

# Glutaraldehyde Fixation of Sodium Transport in Dog Red Blood Cells

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**ABSTRACT** The large increase in passive Na flux that occurs when dog red blood cells are caused to shrink is amiloride sensitive and inhibited when Cl is replaced by nitrate or thiocyanate. Activation and deactivation of this transport pathway by manipulation of cell volume is reversible. Brief treatment of the cells with 0.01–0.03% glutaraldehyde can cause the shrinkage-activated transporter to become irreversibly activated or inactivated, depending on the volume of the cells at the time of glutaraldehyde exposure. Thus, if glutaraldehyde is applied when the cells are shrunken, the amiloride-sensitive Na transporter is activated and remains so regardless of subsequent alterations in cell volume. If the fixative is applied to swollen cells, no amount of subsequent shrinkage will turn on the Na pathway. In its fixed state, the activated transporter is fully amiloride sensitive, but it is no longer inhibited when Cl is replaced by thiocyanate. The action of glutaraldehyde thus allows one to dissect the response to cell shrinkage into two phases. Activation of the pathway is affected by anions and is not prevented by amiloride. Once activated and fixed, the anion requirement disappears. Amiloride inhibits movement of Na through the activated transporter. These experiments demonstrate how a chemical cross-linking agent may be used to study the functional properties of a regulable transport pathway.

## INTRODUCTION

Covalent cross-linking agents can be used to probe functional relationships in intact cells. If these agents are applied in a manner that results in submaximal fixation of the tissue, certain vital functions such as glycolysis, respiration, and the maintenance of transmembrane cation gradients can be preserved, while other cellular processes may be affected by the cross-linker in potentially informative ways (1, 3, 4, 8–10).

Like many other cell types, dog red blood cells possess volume-responsive, specific transport pathways. When the cells are caused to shrink, an amiloride-sensitive transporter is activated that is specific for Na and protons (15). Swelling the cells triggers a Cl-dependent K pathway and also a Ca-Na exchanger (13, 14). The volume-activated transporters are all quickly reversible once the volume stimulus is removed. Among the questions that can be asked about these volume-activated transport pathways is how the cell volume is perceived and how it is translated into a membrane response. As a first approach to this question, it

seemed reasonable to explore whether a volume response could be preserved in a functional state by a covalent cross-linker. The results herein show that this is indeed possible in the case of the shrinkage-activated, amiloride-sensitive Na pathway in dog red blood cells. Glutaraldehyde, among several cross-linkers that were investigated, gave the most satisfactory results.

It is important to point out that dog red blood cells are peculiar among animal cells in that they lack a Na-K pump and have internal Na and K concentrations that are not far from those of extracellular fluid (11). Therefore, when a dog red cell is placed into an all-K medium, as in many of the studies presented here, there are steep gradients favoring Na loss and K entry.

Brief accounts of these results have been presented in abstract form (16, 17).

#### MATERIALS AND METHODS

Glutaraldehyde grade 1 specially purified 25% aqueous solution and bovine albumin fraction V powder were purchased from Sigma Chemical Co., St. Louis, MO.  $^{22}\text{Na}$  was obtained from Amersham International, Ltd., Amersham, England, at a specific activity of 730 Ci/mol.

Blood was drawn from the forearm veins of unanesthetized mongrel dogs into heparin-rinsed syringes within 30 min of the beginning of each experiment. After centrifugation at room temperature at 500 g, the plasma and upper cell layer were discarded. The compositions of the media used for washing and incubations are given in the figure and table legends. In all experiments, the cells were washed at room temperature in phosphate-buffered saline media. Glutaraldehyde exposure was carried out at room temperature with the cells suspended at a cell/medium ratio of 1:20 in phosphate-buffered solutions at pH 7.75 (25°C). The crucial independent variable in these experiments was the volume of the cells at the time of fixation of glutaraldehyde. Cell volume was manipulated by varying the tonicity of the initial wash solutions and the glutaraldehyde-containing medium. After 5 min of gentle swirling in the glutaraldehyde medium, 15 vol of an ice-cold solution designed to stop the interaction between red cells and glutaraldehyde was added to 20 vol of the cell suspension. In all cases, the stopping solution contained: 150 mM NaCl, 10 mM  $\text{NaH}_2\text{PO}_4$ , 5 mM glucose, and 0.5 g/dl albumin. The pH of this solution at room temperature was ~5.0; the pH of the red cell suspension after addition of the stopping solution was 6.0–6.5. The non-glutaraldehyde-treated control cells in each experiment were suspended under identical conditions minus glutaraldehyde but with exposure to the stopping solution. After centrifugation from the glutaraldehyde-stopping mixture, all cells were washed at room temperature in solutions that were designed to prepare them for the next phase of the experiment in which net or isotopic fluxes were measured. Details of the experimental procedure are provided in the figure and table legends.

In Figs. 5 and 6 and Tables I–III, the tonicity of the influx solutions was determined by the concentration of Na salts. Detailed information regarding net water movements and the time course of tracer Na equilibration in dog red cells suspended in various concentrations of NaCl has been published previously (19). If rate constants are calculated by dividing the influx values by the medium Na concentrations, it can be seen that the amiloride-sensitive pathway in glutaraldehyde-fixed cells (e.g., Fig. 6, Table I) behaves as if it were saturated with respect to external Na over the concentration ranges used: the rate constant falls as medium Na concentration is increased.

