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A Basis of the Crenation of Erythrocyte Ghosts by Electrolytes

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Abstract: Erythrocyte ghosts isolated in hemolytic hypotonic buffers, pH 7.0-8.0, 0-4°C were previously observed to be crenated by electrolytes. This shape transformation, which occurred rapidly and reversibly, was attributed to electrostatic interactions of cations with flexible filamentous anionic spectrin, the major protein component of the skeleton and/or anionic phospholipids, since divalent cationic salts crenated at concentrations substantially lower than those of monovalent cationic salts and that crenation appeared unrelated to the anion species. However, crenation by electrolytes was markedly influenced by ionic conditions and temperature. A mechanism of the erythrocyte shape control has been previously suggested in which band 3 (AE1), exchanging the monovalent anions Cl^- and HCO_3^- and linked to spectrin, plays a pivotal role. Briefly, the alternative recruitment of its inward-facing (band 3_i) and outward-facing (band 3_o) conformations contract and relax the skeleton, thereby promoting echinocytosis and stomatocytosis, respectively. Band 3 transports also other anions, including endogenous inorganic phosphate, but at a slow rate. This mechanism would explain the above observations and would lead to some inferences, one of which is a Cl^--dependent crenation by Mg^{2+} and Ca^{2+}, suggesting that they specifically bind on sites on spectrin.

Keywords: Band 3, Buffer, Crenation, Electrolytes, Spectrin.

INTRODUCTION

Ghosts or post-hemolytic plasma membranes of erythrocytes isolated free of hemoglobin in a hemolytic hypotonic buffer, pH 7.0-8.0, 0-4°C since the early 1960`s may have a shape similar to or reminiscent of the resting biconcave disc shape and other familiar shapes of the erythrocyte, but seemingly influenced by the type of the buffer and the pH [1-9]. They are relatively well characterized with respect to their components and assemblies of these components [5] and can be made impermeable to small solutes by resealing under certain conditions in the presence of Mg^{2+} [1, 10. 11]. They have been used for multiple purposes, including monitoring transport of solutes across the membrane [1, 10, 11], probing membrane glucose metabolism and membrane fluctuations in the oxygenation and deoxygenation cycle, altering glucose metabolism and Mg^{2+} level [12-14], testing hypotheses on the erythrocyte shape [2-8], [15-22] and as drug carriers and electrosensors [23-25]. Ghosts isolated in hemolytic hypotonic buffers, pH 7.0-8.0, 0-4°C were previously observed to be crenated by electrolytes (e.g. NaCl, $NaCH_3COO$, $CaCl_2$, $SrCl_2$, $MgSO_4$) [2-6] (see Table **1**). This shape transformation was rapid and reversible [4, 6]. It was generally attributed to electrostatic interactions of cations with flexible filamentous anionic spectrin, the major protein component of the two-dimensional reticulated skeleton, apposed at the inner leaflet of the membrane lipid bilayer, and/or anionic phospholipids, mainly localized in the inner leaflet and composed of phosphatidylserine and phosphatidylinositides [3-5, 17], since divalent cationic salts crenated at concentrations substantially lower than those of monovalent cationic salts (0.1-0.2 mM versus 30 mM) [2-6], and that crenation appeared unrelated to the anionic species [3, 5]. However, this crenation was markedly influenced by ionic conditions and temperature, as indicated by the following observations: a) Ghosts in a 10 mM zwitterionic N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (Hepes) buffer, pH 7.0, 4°C significantly crenated only in the presence of both 0.2 mM $MgCl_2$ and 10 mM NaCl [6]. b) Ghosts in 5 mM sodium phosphate (NaP_i) buffer, pH 8.0, 0°C were strongly crenated by 1 mM $MgSO_4$, but those in 25-30 mM $MgSO_4$ were uncrenated [5]. c) Ghosts in 8 different 10 mM anionic buffers, pH 7.4, 0°C containing 0.25 mM dithioerythritol were crenated by isotonic saline, and remained crenated after raising the temperature to 30°C, but were transformed into smooth discs and cups after the addition of adenosine triphosphate (ATP)-Mg^{2+} complex [22]. On the other hand, ghosts in 14 different 10 mM zwittterionic buffers and 2 different 10 mM cationic buffers, pH 7.4, 0°C containing 0.25 mM dithioerythritol, having a lower ionic strength, were also crenated by isotonic saline, but were spontaneously transformed into smooth discs and cups when the temperature was elevated at 30°C. This spontaneous transformation was not reversed after washing these ghosts with anionic buffers, but ghosts isolated in anionic buffers underwent this spontaneous transformation after being washed with zwitterionic buffers; dithiothreitol appeared to be added in order to exclude the possibility that this spontaneous transformation was caused by oxidation of membrane proteins [6]. Lastly, the spontaneous transformation into discs and cups was minimal when ghosts were in zwitterionic or cationic buffers at pH in the range of 6.5-7.0 or above the pK_a of the zwitterionic buffers at 0°C [22].

