The Effects of Transport Inhibitors on Sodium Outflux and Influx in Red Blood Cells: Evidence for Exchange Diffusion

MICHAEL J. DUNN

From the Division of Medicine, Walter Reed Army Institute of Research, Washington, D. C. 20012 and the University of Vermont College of Medicine, Nephrology Unit, Burlington, Vermont 05401

ABSTRACT Active sodium transport (outflux or efflux) in red blood cells generally has been measured by assessing the amount of outflux inhibited by digitalis glycosides (outflux-fraction I). The presence of a ouabain-uninhibited sodium outflux (outflux-fraction II) attributable either to a second active transport mechanism or to exchange diffusion has been the subject of recent investigations. In the present study a variety of transport inhibitors, including ouabain, ethacrynic acid, furosemide, oligomycin, and amiloride, were studied for their effects on these components of sodium transport in RBC.

In the presence of ouabain both ethacrynic acid and furosemide exerted similar effects on sodium outflux, inhibiting approximately 0.5 mmoles/L of cells per hr. This component of sodium outflux has been called outflux-fraction II. Ethacrynic acid showed no inhibitory potency when ouabain and furosemide were present, thereby suggesting that the same outflux component (fraction II) was affected by ethacrynic acid and by furosemide. In addition, furosemide reduced sodium influx to the same extent that it reduced sodium outflux. Outflux-fraction II, as defined by furosemide, did not contribute a net sodium outflux. These results of sodium outflux and influx experiments confirm the existence of a transport pathway which does not contribute to net flux and which fits the definition of exchange diffusion.

The inhibitory effect of furosemide on outflux-fraction II remained despite the use of a sulfhydryl protective reagent, whereas the effect of ethacrynic acid was obliterated. No combination of inhibitors was found which affected the residual or uninhibited sodium outflux (0.4–0.5 mmoles/liter of cells per hr). Oligomycin possessed an inhibitory potency less than that of ouabain, and it exerted no effect on sodium outflux if it was superimposed upon ouabain inhibition. Amiloride proved to be a very weak inhibitor of sodium outflux in human erythrocytes.

INTRODUCTION

It has been customary to define and to investigate the components of the sodium transport system in erythrocytes through the use of transport inhibitors such as digitalis glycosides. Until recently the digitalis glycosides were thought to inhibit all active sodium transport in erythrocytes, and the uninhibited sodium outflux, observed during tracer studies, was attributed to exchange diffusion. Exchange diffusion is a carrier-mediated transport process, definable through the use of isotopic tracers, which involves the bidirectional exchange of a solute across a membrane for another solute of the same or similar species, without the occurrence of any net transport in either direction (1, 2). In 1966 Hoffman and Kregenow (3, 4) introduced the concept of an ouabain-insensitive pump for sodium in the erythrocyte which was inhibited by ethacrynic acid. Accordingly they defined "pump I" as that component of active sodium outflux from RBC which was inhibited by ouabain, and "pump II" as that additional component of sodium outflux which was inhibited by the addition of ethacrynic acid to maximally inhibitable concentrations of ouabain. Pump II also differed from pump I in other ways: it required extracellular sodium but not extracellular potassium, and it did not appear to utilize

This work was presented, in part, to the American Federation of Clinical Research on 7 December 1968 and on 3 May 1969, and the abstracts appeared in *Clin. Res.* **16**: 565, 1968 and **17**: 428, 1969.

Dr. Dunn's present address is the University of Vermont College of Medicine, Nephrology Unit, Burlington, Vt.

Dr. Dunn is a Scholar in Academic Medicine at the John and Mary R. Markle Foundation.

Received for publication 20 January 1970 and in revised form 22 April 1970.

ATP since pump II persisted for hours after conventional energy stores had been depleted (3, 4). Furthermore Smith, Czerwinski, and Welt have demonstrated that the function of pump II can be dissociated from membrane adenosine triphosphatase (ATPase) and presumably from ATP (5). The evidence is sketchy as to whether pump II actually contributes a net sodium outflux. Since this outflux mechanism depends upon the presence of extracellular sodium, it could be exchange diffusion. Recent studies by Lubowitz and Whittam (6) support this idea that pump II is, in fact, sodium exchange diusion and, therefore, does not contribute to net flux.

The purposes of the present study were to evaluate sodium outflux in RBC through the use of a variety of transport inhibitors and thereby to approach the problem of whether net transport or exchange diffusion is the mechanism of pump II. Because the terminology "pump II" implies an active, uphill, net transport process, which is as yet unproved, this paper will utilize the following terminology: ouabain-inhibited sodium outflux = outflux-fraction I; ouabain-uninhibited ethacrynic acid-inhibited sodium outflux = outflux-fraction II; the remaining, uninhibited sodium outflux = residual outflux. The results suggest that outflux-fraction II does not contribute to net sodium movement and may represent a form of sodium exchange diffusion.

METHODS

Sodium outflux and influx. The sodium outflux (efflux) method has been described previously (7) and was essentially the method of Sachs and Welt (8). Basically, this technique measured the rate of appearance of sodium 22 into the extracellular medium from isotopically labeled fresh, human erythrocytes obtained in heparin immediately before the study. The sodium influx technique has also been recently outlined (7, 9). This measurement quantitated the amount of sodium 22 which appeared in erythrocytes from an isotopically labeled extracellular medium; all influx measurements were corrected for sodium 22 calculated to have been simultaneously lost due to sodium outflux. The sodium influx experiments were conducted over a 7 hr period concomitant with the net outflux studies, rather than the customary 90 min influx period. Sodium outflux was not simultaneously determined during the 7-hr influx and net transport studies. Hence, the correction of the influx curves utilized the outflux rate constants for ouabain plus furosemide, which had been determined previously as shown in Fig. 2. The validity of using flux rate constants from different experiments depends upon the very small variation of outflux data, e.g. outflux rate constants for fraction I and fraction II (furosemide), respectively, are 0.303 ±0.013 and 0.048 ±0.004 (mean ±SEM). In addition the reliability of the bidirectional isotopic flux data is supported by the net flux data in Table I, which shows no net sodium flux attributable to fraction II. This would be expected if the isotopic data showing equal bidirectional fluxes inhibited by furosemide were accurate. Finally, if the corrections applied to the influx equations were inaccurate, the influx plot would not be linear. The rate of appearance (influx) of sodium 22 was shown to be linear over the 7 hr period (see Fig. 6). All flux solutions were of the following composition: 130 mM NaCl; 5.0 mM KCl; glycyl glycine-$MgCO_3$ buffer pH 7.4 at 37°C, 27 mM and 4.4 mM, respectively; 10 mM glucose; 1.2 mM phosphate (as Na_2HPO_4-NaH_2PO_4, pH 7.4) and 0.1 g/100 ml of albumin.