Red cells were analyzed for Na, K, and water content by methods previously reported

from this laboratory (13–15). The procedure for  $^{22}\text{Na}$  influx is detailed in the legend to Fig 5. Isotope counting was done as previously described (15).

The ability of  $^{36}\text{Cl}$  to equilibrate rapidly across the membrane of glutaraldehyde-treated cells was assessed by methods described elsewhere (14).

## RESULTS

Preliminary experiments indicated that the effects of glutaraldehyde to be reported below were best seen when the cells were exposed to the agent for a period of 5 min at room temperature in media buffered with phosphate. Organic buffers appeared to diminish the fixative properties of glutaraldehyde at the low concentrations reported in most of these experiments. Fig. 1 shows the results of two studies aimed at finding a concentration range for glutaraldehyde that would not totally fix the cells but that might inhibit the large Na efflux that occurs when dog red cells are suspended in hypertonic KCl media (12). An

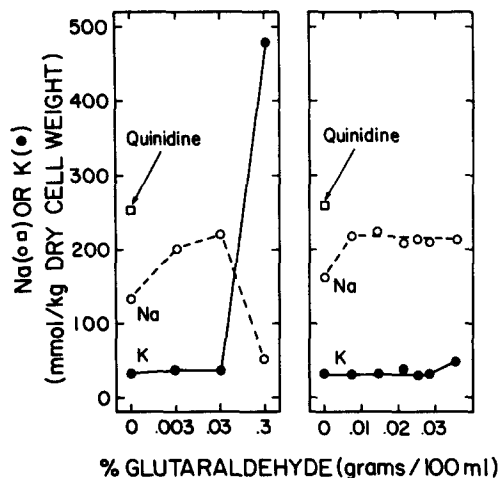


FIGURE 1. Effect of exposure to various concentrations of glutaraldehyde on the Na and K content of cells at the end of a subsequent incubation in hypertonic KCl medium for 20 min. Fresh cells were washed three times with a solution containing (mM): 150 NaCl, 10 Na-phosphate, 5 glucose (pH 7.75 at 25°C). 1 vol of cells was then resuspended in 20 vol of the same solution used for washing to which various concentrations of glutaraldehyde had been added (see abscissae). Suspensions were incubated at room temperature for 5 min with swirling, following which 15 vol of ice-cold stopping solution (see Materials and Methods) was added. Cells were then spun at 4°C and washed three times with original wash solution. Cells were then resuspended in medium containing (mM): 180 KCl, 10 HEPES, 5 glucose, 0.1 Na<sub>2</sub>EDTA (pH 7.4 at 37°C), and incubated for 20 min at 37°C before being centrifuged for analysis of Na, K, and cell water. Two separate studies are shown; notice the log scale on the abscissa of the left panel and the linear scale on the right. Square symbols indicate lots of cells from the control (zero glutaraldehyde) sample in which the subsequent KCl incubation was conducted in the presence of 0.2 mM quinidine, a drug that prevents the net Na movements that occur when dog red cells are shrunken (12). Cell contents at the beginning of the two incubations shown were (mmol or kg per kg dry cell weight): 256–260 Na, 34–35 K, 1.52–1.54 water.

optimum effect of glutaraldehyde is seen at a concentration of 0.01–0.03% (1–3 mM). Higher concentrations of the agent (e.g., 0.3% as shown in Fig. 1) cause a breakdown of the permeability barrier to Na and K; the cells come into equilibrium with their suspending medium. Quinidine is a potent inhibitor of Na movements in dog red cells (12, 15); Fig. 1 shows that although glutaraldehyde pretreatment can retard Na loss into a hypertonic KCl medium, the effect is not as great as that of quinidine. As will be shown, the degree of “protection” that glutaraldehyde pretreatment affords is critically related to the volume of the cells at the time they were exposed to the fixative. In the experiment shown in Fig. 1, the cells were suspended in an isotonic medium when they were treated with glutaraldehyde.

Fig. 2 shows that pre-exposure to 0.03% glutaraldehyde, either in hypo- or hypertonic media, does not affect the cells' ability to respond to a subsequent change of tonicity by gaining or losing water. In other experiments (not shown), exposure of cells to 0.03% glutaraldehyde under the conditions shown in Fig. 2 did not affect their subsequent ATP content (0.35–0.40 mmol/liter cells) or their rate of lactate production (1.0–1.1 mmol/liter cells·h) over a subsequent 90-min incubation.

The experiments that follow are of two kinds. In the “net flux” studies, dog red cells are pre-exposed to hypo- or hypertonic Na media with and without glutaraldehyde, washed, and then placed into K solutions. In this circumstance, there are gradients favoring K entry and Na loss, and the influence of the preincubation conditions is measured on these net movements. In the “isotope fluxes,” cells preincubated under hypo- or hypertonic conditions plus and minus glutaraldehyde are washed and resuspended in Na media;  $^{22}\text{Na}$  is then added,

