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THE COUPLING OF DOWNHILL ION MOVEMENTS ASSOCIATED WITH REVERSAL OF THE SODIUM PUMP IN HUMAN RED CELLS

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SUMMARY

1. Previous work on the incorporation of inorganic phosphate (P_i) into ATP has suggested reversal of the chemical reactions of the Na pump in human red cells. A study has now been made of the associated movements of Na and K.

2. The efflux of K, and the influx and efflux of Na were measured. When the Ringer was without K, the loss of cell K was inhibited by ouabain, and the ouabain-sensitive component of K efflux (0·36 μ-equiv.ml.$^{-1}$.hr^{-1}) required external Na.

3. The exchange of Na was also inhibited by ouabain. When influx and efflux of Na were measured simultaneously in K-free Ringer there was an excess of ouabain-sensitive influx over efflux of about 0·36 μ-equiv.ml.$^{-1}$.hr^{-1}. This difference balanced the ouabain-sensitive K efflux, and was not found with 10 mM-external K. The Na and K movements appear to be coupled and to be mediated by reversal of the Na pump.

4. The net uptake of Na sensitive to ouabain was 0·38 μ-equiv.ml.$^{-1}$.hr^{-1} for red cells incubated in K-free Ringer, and the net loss of K under the same conditions was 0·58 μ-equiv.ml.$^{-1}$.hr^{-1} in rough keeping with the unidirectional flux values.

5. Oligomycin decreased Na influx and efflux to the same extent as ouabain.

6. There appears to be a coupled downhill movement of Na and K that is abolished both by inhibitors of the Na pump and by external K which promotes normal transport of Na outwards and K inwards.

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INTRODUCTION

The Na pump brings about a coupled movement of Na ions outwards and K ions inwards in human red blood cells, and it is this interdependent movement of ions which is inhibited by the cardiac glycoside, ouabain. The factors controlling downhill movements are not so well known as those which regulate active transport, and Na influx and K efflux require further study. One aspect of these ion movements is in relation to the reactions of ATP. Garrahan & Glynn (1967*a*) showed that ATP became labelled with tracer phosphate (P_i) when K-rich ghosts of human red cells were incubated in a Na-Ringer which was K-free. The incorporation was inhibited by ouabain. Concentration gradients of both ions are required, and a low concentration of external K sufficient to activate the normal operation of the Na pump also abolishes the labelling (Lant & Whittam, 1968). Conditions must be appropriate for the ion movements of the Na pump to occur in reverse. If parts of Na influx and K efflux are mediated by the Na pump, as seems likely from the requirements for ATP labelling, the question arises whether there is an interdependence of downhill ion movements comparable to the coupling of Na efflux and K influx. The present results show that in K-free Ringer parts of Na influx and K efflux were sensitive to ouabain. This part of Na influx was greater than the ouabain-sensitive efflux. The main conclusion is that Na influx appears to be coupled to a K efflux in a way sensitive to ouabain and mediated by the Na pump.

A preliminary account of some of the results has been published (Lant & Whittam, 1969; Priestland & Whittam, 1969).

METHODS

Procedure

Human blood, 3–4 weeks old, in acid-citrate-dextrose was kindly supplied by the Sheffield Regional Blood Transfusion Service.

Isotonically resealed ghosts. Erythrocyte ghosts, rich in K and low in Na, were prepared as described previously (Lant & Whittam, 1968).

Flux measurements with tracers. Cells were loaded with [^{24}Na]NaCl or [^{42}K]KCl by incubation for 3–6 hr at 37° C with gentle shaking. The composition of the Ringer solution used for loading was chosen so that the specific activity of the tracer was high and so that the K concentration in the cells was between 60 and 100 μ-equiv/ml. cells. After incubation the cells were centrifuged and washed at least 4 times (1:20) in the same ice-cold non-radioactive medium in which they were subsequently to be incubated.

Na and K efflux. Flasks containing suitable media were forewarmed to 37° C in a water-bath and at the start of the experiment, 1–2 ml. of radioactive, washed cells were added to each flask. Ouabain was sometimes added to give a final concentration of 0·05 or 0·1 mM. Immediately after mixing, a portion of the suspension was with-

drawn (zero time sample) and the remainder was incubated with shaking. At appropriate time intervals, usually after 15, 30, 60, 90 and in some cases 120 min, 3–5 ml. samples were withdrawn from the flasks (through holes in the stoppers). In later experiments only two samples, at 10 and at either 30 or 40 min, were taken.

