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Thiol-Dependent Passive K : Cl Transport in Sheep Red Blood Cells: X. A Hydroxylamine-Oxidation Induced K : Cl Flux Blocked by Diethylpyrocarbonate

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Summary. Hydroxylamine, a potent oxidizing agent used to reverse carbethoxylation of histidine by diethylpyrocarbonate, activated Cl-dependent K flux (K : Cl cotransport) of low K sheep red blood cells almost sixfold. When K : Cl cotransport was already stimulated by N-ethylmaleimide, hydroxylamine caused an additional twofold activation suggesting modification of sites different from those thiol alkylated. This conclusion was supported by the finding that hydroxylamine additively augmented also the diamide-induced K : Cl flux (Lauf, P.K. 1988. *J. Membrane Biol.* **101**:179–188) with dithiothreitol fully reversing the diamide but not the hydroxylamine effect. Stimulation of K : Cl cotransport by hydroxylamine was completely inhibited by treatment with diethylpyrocarbonate also known to prevent K : Cl cotransport stimulation by N-ethylmaleimide, both effects being independent of the order of addition. Hence,although the effect of carbethoxy modification on K : Cl flux cannot be reversed by hydroxylamine and thus excludes histidine as the target for diethylpyrocarbonate, our finding reveals an important chemical determinant of K : Cl cotransport stimulation by both hydroxylamine oxidation and thiol group alkylation.

Key Words sulfhydryls · N-ethylmaleimide · oxidation · carbethoxylation · diethylpyrocarbonate · hydroxylamine · sheep red blood cells · K : Cl cotransport · ATP · GSH

Introduction

Activation in red blood cells of ouabain-resistant, Cl-dependent K transport (K : Cl cotransport) by cell swelling [5, 7, 9, 14, 15, 25, 30, 31] and manipulation of intracellular Mg ions [6, 18, 20] or by chemical modification of thiol groups [16, 17, 19] causes loss of KCl and obligatory water and, hence, cell shrinkage [22]. Interest in this pathway is considerable because K : Cl cotransport constitutes a major component of pathologically elevated passive K fluxes in human red cell disorders such as hemoglobin C and sickle cell disease [2–4, 8]. In sheep, K : Cl cotransport remains active in genetically low K (LK) and obliterates in high K (HK) erythrocytes suggesting a role in the expression of the LK red cell genotype [18, 22].

Based on the low pK_a value of the stimulatory SH group alkylated by N-ethylmaleimide, NEM, we postulated the presence of an acid titrable residue close to the thiol, such as an imidazole group [18], and sought to present evidence for this hypothesis by demonstrating in sheep red cells low pH_i activated K : Cl cotransport [36] also found in human red cells [6]. Indeed, diethylpyrocarbonate, DEPC, reasonably specific for the reaction with the imidazole moiety of histidine between pH 6 and 7.5 [29], inhibited K : Cl cotransport in LK cells and, independent of the order of addition, abolished the NEM-induced K : Cl flux suggesting functional dominance within the transporter of the DEPC-reactive over the thiol-alkylated groups [26].

In order to further identify the DEPC-reactive residue, we subsequently exposed LK cells to hydroxylamine, NH_2OH, known to regenerate histidine, but not to reverse other carbethoxylation products [29]. Instead of a reversal of the DEPC-induced inhibition of K : Cl cotransport, we found that 1 mM NH_2OH alone stimulated K : Cl fluxes in controls about sixfold and in NEM-treated cells twofold, an effect absent when, independent of the order of addition, the cells had been exposed to DEPC. Hence, stimulation of K : Cl cotransport by NH_2OH and NEM occurs through separate sites; however, both effects depend on the same chemical group carbethoxylated by DEPC. Our findings provide new information about chemical sites important for regulation of K : Cl cotransport. Part of this work has been presented in preliminary form [27, 28].

