The Active Transport of Sodium by Ghosts of Human Red Blood Cells

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ABSTRACT The outflux of Na^{24} from prelabeled ghosts was measured under various conditions. Prelabeling was accomplished by hypotonic hemolysis of intact human cells in the presence of tracer Na^{24}. The resultant ghosts when subsequently washed were found to retain 10 to 20 per cent of the initial Na^{24}. Separate experiments indicated that this trapped amount resides in only a portion of ghosts comprising the total population. The characteristics of the outflux of this residual Na^{24} indicated that the ghost system closely resembles intact red cells. The outflux of Na from ghosts could be divided into three components: active and passive transport and exchange diffusion. The active transport system, necessarily driven by metabolism, required the presence of K in the extracellular phase and was blocked by strophanthidin. The concentration dependence of the Na pump flux on the external K and internal Na appeared the same in ghosts as in intact cells. Certain other features of this ghost system are also discussed.

INTRODUCTION

The aim of these experiments was the characterization of a system of ghosts which possess, like intact cells, the ability to transport cations actively. An evaluation of the methodology involved in preparing ghosts is necessary to the development of this system. Therefore the effect of various procedures on the cation distribution and permeability of the resultant ghosts was investigated. A portion of the present work was reported previously (Hoffman, 1960).

MATERIALS AND METHODS

Fresh human blood, obtained from normal adults was used. Following collection into Na_3 citrate$\cdot 2H_2O$ (4 mg/ml blood) the blood was centrifuged for 5 minutes at approximately 15,000 $\times$ *G*. The plasma and buffy coat were removed by aspiration and the remaining unwashed packed red blood cells were treated as described below.

Since the permeability of ghosts to Na and K was investigated under a number of different circumstances, the various procedural routes employed are outlined in

Fig. 1. One volume of packed cells was hemolyzed by rapid mixing with 10 volumes of distilled water (step 1). That complete hemolysis occurred was, in all cases, established by the methods and criteria previously described (Hoffman, 1958). *Reversal*, produced by an increase of the osmotic pressure of the ghost environment (usually to that of plasma), was carried out in several ways: (1) concentrated salt solution was added to the hemolysis mixture (step 3), or (2) the hemolysis mixture was centrifuged and the ghosts washed (step 2) followed by suspension in a solution of increased osmotic pressure (step 4 or step 5). Although the ghosts need not be washed (step 2) for reversal in this manner, it was desirable in the present experiments because of the presence of isotope added at point A or B. Isotope was added in one of the fol-

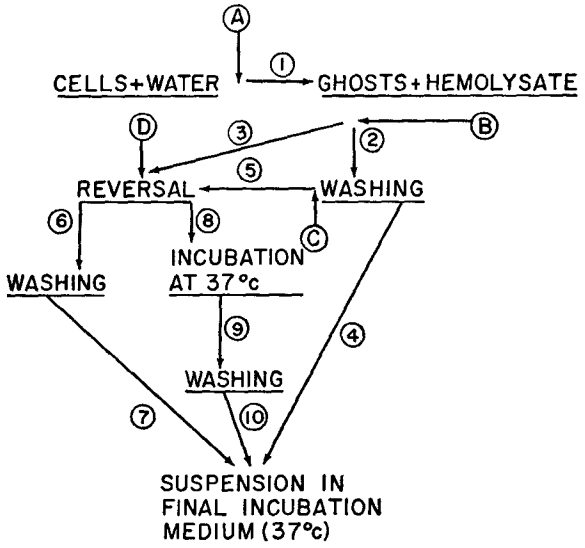

FIGURE 1. Flowsheet for preparation of ghosts. Letters indicate points of introduction of radioactive tracer. Numbers indicate procedural steps.

lowing ways: to the distilled water used for hemolysis (point A), to the hemolysis mixture (point B), after washing but prior to reversal (point C), or after reversal (point D).

For the experiments dealing with the outflux of Na^{24} from prelabeled ghosts (section 2 of Results) the following convenient and reproducible procedure involving steps 1-2-4 (Fig. 1) was adopted. Packed cells were hemolyzed (hemolytic ratio 1 to 10) in the presence of Na^{24} (and, thus, labeled at point A). The resultant hemolysis mixture was allowed to stand 20 minutes at room temperature (23°C) before centrifugation at approximately 20,000 × *G*. The ghosts were resuspended in about 10 volumes of 12 mM $MgCl_2$–tris (pH = 7.5) and left to stand a few minutes before being centrifuged again. At the end of the fourth wash the concentrated ghosts were pooled ready for suspension in the final medium as described below. The supernatant obtained from the final wash appeared in most instances to be Hb-free and indicated that essentially no rehemolysis had taken place. (The use of wash solutions of concentrations less than

1.8 mM $MgCl_2$–tris, however, produced considerable rehemolysis.) One volume of these Na^{24}-labeled ghosts was suspended in about 30 volumes of medium at room temperature (23°C). After mixing and removal of the zero time sample the flask was put at 37°C in a Dubnoff shaker. 5 ml. aliquots were withdrawn at intervals (the zero time sample was taken immediately after mixing), and centrifuged at once at approximately 20,000 × *G*. 3 ml of this ghost-free supernatant, $(R)_s$, as well as 3 ml of the suspension mixture itself, $(R)_{eq}$, was pipetted and counted. The per cent of Na^{24} released from the ghosts for each time, *t*, was calculated as: $(R)_s/(R)_{eq} \times 100$. The time course of the loss of Na from the ghosts is given by a plot of the per cent released against *t*. In a few of the experiments suspension of the ghosts in their final incubation media (at which time they undergo reversal) produced between 5 and 15 per cent hemolysis (estimated by the method given in Hoffman, 1958). Usually this remained constant during the course of the subsequent incubation, but if the rehemolysis continued to increase, then departure from the single exponential rate of loss of Na^{24} (as illustrated in Fig. 3) was noted. In addition, the rate of rehemolysis appeared to be independent of (and less than) the rate of loss of Na^{24}. Data from experiments in which any rehemolysis continued to occur are not used in the present paper.

Concentrations are expressed throughout in millimoles per liter. Buffered salt solutions abbreviated $MgCl_2$–tris, NaCl–PO_4, etc. represent 9:1 mixtures of osmotically equivalent solutions, *e.g.* 12 mM $MgCl_2$–tris contains 9 parts of 12 mM $MgCl_2$ + 1 part of 17 mM tris (hydroxymethylaminomethane). The 12 mM $MgCl_2$–tris solution used in washing (step 2) the ghosts contained not more than 0.065 mM Na and 0.012 mM K as impurities.

$Na_2^{24}CO_3$ and $K_2^{42}CO_3$, obtained from Brookhaven National Laboratory, were converted to their chlorides prior to use. $Na^{22}Cl$ was obtained from Abbott Laboratories.

The analytic methods used were essentially the same as previously described (Hoffman, 1958). Radioactivity was determined using a well-type scintillation counter. Na and K were measured with a Baird flame photometer employing Li as an internal standard. The water content of ghosts was determined gravimetrically.

The abbreviations ATP and EDTA stand for the disodium salts of adenosinetriphosphate and ethylenediaminetetraacetate, respectively.

RESULTS

For convenience the presentation of the results is divided into three sections. Section 1 deals with the effect of the conditions at hemolysis on the exchangeability and trapping of Na and K by ghosts. The outflux of Na from ghosts under different circumstances is dealt with in Section 2. Section 3 is concerned with the heterogeneity of the ghost population.