Samples were rapidly transferred to small Pyrex centrifuge tubes immersed in an ice-bath. After cooling for 2 min, the tubes were spun at 2700 *g*, for 3 min with ghosts, and for 1 min with cells. After immediate centrifugation the supernatant was removed from the cells and the radioactivity was measured in 2 ml. samples of the supernatants. Optical density and K concentration were also estimated in some cases. Total radioactivity (N_t), and the haematocrit (H) were determined on the cell suspensions at zero time. The radioactivity initially present in the volume of ghosts or cells associated with 2 ml. of supernatant (N_0) was calculated from the relationship:

$$N_0 = N_t \frac{100}{100-H}.$$

The natural logarithm of the fraction of counts which remained inside the ghosts or cells at each sample time was plotted against time, and the best fitting straight lines were obtained by the method of least squares (Ezekiel & Fox, 1965). The slope (in hr^{-1}) was multiplied by the K content of ghosts or cells to obtain the K efflux (in μ-equiv.$ml.^{-1}$.hr^{-1}). The ghost or cell K concentrations were determined at the start and end of the incubation and the mean value used.

Na and K influx. Cells were prepared as for efflux experiments, and ^{24}Na or ^{42}K added at the start of incubation for 1 hr at 37° C. The cell suspensions were centrifuged (2 min at 2700 *g*) and the cells washed 3 times in ice-cold unlabelled K-free, Na or choline Ringer and lysed in a small volume of 7 mM-NH_4OH. Radioactivity and absorbance at 540 mμ were determined. Na influx (in μ-equiv.$ml.^{-1}$.hr^{-1}) was calculated according to Glynn (1956).

Measurement of radioactivity. ^{42}K and ^{24}Na were measured with a well-scintillation counter used in conjunction with an automatic sample changer (Ecko Electronics Ltd., Southend-on-Sea or Panax Ltd., Redhill, Surrey). At least 10,000 counts were measured whenever possible. In some experiments ^{42}K activity was measured in an IDL Tritomat Liquid Scintillation Counter (Isotope Developments Ltd., Beenham, Bucks.). Solutions were deproteinized with 6 % (w/v) trichloroacetic acid and counted without the addition of scintillant. This method of determining the radioactivity takes advantage of the Čerenkov phenomenon (see Garrahan & Glynn, 1966).

Haematocrit determinations were made by spinning cell suspensions for 30 min at 2700 *g* in Wintrobe tubes. With ghost suspensions centrifuging was done at 5° C for 1 min at 15,000 *g* in an MSE 'High Speed 18' centrifuge (Measuring & Scientific Equipment Ltd., 25–28 Buckingham Gate, London S.W. 1). Parallel determinations of the optical density at 540 mμ of suspension of ghosts and of packed ghosts were always made to obtain an independent measure of the haematocrit of ghost suspensions.

Haemoglobin (Hb) was estimated as oxyhaemoglobin from the extinction at 540 mμ of suitably diluted samples clarified with 7 mM-NH_4OH as described by Wootton (1964).

Na and K. These elements were measured either by an EEL flame photometer (Evans Electroselenium Ltd., Halstead) or by a Unicam atomic absorption Spectrophotometer (SP 90) (Unicam Instruments Ltd., Cambridge). Samples were diluted to contain less than 0·2 mM-Na or K, and measurements undertaken in triplicate alternating with standard solutions of NaCl or KCl. The cation concentration in cells was calculated by multiplying the concentration in each lysed sample by the ratio of the haemoglobin concentration in unit volume of packed cells or ghosts to that in the sample.

Materials

^{24}Na and ^{42}K were obtained as sterile isotonic solutions of NaCl or KCl from the Radiochemical Centre, Amersham; and oligomycin from Sigma Chemical Co., St Louis, Mo., U.S.A., and ouabain (strophanthin G) from British Drug Houses Ltd. All chemicals were Analar grade whenever possible.

