Biochimica et Biophysica Acta, 772 (1984) 77–83
Elsevier

BBA 72052

TRANSFER OF Tris BUFFER AND EFFECTS ON K^+ LOSS IN HUMAN RED BLOOD CELLS AND RECONSTITUTED GHOSTS

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(Received October 20th, 1983)

Key words: Tromethamine; K^+ efflux; Tris effect; Membrane permeability; Hemoglobin; (Erythrocyte)

(1) The rate of volume changes of human red blood cells in the presence of Tris-HCl is pH-dependent. At 37°C, $t_{1/2}$ is 25–30 min at pH 7.4 and 10–20 min at pH 8.4. Hemolysis in Tris-HCl is delayed by H_2DIDS but is promoted by low concentrations of bicarbonate. This bicarbonate effect has been reversed by inhibiting carbonic anhydrase with acetazolamide. (2) K^+ loss of red blood cells is increased at 37°C in isotonic NaCl solutions containing in addition Tris-HCl. This Tris effect is enhanced from pH 6.4 to 8.4. At pH 8.4 K^+ loss is stimulated about 3-fold by addition of 160 mM Tris-HCl. The onset of the Tris effect is delayed at pH 7.4 and below, but not at pH 8.4. Such a delay is absent after preincubation of the cells with Tris-HCl. After binding H_2DIDS to red cells, no Tris-dependent increase of K^+ loss has been observed. (3) K^+ loss of reconstituted red cell ghosts with equal internal and external chloride concentrations remained unaffected by Tris-HCl added to the external solution. In ghosts containing sucrose for isotonicity instead of choline chloride K^+ loss is smaller but is stimulated by Tris-HCl approaching the rate in those ghosts with equal internal and external chloride concentrations. (4) The transfer of Tris-HCl into red blood cells depends on the pH and on the chloride shift. As there is evidence that Tris-HCl raises the intracellular pH and reduces the Donnan potential at the membrane, K^+ loss of red cells may be increased following an intracellular buffer interaction of hemoglobin and Tris-HCl.

Introduction

Tris (synonyms: tromethamine, THAM) represents an alternative to bicarbonate buffer for regulating acid-base-disturbances. A dose of 3 to 5 mM Tris per kg of body weight administered to healthy probands has been tolerated well [1]. The application of Tris up to 18 mM per kg of body weight was accompanied by a shift of K^+ from the intracellular to the extracellular compartment in dogs as well as in volunteers and in patients with metabolic acidosis due to renal disorders [2]. A similar K^+ shift was observed in studies on unicellular organisms [2]. In human red blood cells Luthra et al. [3] found an increased K^+ and Na^+ loss in the presence of Tris-HCl. These effect coincided or were preceded by a transfer of Tris into the cells [4]. Other effects of Tris include those on enzyme activities [5–7], Ca^{2+} flux [8,9], neuromuscular transmission [10], ventilation [2], blood sugar levels [2], and on structure and function of the membrane in bacteria [11,12] and in liver [13]. None of these latter effects has been related to the K^+ shift in the presence of Tris.

Because intracellular buffering by Tris may be fundamental for its effects on the transfer of K^+ across the membrane this paper examines the diffusion of Tris-HCl into human red blood cells.

Abbreviations: DMO, 5,5-dimethyloxazolidine-2,4-dione; H_2DIDS, 4,4′-diisothiocyanodihydrostilbene-2,2′-disulfonate.

Further analyses concern Tris effects on K^+ loss of red cells and reconstituted red cell ghosts. Based on the experimental evidence the possible mechanism is delineated by which Tris-HCl interferes with K^+ efflux in human red blood cells obviously without damaging the membrane.

Methods

Human red blood cells of freshly drawn blood (with 0.1 mg/dl heparin) were washed three times in 5 vol. of a solution containing isotonic NaCl or $MgCl_2$/sucrose. Reconstituted red cell ghosts were prepared and washed according to the technique described previously [14]. K^+ and Hb were determined at the indicated times in the supernatants and in the suspensions by flame photometry and by extinction at 414 nm (Soret band).

The distribution of 5,5-dimethyloxazolidine-2,4-dione (DMO) was determined 1 h after adding a trace of [^{14}C]DMO to the suspensions. ^{14}C activity was measured in cell water and supernatant after deproteinization by mixing the sample with an equal volume of 10% trichloroacetic acid. Cell water was determined by measuring wet and dry weights of the sediments. The difference between external and internal pH was calculated according to the equation given by Schloerb and Grantham [15].