SECTION 1. EXCHANGEABILITY OF Na AND K DURING AND AFTER HEMOLYSIS AND THE TRAPPING OF CATIONS WITHIN THE GHOST COMPARTMENT The relative exchangeability of Na and K can be estimated from a comparison of the extent of isotopic equilibrium achieved when red cells are hemolyzed in

distilled water (step 1) in the presence of Na^{24} or K^{42} with that achieved when one of these isotopes is added after hemolysis. Table I shows that both Na^{24} and K^{42} added at the time of hemolysis (point A) reach isotopic equilibrium. When K^{42} is added after the occurrence of hemolysis (point B) isotopic equilibrium is also attained. This result is obtained when, for K^{42} added at point B, the ratio of the specific activity of the cells to the medium (*i.e.* X_g/X_o) is determined 5, 30, and 60 minutes after the completion of hemolysis and after the addition of K^{42}. Thus, K is completely exchangeable either during or after hemolysis.

In contrast to the K results, Na^{24} does not reach isotopic equilibrium when it is added after hemolysis takes place (point B). This is so even if, after the addition of Na^{24}, the hemolysis mixture is allowed to stand, at room temperature (23°C), for 1 or 2 hours; that is, the change in X_g/X_o is less than 1.0 per cent during this time period. (The same results are observed when Na^{22} is added at point B using ghosts labeled at point A with Na^{24}.) These results indicate that there is a compartment of Na in the ghosts that is exchangeable only at the time of hemolysis but appears non-exchangeable (or slowly exchangeable) after the completion of hemolysis.

Types of Ghosts Since it will be shown in Section 3 that ghosts resulting from osmotic hemolysis are heterogeneous with respect to their cation perme-

TABLE I

THE DISTRIBUTION AND PER CENT SPECIFIC ACTIVITY EQUILIBRIUM ACHIEVED BETWEEN GHOSTS AND THEIR HEMOLYSATES AS A FUNCTION OF THE LABELING PROCEDURE

The per cent isotopic equilibrium, represented by the ratio, $X_g/X_o \times 100$ where X indicates the specific activity and the subscripts, g and o, refer to the ghost and medium compartments, respectively, was measured on equal volumes of ghosts and hemolysate following their separation at 20,000 × G. The radioactivities (R) and the cation concentrations (see Table II) were determined in identical fashion on both the ghost and supernatant aliquots. Thus, each specific activity is calculated from the relation, $X = \frac{R}{C}$, where C is the molar concentration of the appropriate cation. The ratio, $(R)_g/(R)_o \times 100$, is also given and indicates the relative distribution of tracer between the ghosts and their hemolysate. The average of eleven experiments with Na and five experiments with K is given together with the range of variation enclosed in parentheses. Each experiment was performed at room temperature (23°C) in duplicate with duplicate aliquots from each analyzed.

Hemolysis condition	$X_g/X_o \times 100$		$(R)_g/(R)_o \times 100$	
	Na^{24}	K^{42}	Na^{24}	K^{42}
Isotope at hemolysis (point A)	99.2 (97.4–101.9)	99.2 (98.0–100.2)	106.1 (103.4–109.9)	105.8 (104.7–109.3)
Isotope after hemolysis (point B)	94.6 (93.1–96.5)	99.0 (97.3–100.5)	100.4 (93.3–104.1)	105.6 (102.0–110.7)

ability, it is convenient here to divide the population of ghosts into three groups. Group I is defined as those ghosts which contain the non-exchangeable Na referred to above. Group II is defined as those ghosts which have been induced to recover their relative impermeability to cations as shown below. Group III is defined as those ghosts which remain permeable to Na and K. Group II generally arises from group III. Thus, in the experiment illustrated in Table I the total ghost population is composed of groups I and III. Although group II type ghosts can be occupied non-selectively by both Na and K, group I is selectively occupied by Na. The fraction of Na that is contained in group I can be estimated from the difference between the averages obtained in Table I. It is apparent that approximately 5 per cent of the total ghost Na, resulting from the specified hemolysis condition, resides in group I.

Effect of Washing If the ghosts remained completely permeable to K, then the washing of ghosts hemolyzed and labeled under the above circum-

TABLE II

THE RESULTS OF A TYPICAL EXPERIMENT, REPRESENTATIVE OF FOUR SIMILAR EXPERIMENTS, INDICATING THE EFFECT OF WASHING ON GHOSTS LABELED AT AND AFTER HEMOLYSIS WITH Na^{24} (PART A) OR K^{42} (PART B)

$(Na)_g$, $(Na)_o$, $(K)_g$, $(K)_o$ indicate cation concentration in millimoles/liter of each phase; X signifies the specific activity. Subscripts g and o refer to ghost and outside phase, respectively. Temperature = 23°C. See text for discussion.

Part A

Experiment		Conditions	$(Na)_g$	$(Na)_o$	$(R)_g$	$(R)_o$	X_g
1	Na^{24} at point A	After NaCl–PO_4 wash	16.5	16.6	42794*	578	
2	Na^{24} at point A	Initial mixture	6.36	5.88	10510	9933	1652
		After $MgCl_2$–tris wash	1.45	—	2412	—	1663
3	Na^{24} at point B	Initial mixture	6.32	5.90	9930	9838	1571
		After $MgCl_2$–tris wash	1.46	—	1485	—	1024

* Initial $(R)_g$ = 216737.

Part B

Experiment		Conditions	$(K)_g$	$(K)_o$	$(R)_g$	$(R)_o$	X_g
4	K^{42} at point A	Initial mixture	12.3	11.3	10135	9379	824
		After $MgCl_2$–tris wash	1.75	—	1393	—	812
5	K^{42} at point B	Initial mixture	12.1	11.1	10188	9318	842
		After $MgCl_2$–tris wash	1.58	—	1091	—	691

stances should amplify the differences in the exchangeability recorded in Table I (*i.e.* it might be anticipated that the K should wash out). Although the results presented in Table II show that this expectation is not fulfilled, they demonstrate in a rather direct fashion the characteristics of the trapping of cations by ghosts. These experiments were carried out as follows: In Experiments 1, 2, and 4 the label was added at hemolysis, and in Experiments 3 and 5, after hemolysis occurred. A portion of the hemolysis mixture (called the initial mixture in Table II) was removed and the degree of isotopic equilibrium determined as before. The remaining ghosts were washed 4 times (with very little rehemolysis) with about 10 volumes of 17 mM NaCl–PO_4 in Experiment 1 and 12 mM $MgCl_2$–tris in Experiments 2, 3, 4, and 5.

TABLE III

THE PER CENT OF THE INITIAL LABELING CONCENTRATION OF ISOTOPE TRAPPED IN GHOSTS AFTER WASHING WITH $MgCl_2$–TRIS

Calculated from data of Table II according to:

$$\frac{(R)_g}{(R)_g \text{ initial}} \times 100 = \text{per cent remaining.}$$

Condition	Per cent of initial label remaining after washing	
	Na^{24}	K^{42}
Isotope at point A	22.9	13.7
Isotope at point B	14.9	10.7

Direct evidence that Na^{24} is trapped by ghosts is given in Experiment 1 of Table II. Since these ghosts were washed with NaCl solutions, it was possible to measure, after washing, X_o as well as X_g. It is apparent that the specific activity of the Na^{24} retained by the ghosts was nearly 75 times (7450 per cent) the specific activity of the final wash. In Experiments 2 and 4, in which the ghosts were washed with Na-free solutions, the results show that for both Na and K the specific activity of the ghosts remained unchanged during washing, reflecting the fact that specific activity equilibrium was reached at hemolysis. On the other hand, when the label was added after hemolysis (Experiments 3 and 5) X_g is seen to decrease significantly. The percentage of the initial radioactivity remaining after washing is summarized in Table III. It can be seen in Table III that when the label is added at hemolysis a greater percentage of Na^{24} than of K^{42} is retained by the ghosts. The fact that appreciable K^{42} remained, whether presented to the ghosts at or after hemolysis, is contrary to the expectation based on the results in Table I.