RESULTS

K efflux from high K, low Na ghosts in the absence of external K. Labelling of ATP from P_i has been followed in ghosts, so K efflux was also measured in ghosts under optimal conditions for P_i incorporation. Ouabain was added to see if it decreased K efflux. High K, low Na ghosts were loaded with ^{42}K, and the migration of isotope followed into Na-rich, K-free Ringer solution. Preliminary experiments showed that the appearance of ^{42}K was approximately linear with time during $1\frac{1}{2}$–2 hr indicating first order kinetics. However, values for K efflux from ghosts containing 10 mM-P_i varied considerably from experiment to experiment and ranged from 5·2 to 11·7 μ-equiv K.ml.$^{-1}$.hr^{-1}. Despite the considerable scatter of results, addition of ouabain caused a reduction in K efflux in four out of five experiments, the mean decrease being 1·2 μ-equiv.ml.$^{-1}$.hr^{-1}. The decrease with ouabain was somewhat less consistent when ghosts contained less phosphate (1 or 5 mM). The scatter of results is not surprising in view of Hoffman's (1962) work, and in order to overcome the variability subsequent experiments were made with intact cells rendered low in internal Na and high in internal K by pre-incubation in a high K medium usually containing 10 mM-inosine.

K efflux from cells. Glynn & Lüthi (1968) have described a fall in K efflux with ouabain when cells are incubated in K-free, Na-Ringer. We find the same decrease. Table 1 shows that ouabain caused a small but significant reduction of approximately 20% in K efflux amounting to 0·36 μ-equiv.ml.$^{-1}$.hr^{-1} (S.E. = 0·05, n = 19 values) with a range of internal K from 60 to 98 μ-equiv/ml. cells. There was no correlation between the ouabain-sensitive efflux and the internal K concentration. When external Na was replaced by either choline or magnesium, ouabain had no longer inhibited K efflux as Glynn & Lüthi (1968) previously found when choline was the substituent for Na. These results indicate that under the same conditions in which there was ouabain-sensitive incorporation of P_i into ATP, about 20% of K efflux was inhibited by ouabain and depended on the availability of external Na. The possibility therefore arises that ouabain-sensitive K efflux might be balanced by Na influx.

TABLE 1. Inhibition by ouabain of K efflux from red cells in K-free Na-Ringer

K concentration (μ-equiv/ml. cells)	K efflux (μ-equiv. ml.$^{-1}$. hr^{-1}) Control (a)	With ouabain (b)	Ouabain-sensitive (a) − (b)
60	1·82	1·45	0·37
62	1·46	1·34	0·12
63	1·50	1·45	0·05
64	1·52	1·13	0·39
66	2·06	1·83	0·23
66	2·20	1·99	0·21
69	1·58	1·42	0·16
69	1·96	1·58	0·38
72	1·95	1·76	0·19
74	2·45	1·88	0·57
74	1·87	1·49	0·38
75	2·68	1·77	0·91
75	1·65	1·51	0·14
78	2·08	1·79	0·29
78	1·76	1·51	0·25
78	2·85	2·17	0·68
79	1·79	1·37	0·42
91	1·55	1·16	0·39
98	2·84	2·16	0·68
		Mean difference	0·36
		S.E. of mean	0·05

Red cells (3 weeks old containing 8–10 mM-P_i) were loaded with [^{42}K]KCl and incubated in K-free Na-Ringer at 37° C. No substrate was added. Ouabain was 0·05–0·1 mM. The internal K concentration was sometimes varied before the tracer experiment as described in Methods.

Comparison of Na influx and efflux

Garrahan & Glynn (1967*b*) have shown that there is an ouabain-sensitive portion of Na influx which is linked and balanced by an equal ouabain-sensitive portion of Na efflux when fresh red cells are incubated in K-free Na-Ringer. However, if part of the ouabain-sensitive Na influx is associated with backward running of the Na pump, then Na influx should exceed Na efflux to an extent equal to the ouabain-sensitive K efflux. To test this point, measurements of Na influx and efflux were made with cells from 3 weeks old blood on the same day both with and without ouabain.

The conditions were identical for measuring both influx and efflux, except that the tracer Na was initially either in the Ringer solution or in the cells. In the absence of external K it is not surprising that the ouabain-sensitive efflux is a low value and much less than the values for Na efflux

in the presence of substrate and of external K (Table 2). Both with and without ouabain, influx exceeded efflux in keeping with the observed net increase of cell Na (see Table 4). The results (Table 2) show further that ouabain inhibited both influx and efflux by a similar amount as previously described by Garrahan & Glynn (1967*b*). Nevertheless, in all but one of the experiments the ouabain-sensitive part of Na influx exceeded the ouabain-sensitive efflux. The difference between the two fluxes was small and arises from comparisons between four values, each of which has an experimental

