

# Passive Potassium Transport in LK Sheep Red Cells

## *Modification by N-Ethyl Maleimide*

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**ABSTRACT** Passive K transport, as modified by *N*-ethyl maleimide (NEM), was studied in erythrocytes of the low-K (LK) phenotype of sheep. Brief (5-min) treatment with NEM at  $<0.5$  mM caused inhibition of passive K influx; NEM at concentrations  $>0.5$  mM caused stimulation of K influx. NEM had similar effects on K efflux. The treatments with NEM did not affect cell volumes (passive K transport in LK cells is sensitive to changes in cell volume). The stimulation of K transport by high [NEM] was also not a consequence of an effect on the metabolic state of the cells. Passive K transport in LK cells is dependent on Cl (it is inhibited in Cl-free media; it may be K/Cl cotransport). NEM had no effect on K influx in Cl-free ( $\text{NO}_3$ -substituted) media. Pretreatment of the cells with anti-L antiserum (L antigen is found on LK cells and not on HK cells) prevented stimulation of K influx by NEM, but did not prevent inhibition. Therefore, NEM modifies the Cl-dependent K transport pathway at two separate sites, a low-affinity site, at which it stimulates, and a high-affinity site, at which it inhibits. Anti-L antibody prevents NEM's action, but only at the low-affinity site.

### INTRODUCTION

Membrane transport of Na and K independent of the Na/K pump is mediated by an array of specific, complex pathways (cf. Lew and Beaugé, 1979). The high specificity of the voltage-sensitive pathways for Na and K in electrically excitable cells has been known for decades. It now appears that passive transport of Na and K in nonexcitable cells is also highly specific, though the turnover rates per site are probably much lower than through voltage-gated channels. On the other hand, the non-voltage-gated channels have a greater complexity in terms of coupling between flows of different solutes.

An early report suggesting this complexity in human red cells was Glynn's demonstration (1957) of saturation kinetics of the passive K influx (passive

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meaning insensitive to cardiotonic steroids). Later it was shown that a portion of this passive K influx was dependent on the Na concentration in the medium (Lubowitz and Whittam, 1969; Glynn et al., 1970; Beaugé and Adragna, 1971). Dependence of Na influx on K was also demonstrated (Garrahan and Glynn, 1967; Hoffman and Kregenow, 1966; Sachs, 1971). It was suggested that these mutually interdependent Na and K fluxes, sensitive to diuretics such as ethacrynic acid and furosemide, represent a coupled cotransport system for Na and K (Wiley and Cooper, 1974). More recently, dependence of these passive fluxes on Cl has been shown, which suggests cotransport of cations and anions (Dunham et al., 1980; Chipperfield, 1981), similar to systems proposed for avian red cells (Kregenow, 1981; Haas et al., 1982) and Ehrlich ascites cells (Geck et al., 1980), in which there is cotransport of Na, K, and Cl in an electroneutral fashion (stoichiometry 1 Na:1 K:2 Cl). Perhaps the earliest proposal for a complex cotransport system for anions and cations in red cells was the demonstration of an interdependence of the influxes of glycine, Na, and Cl in pigeon red cells (Imler and Vidaver, 1972). For a recent brief review on Cl-dependent cation transport in red cells, see Ellory et al. (1982). Evidence for similar Na/K/Cl cotransport systems has been presented for squid axon (Russell, 1979, 1981), mammalian kidney in both nephron segments (Greger and Schlatter, 1981) and cultured cells (McRoberts et al., 1982), and flounder intestine (Musch et al., 1982).

In LK sheep red cells there is no Na/K cotransport (Dunham, 1976*a*). There is, however, Cl-dependent K transport (Dunham and Ellory, 1981), which may be K/Cl cotransport, a system proposed to coexist with Na/K/Cl cotransport in avian red cells (Kregenow, 1981). Na/K/Cl and K/Cl cotransport probably also coexist in human red cells. The evidence is that (*a*) Cl-dependent K transport exceeds Cl-dependent Na transport by severalfold (Dunham et al., 1980); (*b*) furosemide, which inhibits Na/K cotransport, also inhibits a portion of K transport in the absence of Na (Wiley and Cooper, 1974; Wiater and Dunham, 1983); and (*c*) the stoichiometric ratio of apparent Na/K cotransport varies with changes in cell volume (which suggests separate pathways with different sensitivities to changes in cell volume; Adragna et al., 1980; Logue and Dunham, unpublished results).<sup>\*</sup> K/Cl cotransport has also been proposed recently for crayfish neurons (Aickin et al., 1982).

The passive K transport system in sheep red cells is particularly interesting because of the variety of factors controlling it. First of all, there is the genetically controlled HK-LK polymorphism in which HK (high-K) red cells have relatively high active fluxes of Na and K and relatively low passive cation fluxes, while LK (low-K) cells have low pump fluxes and high passive fluxes (see Ellory, 1977, and Lauf, 1982*a*, for reviews).

Second, passive transport of K (but not Na) in LK cells is controlled by three otherwise unrelated properties of the cells: cell volume, Cl concentration, and a membrane antigen. Regarding cell volume, K transport increases when

<sup>\*</sup> The high rate of Cl transport in mammalian red cells through the anion exchanger (Knauf, 1979), which is independent of cation transport, has so far prevented unequivocal demonstration of cation/Cl cotransport.

cells are osmotically swollen and decreases in shrunken cells; the volume-sensitive pathway is identical with one requiring Cl (Dunham and Ellory, 1981). Regarding the membrane antigen, passive K transport is inhibited by anti-L<sub>1</sub> antibody (Dunham, 1976*a, b*). This alloimmune antibody is raised in HK sheep (which lack the blood group L<sub>1</sub> antigen) immunized with LK cells, which possess the L<sub>1</sub> antigen. (A related antibody, anti-L<sub>p</sub>, stimulates active Na/K transport [Ellory and Tucker, 1969].) The anti-L<sub>1</sub>-inhibitable pathway is identical with the volume-sensitive, Cl-dependent one (Dunham and Ellory, 1981).

A useful approach to identifying and characterizing this transport pathway in LK cells may be through the use of sulfhydryl-binding reagents. Iodoacetamide (IAA) reduced the volume sensitivity of K transport (in swollen cells IAA inhibited passive K transport, and in shrunken cells it enhanced it; Ellory and Dunham, 1980). Lauf and Theg (1980) showed stimulation of passive K transport in LK cells by another sulfhydryl-binding agent, *N*-ethyl maleimide (NEM). The effect of NEM was specific for K and was not observed in HK cells. While IAA's effect was initially thought to be metabolic (Ellory and Dunham, 1980), the rapidity of the onset of NEM's effect suggested an action on sulfhydryl groups of membrane proteins rather than on metabolism. Although substitution for Cl with other anions had little or no effect in these experiments on passive K transport (measured as efflux), only with Cl and Br (and not with NO<sub>3</sub>, SO<sub>4</sub>, or PO<sub>4</sub>) did NEM stimulate K transport (Lauf and Theg, 1980). Thus, the NEM-stimulated K transport appeared to share some of the properties of the volume-sensitive pathway that we described at about the same time (Ellory and Dunham, 1980; Dunham and Ellory, 1981). More recently, Lauf (1982*b*) has shown that pretreatment with IAA blocks the stimulation by NEM. Lauf and Theg (1981) have also provided preliminary evidence that pretreatment with anti-L also may prevent stimulation by NEM (one of the seven anti-L sera tested was effective).

NEM has been employed to advantage in studies of other transport systems. Recent examples include the anion exchanger of human red cells (Rao and Reithmeier, 1979; Solomon et al., 1983), Na,K-ATPase of dog kidney (Winslow, 1981), and proton ATPase of *Neurospora* (Brooker and Slayman, 1983). NEM binds with high specificity to sulfhydryl groups (Benesch and Benesch, 1962), so in membranes it alkylates cysteine residues of proteins. NEM is permeant, so it can bind intracellularly as well as externally; it has access to many, if not all, membrane proteins. The effects of NEM on K transport were not predictable; indeed, other sulfhydryl active agents (e.g., iodoacetamide and *p*-chloromercuribenzenesulfonate) have different effects on K transport.