It will be noted in Table III that the percentage of the isotope trapped when added after hemolysis is approximately the same for both K^{42} and Na^{24}

and approximates the percentage of K^{42} trapped when this isotope is added at hemolysis. These data provide the basis for defining group II as the ghosts that contain the K^{42} and Na^{24} trapped after hemolysis. Presumably, exposure of the ghosts to the washing solution induced this retention of K^{42} and a like amount of Na^{24}, in addition to the Na contained in group I, defined above. Since the osmotic pressure of the wash solution is somewhat greater than the resultant osmotic pressure of the hemolysate, this additional trapping of Na^{24} and K^{42} could be a reflection of incipient *reversal* (*cf.* Hoffman, 1958). This interpretation is supported by the fact that the degree of retention (the size of group II) appears to depend on the osmotic pressure of the hemolyzing solution (see below). Therefore, ghosts, labeled at hemolysis with Na^{24} and subsequently washed, contain isotope in both groups I and II; whereas ghosts labeled after hemolysis with Na^{24} or K^{42} (or labeled at hemolysis with K^{42}) retain isotope only in group II. Thus, the total population of ghosts hemolyzed in the presence of Na^{24} and washed is comprised of groups I + II + III. It is apparent that washing, by definition, removes all cations from group III type ghosts.

The fact that the ratio, $(R)_g/(R)_o$, was greater than 100 per cent as in Table I indicates either that sites exist within the ghost membrane which bind cations or that the ghosts shrink after hemolysis so as to concentrate the cations. The latter possibility appears unlikely since it has been found that the hemolytic volume is the same as the volume the ghost occupies after hemolysis (Hoffman *et al.*, 1958). In addition, the comparison of the water content (or the concentration of hemoglobin, *cf.* Hoffman, 1958) of ghosts and their equilibrium hemolysate does not reflect this difference: Ghosts contain 95.5 ± 0.3 per cent water; hemolysate contains 96.4 ± 0.3 per cent water.

That portion of the total ghost volume occupied by group I can also be estimated from the differences in the fraction of the initial Na^{24} retained by ghosts labeled at point A and at point B (Table III) but washed with dilute $MgCl_2$. Although a difference of 8 per cent is seen here, the average obtained from four experiments is 7.2 per cent (range is 4.1 to 9.2) and approximates the value of 5 per cent noted before. Isotope (Na^{24} or Na^{22}) added after washing (point C) shows a decreased sodium distribution in accordance with an increase in the proportionate size of group II. It must be recognized that the 5 per cent value which has been found and discussed above for the characterization of group I applies only for the experimental condition in which the hemolytic ratio (volume of cells to volume of solution) equals 1 to 10 and hemolysis occurrs in solutions containing less than 4 mM NaCl.

The proportion of ghosts in group I can be increased by increasing the osmotic pressure of the hemolyzing solution. This was demonstrated by hemolyzing cells at a given hemolytic ratio (*i.e.*, 1 to 10) in 0, 5, 11, and 18

mM NaCl (or KCl). The size of group I (estimated as the difference in the ratio, $(R)_0/(R)_\infty$, obtained for Na^{24} added at point A and point B, as in Table I) was found to be 4.8, 10.4, 16, and 24 per cent, respectively. The explanation for the increase in the proportion of ghosts in group I may be either that the injury of hemolysis is diminished or that the rate of recovery is enhanced by increasing the tonicity of the hemolyzing solution. As pointed out earlier increasing the osmotic pressure *after* hemolysis does not appreciably influence

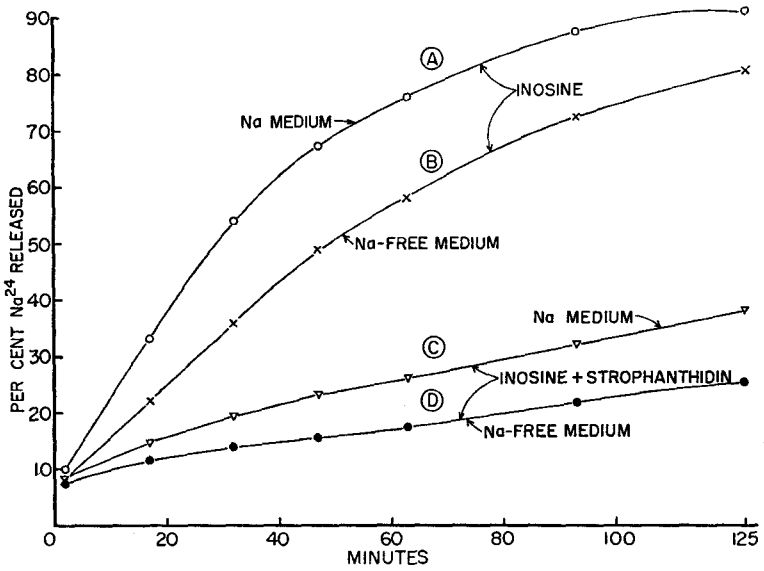

FIGURE 2. The rate of release of Na^{24} from prelabeled ghosts into various media at 37°C. Na medium contains 155 mM NaCl + 5 mM KCl: Na-free medium contains 110 mM $MgCl_2$ + 5 mM KCl. Both media contain 10 mM tris buffer (pH = 7.45). Concentration of inosine is 5 mM; strophanthidin, 5×10^{-5} M.

the size of group I although group II is increased (at the expense of group III). Apparently the important factor in affecting the size of group I is the hemolysis condition itself.

SECTION 2. RELEASE OF Na^{24} FROM PRELABELED GHOSTS The results presented in the previous section show that ghosts can be labeled at hemolysis with Na^{24} and that these ghosts retain a portion of the initial Na^{24} activity to which they were exposed when subjected to subsequent washing. This section deals with the characteristics of the release of this trapped Na^{24} from ghosts prepared as described under Materials and Methods.

The Active Transport of Na The release of Na^{24} from washed prelabeled ghosts into media of different composition is illustrated in Fig. 2. Under the conditions of these experiments the loss of Na is at a minimum when Na is

absent from the external medium and strophanthidin is present (curve D). Upon the addition of Na to the external medium Na outflux increases (curve C). The difference between these two curves is taken to represent exchange diffusion (Ussing, 1948), that is, the exchange of Na^{23} for Na^{24}. (No net movement of Na can occur by this mechanism.) Exchange diffusion also predominates in the difference between curves A and B. When strophanthidin is present, the rate of loss of Na^{24} is unaffected by the removal of inosine or K or both from the medium. In the absence of strophanthidin and inosine the rate of loss is not appreciably altered by the presence or absence of K. But when both inosine and K are added to the external medium the outflux of Na is markedly stimulated as represented by curves A and B. The addition of strophanthidin (curves C and D) (or the removal of K) inhibits this stimulation. The "insensitive" outflux seen in curve D of Fig. 3 is taken as representing the passive loss by diffusion; *i.e.*, leak. Thus, curve A represents the composite of pump + leak + exchange diffusion; curve B, pump + leak; curve C, leak + exchange diffusion; and curve D, leak only.