TABLE 2. Simultaneous influx and efflux of Na in red cells

Na fluxes (μ-equiv.ml.$^{-1}$.hr^{-1})						
Influx			Efflux			Excess of ouabain-sensitive influx over efflux $[(a)-(b)]-[(c)-(d)]$
Control (a)	With ouabain (b)	Ouabain-sensitive $(a)-(b)$	Control (c)	With ouabain (d)	Ouabain-sensitive $(c)-(d)$	
3·59	1·83	1·76	2·69	1·60	1·09	0·67
3·14	1·69	1·45	2·28	1·29	0·99	0·46
3·44	2·04	1·40	2·07	1·25	0·82	0·58
3·22	2·25	0·97	2·60	1·78	0·82	0·15
4·50	2·76	1·74	3·68	2·40	1·28	0·46
2·78	1·88	0·90	1·95	1·26	0·69	0·21
3·53	2·52	1·01	3·05	2·29	0·76	0·25
3·86	2·33	1·53	2·82	1·86	0·96	0·57
3·57	2·05	1·52	3·07	1·98	1·09	0·43
3·34	2·05	1·29	3·08	1·64	1·44	−0·15
	Mean	1·36			0·99	0·36
	S.E. of mean	0·09			0·07	0·07

Influx and efflux of Na were measured with cells from the same sample of cold-stored blood with and without ouabain on the same day. Incubations were at 37° C in K-free Na-Ringer (concentrations (mM) were NaCl, 150; Tris Cl, 10; pH 7·6). No substrate was added. Ouabain concentration was 0·1 mM.

error of about 5% (see last column of Table 2). As expected, therefore, the excess of influx over efflux shows some variation. The difference of $0{\cdot}36 \pm 0{\cdot}07$ μ-equiv.ml.$^{-1}$.hr^{-1} (S.E. for ten observations) is significant and in close agreement with that of 0·36 found for the part of K efflux which is sensitive to ouabain. Despite the inevitable experimental errors, these results suggest that the K efflux is balanced by Na influx, both ion movements being sensitive to ouabain and occurring by reversal of the Na pump. In addition to this downhill Na–K exchange there is the rest of the ouabain-sensitive influx and efflux of Na which occurs by exchange diffusion in the way described by Garrahan & Glynn (1967*b*).

Net cation movements

Since net movements of ions arise from a difference in the rates of unidirectional fluxes, it seems that there should be a net gain of cell Na which is sensitive to ouabain. Similarly a net loss of K which is sensitive to ouabain would also be expected. Measurements of net changes in Na and K content of cells were therefore undertaken in an attempt to check these predictions.

Net potassium loss. Measurement of net K loss was determined from the K content of the cell-free supernatants after incubation, allowance being made for any small K loss that might arise from lysed cells. The K concentration in the supernatants at the end of 2 hr incubation ranged from

TABLE 3. Net loss of K from red cells incubated in a high Na, K-free Ringer in the absence and presence of ouabain

Time of incubation (hr)	Initial cell potassium (μ-equiv/ml.)	Net potassium loss (μ-equiv.ml^{-1}.hr^{-1}) Control	+ ouabain	Difference due to ouabain (μ-equiv.ml.$^{-1}$.hr^{-1})
2	62	1·10	1·10	0
2	69	2·59	1·74	0·85
2	78	1·31	1·00	0·31
2	74	2·34	1·38	0·96
2	74	2·92	2·16	0·76
			Mean difference	0·58
			S.E. of mean	0·18

Net changes were derived from the losses of K to the medium, allowance being made for loss due to lysis during incubation. The concentration of ouabain was 50 μM. The results are means of incubations done in triplicate.

0·18 to 0·25 mM. It was important to keep the level of extracellular K as low as possible, so when incubation was continued for 4 hr, an equal volume of K-free, Na-Ringer at 37° C was added to each flask after 2 hr. The final concentration of K in the medium at the end of 4 hr incubation ranged between 0·17 and 0·19 mM. The K loss to a K-free, Na-Ringer was significantly smaller with ouabain than in the control (Table 3). The mean difference (μ-equiv.ml.$^{-1}$.hr^{-1}) was $0{\cdot}58 \pm 0{\cdot}18$ (mean $\pm$ S.E. of mean; $n = 5$).

