Biochimica et Biophysica Acta, 983 (1989) 65–76
Elsevier

BBAMEM 74483

Cation transport in oxidant-stressed human erythrocytes: heightened *N*-ethylmaleimide activation of passive K^+ influx after mild peroxidation

Harland E. Sheerin [1], L. Michael Snyder [2] and Grant Fairbanks [1]

[1] *Cell Biology Group, Worcester Foundation for Experimental Biology, Shrewsbury and* [2] *Division of Hematology, St. Vincent Hospital, Worcester, MA (U.S.A.)*

(Received 12 April 1989)

Key words: Potassium ion transport; *N*-Ethylmaleimide; Peroxide; Hemoglobin; Thiol; Erythrocyte; (Human)

Normal and chronically dehydrated (hereditary xerocytosis) human red cells were subjected to mild peroxidative treatment (315 μM hydrogen peroxide (H_2O_2), 15 min) in the presence of azide. The subsequent expression of passive (ouabain-resistant) K^+ transport activities was analyzed by measurement of $^{86}Rb^+$ influx. Peroxidation of normal red cells did not affect basal K^+ transport activity, but the increment in K^+ influx elicited by 0.5 mM *N*-ethylmaleimide (NEM) was increased 3-fold. The enhanced K^+ influx was chloride-dependent, but only partially inhibited by 0.1 mM furosemide. Stimulated activity declined progressively after NEM activation, but could be restored by a second NEM treatment. Prior conversion of hemoglobin to the carbonmonoxy form abolished the response to peroxide, while 200 μM butylated hydroxytoluene (BHT) exerted only partial inhibition, suggesting that the effect of H_2O_2 requires interaction of activated, unstable hemoglobin species with the membrane, but that lipid peroxidation is not sufficient. Peroxidation following NEM treatment also enhanced NEM activation, indicating that enhancement does not require altered NEM reactions with stimulatory or inhibitory sites. Passive K^+ transport in hereditary xerocytosis red cells was not activated by NEM, with or without H_2O_2 pretreatment. The results demonstrate that modest peroxidative damage to red cells can heighten the activation of a transport system that is thought to be capable of mediating net K^+ efflux and volume reduction in cells that express it. Models are proposed in which the effects of NEM, H_2O_2, cell swelling and other factors are mediated by conformational changes in a postulated subpopulation of anion channel (Band 3) molecules that bind the K^+ transporter.

Introduction

In its indispensable role as oxygen carrier, the red blood cell is subjected to continuous oxidative stress. The cell is equipped with enzymatic detoxification mechanisms that provide significant protection against oxidative insults, but oxidative damage accumulates and contributes to the decline of cellular functions in vivo in both normal and disease states [1–5]. Model systems in which toxic oxygen species are delivered to red cells in vitro have been used by many laboratories to identify sites of oxidant injury, to delineate mechanisms in damage and detoxification pathways, and to analyze effects on cellular properties. In studies of the effects of externally applied H_2O_2, Snyder and associates have observed an apparent relationship between peroxide-induced spectrin-hemoglobin cross-linking in intact red cells [6,7] and membrane functional deterioration represented by decreased membrane deformability and increased recognition for phagocytosis by monocytes [8,9]. They have also observed that the susceptibility of red cells to peroxide is greater in hereditary xerocytosis (HX), a mild, chronic hemolytic anemia in which red cells suffer dehydration due to accelerated leakage of intracellular K^+ [10–14]. Thus, peroxide-stressed HX red cells undergo shape changes and generate membrane protein aggregates at lower peroxide concentrations, and, at high peroxide concentrations, they exhibit exaggerated K^+ leakage [6,15].

There is considerable evidence that protein sulfhydryl groups participate in both reversible and irreversible

Abbreviations: BHT, butylated hydroxytoluene; DIDS, 4,4′-diisothiocyanatostilbene-2,2′-disulfonic acid; HX, hereditary xerocytosis; NEM, *N*-ethylmaleimide; MCHC, mean corpuscular hemoglobin concentration.

Correspondence: G. Fairbanks, Cell Biology Group, Worcester Foundation for Experimental Biology, Shrewsbury, MA 01545, U.S.A.

membrane protein cross-linking, as well as in other manifestations of membrane damage in oxidatively-stressed red cells [6,9,16–18]. Membrane thiols have also been implicated in the regulation of K^+ transport activities, in that there are NEM-stimulated, chloride-dependent, ouabain-resistant K^+ transport pathways in both sheep and human red cells [19–27]. The activities disclosed by NEM are also stimulated by cell swelling. Interestingly, dehydrated HX red cells, which appear defective in volume regulation, do not exhibit this response to NEM [27]. These observations led us to explore the effect of mild peroxidation on the passive cation-transport pathways of normal and hereditary xerocytosis red cells. We report here that mild peroxide damage to normal cells that does not itself alter transport has the effect of heightening the stimulation in chloride-dependent K^+ transport activity produced by NEM treatment. The dehydrated hereditary xerocytosis red cells do not display this phenomenon. Some of these results have been presented earlier in preliminary form [28].

Methods and Materials

Cell preparation. Venous blood was obtained from normal healthy volunteers and HX patients from a Worcester-area family [11–13]. The heparinized blood was centrifuged and the buffy coat and plasma removed. The red cells were washed three times in saline containing 10 mM phosphate (pH 7.4) and 5 mM glucose. In some cases, white cells were removed by filtration through cellulose [29] with elution in Hanks' balanced salt solution followed by washing without aspiration from the top of the red cell pellet. In a few experiments, red cell subpopulations differing in buoyant density were obtained by fractionation on Percol/Hypaque gradients as described previously [30].

Pretreatments with H_2O_2 were carried out in phosphate-buffered saline with washed red cells at 20% hematocrit. Sodium azide was present at 1 mM to inhibit catalase. Following addition of H_2O_2 to the desired final concentration, normally 315 μM, the suspensions were incubated at 37°C in a shaking water bath for 15 min. The color of the suspensions deepened slightly immediately upon addition of H_2O_2, consistent with the rapid formation of small amounts of methemoglobin that persisted through subsequent steps [8]. Control cells underwent the same regimen without added peroxide. In preparation for subsequent NEM treatment and K^+ influx measurements, the cells were washed three times in phosphate-buffered saline and suspended at 10% hematocrit in 150 mM NaCl/10 mM Tris-HCl/5 mM glucose (pH 7.5) or in the same medium with $NaNO_3$ substituted for NaCl. These suspensions were incubated at 37°C for 30 min. The cells were then sedimented, subjected to a second incubation in fresh medium under the same conditions, and washed once. The incubations with $NaNO_3$ have been shown to deplete the cells of chloride and eliminate the chloride-dependent passive cation fluxes [31]. NEM activation of K^+ transport was begun by adding portions of a fresh concentrate of the reagent to the washed, equilibrated cells to yield 10% cell suspensions with the desired final NEM concentration, normally 0.5 mM. Treatment at 37°C was terminated by addition of dithioerythritol to 1 mM followed by washing three times in chilled influx assay medium. In earlier experiments, this NEM treatment regimen produced maximal stimulation of chloride-dependent K^+ influx in normal red cells [27]. In five experiments examining the effects of changing the order of treatments (Table III, Fig. 8), times of exposure to NEM and H_2O_2 were shortened to 15 and 10 min, respectively.

BHT treatment: in some experiments, washed cells were preincubated for 30 min with the antioxidant, BHT, at 200 μM before addition of H_2O_2 [8].