TABLE I

*Comparison of Increases in Intracellular Sodium in Erythrocytes Exposed to Ouabain and to Ouabain Plus Furosemide**

	Ouabain			Ouabain and furosemide		
Exp.	3–5 hr	5–7 hr	3–7 hr total	3–5 hr	5–7 hr	3–7 hr total
62	3.0‡	2.4	5.4	2.8	3.1	5.9
64	1.5	2.0	3.5	2.0	1.6	3.6
71	2.1	1.8	3.9	2.6	0.8	3.4
71	1.9	1.7	3.6	1.6	1.9	3.5
72	2.2	1.5	3.7	1.7	2.8	4.5
72	2.5	1.2	3.7	2.5	1.4	3.9
Mean	2.2	1.8	4.0	2.2§	1.9§	4.1§
±SEM	0.2	0.2	0.3	0.2	0.4	0.4

* The ionic composition of the incubation solutions was identical with that of the flux solutions described under methods.
‡ Net change of Na_e in mmoles/liter cells.
§ No significant difference from ouabain-group (paired *t* test).

Transport inhibitors. Ouabain was used in a 1×10^{-4} M concentration, and ethacrynic acid [2-3 dichloro-4-(2-methylene butyryl)phenoxyacetic acid],[1] furosemide [4 chloro-*N*(furylmethyl)-5-sulfamyl-anthranilic acid] [2] and amiloride [n-amidino 3,5 diamino-6-chloropyrazine carboxamide] [1] were added, as the dry chemical, to a final concentration of 1×10^{-3} M in all studies except the sulfhydryl reagent experiments in which the final molarity was unchanged but ethacrynic acid was solubilized in 0.5 ml ethyl alcohol and furosemide was suspended in 1.0 ml of 0.1 *N* NaOH. Whenever ethyl alcohol was used as a solvent, an equivalent amount of alcohol was added to all flux solutions in the experiments. Oligomycin [3] was dissolved in ethyl alcohol, before the addition and was used in 10 μg/ml concentrations. This preparation of oligomycin is approximately 15% oligomycin A and 85% oligomycin B, and therefore 10 μg/ml is approximately 2.5×10^{-7} M.

Erythrocyte sodium. The concentrations of sodium in the RBC (Na_c) were determined on $MgCl_2$-washed cells with the values expressed as mmoles of cation per liter of cells. For those experiments in which Na_c was increased, fresh cells were incubated at 37°C for 12–24 hr in the following zero potassium-solution: 142 mM NaCl, glycyl glycine-$MgCO_3$ buffer 54 mM and 9 mM, respectively, 1.2 mM phosphate 24 mM glucose, 3 mM adenine, 10 mM inosine, 0.1 g/100 ml of albumin, 200,000 U/100 ml of penicillin, and 0.2 g/100 ml of streptomycin. These solutions were replaced after 6–8 hr of incubation to maintain a low extracellular potassium and normal RBC ATP stores.

Sulfhydryl reagent studies. Clelands reagent (dithiothreitol)[4] was used because of its superior properties as a

[1] Merck, Sharp & Dohme, West Point, Pa.
[2] Hoechst Pharmaceuticals Co., Kansas City, Mo.
[3] Sigma Chemical Co., St. Louis, Mo.
[4] Calbiochem, Los Angeles, Calif.

sulfhydryl (SH) protective reagent (10). This reagent was prepared freshly for each experiment to prevent deterioration of stored solutions. Clelands reagent was always used in 2×10^{-3} concentrations. The erythrocytes were incubated for 10–15 min in the flux solutions containing the SH protective reagent before the ouabain, ethacynic acid, or furosemide were added.

RESULTS

Ouabain, ethacrynic acid, and furosemide. Fig. 1 shows the in vitro inhibitory effects of ouabain, and ouabain plus ethacrynic acid on sodium outflux (efflux) in the erythrocytes from 16 normal human subjects. Total sodium outflux in the absence of inhibitors was 3.48 ±0.16 (mean ±SEM) mmoles/L of cells per hr. Ouabain (1×10^{-4} M) inhibited 2.52 ±0.11 mmoles or 72% of the total outflux; this is depicted as outflux-fraction I. The addition of ethacrynic acid (1×10^{-3} M) to ouabain produced a further decrement of 0.50 ±0.04 mmoles; this is depicted as outflux-fraction II. A residual or uninhibited fraction of sodium outflux of 0.46 ±0.03 mmoles persisted despite the combined use of ouabain and ethacrynic acid.

Fig. 2 depicts the data from six paired experiments which evaluated the inhibitory actions of furosemide in the human RBC. The third column of Fig. 2 shows that furosemide (1×10^{-3} M) produced a decrement of 0.38 ±0.04 mmoles of sodium outflux when it was added to ouabain inhibition. This value of 0.38 mmoles, which is plotted as outflux-fraction II, compared closely with the value of 0.40 ±0.05 mmoles for the ethacrynic acid-inhibited outflux-fraction II, obtained in these paired studies. The similarities between the inhibitory actions of ethacrynic acid and furosemide, when either drug was superimposed upon ouabain inhibition, did not carry over to the effects of either drug when used alone. Ethacrynic acid inhibited 2.55 ±0.15 mmoles of sodium outflux in 10 experiments when it was used in the

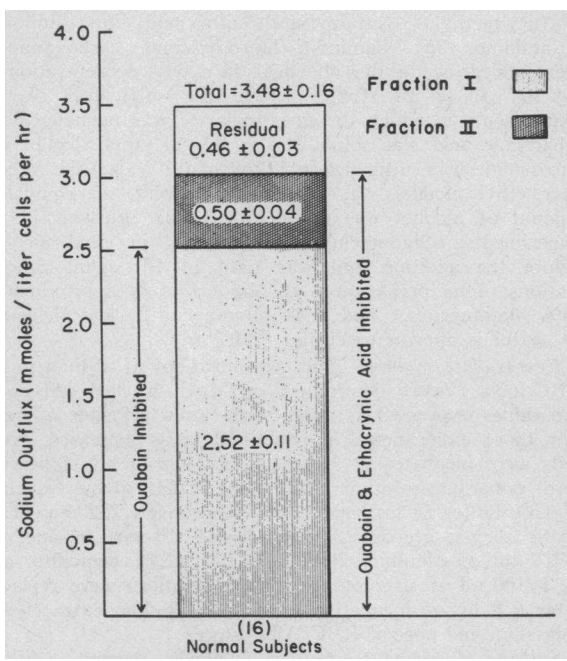

FIGURE 1 The addition of ethacrynic acid (1×10^{-3} M) to ouabain (1×10^{-4} M) further reduced sodium outflux by 0.50 ±0.04 mmoles. This component has been called outflux-fraction II in order to distinguish it from the conventional, ouabain-inhibited component, referred to as fraction I. The mean Na_e in these 16 studies was 8.42 ±0.38 mM.