Net Na uptake. The increase in Na content of unwashed packed cells was determined after centrifugation for 20 min at 2700 *g* in haematocrit tubes. The supernatant medium was carefully removed by suction and the cells mixed thoroughly before analysis. The cell Na content was always

raised, and in four out of seven experiments the gain with ouabain was less than in the control. The ouabain-sensitive net uptake (μ-equiv.ml.$^{-1}$.hr^{-1}) was $0{\cdot}38 \pm 0{\cdot}16$ (means $\pm$ S.E. of mean; $n = 7$) (Table 4). The results suggest that, in the absence of external K, net downhill movements of Na inwards and of K outwards partly occur through backwards running of the Na pump.

TABLE 4. Net uptake of Na into red cells incubated in a high Na-Ringer medium in the absence and presence of ouabain

Time of incubation (hr)	Cell sodium (μ-equiv/ml.)			Net sodium uptake (μ-equiv/ml.)		
	Initial	After incubation				Differences due to ouabain (μ-equiv.ml^{-1}.hr^{-1})
		Control	+ouabain	Control	+ouabain	
5	18·5*	20·5	19·0	2·0	0·5	0·30
3	41·0	44·0	41·5	3·0	0·5	0·83
4	53·5	58·0	54·0	4·5	0·5	1·00
4	41·5	50·0	50·0	8·5	8·5	0
4	34·0	42·5	40·5	8·5	6·5	0·50
4	41·0	46·0	46·0	5·0	5·0	0
4	36·0	44·0	44·0	8·0	8·0	0
					Mean difference	0·38
					S.E. of mean	$\pm$ 0·16

* Cells pre-incubated in 140 mM-KCl: 10 mM-P_i for 42 hr at 7° C.

Cell Na content was measured before and after incubation at 37° C for 3–5 hr in a K-free, Na-Ringer. The concentration of ouabain was 50 μM. The results are means of incubations done in triplicate.

Inhibition of Na influx and efflux by oligomycin. Oligomycin is like ouabain in inhibiting the active transport of Na and K in red cells (see Blake, Leader & Whittam, 1967). Measurements were therefore made to see whether it is like ouabain in its effect on ion movements in the absence of external K. A comparison was made between the inhibition by ouabain (0·1 mM) and oligomycin (20 μg/ml.) on the fluxes of Na in cells in the absence of external K. These concentrations of inhibitors were used because in the presence of external K, they produce maximum inhibition of Na and K movements.

Figure 1 shows that oligomycin and ouabain each reduced Na influx to the same extent (by $1{\cdot}50 \pm 0{\cdot}09$ and $1{\cdot}63 \pm 0{\cdot}07$ μ-equiv.Na.ml.$^{-1}$.hr^{-1} respectively; means $\pm$ S.E. of mean; $n = 4$). In the same four experiments, oligomycin and ouabain also caused a similar decrease in Na efflux but the fall was somewhat less than was found for influx, viz. $1{\cdot}11 \pm 0{\cdot}04$ and $1{\cdot}01 \pm 0{\cdot}11$ μ-equiv.Na.ml.$^{-1}$.hr^{-1} respectively. Other experiments showed

that the two inhibitors together had only the same effect as one of them alone. The inhibition of influx was thus greater than that of efflux, the differences (in μ-equiv.ml.$^{-1}$.hr^{-1}) being 0·63 with ouabain and 0·39 with oligomycin. The results suggest that these two compounds have a similar effect on those Na fluxes which occur in the absence of external K, just as they do with external K.