CO treatment: some cell preparations were exposed to carbon monoxide for 10 min before addition of H_2O_2. Under these conditions, the hemoglobin was converted to the stable carbonmonoxy form and methemoglobin formation during H_2O_2 pretreatment was prevented [8].

Influx measurements. K^+ influx was measured using $^{86}Rb^+$ as tracer by a modification [27] of the method of Dunham and Ellory [32]. The cells were suspended at 3–5% hematocrit in the Tris-buffered chloride or nitrate medium containing 0.1 mM ouabain, 5 mM KCl and $^{86}Rb^+Cl$. In some experiments, the flux media also contained 0.1 mM furosemide and/or 0.5 mM DIDS. The samples were incubated at 37°C in 1.5 ml microcentrifuge tubes. After 30–45 min, uptake of $^{86}Rb^+$ was terminated by addition of ice-cold 0.106 M $MgNO_3$/10 mM Tris-HNO_3 (pH 7.45), followed immediately by centrifugation and four washings in the same termination buffer. Each pellet of washed cells was sampled for measurement of $^{86}Rb^+$ radioactivity in a planchet counter or liquid-scintillation spectrometer. Results were normalized to the hemoglobin contents of the corresponding sample volume. Except where noted otherwise, influx was measured in triplicate assays, values shown are means ± S.D., and two-tailed paired *t*-tests were used to assess the significance of differences. When influx was assayed in both nitrate and chloride media, the chloride-dependent component was calculated as the difference in the means of the normalized values.

Other assays. Hemoglobin was measured using the cyanmethemoglobin method as described by Beutler [29]. Hematocrits and hemoglobin concentration values were used to calculate the mean corpuscular hemoglobin concentrations (MCHCs) in g Hb/100 ml packed cells. Levels of ATP and reduced glutathione (GSH) in extracts of washed incubated red cells were measured as

described by Beutler [29], using the coupled hexokinase/glucose-6-phosphate dehydrogenase reaction and reaction with 5,5'-dithiobis(2-nitrobenzoic acid) (Ellman's reagent), respectively. Cellular contents of Na^+ and K^+ were determined using an Instrumentation Laboratories S12 AA/AE spectrophotometer. The cells were washed four times in chilled 0.11 M $MgCl_2$ and sampled in triplicate with dilution and lysis in deionized water.

Chemicals. $^{86}RbCl$ was obtained from NEN Research Products (Du Pont Company, Boston, MA). Furosemide was a gift from Hoechst-Roussel Pharmaceuticals (Somerville, NJ). ATP-assay chemicals (hexokinase, glucose-6-phosphate dehydrogenase, NADP), hemoglobin assay kits, Ellman's reagent, Tris, ouabain, NEM, dithioerythritol, DIDS and hydrogen peroxide were purchased from Sigma (St. Louis, MO). Hanks' balanced salt solution (Ca^{2+}- and Mg^{2+}-free) was obtained from Gibco (Grand Island, NY).

Results

Effects of peroxide and NEM

Cells were pretreated with H_2O_2 with azide present to inhibit catalatic decomposition of the oxidant. Fig. 1A and Table I show the resulting enhancement of NEM activation of ouabain-resistant K^+ influx in normal red cells. Mild peroxidation (315 μM H_2O_2) did not significantly alter the basal level of passive K^+ influx, but produced a three-fold increase in the increment in activity elicited by NEM. K^+ influx activated by NEM is only partially inhibited by furosemide at 0.1 mM [22,24,25], so the proportion of furosemide-sensitive influx was decreased by NEM treatment. Similarly, the enhanced NEM activation after H_2O_2 pretreatment was reflected in increases in both furosemide-sensitive and furosemide-insensitive components (Table I).

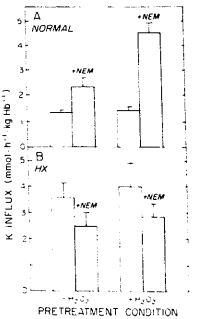

Fig. 1. Effect of peroxidation and NEM treatment on ouabain-resistant K^+ influx. (A) Normal red cells. (B) HX red cells. Cells were pretreated with 315 μM H_2O_2 or incubated in buffer without H_2O_2, then treated with 0.5 mM NEM (shaded bars) or incubated in parallel without NEM (open bars). K^+ influx values are means ± S.E. of measurements in chloride medium from ten experiments on normal cells and four experiments on HX cells. For normal cells, effects of NEM and enhancement of activation by H_2O_2 pretreatment were highly significant: $P < 0.005$ for $-H_2O_2$, $-$NEM vs. $-H_2O_2$, +NEM; $P < 0.0005$ for $+H_2O_2$, $-$NEM vs. $+H_2O_2$, +NEM; $P < 0.0005$ for $-H_2O_2$, +NEM vs. $+H_2O_2$, +NEM in paired *t*-tests. Basal K^+ influx was significantly elevated in HX cells: $P < 0.0005$ for normal $-H_2O_2$, $-$NEM vs. HX $-H_2O_2$, $-$NEM; $P < 0.001$ for normal $+H_2O_2$, $-$NEM vs. HX $+H_2O_2$, $-$NEM in unpaired *t*-tests.

The NEM concentration-dependence of K^+ influx with and without H_2O_2 pretreatment is illustrated in Fig. 2. Pretreated normal cells exhibited enhanced

TABLE I

Effects of NEM and H_2O_2 on ouabain-resistant K^+ influxes in normal red cells

Results are from a representative experiment with 315 μM H_2O_2 pretreatment followed by 0.5 mM NEM treatment (or control incubations) as described in Methods and Materials. K^+ influx was assayed in triplicate in chloride and nitrate media ± 0.5 mM DIDS and ± 0.1 mM furosemide ('Fur'), as indicated.

NEM and assay conditions			K^+ influx (mmol·h^{-1}·kg Hb^{-1})					
			pretreatment controls			H_2O_2-pretreatment		
DIDS	NEM	Fur	$+Cl^-$	$-Cl^-$ (NO_3^-)	Cl^--dependent	$+Cl^-$	$-Cl^-$ (NO_3^-)	Cl^--dependent
−	−	−	1.8 ± 0.1	0.38 ± 0.15	1.4 ± 0.2	1.8 ± 0.4	0.29 ± 0.02	1.5 ± 0.4
−	−	+	0.82 ± 0.08	0.23 ± 0.01	0.59 ± 0.08	0.54 ± 0.14	0.25 ± 0.03	0.29 ± 0.14
−	+	−	4.8 ± 0.8	0.40 ± 0.03	4.4 ± 0.8	8.3 ± 2.9	0.59 ± 0.13	7.7 ± 3.0
−	+	+	3.3 ± 0.6	0.40 ± 0.16	2.9 ± 0.6	4.2 ± 0.5	0.51 ± 0.02	3.7 ± 0.5
+	−	−	1.7 ± 0.1	0.59 ± 0.06	1.1 ± 0.1	2.2 ± 0.3	0.63 ± 0.13	1.6 ± 0.4
+	−	+	0.82 ± 0.13	0.34 ± 0.05	0.48 ± 0.14	1.02 ± 0.18	0.41 ± 0.02	0.60 ± 0.18
+	+	−	1.8 ± 0.2	0.55 ± 0.06	1.2 ± 0.2	2.1 ± 0.3	0.76 ± 0.09	1.3 ± 0.4
+	+	+	2.0 ± 0.2	0.37 ± 0.04	1.7 ± 0.2	2.0 ± 0.2	0.62 ± 0.07	1.4 ± 0.2

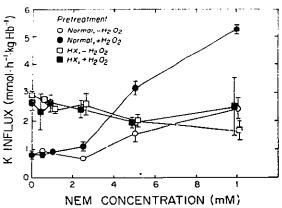

Fig. 2. NEM concentration-dependence of ouabain-resistant K^+ influx. Normal (○,●) and HX (□,■) red cells were treated with NEM at the concentrations indicated after pretreatment with 315 μM H_2O_2 (●,■) or after control preincubation in the absence of H_2O_2 (○,□).