In this report we confirm the stimulation of passive K transport (measured as ouabain-insensitive unidirectional K influx and unidirectional K efflux) in LK cells by brief pretreatment with NEM at  $\geq 0.5$  mM. However, lower concentrations of NEM inhibit passive K fluxes, which indicates two separate sites accessible to NEM that control the K transport (though it does not necessarily show two separate pathways). We show that pretreatment with anti-L prevents stimulation by NEM, but not inhibition by low NEM

concentrations. Finally, NEM had no effect on K influx in Cl-free cells. A preliminary report of a few of these results has been published (Ellory et al., 1982).

#### MATERIALS AND METHODS

##### *Animals and Cells*

Adult sheep (Dorset breed) were maintained at the farm of Krutulis Laboratories, Inc., Bridgeport, NY. Blood was drawn into heparin from the jugular vein. The red cells were washed by centrifugation and resuspension in a solution containing 145 mM NaCl, 5 mM KCl, 5 mM glucose, and 10 mM Tris-HCl, pH 7.4. This standard incubation medium was used in all experiments unless otherwise specified. The phenotype of the sheep (HK or LK) was determined by flame photometric analysis of Na and K concentrations in lysates of cells that had been washed in 116 mM isotonic MgCl<sub>2</sub>. The genotype of LK sheep (the allele for LK is dominant) was determined by testing for complement-dependent lysis (Tucker, 1965) with anti-L or anti-M sera (cells from heterozygous LK sheep have both M and L antigens; cells from homozygotes have only L antigens; Rasmusen and Hall, 1966; Rasmusen, 1969). In most of the experiments on LK cells (except where indicated), the cells were from heterozygous LK sheep.

##### *Antisera*

Alloimmune anti-L antisera were raised in HK sheep by immunization with washed LK sheep cells suspended in Freund's complete adjuvant; the method has been described before (Dunham, 1976a). Samples of purified alloimmune anti-M antibody were kindly provided by Dr. P. K. Lauf, Duke University Medical Center, Durham, NC, and by Dr. B. A. Rasmusen, University of Illinois, College of Agriculture, Urbana, IL.

In some of the experiments on K influx described below, cells were first sensitized with anti-L antibody by incubation with whole serum at 10% hematocrit for 30 min at 37°C. Anti-L sera from three different HK sheep were employed with no difference in the results.

##### *Unidirectional Influxes*

These were measured using <sup>86</sup>Rb as a tracer for K and <sup>22</sup>Na for Na by a modification of a method described previously (Dunham and Ellory, 1980). The fluxes were carried out in microcentrifuge tubes (polypropylene, 1.5 ml; Fisher Scientific Co., Pittsburgh, PA). After exposure to the tracer (5% hematocrit, 1 ml total volume), the cells were washed by four brief centrifugations in Fisher microcentrifuges (model 235A). The washed cells were lysed in 1 ml of distilled water. Samples were taken for determination of radioactivity in an autogamma counter (Nuclear Chicago, Des Plaines, IL) and for hemoglobin concentration (as cyanmethemoglobin). The latter measurement was used to calculate the volume of cells in each sample. The fluxes (in moles per liter cells per hour) were calculated from the radioactivities of cells and media and from the volume of cells. Active and passive transport were distinguished by the inclusion of ouabain (0.1 mM) with some aliquots of cells. In most experiments, ouabain was included with all aliquots.

##### *Relative Cell Volumes*

These were determined from hematocrits and hemoglobin concentrations of lysates as described previously (Dunham and Ellory, 1981). Fresh cells (in serum) were used to define a relative cell volume of 1.00.

### *Unidirectional Effluxes*

These were measured by a modification of the method described previously (Dunham and Hoffman, 1971). The cells were loaded with tracer ( $^{86}\text{Rb}$  or  $^{42}\text{K}$ ) by incubation overnight at  $4^{\circ}\text{C}$ , 40% hematocrit. During the efflux, samples were taken from duplicate flasks at times 0, 30, and 60 min. Rate constants for efflux were calculated from the radioactivities of these samples and from the radioactivity of a lysate of the suspensions of cells.

Although  $^{86}\text{Rb}$  has become accepted as a suitable tracer for measuring K influx, it may not be suitable for K efflux. To determine whether it is, effluxes of  $^{42}\text{K}$  and  $^{86}\text{Rb}$  were measured simultaneously after loading cells with both tracers. Radioactivities of samples were determined immediately after the experiment (for  $^{86}\text{Rb}$  and  $^{42}\text{K}$ ) and after 1 wk (for  $^{86}\text{Rb}$ ).  $^{42}\text{K}$  levels were obtained after subtracting the later counts and correcting for decay of  $^{42}\text{K}$  during the counting. The results of this experiment will be shown below (Table III, part B).

### *Sources of Materials*

$^{86}\text{Rb}$ ,  $^{22}\text{Na}$ , and  $^{42}\text{K}$  were obtained as chlorides in neutral aqueous solution from New England Nuclear Corporation (Boston, MA). Ouabain, iodoacetamide, and NEM were obtained from Sigma Chemical Co. (St. Louis, MO). Highly purified choline chloride was obtained from the chemical division of Syntex Agri-Business Inc. (Springfield, MO) and was purified further by recrystallization from hot ethanol solutions. Furosemide was a gift of Hoechst-Roussel Pharmaceuticals, Inc. (Somerville, NJ). All other compounds used were analytical reagent grade.

### *Abbreviations*

NEM: *N*-ethyl maleimide; IAA: iodoacetamide; Tris: tris(hydroxymethyl)aminomethane.

## RESULTS

### *Effects of NEM on Passive K Influx: Dependence on NEM Concentration*

Fig. 1 shows unidirectional K influxes in LK sheep red cells that had been treated briefly with various concentrations of NEM; after incubation with NEM for 5 min, the cells were washed by centrifugation, and the tracer influx was carried out in the absence of NEM. Just before initiating the flux (and after NEM treatment), the cells were incubated with ouabain. Except where indicated otherwise, treatment with NEM was carried out in this manner in all subsequent experiments.

The results in Fig. 1 show that brief treatment with NEM at concentrations  $>0.5$  mM enhances passive K influx (in confirmation of the findings of Lauf and Theg [1980]). However, Fig. 1 also shows that NEM at concentrations below 0.5 mM inhibits passive K influx. As subsequent figures will show, this result was a consistent finding. It was observed with cells from eight different LK sheep. In their first report, Lauf and Theg (1980) did not investigate concentrations of NEM below 1 mM. More recently, they have examined effects of lower concentrations of NEM on K efflux, but no indication of inhibition was reported (Lauf and Theg, 1981).