Materials and Methods

Blood from LK or HK phenotype sheep identified by cation analysis of their red blood cells was obtained by jugular venipuncture into heparin (10 IU/ml blood), kept on ice and used within hours. Red blood cells were washed in isosmotic (295 mOsM)

NaCl solutions in order to remove buffy coat and white cells. Chemical treatments with NEM (N-ethylmaleimide, Sigma Chemical, St. Louis, MO), diamide (Sigma Chemical, St. Louis, MO), DTT (dithiothreitol, Sigma Chemical, St. Louis, MO), and with DEPC (diethylpyrocarbonate, Aldrich Chemical, Milwaukee, WI) were carried out at a 5% (vol/vol) hematocrit as described earlier [16, 20, 21]. It should be pointed out that over the time period of chemical manipulations following the 15-min NEM treatment (i.e., 5 min DEPC and 5–30 min NH_2OH) and during the short fluxes (30–40 min), the NEM effect remained stable. In fact, in sheep red cells the NEM effect is not transient as LK red cells treated with NEM for 15 min [16] maintain a stimulated K : Cl flux for more than 3 hr (*data not shown*). In contrast, in human red cells the NEM effect has been reported to be transient, with a time constant of hours [35]. Treatment with hydroxylamine (NH_2OH, Fisher Scientific, Fair Lawn, NJ) was done at 37°C, for the time periods and concentrations indicated, and at a hematocrit of 5% (vol/vol). Immediately before the experiment, all chemicals were dissolved in isosmotic $NaNO_3$ buffered with 5 mM PO to pH 7.4 and mixed with the cells which were washed in the same cold medium without additions between treatments. Zero-*trans* K effluxes in 5 mM phosphate-buffered NaCl or $NaNO_3$ media (295 mOsM) were performed as previously described and the rate constants of the passive zero-*trans* K efflux, ${}^0k_K(hr^{-1})$, were determined from the appearance, at four time points, of K in the supernatant under initial velocity conditions [16]. In general, we did not add ouabain which, when present, did not alter the ${}^0k_K$ values. Hemoglobin was quantitated either at 527 nm or by a modified cyanomethemoglobin method. For assessment of methemoglobin formation relative to oxyhemoglobin, we determined the 500/540 nm absorbance ratio which reliably permitted to follow time course and dependence on the concentration of the oxidant. Cellular ATP and glutathione, GSH, were determined as reported before [17, 24] and expressed in concentration (mmol) per liter original cells (LOC).

Results

Diethylpyrocarbonate is known to abolish completely the NEM-induced stimulation of ouabain-resistant K efflux [26]. Figure 1 shows this effect of 4 mM DEPC on the rate constant, ${}^0k_K$, of the NEM-induced K efflux in Cl media, but in addition, reveals that DEPC also lowered control fluxes. Furthermore, while the NEM-induced K efflux was similar in Cl or NO_3, DEPC preincubation in NO_3 instead of in Cl exhibited a greater inhibitory effect on NEM-treated cells, suggesting that the DEPC-sensitive group is also responsive to Cl replacement [28].

Because NH_2OH reverses the carbethoxylation causing hydroxamates and regenerating histidine [29], we expected that NH_2OH would restore the DEPC-induced inhibition of the NEM-activated K efflux seen in Fig. 1 if indeed a histidine was involved. However, when we tested the effect of NH_2OH on untreated LK control cells (Fig. 2), we observed a dose-dependent *stimulation* of Cl-dependent K efflux reaching at about 0.4 mM NH_2OH (equivalent to 8 mmol $(LOC)^{-1}$) a greater than five-

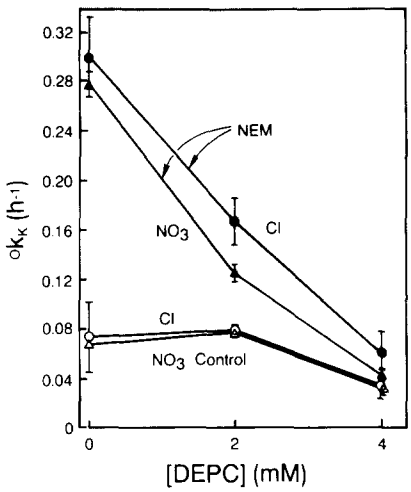

Fig. 1. The effect of DEPC treatment in Cl or NO_3 media on the rate constants of zero-*trans* K efflux, ${}^0k_K$, measured in Cl from LK sheep red blood cells before and after treatment with NEM. Note that 4 mM DEPC inhibited K efflux of both controls and NEM-treated cells, and that in the latter, the effect was greater in NO_3. $n = 3$, bars indicate ± SE