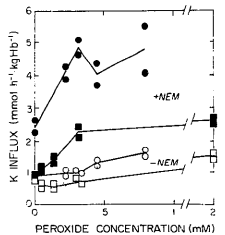

Fig. 3. Dependence of basal and NEM-activated, chloride-dependent K^+ influx on peroxide concentration during pretreatment. Normal red cells were exposed to H_2O_2 at the concentrations indicated, then treated with 0.5 mM NEM (●,■) or incubated without NEM (○,□). Individual values from duplicate assays in two experiments are plotted.

activation at NEM concentrations of 250 μM and above. At 0.5 mM, NEM stimulates mainly the chloride-dependent components of total ouabain-resistant K^+ influx [21,24–27] (Tables I and III), but, above 0.5 mM NEM, the chloride-independent 'leak' flux rises and contributes to the progressive increase in total passive K^+ influx [27].

Fig. 3 shows the dependence of ouabain-resistant K^+ influx on the H_2O_2 concentration during pretreatment. In these experiments, enhanced NEM stimulation was detectable at H_2O_2 concentrations below 200 μM. The effect was maximal at 315 μM, the standard condition adopted for our studies. The leak permeability assayed in nitrate medium was minimally affected at this H_2O_2 concentration (increments ranging from -0.03 to $+0.25$ mmol · h^{-1} · kg Hb^{-1} –see Tables I and III). Exposure to 2 mM H_2O_2 increased chloride-independent influx by 0.45 mmol · h^{-1} · kg Hb^{-1}.

Mechanism of peroxide enhancement

Table II summarizes results of measurements of red cell MCHC, cations and metabolites made during this study. Aside from the high MCHC and low K^+ content characteristic of hereditary xerocytosis red cells [10–13], the most conspicuous observation is the virtual absence of GSH in NEM-treated cells. Destruction of GSH is consistent with the early finding of Jacob and Jandl [33] that NEM reacts nearly stoichiometrically with red cell GSH and that 2.5 μmol NEM suffices to block GSH in 1 ml of cells (our condition being 0.5 μmol NEM/ml 10% suspension). In most experiments, NEM treatment also reduced ATP levels. The average decrement was only 15–20%, but significant statistically ($P < 0.05$ for pooled pair data in Table II ($N = 8$) – not including data for later times plotted in Fig. 7). Jacob and Jandl [33] have noted impaired glycolysis when NEM was applied at levels that completely blocked GSH. NEM treatment did not affect K^+ levels significantly under typical experimental conditions (< 1.5 h after NEM treatment, see Table II), but loss of K^+ from normal cells was noted at later times (Fig. 6 and data not

TABLE II

Effects of NEM and H_2O_2 on red cell MCHC, cations and metabolites

Cells were incubated ±315 μM H_2O_2, then ±0.5 mM NEM, under standard conditions. Values tabulated are means ± S.D. for pooled data from cells in chloride and nitrate media. The number of preparations represented in each mean is indicated in parenthesis.

Cells	Parameter assayed	Pretreatment controls		H_2O_2-pretreated	
		−NEM	+NEM	−NEM	+NEM
Normal	MCHC (g Hb/dl RBC)	33 ± 3 (22)	34 ± 2 (23)	34 ± 3 (12)	35 ± 3 (12)
	$[K^+]$ (mmol/l RBC)	94 ±13 (19)	96 ±14 (20)	84 ±18 (9)	86 ±17 (9)
	$[Na^+]$ (mmol/l RBC)	6.4 ± 1.2 (4)	7.2 ± 2.0 (4)	9.4 (1)	8.6 (1)
	ATP (μmol/g Hb)	2.7 ± 0.5 (4)	2.2 ± 0.4 (4)	2.0 (1)	2.5 (1)
	GSH (μmol/g Hb)	5.4 ± 1.3 (5)	0.11 ± 0.15 (5)	4.9 ± 0.7 (6)	0.03 ± 0.04 (5)
HX	MCHC (g Hb/dl RBC)	40 ± 2 (7)	39 ± 4 (7)	42 (1)	44 (1)
	$[K^+]$ (mmol/l RBC)	64 ±11 (7)	67 ±14 (7)	43 (1)	47 (1)
	$[Na^+]$ (mmol/l RBC)	13.5 ± 3.3 (5)	16.3 (1)	n.d.	n.d.
	ATP (μmol/g Hb)	2.1 ± 0.3 (2)	1.4 ± 0.01 (2)	1.8 (1)	1.4 (1)

TABLE III

Effects of changes in the sequence of peroxide and NEM treatments

In five experiments with different normal donors, cells were incubated first ± NEM, then ± H_2O_2. In three of these experiments, a third incubation ± NEM was carried out. Incubation times were 15 min with NEM, 10 min with H_2O_2. Ouabain-resistant K^+ influxes were measured with 30 min incubations in chloride medium. Mean values are tabulated, with ranges (values from individual experiments based on triplicate assays) shown in parentheses. NEM treatment before peroxidation or control incubation increased activity significantly above basal levels ($P = 0.02$ and 0.03, respectively; $N = 5$). Stimulation by NEM treatment at the third step did not differ significantly from that observed with NEM treatment at the first step ($P = 0.5$ for cells exposed to H_2O_2, $P = 0.4$ for oxidation controls; $N = 3$). Hencement of NEM increments by exposure to peroxide was signficiant ($P = 0.003$ for pooled data on both orders of treatment; $N = 8$). Significantly greater increments were produced by two NEM treatments than by the single treatments ($P = 0.01$ for pooled data on cells incubated ± H_2O_2; $N = 6$). Diffusional leak fluxes were measured in nitrate medium in two experiments. These averaged: 0.29 mmol·h^{-1}·kg Hb^{-1} (0.28–0.30) for untreated controls or cells treated once with NEM ($N = 6$); 0.32 mmol·h^{-1}·kg Hb^{-1} (0.30–0.33) for H_2O_2-treated cells with and without a single NEM treatment ($N = 6$); and 0.36 mmol·h^{-1}·kg Hb^{-1} (0.33–0.39) for cells treated twice with NEM ($N = 4$). The differences in the leak fluxes were significant statistically ($P < 0.05$ in unpaired *t*-tests) but too small to diminish the significance of the increased stimulation observed with two NEM treatments.