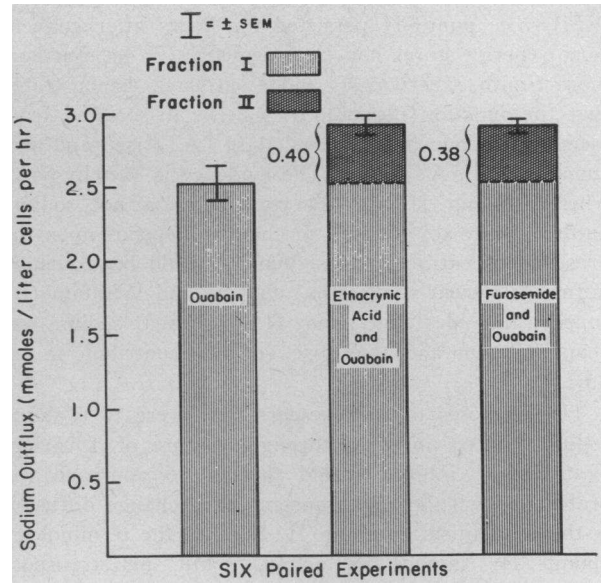

FIGURE 2 These data from six paired experiments show that outflux-fraction II can be operationally defined through the use of furosemide, 0.38 ±0.04, as well as ethacrynic acid, 0.40 ±0.05, when either agent is superimposed upon ouabain.

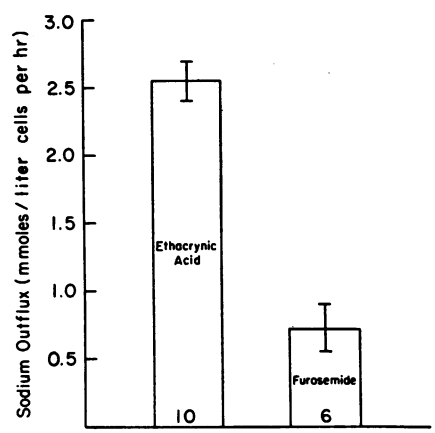

FIGURE 3 Whereas ethacrynic acid, used alone, inhibited a large fraction of outflux, 2.55 ±0.15 mmoles, furosemide alone only inhibited 0.72 ±0.18 mmoles.

absence of other transport inhibitors (Fig. 3). However, furosemide inhibited only 0.72 ±0.18 mmoles of outflux when it was studied in six sets of RBC in the absence of ouabain (Fig. 3). From these experiments it appeared that furosemide may have inhibited an amount of sodium outflux roughly equivalent to outflux-fraction II, whereas ethacrynic acid obviously affected a considerable portion of outflux-fraction I. The difference between the inhibitory effects of furosemide alone (0.72 ±0.18) and furosemide superimposed upon ouabain (0.38 ±0.04) are not statistically different because of the large SEM for the "furosemide alone" data.

If sodium outflux-fraction II as operationally defined by ethacrynic acid was the same transport mechanism defined by furosemide then it would be expected that ethacrynic acid would not exert any effect when studied with ouabain and furosemide. Fig. 4 shows the data from six studies which examined this hypothesis. The data in the fourth column of Fig. 4 will be discussed later (see below). The addition of ethacrynic acid to ouabain and furosemide did not inhibit any more sodium outflux (2.93 ±0.24 mmoles/liter of cells per hr) than did ouabain and furosemide (2.91 ±0.18) or ouabain and ethacrynic acid (3.06 ±0.17). There are no statistically significant differences among these three groups. Hence, these data supported the conclusion that both of these diuretics interfered with the same transport component, outflux-fraction II, since no additional inhibition was observed when ethacrynic acid was added to ouabain plus furosemide.

In order to examine further the specificity of furosemide inhibition on outflux-fraction II four experiments were conducted in which intracellular sodium (Na_c) was increased from a mean of 8.4 mM to 17.1 mM, and the response of outflux-fraction II and of furosemide inhibition without ouabain was measured. Fig. 5 shows that whether outflux-fraction II was defined with ethacrynic acid plus ouabain (0.64 ±0.06) or with furosemide plus ouabain (0.68 ±0.19), there was only a slight increase of this portion of sodium outflux concomitant with the doubling of Na_c. But, when furosemide's effect was studied in the high-sodium cells, without ouabain present, the amount of outflux which was inhibited increased from 0.68 ±0.19 mmoles in the controls to 1.86 ±0.44

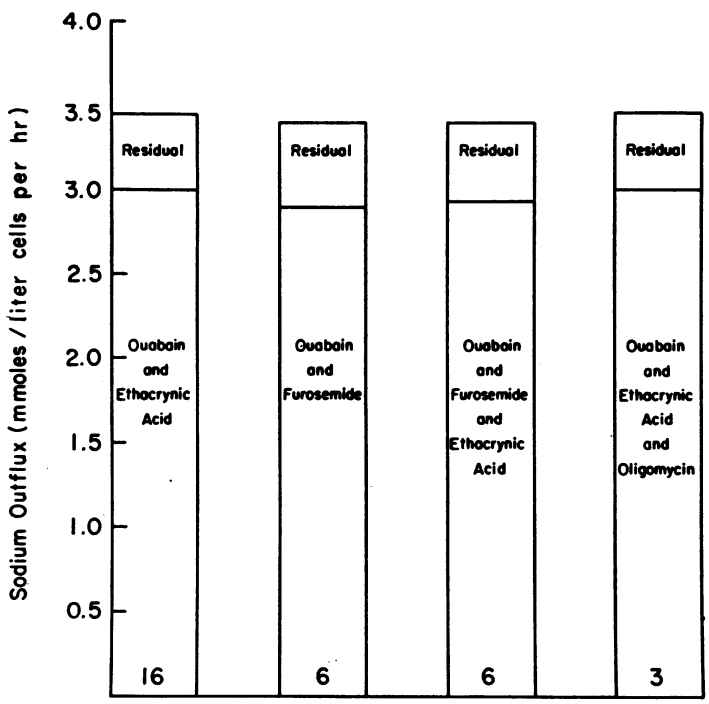

FIGURE 4 The data in columns one and two have been previously presented in Figs. 1 and 2, respectively, and are shown here for comparison with columns three and four. The number of experiments is listed at the base of each column. The third column shows that ethacrynic acid had no inhibitory potency when ouabain and furosemide were present, thereby confirming a similar locus of action for these diuretics. As depicted in the fourth column, oligomycin was also without effect when added to ouabain and ethacrynic acid. There were no statistically significant differences among any of these four combinations of inhibitors. See text for detailed data.

