entered the cells; for when allowance was made for glutamate in the intercellular fluid, the glutamate of cells suspended for 4 hr at 37° C in a medium containing 10 mM glutamate, was only 0·4 mM/l. cells in one experiment and 0·6 mM/l. in a second.

TABLE 4. Effects of temperature, calcium and various respiratory poisons on cation transport in tortoise erythrocytes kept at 4° C for 7 days and then incubated

(Initial external concentrations of incubated systems, K 10 and Na 160 m.equiv/l., glucose 11 mM)

	Incubation			Erythrocytes						
							Contents m.equiv/l. cells*		Concentrations m.equiv/l. cell water	
Expt.	Temp. (° C)	Time (hr)	Additions mM/l. cell suspension	pH at 20° C	*V*	Haemolysis	K	Na	K	Na
1 *a*		0	(Unincubated)	6·90	105	0	102	18	136	25
b	23	3	No addition	6·78	103	0	104	16	142	22
c	23	3	$CaCl_2$ 5	6·76	101	0	106	11	150	15
d	23	3	NaCN 2	6·65	112	0	94	30	115	37
e	23	3	NaCN 2, $CaCl_2$ 5	6·66	103	0	98	18	134	25
f	37	3	No addition	6·67	178	Marked	53	176	36	120
g	37	3	$CaCl_2$ 5	6·72	100	0	105	11	151	16
h	37	3	$MgCl_2$ 5	6·69	180	Marked	53	182	35	122
2 *a*		0	(Unincubated)	6·94	111	0	88	42	108	52
b	23	3¼	No addition	7·06	108	0	90	37	116	47
c	23	3¼	Dinitrophenol 2·5	6·82	116	0	79	56	92	65
d	23	3¼	Carbon monoxide	6·92	114	0	73	58	87	69

V = cell volume as a percentage of the original cell volume.

* Contents corrected for changes in volume by reference to the original cell volume (see Methods).

Expt. 1, anticoagulant was heparin; Expt. 2, anticoagulant was trisodium citrate.

Metabolism and cation transport in the erythrocytes of the African tortoise

When heparinized blood was stored for 7 days at 4° C, change in cell cation was slight, Na rising and K falling by 2 or 3 m.equiv. If citrate were added, however, passive change in cation distribution was much more marked

(Table 4, Expt. 2). In either case incubation at 23° C resulted in a small amount of transport (Expts. 1, *a* and *b*; 2, *a* and *b*), but at 37° C there was a moderate decrease of K, a marked increase in Na and volume, with a tendency to haemolysis (Expt. 1, *a* and *f*). The response of cells from different individuals varied; in some cases, cell swelling was 30% or less and lysis was absent; in others swelling exceeded 70% and lysis was marked or even almost complete. The addition of calcium to the standard calcium-free suspending medium (3–5 mM was used, though less might suffice) inhibited swelling and haemolysis, and made cation transport more evident (Expt. 1, *a–c*; 1, *a*, *f* and *g*). The addition of carbon monoxide, dinitrophenol and cyanide to calcium-free cell suspensions incubated at 23° C inhibited transport, but in the presence of calcium the effect of cyanide was much less marked (Expts. 1, *a*, *b*, *d* and *e*): presumably, calcium acts by lowering permeability and delaying the passive penetration of cations; magnesium is without effect.

Metabolism and cation transport in the erythrocytes of the grass snake

Very few experiments were done with the grass snake because the yield per snake was only about 5 ml. blood and 1·2 ml. cells. Sodium alone was estimated and in three pools of fresh blood values of 10·0, 10·6 and 11·2 m.equiv/l. cells were found. The cells were resistant to increase of Na content during periods of cold-storage up to 1 week, the rise varying between 3 and 6 m.equiv/l. cells. Incubation with or without added glucose between 16 and 37° C usually restored cell Na to the value found in fresh erythrocytes. Fall in cell Na during incubation was prevented by the addition of cyanide. Thus, in one instance cell Na in fresh blood was 10·6 m.equiv and rose to 14·8 m.equiv after 1 week at 4° C; incubation at 25° C in the absence of cyanide then caused cell Na to fall to 9·6 m.equiv, while in the presence of cyanide (2 mM/l. cell suspension) cell Na rose from 14·8 to 20·2 m.equiv in 8 hr.