Treatment schedule			N	K^+ influx (mmol·h^{-1}·kg Hb^{-1})		
NEM at first step	H_2O_2 at second step	NEM at third step		Total	NEM stimulation [a]	Increment due to peroxidation [b]
–	–	–	5	1.9 (1.5– 2.3)		
+	–	–	5	5.3 (3.8– 9.1)	3.4 (1.4– 7.4)	
–	+	–	5	2.1 (1.8– 2.4)		0.2 (0.0–0.6)
+	+	–	5	7.4 (5.0–12.8)	5.3 (2.6–10.5)	2.1 (1.2–3.7)
–	–	+	3	5.0 (4.3– 5.7)	3.0 (2.0– 3.8)	
+	–	+	3	6.2 (4.8– 8.2)	4.2 (2.4– 6.2)	
–	+	+	3	7.4 (5.6–10.6)	5.3 (3.3– 8.5)	2.4 (1.1–4.9)
+	+	+	3	9.2 (7.8–11.8)	7.1 (5.4– 9.6)	3.0 (2.5–3.5)

[a] Mean (range) of increments above values for cells not exposed to NEM.
[b] Mean (range) of increments above values for the same NEM schedule without H_2O_2.

shown), presumably reflecting passive movement down the K^+ concentration gradient promoted by NEM activation of the latent chloride-dependent K^+ transport activity (Fig. 1, Table I; Refs. 19–27).

Red cells subjected to the peroxide pretreatment regimen suffered small losses of K^+, averaging 10–15% ($P < 0.05$ for 18 paired normal preparations; $P < 0.002$ including two HX pairs). This reduction in K^+ was associated with a small, but statistically significant, increase in the MCHC of H_2O_2-treated cells ($P < 0.05$ for 24 normal pairs). The limited data for Na^+ suggest that increased Na^+ may have partially compensated for the K^+ loss from normal cells. The average GSH content of peroxide-treated normal cells was lower than that of untreated controls, but the effect is not significant statistically for this data set.

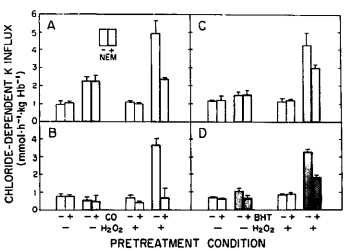

Fig. 4. Selective inhibition of H_2O_2 enhancement of NEM-activated K^+ influx in normal red cells. (A, B) Carbon monoxide. (C, D) 200 μM BHT. Cells were treated with carbon monoxide or BHT before addition of H_2O_2. Chloride-dependent K^+ influxes were measured after subsequent NEM activation (shaded bars) or control incubation (open bars). Results in (A) and (C) are comparable to those in (B) and (D), respectively, but show inhibitor effects on cells from different donors studied in separate experiments.

The nature of the reactions leading to enhancement of NEM activation in H_2O_2-treated cells was explored by analyzing the effects of selective inhibitors (Fig. 4). When the cells were saturated with carbon monoxide prior to pretreatment with H_2O_2, enhancement of NEM-activated, chloride-dependent passive K^+ transport was abolished (left panels, Figs. 4A and B). The enhancement was also reduced by including 200 μM BHT with H_2O_2 in the pretreatment step, but the reduction by BHT was only about 50% relative to the heightened response to NEM in controls (right panels, Figs. 4C and D). Earlier work established that carbon monoxide loading of hemoglobin does not inhibit lipid peroxidation, but prevents methemoglobin formation and eliminates other forms of membrane damage that are thought to involve reactions of the activated hemoglobin in H_2O_2-treated cells. The effect of the antioxidant, BHT, in this system differs, in that it partially inhibits lipid peroxidation but has no known effect on the other oxidative reactions in H_2O_2-stressed cells [8,15].

TABLE IV

Reactivation by NEM after decay of stimulated K^+ transport activity

Cells were subjected to three treatments under standard incubation conditions: (1) ±NEM; (2) ±H_2O_2; (3) ±NEM. K^+ influx in chloride medium was measured for samples taken after the H_2O_2 incubation step (2 h) and for cells subjected to the second NEM incubation (3.3 and 6 h). Values are means ± S.D. of triplicate (2 h) or duplicate (3.3 and 6 h) measurements from one experiment.

Treatment conditions			K^+ influx (mmol·h^{-1}·kg Hb^{-1})		
NEM at first incubation	H_2O_2 at second incubation	NEM at third incubation	before third incubation (2 h)	after third incubation (3.3 h)	after third incubation (6 h)
–	–	–	2.0 ± 0.1	1.4 ± 0.3	1.1 ± 0.2
		+		4.1 ± 0.4	2.2 ± 0.2
+	–	–	2.9 ± 0.4	1.2 ± 0.05	1.2 ± 0.04
		+		4.6 ± 0.4	2.9 ± 0.3
–	+	–	2.2 ± 0.2	1.4 ± 0.1	1.4 ± 0.1
		+		4.9 ± 0.5	2.6 ± 0.3
+	+	–	3.9 ± 0.2	1.9 ± 0.1	1.9 ± 0.2
		+		6.6 ± 1.4	3.6 ± 0.5

If peroxide were to exert its effect by modifying the reactivity of protein thiol groups (for example, by making stimulatory sites more accessible to NEM or by causing inhibitory sites to be masked), we would expect peroxidation to be effective only when it precedes NEM treatment. Findings directly opposed to this prediction emerged from experiments in which the sequence of treatments was varied systematically. Thus, results displayed in Table III and Fig. 8 demonstrate that peroxide also enhanced NEM activation when it was applied last. In order to address this issue effectively, it was necessary to reduce the durations of cell treatments and influx assays (Table III). During multiple treatment regimens with longer intervals, decay in the stimulated K^+ influx activity of cells treated first with NEM (see below) often virtually obscured the enhancement produced by later exposure to H_2O_2 (e.g., Table IV). In all of these experiments, K^+ influx via the chloride-independent leak pathway constituted < 20% of the total influx in chloride medium and was not significantly affected by reversing the order of incubations. These results imply that the peroxide effect involves membrane perturbations that alter expression of the NEM-activated, chloride-dependent system, as opposed to modification of protein thiol target activity in cells undergoing NEM treatment.

We observed repeatedly that methemoglobin formation during exposure to H_2O_2 was reduced in cells first treated with NEM (visual observations, data not shown). In a separate study, NEM pretreatment also markedly reduced subsequent lipid peroxidation under comparable conditions [9]. Thus, the observation that H_2O_2 enhanced activation even when it followed NEM treatment adds weight to the evidence from the BHT study (Figs. 4C and D) indicating that lipid peroxidation is not involved.

Decay of NEM activation

In most experiments, the magnitude of NEM activation of ouabain-resistant K^+ influx in normal cells was lower than that observed in earlier studies in our laboratories [27] and elsewhere [21,25,26]. In addition, there was relatively high variability in the response to NEM, even among repeated studies on red cells from the same normal donors. The results illustrated in Fig. 5 suggest

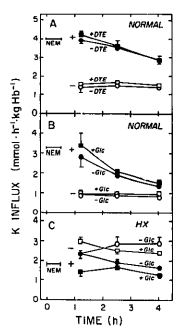

Fig. 5. Decay of NEM-activated ouabain-resistant K^+ transport activity under various conditions. (A) ±1 mM dithioerythritol (DTE), normal cells. (B) ±5 mM glucose, normal cells. (C) ±5 mM glucose, HX cells. K^+ influx in NEM-treated cells (●,■) and pretreatment incubation controls (○,□) was measured in chloride medium at intervals during incubation at room temperature in the presence (□,■) or absence (○,●) of the indicated supplements. Results in panels A and B are from separate experiments on normal cells from different donors.