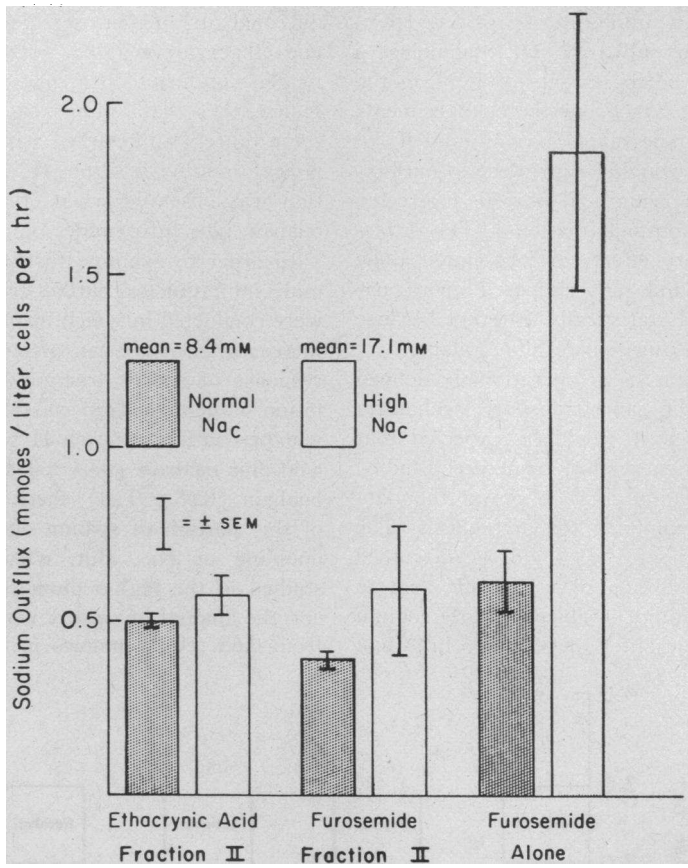

FIGURE 5 These results are the mean data from four studies. Na_e was increased through overnight incubation of cells in a high-sodium, no-potassium media. The results for the first two sets of columns depicting fraction II were obtained in the presence of ouabain. It is apparent that outflux fraction II increases slightly when Na_e is doubled in this range and that both ethacrynic acid and furosemide exert similar quantitative effects when used with ouabain. Furosemide, studied alone, appears to affect a considerable portion of outflux fraction I under circumstances of high cell sodium. See text for detailed data.

mmoles in the high-sodium group. The conventional, ouabain-inhibited pump (outflux-fraction I), increased almost 50% from 2.52 to 3.85 mmoles (not shown in Fig. 5), as would be predicted from many previous studies. The striking increase of the inhibitory potency of furosemide used alone, when Na_e is elevated, indicated that this agent affected more than outflux-fraction II. Clearly, from the data shown in Figs. 3 and 5, it can be concluded that the inhibitory actions of both ethacrynic acid and furosemide in the human erythrocyte are not specific for, or discretely restricted to, the outflux-fraction II.

Outflux-fraction II and net flux. Hoffman and Kregenow (3) have concluded that pump II does contribute to net flux and therefore is not exchange diffusion. Since ethacrynic acid is toxic to intact erythrocytes, causes hemolysis,[5] and increases sodium influx (3), it is very difficult to assess net sodium gain in erythrocytes exposed to ouabain and ethacrynic acid since a net sodium accumulation under these circumstances may reflect increased sodium influx rather than decreased sodium outflux. Furosemide offers advantages for such a study since it is not as hemolytic[5] and does not increase sodium influx (see below, tracer influx decreases). Accordingly, six experiments were conducted (concomitant with the influx studies described below) to evaluate net sodium flux in RBC incubated with ouabain alone and with ouabain and furosemide.

[5] Personal observations.

If outflux-fraction II contributes a net sodium outflux, rather than an exchange diffusion, Na_c should increase more rapidly in the cells incubated with ouabain and furosemide than in the group with ouabain alone. Table I shows the results of studies in which net accumulation of erythrocytic sodium was measured after 3, 5, and 7 hr of incubation. The increase in Na_c was not greater, during a 7 hr incubation, in RBC exposed to ouabain plus furosemide than in those cells exposed only to ouabain. Both groups of cells gained approximately 4 mmoles of sodium/liter of cells over the last 4 hr of this 7 hr incubation. Since this method of determination of Na_c has 3–5% reproducibility, and since inhibition of fraction II would raise Na_c 3.5 mM over 7 hr if this were a net transport step, these experiments would be expected to detect an accelerated accumulation of intracellular sodium due to the use of furosemide. Cell water content and mean cellular hemoglobin concentration do not change during such an incubation with ouabain or ouabain plus furosemide; hence, sodium content could be expressed per liter of intracellular water without affecting the conclusions. Therefore, it appeared from these studies that the furosemide-inhibited outflux-fraction II did not contribute to net flux.

Furosemide and sodium influx. The question of net flux versus exchange diffusion was also investigated by looking for a furosemide-inhibited fraction of sodium influx. If outflux-fraction II is a form of soduim exchange diffusion, then furosemide should inhibit an equal amount of sodium influx. The following studies