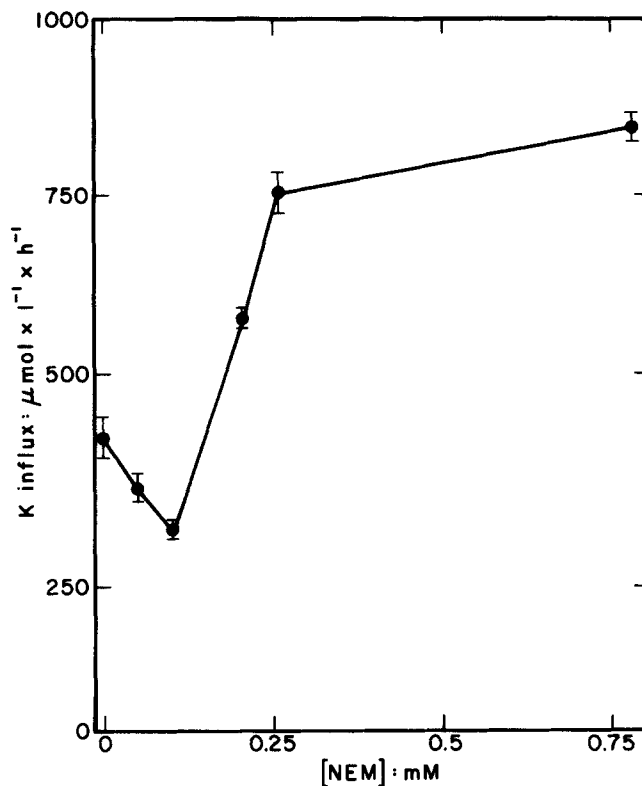
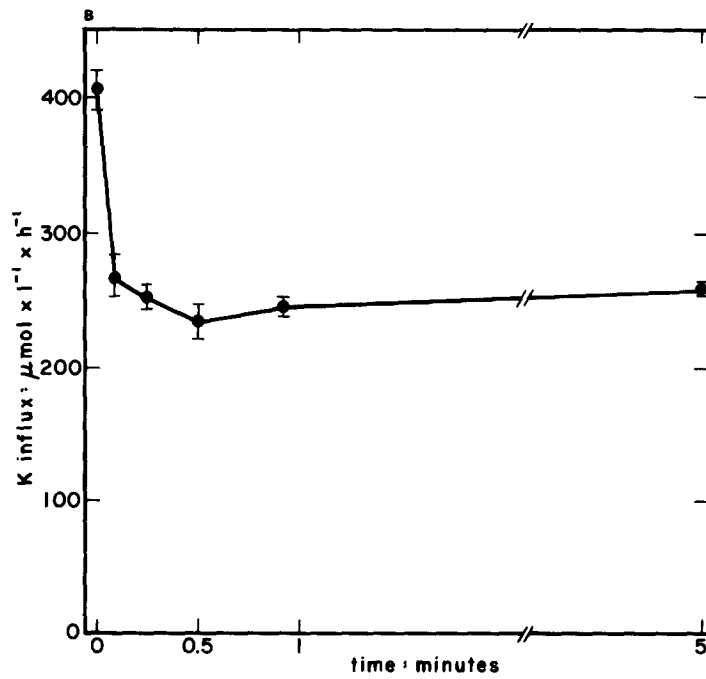
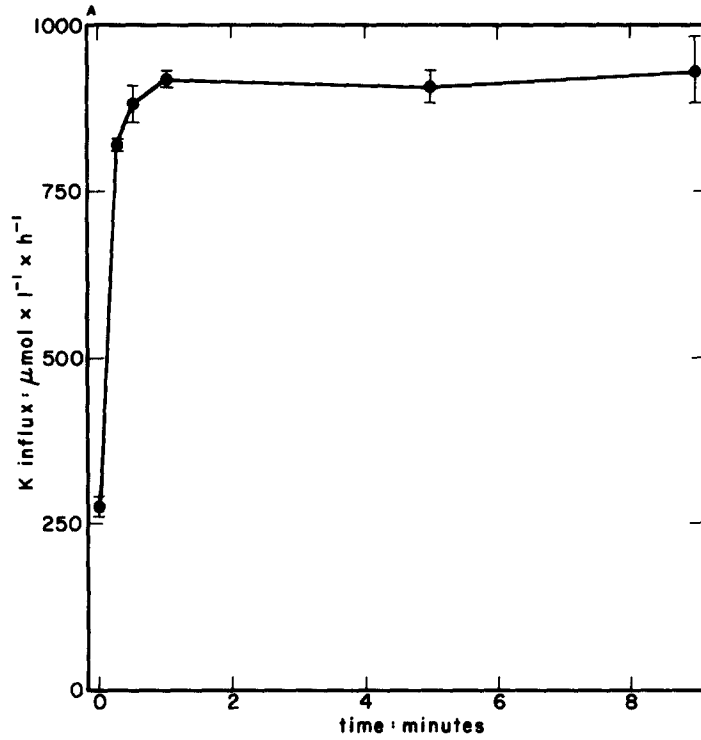


FIGURE 1. Effects of brief treatment with NEM at various concentrations on passive K influx in LK sheep red cells. The cells were treated with the concentrations of NEM indicated in the standard incubation medium at 5% hematocrit for 5 min at 37°C. The cells were then washed three times by centrifugation and resuspension in the same medium. After treatment with ouabain, unidirectional K influxes were measured as described in Materials and Methods. The fluxes are given in  $\mu\text{mol}/\text{liter}/\text{h}$ ; are mean values  $\pm\text{SD}$  ( $n = 3$ ). Similar results were obtained in experiments on seven other LK sheep, including one homozygous LK sheep.

*Time Course of Stimulation and Inhibition of Passive K Influx by NEM*

Fig. 2 shows ouabain-insensitive K influx in LK sheep red cells at various times after suspension at 37°C (5 min) in media containing either a high (0.8 mM; Fig. 2A) or a low concentration of NEM (0.2 mM; Fig. 2B), concentrations which either stimulated or inhibited the K influx, respectively (see Fig.

FIGURE 2. (*opposite*) Time course of effects of low and high NEM concentrations on passive K influx in LK sheep red cells measured at 37°C. In A, the NEM concentration was 0.8 mM; in B, it was 0.1 mM. Incubations were carried out at 5% hematocrit. At times indicated, samples were removed and washed three times; after ouabain treatment K influxes were measured. Fluxes (in  $\mu\text{mol}/\text{liter}/\text{h}$ ) are means  $\pm\text{SD}$  ( $n = 3$ ). Similar results were obtained in three other experiments.



1). The onset of both stimulation and inhibition were complete, or nearly so, in 1 min.

If there is both inhibition and stimulation of K influx by NEM, inhibition at high-affinity (or high-accessibility) sites and stimulation at low-affinity (or low-accessibility) sites, then it should be possible to see both effects in the same experiment, i.e., inhibition early during the incubation and stimulation later. Fig. 3 shows the results of two experiments designed to test this prediction, with the rates of the reactions with NEM slowed by lowering the temperature or lowering [NEM]. In the experiment shown in Fig. 3A the cells had been incubated at 37°C with 0.1 mM NEM; in Fig. 3B the incubation had been with 0.4 mM NEM at 1°C. In both experiments the results were as predicted: inhibition early and stimulation later. The results support the notion of two classes of NEM binding sites with either different affinities or different accessibilities.

It is possible that the early inhibition is a transient effect and would reverse in time even if [NEM] were not high enough to stimulate K influx. To test this possibility, cells were incubated at 37°C with 0.01 mM NEM for 6 h (hematocrit, 5%). To maintain constant [NEM], each hour the cells were spun and suspended in fresh solution. Inhibition of ~50% was observed at 1 h (first sample taken; results not shown). After 6 h, K influx was still inhibited. Therefore, the low-affinity sites don't bind significantly in 6 h at 0.01 mM, and the inhibition is not transient.

In early experiments variability was noted in the effects of concentrations of NEM between 0.1 and 0.4 mM. It was then demonstrated that the variability was correlated with the hematocrit during the incubation with NEM, as shown in Table I. After incubation with NEM at 0.75 mM at 5, 10, and 20% hematocrit, inhibition was observed at 20% and stimulation at 5%. Therefore, at 20% hematocrit, the binding of NEM to surface sites and to hemoglobin after permeation of the cells lowers the NEM concentration to the extent that the major effect is inhibition. In all subsequent experiments incubations with NEM were at 5% hematocrit.

#### *Effect of NEM on Cell Volume*

Passive K transport in LK cells is particularly sensitive to changes in cell volume (Dunham and Ellory, 1981). Therefore, it was important to determine if the treatments with NEM were modifying cell volume. Fig. 4 shows the results of six experiments in which LK cells were treated with NEM at 0.75 mM for 5 min and then washed. Relative cell volumes were measured (by measuring hematocrit and hemoglobin concentrations) at various intervals up to 1 h and compared with the volumes of untreated cells. There was no significant effect of NEM on cell volume. If there was any effect at all of NEM, it was a transient decrease (~2%), but this could not account for the effect of NEM on K influx because cell shrinkage inhibits influx and 0.75 mM NEM stimulates it.

#### *Interaction Between NEM and IAA in Modifying Passive K Influx*

Experiments were carried out to determine the effects of high and low NEM concentrations on cells pretreated with another sulfhydryl-binding agent, IAA.