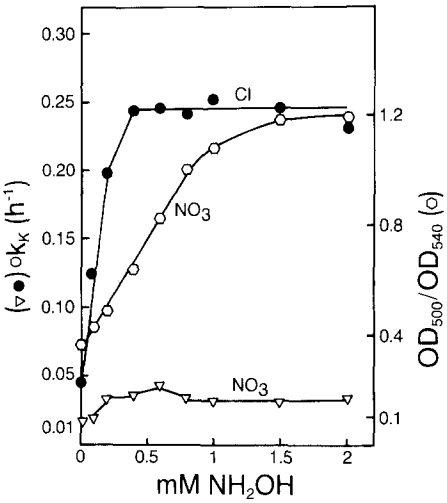

Fig. 2. Activation by NH_2OH of Cl-dependent K efflux based on measurement of ${}^0k_K$ in Cl or NO_3 (left ordinate) and methemoglobin formation in NO_3 as determined by the OD_{500}/OD_{540} ratio (right ordinate). Note that the NH_2OH effect on K efflux, but not on methemoglobin formation, was Cl dependent

fold maximum above controls while there was no effect in NO_3 media. We also noted a NH_2OH dose-dependent formation of methemoglobin as measured by the OD_{500}/OD_{540} ratio which, in NO_3, did not match the dose-response curve of the Cl-dependent K efflux. As shown in Fig. 3, the stimulation of Cl-dependent K efflux and the oxidation of hemoglobin to methemoglobin by 1 mM NH_2OH were completed

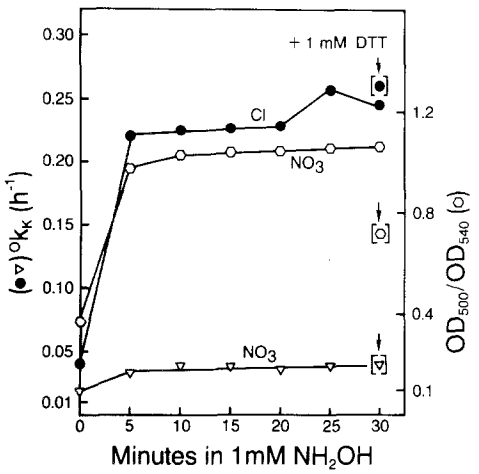

Fig. 3. Time course of the NH_2OH effect on the rate constants, ${}^0k_K$, of K efflux in Cl or NO_3 (left ordinate) and on methemoglobin formation (right ordinate). The action of NH_2OH on both parameters was fully expressed by 5 min at 37°C. Addition of 1 mM dithiothreitol (DTT) partially reversed the effect of NH_2OH on hemoglobin, but had no influence on the NH_2OH-induced zero-*trans* K efflux

Table 1. Effect of Cl replacement on methemoglobin formation due to oxidation by NH_2OH

Time (min)	NH_2OH 1 mM	Cl	NO_3	OD_{500}/OD_{540}	± SD (n = 3)
1	−	+	−	0.39	0.0014
1	−	−	+	0.38	0.0005
5	+	+	−	0.97	0.0039
5	+	−	+	0.96	0.0054
15	+	+	−	1.10	0.0060
15	+	−	+	1.07	0.0049
30	+	+	−	1.14	0.0010
30	+	−	+	1.09	0.0021

in less than 5 min. The effect of NH_2OH on hemoglobin was Cl independent. Table 1 shows that the development of methemoglobin as measured by the OD_{500}/OD_{540} ratio was not different in Cl and NO_3, respectively. The oxidation of hemoglobin by NH_2OH was, in part, reversible when cells were subsequently incubated in 1 mM dithiothreitol, DTT, which, however, did not affect the altered K flux rates in Cl. We conclude that NH_2OH permeated the plasma membrane and, independently, oxidized the K : Cl cotransport protein, as well as the hemoglobin to methemoglobin. Another primary amine, monochloramine, is known to oxidize hemoglobin and reduced glutathione (GSH, ref. [11]). Since thiol activation of K : Cl cotransport is preceded by alkylation [23] or oxidation of cellular GSH [24], we explored whether NH_2OH would still affect NEM-stimulated K : Cl cotransport, and furthermore, whether DEPC reported to abrogate the NEM-effect [26] would also influence the NH_2OH-stimulated K fluxes.