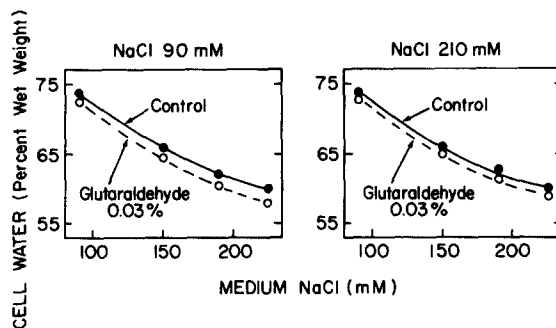


FIGURE 2. Effects of exposure to 0.03% glutaraldehyde on the subsequent osmometric behavior of dog red cells. Conditions of exposure to glutaraldehyde were similar to those noted in the legend to Fig. 1 except that the NaCl concentration of the glutaraldehyde pretreatment medium was 90 mM in the study shown in the left panel and 210 mM in study shown on the right. After application of the stopping solution and washing, the cells were resuspended in media containing (mM): 90–225 mM NaCl as noted on abscissae, 10 HEPES, 5 glucose (pH 7.4 at 37°C). After 5 min incubation at 37°C, the cells were centrifuged for determination of their water content. Solid symbols and lines indicate controls, open symbols and dashed lines indicate glutaraldehyde-treated cells. Each graph shows data from a single study, but the results of each are representative of four studies.

and the influx of the tracer is measured under conditions that more nearly approximate a steady state. The net flux experiments are presented to emphasize that the effects are not simply due to Na-Na exchange, while the tracer fluxes illustrate that the effects are observable under the more physiological circumstance in which Na is high on both sides of the membrane.

Fig. 3 shows net movements of Na, K, and water in cells suspended in a hypertonic (180 mM) KCl medium. Before the flux measurements were made, the cells were pretreated with hypotonic (90 mM) NaCl, in the presence or absence of 0.03% glutaraldehyde. Notice that the unfixed cells respond to hypertonic KCl by losing Na and water at a fast rate; the Na loss is substantially retarded by amiloride. But if during the hypotonic pretreatment the cells are exposed to glutaraldehyde, the subsequent incubation in hypertonic KCl results in only a slow rate of Na loss and very little amiloride effect.

Fig. 4 shows an experiment similar in form to that of Fig. 3, but in this case the net flux measurements are made in a hypotonic, 90 mM KCl medium. Before the flux measurements, the cells were pretreated with hypertonic (200 mM) NaCl, in the presence and absence of glutaraldehyde. As expected, the cells that were unfixed responded to the hypotonic KCl incubation with only a slow Na efflux that was barely inhibited by amiloride. In contrast, the cells that were exposed to glutaraldehyde during the hypotonic preincubation had a brisk loss of Na into the hypotonic KCl medium, and amiloride had a strong inhibitory

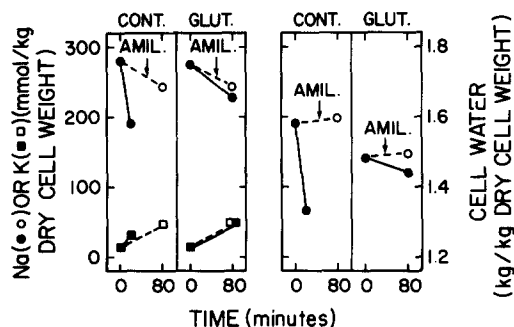


FIGURE 3. Na, K (left panel), and water (right panel) content of cells suspended in 180 mM KCl after pre-exposure to hypotonic solutions that contained (right half of each panel) or did not contain (left half of each panel) 0.03% glutaraldehyde. Fresh cells were washed three times at room temperature with a hypotonic solution containing (mM): 90 NaCl, 10 Na-phosphate, 5 glucose, 0.2 Na<sub>2</sub>EDTA (pH 7.75 at 25°C). Cells were then divided into two lots and resuspended in 20 vol of the same wash solution in the presence or absence of 0.03% glutaraldehyde, incubated for 5 min at room temperature, treated with 15 vol of ice-cold stopping solution, and washed twice with a hypertonic solution containing (mM): 180 NaCl, 10 HEPES, 5 glucose, 0.2 Na<sub>2</sub>EDTA (pH 7.4 at 37°C). Cells were then suspended in hypertonic KCl solution containing (mM): 180 KCl, 10 HEPES, 5 glucose, 0.2 Na<sub>2</sub>EDTA, plus (open symbols and dashed lines) or minus (solid symbols and lines) 0.2 amiloride (pH 7.4 at 37°C), and placed at 37°C. Samples were taken at zero time and at 15 or 80 min for cell ion and water content. Means  $\pm$  SEM for four studies. In some cases, the error bars are smaller than the data points.