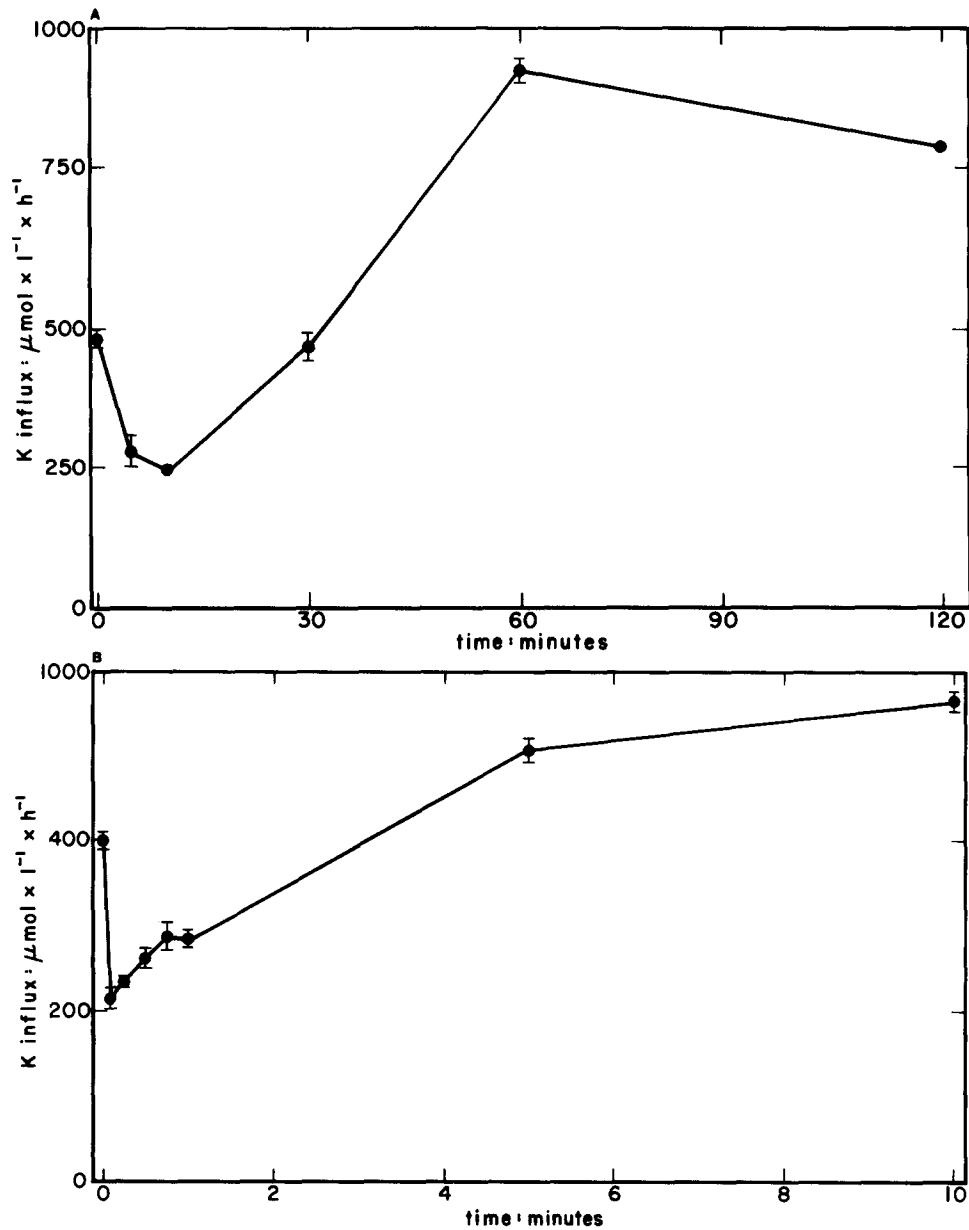


FIGURE 3. Time courses of effects of low NEM concentrations on passive K influx in LK sheep red cells. In A, the NEM concentration was 0.1 mM and the temperature of the incubation was 37°C. In B, the NEM concentration was 0.4 mM and the temperature was 1°C. The procedure for the experiment and the meanings of the symbols are the same as in Fig. 2. (In this figure and in subsequent ones, error bars were omitted when their extent was smaller than the symbols.) In the experiment in B, the flux in cells incubated at the same NEM concentration (0.4 mM) at 37°C for 5 min was 717  $\mu\text{mol}/\text{liter}/\text{h}$ . Therefore, the flux after 10 min at 1°C was 78% of the maximum.

TABLE I  
PASSIVE K INFLUX IN NEM-TREATED LK SHEEP RED CELLS  
AT VARIOUS HEMATOCRITS

	Passive K influx Hematocrit		
	5%	10%	20%
Control	262±7	268±6	271±18
NEM	517±24	185±5	178±12

Cells were incubated 5 min with NEM at various hematocrits. Incubations were in the standard incubation medium containing 0.75 mM NEM at 37°C. The hematocrits were adjusted to 5, 10, or 20%; the total volumes were all 5 ml. After 5 min, the cells were washed three times by centrifugation and resuspension in the standard medium. The cells were then treated with ouabain (0.1 mM, 5 min, 37°C), and unidirectional K influxes were measured as described in Materials and Methods. Fluxes were also measured on cells treated in the same manner, but without NEM (controls). Fluxes (micromoles per liter per hour) are means ± SD ( $n = 3$ ).

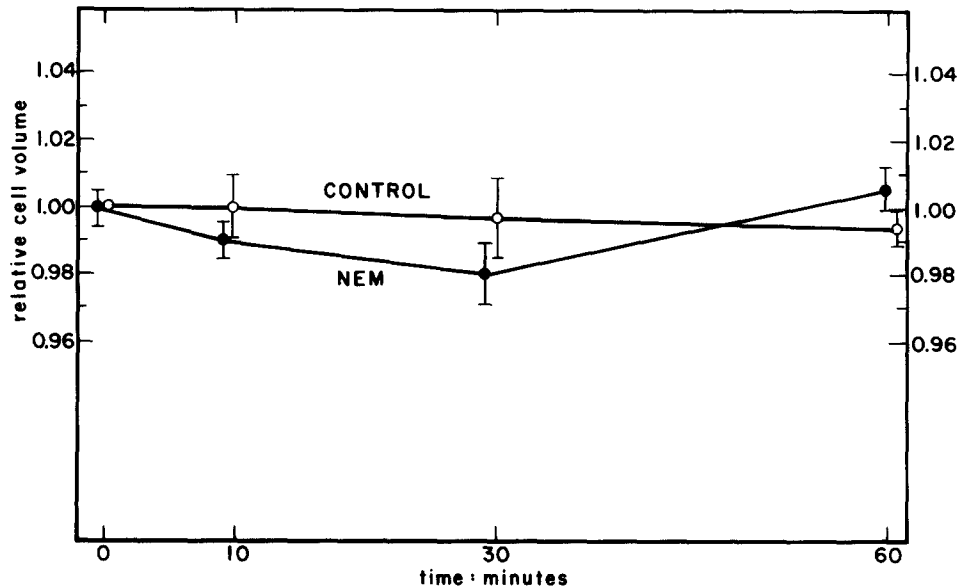


FIGURE 4. Effects of brief treatment with NEM on mean volumes of LK sheep red cells. Cells were incubated in the standard medium with NEM at 0.75 mM (5% hematocrit) for 5 min and then washed three times by centrifugation and resuspension in the same medium without NEM. Control suspensions were also incubated and washed. Control (○) and NEM-treated (●) cells were then incubated with shaking at 5% hematocrit. Duplicate samples were taken at 0, 10, 30, and 60 min for determination of relative cell volume as described in Materials and Methods. Time zero was 20 min after the beginning of the NEM incubation. The results shown are means of six separate experiments (±SEM).

The results of one such experiment are shown in Fig. 5. Cells treated with IAA (5 mM, 5 min, 37°C) were then incubated with [NEM] at either 0.1 or 0.75 mM. As control, cells first treated with NEM at both concentrations were subsequently incubated with IAA. K influxes were measured after the initial, or primary treatments, as well as after the secondary treatments. The primary treatments with IAA (5 mM) and low NEM (0.1 mM) inhibited K influx to the same extent; after neither of the secondary treatments of these cells was influx inhibited any further. In the simplest interpretation, these results suggest that IAA and NEM exert their inhibitor effects at the same class of sites.

The primary treatment with high NEM (0.75 mM) stimulated K influx nearly twofold, which is consistent with results shown above. Secondary treatment of IAA-treated cells with high NEM resulted in little, if any, stimulation of K influx, which shows that IAA prevents the stimulatory effect of NEM at low-affinity sites. These results on the interactions between NEM and IAA show that IAA affects NEM's binding with both class sites (low and high affinity), but with different effects. At the high-affinity site the effects of NEM and IAA were the same in that both agents were inhibitory and the effects were not additive. At the low-affinity NEM site, IAA prevented stimulation by NEM but did not itself stimulate.