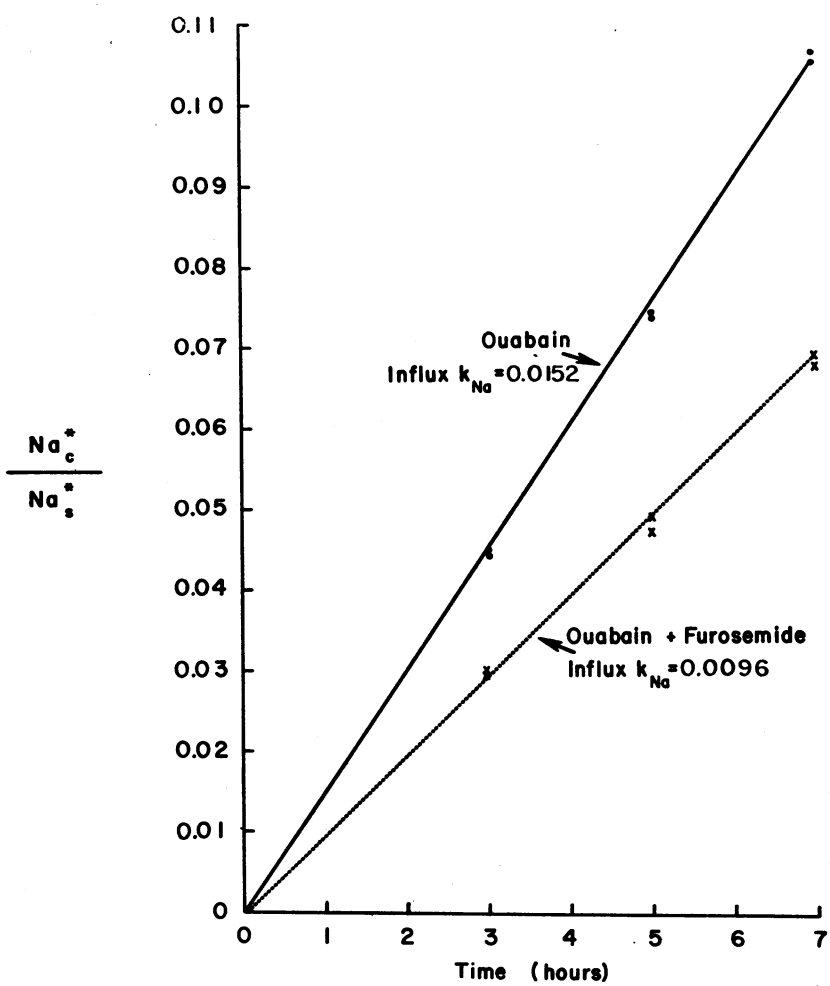

FIGURE 6 The results of one of six experiments are plotted to demonstrate the inhibitory action of furosemide on sodium influx. Na_c^*/Na_s^* represents the concentration of sodium-22 in cpm/ml of cells factored by the cpm/ml of supernatant (media) solution. The slope of the lines was calculated through the sum of least squares method. Extracellular sodium was 130 mM in all studies. See text for data from all six studies.

TABLE II

Effects of Oligomycin on Sodium Outflux*
(Mean Na_e = 8.4 mM)

Exp. No.	Sodium outflux: Ouabain sensitive	Oligomycin sensitive	Ouabain and oligomycin sensitive	Total
22	2.37	2.10	2.41	3.16
39	2.20	1.98	2.33	3.10
48	1.68	1.20	1.66	2.42
49	3.00	2.69	3.00	4.08
Mean	2.31‡	1.99‡	2.35	3.19
SEM	0.27	0.31	0.27	0.34

* 10 μg/ml.
‡ Paired *t* test, $P < 0.02$.

were conducted upon the erythrocytes during the net outflux experiments described in Table I. Fig. 6 shows, in a representative experiment, the inhibitory effect of furosemide upon the sodium influx rate constant in the presence of ouabain. In six experiments the addition of furosemide to ouabain lowered sodium influx rate constants to 0.011 ±0.0006 (mean ±SEM) from a value of 0.016 ±0.001 in the presence of ouabain alone, $P <$ 0.001. Since extracellular sodium was 130 mM it can be calculated that furosemide reduced sodium influx by 0.73 ±0.04 mmoles/L of cells per hr. The sodium influx in the absence of ouabain was not measured but other authors (3, 6, 11) have been unable to demonstrate an effect of ouabain on sodium influx under these circumstances, i.e. in the presence of extracellular potassium. The sodium outflux was not directly measured in these studies, but it was calculated using the measured Na_e and the rate constant for furosemide-sensitive fraction II from previous experiments (see methods section for justification). The calculated mean sodium outflux sensitive to furosemide, at an Na_e of 13 mM (measured 5 hr value) would have been 0.62 mmoles, which compared closely with the influx value of 0.73 mmoles.

Residual outflux, oligomycin, and amiloride. The residual sodium outflux is that sodium movement out of RBC which persists in the presence of ouabain and ethacrynic acid or ouabain and furosemide. It is unknown whether this residual flux represents an active or a passive process. It seemed important to ascertain whether any combination of inhibitors would decrease this residual or uninhibited outflux. Fig. 4 depicts the results of these experiments. The first three columns show previously discussed results with ouabain plus ethacrynic acid, ouabain plus furosemide, and ouabain plus furosemide and ethacrynic acid. These combinations of transport inhibitors did not reduce the residual outflux below 0.5 mmoles/liter of cells per hr. The fourth column of Fig. 4 shows that oligomycin, 10 μg/ml, in the presence of ouabain and ethacrynic acid, had no inhibitory effect and therefore did not reduce the residual outflux of sodium. When oligomycin was studied in four experiments, without other transport inhibitors, it inhibited less sodium outflux than did ouabain (Table II). In cells with a normal Na_e (8.4 mM) oligomycin inhibited 63% of sodium outflux (1.99 mmoles), whereas ouabain inhibited 72% of outflux (2.31 mmoles). Table II also shows that ouabain and oligomycin inhibited no more outflux than did ouabain alone. Amiloride, 1×10^{-3} M, exerted no effect on sodium outflux when it was used in the presence of ouabain, although it reduced sodium outflux by 0.5 to 0.6 mmoles in three studies when it was used in the absence of ouabain.

Effects of a sulfhydryl-protective reagent on the actions of transport inhibitors. Since ethacrynic acid is a potent inhibitor of SH groups (12), the possible protective effect of a strong SH reagent was investigated. Clelands reagent (dithiothreitol) was used in a concentration of 2×10^{-3} M, which was equivalent to twice the molarity of the ethacrynic acid and the furosemide and twenty times the molarity of the ouabain. Table III presents the results of these experiments. Whereas the effect of ethacrynic acid on outflux-fraction II was obliterated, the SH protective reagent interfered minimally with the furosemide action on outflux-fraction II, and the action of ouabain on outflux-fraction I was enhanced slightly. These results suggested that ethacrynic acid affected the sodium outflux mechanism in a fashion different from the action of furosemide and that ethacrynic acid's effect depended upon intact SH-reactive moieties in the drug.

Species specificity. The effects of transport inhibitors on simian erythrocytes were examined in the course of previously reported work on the impact of malaria on

TABLE III

Effects of a Sulfhydryl-Protective Reagent on the Action of Transport Inhibitors on Sodium Outflux*

	Sodium outflux: Without SH‡ reagent	With SH reagent
	mmoles/liter of cells per hr	
Outflux-fraction I (Ouabain)	2.52 ±0.11	2.99 ±0.43 (6 exps.)§
Outflux-fraction II (Ethacrynic acid)	0.50 ±0.04	0.03 ±0.02 (4 exps.)‖
Outflux-fraction II (Furosemide)	0.38 ±0.04	0.30 ±0.08 (4 exps.)§

* Clelands reagent (dithiothreitol).
‡ Data from Figs. 1 and 2.
§ No significant difference, nonpaired *t* test.
‖ Nonpaired *t* test, $P < 0.001$.