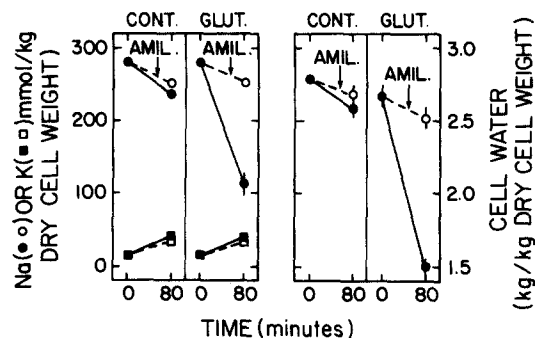


FIGURE 4. Na, K (left panel), and water (right panel) content of cells suspended in 90 mM KCl after pre-exposure to hypertonic solutions that contained (right half of each panel) or did not contain (left half of each panel) 0.03% glutaraldehyde. Fresh cells were washed three times at room temperature with a hypertonic solution containing (mM): 200 NaCl, 10 Na-phosphate, 5 glucose, 0.2 Na<sub>2</sub>EDTA (pH 7.75 at 25°C). Cells were then divided into two lots and resuspended in 20 vol of the same wash solution in the presence or absence of 0.03% glutaraldehyde, incubated for 5 min at room temperature, treated with 15 vol of ice-cold stopping solution, and washed twice with a hypotonic solution containing (mM): 89 NaCl, 10 HEPES, 5 glucose, 0.2 Na<sub>2</sub>EDTA (pH 7.4 at 37°C). Cells were then suspended in hypotonic KCl solution containing (mM): 90 KCl, 10 HEPES, 5 glucose, 0.2 Na<sub>2</sub>EDTA, plus (open symbols and dashed lines) or minus (solid symbols and lines) 0.2 amiloride (pH 7.4 at 37°C), and placed at 37°C. Samples were taken at zero time and at 80 min for cell ion and water content. Means  $\pm$  SEM for four studies. In some cases, the error bars are smaller than the data points.

effect. That the cells' ability to swell and shrink was unaffected by the glutaraldehyde treatment can be seen from Fig. 2, as well as from an inspection of the "zero time" values for cell water in Figs. 3 and 4.

The same general phenomena were observable in studies of tracer Na movements. Fig. 5 shows Na influx, done in Na media of varying tonicities, as a function of cell water in cells pretreated under hypo- or hypertonic conditions in the presence or absence of glutaraldehyde. As shown previously (19), shrinkage of dog red cells progressively increases their Na influx. This effect does not seem, from the results of Fig. 5, to be influenced by the previous volume history of the cells unless glutaraldehyde was present during the preincubation phase. Cells preincubated in the presence of glutaraldehyde show a much-reduced response of Na influx to alterations in cell volume. The cells that were "fixed" in hypotonic medium have a low Na influx that does not increase normally as the cells are shrunken; the cells that were "fixed" in hypertonic medium have a high Na influx that does not decrease normally as the cells are swollen.

Fig. 6 shows a variation on the experiment of Fig. 5 in which amiloride-sensitive Na influxes were measured at low and high cell volumes. As in Fig. 5, the cells were pre-exposed to hypo- or hypertonic NaCl in the presence or absence of glutaraldehyde. So long as glutaraldehyde was omitted, the tonicity of the preincubation medium had little influence on the subsequent behavior of the cells; both hypo- and hypertonically preconditioned cells had a brisk response

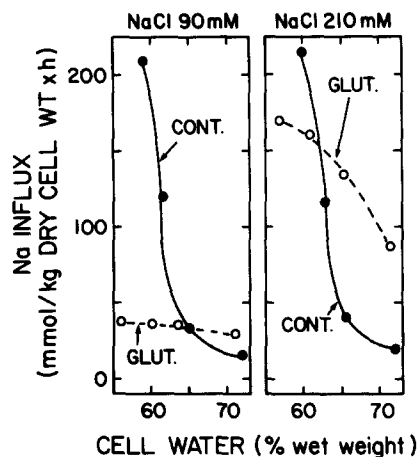


FIGURE 5. Na influx as a function of cell water content in cells that were pre-exposed to hypotonic (left panel) or hypertonic (right panel) preincubation in the presence (clear symbols, dashed lines) or absence (solid symbols and lines) of 0.03% glutaraldehyde. Fresh cells were washed three times in media that contained (mM): 90 or 210 NaCl, 10 Na-phosphate, 5 glucose, 0.2 Na<sub>2</sub>EDTA (pH 7.75 at 25°C). Cells were then resuspended in 20 vol of wash medium plus or minus 0.03% glutaraldehyde. After 5 min at room temperature, 15 vol of stopping solution was added and the cells were spun and washed three times with a solution containing (mM): 150 NaCl, 10 HEPES, 5 glucose, 0.2 Na<sub>2</sub>EDTA (pH 7.4 at 37°C). Glutaraldehyde-treated and control cells were each divided into four lots and resuspended in one of four solutions in which the tonicity was varied by adjusting the NaCl concentration. These contained (mM): 90, 150, 190, or 225 NaCl, 10 HEPES, 5 glucose, 0.2 Na<sub>2</sub>EDTA (pH 7.4 at 37°C). After 4 min incubation at 37°C, <sup>22</sup>Na (2 μCi/ml) was added to each suspension and samples were centrifuged at 0 and 60 min. Cells were assayed for wet and dry weight and isotope content. Influx was calculated as the increase in radioactivity per kilogram dry cell weight in 1 h divided by the specific activity of the medium. Each panel shows a single experiment representative of three others.

of their Na influx to cell shrinkage, and the effect was inhibited by amiloride. In contrast, the glutaraldehyde-treated cells behaved differently in the subsequent influx study, depending on the tonicity of the medium in which the glutaraldehyde treatment occurred. Cells "fixed" in 90 mM NaCl had a low Na influx at all volumes of which 10–20 mmol/kg dry cell weight · h was amiloride sensitive, while cells "fixed" in 210 mM NaCl had a high Na influx at all subsequent cell volumes of which 125–150 mmol/kg dry cell weight · h was amiloride sensitive.