Table 2A shows that NH_2OH stimulated Cl-dependent K fluxes of untreated controls 5.8-fold, and of NEM-treated cells twofold. However, the absolute change of Cl-dependent K efflux in untreated and NEM-pretreated LK cells were identical, as seen in Table 2B (*compare* a2 and b2), suggesting that NH_2OH activated K : Cl cotransport through a mechanism different from that targeted by NEM. Table 2A also shows that 3.5 mM DEPC abolished the Cl-dependent K efflux in controls and completely abrogated the NEM-stimulated component (*compare also* the canceling of the absolute changes of a1 and b3 in Table 2B). When treatment with 3.5 mM DEPC preceded exposure of control or NEM-treated LK red cells to 1 mM NH_2OH, the absolute K flux rates were not different from zero in controls (a4 of Table 2B), and were in NEM-treated cells identical to those observed with DEPC alone (*compare* b3 with b4 in Table 2B). Furthermore, DEPC applied after NH_2OH also inhibited the NH_2OH-induced stimulation of K efflux in both controls and NEM-modified cells (*see* a5 and b5). These findings strongly suggest that DEPC not only alters the NEM effect on K transport as we previously showed [26], but also prevents the stimulation of K : Cl cotransport through oxidation by NH_2OH at a site separate from that alkylated by NEM. Since the DEPC effect was elicited whether or not the cells were DEPC treated prior to NH_2OH exposure, the DEPC-sensitive site must be functionally dominant over the NEM- and NH_2OH-reactive residues.

In order to assign further the NH_2OH effect to thiols or groups different from the thiols alkylated by NEM [16], we studied the action of NH_2OH after diamide-induced oxidation of the stimulatory thiols to dithiols which is reversed by DTT [24]. Tables 3A and B show that the effect of NH_2OH to stimulate K : Cl cotransport in controls is only partially reversed by DTT, probably due to reversible oxidation of some thiols reached also by NEM. However, when diamide, through reversible oxidation to dithiols, had activated K : Cl cotransport, NH_2OH still doubled the K flux activity to the remarkable values of 0.46 hr^{-1}, which is equivalent to a K efflux of about 10 mmol $(LOC)^{-1} \cdot hr^{-1}$. Following NH_2OH treatment of diamide-modified cells, DTT was not able to restore the flux to values close to baseline as in the case of diamide alone, which we like to interpret as an indication for a largely irreversible effect of NH_2OH on chemical residues different from those thiols ordinarily attacked by NEM or IAAM (iodoacetamide, *see* ref. [21]).

Table 2A. Effect of hydroxylamine[a] and diethylpyrocarbonate[b] on the rate constants of passive, zero-*trans* K efflux in control and N-ethylmaleimide[c]-treated low K (LK) sheep red blood cells in Cl or NO_3 media

First treatment	Second → third treatment	^0k_K (hr^{-1}) Cl ± SE	NO_3 ± SE	Δ Cl ± SE
None	None	0.062 ± 0.008	0.018 ± 0.003	0.044 ± 0.008
None	NH_2OH (1 mM)	0.288 ± 0.022	0.018 ± 0.005	0.255 ± 0.020
None	DEPC (3.5 mM)	0.024 ± 0.002	0.016 ± 0.004	0.008 ± 0.002
None	DEPC → NH_2OH	0.082 ± 0.014	0.021 ± 0.005	0.062 ± 0.019
None	NH_2OH → DEPC	0.090 ± 0.008	0.021 ± 0.002	0.069 ± 0.010
NEM (1 mM)	None	0.197 ± 0.014	0.019 ± 0.002	0.178 ± 0.012
NEM (1 mM)	NH_2OH (1 mM)	0.403 ± 0.012	0.039 ± 0.001	0.365 ± 0.011
NEM (1 mM)	DEPC (3.5 mM)	0.067 ± 0.004	0.027 ± 0.005	0.040 ± 0.010
NEM (1 mM)	DEPC → NH_2OH	0.064 ± 0.004	0.028 ± 0.004	0.036 ± 0.008
NEM (1 mM)	NH_2OH → DEPC	0.094 ± 0.009	0.026 ± 0.002	0.068 ± 0.008

[a] NH_2OH.
[b] DEPC.
[c] NEM.
n = three experiments on the same animal (LK6,LL).

Table 2B. Absolute changes in Cl-dependent K efflux rate constants of LK sheep red blood cells caused by NEM, DEPC and NH_2OH

Cells	Chemical and ^0k_K (hr^{-1}) 1 NEM	2 NH_2OH	3 DEPC	4 DEPC → NH_2OH	5 NH_2OH → DEPC
a) Untreated	+0.134 (0.011)	+0.211 (0.014)	−0.040 (0.005)	+0.018 (0.013)	+0.025 (0.017)
b) NEM-treated		+0.187 (0.003)	−0.138 (0.009)	−0.141 (0.008)	−0.110 (0.018)

Values for n = 3 ± SE (in parenthesis) calculated from Table 2A.