RBC cation transport in the Rhesus monkey (7). The results of five studies showed that ouabain alone reduced sodium outflux to a baseline level which could not be lowered further through the use of ethacrynic acid or furosemide. The ouabain-inhibited fraction of outflux was 3.30 ±0.22 mmoles/liter cell per hr whereas outflux fraction II, whether studied with ethacrynic acid or with furosemide was not measurable (0.09 ±0.04). The residual outflux of 0.54 ±0.08 closely resembled the values obtained with human cells. These results were not ascribable to a complete lack of inhibitory potency of ethacrynic acid or furosemide in simian RBC since these compounds were active when used in the absence of ouabain.

DISCUSSION

Ethacrynic acid, a potent sulfhydryl inhibitor and diuretic agent in humans (13), has been shown to interfere with active cation transport in renal tubules (14), kidney slices (15, 16), toad bladder (17), smooth muscle (18), striated muscle (19), toad oocytes (20), and human erythrocytes (3, 4, 20–23). As such it appears to have widespread inhibitory potency for active cation transport. Furosemide has received less attention than ethacrynic acid as an in vitro transport inhibitor (24). The inhibitory effects of furosemide upon sodium transport in erythrocytes have not been reported previously although Welt (22) has recently referred to unpublished data showing an effect of furosemide on outflux fraction II, and Wessels (23) alludes to work, in press, which demonstrates furosemide-inhibition of active and passive sodium transport in erythrocytes. Herms and Jaedicke (25) have recently reported that furosemide had no effect upon the intracellular Na : K ratio in erythrocytes; their study did not measure sodium flux and therefore would not have been expected to detect the furosemide effect.

Before discussing the evidence that outflux-fraction II is exchange diffusion, it is necessary to establish that furosemide inhibits the same component of outflux which is affected by ethacrynic acid (in the presence of ouabain). This conclusion is suggested but not proved by the quantitative similarities between outflux-fraction II defined by ethacrynic acid (0.50 mmoles in 16 studies, Fig. 1; or 0.40 mmoles in six paired studies, Fig. 2) and by furosemide (0.38 mmoles, Fig. 2). It is more important to note that ethacrynic acid has no inhibitory effect on sodium outflux when ouabain and furosemide have already been added to the media (cf. Fig. 4). Therefore, it seems likely that the addition of either agent to ouabain inhibits the same transport mechanism. The data concerning ethacrynic acid and sodium outflux in the present paper are quantitatively similar to Hoffman and Kregenow's (3, 4), although the conclusions drawn are different. The value of 0.50 mmoles/liter cells per hr for outflux-fraction II (i.e., the inhibitory effect of ethacrynic acid when it is superimposed upon ouabain inhibition) is identical with the value reported by Hoffman and Kregenow (3) and is similar to the decrements reported by Balfe, Cole, and Welt (26) and Lubowitz and Whittam (6).

The sine qua non of an active transport process is the ability to accomplish net transport of the solute against its electrochemical gradient. Therefore it is critical in any examination of outflux-fraction II to ascertain whether it contributes any net outflux of sodium. Hoffman and Kregenow's conclusions (3), positing net transport due to pump II, were based upon studies which showed that 15-hr depleted cells (presumably reducing pump II) gained more intracellular sodium than did fresh cells during a 12 hr incubation when extracellular sodium was high (136 mM) but not when extra cellular sodium was low (30 mM). Net flux studies would be easier to interpret if an inhibitor of outflux-fraction II were used with fresh cells and a normal extracellular sodium. Since ethacrynic acid disrupts membrane integrity and causes hemolysis, it increases sodium influx (3) and thereby obscures the interpretation of any studies which compare the net sodium gain of erythrocytes exposed to ouabain vs. ouabain and ethacrynic acid. Wessels has recently published data suggestive of a small inhibitory effect of low concentrations of ethacrynic acid (0.3×10^{-5}–0.3×10^{-4} M) on sodium influx in human erythrocytes (23). Leblanc and Erlij (19), using frog sartorius muscle, have shown an inhibitory action of ethacrynic acid on sodium influx in strophanthidin-treated muscles; this component of sodium influx was considered to be the sodium exchange diffusion described by Keynes and Steinhardt (27). Since furosemide does not seem to have these disadvantages of increased membrane permeability, net flux studies, as shown in Table I, can be done. The results indicate that the furosemide-inhibited outflux-fraction II does not contribute a net outflux of sodium. Lubowitz and Whittam (6) also could not demonstrate net sodium outflux due to a ouabain-insensitive mechanism. These authors measured the ability of high-sodium cells to reduce their sodium content, in the presence and absence of ouabain and in media with varying extracellular sodium concentrations (30–150 mM). They could not document any net sodium outflux which was stimulated by extracellular sodium in the presence of ouabain. Their net outflux results address the question more indirectly than the data in Table I since they noted that sodium influx undoubtedly increased as extracellular sodium was increased, and any outflux mechanism stimulated by extracellular sodium could be obscured by this concomitant increase of influx, when the measured parameter is the intracellular sodium.

The sodium influx experiments which demonstrated a component of influx (0.73 mmoles/liter of cells per hr) inhibited by furosemide in the presence of ouabain extend and support the conclusions drawn from the net transport experiments. It would be predicted that this effect on influx would be seen if outflux-fraction II were exchange diffusion, since by definition exchange diffusion involves the bidirectional movement of a cation across a membrane without net transport. Hoffman and Kregenow (3) were unable to document any inhibitory effect of ethacrynic acid upon sodium influx, but it is entirely possible that such an inhibitory effect was present and was obscured by the enhanced permeability of the cellular membrane with the attendant increase of influx. Lubowitz and Whittam (6) demonstrated a mean increase of sodium influx of 0.49 ±0.13 mmoles/liter of cells per hr after increases of intracellular sodium of 29 mM. This sodium influx was not inhibited by ouabain. Since raising intracellular sodium diminishes the chemical gradient for "passive" sodium influx, these results were interpreted as proof of exchange diffusion. It seems quite likely that the furosemide-inhibited sodium influx described in the present study is the same component of influx which Lubowitz and Whittam found stimulated