The ability to "lock" the volume-activated, amiloride-sensitive Na transporter into a functional or nonfunctional state offered a means to approach the problem of the anion requirement of the pathway. It was shown previously (15) that replacement of Cl by NO<sub>3</sub> or SCN caused a substantial inhibition of shrinkage-activated Na movements in dog red cells. No information was available regarding the role of Cl in the functioning of the transporter or the mechanism of the anion effects. In Fig. 7, the basic effect is recapitulated, together with evidence

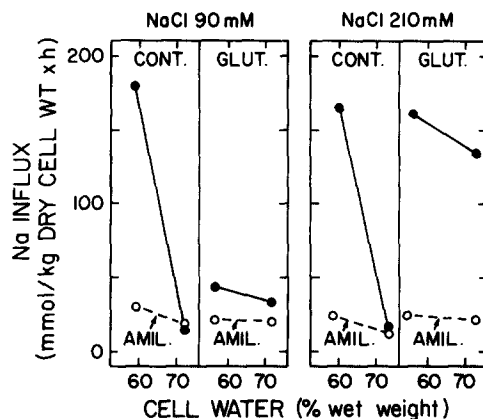


FIGURE 6. Na influx as a function of cell water content in cells that were pre-exposed to hypotonic (left panel) or hypertonic (right panel) preincubation in the presence (right half of each panel) or absence (left half of each panel) of 0.03% glutaraldehyde. Flux determinations were done in the presence (open symbols, dashed lines) or absence (solid symbols and lines) of 0.2 mM amiloride. Fresh cells were washed three times in media that contained (mM): 90 or 210 NaCl, 10 Naphosphate, 5 glucose, 0.2 Na<sub>2</sub>EDTA (pH 7.75 at 25°C). Cells were then resuspended in 20 vol of wash medium plus or minus 0.03% glutaraldehyde. After 5 min at room temperature, 15 vol of stopping solution was added and the cells were spun and washed three times with a solution containing (mM): 150 NaCl, 10 HEPES, 5 glucose, 0.2 Na<sub>2</sub>EDTA (pH 7.4 at 37°C). Glutaraldehyde-treated and control cells were each divided into four lots and resuspended in one of four solutions in which the tonicity was varied by adjusting the NaCl concentration and to which amiloride was or was not added. These contained (mM): 90 or 225 NaCl, 10 HEPES, 5 glucose, 0.2 Na<sub>2</sub>EDTA, plus or minus 0.2 amiloride (pH 7.4 at 37°C). After 4 min incubation at 37°C, <sup>22</sup>Na (2 μCi/ml) was added at each suspension and samples were centrifuged at 0 and 60 min. Cells were assayed for wet and dry weight and isotope content. Influx was calculated as the increase in radioactivity per kilogram dry cell weight in 1 h divided by the specific activity of the medium. Each panel shows a single experiment representative of three others.

for its reversibility. The figure shows net movements of Na and water out of dog red cells in hypertonic K media that contained Cl or SCN as the principle anion. Before the net flux measurements, the cells were pre-exposed either to NaCl or NaSCN 200 mM, but no glutaraldehyde was used. The results indicate that the rapid Na and water loss from cells incubated in hypertonic K media is retarded when SCN is substituted for Cl. Moreover, pre-exposure of cells to hypertonic NaCl or NaSCN media has little influence on the subsequent net fluxes or their response to anion replacement.

Fig. 8 shows the result of adding glutaraldehyde to the hypertonic preincubation media. The net fluxes in this figure are done in hypotonic K media to emphasize the point that the Na and water movements are due to the conditions that prevailed at the time of glutaraldehyde exposure (see Figs. 3 and 4). Notice that cells fixed in hypertonic Cl media lost Na rapidly, even though they were



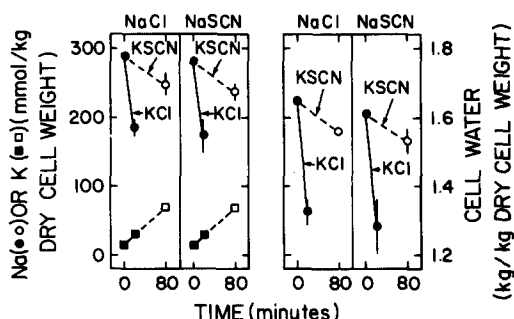
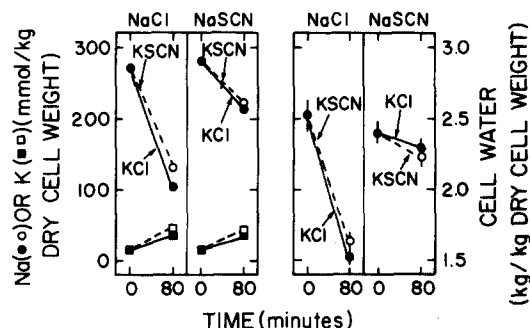