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Table 1. Observations of Electrolytes and Buffers on Ghost and Erythrocyte Shapes

Buffer	Electrolytes	[Electrolyte] (mM)	Crenation	Ref.
Hemolytic hypotonic buffer (ghosts)				
5.6 mM Tris-HCl-40 mM Tes-Tea, pH 7.4 [a]				
	-	-	Monoconcave discs	[2]
	NH_4Cl	50 [b]	+	
	$MgCl_2$	1 [b]	+	
	$NiCl_2$	0.4 [b]	+	
5 mM Tris-HCl, 7 mM NaCl, pH 7.4				
	-	-	Discs, Cups	[3,4]
	$CaCl_2$	1	+	
	$MgCl_2$	1	+	
	$SrCl_2$	1	+	
	NaCl	30	+	
5 mM NaP_i, pH 8.0				
	-	-	Plump bowls with one shallow dimple	[5]
	NaCl	150	+	
	NaCl, glycerol	150, 300	+	
	NH_4HCO_3	150	+	
	$CaCl_2$	1	+	
25-30 mM $MgSO_4$	$MgSO_4$	1		
10 mM Hepes, pH 7.0	$MgSO_4$	25-30	Uncrenated	[5]
	-	-	Spheres	[6]
	$CaCl_2$	0.1-1	+	
	$MgCl_2$	0.2	Some crenation	
	NaCl	10	Some crenation	
	$MgCl_2$, NaCl	0.2, 10	+	
	NaCl or KCl	70	+	
Hemolytic isotonic medium (ghosts)				
150 mM NH_4HCO_3	NH_4HCO_3	150	Globoid	[5]
300 mM Glycerol	-	-	Globoid	[5]
	NaCl	150	Globoid	
Buffered isotonic sucrose (erythrocytes)				
300 mM Sucrose, NaCl, Hepes, pH 6.0				
	NaCl, Hepes	0.52, 0.0175	Crenation ->	[34]
			Discocytosis ->	
			Cupping [c]	

a, Ghosts in 28 mM Tris-HCl buffer, pH 7.4 were diluted with 4 volumes of 50 mM Tes-Tea (N-tris(hydroxymethyl)-2-aminoethanesulfonic acid- triethylamine) buffer, pH 7.4; b, electrolyte concentration causing 50 % crenation; c, the rapid morphological transformation crenation -> discocytosis -> cupping occurred in different zwitterionic buffers.