Inosine in ghost systems has been shown by Lionetti *et al.* (1957) and by Hoffman, Tosteson, and Whittam (unpublished experiments) to initiate and sustain glycolysis in addition to replenishing the phosphorylated metabolic intermediates as previously described in intact red cells by Dische (1951) and Gabrio *et al.* (1956). Strophanthidin, a cardiac aglycone, shown by Schatzmann (1953) to inhibit completely the active transport of both Na and K, was used in the present experiments to aid in the differentiation of the several components of the Na release. It should be mentioned that with intact cells, strophanthidin has also been found (*cf.* Glynn, 1957) to bring about a partial inhibition of the passive or "downhill" movements of K and Na. While this action of strophanthidin has not been observed in ghost systems (see below) the results would not be appreciably altered were such an effect present. The production of lactate by ghosts is not influenced by strophanthidin or by the inorganic cation composition of the external medium.

Fig. 3 is a plot of the curves from Fig. 2 according to the equation:

$$\ln\left(1 - \frac{(R)_s}{(R)_{eq}}\right) = o_{k_{Na}} \cdot t$$

where the ratio, $(R)_s/(R)_{eq}$, represents the fraction of Na^{24} released in time, t. $o_{k_{Na}}$ is the outward rate constant (in units of reciprocal hours). The curves when so plotted are linear and show that the loss of Na^{24} follows a single exponential. The rate constants corresponding to curves A, B, C, and D are 1.29, 0.77, 0.19, and 0.11 $hours^{-1}$, respectively.

Since the effect of strophanthidin in inhibiting the release of Na is essentially equivalent to the removal of K from the medium, the strophanthidin-sensitive portion is taken as the active transport component of the Na outflux; *i.e.*, the

pump flux. Thus, the difference between curves A and C (1.10) or B and D (0.66) represents the contribution of the pump.

The difference in the magnitude of the pump flux for the two conditions just referred to can be explained either as a depression of the pump by Mg or stimulation of the pump in the presence of exchange diffusion. It is un-

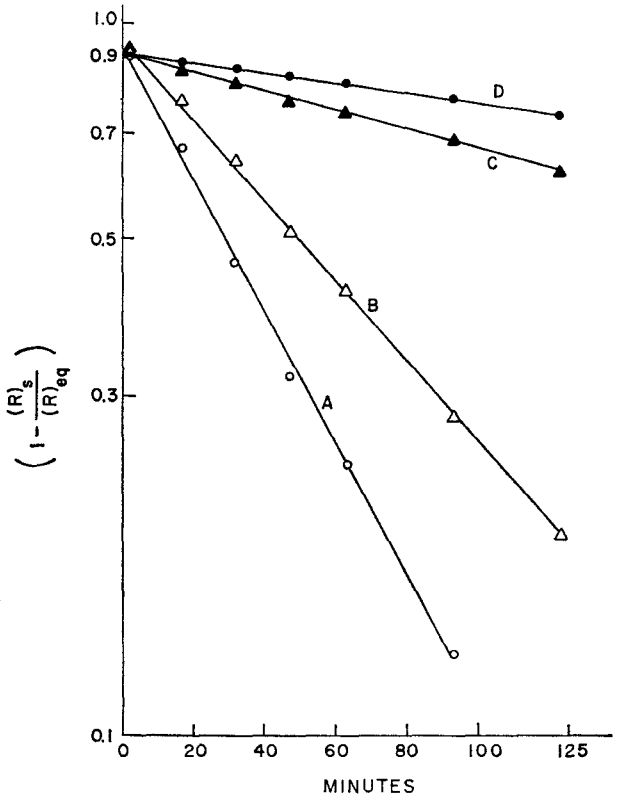

FIGURE 3. Plot of the curves from Fig. 3 showing that the outflux of Na^{24} is single exponential in all cases. See text for discussion.

likely that this difference represents a direct stimulation of the pump by an increase in the internal Na concentration since the ghosts containing the tracer do not appear to be permeable to Na (see Section 1). It cannot be determined whether the increased pump flux in the presence of external Na results in a net loss of Na from the ghosts without estimates of either the net change or the influx. Measurement of either of the latter quantities is difficult due to the heterogeneity described in Section 3.

It should be noted that ghosts undergo *reversal* when they are added to the suspension medium (refer to Fig. 1, steps 1, 2, and 4). This results in the liberation of a small portion of the trapped Na indicated in Figs. 2 and 3 by

release of about 10 per cent of the radioactivity at zero time. The reason for this initial liberation is not clear. The data given in subsequent figures and used in the computation of the fluxes have been corrected for this initial release (by subtraction of the radioactivity present in the supernatant of the zero time sample from all other samples taken from each flask).

The Effect of External K The effect of the K concentration in the external medium on the rate of release of Na^{24} from ghosts is shown in Fig. 4. The four curves represent conditions comparable to those in Fig. 2 but with K

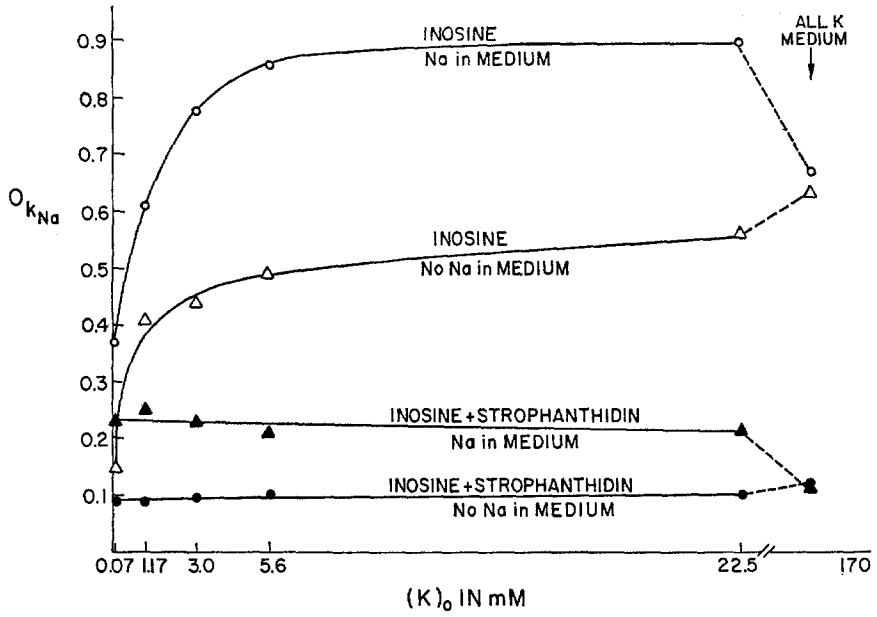

FIGURE 4. The activation of Na^{24} outflux by K in the external medium. Same conditions as described for Fig. 3 except that $(K)_o$ varied as indicated. See text for discussion.

substituted for an osmotically equivalent amount of cation in each of the media. For each $(K)_o$ the time-course of Na outflux was measured for each of the four conditions. As in intact cells (Harris and Maizels, 1951; Glynn, 1954) the outflux of Na from ghosts is apparently related to the external concentration of K. From the difference between the appropriate pairs of curves the extent of the activation of the pump by K is obtained. The $(K)_o$ giving a half-maximal flux is between 0.3 and 0.85 mM in the presence or absence of external sodium and is in fair agreement with the value of 1.8 found for intact human cells (Glynn, 1956; see also Post *et al.*, 1960). These results again indicate the similarity of the active transport of Na in ghosts to that in intact cells. In addition, the relation between the inhibition of the pump produced by strophanthidin compared to the removal of K can also

be evaluated. Here, the increased inhibition obtained with strophanthidin (Fig. 4) may reflect the fact that strophanthidin acts to inhibit more than the pump component of the Na outflux, as mentioned before. On the other hand, if the pump is slightly stimulated in the presence of 0.07 mM K this activity could be inhibited by strophanthidin. These results indicate the justification for the use of the strophanthidin sensitivity to denote the magnitude of the pump flux.