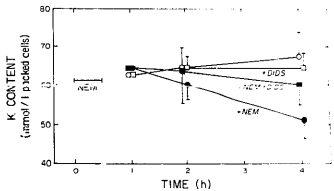

Fig. 6. Loss of intracellular K^+ after NEM treatment. Measurements were made at intervals after NEM treatment (●,■) or control incubation (○,□) of normal red cells. The cells were held at room temperature in the presence (□,■) or absence (○,●) of 0.5 mM DIDS. For values at 2 and 4 h, $N = 4$; values at 1 h are from duplicated measurements.

that these features of the data can be attributed in part to decay in the NEM-activated K^+ influx. This was a significant factor because the interval between NEM treatment and measurement of transport activities varied depending on the complexity of the experiment. The decline in NEM activation was not affected by the inclusion of DTE (Fig. 5A) or glucose (Fig. 5B) in the incubation medium after NEM treatment. The basal passive K^+ transport activity of untreated normal cells did not change significantly under these conditions.

This decay is probably not attributable to reductions in cellular levels of K^+ or ATP, which declined relatively slowly after NEM treatment (Figs. 6 and 7). Lauf and associates [22] have described a relationship between red cell ATP content and responsiveness to NEM; they estimated that 50% activation of chloride-dependent Rb^+ influx was supported by 0.3 mM red cell ATP. In our experiments, the minimum ATP level observed with normal cells was 0.5 mM (approx. 1.5

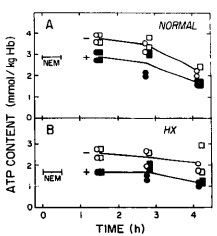

Fig. 7. Effects of NEM treatment and subsequent incubation on levels of ATP. (A) Normal red cells. (B) HX red cells. Individual values are plotted for duplicate assays on the cell preparations of Figs. 5A and C. The cells were treated with NEM (●,■) or without NEM (○,□) and incubated with 5 mM glucose (□,■) or without glucose (○,●).

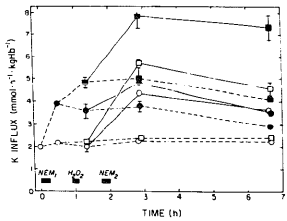

Fig. 8. Development and decay of stimulated passive K^+ transport activity with NEM treatment before and/or after H_2O_2 enhancement. Conditions are outlined in the legend to Table III. The cells were treated in nitrate media; samples were washed in chloride or nitrate influx assay medium before each set of assays. Labeled bars show the treatment schedule. At the first step ('NEM_1'), cells were incubated with NEM (●,■) or without NEM (○,□). The preparations were then divided for treatment ('H_2O_2') with H_2O_2 (□,■) or control incubation (○,●). At the third step ('NEM_2') portions were subjected either to NEM treatment (———) or control incubation (– – –). The cell suspensions were then kept at room temperature. Values plotted are means ± S.D. of K^+ influx in chloride medium assayed at each stage. (Values for leak fluxes measured in nitrate medium at 2.8 h are included in the results summarized in the legend to Table III.)

mmol/kg Hb, see Fig. 7A). At early times, when passive transport activity was stimulated maximally, the NEM-treated cells had already suffered significant ATP depletion (Fig. 7A; Table II).

Decay in the stimulated K^+ transport activity does not reflect loss of the capaci to respond to NEM. As illustrated in Tables III and IV and Fig. 8, cells treated with NEM were markedly stimulated by a second NEM treatment more than 2 h later, regardless of the conditions of treatment in the interim. In some cases, stimulation by NEM treatment at the first step was demonstrable after incubation with or without H_2O_2 at the second step, but activity declined to near basal levels during a third incubation step unless the cells were treated again with NEM (Table IV). The highest levels of K^+ influx were obtained with regimens involving these dual NEM stimulations. Increases in the chloride-independent fluxes were detectable (Table III), but the contributions of these leak fluxes to the large increments in chloride-dependent K^+ influx were negligible. These results imply that the expression of the NEM-activated transport activity was not limited by the concentrations of ATP or K^+ in the treated cells, because the levels of these metabolites presumably stabilized or declined further after the first incubation step. In all cases, exposure to H_2O_2 did not prevent the progressive loss of NEM-activated K^+ transport, indicating that enhancement of the NEM affect is not based on H_2O_2 stabilization of the activity against decay (Table IV, Fig. 8).

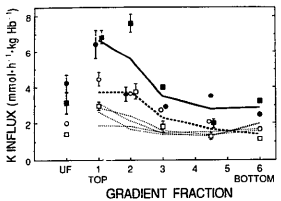

Fig. 9. NEM-activated ouabain-resistant K^+ transport activity in red cells from Percoll/Hypaque density gradient fractions. Cells were fractionated after pretreatment with H_2O_2 or control incubation without oxidant. Most cells were recovered in fractions of intermediate density, Fraction 3 and Fractions 4 and 5 (combined). Results from two separate experiments (□,■ and ○,●) are plotted. ●,■ represent NEM-stimulated K^+ influx enhanced by H_2O_2 pretreatment; the bold solid line traces the average activity profile for the two experiments. ○,□ represent NEM-stimulated activity without enhancement; the bold dashed line traces the corresponding average profile. Fine dashed lines indicate activity profiles for samples not exposed to NEM. NEM-stimulated K^+ influxes for unfractionated cells ('UF') are shown on the left.

Effects on red cell subpopulations differing in density

Fig. 9 shows the results of two experiments demonstrating that red cell fractions from Percoll-Hypaque density gradients differ in their capacity to respond to NEM and H_2O_2. As shown recently by Brugnara and Tosteson [34], NEM responsiveness declines with increasing cell density and is lowest in fractions from the bottom of the gradients that are depleted in reticulocytes. After pretreatment with H_2O_2, significant NEM stimulation was demonstrable even in dense cells from the bottom of the gradient. This pattern suggests that, although NEM responsiveness is suppressed as cells undergo aging and dehydration, the transport pathway and response mechanism persists in latent form. Alternatively, the results could reflect density shifts in a subpopulation of responsive cells due to K^+ loss during the H_2O_2 treatment (Table II), which preceded the gradient fractionation.

DIDS inhibition

Both activation by NEM and enhancement of activation by H_2O_2 pretreatment were inhibited by 0.5 mM DIDS (Table I). DIDS is an anion-channel blocker that binds to the predominant membrane protein, Band 3 [35]. It exerted its maximal effect only when present during the K^+ influx assay (results for other conditions not shown). The results shown in Fig. 6 suggest that DIDS also inhibits progressive loss of intracellular K^+ after NEM treatment. Similar effects of DIDS have been noted by Orringer [36].

Unresponsiveness of HX red cells

In HX red cells, basal passive K^+ influx is elevated and the response to NEM is absent or obscured (Refs. 27 and 28 and unpublished observations). In most experiments, the net effect of NEM on these dehydrated cells was inhibitory (Figs. 1B, 5C) and pretreatment with H_2O_2 did not yield the enhancement of NEM responsiveness demonstrable with normal cells under the same conditions (Fig. 1A). The passive transport activity of the HX red cells did not exhibit any consistent shifts as a function of time after exposure to NEM (Fig. 5C). Although the ATP levels of these cells in vitro tend to be depressed [11,12], in these experiments they remained well within the range [22] that would have supported expression of NEM-activated K^+ transport activity (Fig. 7B).