DISCUSSION

It has been concluded from the effects of metabolic poisons and other agents that active cation transport in incubated chicken erythrocytes is based mainly, if not entirely, on respiratory activity and not, as in human cells, on glycolysis. Thus, it is inhibited by cyanide, azide, malonate, 2:4-dinitrophenol and by the simple removal of oxygen. It is energized by glucose and also by lactate, the former especially being adversely affected by the presence of fluoride or iodoacetate. Presumably, cyanide and azide inhibit through their action on cytochrome oxidase, malonate by competing for succinic dehydrogenase and DNP by its restraint on oxidative phosphorylation (Judah, 1951). There is no evidence that the respiratory poisons decrease apparent transport by increasing passive permeability to cations: on the contrary, preliminary observations show quite consistently, that cyanide, azide and anaerobiosis cause

a decrease in permeability to ^{24}Na. Fluoride and monoiodoacetate probably inhibit cation transport through their respective actions on the enolase and triosephosphate dehydrogenase of the glycolytic system, but the effects are not easy to demonstrate because of lactate naturally present in cell suspensions energizing transport even in the presence of these poisons. It follows that the effects of fluoride and MIA will be more evident when they act on cells which have been largely freed of lactate by washing; yet even here, inhibition is not complete and this may arise from a relatively high content of energy-yielding reserves within the cell. In this connexion it is relevant to recall the human erythrocyte which does not respire and whose content of acid soluble phosphorus is only half that found in avian cells (Rapoport & Guest, 1941); yet even this cell when incubated with fluoride maintains for some time a constant cation level in the face of an adverse concentration gradient; it is not until about 2 hr have elapsed that cell Na begins to rise (Harris & Maizels, 1951). It has been suggested that these findings might be due to a small reserve of energizing compounds within the cell. Another reason for the high resistance of chicken erythrocytes to fluoride (but not to MIA) may lie in the high magnesium content of these cells. It may be said therefore that in suspensions of washed cells to which glucose has been added, NaF and MIA cause considerable inhibition of cation transport (Table 3), but when lactate itself is added to such poisoned suspensions transport is more free, though it is not quite as great as the transport effected by lactate in the absence of these poisons (Table 3). It is likely that this is due to an inhibitory effect of fluoride and MIA on respiration as well as on glycolysis. Such an inhibition by fluoride of the aerobic phosphorylation effected by cell-free heart extract was described by Ochoa (1943) and is presumably due to inactivation of magnesium by fluoride added. The importance of magnesium in respiring systems is well recognized since the work of Lipmann (1939) on *Bacillus delbrückii* and its effect on the Krebs cycle is illustrated by the observations of Stern & Ochoa (1949) which show that magnesium (and manganese) greatly accelerate the synthesis of citrate from acetate, oxaloacetate and adenosine triphosphate, by enzymes prepared from chicken liver. In the case of MIA it is the sulphydryl groups of certain enzymes which are affected and though triosephosphate dehydrogenase is especially sensitive, action on the respiratory dehydrogenases is not negligible.

Glutamate and cation transport. Reference has already been made to a paper by Krebs and his co-workers (Terner *et al.* 1950) which shows that besides glucose, L-glutamate is necessary for the active transport of sodium and potassium in brain and retina. In the case of retina it appears that after 1 or 2 hr in the cold, the tissue loses about half its potassium and gains an equivalent amount of sodium, in accordance with the concentration gradients. If it be now incubated either with glucose, lactate, pyruvate or glutamate a small

increase of K occurs, but if any of the first three be added to the saline medium together with glutamate, the active uptake of K against the gradient is much greater and there is also an active accumulation of glutamate itself, so that with a concentration of 5 mM/l. external medium, glutamate in the retina may rise from a normal value of 6 to about 30 mM/kg tissue. Terner *et al.* found that with suitable experimental conditions the respective gains of glutamate and potassium were between 10 and 20 mM/kg hr^{-1} and were roughly equal. They suggested that glucose, lactate or pyruvate supply energy for cation transport and that glutamate acts in some other way, though they have not cared to advance the explicit suggestion that it may be directly concerned in a cation carrier system. It follows from what has been said, that to support a large rise of K in the K-depleted retina sufficient extra glutamate must be made available to permit a correspondingly great accumulation in the tissues, though it must be presumed that for the maintenance of cation equilibrium in the intact retina considerably less glutamate suffices, since the content of fresh tissues is only about 6 mM/kg.

Transport in chicken cells may now be considered in the light of the preceding observations. It has been seen that the glutamate content of chicken erythrocytes stored at 4° C for 7 days and then incubated for 4 hr is usually less than 1 mM/l. cells and that even when cells are suspended in a medium rich in glutamate, their content of glutamate increases little if at all. Glutamate was estimated by the chromatographic method of Fowden (1951) which is unlikely to give results identical with the decarboxylase method used by Terner *et al.*, but the above results suffice to show clearly that glutamate in chicken erythrocytes is very much less than it is in ox retina, that no active uptake of glutamate occurs even in the presence of glucose and that, indeed, glutamate penetrates these cells with difficulty. It follows that uptake of K by chicken erythrocytes (which may exceed 10 m.equiv/l. cells during the first hour's incubation) cannot be accompanied by an equimolar uptake of glutamate and that if glutamate be at all concerned in cation transport by these cells, it must act in some cyclical manner.