For binding 4,4′-diisothiocyanodihydrostilbene-2,2′-disulfonate (H_2DIDS), an inhibitor of the anion exchange system [16], red cells were washed four times in 10 parts of an unbuffered solution containing 166 mM KCl, and were suspended at 10% hematocrit in a solution containing additionally 20 μM of H_2DIDS. Incubation was performed for 50 min at 37°C. Control cells were treated in the same way but without H_2DIDS.

All solutions were controlled for osmolarity and were adjusted to the specified pH at room temperature. The pH of Tris buffer was approx. 8.2 at 20°C [17] and was shifted to approx. 7.8 at 37°C. When indicated the solutions contained 10^{-4} M ouabain.

^{36}Cl was obtained from NEN-Chemicals, [^{14}C]DMO from Amersham Buchler. H_2DIDS was kindly made available by Dr. H. Passow. Acetazolamide (Diamox® parenteral) was obtained from Cyanamid.

Results

I. Volume changes and hemolysis of red cells in the presence of Tris-HCl

Red cells when placed into hypertonic solution containing isotonic salt concentrations and additional Tris-HCl, initially shrink and then slowly swell up. After uptake of Tris-HCl the cells initially swell up in a salt solution without Tris-HCl and then slowly shrink.

Fig. 1 shows the time-course of swelling (A) in an isotonic $MgCl_2$/sucrose solution plus 170 mM Tris-HCl, and of shrinkage of the cells after the uptake of Tris-HCl (B) in a solution containing the same concentrations of $MgCl_2$ and sucrose but only 10 mM Tris-HCl. $t_{1/2}$ of swelling and shrin-

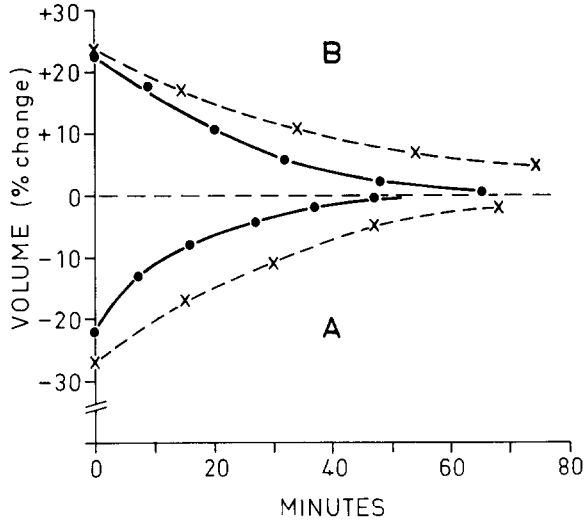

Fig. 1. Volume changes of red cells due to movements of Tris buffer across the membrane. Washed red cells (3 g) were suspended in a medium (7 ml) containing 85 mM $MgCl_2$, 60 mM sucrose and 10 mM Tris-HCl, and were incubated for about 5 min at 37°C. (A) At zero-time, 0.4 ml of a solution containing 3 M Tris-HCl were added to the suspension. The resulting concentrations of Tris-HCl in the incubation medium was approx. 170 mM. The hematocrit was determined immediately and after the indicated times of incubation at 37°C in quadruplicate and was correlated with the values at equilibrium obtained after 3 h of incubation. The pH of the solution was either 7.4 (×- -×) or 8.4 (●———●). (B) After completing part (A), the cells equilibrated with Tris-HCl were spun down. 80% of the clear supernatant was taken off and replaced at zero-time by the same volume of the original medium containing 85 mM $MgCl_2$, 60 mM sucrose and 10 mM Tris-HCl. The pH was 7.4 (×- -×) or 8.4 (●———●).