Alteration of Internal Na Fig. 5 and Table IV show the effect of altering the sodium content of the ghosts on the outflux of Na at constant $(K)_o$. $(Na)_g$ was varied by carrying out the hemolysis in solutions of varying NaCl concen-

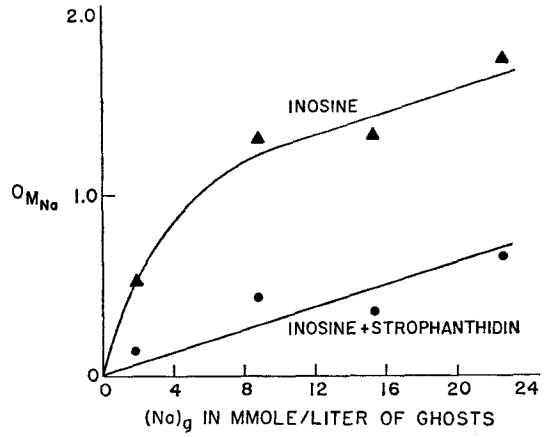

FIGURE 5. The relation between the outflux of Na^{24} ($o_{M_{Na}}$) and the concentration of Na inside ghosts. See text for discussion.

tration and containing Na^{24}. The $(Na)_g$ resulting from hemolysis under these conditions is given in Table IV. To minimize rehemolysis the ghosts were washed with $MgCl_2$–tris of appropriately increased concentration commensurate with the increased osmotic pressure of the hemolyzing solution. As the sodium concentration of the hemolyzing solution is increased there is an increase in the fraction of the labeling Na^{24} trapped within the ghosts (representing an increase in group I as indicated before). The rate of release of this trapped Na^{24} was measured in each case and the outward rate constant, $o_{k_{Na}}$, computed as before. The fluxes, plotted on the ordinate of Fig. 5, are calculated from the relation: $o_{M_{Na}} = o_{k_{Na}}.(Na)_g$ where $o_{M_{Na}}$ is the total outflux of Na in mmoles/(liter of ghosts × hour). $(Na)_g$ was determined immediately following hemolysis, and, therefore represents the concentration of Na per liter of ghosts at the time of hemolysis. ($(K)_g$ determined in a similar fashion was found to be about 12 mmoles/liter of ghosts, in all cases.) The general

form of the outflux curves suggests a saturable pump and linear leak. In this behavior it is similar to the influx of K into intact cells (Shaw, 1955).

Since the ghosts shrink as a result of washing and reversal (their water content changes from 95.9 ± 0.4 per cent to 86.4 ± 0.3 per cent) the actual value of $(Na)_g$ is not known under the conditions of assay; thus, the value of $(Na)_g$ giving half-saturation of the pump cannot be obtained directly from the data as presented in Fig. 5. If the extent of the shrinkage were known then the actual intracellular sodium concentration would be given by $\gamma \cdot (Na)_g$, where γ represents the volume correction factor. Presumably, the limits of γ can be estimated by assuming that the ghosts that trap Na^{24} at hemolysis are

TABLE IV

THE RESULTS OF A REPRESENTATIVE EXPERIMENT (ONE OF THREE) SHOWING THE OUTFLUX OF Na ($o_{M_{Na}}$) AS A FUNCTION OF THE CONCENTRATION OF $(Na)_g$ WITHIN THE GHOSTS

$o_{M_{Na}}$ is in millimoles Na/(liter of ghosts × hour). $o_{k_{Na}}$ is the outward rate constant in reciprocal hours. All incubations were carried out in a medium containing 153 mM NaCl–tris + 17 mM KCl–PO_4. Concentration of inosine is 5 mM. Stroph. is abbreviation for strophanthidin (5×10^{-5} M). These data are plotted in Fig. 5.

$(Na)_g$ after hemolysis *mM/L*	Milliosmols (as NaCl) Hemolysate	Wash solution	$o_{k_{Na}}$ Inosine	$o_{k_{Na}}$ Inosine + stroph.	$o_{M_{Na}}$ Inosine	$o_{M_{Na}}$ Inosine + stroph.
1.9	23	31	0.280	0.078	0.53	0.15
8.7	33	47	0.153	0.051	1.33	0.44
15.2	47	61	0.089	0.025	1.35	0.38
22.5	61	76	0.078	0.030	1.76	0.68

osmometers after hemolysis and respond accordingly to any alteration in the tonicity of their environment. This assumption appears to be justified from the properties referred to in Sections I and III. Thus, the limits of γ can be estimated, by one of two methods which differ only with respect to the particular volume at which the ghosts become impermeable; *i.e.*, osmometers. In both instances, let the reference point be $\gamma = 1$; thus, after hemolysis and prior to any alteration in the osmotic pressure of the ghosts' environment $(Na)_g$ would be given by the values of $(Na)_g$ measured in the hemolysis mixture, as listed in Table IV. The first method assumes that the ghosts become osmometers at their *hemolytic volume*, V_h. Then for the condition of 1 volume of cells hemolyzed with 10 volumes of solution, as in this experiment, the actual hemolytic ratio at the point of hemolysis is 1/7 assuming a $V_h = 1.57$ (Hoffman *et al.*, 1958). Thus, ghosts that become osmometers under these conditions would have one-seventh their original tonicity. Subsequent

suspension in a medium isotonic with plasma would induce an approximate sevenfold shrinkage and correspondingly $\gamma = 7$. In the second method, if the ghosts become osmometers after hemolysis but after return to their *initial volume*, V_o, as Teorell (1952) found, then the upper limit of γ would be 11 rather than 7, where $V_o = V_h = 1$. This distinction in the volume at which ghosts become osmometers has been considered previously by Hoffman (1958) and indicates that the second method offers the best estimate. The actual value of γ, in the present experiment, must necessarily be considered approximate since the osmotic pressure differential between the total concentration

TABLE V

REPRESENTATIVE RESULTS FROM ONE OF TWO SIMILAR EXPERIMENTS ILLUSTRATING THE EFFECT OF TONICITY ON THE PERMEABILITY OF THE GHOST TO Na

Experiment A is graphed in Fig. 6. See text for discussion.

Experiment	Hemolysis solution	$(Na)_g$ after hemolysis	Wash solution $MgCl_2$-tris	Incubation medium mM KCl-PO_4		$^{o}k_{Na}$ Inosine	$^{o}k_{Na}$ Inosine + stroph.	γ
		mM	*mM*					
A	Distilled H_2O	1.34	12	17	(36)	0.13	0.08	1
		(21)	(33)	51	(93)	0.67	0.12	3
				102	(184)	0.94	0.15	6
				170	(305)	0.99	0.20	10
B	14 mM/liter NaCl	15.0	24	51	(93)	0.17	0.09	1.5
		(47)	(62)	102	(184)	0.30	0.11	3
				170	(305)	0.33	0.12	5
C	14 mM/liter KCl	1.28	24	51	(93)	0.24	0.09	1.5
		(47)	(62)	102	(184)	0.39	0.11	3
				170	(305)	0.48	0.11	5

of salt after hemolysis and a solution isotonic with plasma varies inversely with γ for the series presented in Table IV. Compensation of these differences in the osmotic pressure occurring at hemolysis by the addition of KCl (so that all hemolyses take place at the same osmotic pressure) does not significantly alter the relative shapes of the curves given in Fig. 5 but the magnitudes of the calculated fluxes are reduced in the instances in which K was added. The reason for this is not clear but perhaps this reflects an inhibition of Na outflux by high intracellular K. Evidence for this point is given in Table V.