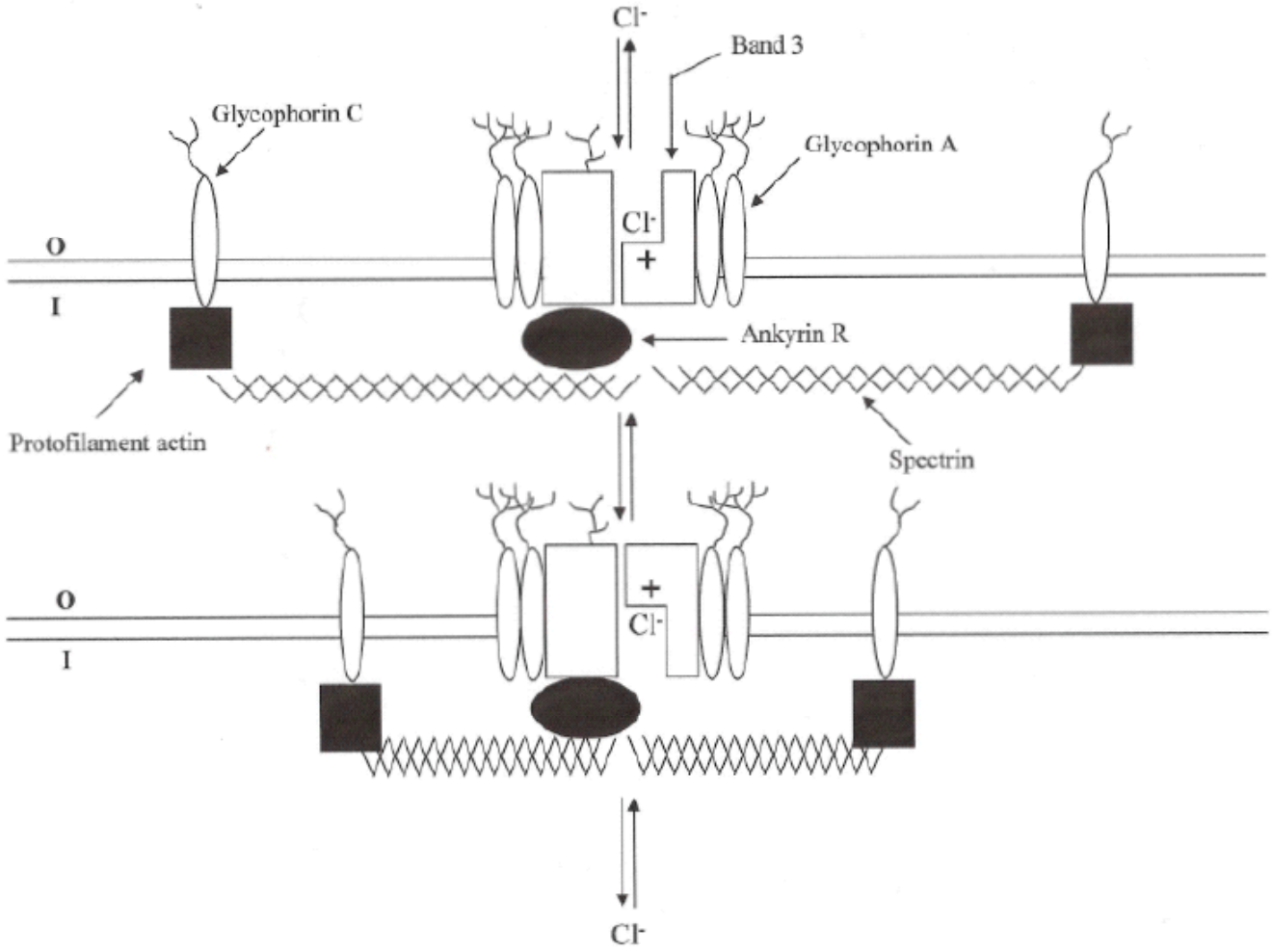

Fig. (1). Scheme of a mechanism of control of the erythrocyte shape. The transmembrane glycoprotein band 3 (AE1), which exchanges Cl^- and HCO_3^- and is linked to spectrin, the major skeleton protein, by the intermediary of ankyrin R, plays a central role in the mechanism. The alternative recruitment of band 3_i and band 3_o by the exchange of Cl^- and HCO_3^- folds and unfolds spectrin, thus promoting echinocytosis and stomatocytosis, respectively. The band 3_o/band 3_i ratio is determined by the Donnan ratio ($r = Cl^-_i/Cl^-_o = HCO_3^-_i/HCO_3^-_o = H^+_o/H^+_i$), but this ratio is 0. 1 and 10 in the self-exchanges of Cl^- and HCO_3^- indicating that the latter are intrinsically echinocytogenic and stomatocytogenic, respectively.