FIGURE 7. Na, K (left panel), and water (right panel) content of cells suspended in 180 mM KCl (solid symbols and lines) or KSCN (open symbols, dashed lines) after pre-exposure to hypertonic NaCl (left half of both panels) or NaSCN (right half of both panels). No glutaraldehyde was used in this experiment. Fresh cells were washed twice at room temperature in a medium containing (mM): 200 NaCl, 10 Na-phosphate, 5 glucose, 0.2 Na<sub>2</sub>EDTA (pH 7.75 at 25°C). Cells were divided into two equal lots, one half washed twice more with the same wash (200 mM NaCl), and the other half washed with a medium identical to the original solution except for the equimolar replacement of NaCl by NaSCN. Both lots of cells were then suspended in 20 vol of their respective wash solutions without glutaraldehyde and incubated 5 min at room temperature. 20 vol of stopping solution was then added to both suspensions, the cells were centrifuged, and each group of cells was divided into two equal portions. Half the Cl-preincubated cells were washed with 180 mM NaCl and half with 180 mM NaSCN. Half the SCN-preincubated cells were washed with 180 mM NaCl and half with 180 mM NaSCN. The cells were placed into 180 mM K media containing either Cl or SCN so that the four lots of cells had the following histories: preincubated in 200 mM NaCl, washed in 180 mM NaCl, incubated in 180 mM KCl (left half of each panel, solid symbols); preincubated in 200 mM NaCl, washed in 180 mM NaSCN, incubated in 180 mM KSCN (left half of each panel, open symbols); preincubated in 200 mM NaSCN, washed in 180 mM NaCl, incubated in 180 mM KCl (right half of each panel, solid symbols); preincubated in 200 mM NaSCN, washed in 180 mM NaSCN, incubated in 180 mM KSCN (right half of each panel, open symbols). Other ingredients of the wash and flux media were (mM): 10 HEPES, 5 glucose, 0.2 Na<sub>2</sub>EDTA (pH 7.4 at 37°C). Suspensions were placed at 37°C and cells were sampled at 0 and 15 or 80 min for Na, K, and cell water. Means  $\pm$  SD for three studies.

swollen. Also notice that replacement of Cl by SCN after fixation had very little inhibitory effect. Cells fixed in hypertonic SCN solution had a low rate of Na and water loss, and replacement of SCN by Cl after fixation caused no increase in the movement of Na or water.

These points are pursued in Tables I–III, which show the results of <sup>22</sup>Na influx measurements as influenced by anion replacement. Table I shows an experiment similar to that of Fig. 8 but with amiloride data that further characterize the post-glutaraldehyde fluxes. Cells were pre-exposed to hypertonic NaCl in the presence and absence of glutaraldehyde and then washed, and Na influx was determined in hypo- and hypertonic Na media in the presence and absence of amiloride and with Cl or SCN as the major anions. Unfixed cells incubated in



**FIGURE 8.** Na, K (left panel), and water (right panel) content of cells suspended in 90 mM KCl (solid symbols and lines) or KSCN (open symbols, dashed lines) after pre-exposure to hypertonic NaCl (left half of both panels) or NaSCN (right half of both panels). Glutaraldehyde 0.03% was added to both preincubation media. Fresh cells were washed twice at room temperature in a medium containing (mM): 200 NaCl, 10 Na-phosphate, 5 glucose, 0.2 Na<sub>2</sub>EDTA (pH 7.75 at 25°C). Cells were divided into two equal lots; one half was washed twice more with the same wash (200 mM NaCl), and the other half was washed with a medium identical to the original solution except for the equimolar replacement of NaCl by NaSCN. Both lots of cells were then suspended in 20 vol of their respective wash solutions to which 0.03% glutaraldehyde had been added, and incubated 5 min at room temperature. 20 vol of stopping solution was then added to both suspensions, the cells were centrifuged, and each group of cells was divided into two equal portions. Half the Cl-preincubated cells were washed with 90 mM NaCl and half with 90 mM NaSCN. Half the SCN-preincubated cells were washed with 90 mM NaCl and half with 90 mM NaSCN. The cells were placed into 90 mM K media containing either Cl or SCN, so that the four lots of cells had the following histories: preincubated in 200 mM NaCl plus glutaraldehyde, washed in 90 mM NaCl, incubated in 90 mM KCl (left half of each panel, solid symbols); preincubated in 200 mM NaCl plus glutaraldehyde, washed in 90 mM NaSCN, incubated in 90 mM KSCN (left half of each panel, open symbols); preincubated in 200 mM NaSCN plus glutaraldehyde, washed in 90 mM NaCl, incubated in 90 mM KCl (right half of each panel, solid symbols); preincubated in 200 mM NaSCN plus glutaraldehyde, washed in 90 mM NaSCN, incubated in 90 mM KSCN (right half of each panel, open symbols). Other ingredients of the wash and flux media were (mM): 10 HEPES, 5 glucose, 0.2 Na<sub>2</sub>EDTA (pH 7.4 at 37°C). Suspensions were placed at 37°C and cells were sampled at 0 and 80 min for Na, K, and cell water. Means  $\pm$  SD for three studies.

hypertonic NaCl show a Na influx of 167–183 mmol/kg dry cell weight·h. The flux is inhibited by amiloride and also by replacing Cl with SCN. Unfixed cells incubated in hypotonic NaCl show a smaller Na influx (16–19 mmol/kg dry cell weight·h), no amiloride effect, and no inhibition by substitution of SCN for Cl. The fixed cells have about the same Na influx as the unfixed cells when incubated in 225 mM NaCl, and the amiloride sensitivity is preserved. However, replacement of Cl by SCN in the fixed cells causes only a small inhibition of Na influx in a hypertonic medium. In hypotonic Na media, the Na influx of fixed cells is higher than that of unfixed cells and is amiloride sensitive, and there is little effect of anion substitution. Fixation of the cells in a hypertonic NaCl medium