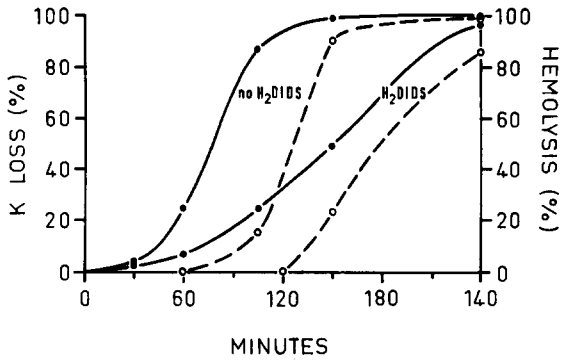

Fig. 2. Effects of H_2DIDS on K^+ loss and hemolysis of red cells in isotonic Tris-HCl solution. K^+ loss (●———●) and hemolysis (○- - -○) were measured in a solution containing 200 mM Tris-HCl, pH 7.4, at 37°C. The hematocrit was 5%. For binding H_2DIDS, the cells were preincubated for 50 min at 37°C in an unbuffered isotonic KCl solution containing in addition 20 μM H_2DIDS. The same type of results was obtained in another experiment of similar design.

kage at pH 8.4 and 7.4 is 10 to 20 min and 25 to 30 min, respectively. Almost identical results have been obtained when using reconstituted ghosts instead of intact red cells.

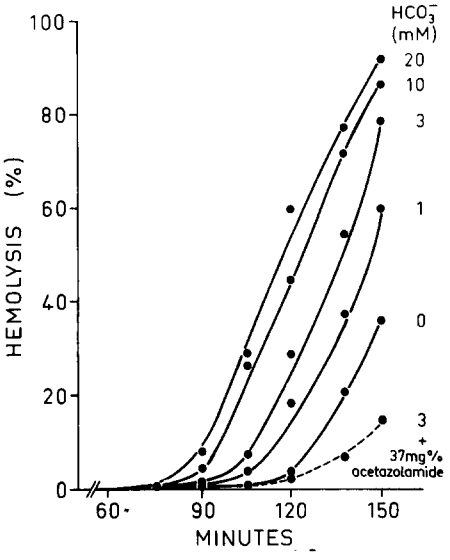

Fig. 3. Effect of bicarbonate on hemolysis in isotonic Tris-HCl solution. Red cells were incubated at 37°C in solutions containing 200 mM Tris-HCl at pH 7.7 and the indicated concentrations of $NaHCO_3$. NaCl was added to keep (NaCl + $NaHCO_3$) at 20 mM. The hematocrit was 8%. Hemoglobin was determined in duplicates at the indicated times, and is expressed in percentage of the original hemoglobin content of the cells. The dotted line indicates hemolysis in the presence of bicarbonate and acetazolamide.

Incubation of red cells in isotonic Tris-HCl solutions terminates into osmotic hemolysis which is preceded by a rapid K^+ loss (Fig. 2). K^+ loss and hemolysis are delayed in red cells after inhibition of the anion exchange system with H_2DIDS (Fig. 2), but can be promoted by the addition of relatively low concentrations of bicarbonate (Fig. 3). This bicarbonate effect is reversed with acetazolamide (Fig. 3) presumably by the inhibition of carbonic anhydrase.

II. Effects of Tris-HCl on K^+ loss of red cells

In isotonic NaCl hemolysis remains negligibly small when adding Tris-HCl. Nevertheless, K^+ loss is increased in relation to the Tris concentration and the pH (Fig. 4) and is raised about 3-fold in the presence of 160 mM Tris-HCl at pH 8.4.

At pH 7.4, but not at 8.4, the onset of the Tris effects is delayed. The delay indicates the possibility that Tris-HCl enters the cell first before it stimulates K^+ loss and does so at a slower rate below pH 8.4. Therefore, further red cells were equilibrated with 220 mM Tris-HCl by preincubation at pH 8.4, or were preincubated with 10 mM Tris-HCl for control. By preincubation with 220 mM Tris-HCl instead of 10 mM Tris-HCl, under

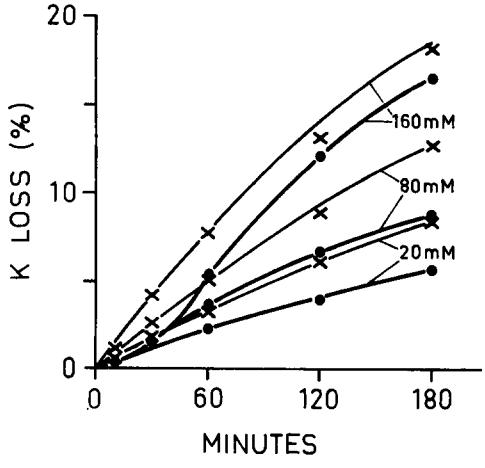

Fig. 4. Effects of Tris-HCl on K^+ loss of red cells in isotonic NaCl solution. Red cells were incubated at 37°C (hematocrit 9%) in solutions containing 165 mM NaCl, 10^{-4} M ouabain, and the indicated concentrations of Tris-HCl. The pH was 7.55 (●———●) or 8.4 (×——— ×). The analyses of the experiment were performed in duplicate. The same type of results was obtained in several experiments of similar design.