A mechanism of control of the erythrocyte shape has been previously proposed in which the anion exchanger band 3 (AE1) plays a central role (see Fig. **1**) [26]. Briefly, band 3 is a 90-100-kDa transmembrane glycoprotein which rapidly exchanges the monovalent anions Cl^- and HCO_3^- and is linked via ankyrin R to flexible anionic spectrin, the major protein component of the two-dimensionsional reticulated skeleton. It transports also endogenous P_i and a wide variety of other inorganic and organic anions, including bulky ones, but at a slow rate [27]. The alternative recruitment of its inward-facing (band 3_i) and outward-facing (band 3_o) conformations by exchange of Cl^- and HCO_3^- folds and unfolds spectrin (or contracts and relaxes the skeleton), thus promoting echinocytosis and stomatocytosis, respectively. The band 3_o/band 3_i equilibrium ratio increases with the increase of the Donnan equilibrium ratio of anions Cl^- and HCO_3^- and H^+ ($r = Cl^-_i/Cl^-_o = HCO_3^-_i/HCO_3^-_o = H^+_o/H^+_i$), which is determined by hemoglobin and the major organic phosphate intermediate 2,3-bisphophoglycerate. Substrates slowly transported by band 3, preferentially inwardly and outwardly are echinocytogenic and stomatocytogenic, respectively. Recently, a process of echinocytosis by glucose depletion has been suggested based on this mechanism [28]. As was indicated, this process would be compatible with the reversal of ghost crenation by buffered isotonic saline by ATP-Mg^{2+} complex, since ATP is hydrolyzed by a vanadate-sensitive Mg^{2+}-ATPase which would promote the stomatocytogenic outward transport of divalent P_i with a hydrogen ion (H^+) (or monovalent P_i) by band 3 [18, 20-22]. The plausibility of this process would also be further supported by two previous observations. a) Ghosts isolated in 5 mM NaP_i buffer, pH 8.0, 0°C were morphologically indistinguishable before and after ATP depletion of erythrocytes by glucose depletion in a buffered isotonic saline at 37°C, and appeared as plump bowls with a single shallow dimple [5]. b) Rabbit erythrocytes suspended in phosphate-buffered isotonic saline, pH 7.7 were crenated after a blood storage of 1 day, but had a biconvave disc shape after suspending them in phosphate-buffered isotonic saline, pH 8.5, a pH markedly opposing the transport of divalent P_i with a H^+ by band 3, since its pK_a is 7.31-7.18, 0-40°C [29, 30]. We have examined whether the mechanism could be the basis of the marked influence of the ionic composition and temperature on crenation of ghosts by

divalent and monovalent cationic salts. This appears plausible since it can explain the following observations.