#### *Anion Dependence of NEM-sensitive Fluxes*

Much of the passive K influx in LK sheep red cells is abolished by substituting any of a number of permeant, monovalent anions for Cl (except for Br, which enhances the flux; Dunham and Ellory, 1981). Therefore, it seemed worthwhile to examine the concentration dependence of the effect of NEM in Cl-free cells.

Fig. 6 shows passive K influx in LK sheep red cells preincubated briefly in various concentrations of NEM. After incubation with the drug, some of the cells were washed in a medium with NO<sub>3</sub> (or Br; Fig. 6B) substituted for all of the Cl (the solutions were buffered with Tris-HNO<sub>3</sub>). Fig. 6A shows the results of the experiment carried out with Cl cells and NO<sub>3</sub> cells and a series of NEM concentrations. Fig. 6B shows a similar experiment with Cl, NO<sub>3</sub>, and Br cells at just two NEM concentrations. In addition, in the latter experiment the flux for each type of cells was determined in the presence of furosemide, an inhibitor of the Na/K/Cl transport system in human red cells (Dunham et al., 1980; Chipperfield, 1981) and other cell types as well. Figs. 6A and B show the same NEM sensitivity of K influx in Cl cells as shown in Fig. 1: inhibition at low concentrations and stimulation at high concentrations. In the NO<sub>3</sub> cells, K influx was inhibited in the absence of NEM and was completely unaffected by NEM. The minimum flux in Cl-containing, NEM-treated cells was nearly as low as the flux in NO<sub>3</sub> cells, which suggests the identity of the Cl-dependent and NEM-inhibitable portions of passive K influx (Lauf and Theg [1980] had shown preliminary results in which NEM did not enhance K efflux in cells with Cl replaced by NO<sub>3</sub>, SO<sub>4</sub>, or PO<sub>4</sub>).

Fig. 6B, in addition to confirming the results in Fig. 6A, shows the enhancement of K influx in Br media as compared with Cl. The effect was observed in the absence of NEM (confirming an earlier finding; Dunham and Ellory, 1981) and at both high and low [NEM]. Fig. 6B also shows the effects

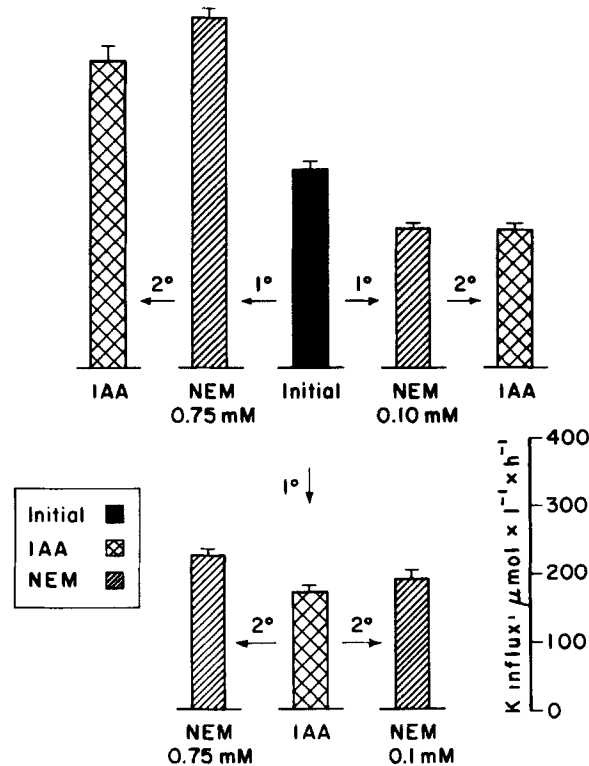


FIGURE 5. Interaction between NEM and IAA in modifying passive K influx in LK sheep red cells. In the primary treatments (1°), cells were incubated with either IAA (5 mM) or NEM at one or two concentrations (0.1 or 0.75 mM). In the secondary treatments (2°), aliquots of IAA-treated cells were incubated with NEM at either the high or the low concentration. Likewise, in the secondary treatments aliquots of NEM-treated cells (both concentrations) were incubated with IAA (5 mM). All of the incubations with the drugs were for 5 min at 37°C. (IAA at 5 mM and 10 mM had the same effect after a 5-min incubation.) After each incubation the cells were washed three times by centrifugation and resuspension. After secondary treatments with IAA or NEM, cells were incubated with ouabain, and the K influxes were determined. The standard incubation medium was used throughout. The heights of the bars represent mean fluxes (the scale at the lower right is for the entire figure); brackets at the top of the bars show SD ( $n = 3$ ). Similar results were obtained in two other experiments.

of furosemide. This loop diuretic at 1 mM completely inhibits the Na/K/Cl cotransport system in a number of cells types, but only partially inhibits Cl-dependent K transport (Na-independent) in sheep red cells at 2 mM (Ellory and Dunham, 1980). Fig. 6B shows partial inhibition of Cl-dependent K

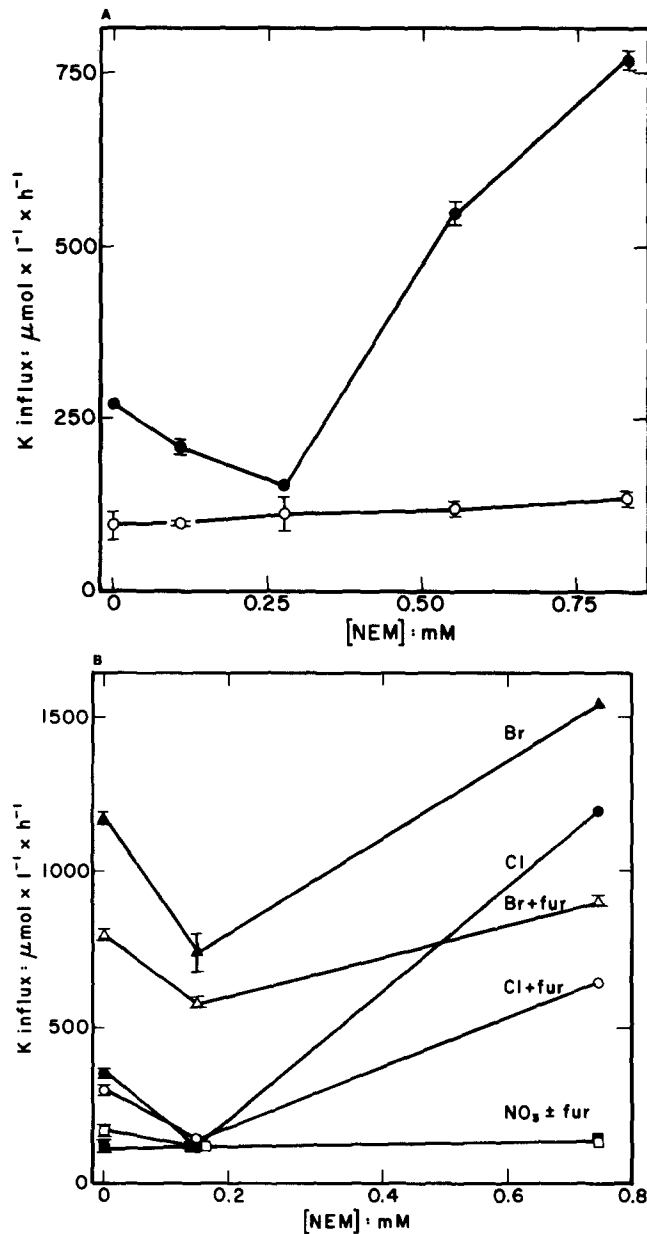


FIGURE 6. Effect of brief treatments with various concentrations of NEM on passive K influx in LK sheep red cells either in the standard incubation medium or equilibrated in Cl-free medium (with NO<sub>3</sub> substituted for Cl in A and NO<sub>3</sub> or Br in B). First, cells were incubated with NEM as in the experiment in Fig. 1. Then cells were washed in either the standard medium or the NO<sub>3</sub> or Br medium (pH adjusted with Tris-HNO<sub>3</sub> or Tris-HBr as appropriate). In the experiment in B, aliquots of cells were incubated with furosemide (1 mM) for 5 min at 37°C before measuring the flux. The appropriate flux media also contained furosemide. The measurement of the fluxes was the same as in Fig. 1. In A, the fluxes in Cl cells are shown by solid circles (●); in NO<sub>3</sub> cells by open circles (○). In B, the circles are for Cl cells, the squares (□, ■), NO<sub>3</sub> cells, and the triangles (△, ▲), Br cells. The open symbols (○, □, △) show fluxes in furosemide-treated (fur) cells and the solid symbols (●, ■, ▲) in control cells. In A, the symbols represent means ± SD (*n* = 3). In B, the brackets show total range (*n* = 2). Similar results were obtained in three other experiments.

influx at 1 mM. The relatively low sensitivity to furosemide is perhaps correlated with the independence of the transport system of Na. There may be a molecular commonality between Na/K/Cl and K/Cl cotransport systems, with differences reflected in differences in transport of Na, sensitivity to furosemide, and perhaps sensitivity to changes in cell volume (Haas et al., 1982; Adragna et al., 1980; Dunham and Ellory, 1981).