TABLE IV

*Sodium Exchange Diffusion in the Erythrocytes of Various Species**

Species	Rate of exchange	Ratio of exchange diffusion to total Na outflux	Method of demonstration	Reference
	mmoles/liter cells per hr			
Man	a) 0.50	0.14	Furosemide-inhibited bidirectional Na flux without net transport.	Present study
	b) 0.50–0.75	0.18	Na-stimulated bidirectional Na flux without net transport; ethacrynic acid inhibition of Na outflux dependent on extracellular Na (Na_0).	Lubowitz and Whittam (6)
	c) 0.70‡	0.39	Ouabain-inhibited bidirectional Na flux in absence of extracellular K (K_0) and dependent on Na_0.	Garrahan and Glynn (29)
Sheep	3.3–4.3§	0.94–0.88	Na outflux, in absence of K_0, dependent on Na_0.	Tosteson and Hoffman (30)
Turtle	0.31–0.60‖,¶	0.15–0.22	Na outflux, uninhibited by ouabain, dependent on Na_0 and inhibited by ethacrynic acid.	Klahr et al. (31)
Rabbit	4.6	0.50	Na stimulated bidirectional Na flux, uninhibited by ouabain; also a ouabain-inhibited bidirectional flux in absence of K_0.	Rettori et al. (32); Villamil and Kleeman (33)
Swine	0.5–1.0	0.20	Na-stimulated bidirectional Na flux uninhibited by strophanthidin.	Sorenson et al. (34)
Ox (Beef)	10	0.85	Na outflux uninhibited by strophanthidin and dependent upon Na_0.	Sorenson et al. (34)

* Data are valid at normal intracellular and extracellular sodium for each species.
‡ Differs from a and b since this exchange is inhibitied by ouabain and extracellular potassium.
§ High potassium and low potassium sheep cells, respectively.
‖ Expressed as mmoles/kg RBC per hr.
¶ Aerobic and anaerobic, respectively.

by intracellular sodium. Studies are in progress examining the effect of varied intra and extracellular sodium concentrations upon the furosemide-inhibited sodium outflux and influx. The inhibitory effects of ethacrynic acid and of furosemide upon outflux fraction II seem nonspecific and somewhat disimilar. This impression is derived from three observations. First, the large effect of ethacrynic acid when used alone demonstrates substantial inhibition of outflux fraction I. Second, the increased inhibitory action of furosemide in high-sodium cells also suggests overlap of the effects into fraction I. Third, the different responses of these inhibitors in the presence of a thiol reagent suggests but does not prove different modes of action. Whereas it is known that ethacrynic acid is a potent sulfhydryl inhibitor (12), furosemide is not thought to have a significant effect upon membrane sulfhydryl integrity (24). The data in Table III indicate that Clelands reagent obliterated the action of ethacrynic acid upon outflux-fraction II whereas furosemide's effect was barely reduced. Two alternative explanations are possible. The more likely explanation is that the thiol reagent saturated the SH reactive sites of the ethacrynic acid and that the SH reactive moiety of ethacrynic acid is necessary for the inhibitory effects of the drug on outflux fraction II. Alternatively, the Clelands reagent may complex or chelate the ethacrynic acid and thereby form a complex which does not have access to the necessary membrane sites. These data extend the results of Smith et al. (5, 28), who have shown that two analogues of ethacrynic acid, which have no sulfhydryl-inhibitory potency and which do not affect ATPase, will eliminate outflux-fraction II. Smith and his coworkers (5, 28) could not eliminate the effect of ethacrynic acid upon outflux-fraction II through the use of the thiol reagent cysteine. Their results are at variance with the data presented in Table III. These differences may be attributable to the weaker SH protective effect of cysteine (10).

Sodium exchange diffusion has been found in the RBC of many species. Table IV lists those species and summarizes the magnitude of the exchange which ranges from 15% of total efflux in man to 85–95% in cattle and sheep. The sodium exchange described by Garrahan and Glynn (29) differs mechanistically from the transport system described in the present paper as well as from those in other species. Their exchange diffusion mechanism (carrier?) appeared to be the active transport mechanism since exchange diffusion occurred only in the absence of external potassium and was inhibited by digitalis glycosides (29). A fraction of the exchange diffusion of sodium in rabbit erythrocytes resembles the exchange described by Garrahan and Glynn in human cells since it is inhibited by ouabain and is seen only in the absence of extracellular potassium (33). Rhesus monkey erythrocytes differ from human erythrocytes since an inhibitory effect of the diuretics beyond the ouabain effect could not be found nor could a ouabain-uninhibited sodium outflux dependent upon extracellular sodium be demonstrated (7). It is of importance to clarify the issue of exchange diffusion in human erythrocytes since studies of human disease have revealed conditions with high-sodium cells (22, 28, 35, 36) and with normal-sodium cells (26) in which both components of sodium outflux show diminished rate constants. In addition the ouabain-inhibited exchange diffusion mechanism described by Garrahan and Glynn (29) has recently been utilized as a "marker" for the sodium pump, and increased activity of this component of influx has been found in hereditary sperocytes (37) in which sodium transport is increased whereas diminished activity of this exchange diffusion has been described in erythrocytes with diminished active sodium transport due to uremia (22).

Oligomycin, an inhibitor of oxidative phosphorylation, has been shown to exert an inhibitory effect upon membrane ATPase and sodium transport in anaerobic cells, i.e. human erythrocytes (38, 39) and more recently in turtle erythrocytes (40). Our results showing less oligomycin than ouabain effect may be a dose-related observation since Whittam, Wheeler, and Blake (39) found ouabain and oligomycin to be equipotent with twofold greater oligomycin concentrations. These studies are in agreement, however, that oligomycin has no effect upon sodium outflux if maximally inhibitable concentrations of ouabain are used. The weak effect of amiloride on sodium outflux agrees with earlier data showing only minimal inhibition of potassium influx in human erythrocytes (21) and with more recent data showing a small effect on sodium outflux in the absence of ouabain (23).

ACKNOWLEDGMENTS

I am grateful to Robert Bevins for expert technical assistance. The ethacrynic acid and amiloride were kindly supplied by Merck, Sharp & Dohme. The furosemide was provided by Hoechst Pharmaceuticals.

REFERENCES

1. Ussing, H. H. 1949. Transport of ions across cellular membranes. *Physiol. Rev.* **29**: 127.
2. Ussing, H. H. 1960. The alkali metal ions in isolated systems and tissues. *Hand. Exper. Pharmacol.* **13**: 1.
3. Hoffman, J. F., and F. M. Kregenow. 1966. The characterization of new energy dependent cation transport processes in red blood cells. *Ann. N. Y. Acad. Sci.* **137** (2): 566.
4. Hoffman, J. F. 1966. The red cell membrane and the transport of sodium and potassium. *Amer. J. Med.* **41**: 666.
5. Smith, E. K. M., A. Czerwinski, and L. G. Welt. 1967. A dissociation between erythrocyte pump II and membrane ATPase. *Clin. Res.* **15**: 372.