1) Ghosts in 5 mM NaP_i buffer, pH 8.0, 0°C were crenated by hemolytic isotonic salt 150 mM NH_4HCO_3 or 1 mM 2,4-dinitrophenol (DNP), but ghosts isolated in this hemolytic salt at 0°C were globoid and not crenated by DNP [5]. It was previously shown that the band 3_o/band 3_i equilibrium ratio in erythrocytes was 0.1 in the self-exchange of Cl^- at pH 7.8, 0°C, but this ratio was 10 in the self-exchange of HCO_3^- [31]. The large difference between these two ratios does not appear to present an advantage in HCO_3^- transport in blood, but with respect to the mechanism of control of the erythrocyte shape this difference would indicate that the Cl^- and HCO_3^- self-exchanges by band 3 are intrinsically echinocytogenic and stomatocytogenic, respectively. This is plausible since it provides an explanation for the Cl^--dependent crenation of ghosts by 1 mM DNP and by 0.11 mM lysolecithin [5] as well as the appearance of ghosts as globoid and of an absence of crenation by 1 mM DNP isolated in hemolytic isotonic 150 mM NH_4HCO_3. The band 3_o/band 3_i equilibrium ratio in the self-exchange of divalent P_i with a H^+ by band 3 at pH 8.0, 0°C would probably to be similar to that of Cl^-, since ghosts became crenated when the rate of this self-exchange was increased by decreasing the pH [5]; hence crenation of ghosts in 5 mM NaP_i , pH 8.0, 0°C by isotonic 150 mM NH_4HCO_3 and by 1 mM DNP. Crenation of ghosts in this buffer by isotonic 150 mM NH_4HCO_3 would not be precluded by the opposition of HCO_3^- to crenation if the crenated ghosts were stabilized by dehydration by this salt [4]. Isolation of ghosts in this hemolytic salt at 0°C would not be accompanied by dehydration since the diffusion of this salt across the membrane would be accompanied by an increase of cell water. Ghosts isolated in hemolytic isotonic 300 mM glycerol at 0°C also had a globoid shape and were not crenated by isotonic 150 mM NaCl [5]. However, these ghosts are not equivalent to those isolated in hemolytic isotonic 150 mM NH_4HCO_3 because of the destabilization of the skeleton in the absence of Cl^- (see later), and ghosts isolated in 10 mM $NaHCO_3$, pH 7.4, 0°C were as stable as those isolated in other 10 mM anionic buffers, pH 7.4, 0°C [22].

2) Crenation of ghosts in 10 mM Hepes buffer, pH 7.0, 4 °C occurred significantly only in presence of both 0.2 mM $MgCl_2$ and 10 mM NaCl [6]. Moreover, ghosts in 5 mM NaP_i buffer, pH 8.0, 0°C were strongly crenated by 1 mM $MgSO_4$, but those in 25-50 mM $MgSO_4$ were uncrenated [6]. These observations can be rationalized as follows. The self-exchanges of Cl^- or divalent P_i with a H^+ by band 3 are intrinsically echinocytogenic. The divalent SO_4^{2-} can not be self-exchanged by band 3 at basic pH, in contrast to divalent P_i. However, divalent SO_4^{2-} with a H^+ can be self-exchanged by band 3 at acid pH, which would favor crenation of ghosts by Mg^{2+} [32]. An implication of a Cl^- or P_i -dependent crenation by Mg^{2+} would be that this cation binds specifically at a site on spectrin, the major protein of the skeleton (see Fig. **2**). The basis of the crenation of ghosts by other divalent cations appears to be the same, since crenation of ghosts in 10 mM Hepes buffer, pH 7.0, 4°C also became significant only in the presence of both chloride salts of these other divalent cations and NaCl at relatively low concentrations [6]. It is plausible that the basis of the crenation of ghosts by monovalent cationic salts at the relatively low concentration of 30 mM would be the same [4].

3) The dependence of Cl^- on the skeleton conformation can also explain the spontaneous transformation into smooth discs and cups when the temperature was raised to 37 or 30°C after crenation by an isotonic saline of ghosts in 10 mM zwitterionic and cationic buffers, pH 7.0 or 7.4, 0-4°C [6, 22], since Cl^- was absent or present at relatively low concentrations in these hypotonic buffers. This explanation is supported by the following observations. a) The irreversibility of the spontaneous transformation to discs and cups at 30°C of ghosts isolated in hypotonic zwitterionic buffers, pH 7.4, 0°C [22]. b) The stabilization at 37°C of ghosts isolated in hypotonic zwitterionic and cationic buffers, pH 7.4, 0°C supplemented with NaCl [9]. c) The previous unexpected observation that the skeleton in ghosts was less stable than that isolated at a very low ionic strength of the medium (very low concentrations of Cl^- or P_i in the medium) [33].