From Fig. 5 the half-saturation value for the pump component of Na outflux occurs at $(Na)_g = 2.0$. The limits of γ are between 7 and 11 giving an estimate of the actual value of $(Na)_g$ between 14 and 22 mM, respectively.

The half-saturation value estimated in this fashion agrees reasonably well with the measurements made by Post and Jolly (1957) for intact cells.

Effect of Medium Tonicity on the Pump The influence of the osmotic pressure of the final medium on the capacity of the ghosts to transport Na actively is illustrated in Table V and the data from Experiment A (Table V) are represented in Fig. 6. The ghosts used in Experiment A were hemolyzed in the usual manner and washed with 12 mM $MgCl_2$–tris solution. These ghosts were then suspended in KCl–PO_4 media at the various concentrations indicated. (Similar results are obtained when the osmotic pressure is increased

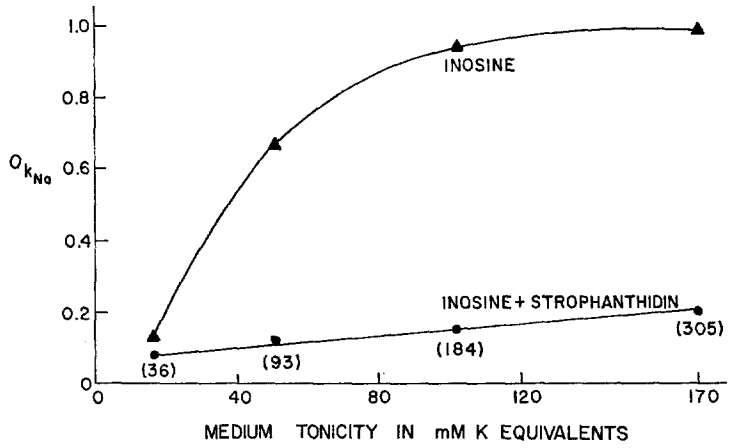

FIGURE 6. The effect of the tonicity of the suspension medium at the time of assay on the outflux of Na^{24}. See text for discussion.

with $MgCl_2$ with the K concentration constant at 6 mM.) The numbers in parentheses represent the milliosmolality of each solution calculated from its freezing point depression. It is seen that the capacity of the ghosts to transport Na increases as the osmotic pressure of the medium approaches that of plasma. The results of this experiment presumably depend upon the operation of two separate factors: (*a*) the increase in the $o_{k_{Na}}$ brought about by the increase in internal concentration of Na that results from the shrinkage induced by the increased tonicity and (*b*) an effect of the tonicity itself on the integrity of the membrane. The extent of the influence of the internal Na concentration can be evaluated from the data presented in Table V. Experiment B differs from A in that the Na concentration at hemolysis was increased so that the total resulting osmotic pressure was approximately double that in A. After hemolysis the ghosts were washed and suspended in the media indicated and the $o_{k_{Na}}$ measured. γ was estimated, assuming the ghosts that trap Na^{24} are osmometers, from the ratio of the tonicity at hemolysis to the

tonicity of the final suspension medium. Inspection of the table shows that no simple relation exists between the tonicity of the medium and the $o_{k_{Na}}$. The $o_{k_{Na}}$ for the leak (*e.g.* inosine + strophanthidin) is less than double for a five- to sixfold change in the tonicity. Further, the $o_{k_{Na}}$ for the pump (the difference between the two values of $o_{k_{Na}}$ for each incubation medium) is approximately the same for A and B at $\gamma = 1$ and 1.5, respectively, whereas the difference in internal Na concentration is at least thirteenfold. Or, alternatively, the comparison of the $o_{k_{Na}}$ for $\gamma = 10$ of A to $\gamma = 1.5$ of B shows a marked difference in the respective values of $o_{k_{Na}}$ whereas the calculated internal Na concentration is about the same for both cases. Evidently another factor, such as an effect of the tonicity on the pump, is involved. These data indicate that the pump cannot operate optimally until the relevant membrane parameters become reestablished. This appears to be controlled by the ionic strength of the medium.

Inhibition of Na Outflux by Internal K Table V is of interest in another connection. Experiment C was performed in the same manner as Experiment B except that the hemolysis solution contained KCl rather than NaCl. Thus, the ghosts of Experiments A and C contain essentially the same internal Na concentration but the ghosts of Experiment C contain, in addition, more K. Comparing those incubations in which there were equivalent values of γ (either 3 to 3 or 6 to 5), the $o_{k_{Na}}$ for the pump in the ghosts of Experiment C is less than one-half the pump $o_{k_{Na}}$ of the ghosts in Experiment A. Thus, the comparison of Experiments A and C in Table V indicates that a high intracellular K inhibits the outflux of Na from ghosts. Post *et al.* (1960) have shown in intact cells that the influx of K is inhibited by high extracellular Na. These results show the converse relationship in that the outflux of Na is affected by the intracellular concentration of K. These reciprocal dependencies at the two surfaces are important in the consideration of the mechanism of a coupled Na-K pump (see Hoffman, 1961).

SECTION 3. HETEROGENEITY AND THE LOCALIZATION OF GROUP I As discussed in Section 1, the population of ghosts resulting from hemolysis could be characterized as containing three compartments the observation of which depended upon the particular labeling procedure used and the ion in question. Group I could be labeled only at the time of hemolysis and could be observed only for Na. Group II could be occupied, non-selectively, by both Na and K. These differences are used below to demonstrate that groups I and II are contained in different and distinct portions of the total ghost population. Two types of experiments have been performed which provide direct evidence for this heterogeneity. Both types utilize ghosts which have groups I and II separately labeled so that the characteristics of each group can be directly compared. Measurements of the osmotic resistance of ghosts and their capacity

to transport Na actively have been made. From the data presented below it will become apparent that the ghosts comprising group I are more osmotically resistant than those ghosts making up group II. In addition, the ghosts comprising group I actively transport Na under experimental circumstances in which the ghosts of group II have lost this capacity.