Discussion

Relationship to previous studies

Several earlier studies have demonstrated that the permeability of the human erythrocyte membrane to K^+ is increased by exposure to oxidizing agents that can attack membrane proteins and lipids directly or damage the membrane indirectly by stimulating the intracellular generation of free radicals [18,36–40]. Orringer [36] has observed that treatment with 20 mM acetylphenylhydrazine selectively augmented efflux of K^+ from human erythrocytes via a pathway dependent on chloride, independent of Na^+, and partially inhibited by furosemide. That activity is probably identical to the component of K^+ transport considered here, but its response to NEM treatment or cell swelling was not studied. In another study [38], free radicals generated by low levels of phenazine methosulfate increased the permeability of human erythrocyte membranes. However, the bumetanide-sensitive Na^+/K^+ cotransport activity was inhibited by this oxidant damage and all of the increased K^+ permeability could be attributed to the diuretic-insensitive, nonsaturable electrodiffusional leak. Similarly, K^+ leakage was produced by treatment with the lipid hydroperoxide analogue, *t*-butyl-hydroperoxide [18,39,40], but the oxidant-induced leak pathway mediated DIDS-insensitive permeation of chloride and had other properties of a continuous aqueous channel [39].

Our study differs from most of the previous work in that relatively low levels of peroxide have been used and the increased transport activity was latent until activated by treatment with the thiol alkylating agent, NEM. In its insensitivity to ouabain, dependence on NEM activation and requirement for chloride, that activity resembles the Na^+-independent K^+ transport pathway that has recently been distinguished from both Na^+/K^+ cotransport and basal Na^+-independent, chloride-dependent transport [41] on the basis of several of its

properties, including its relative insensitivity to the loop diuretics, its independence from Na^+, its affinity for K^+, and its activation by NEM and cell swelling [21,22,24,25,34,42–44]. NEM activation of K^+ transport is characterized by a selective increase in the V_{max} of the Na^+-independent, chloride-dependent, saturable component [21,26]. In contrast to chloride-dependent Na^+/K^+ cotransport, which is highly sensitive to loop diuretics [24,41,45], Na^+-independent, NEM-activated K^+ transport is relatively insensitive to bumetanide and furosemide [21,24,25,43]. Thus, it is not surprising that the heightened response to NEM we observed following mild peroxidation appeared to have both furosemide-sensitive and -insensitive components. However, additional work, encompassing analysis of kinetic parameters, diuretic sensitivity and dependence on Na^+, pH and cell volume, will be required to establish definitively the relationship of the K^+ influx component augmented by H_2O_2 to NEM-activated transport in unmodified cells.

Mechanism

Oxidation of thiols can yield disulfides where steric factors favor cross-linking, but peroxide and other strong oxidizing agents tend to generate sulfenic, sulfinic and sulfonic acids, successively [46–49]. Red cells exposed to high levels of H_2O_2 (6.7 mM [15]; 30 mM [18]) exhibit excessive K^+ leakage. Van der Zee et al. [18] have found that the magnitude of the induced leak could be inhibited by diamide but not by pretreatment with carbon monoxide, suggesting that direct oxidation of membrane thiols could contribute to the formation of leak pathways under severe conditions of peroxidative stress. However, these reactions probably do not make a major contribution to the modification in transport properties that we observe at much lower levels of oxidant. The observation that carbon monoxide pretreatment eliminates the heightened NEM responsiveness of the treated cells instead implicates hemoglobin or hemoglobin-catalyzed reactions. Catalytic activities of heme proteins in both thiol oxidation [48] and lipid peroxidation [50–52] have been demonstrated. Trotta et al. [53] have observed that carbonmonoxyhemoglobin was an effective initiator of lipid peroxidation in red cells exposed to *t*-butylhydroperoxide, and Snyder et al. [8] have found that carbon monoxide inhibition of spectrin-hemoglobin cross-linking in cells exposed to low levels of H_2O_2 was not associated with inhibition of lipid peroxidation. Conversely, BHT is an effective inhibitor of lipid peroxidation but is relatively ineffective in preventing spectrin-hemoglobin cross-linking [8] and heightened NEM responsiveness of K^+ transport in peroxide-stressed cells (Fig. 4). Furthermore, we have also observed enhancement when H_2O_2 was applied after NEM treatment, despite the fact that NEM-pretreated cells generate much lower levels of lipid peroxidation products under these conditions [9]. Overall, our results strongly suggest that the latent effect of mild peroxidation involves the cascade of activated, unstable hemoglobin species leading from methemoglobin to hemichromes [1,2], but that the concomitant lipid peroxidation reactions are not responsible for the altered responsiveness to NEM.

This proposition is consistent with the observation that membranes isolated from red cells subjected to mild peroxidation retain much higher levels of hemoglobin [9]. Binding of partially denatured hemoglobin molecules to the cytoplasmic domain of Band 3 [54,55] might induce conformational changes that promote expression of K^+ transport activated by NEM. Alternatively, enhancement by H_2O_2 could be mediated by membrane thiol oxidation catalyzed by the activated intermediates derived from methemoglobin [48]. The appearance of reducible high-molecular-weight complexes of membrane proteins in cells exposed to H_2O_2 [15] is direct evidence that thiol oxidation occurs under these conditions. The thiol oxidation and alkylation reactions proceed in the face of a large excess of reactive intracellular thiol groups presented by GSH and hemoglobin [33], and the critical reactions probably take place in a zone of depletion at the membrane-cytosol interface where the diffusible reactants meet and are mutually consumed. In this microenvironment, the effective concentration of membrane-bound hemoglobin [54,55] may favor selective attack on membrane protein thiols over reaction with GSH during exposure to H_2O_2. Red cell membrane protein thiol groups are known to vary markedly in their reactivity with NEM [56], but the pattern of modification corresponding to optimum stimulation of the chloride-dependent K^+ transport pathway has not yet been described biochemically.

Despite the sequestration of thiol groups that results from disulfide formation and other oxidative reactions, overall labeling of membrane proteins by [^{14}C]NEM applied to intact cells is markedly increased by pretreatment with 315 μM H_2O_2 [9]. Considering that intracellular GSH levels are maintained in the face of mild oxidative stress during pretreatment (Table II and Ref. 9), these labeling results imply that the enhancement of NEM-activated transport is associated with a global change in membrane proteins. There is evidence for inhibitory [23,25] as well as stimulatory [19–27] sites of NEM action on the Na^+-independent, chloride-dependent K^+ transport system. However, our observation that NEM activation is augmented by a subsequent exposure to H_2O_2 (Tables III and IV, Fig. 8) demonstrates that H_2O_2 enhancement does not result from a shift in the balance of inhibitory and stimulatory NEM reactions.

To our knowledge, the decay of NEM activation has not been studied previously. Since reaction of NEM with thiols yields mainly stable thioethers [57], it is

unlikely that the decline represents restoration of themodified thiols. (The possibility that other reactions of NEM [58] are facilitated in the microenvironment of the site of activation is remote, but cannot be excluded entirely.) Our observation that a second treatment with NEM can elicit a second increment in activity (Table III, Fig. 8), even while the stimulated activity is decaying (Table IV), implies, in general, that dissipative processes – such as leakage of K^+ and depletion of ATP – cannot be responsible for the short-term attenuation of NEM activated K^+ influx.