TABLE I  
*Na Influx (mmol/kg dry cell weight · h) in Cells Preincubated in Hypertonic NaCl Media*

		Preincubated in 210 mM NaCl			
		No glutaraldehyde		Plus glutaraldehyde	
		Experiment 1	Experiment 2	Experiment 1	Experiment 2
Flux media (± amiloride)					
225 mM NaCl	-	167	183	167	175
	+	40	34	31	25
225 mM NaSCN	-	27	31	127	127
	+	29	28	43	48
90 mM NaCl	-	16	19	79	82
	+	17	15	29	24
90 mM NaSCN	-	22	19	70	70
	+	23	19	36	32

Fresh cells were washed in a medium containing (mM): 210 NaCl, 10 Na-phosphate, 5 glucose, 0.2 Na<sub>2</sub>EDTA (pH 7.75 at 25°C). Cells were then incubated 5 min at room temperature in 20 vol of wash solution, plus or minus 0.03% glutaraldehyde. 15 vol of ice-cold stopping solution was then added, and each lot of cells was washed three times in 150 mM NaCl or NaSCN plus (mM): 10 HEPES, 5 glucose, 0.2 Na<sub>2</sub>EDTA (pH 7.4 at 37°C). Cells were then resuspended in flux media containing (mM): 225 or 90 NaCl or 225 or 90 NaSCN, 10 HEPES, 5 glucose, 0.2 Na<sub>2</sub>EDTA, plus or minus amiloride 0.2 (pH 7.4 at 37°C). After 4 min at 37°C, <sup>22</sup>Na (2 μCi/ml) was added to each suspension and the samples were centrifuged at 0 and 60 min. See legend to Fig. 5 for further details of influx procedure. Results of two separate experiments.

preserves the amiloride-sensitive flux pathway, even though the cells are subsequently swollen, and the fluxes are no longer sensitive to Cl replacement.

Table II examines the effect of fixing cells in a hypertonic SCN medium. The control studies show that after exposure of cells to hypertonic NaSCN in the absence of glutaraldehyde, there is no suppression of amiloride-sensitive Na influx as measured subsequently in a hypertonic NaCl medium. Pre-exposure of the cells to SCN in the presence of glutaraldehyde, however, is associated with a marked suppression of the amiloride-sensitive Na flux pathway measured in cells that are washed and incubated in hypertonic NaCl.

In regard to the anion replacement experiments, it was important to evaluate the effect of mild glutaraldehyde fixation on the ability of Cl-SCN exchange to occur promptly during cell washes at room temperature. Cells were exposed to 210 mM NaCl or NaSCN in the presence or absence of 0.03% glutaraldehyde under conditions that were exactly as noted in the legends to Tables I and II. The Cl-treated cells were then washed with 90 mM NaSCN media, and the SCN-treated cells with 90 mM NaCl media at room temperature. All groups of cells were exposed to <sup>36</sup>Cl tracer for 2 min at room temperature in their new media and then centrifuged for determination of the cell/medium Cl ratios. In three such experiments, the tracer equilibrated to a value of 0.68 ± SD 0.03 cpm per kg cell water/cpm per kg medium water. There were no differences in the <sup>36</sup>Cl

TABLE II  
*Na Influx (mmol/kg dry cell weight · h) in Cells Preincubated in Hypertonic Media  
 Containing Cl or SCN*

Flux media (± amiloride)		Preincubated in 210 mM NaCl or NaSCN			
		No glutaraldehyde		Plus glutaraldehyde	
		NaCl	NaSCN	NaCl	NaSCN
210 mM NaCl	-	137±19	163±15	167±13	35±6
	+	32±2	28±2	36±3	18±1

Fresh cells were washed in media containing (mM): 210 NaCl or NaSCN, 10 Na-phosphate, 5 glucose, 0.2 Na<sub>2</sub>EDTA (pH 7.75 at 25°C). Cells were then incubated 5 min at room temperature in 20 vol of wash solution, plus or minus 0.03% glutaraldehyde. 15 vol of ice-cold stopping solution was added, and each lot of cells was washed three times in a solution containing (mM): 150 NaCl, 10 HEPES, 5 glucose, 0.2 Na<sub>2</sub>EDTA (pH 7.4 at 37°C). Cells were then resuspended in flux media containing (mM): 210 NaCl, 10 HEPES, 5 glucose, 0.2 Na<sub>2</sub>EDTA, plus or minus 0.2 amiloride (pH 7.4 at 37°C). After 4 min at 37°C, the isotope was added and influx was measured as described in the legend to Fig. 5. Means ± SD for three studies.

ratio between glutaraldehyde-treated cells and controls. The ability of band 3 to mediate rapid exchanges of anions was thus judged to be unaffected by the glutaraldehyde treatment.