#### *Sensitivity of Passive Na Transport to NEM in LK Cells*

The volume-sensitive cation transport pathway in LK cells is specific for K (Na transport is insensitive to changes in cell volume; Dunham and Ellory, 1981). Therefore, the concentration dependence of the effect of NEM on unidirectional Na influx was examined in LK cells; the results of such an experiment are shown in Fig. 7, along with the corresponding measurements obtained simultaneously on K influx, which are shown for comparison. The biphasic effect of NEM on ouabain-insensitive K influx is the same as shown above in Figs. 1 and 3. NEM inhibited Na influx in the same range of concentrations that stimulated K influx. The failure of NEM to stimulate passive Na transport is in confirmation of the results of Lauf and Theg (1980). The inhibition of Na transport by NEM (like the inhibition of K influx) is at variance with the results of Lauf and Theg, who saw no effect of NEM on passive Na transport.

#### *Effects of NEM on K Efflux*

Most of the experiments from Lauf's laboratory on the effects of NEM involved measurements of efflux. Therefore, it was important to determine whether the apparently conflicting results, i.e., the inhibition of influx by low [NEM] in our experiments, reflects an effect of NEM on influx alone, and not efflux. Accordingly, effluxes and influxes were measured simultaneously on cells treated with NEM at various concentrations. After loading overnight with  $^{86}\text{Rb}$  of an aliquot of cells (for the efflux), samples of both  $^{86}\text{Rb}$ -labeled and unlabeled cells (also incubated overnight) were incubated at 37°C with NEM at either 0.75 or 0.075 mM. Control samples were also incubated at 37°C. Then influx or efflux was measured on each sample of cells. Table II shows the results of a representative experiment. NEM at 0.075 mM inhibited efflux by 39% and influx by 23%; NEM at 0.75 mM stimulated efflux by 71% and influx by 59%. Therefore, the effects of brief treatment with NEM on efflux and influx are about the same: 30% inhibition at low [NEM] and 60% stimulation at high [NEM].

In cells at steady state, efflux equals influx. The concentrations of K in the cells were not determined in the experiment in Table II. Assuming steady state, the intracellular concentration (in millimoles per liter) is given by the ratio of the influx to the efflux in the units given in Table II. The values so obtained for the three conditions were 4.2, 5.3, and 3.9 mmol/liter. These are more than a factor of two lower than the concentrations in fresh cells. However, the cells had been incubated overnight at 4°C; furthermore, they may not have been quite at steady state during the experiment. If one assumes

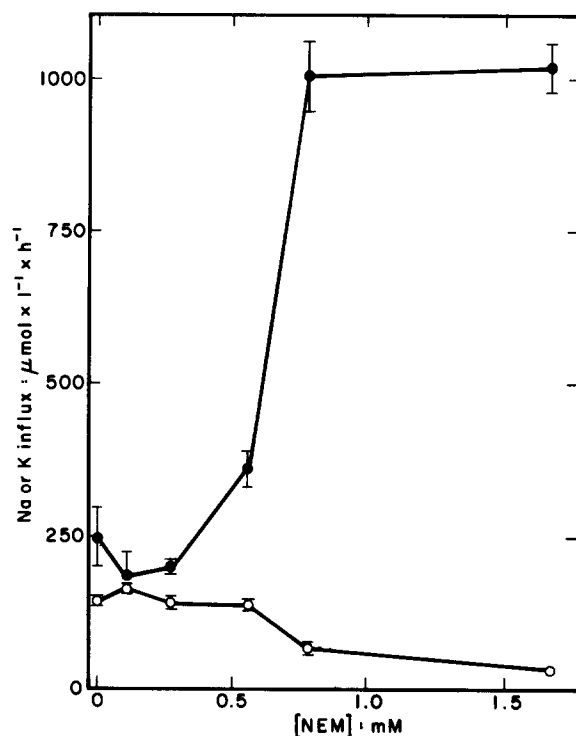


FIGURE 7. Effects of brief treatment with various concentrations of NEM on unidirectional influxes of Na in LK sheep red cells. For comparison, influxes of K measured at the same time are shown. The cells were incubated with NEM, washed, and treated with ouabain as described for the experiment in Fig. 1. Then influxes of Na and K were measured simultaneously on identical aliquots of cells in a medium containing (mM): 5 KCl, 5 NaCl, 140 choline Cl, 5 glucose, 10 Tris-HCl, pH 7.4. K influxes are shown by solid circles (●) and Na influxes by open circles (○); the brackets indicate  $\pm$  SD ( $n = 3$ ). Similar results were obtained in two other experiments.

TABLE II  
INFLUX AND EFFLUX OF K IN NEM-TREATED LK SHEEP  
RED CELLS

Pretreatment	Influx $\mu\text{mol/liter/h}$	Efflux $k, 1/h \times 10^3$
Control	$567 \pm 28$	$135 \pm 19$
0.075 mM NEM	$438 \pm 10$	$83 \pm 7$
0.75 mM NEM	$903 \pm 20$	$231 \pm 5$

Simultaneous measurements of unidirectional K influx and K efflux in NEM-treated LK sheep red cells. An aliquot of cells was incubated with  $^{86}\text{Rb}$  for measurement of efflux (see Materials and Methods). Then these cells and unlabeled cells were incubated with NEM at 0.075 or 0.75 mM (control samples were also incubated). The incubation was for 10 min (not 5 min, as in most experiments) at 37°C. After washing, cells were exposed to ouabain, and influx or efflux was measured as described in Materials and Methods. The medium was the standard incubation medium. Influxes are expressed as micromoles per liter per hour ( $\pm$ SD;  $n = 3$ ); effluxes are given as  $k$ , the rate constant in reciprocal hours  $\times 10^3$  ( $\pm 1/2$  of total range). Effluxes were carried out in duplicate flasks; samples were taken at three times from each. Values given are means of two values (one from each flask).

intracellular [K] was 8 mmol/liter, then efflux would have exceeded influx by ~80%.

Experiments on NEM-sensitive effluxes provide a convenient opportunity to determine whether the transport pathway has a *trans* dependence on K. Accordingly, K efflux was measured in control and NEM-treated cells in the absence and presence of K (15 mM). The results are shown in Table III, part A. First of all, the results confirm the biphasic effect of NEM on K efflux. Second, there was no stimulation of K efflux by external K under any of the conditions. (The hint of an inhibition by external K after 0.75 mM NEM is probably not significant statistically.) A similar result was reported previously (Dunham, 1976a).

TABLE III  
K EFFLUX FROM NEM-TREATED CELLS: EFFECT OF [K]<sub>o</sub>;  
COMPARISON OF <sup>86</sup>Rb AND <sup>42</sup>K

Experiment	Pretreatment	K efflux ( <i>t</i> )	
		[K] <sub>o</sub>	
		15 mM	0 mM
A	Control	302±33	300±20
	0.075 mM NEM	138±18	155±25
	0.75 mM NEM	338±13	433±58
		Tracer	
		<sup>86</sup> Rb	<sup>42</sup> K
B	Control	208±60	193±35
	0.75 mM NEM	245±56	240±4

Experiment A: effect of external [K] on K efflux. Experiment B: comparison of K efflux measured using <sup>86</sup>Rb and <sup>42</sup>K as tracers. The experiments were carried out in the same way as the efflux in Table II except that in experiment A the flux media contained either 15 mM K and 135 mM Na or 150 mM Na (K-free) as indicated. In experiment B the cells were loaded with both <sup>86</sup>Rb and <sup>42</sup>K. The flux medium contained 5 mM K and 145 mM Na. The samples in experiment B were counted twice, immediately after the experiment and 1 wk later. The final count gave radioactivities of <sup>86</sup>Rb; the differences between initial and final counts gave radioactivities of <sup>42</sup>K. Appropriate corrections were made for decay of both <sup>86</sup>Rb and <sup>42</sup>K. The rate constants (*t*) and the errors have the same meanings as in Table II.