6. Lubowitz, H., and R. Whittam. 1969. Ion movements in human red cells independent of the sodium pump. *J. Physiol. (London).* **202:** 111.
7. Dunn, M. J. 1969. Alterations of red blood cell sodium transport during malarial infection. *J. Clin. Invest.* **48:** 674.
8. Sachs, J. R., and L. G. Welt. 1967. The concentration dependence of active potassium transport in the human red blood cell. *J. Clin. Invest.* **46:** 65.
9. Sachs, J. R., and M. E. Conrad. 1968. Effect of tetraethylammonium on the active cation transport system of the red blood cell. *Amer. J. Physiol.* **215:** 795.
10. Cleland, W. W. 1964. Dithiothreitol, new protective reagent for SH groups. *Biochem. J.* **3:** 480.
11. Glynn, I. M. 1957. The action of cardiac glycosides on sodium and potassium movements in human red cells. *J. Physiol. (London).* **136:** 148.
12. Duggan, D. E., and R. M. Noll. 1965. Effects of ethacrynic acid and cardiac glycosides upon a membrane adenosinetriphosphatase of renal cortex. *Arch. Biochem. Biophys.* **109:** 388.
13. Peters, G., and F. Roch-Ramel. 1969. Ethacrynic acid and related drugs. *Hand. Exper. Pharmacol.* **24:** 406.
14. Bank, N. 1968. Physiological basis of diuretic action. *Ann. Rev. Med.* **19:** 103.
15. Whittembury, G. 1968. Sodium and water transport in kidney proximal tubular cells. *J. Gen. Physiol.* **51:** 303s.
16. Whittembury, G., and J. Fishman. 1969. Relation between cell Na extrusion and transtubular absorption in the perfused toad kidney: the effect of K, ouabain and ethacrynic acid. *Pfluegers Arch. Gesamte Physiol. Menschen Tiere.* **307:** 138.
17. Lipson, S., and R. M. Hays. 1966. The effect of ethacrynic acid and furosemide on sodium transport and ionic permeability in the toad bladder. *J. Clin. Invest.* **45:** 1042.
18. Daniel, E. E. 1967. The effects of new diuretics on net ion movements in sodium-rich smooth muscle. *Can. J. Physiol. Pharmacol.* **45:** 149.
19. Leblanc, G., and D. Erlij. 1969. Effects of ethacrynic acid on sodium fluxes in frog sartorius muscle. *Biochim. Biophys. Acta* **173:** 149.
20. Bittar, E. E., D. A. T. Dick, and D. J. Fry. 1968. The action of ethacrynic acid on sodium efflux from single toad oocytes. *J. Physiol. (London).* **196:** 693.
21. Sachs, J. R., and L. G. Welt. 1968. Concentration dependence of active potassium transport in the human red blood cell in the presence of inhibitors. *J. Clin. Invest.* **47:** 949.
22. Welt, L. G. 1969. A further evaluation of erythrocyte sodium transport in control subjects and patients with uremia. *Nephron.* **6:** 406.
23. Wessels, V. F. 1969. Der einfluss von etacrynsaeure und amilorid auf den natriumtransport der erythrocyten des menschen in vitro. *Arzneimittel-Forschung. (Drug Res.)* **19:** 1959.
24. Peters, G., and F. Roch-Ramel. 1969. Furosemide. *Hand. Exper. Pharmacol.* **24:** 386.
25. Herms, W., and W. Jaedicke. 1968. Der einfluss von diuretica auf electrolyt-und wassergehalt menschlicher erythrocyten. *Klin. Wochenschr.* **46:** 194.
26. Balfe, J. W., C. Cole, and L. G. Welt. 1968. Red cell transport defect in patients with cystic fibrosis and in their parents. *Science (Washington).* **162:** 689.
27. Keynes, R. D., and R. A. Steinhardt. 1968. The components of sodium efflux in frog muscle. *J. Physiol. (London).* **198:** 581.
28. Smith, E. K. M. 1968. Studies of the membrane adenosine triphosphatase of human red cells in health and disease. Doctorate Thesis. University of London.
29. Garrahan, P. J., and I. M. Glynn. 1967. The behavior of the sodium pump in red cells in the absence of external potassium. *J. Physiol. (London).* **192:** 159.
30. Tosteson, D. C., and J. F. Hoffman. 1960. Regulation of cell volume by active cation transport in high and low potassium sheep red cells. *J. Gen. Physiol.* **44:** 169.
31. Klahr, S., A. B. Shaw, K. H. Hwang, and C. L. Miller. 1969. Sodium transport in turtle erythrocytes. Apparent stimulation of exchange diffusion by anaerobiosis. *J. Gen. Physiol.* **54:** 479.
32. Rettori, O., V. Rettori, J. V. Maloney, and M. F. Villamil. 1969. Sodium efflux in rabbit erythrocytes. *Amer. J. Physiol.* **217:** 605.
33. Villamil, M. F., and C. R. Kleeman. 1969. The effect of ouabain and external potassium on the ion transport of rabbit red cells. *J. Gen. Physiol.* **54:** 576.
34. Sorenson, A. L., L. B. Kirschner, and J. Barker. 1962. Sodium fluxes in the erythrocytes of swine, ox and dog. *J. Gen. Physiol.* **45:** 1031.
35. Dunn, M. J., E. K. M. Smith, A. Czerwinski, H. J. Gitelman, and L. G. Welt. 1966. Cellular illness: a prelude to death? *J. Clin. Invest.* **45:** 1002.
36. Welt, L. G., E. K. Smith, M. J. Dunn, A. Czerwinski, H. Procter, C. Cole, J. W. Balfe, and H. J. Gitelman. 1967. Membrane transport defect: the sick cell. *Trans. Ass. Amer. Physicians Philadelphia.* **80:** 217.
37. Wiley, J. S. 1969. Inheritance of an increased sodium pump in human red cells. *Nature (London).* **221:** 1222.
38. Glynn, I. M. 1963. Transport adenosinetriphosphatase in electric organ. The relation between ion transport and oxidative phosphorylation. *J. Physiol. (London).* **169:** 452.
39. Whittam, R., K. P. Wheeler, and A. Blake. 1964. Oligomycin and active transport reactions in cell membranes. *Nature (London).* **203:** 720.
40. Guerra, L., S. Klahr, W. Beauman, C. Marchena, J. Bourgoignie, and N. S. Bricker. 1969. Effects of oligomycin on anaerobic sodium transport and metabolism in shark erythrocytes. *Amer. J. Physiol.* **217:** 1292.