Table III presents an experiment similar in form to that of Table II. The question asked here is whether the inhibitory effect of amiloride is reversible, particularly if the cells are exposed to the drug during glutaraldehyde treatment. The results show that cells pre-exposed to a hypertonic NaCl solution plus amiloride, then washed in drug-free medium, have the same amiloride-sensitive

TABLE III  
*Na Influx (mmol/kg dry cell weight · h) in Cells Preincubated in Hypertonic NaCl Media Plus or  
 Minus 0.2 mM Amiloride*

Flux media (± amiloride)		Preincubated in 210 mM NaCl			
		No glutaraldehyde		Plus glutaraldehyde	
		Control	Amiloride	Control	Amiloride
210 mM NaCl	-	158±13	157±11	156±21	169±15
	+	37±3	35±4	31±6	32±3

Fresh cells were washed in media containing (mM): 210 NaCl, 10 Na-phosphate, 5 glucose, 0.2 Na<sub>2</sub>EDTA, plus or minus 0.2 amiloride (pH 7.75 at 25°C). Cells were then incubated 5 min at room temperature in 20 vol of wash solution, plus or minus 0.03% glutaraldehyde. 15 vol of ice-cold stopping solution was added, and each lot of cells was washed three times in a solution containing (mM): 150 NaCl, 10 HEPES, 5 glucose, 0.2 Na<sub>2</sub>EDTA (pH 7.4 at 37°C). Cells were then resuspended in flux media containing (mM): 210 NaCl, 10 HEPES, 5 glucose, 0.2 Na<sub>2</sub>EDTA, plus or minus 0.2 amiloride (pH 7.4 at 37°C). After 4 min at 37°C, the isotope was added and influx was measured as described in the legend to Fig. 5. Means ± SD for three studies.

flux as cells that were not pre-exposed to the drug. This is true whether or not glutaraldehyde was present during the drug pre-exposure. Quinidine, a drug that also inhibits shrinkage-activated Na movements in dog red cells, behaves in these experiments in a manner similar to amiloride in Table III: addition of glutaraldehyde to cells in the presence of quinidine does not prevent the Na pathway from being activated. After the quinidine and glutaraldehyde are washed off, the cells respond as if they had never been exposed to quinidine before (data not shown).

Thus, glutaraldehyde fixation discloses a difference between two inhibitory influences on the shrinkage-activated pathway in dog red cells. Removal of Cl or substitution of Cl by SCN causes an alteration in the transport mechanism that can be preserved by glutaraldehyde fixation. Once activated in a Cl-containing hypertonic medium and "locked in" by glutaraldehyde, the mechanism is thereafter insensitive to the replacement of Cl by SCN. On the other hand, amiloride, although it is a potent inhibitor of the Na transport pathway, exerts no irreversible effect even when the cells are exposed to the drug before and during glutaraldehyde exposure. Fixation of the transport pathway in an active form does not render the cells refractory to amiloride.

#### DISCUSSION

The mechanisms by which cell volume changes activate transport pathways are not well understood. We do not know how the cell senses its volume, or how the message is conveyed to the membrane that a given transporter should begin to function in response to a volume change. It has been proposed (2) that swelling or shrinkage of cells might cause changes in cytoplasmic pH that could trigger proton-alkali metal counterflow. Cytoskeletal components have been implicated as possible mediators of volume-activated transport (6). There is evidence (5, 18) that in dog red cells volume-responsive Na fluxes are in some way dependent on a functioning glycolytic pathway. The present studies may provide a methodological approach to some of these questions. For example, we found (15) that movements of Na in response to shrinkage of dog red cells were influenced by the predominant anion present. The apparent Cl requirement raised the question whether Cl is necessary for the activation (by cell shrinkage) of the Na transporter, or whether Cl is necessary for the transport of Na by the mechanism after it has been activated. The same could be asked of the amiloride effect: does amiloride interfere with the function of the activated Na transporter or with the triggering of the mechanism by cell volume perturbation?

Because the Na transporter becomes fixed in the state it was in at the time of exposure to glutaraldehyde, experiments are possible that allow some clarification of the influences on Na flux of cell volume, anion environment, and specific inhibitors. The transporter assumes the active state when the cells are shrunken and Cl is present. If glutaraldehyde is applied to the cells under these conditions, the transporter remains active despite subsequent alterations in cell volume or changes in anion composition of the medium (Figs. 4-6 and 8; Table I). The transporter is in the inactive state when the cells are swollen or when Cl is

replaced by SCN<sup>1</sup> (Figs. 3 and 7; Table I). Exposure to glutaraldehyde under these circumstances causes permanent inactivation of the system, despite subsequent cell shrinkage or replacement of SCN by Cl (Fig. 3, Table II). Amiloride has no effect on the conformation (active or inactive) of the transporter; this drug appears to exert its inhibitory effect at the level of the translocation step (Table III).

A weakness of the data presented here is that the specific reactions of glutaraldehyde with membrane components are not clear-cut. Although cross-linking of proteins and peptides occurs, in part by reactions involving the ε-amino groups of lysine residues (20), other reactions with proteins and lipids are likely to take place that are less well characterized (7).

The type of approach described here with glutaraldehyde might be extended to other, more specific cross-linking agents, other volume-responsive transport pathways, and other membrane functions that are susceptible to influence by chemical or physical stimuli. Ultrastructural and macromolecular correlations might also be possible using covalent cross-linking agents under conditions that result in the preservation of specific membrane physiological properties.

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<sup>1</sup> Although SCN was used as a Cl replacement in the experiments shown here, previous work has demonstrated that nitrate is also an effective alternative anion. The inhibitory effect of nitrate substitution is not as great as that of SCN (15).

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