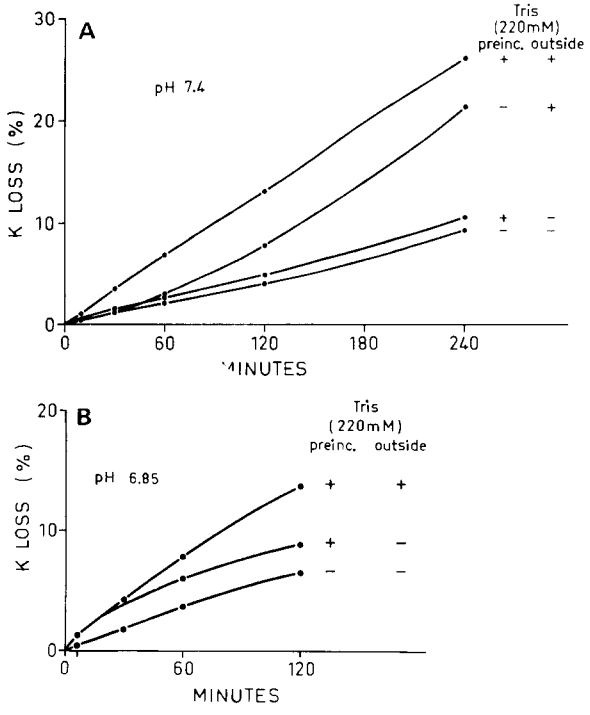

Fig. 5. K^+ loss of red cells after preincubation with high or with negligible Tris. Red cells were preincubated at 37°C for 15 min in a medium containing 110 mM KCl, 40 mM NaCl, and either 10 mM Tris-HCl (preinc. −) or 220 mM Tris-HCl (preinc. +) at pH 8.4 (hematocrit 10%). Then the cells were washed three times in ice-cold solutions containing 150 mM NaCl plus 10 mM Tris-HCl or 150 mM NaCl plus 220 mM Tris-HCl and resuspended at hematocrit 9% in solutions containing either 330 mM NaCl and 15 mosM imidazole-HCl (Tris: outside −) or 165 mM NaCl, 220 mM Tris-HCl and 15 mosM imidazole-HCl (Tris: outside +). K^+ loss was followed at 37°C in the presence of 10^{-4} ouabain and was determined in the total suspension and in the supernatants after centrifugation. Original K^+ content of the cells (mmol/l cells): 'Tris: inside −' 86.3; 'Tris: inside +' 91.2. (A) pH 7.4. (B) pH 6.85. The points in the figure represent the mean values obtained in four experiments.

the given experimental conditions, the ratio of internal versus external [^{14}C]DMO indicates a shift of the difference between external and internal pH from 0.224 to 0.060. Fig. 5 reveals that, after preincubation with 220 mM Tris-HCl, K^+ loss is increased without delay in the presence of the same concentrations of external Tris-HCl at pH 6.85, as well as pH 7.4. At pH 6.85 stimulation occurs initially after preincubation with 220 mM Tris-HCl even with low external Tris-HCl. This stimulation which depends on the preincubation with 220 mM Tris-HCl, is significant at pH 6.85 and 7.4 (Table I). At pH 8.4, K^+ loss is unaffected by the preincubation with 220 mM Tris-HCl and is increased only in the presence of 220 mM Tris-HCl in the external solution (data not shown).

