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Relationship of hemolysis buffer structure, pH and ionic strength to spontaneous contour smoothing of isolated erythrocyte membranes

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Isolated human erythrocyte membranes crenate when suspended in isotonic medium, but can use MgATP to reduce their net positive curvature, yielding smooth discs and cup forms that eventually undergo endocytosis. An earlier report from this laboratory (Patel, V.P. and Fairbanks, G. (1981) J. Cell Biol. 88, 430–440), has described a phenomenon of ATP-independent shape change in which ghosts prepared by hemolysis and washing in synthetic zwitterionic buffers crenated at 0°C, but underwent conversion to smooth discs and cups when warmed in the absence of MgATP. We have further explored the effect of the hemolysis condition on the requirement for ATP in ghost shape change. 25 hemolysis buffers were applied at 10 mM (pH 7.4, 0°C). Eight anionic buffers with relatively high ionic strength (e.g., phosphate and diethylmalonic acid (DMA)) yielded ghosts requiring ATP for shape change, while two cationic buffers (Bistris and imidazole) and ten synthetic zwitterionic buffers (e.g., Tricine and Hepes) with lower ionic strength produced ghosts that smoothed spontaneously at 30°C. Hemolysis at intermediate ionic strength yielded mixed populations in which spontaneous smoothing was expressed in all-or-none fashion. Maximal ATP-independent shape change was induced by hemolysis at pH 7.3–7.7, while ATP was required after hemolysis at pH ≤ 7.1 even when the ionic strength at hemolysis was low. Ghosts requiring ATP could be converted to ATP independence by washing at low ionic strength, but ATP independence could not be reversed readily by washing at high ionic strength. Exposure to low ionic strength at pH > 7.1 presumably changes membrane organization in a way that alters the temperature dependence of tensions within the bilayer or skeleton of the composite membrane.

Introduction

Membranes isolated from mammalian erythrocytes can undergo ATP-dependent shape transformations in which echinocytic (crenated) forms smooth to discocytic and stomatocytic (cupped) forms [1–3]. This process, which can culminate in endocytosis with the formation of inverted vesicles [4,5], is associated with a progressive reduction in the net positive curvature of the membrane [5] and an increase in the area of the inner surface relative to the outer surface [5–8]. Various mechanisms have been postulated to explain these shape transformations, including the operation of an actomyosin-like contractile system [2,9–14], changes in the compressibility of the two-dimensional spectrin/actin 'gel' underlying the membrane [2,15,16], spectrin and/or lipid phosphorylations [17–25] and aminophospholipid flipping [26,27]. Patel and Fairbanks [19,22] have shown that phosphorylation of spectrin and phosphoinositides is neither necessary nor sufficient for shape change in the ghost system and have suggested that the vanadate-sensitive Mg^{2+}-ATPase is involved [22,28,29]. Recent work suggests that this activity may correspond to the enzyme system that mediates translocation of aminophospholipids from the outer to the inner leaflet [26,27,30].

Most of the earlier biochemical studies were done using ghosts prepared by hemolysis in Tris buffer. These Tris ghosts normally required ATP for conversion to smooth forms, but, occasionally, a large fraction (up to 50%) of the population changed shape without ATP [22]. It was also noted that hemolysis in Hepes or Tes buffers yielded ghosts that smoothed their contours rapidly without MgATP on elevation of the temperature

Abbreviations: DMA, diethylmalonic acid; DTE, dithioerythritol. Abbreviations for synthetic buffers are given in Table I.

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from 0 to 37 °C [19]. This paper represents an extension of these earlier observations. We demonstrate that the capacity of ghosts to exhibit spontaneous contour smoothing is dependent on the ionic strength, pH and structure of the hemolysis buffer. Some of the results have been summarized in a preliminary report [31].

Reagents

Vanadate-free disodium ATP from equine muscle (No. A-5394), Tris, Epps, Hepes, Tes, cacodylic acid, imidazole, Tricine, and cell culture grade water (No. W 3500) were supplied by Sigma Chemical, St. Louis, MO. DMA was purchased from Calbiochem, San Diego, CA. Aces, Ada, Ampso, Bistris, Capso, Dipso, Mes, Mops, Mopso, Pipes, Popso, Taps and Tapso were purchased from Research Organics, Cleveland, OH. All other reagents were from Mallinckrodt Chemical Works (AR grade), Paris, KY or from Sigma Chemical.

Methods

Buffer preparation

Buffers were prepared in deionized water from 100 mM stock solutions and adjusted to pH near the working temperature. Aces, Ada, Ampso, Capso, DMA, Dipso, Epps, Hepes, Heppso, Mes, Mops, Mopso, Pipes, Popso, Taps, Tapso, Tes and Tricine (Table I) were adjusted to pH with NaOH. Borate, Bistris, cacodylate, imidazole and Tris were adjusted to pH with HCl. Bicarbonate buffer was made by bubbling 5% CO_2 through $NaHCO_3$ for 1 h at room temperature. Phosphate buffer was made by mixing Na_2HPO_4 with NaH_2PO_4.