The difference in the osmotic resistance between group I and group II is shown in Fig. 7. In this experiment a single preparation of ghosts was doubly labeled so that group I contained Na^{24} and group II, Na^{22}. This was accom-

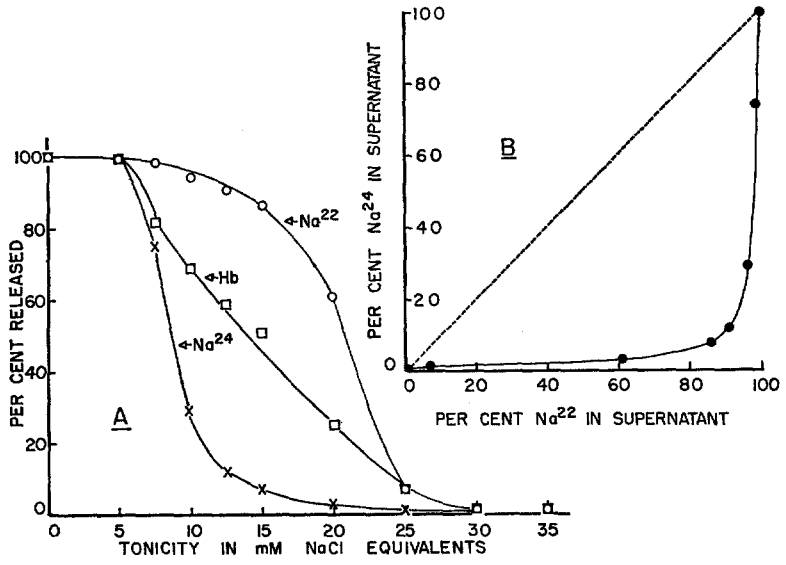

FIGURE 7. The results of a typical experiment illustrating the osmotic resistance of ghosts labeled at hemolysis with Na^{24} and after hemolysis with Na^{22}. See text for discussion.

plished using the following procedure: cells labeled at point A with Na^{24} were washed four times with 12 mM $MgCl_2$–tris (to remove the excess Na^{24}). The ghosts were at once resuspended and reversed with a solution containing Na^{22} thereby labeling the ghosts at point D (Fig. 1). (The composition of the reversing solution was 170 mM KCl–PO_4 at pH 7.4.) The suspension was then incubated at 37°C for 65 minutes. After this preincubation the ghosts were washed three times with the reversal solution (to remove the excess Na^{22}). These ghosts were dispensed into tubes containing various dilutions of the wash solution to give the osmotic series noted on the abscissa (Fig. 7, A). These final suspensions were incubated for 10 minutes at 37°C during which time aliquots of the mixtures were removed. The tubes were then centrifuged. Using the appropriate mixture and supernatant from each tube the per cent Hb released (per cent rehemolysis) was determined and in like manner the

percentage of the Na^{24} and Na^{22} released (by the measurement of the radioactivity twice with an interval of 10 days). Since it was found that in distilled water only 86.9 per cent rehemolysis occurred although 100 per cent of the Na^{24} and Na^{22} was released, the hemoglobin scale was expanded to 100 per cent for comparative purposes. These data are presented in Fig. 7, A. The preincubated ghosts labeled with Na^{24} (group I) comprise the most osmotically resistant group whereas ghosts labeled with Na^{22} (group II) show the lowest resistance to rehemolysis. These differences are emphasized in the graph shown in Fig. 7, B. This method of plotting has been used previously (Hoffman, 1958 *a*) to differentiate heterogeneity. The dashed line indicates the expected relationship between the amounts of the two isotopes released if Na^{24} and Na^{22} were to occupy the same space. The actual results show that there is little, if any, overlap between groups I and II.

It should be emphasized that preincubation in the above experiment serves a twofold purpose: (*a*) it promotes the continued and essentially complete loss of Na^{24} trapped in group II (that resulted from the washing required before labeling at point D) and, therefore, provides a system in which Na^{24} is contained almost exclusively in group I; (*b*) it promotes the trapping of Na^{22} by group II type ghosts while at the same time increasing the size (number) of group II, therefore, providing a system in which Na^{22} is contained only in group II. Evidence for these effects of preincubation is presented below in connection with an experiment differentiating group I from group II with regard to Na transport.

Fig. 8 illustrates the loss of the capacity of group II but not group I ghosts to transport Na actively. This experiment is divided into two parts: Part A deals with the outflux of Na from groups I and II before preincubation; part B, after preincubation. The particular labeling procedures used here differ from those described in the osmotic resistance experiment in that groups I and II are each separately labeled with Na^{24}. This is carried out by using paired flasks handled in identical fashion except for the point of addition of Na^{24}.

In part A (Fig. 8) the cells were hemolyzed in two flasks, Na^{24} added at point A in one and at point B in the other. Following hemolysis the ghosts were washed with 12 mM $MgCl_2$–tris, suspended in the final incubation medium, and the rate of release of Na^{24} measured (*cf.* Fig. 3). This corresponds to flowsheet steps 1–2–4 (Fig. 1). The results show that the outflux of Na from ghosts labeled at hemolysis is completely comparable to that from ghosts labeled after hemolysis.

In part B (Fig. 8), as part of the same experiment, two additional flasks of ghosts were prepared. One flask was labeled at point A as before but the other was labeled at point C (that is, after washing with 12 mM $MgCl_2$–tris but before suspension in the reversing solution). While suspended in this

reversing solution (140 mM NaCl, 20 mM KCl, 17 mM tris) the ghosts of both flasks were preincubated at 37°C for 50 minutes. They were then washed four times with fresh solution, suspended in the final incubation medium, and the rate of release of Na^{24} measured as before. This corresponds to procedural steps 1–2–5–8–9–10 (Fig. 1). As in the osmotic resistance experiment ghosts labeled at point A represent group I ghosts; ghosts labeled at point C, group II ghosts. It is seen that group I ghosts retain the capacity to transport

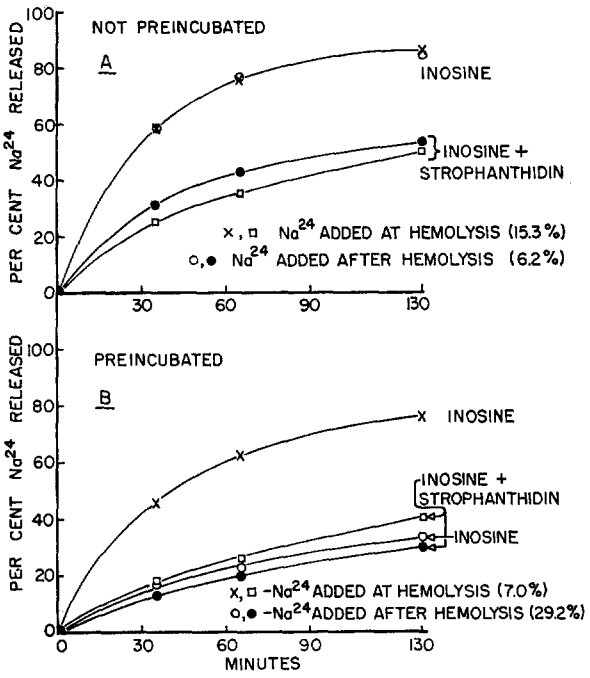

FIGURE 8. The outflux of Na^{24} from ghosts labeled at and after hemolysis with Na^{24} and the influence of preincubation. See text for discussion.

Na actively whereas group II ghosts have lost this capacity. The reason for this is indicated in the Discussion.

The numbers in parentheses in Fig. 8 represent the per cent of the original label trapped in the ghosts at the time of assay. Comparison of these figures demonstrates the effect of preincubation emphasized earlier. These values are based on the counts per unit Hb of ghosts obtained after reversal to ghosts obtained from the initial labeling mixture. It will be noted that ghosts labeled at point A (groups I + II) lost about half of their trapped Na^{24} upon preincubation (15.3 per cent compared to 7.0 per cent). Ghosts labeled at point B (group II) must either lose or reduce their trapped Na^{24} upon preincubation so that the assay given in part B completely masks their presence. This is to say that the Na^{24} trapped in group II by washing ghosts labeled at point A

is lost upon preincubation leaving only group I type ghosts labeled. On the other hand, ghosts labeled at point C and preincubated in the presence of label are found to trap considerably more Na^{24} than was possible by labeling at point B (29.2 per cent compared to 6.2 per cent). This effect of temperature has been shown by Hoffman *et al.* (1960) to accelerate the recovery of cation impermeability of ghosts and is used in the above experiments to label specifically group II type ghosts.