Lauf [59] has schematized the regulation of NEM-activated K^+ transport in red cells in terms of closely coupled but functionally distinct domains responsive to NEM, metal ions and changes in cell volume. At this stage, these entities are abstractions and do not necessarily correspond to physical domains of the transporter molecule itself. On the basis of our observations, we propose, as a working hypothesis, that the locus of action of NEM, H_2O_2, cell swelling and other factors is a subpopulation of Band 3 molecules that are associated through their cytoplasmic domains with membrane skeletal proteins, glycolytic enzymes and/or hemoglobin [54,55,60], and to which the NEM-responsive K^+ transporter is also bound. Reaction 1 outlines a scheme in which membrane modification by NEM causes Band 3 (the anion channel, A) to adopt an altered conformation, A′, in which its postulated affinity for the K^+ transporter, T, is markedly reduced. In this scheme we speculate that the transporter is inactive when bound, but converted to an active form, T′, when dissociated.

$$A + T \rightleftharpoons (A \cdot T) \xrightarrow{NEM} (A' \cdot T) \rightleftharpoons A' + T^* \quad (1)$$

In the case of activation induced by swelling, the effect might be translated via the cytoplasmic domain of Band 3 in response to altered tension in the membrane skeleton. The effect of NEM might involve reaction with Band 3 directly, modification of associated peripheral proteins [9], or both. Activation of transport activity by induced dissociation of a membrane protein complex, as in Reaction 1, has been proposed by Farquharson and Dunham [61] as the basis for anti-L stimulation of Na^+/K^+-pump activity in sheep red cells.

An alternative scheme outlined in Reaction 2

$$A + T \rightleftharpoons (A \cdot T) \xrightarrow{NEM} (A' \cdot T^*) \rightleftharpoons A' + T \quad (2)$$

envisions that the transporter is inactive when free, but active in the putative complex, $(A' \cdot T^*)$ when the conformation of Band 3 is altered by NEM treatment. As in Reaction 1, conversion of A to A′ need not require direct modification of Band 3, but might be induced indirectly through the cytoplasmic domain of Band 3 in response to modification of associated proteins by NEM or cell swelling. The concept of Band 3 centered complexes, in which transport systems interact with proteins associated with the cytoplasmic domains has been developed by Fossel and Solomon [60] and is based on studies of transport-inhibitor effects on states of metabolic intermediates assessed by ^{31}P nuclear magnetic resonance.

The phenomenon of H_2O_2 enhancement can be rationalized in either scheme by considering the hypothesis that mild peroxidation is not sufficient in itself to induce the putative changes in Band 3 conformation that regulate transport activity, but that it amplifies the response to NEM modification. For example, tight binding of hemichromes to the cytoplasmic domain [54,55] might alter the equilibria in Reactions 1 and 2. DIDS inhibition can be understood if the conformational changes produced by its tight binding to Band 3 [62] prevent dissociation (Reaction 1) or binding (Reaction 2) of the transporter. This general line of thinking also provides straightforward explanations for the phenomena of decay and repeated activation. In Reaction 1, decay of stimulated activity would occur as active transporter molecules, T*, diffuse in the plane of the membrane and bind to unmodified Band 3 molecules. In Reaction 2, decay would result from dissociation of the active complex, $(A' \cdot T^*)$. In both schemes, reassortment of peripheral proteins associated with A′ might also be the basis for conversion to an inactive configuration of Band 3. (Similarly, assembly of complexes of glycolytic enzymes with Band 3 under conditions of metabolic depletion [60] might underlie the metabolic requirement for NEM activation [22].) In all cases, the system would retain the capacity to undergo a second cycle of activation after an episode of decay. Because of this feature, in particular, these schemes merit consideration in future studies of the biochemical basis for NEM activation.

Passive K^+ transport in HX

The results presented here demonstrate that peroxide pretreatment does not make HX erythrocytes responsive to NEM. In these dehydrated cells, the K^+ transport pathway activated by NEM and cell swelling is suppressed [27]. (HX in this kindred is also characterized by subnormal levels of 2,3-DPG [10–13] and increased membrane retention of glyceraldehyde-3-phosphate dehydrogenase [12], findings consistent with our speculation that peripheral associations with Band 3 are involved in regulating the activity.) There is an elevation in total K^+ influx (Fig. 1 and Ref. 27), but this abnormality reflects high activity of the furosemide-sensitive Na^+/K^+ cotransport system [41], which is partially inhibited by NEM (unpublished observations). Interestingly, K^+ transport activation by NEM is slight in the denser subpopulations of normal red cells, sug-

gesting that responsiveness to NEM is a property of the younger, relatively hydrated cells (Fig. 9 and Ref. 34). Reticulocytosis in these patients is usually about 4% [63]. Cells that are chronologically young predominate [10,11], and NEM activation can be demonstrated in the most reticulocyte-enriched, buoyant fractions (unpublished observations). However, because of their rapid dehydration in vivo and increased NEM-inhibited, furosemide-sensitive K^+ transport activity, unfractionated hereditary xerocytosis cells appear to be unresponsive. We have found that the NEM-responsive component is not destroyed or removed altogether, as it reappears in HX red cells that have been rehydrated (unpublished observations).

Significance in normal red cells

The physiological significance of the effect of mild peroxidation on NEM activation depends on the function served by that component of passive K^+ transport and on the extent to which the peroxidation in vitro can be considered to mimic endogenous oxidative stress in vivo. Chloride-dependent K^+ transport activated by NEM or cell swelling appears to be capable of mediating net efflux of K^+ [34,42,64]. Its expression in young red blood cells may bring about net K^+ loss and cell shrinkage to surface/volume ratios appropriate for mature cells [34,65]. Our results suggest that, under conditions that elicit the activity, cells that have suffered subacute oxidative damage might respond with hyperactivation or activation at normal volume, leading to excessive loss of K^+ and dehydration. Considering that cell dehydration appears to make red cells more vulnerable to some forms of oxidative damage [15], the heightened activation of K^+ transport following such damage could be a link in a degenerative cycle shortening the functional life of the cell.

Acknowledgments

This work was supported by National Institutes of Health grants (HL19933 and CA12708) and by the Harold G. and Leila Y. Mathers Charitable Foundation of White Plains, New York. We are grateful to Kevin J. Gaffney and Bonnie J. Germain for excellent technical assistance and thank Dr. Carol A. Ziomek for guidance and encouragement in the use of her computer and software for statistical analysis.