The effect of DEPC to prevent the action of NEM and NH_2OH cannot be explained by a drop in cellular ATP levels known to interfere with the chemical stimulation of NEM [17]. Accordingly, Table 4 shows that 4 mM DEPC had no effect on cellular ATP levels in LK, nor in HK red cells which normally have higher ATP concentrations [10] as also shown here. There was only a 10% reduction of cellular ATP after treatment with 1 mM NH_2OH which fully stimulated K : Cl cotransport. On the other hand and as expected, 1 mM NH_2OH reduced GSH to very low levels consistent with its action as an oxidant and the strong methemoglobin formation. Note that DEPC also diminished the GSH levels significantly, an effect not readily explained.

Discussion

In this study we attempted to use the oxidizing chemical NH_2OH to reverse the inhibitory effect of DEPC on the basal or the thiol-activated K : Cl cotransport reported by us earlier (Fig. 1) [26]. The choice of NH_2OH was logical because its known capability to reverse the carbethoxylation of histidine but not of adducts between DEPC and other amino acids or groups makes it a diagnostic tool to assess the presence of imidazole moieties [29]. We were very much interested to demonstrate the existence of such imidazole residues functionally important for K : Cl cotransport since in an earlier hypothesis we proposed that such a residue may take part in a protonation-mediated activation of the K : Cl cotransporter [18]. Indeed, we have independent evidence for activation of K : Cl cotransport in sheep red cells by low pH_i [36].

However, when we applied NH_2OH alone we discovered that it stimulated K : Cl cotransport almost sixfold in controls (Figs. 2 and 3). The target groups for the oxidative action of NH_2OH could be a variety of amino acids including thiol amino acids [11]. Thus, it was important to distinguish the NH_2OH effect from the NEM effect reported earlier [16]. As shown in Tables 2A and B, NH_2OH stimu-

Table 3A. Effect of hydroxylamine[a] and dithiothreitol[b] on the rate constants of passive, zero-*trans* K efflux in control and diamide[c]-treated low K (LK) sheep red blood cells

First treatment	Second → third treatment	^0k_K (hr^{-1}) ± SE
None	None	0.050 ± 0.007
None	DTT (2 mM)	0.039 ± 0.005
None	NH_2OH (1 mM)	0.315 ± 0.040
None	NH_2OH → DTT	0.221 ± 0.030
DM (2 mM)	None	0.205 ± 0.030
DM (2 mM)	DTT (2 mM)	0.050 ± 0.006
DM (2 mM)	NH_2OH (1 mM)	0.455 ± 0.056
DM (2 mM)	NH_2OH → DTT	0.277 ± 0.039

[a] NH_2OH.
[b] DTT.
[c] DM.
n = three experiments on the same animal (LK6,LL).

Table 3B. Absolute changes in the K efflux rate constants of LK sheep red blood cells caused by DM, NH_2OH and DTT

Cells	Chemical and ^0k_K (hr^{-1})			
	1 DM	2 DTT	3 NH_2OH	4 NH_2OH → DTT
a) Untreated	+0.155	+0.011	−0.265	+0.171
b) DM-treated	—	−0.155	+0.250	+0.072

Calculated from Table 2A.

lated control as well as NEM-treated cells to similar degrees, and additively in the latter case, suggesting action of NH_2OH at chemical groups very different from those alkylated by NEM. A similar conclusion can be reached on the basis of our experiments with diamide-pretreated LK cells (Tables 3A and B). Here, diamide through dithiol formation, did not block the oxidation by NH_2OH, rather causing an additional stimulation of K efflux. When these doubly treated cells were exposed to DTT, which we have previously shown to fully reverse the diamide effect on K : Cl cotransport [24], a sizable stimulation of K : Cl cotransport remained. This means that NH_2OH, mostly irreversibly, activated the transporter through very different chemical groups (Tables 3A and B, *see also* Fig. 3). Interestingly, the anti-malaria agent dapsone-hydroxylamine, an apparent conjugate compound of NH_2OH, has been shown to activate fivefold K : Cl cotransport in human red cells, an effect explained by thiol oxidation [12]. This effect could be very close to the one described here if one assumes that the conjugate hydrolyzed within the cells to dapsone and NH_2OH. The present study unequivocally proves that NH_2OH in sheep red cells does not react with those thiols readily alkylated by NEM or oxidized by diamide, in particular because of its additive and DTT-irreversible effects.