Table III, part B, shows the results of an experiment in which efflux of <sup>86</sup>Rb and <sup>42</sup>K were measured simultaneously from the same samples of cells, both control and treated with NEM (0.75 mM). The two tracers gave the same results.

#### *Effects of NEM on Influxes of Na and K in HK Cells*

Fig. 8 shows measurements of unidirectional influxes of both Na and K in cells from several HK sheep (A: Na influx; B: K influx) pretreated for 5 min with various concentrations of NEM. The Na influx was inhibited in cells from all three sheep with a concentration dependence similar to that observed



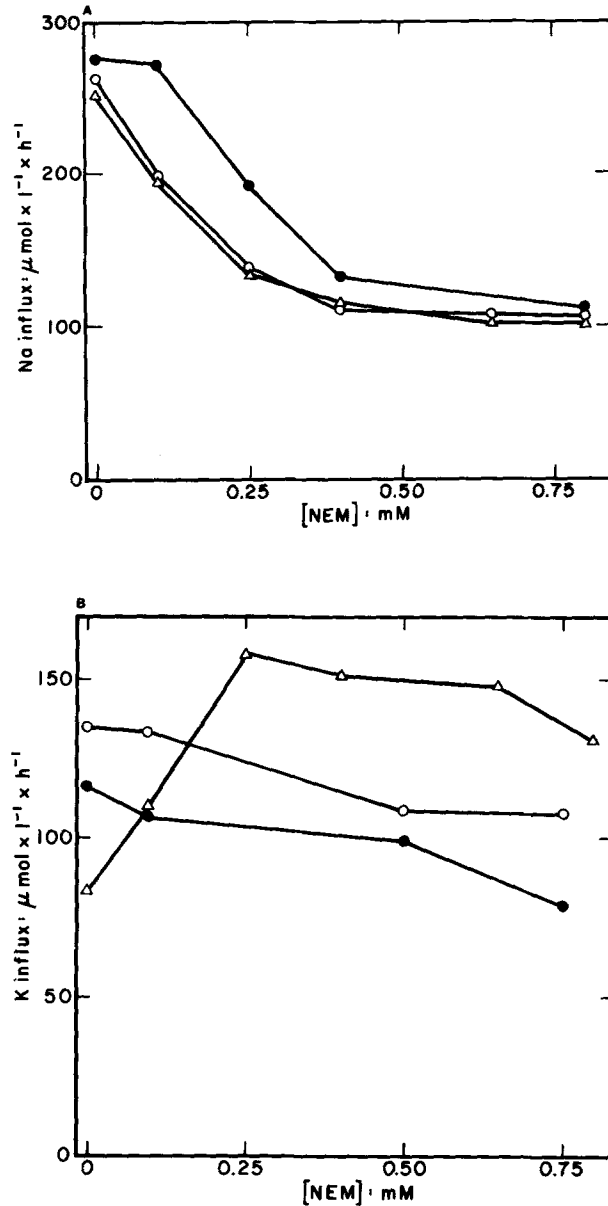


FIGURE 8. Effects of brief treatment with NEM on unidirectional influxes of Na and K in HK sheep red cells. The cells were incubated with NEM, washed, and treated with ouabain as described for the experiment in Fig. 1. Influxes of Na (A) and K (B) were measured as described for the experiment in Fig. 7. The three curves in the two figures are from experiments on three different HK sheep. The triangles ( $\Delta$ ) and the solid circles ( $\bullet$ ) in the two figures each represent results from the same sheep. The results shown by the open circles ( $\circ$ ) are for two different sheep in the two figures.

for Na influx in LK cells (see Fig. 7). With K influx there was variability between HK sheep in response of their cells to NEM, as shown in Fig. 8B. In some there was only inhibition, and in others, only stimulation (similar to the response to NEM in human red cells; Wiater and Dunham, 1983). Lauf and Theg (1980) reported that there were no effects of NEM on either K or Na influx in HK cells.

NEM has been reported to inhibit ouabain-insensitive Na/Na exchange in sheep red cells (Duhm and Becker, 1979). This is probably the explanation for the inhibition of the Na influx by NEM in both LK and HK cells (Figs. 7 and 8a).

#### *Effect of Pretreatment with Anti-L in LK Cells*

Because of the similarities between anti-L-dependent passive fluxes and NEM-affected fluxes (specificity for K, dependence on Cl, and specificity for LK cells), the effect of NEM on anti-L-pretreated cells was investigated. Fig. 9 shows K influx in an experiment in which cells sensitized with anti-L (as described in Materials and Methods) were treated with various concentrations of NEM as in the experiments shown above. For comparison, aliquots of cells were also treated with NEM before sensitization with anti-L. The upper curve (filled circles) shows the typical inhibition of K influx at low [NEM] and enhancement at a higher NEM concentration. The triangles show influxes in cells treated with NEM after anti-L. Without NEM and at [NEM] up to ~0.5 mM there is nearly a constant difference between this curve and the upper curve, i.e., the typical inhibition of passive K influx by anti-L (Dunham, 1976a), and no interaction between anti-L and NEM. However, at higher NEM concentrations, at which enhancement of influx is observed in the upper curve, there is no enhancement by NEM in cells pretreated with anti-L (influx is more or less unchanged from 0.2 mM NEM to 0.8 mM). Therefore, anti-L prevented the enhancement by NEM. In contrast, pretreatment with NEM did not prevent subsequent inhibition of K influx by anti-L (open circles).

Thus, although anti-L prevents stimulation by NEM, NEM does not prevent inhibition by anti-L. These observations can be understood in terms of binding of the two ligands at adjacent sites. The immunoglobulin is large enough to prevent sterically access of NEM, but not vice versa.

Another conclusion that may be drawn is that the site at which NEM stimulates transport is at the external surface of the membrane. Anti-L blocks NEM binding to the sites at which it stimulates; an antibody is unlikely to block access to intracellular sites. Therefore, this class of sites has a relatively low affinity for NEM rather than a low accessibility.

#### *Effect of Metabolic Depletion*

Changes in the metabolic state of red cells by long-term incubation or by metabolic inhibitors may alter passive cation transport (cf. Sachs, 1971); the mechanism of action is unclear. Although in most of our experiments the incubation time with NEM was short (5 min), an indirect effect of NEM on passive fluxes through an alteration of metabolic state remains possible since

NEM readily permeates red cells. The experiment in Table IV was carried out in an attempt to test this possibility. Control and NEM-treated cells were incubated 2 h in media either containing (fed cells) or lacking (depleted cells) nutrients as indicated. There were no active K influxes in depleted cells (NEM did not inhibit active influx in fed cells).

As shown at the bottom of Table IV, depletion led to an increased passive K influx in both control and NEM cells (to a slightly lesser extent in NEM

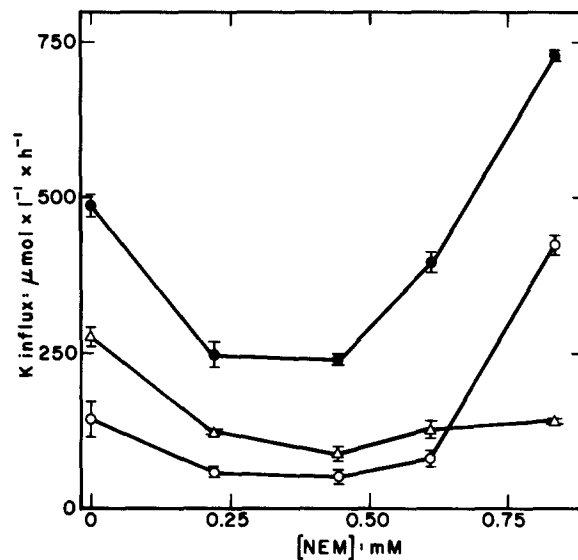


FIGURE 9. Interaction between anti-L antiserum and NEM in modifying passive K influx in LK sheep red cells. The upper curve (●) shows fluxes for cells treated with various concentrations of NEM as described for the experiment in Fig. 1. Aliquots of NEM-treated cells, after washing, were exposed to alloimmune antiserum as described in Materials and Methods (○). Other aliquots of cells were exposed first to antiserum, washed, and then incubated with the various concentrations of NEM (Δ). All aliquots of cells were incubated with ouabain before measuring unidirectional K influxes. The symbols represent means  $\pm$  SD ( $n = 3$ ). Similar results were obtained in three other experiments.

cells), and passive influx was enhanced in NEM cells compared with controls, both fed and depleted (slightly less in depleted cells). Therefore, the stimulation of passive K influx cannot be ascribed to an alteration of metabolic state by NEM.