TABLE I

SIGNIFICANCES OF INCREASES OF K^+ LOSS AFTER PREINCUBATION OF RED CELLS WITH HIGH Tris-HCl

The experimental details are those described in the legend to Fig. 5. The table contains the differences in the average K^+ loss ($\Delta \bar{K}$) and the standard deviations (S.D.) of cells preincubated with 10 mM or 220 mM Tris-HCl, respectively. The final flux media contained either 330 mM NaCl plus 15 mosM imidazole-HCl (= NaCl) or 165 mM NaCl, 15 mosM imidazole-HCl and 220 mM Tris-HCl (= NaCl + Tris). All solutions contained in addition 10^{-4} M ouabain. The significance of the differences was calculated with the *t*-test and is indicated by *P*; n.s. = not significant ($P > 0.025$). The values are derived from eight experiments. The average K^+ content of the cells at the onset of the flux measurements was 94.1 mmol/l cells after preincubation in 220 mM Tris-HCl, and 86.3 mmol/l cells after preincubation in 10 mM Tris-HCl.

pH	Time (min)	Outside NaCl + Tris		Outside NaCl	
		$\Delta \bar{K} \pm$S.D. (mM)	*P*	$\Delta \bar{K} \pm$S.D. (mM)	*P*
6.85	10	0.2 ± 0.2	n.s.	0.3 ± 0.2	< 0.005
	30	0.9 ± 0.4	< 0.005	0.7 ± 0.6	< 0.025
	60	2.0 ± 1.0	< 0.005	0.9 ± 0.6	< 0.005
	120	3.8 ± 1.8	< 0.005	1.2 ± 0.9	< 0.005
7.4	10	0.3 ± 0.4	n.s.	0.1 ± 0.4	n.s.
	30	1.8 ± 1.0	< 0.005	0.3 ± 0.5	n.s.
	60	3.2 ± 1.6	< 0.005	0.4 ± 0.5	n.s.
	120	4.1 ± 1.9	< 0.005	0.6 ± 1.1	n.s.

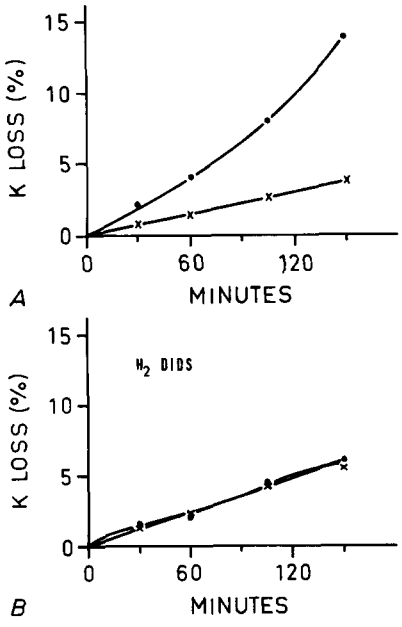

Fig. 6. Effects of Tris-HCl on K^+ loss of red cells treated with H_2DIDS and of control cells without H_2DIDS. K^+ loss was measured at 37°C in solutions containing 160 mM NaCl, 10 mM Tris-HCl, pH 7.4 (×——×) or 160 mM NaCl, 200 mM Tris-HCl, pH 8.4 (●——●). All solutions contained in addition 10^{-4} M ouabain. The hematocrit was 5%. (A) Control cells without H_2DIDS. (B) H_2DIDS cells. Analogous results were obtained in another experiment of similar design.

After treating the cells with H_2DIDS the stimulation of K^+ loss by Tris-HCl, which is seen in untreated cells (Fig. 6A), is abolished (Fig. 6B).

III. Effects of Tris-HCl on K^+ loss of reconstituted ghosts

K^+ loss of reconstituted ghosts is considerably higher than that of red cells and displays a distinct pH-dependency (Fig. 7). It remains unaffected by Tris-HCl if the internal and external chloride concentrations are about the same (Fig. 7). By lowering the chloride concentrations inside the ghosts with sucrose the following K^+ loss is considerably diminished (Fig. 7). In this situation stimulation of K^+ loss by Tris-HCl reappears and approaches after some delay the rate of K^+ loss in ghosts with equal internal and external chloride concentrations. The distribution of [^{14}C]DMO under these conditions reveals that the difference between external and internal pH is higher in ghosts containing sucrose instead of choline chloride (Table II) but that this difference is diminished in the presence of Tris-HCl.