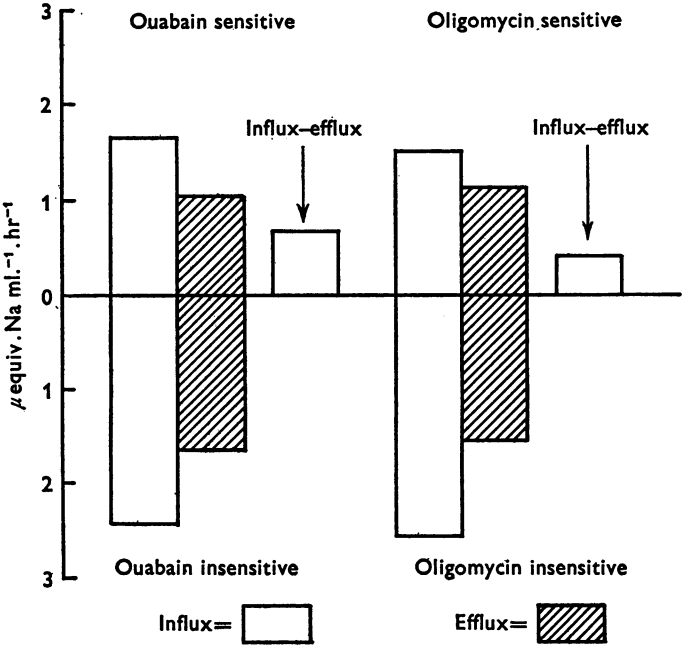

Fig. 1. Inhibition of sodium influx and efflux by ouabain and oligomycin. Influx and efflux of sodium were measured with cells from the same sample of cold-stored blood without addition of inhibitor, with ouabain (0·1 mM) and with oligomycin (20 μg/ml.). All the flux measurements on each sample of red cells were made on the same day. Incubations were in K-free Na-Ringer (concentrations (mM): NaCl, 150; Tris Cl, 10; pH 7·6) at 37° C without addition of substrate. The results shown are the mean values of four experiments.

The total height of the open bars represents influx under control conditions; the total height of the shaded bars represents efflux under control conditions. Fluxes in the presence of ouabain or oligomycin are shown below the line. The portions of the fluxes inhibited by ouabain or oligomycin, obtained by difference, are shown above the line.

DISCUSSION

The Na pump stops splitting ATP and extruding Na in the absence of external potassium. In this situation P_i can be incorporated into ATP in ghosts of human red cells, suggesting that the ATPase reaction is revers-

ible (Garrahan & Glynn, 1967*a*). This kind of labelling of ATP is not found with fragmented membranes and a coupling to an energy-giving reaction would seem to be necessary, as happens when ATP is formed in the course of cell metabolism. There was no chemical source of energy in the red cell ghosts and the labelling depended on having Na-rich, K-free Ringer and K-rich ghosts. Indeed, the conditions have to be right to allow movements down the concentration gradients of both Na inwards and K outwards since movement of one ion alone does not support the incorporation (Lant & Whittam, 1968). Our present results have most bearing on ion movements in relation to the reactions of ATP catalysed by the Na pump.

The Na and K fluxes have been measured when there was no external K, and therefore no normal operation of the Na pump. There appear to be two kinds of ion movement mediated by the pump which are different from the normal operation in not being against concentration gradients.

The first has been described before by Garrahan & Glynn (1967*b*) and is exchange diffusion of Na. This accounts for most of the ouabain-sensitive exchange of Na. The second kind of ion movement is suggested by the results described above and seems to be of Na inwards and K outwards. Simultaneous measurements of Na influx and efflux showed that the influx was greater than the efflux and that this was true also for the ouabain-sensitive components (Table 2). This difference in sodium entry and exit was also in rough agreement, in spite of big experimental errors, with the retardation by ouabain of the net Na gain. The disparity of Na influx and efflux shows that exchange diffusion does not completely account for the Na exchange although it represents the major part. As regards K, Glynn & Lüthi (1968) and the above results show that a part of K efflux was also inhibited by ouabain provided there was Na in the Ringer. A comparison can therefore be made of Na and K movements. The excess of ouabain-sensitive influx over efflux for Na may be compared with the ouabain-sensitive parts of K efflux and the net loss of K. The results show that the values for changes in Na moving inwards and K moving outwards were about the same. There appears to be a K loss and Na gain such that the movements are both inhibited by ouabain and evidently brought about by reverse operation of the Na pump.

The dependence of K efflux on external Na in K-free Ringer is the opposite of the familiar dependence of Na efflux on external K. This suggests that the ion movements down concentration gradients are coupled in a similar way but in an opposite direction to those of active transport against concentration gradients. It is striking that the changes in experimental conditions which abolish the linked movements of Na inwards and K outwards are those which also stop the labelling of ATP. Both ouabain

and external K had this effect but for different reasons; ouabain inhibits whereas external K stimulates the normal activity of the Na pump. When the external sites of the pump are occupied by K there is then no reversal of the chemical reaction and ion movements. While this finding does not throw light on the molecular mechanism of the Na pump it does suggest that the sites which normally take up Na on the inside and K on the outside are not absolute in their specificity. The site for external K is subject to competition from external Na and Na begins to enter the cell on the K carrier when external K is very low. Na appears to take the place of K so that the ions move and the chemical reaction occurs in the direction opposite from normal.

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