Ghosts in buffers containing relatively high concentrations of Cl^- or P_i were also unstable as indicated by their loss of the ability to be crenated by different agents over time depending of the pH and temperature [5, 17]. The agents included 10^{-6}-10^{-7} M Ca^{2+}, 150 mM NaCl and 1 M NaCl plus 1 mM DNP. This instability would be attributed to destabilization of the skeleton as a result of band 3 losing the ability of transporting anions, as indicated by this observation. The rate of the loss of crenatability of ghosts in 5 mM NaP_i buffer by 150 mM NaCl increased significantly as the pH increased from 6 to 8, as would be expected since band 3 transports divalent P_i with a H^+ [5].

4) The minimal spontaneous transformation into smooth discs and cups observed when ghosts were isolated in zwitterionic and cationic buffers between pH 6.5-7.0, 0°C [22] could be due to the presence of sufficient amounts Cl^- in these cationic buffers binding to band 3, or to the transport by band 3 of the anionic zwitterionic species with a H^+ of zwitterionic buffers stabilizing the skeleton, as indicated by the following observations: a) The pKa of the 2 cationic buffers used, 2-[bis (-hydroxyethyl) amino]-2-(hydroxy-methyl) propane-1,3-diol (Bistris) and imidazole, are 6.66 and 7.38 at 0°C, respectively [22]. b) Different zwitterionic buffers crenated erythrocytes at a low hematocrit (0.06 %) in a low ionic strength isotonic sucrose, pH 6.0, 20-22°C [34], which is likely due to their inward transport with a H^+ by band 3, since the zwitterionic amino acid glycine and the bulky divalent band 3 high affinity inhibitor 4,4'-dinitro-2,2'-stilbenedisulfonate (DNDS) were transported with a H^+ by band 3 at acid pH [35, 36]. Erythrocytes after crenation underwent a discocytosis followed by a cupping which may be due to the opposite transport of the buffer. c) Finally, the percentage of smooth discs and cups as a function of the pH of different zwitterionic buffers increased significantly when the pH increased from pH 7.0 to a certain pH, and then decreased significantly as the pH was further increased [33]. The transition of each of the zwitterionic buffers occurred at a pH which was consistent with its pK_a, thus indicating that the skeleton was stabilized by the transport of its anionic species by band 3. However, the percentage of smooth discs and cups at pH 7.4 in a given zwitterionic buffer may be