Discussion

This work confirms findings of Omachi et al. [4] that Tris buffer predominantly enters the red cells in the uncharged form. Since about two-thirds of Tris in the solution exists in the uncharged form at pH 8.4 and 37°C, in contrast to about one-third at

TABLE II

DIFFERENCE BETWEEN EXTERNAL AND INTERNAL pH, pH_e AND pH_i, IN RECONSTITUTED RED CELL GHOSTS DERIVED FROM THE RATIO OF INTERNAL VERSUS EXTERNAL DMO

Reconstituted ghosts contained the indicated concentrations and in addition (mM) 4 $MgCl_2$, 2 Na_2ATP, 7 Tris-HCl (pH 7.4) and 40 KCl in the original hemolysates. After washing the ghosts were suspended in hypertonic solutions containing the indicated concentrations and in addition 140 mM $MgCl_2$ and a trace amount of [^{14}C]DMO. After incubation at 37°C for 1 h ^{14}C activities were determined in cell water and external solution in duplicates.

External pH	n	Internal (mM)		External (mM)		$pH_e - pH_i$
		Choline Cl	Sucrose	NaCl	Tris-HCl	
7.4	4	125	0	150	10	0.103
	4	125	0	0	200	0.065
	4	0	250	150	10	0.240
	4	0	250	0	200	0.155
8.4	2	125	0	110	10	0.070
	2	125	0	0	200	0.035
	2	0	250	110	10	0.335
	2	0	250	0	200	0.175

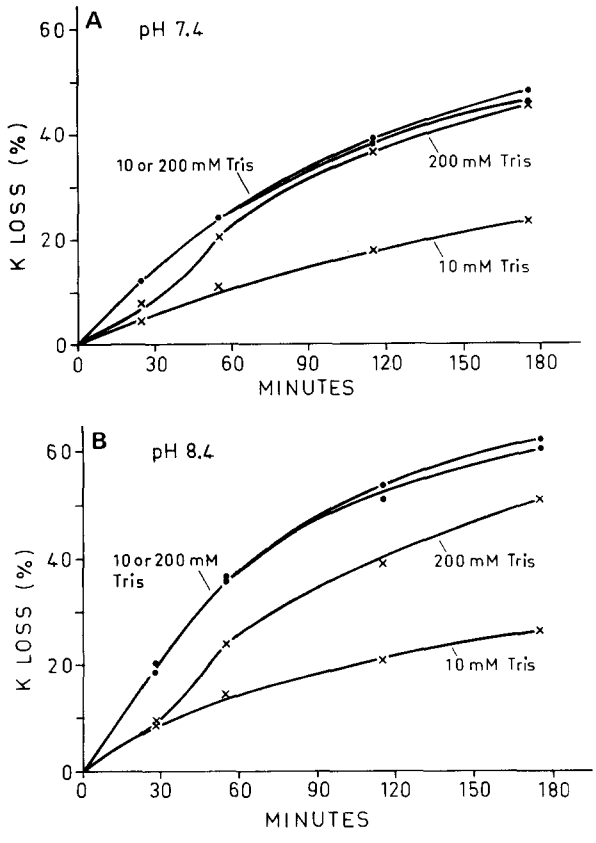

Fig. 7. Effects of Tris-HCl on K^+ loss of reconstituted red cell ghosts containing choline Cl or sucrose for isotonicity. Reconstituted ghosts contained 4 mM $MgCl_2$, 2 mM Na_2ATP, 7 mM Tris, 43 mM KCl and either 125 mM choline chloride (●———●) or 250 mM sucrose (×——— ×) in the original hemolysates. K^+ loss was measured at pH 7.4 (A) and at pH 8.4 (B). All solutions contains 140 mM $MgCl_2$, 10^{-4} M ouabain, and either 10 mM or 200 mM Tris-HCl. 150 mM NaCl were added at pH 7.4 or 110 mM NaCl at pH 8.4 in exchange for Tris-HCl. The hematocrit was 6%, the temperature 37°C. The data in the figure represent the results of two identical experiments.