DISCUSSION

Although the experiments reported above illustrate that many of the features of the outflux of Na characteristic of intact cells are retained by their ghosts, the most significant finding is the demonstration that ghosts have the capacity to transport Na actively. This property was established in ghosts using the same criteria of response that have been applied to intact red cells (*cf.* Glynn, 1957). Thus, the Na pump in ghosts was found to depend upon metabolism, to be linked to the influx of K, to show saturation kinetics, and to be inhibited by strophanthidin. These results provide the basis for using the ghost system to study in more detail the mechanism of Na transport. This system has been used to assay various metabolic substrates for their possible role in activating and sustaining the Na pump. The immediate substrate and energy source for transport was found to be ATP (Hoffman, 1960 *a*). Further studies have been carried out (*cf.* Hoffman, 1961) in which the ghost system was employed to correlate directly the utilization of ATP by the pump with the activity of an ATPase present in the membrane as described by Post *et al.* (1960).

From the data found in Sections 1 and 3 it was shown that three different types of ghost constitute the total population. Two types of ghosts, identified as group I and group II, were shown to trap cations. The particular property which distinguishes these two groups resides in their ability to recover relative cation impermeability following hemolysis. Group I ghosts appear to recover spontaneously; this is evidenced by their relative impermeability to Na following hemolysis (Table I), retention of Na upon washing (Table II), and the lack of response to reversal. Group II, on the other hand, responds to and requires reversal for the restitution of relative cation impermeability; thus, after hemolysis group II ghosts remain permeable to K and Na (Table I) and their internal cation composition changes in response to alterations in the composition of the external environment (*cf.* Hoffman, 1958; Hoffman *et al.*, 1960). In addition, relative impermeability can be induced by increasing the tonicity of the external environment (incipient reversal) as shown by the retention of K illustrated in Table III; this retention can be enhanced by incubation at 37° (Hoffman *et al.*, 1960). Group I ghosts appear to be unaffected by this procedure.

Thus, the property of spontaneous as opposed to induced reconstitution serves to characterize the difference between group I and group II ghosts. In this respect, group I ghosts are indistinguishable from the ghosts investigated by Teorell (1952) whereas group II ghosts represent essentially those that have been studied by Hoffman (1958). Attention has previously been directed to the differences between these two types of ghosts (Hoffman, 1958). The data presented in Fig. 6 indicate that the type of ghosts used by Teorell (1952) would actively transport cations if the ionic strength of the medium were increased and an appropriate substrate supplied.

The measurement in ghosts of Na outflux rather than K influx has the advantage that the kinetics describing the loss of Na are first order whereas the tracer influx of K is considerably more complicated. In addition, Hoffman, Tosteson, and Whittam (data to be published) have found that the procedure leading to a satisfactory system of ghosts for the measurement of K influx required reversal and incubation at 37°C (see Hoffman *et al.*, 1960). This places limitations on the applicability of this system for such purposes as the assay of labile intermediates for their role in the active transport of cations (Hoffman, 1960 *a*).

Hoffman and Tosteson (1959) have shown that induced reconstitution of cation impermeability is not possible if, in the course of the preparation of ghosts, the initial hemolysis takes place in the presence of a molecule which complexes divalent cations (such as ATP or EDTA). They have shown that the ability of ghosts to reconstitute depends upon the presence, within the membrane, of a divalent ion presumably Mg^{++}. In the present studies these observations were extended to apply to group I ghosts. Hemolysis in the presence of 2 mM Na_2EDTA prevents completely the retention of Na^{24}. This effect of hemolysis in the presence of a complexing agent very likely applies also to the ghosts studied by Straub and his colleagues (see Gardos, 1954, for references); this suggests that the K accumulation noted in their system may have been due to the presence of intact cells rather than to ghosts.

It was mentioned before that the proportion of group I ghosts increases as the ionic strength of the hemolysis solution increases (within the range of complete hemolysis). This can be interpreted that the injury suffered at hemolysis, estimated by the capacity to reconstitute spontaneously, is caused by reduced ionic strength. This suggests that group I ghosts may be the most osmotically resistant cells. In a hemolysis system of one part cells to ten parts water the salt liberated by the hemolyzing cells would increase the salt concentration of the medium and yield the greatest protection to the last cells to hemolyze. Although it can be shown that the most osmotically resistant cells are the youngest cells (*cf.* Hoffman, 1958 *a*), it appears that the ability of the ghost to reconstitute spontaneously does not correlate with the age of the cell. Young cells can be easily separated from old cells by centri-

fugation, the cells at the top of the packed column being the youngest, those at the bottom, the oldest (Hoffman, 1958 *a*). The proportion of group I ghosts was found to be approximately the same in ghosts made from intact cells harvested from the top and bottom.

Two factors appear to affect the capacity of ghosts to reconstitute spontaneously: the ionic strength of the medium and the presence of Mg^{++}. If intact cells are washed prior to hemolysis the size of group I diminishes as the ratio of cells to hemolyzing solution decreases (at constant ionic strength) unless Mg^{++} is provided at hemolysis. If adequate Mg^{++} is present, the size of group I is independent of the ratio of cells to hemolyzing solution. It should be added that the induced reconstitution of group II ghosts also appears to require Mg^{++}. Consideration of these relationships provides a basis for understanding the fact that the ghosts prepared by Davson and Ponder (1938) were almost completely permeable to cations and that the behavior of the ghosts used by Teorell (1952) was dependent upon the ratio of cells to hemolyzing solution.

In Section 3 it was shown that group II ghosts pump Na but that, in contrast to the behavior of group I, this capacity is lost upon preincubation at 37°C. This difference is brought about by the use of inosine as substrate. If adenosine is substituted for inosine in the final incubation medium, group II ghosts are found to transport Na actively after preincubation. The basis for this effect apparently lies in a specific loss of adenine nucleotides that can be reversed by providing a source of adenine (Hoffman, 1961).

Two further points require comment. The first deals with the difference between the permeability of group I ghosts to Na and their permeability to K (Table I). These ghosts are considerably more permeable to K but much less permeable to Na than the ghosts investigated by Teorell (1952). Perhaps a part of the difference lies in the fact that Teorell's preparation contained a greater proportion of the population than is represented by group I in the present study. Nevertheless, the large differential in the permeability to Na and K displayed by group I ghosts remains unexplained. Preliminary experiments indicate that the factor of age may contribute to this difference: ghosts from old cells seem to be more permeable to Na than to K. The second point concerns the rate of loss of Na from group I ghosts. This was shown (Fig. 3) to follow a single exponential. But alterations in the conditions at hemolysis apparently change this relationship. Preliminary experiments indicate that the loss of Na from ghosts prepared by hemolysis in 1 mM $MgCl_2$ is described by a complex exponential and that this is associated with an increase in the size of group I. It is known (Solomon, 1952; see also Maizels and Remington, 1959) that the flux of Na in intact cells is described by a double exponential; this may be interpreted as indicating either that there are two compartments of Na in each cell or that the intact cell population is heterogeneous with

respect to Na. The question is not resolved by the present studies because, although heterogeneity in the intact cell population is implied by the properties of their ghosts, the origin of the ghosts and their relation to the intact cell distribution are yet to be established.

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