In the case of human erythrocytes, Terner *et al.* (1950) themselves suggested the possibility that glutamate may be concerned in cation transport, and remarked that plasma glutamate in relevant experiments (Danowski, 1941; Harris, 1941; Maizels, 1949) would be about 0·5 mM/l., though in some of Flynn & Maizels's (1949) experiments, plasma was diluted 40 times and the glutamate content of the suspending medium was very low. Moreover, during 6 hr incubation of a cell suspension (cold-stored blood, 2·5 ml.; NaCl 0·15 M, 6·85 ml.; KCl 0·15 M, 0·6 ml.; glucose 2·75 M, 0·05 ml.) cell glutamate changed from 0·25 to 0·3 mM/l. with a rise in cell K of 10 m.equiv/l. in 4 hr. Here, as in chicken cells, there is no correspondence between the glutamate and potassium changes, though the possibility exists that in spite of the low cell content,

glutamate acts in a cyclical fashion in some cation carrier system. However this may be, the contrast between cation transport in brain and retina where equimolar movements of K and glutamate occur, and cation transport in human and chicken erythrocytes where no such correspondence exists, is quite definite.

Tortoise erythrocytes. Tortoise erythrocytes differ from human and chicken cells in that they swell in calcium-free media. The process, which is due to gain of Na exceeding loss of K, is accelerated by rise of temperature and at 37° C swelling is considerable and haemolysis may be marked (Table 4). Since it is unlikely that rise of temperature decreases cation transport, it is probable that the effect is due to increased cell permeability, and as calcium inhibits swelling and lysis at 37° C it is likely that this action is also due to an effect on permeability. This view is supported by the observation that the increase in Na which occurs when tortoise cells are poisoned with cyanide, is delayed by the addition of Ca. However, the possibility that Ca acts in part on the metabolic cycle has not yet been excluded.

These observations apply to the African tortoise (the only species used): Black & Irving (1938) have observed haemolysis in the oxalated blood of the carp, and Hamdi & Ferguson (1940) in several teleosts and elasmobranchs whose blood had been treated with fluoride. Hamdi & Ferguson suggest that the action is due to the removal of ionized magnesium, though the present paper shows that Mg is without action on tortoise cells (Table 4, Expt. 1, *h*). Lyman (1945) showed that when ionized Ca in suspensions of erythrocytes from the snapping turtle fell below 1 mM/l. haemolysis occurred: blood from four other varieties of turtle showed no lysis when plasma Ca was reduced by oxalate or by dilution. Erythrocytes from the chicken and grass snake transport efficiently and are not haemolysed by calcium-free media.

Respiration, glycolysis and cation transport. The marked inhibitory effect of respiratory poisons and of anaerobiosis, and also the ability of lactate to promote cation transport in chicken erythrocytes, show that the requisite energy for transport is largely derived from respiratory processes. It has been seen that after similar conditions of cold-storage, chicken cells incubated with cyanide liberate about half the amount of lactate formed by incubated human cells (whose glycolysis is only slightly affected by the presence of respiratory poisons). Nevertheless, lactic acid formation by poisoned chicken erythrocytes is considerable, though it still seems to be insufficient to overcome the resistance of the cell to transport. Alternatively, it is possible that in the respiring cell glycolysis cannot be coupled to cation transport or else that so much of the energy derived from glycolysis is diverted to other metabolic requirements of the poisoned cell, that insufficient remains for the needs of cation transport.

SUMMARY

1. Chicken and tortoise erythrocytes are larger than human cells, but the potassium, sodium and water contents are similar; the magnesium content is about twice as great.

2. The respiring chicken erythrocyte produces little lactic acid, but when respiration is suppressed by absence of oxygen or the presence of cyanide or 2:4-dinitrophenol, lactic acid production is much increased and is about half as great as that of human erythrocytes under similar conditions.

3. Active cation transport in chicken erythrocytes may be energized by glucose or lactate. Fluoride and monoiodoacetate tend to inhibit transport, their action being antagonized by lactate. Cation transport is strongly inhibited by anoxia and by low concentrations of cyanide, azide and dinitrophenol, although these agencies increase lactic acid production. Inhibition of transport by malonate and fluoroacetate is less powerful.

4. It is concluded that cation transport in chicken erythrocytes derives its energy from respiration, and that glycolysis contributes but little, either because it is quantitatively insufficient or because it cannot be coupled directly to transport. This contrasts with cation transport in the non-nucleated human erythrocyte, which is energized solely by glycolysis.

5. There is no evidence that glutamate is a factor in the cation transport of chicken erythrocytes.

6. Cyanide and dinitrophenol inhibit cation transport in tortoise erythrocytes and cyanide inhibits transport in snake erythrocytes; the effects of other metabolic poisons were not investigated. It was concluded that respiration was concerned in the cation transport of these cells.

7. Chicken and snake erythrocytes are unaffected by the external calcium concentration, but when tortoise cells are incubated at 37° C in calcium-free media, sodium and potassium move with the concentration gradients and active transport is not apparent: swelling and haemolysis result.

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