#### DISCUSSION

We have demonstrated a biphasic effect of NEM on passive transport of K in LK sheep red cells: enhancement at higher concentrations ( $>0.5$  mM) and reduction at lower concentrations ( $<0.5$  mM). Preincubation with anti-L antibody prevented the enhancement by NEM, but not the inhibition. In

chloride-free cells ( $\text{NO}_3$  substituted for Cl), passive K transport was inhibited as compared with Cl cells, and NEM was without effect.

Therefore, there are two separate classes of sites with different affinities for NEM, high-affinity sites and low-affinity sites, at which NEM can modify K transport, and one class, the low-affinity sites, may be part of (or near) the L-antigen. (Anti-L blocked stimulation of K transport at high [NEM], which

TABLE IV  
ACTIVE AND PASSIVE K INFLUXES IN METABOLICALLY  
DEPLETED, NEM-TREATED CELLS

Cells	K influx	
	Active	Passive
	$\mu\text{mol/liter/h}$	
Control, fed	60±10	163±9
NEM, fed	80±21	763±20
Control, depleted	3±35	466±21
NEM, depleted	6±4	955±3
Treatment	Cells	$\Delta$ :stimulation (passive influx)
I. Depletion	Control	303±23
	NEM	191±20
II. NEM	Fed	600±22
	Depleted	489±21

An aliquot of cells was incubated with 1 mM NEM and washed as described for the experiment in Fig. 1. A control aliquot was also incubated and washed. Each aliquot was then divided and incubated for 2 h in standard medium containing either 5 mM glucose and 20 mM phosphate (as  $\text{Tris-H}_2\text{PO}_4$ ; fed cells) or 5 mM sucrose replacing the glucose (and 20 mM  $\text{Tris-HCl}$  replacing  $\text{Tris-H}_2\text{PO}_4$ ; depleted cells). At the end of this incubation, half of each aliquot was treated with ouabain and active and passive K influxes were measured. Shown at the bottom of the table are the increases in passive influx caused by depletion in control and NEM cells and the increase in influx caused by NEM in fed and depleted cells. Depleted cells were swollen 11% compared with fed cells. NEM had no effect on cell volume in either fed or depleted cells. The stimulation ( $\Delta$ ) of passive K influx was caused by (I) depletion in control and NEM cells and (II) by NEM in fed and depleted cells. Means are shown  $\pm$  SD ( $n = 3$ ) for passive influxes. The errors for the active fluxes and for the difference between passive fluxes are standard deviations of differences calculated from the expression:  $\sqrt{\text{SD}_1^2 + \text{SD}_2^2}$ , where the subscripts 1 and 2 designate the two different standard deviations. Similar results were obtained in two other experiments.

demonstrates low affinity rather than low accessibility.) However, the two types of NEM binding sites may be associated with the same K transport pathways. This conclusion is a tentative one, based upon the dependence of both the inhibitable and stimuable K pathways on Cl, the insensitivity of K transport to NEM in  $\text{NO}_3$  cells, and the near-identity of the minimum flux in NEM-(Cl) cells and the flux in  $\text{NO}_3$  cells.

The enhancement of passive K transport confirms the observations of Lauf and Theg (1980, 1981). They saw an increase in unidirectional passive K

influx (1980) and in net K efflux (1980, 1981) induced by NEM at 1–2 mM. In investigating the effects of lower NEM concentrations on net K efflux, Lauf and Theg (1981) observed no inhibition. Two differences between our experiments and theirs might explain the apparent discrepancy. (a) After brief exposures to NEM, we washed the cells by centrifugation and measured the fluxes in the absence of NEM; Lauf and Theg conducted the fluxes in the presence of NEM. (b) We measured unidirectional influxes and Lauf and Theg usually measured net effluxes.

The rapid onset of the effects of NEM is consistent with involvement of surface sulfhydryl groups rather than an effect on metabolic enzymes or on amino groups of proteins. Lauf and Theg had arrived at the same conclusion, though they presented no time courses of the effect of NEM. The action of anti-L in blocking the enhancement by NEM also confirms a preliminary finding of Lauf and Theg (1981), though they found only one effective sample of serum among seven. As mentioned above this effect of anti-L is direct evidence for the external location of the site at which NEM stimulates.

The NEM-sensitive K pathway is independent of Na, and is not, therefore, a Na/K cotransport pathway, as has been described in numerous cell types. In fact, sheep red cells appear to lack Na/K cotransport (Dunham, 1976*a*). It follows that the inhibition of Na transport by NEM represents an effect on a separate pathway, probably Na/Na exchange (Duhm and Becker, 1979). Consistent with this conclusion is the very similar effect of NEM on Na transport in LK and HK cells. In cells from some HK sheep there is inhibition of K transport by NEM, but higher concentrations are required than for inhibition in LK cells. Therefore, this pathway, NEM-inhibitable K influx in HK cells, is also a distinct pathway, and it is not clear if it exists in LK cells because the concentration range required to give a small inhibition of K influx in HK cells is the same as the range that causes a large stimulation in LK cells. (The stimulation of K influx by NEM in cells from some HK is unexplained.)

There are similarities between NEM-sensitive pathways of cation transport between sheep and human red cells. In human cells, NEM stimulates K influx just as it does in LK sheep cells (and in some HK cells), and in a similar concentration range (Wiater and Dunham, 1983). There is, in addition, a slight inhibition of K influx in human red cells at low NEM concentrations, but it is not as striking as in LK sheep cells. Na influx is inhibited slightly in human cells, just as it is in both phenotypes of sheep cells.

It has been proposed that passive cation fluxes in human red cells are mediated by the anion exchanger of band 3 (Solomon et al., 1983). It is not possible to rule out that the NEM-modulated K flux in sheep cells is through the anion exchanger, but it seems unlikely. There are five NEM binding sites on band 3 protein of human cells, and they are all "intracellular" (Rao and Reithmeier, 1979). We have shown here that the site at which NEM stimulates K influx is external since it is protected by the antibody.

The functional significance of transport systems in which the flow of one solute is coupled to the flow of another (cotransport or countertransport) lies in the downhill (thermodynamically) flow of one solute driving the flow of

another solute uphill. Net solute flow by cotransport can also move water against its chemical potential gradient. The proximate energy source for those secondary active transport processes is the gradient of a solute (Na and/or K) subject to primary active transport, i.e., transport directly coupled to metabolism. In epithelial tissues two functions of cotransport of inorganic ions (Na, K, and Cl) can be proposed: participation in transcellular movement of solutes and water, and regulation of volume of the epithelial cells during osmotic challenges, e.g., during diuresis or antidiuresis in the kidney. (It has recently been proposed that only one of these functions is served by Na/Cl cotransport in gall bladder, namely transepithelial fluid transport, and that regulation of cell volume following hyperosmotic challenge is accomplished by Na/H and Cl/HCO<sub>3</sub> countertransport systems [Ericson and Spring, 1982*a, b*]).

In nonepithelial cells the functional significance of inorganic ion cotransport processes is less clear. In avian red cells it has been demonstrated that Na/K/Cl and K/Cl cotransport can restore cell volume after hyper- or hypo-osmotic challenges. Constant volume is maintained through expenditure of metabolic energy by the Na/K pump (Tosteson and Hoffman, 1960; MacKnight and Leaf, 1978). The sustained osmotic stresses confronting epithelial cells, and inducing volume regulation by cotransport, probably do not affect red cells. Although passive cotransport systems in mammalian red cells could conceivably play a role in steady-state volume regulation, it is more likely that they are relics from precursor cells, where they may have functioned in regulating growth. Cell growth may require these transport mechanisms for control of net fluxes of water and ions during the concomitant increase in volume.

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