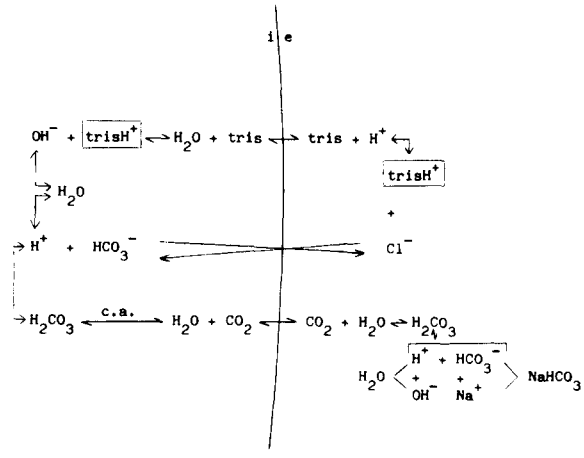

Fig. 8. Catalyzed diffusion of Tris-HCl into the red blood cell in the presence of bicarbonate. The figure shows a modified version of the concept of Jacobs and Stewart [20] for ammonium chloride, 'i' and 'e' refer to the inside and outside of the membrane. c.a., carbonic anhydrase.

pH 7.4 and 37°C [17], the observed ratio of the rates of volume changes at pH 7.4 and 8.4 appears to reflect roughly the ratio of the concentrations of uncharged Tris in the solutions. As the uncharged Tris raises the intracellular pH, the rate of transfer of Tris-HCl into the cells will also depend on the exchange of internal OH^- for external Cl^- or H^+/Cl^- cotransport [18,19]. According to the concept of 'catalyzed diffusion' of ammonium chloride, which was proposed by Jacobs and Stewart in 1942 [20], the addition of small concentrations of bicarbonate to the external medium stimulates HCO_3^-/Cl^- exchange across the membrane and increases the shift of Cl^- and consequently of Tris-HCl into the cells. Fig. 8 shows the presumed mechanism of the transfer of Tris-HCl into the red cells. A half-maximal bicarbonate effect was obtained with about 2 mM bicarbonate. This value may reflect the K_m of bicarbonate for the activation of HCO_3^- exchange. K_m values of 0.4 mM [21], as well as of 10 mM [22], have been reported. The reversion of the bicarbonate effect by inhibiting carbonic anhydrase fits into this concept (Fig. 3).

Although alterations of membrane structure and function in the presence of Tris-HCl have been observed in bacteria and rat liver [11–13], direct changes of the membrane barrier of red cells for K^+ in the presence of Tris are unlikely. No effects were seen in reconstituted ghosts containing isotonic chloride solutions. Also, the experiments have been too short for the accumulation of a phosphorylated Tris-intermediate [23] which might be responsible for an increase of K^+ loss. Changes of active transport of K^+ can be excluded because the Na^+,K^+-pump was already completely inhibited in the presence of ouabain. However, an increased loss of Na^+ in the absence of ouabain which was observed by Luthra et al. [3] might well reflect an activation of the Na^+,K^+-pump by K^+,

leaking out of the cells in the presence of Tris, rather than the depletion of a rapidly exchangeable Na^+ pool as suggested by these authors. Volume changes appear not to be responsible for Tris effects on K^+ loss because stimulation of K^+ loss in the presence of Tris-HCl was seen first in cells after regaining their original volume (Fig. 1).

The delayed onset of the Tris effect on K^+ loss at pH 7.4 and below, and the absence of such a delay after preincubation of the cells with Tris-HCl reflect a requirement of Tris-HCl inside the cells. Provided effectively impermeable red cells for Na^+ and K^+ intracellular Tris-HCl raises the cellular pH and chloride concentration and decreases the Donnan potential at the membrane, parameters, which are controlled by intracellular hemoglobin [24,25]. These changes favour K^+ leakage [26]. Following to this mechanism binding of H_2DIDS to the cells prevents the accumulation of intracellular Tris-HCl and hence effects of Tris-HCl on K^+ loss (Figs. 2 and 6). Reconstituted ghosts which contain little hemoglobin and isotonic chloride solutions lack a significant Donnan potential. In this situation, K^+ loss is unaltered in the presence of Tris-HCl. The addition of sucrose to the internal medium establishes a negative membrane potential. K^+ loss of these ghosts is considerably lowered but approximates the rate observed in ghosts containing isotonic chloride when Tris-HCl is added. Therefore, an interaction of the buffer hemoglobin and Tris-HCl appears to be responsible for an increased K^+ loss of red cells.

Acknowledgement

The authors thank Mrs. Hella Wachter and Mrs. Regine Baur for their excellent technical assistance. This study was supported by the Deutsche Forschungsgemeinschaft.

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