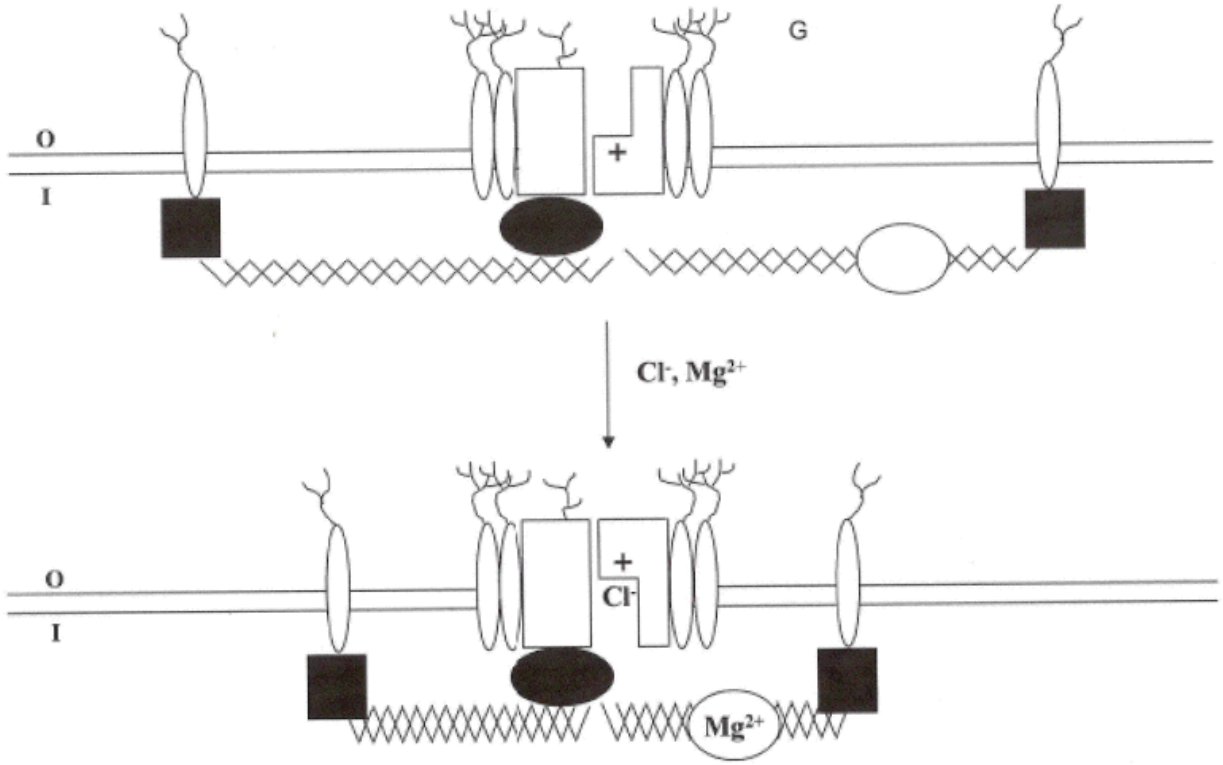

Fig. (2). The basis of the crenation of ghosts by Mg^{2+}. Ghost crenation by Mg^{2+} occurs only in the self-exchange of Cl^- by band 3, suggesting that Mg^{2+} binds at a specific site on spectrin. Other observations on ghosts cited in the text can be explained by self-exchanges of Cl^-, HCO_3^- or P_i by band 3, a destabilization of the skeleton or an ability of band 3 of transporting zwitterionic buffers.

significantly different from that of another with the same pK_a (e.g. Tes (43 %) vs Hepes (100 %) (Fig. 2 of ref. 22). This difference may be due to binding to the transport site of band 3 of zwitterionic buffers with a lower percentage of smooth discs and cups with a lower steric hindrance.

In conclusion, the marked influence of ionic conditions and temperature on the crenation of ghosts by electrolytes can be explained by a mechanism of control of the erythrocyte shape, which would have the following major implications. a) The shape of ghosts isolated in hemolytic hypotonic buffers is specifically influenced by the type of the buffer and the pH. b) The instability of ghosts isolated in hypotonic zwitterionic and cationic buffers at some pH and temperature is the result of an absence of or of a relatively low concentration of Cl^-, which would destabilize the skeleton. c) The instability of ghosts in buffers containing a relatively high concentration of Cl^- or P_i is caused by a destabilization of the skeleton as a result of band 3 of losing the ability to transport anions. d) Crenation by divalent cations Mg^{2+} and Ca^{2+} depends on Cl^- thus suggesting that these cations crenate by binding at specific sites on spectrin, the major protein of the skeleton. The Cl^- dependent crenation by these two divalent cations as well as by amphiphiles can be explained by the Cl^- and HCO_3^- self-exchanges by band 3 being intrinsically echinocytogenic and stomatocytogenic, respectively. e) Lastly, the ability of resealing ghosts under certain conditions in the presence of Mg^{2+} [1, 10, 11] is likely to be the result of a stabilization of the skeleton by a high Cl^- concentration, a slow transport by band 3 of an anionic species of a buffer or of an inorganic salt and a specific binding of Mg^{2+} to a spectrin site.

CONFLICT OF INTEREST

The authors confirm that this article content has no conflicts of interest.

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