References

1 Carrell, R.W., Winterbourn, C.C. and Rachmilewitz, E.A. (1975) Br. J. Haematol. 30, 259–264.
2 Hebbel, R.P. (1985) Clin. Haematol. 14, 129–140.
3 Rachmilewitz, E.A., Shinar, E., Shalev, O., Galili, U. and Schrier, S.L. (1985) Clin. Haematol. 14, 163–182.
4 Clark, M.R. and Shohet, S. (1985) Clin. Haematol. 14, 223–257.
5 Kay, M.M.B., Bosman, G.J.C.G.M., Shapiro, S.S., Bendich, A. and Bassel, P.S. (1986) Proc. Natl. Acad. Sci. USA 83, 2463–2467.
6 Sauberman, N., Fortier, N.L., Joshi, W., Piotrowski, J. and Snyder, L.M. (1983) Br. J. Haematol. 54, 51–28.
7 Snyder, L.M., Fortier, N.L., Sauberman, N., Leb, L., Trainor (Piotrowski), J. and Fairbanks, G. (1984) Prog. Clin. Biol. Res. 159, 227–242.
8 Snyder, L.M., Fortier, N.L., Trianor, J., Jacobs, J., Leb, L., Lubin, B., Chiu, D., Shohet, S. and Mohandas, N. (1985) J. Clin. Invest. 76, 1971–1977.
9 Snyder, L.M., Fortier, N.L., Leb, L., McKenney, J., Trainor, J., Sheerin, H. and Mohandas, N. (1988) Biochim. Biophys. Acta 937, 229–240.
10 Glader, B.E., Fortier, N.L. and Snyder, L.M. (1976) Clin. Res. 24, 309A.
11 Snyder, L.M., Lutz, H.U., Sauberman, N., Jacobs, J. and Fortier, N.L. (1978) Blood 52, 750–761.
12 Fairbanks, G., Dino, J.E., Fortier, N.L. and Snyder, L.M. (1978) Prog. Clin. Biol. Res. 20, 173–184.
13 Harm, W., Fortier, N.L., Lutz, H.U., Fairbanks, G. and Snyder, L.M. (1979) Clin. Chim. Acta 99, 121–128.
14 Lande, W.M. and Mentzer, W.C. (1985) Clin. Haematol. 14(1), 89–103.
15 Snyder, L.M., Sauberman, N., Condara, H., Dolan, J., Jacobs, J., Szymanski, I. and Fortier, N.L. (1981) Br. J. Haematol. 48, 435–444
16 Heller, K.B., Poser, B., Haest, C.W.M. and Deuticke, B. (1984) Biochim. Biophys. Acta 777, 107–116.
17 [illegible] (1985) J. Clin. Invest. 75, 1931–1937.
18 Van der Zee, J., Dubbelman, T.M.A.R. and Van Steveninck, J. (1985) Biochim. Biophys. Acta 818, 38–44.
19 Lauf, P.K. and Theg, B.E. (1980) Biochem. Biophys. Res. Commun. 92, 1422–1428.
20 Lauf, P.K. (1983) J. Membr. Biol. 73, 237–246.
21 Lauf, P.K., Adragna, N.C. and Garay, R.P. (1984) Am. J. Physiol. 246, C385–C390.
22 Lauf, P.K., Perkins, C.M. and Adragna, N.C. (1985) Am. J. Physiol. 249, C124–C128.
23 Logue, P., Anderson, C., Kanik, C., Farquharson, B. and Dunham, P. (1983) J. Gen. Physiol. 81, 861–885.
24 Ellory, J.C., Dunham, P.B., Logue, P.J. and Stewart, G.W. (1982) Phil. Trans. R. Soc. Lond. Ser. B, 483–495.
25 Wiater, L.A. and Dunham, P.B. (1983) Am. J. Physiol. 245, C348–C356.
26 Kaji, D. and Kahn, T. (1985) Am. J. Physiol. 249, C490–C496.
27 Fairbanks, G., Dino, J.E. and Snyder, L.M. (1984) Prog. Clin. Biol. Res. 159, 205–217.
28 Sheerin, H.E., Fairbanks, G., Trainor, J. and Snyder, L.M. (1985) Blood 66, Suppl. 1, 39a
29 Beutler, E. (1975) Red Cell Metabolism: A Manual of Biochemical Methods, Grune and Stratton, New York.
30 Snyder, L.M., Leb, L., Piotrowski, J., Sauberman, N., Liu, S.C. and Fortier, N.L. (1983) Br. J. Haematol. 53, 379–384.
31 Dunham, P.B., Stewart, G.W. and Ellory, J.C. (1980) Proc. Natl. Acad. Sci. USA 77, 1711–1715.
32 Dunham, P.B. and Ellory, J.C. (1980) J. Physiol. 301, 25–37.
33 Jacob, H.S. and Jandl, J.H. (1962) J. Clin. Invest. 41, 779–792.
34 Brugnara, C. and Tosteson, D.C. (1987) Am. J. Physiol. 252, C269–C276.
35 Knauf, P. (1979) in Current Topics in Membranes and Transport, Vol. 12 (Bronner, F. and Kleinzeller, A., eds.), pp. 249–363, Academic Press, New York.
36 Orringer, E.P. (1984) Am. J. Hematol. 16, 355–366.
37 Orringer, E.P. and Parker, J.C. (1977) Blood 50, 1013–1021.
38 Maridonneau, I., Braquet, P. and Garay, R.P. (1983) J. Biol. Chem. 258, 3107–3113.
39 Deuticke, B., Heller, K.B. and Haest, C.W.M. (1986) Biochim. Biophys. Acta 854, 169–183.
40 Deuticke, B., Heller, K.B. and Haest, C.W.M. (1987) Biochim. Biophys. Acta 899, 113–124.

41 Wiley, J.S. and Cooper, R.A. (1974) J. Clin. Invest. 53, 745–755.
42 Kaji, D. (1986) J. Gen. Physiol. 88, 719–738.
43 Duhm, J. (1987) J. Membr. Biol. 98, 15–32.
44 O'Neill, W.C. (1987) Am. J. Physiol. 253, C883–C888.
45 Chipperfield, A.R. (1985) J. Physiol. 369, 61–77.
46 Cecil, R. (1963) in The Proteins: Composition, Structure, and Function, 2nd Edn. (Neurath, H., ed.), Vol. 1, pp. 379–476, Academic Press, New York.
47 Little, C. and O'Brien, P.J. (1967) Arch. Biochem. Biophys. 122, 406–410.
48 Little, C. and O'Brien, P.J. (1969) Eur. J. Biochem. 10, 533–538.
49 Neumann, N.P. (1972) Methods Enzymol. 25, 393–409.
50 Sadrzadeh, S.M.H., Graf, E., Panter, S.S., Hallaway, P.E. and Eaton, J.W. (1984) J. Biol. Chem. 259, 14354–14356.
51 Szebeni, J., Winterbourn, C.C. and Carrell, R.W. (1984) Biochem. J. 220, 685–692.
52 Clemens, M.R., Einsele, H., Remmer, H. and Waller, H.D. (1985) Biochem. Pharmacol. 34, 1339–1341.
53 Trotta, R.J., Sullivan, S.G. and Stern, A. (1983) Biochem. J. 212, 759–772.
54 Low, P.S. (1985) Biochim. Biophys. Acta 864, 145–167.
55 Waugh, S.M. and Low, P.S. (1985) Biochemistry 24, 34–39.
56 Haest, C.W.M., Kamp, D. and Deuticke, B. (1981) Biochim. Biophys. Acta 643, 319–326.
57 Riordan, J.F. and Vallee, B.L. (1972) Methods Enzymol. 25, 449–456.
58 Brewer, C.F. and Riehm, J.P. (1967) Anal. Biochem. 18, 248–255.
59 Lauf, P.K. (1985) J. Membr. Biol. 88, 1–13.
60 Fossel, E.T. and Solomon, A.K. (1981) Biochim. Biophys. Acta 649, 557–571.
61 Farquharson, B.E. and Dunham, P.B. (1986) Biochem. Biophys. Res. Commun. 134, 982–988.
62 Macara, I.G., Kuo, S. and Cantley, L.C. (1983) J. Biol. Chem. 258, 1785–1792.
63 Sauberman, N., Fortier, N.L., Fairbanks, G., O'Connor, R.J. and Snyder, L.M. (1979) Biochim. Biophys. Acta 556, 292–313.
64 Berkowitz, L.R. and Orringer, E.P. (1987) Am. J. Physiol. 252, C300–C306.
65 Hall, A.C. and Ellory, J.C. (1986) Biochim. Biophys. Acta 858, 317–320.