From recent work on establishing the presence of K : Cl cotransport in human white ghosts [34], it can be concluded that hemoglobin is not necessary for the expression of transport function. This conclusion is supported by our finding that the concentration dependence of NH_2OH-induced methemoglobin formation was different from that of K : Cl transport stimulation (Fig. 2), which occurred in NO_3 (Figs. 2 and 3), and equally well in Cl (Table 1), and was to some extent reduced by DTT which did not affect K : Cl transport (Fig. 3). Thus the oxidative effect of hydroxylamine on K : Cl transport is direct, not involving hemoglobin. This conclusion differs from that reached by others on human red cells using hydrogen peroxide oxidation in addition to NEM treatment and postulating a role of unstable hemoglobin species in the additionally observed activation of K : Cl cotransport [35].

Does NH_2OH activate K : Cl flux by depleting other metabolites such as GSH or ATP? Indeed 1 mM NH_2OH reduced cellular GSH to almost zero (Table 4). Transport stimulation in GSH-depleted LK cells is reminiscent of the diamide-induced K : Cl flux which was reversed by metabolic repletion of GSH, thus suggesting for the first time a metabolic (redox) control of a membrane transporter [22, 24]. This possibility cannot be tested easily since the NH_2OH effect on K : Cl cotransport did not respond to metabolic restoration attempts (*data not shown*). On the other hand, NH_2OH only slightly reduced cellular ATP levels (in both LK and HK cells), and this effect was already pronounced at 0.5 mM NH_2OH (Table 4). Thus, if, like the NEM effect [17], the mechanism of NH_2OH oxidation requires the presence of ATP, less stimulation by NH_2OH should be expected in ATP-depleted cells, a hypothesis to be tested in the future. If this indeed is the case, one could explain the NH_2OH effect in terms of a dephosphorylation of ATP supported acylphosphates [32]. Interestingly, it has been recently proposed that the resting state of K : Cl cotransport in rabbit red cells is associated with phosphorylation and the activated state with dephosphorylation [13].

Whatever the target groups for NH_2OH and the mechanism of their oxidation may be, our finding that DEPC, independent of the order of addition, prevents this effect is most interesting as DEPC modified the NEM- and swelling-induced K : Cl pathway in the same manner [26]. The question then to be asked is whether the chemical inhibitor DEPC has the same kinetic effect as the chemical activator NEM to simply alter (reduce) the number of K : Cl

Table 4. Cellular ATP and GSH levels after treatment of sheep erythrocytes with NH_2OH or DEPC

Chemical (concentration)	GSH [mmol (LOC)$^{-1}$ ± SE] LK (n = 3)	ATP [mmol (LOC)$^{-1}$ ± SE] LK (n = 3)	HK (n = 3)
		LK (n = 4)	HK (n = 3)
Control	2.55 ± 0.21	1.08 ± 0.05	1.47 ± 0.14
NH_2OH (0.5 mM)	—	0.90 ± 0.05	1.28 ± 0.15
(1.0 mM)	0.09 ± 0.003	0.94 ± 0.03	1.32 ± 0.15
(2.0 mM)	—	0.97 ± 0.04	1.30 ± 0.10
Control	—	0.87 ± 0.04	1.22 ± 0.06
DEPC (4.0 mM)	0.87 ± 0.12	0.93 ± 0.04	1.14 ± 0.09

The determination of GSH was done in three experiments measuring simultaneously the NH_2OH and DEPC effects. The ATP determinations were done on two separate sets of three experiments for NH_2OH and DEPC, each having its own control.

transporters in operation. Alternatively, chemical inhibitors and activators may work through regulatory or set point sites. DEPC is known to alter a modulator site on the Na/H exchanger [1]. Moreover, the independence of the actions of DEPC (Tables 2A and B and ref. [26]) from the NH_2OH and NEM effects underscores the importance of the DEPC-reactive group for the overall K : Cl transport function and its functional dominance over chemical modulation through activating [18] or inhibitory [33] thiols or NH_2OH oxidizable residues. Thus, it is conceivable that DEPC modulates transport by varying the number of transporting units directly while all the other stimulatory chemicals affect K : Cl cotransport on the regulatory level.

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