Cell and membrane preparations

Fresh blood samples were drawn by venipuncture from healthy donors, mixed immediately with heparin (10 USP U/ml, final concentration) and filtered through a cellulose pad to deplete the preparation of leucocytes and platelets [32,33]. Red cells were eluted with Hanks' balanced salt solution (calcium- and magnesium-free), sedimented at $950 \times g$ for 5 min and washed three times with 0.15 M NaCl/20 mM Tris-HCl/0.25 mM DTE (pH 7.4) at 0–4 °C. In the final washing the red cells were sedimented by centrifugation for 10 min. In some experiments, the first cell pellet was divided and small portions (typically 0.15 ml packed cells) were washed twice with 10 ml 0.15 M NaCl/20 mM test buffer/0.25 mM DTE, at 0–4 °C.

Packed washed erythrocytes were hemolyzed by rapid 1 : 60 dilution in ice-cold 10 mM test buffer/0.25 mM DTE, with stirring at 0–4 °C. The membranes were pelleted at $11\,000 \times g$ (r_{max}) in 10 min using a Sorvall SE-12 rotor at 0–4 °C, and were washed twice (1 : 30) in the hemolysis buffer. Washed membranes were held on ice for up to 2 h before initiation of shape-change incubations.

Shape-change assay

Assay mixtures were made up on ice in glass tubes that had been rinsed with 10 mM Tris-HCl (pH 7.4). Packed washed membranes were diluted 1 : 10 with a solute mixture concentrate to yield 0.125 M NaCl/2 mM $MgCl_2$/0.2 mM EGTA/0.5 mM DTE/10 mM Tris-HCl (pH 7.4) (25 °C). Hemolysis test buffer carried over with the membranes was present at 1 mM. ATP-dependent shape change was assayed in the same medium with addition of 2 mM ATP.

After 3 min on ice, the tubes were plunged into a water bath at 30 °C, and, at intervals from 0 to 30 min, 25 µl portions of the suspension were added to 75 µl of 1.25% glutaraldehyde/0.14 M KCl/10 mM sodium phosphate (pH 7.5) (0 °C) on ice. The shapes of the fixed membranes were assayed by counting with dark-field microscopy as described previously [19]. This assay gives the percentage of membranes that have lost their spicules to yield smooth forms, including discs and cups as well as membranes that have progressed through the initial smoothing stage of shape change and are undergoing endocytosis. ATP-dependent shape change is defined as the difference in the percent smooth in the presence and absence of ATP. All buffers tested yielded preparations in which the entire population of ghosts underwent smoothing under standard assay conditions (i.e., the sum of ATP-independent and ATP-dependent components was always near 100%).

Darkfield images [19] of ghosts fixed at various stages in shape change were recorded using a video camera and enhanced (eight-frame averaging, background subtraction, 'auto-enhancement' and 2X zoom) with the Image-1/AT Image Processing System (Universal Imaging, Media, PA). The processed images were printed using a SONY UP-811 Video Graphic Printer.

Results

Effect of hemolysis buffer

The results of experiments in which Tris ghosts or Hepes ghosts were crenated by suspension in isotonic medium and incubated at 30 °C with and without ATP closely resemble those previously published from our laboratory [19,22]. As illustrated in Fig. 1A, the shape change of Tris ghosts is usually ATP-dependent. In this experiment, 41% of the ghosts had progressed from the crenated form to smooth discs after 5 min and, at 30 min, the entire membrane population had converted to smooth forms, including stomatocytic and endocytic forms. The background of ATP-independent shape change (15% after incubation for 5 min, progressing to 18% at 30 min) was slightly elevated. Much higher

backgrounds are sometimes encountered with Tris hemolysis (Ref. 22 and below).

Hemolysis in Hepes buffer (Fig. 2B) yielded ghosts that crenated in isotonic medium at 0°C but rapidly lost their spicules at 30°C in the absence of ATP to yield smooth discs and cups. Based on similar observations, Patel and Fairbanks [19] have suggested that the predominant factor determining the degree of ATP dependence and the initial rate of the shape-change process is the buffer used in hemolysis and initial washing.

As illustrated in Fig. 2, 25 hemolysis buffers tested differed markedly in the extent to which they yielded populations of ghosts smoothing their contours spontaneously in the standard shape-change assay system. Based on these results, the buffers can be classified into three groups:

Group 1: < 25% spontaneous smoothing (ATP dependence). Pipes, cacodylate, phosphate, bicarbonate, borate, Popso, DMA, Ada and Tris.

Group 2: 25–77% spontaneous smoothing (intermediate). Mes, Tes, Mopso and Aces.

Group 3: > 77% spontaneous smoothing (ATP independence). Mops, Heppso, Dipso, Taps, Ampso, Epps, Tapso, Tricine, imidazole, Hepes, Capso and Bistris.

The shape changes of ghosts prepared in DMA, Mes and Tricine, representing these three groups, are illustrated in Fig. 3. When suspended in shape-change medium containing 125 mM NaCl, all ghosts crenated at 0°C, assuming shapes resembling echinocytes II and

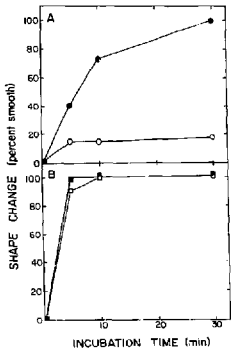

Fig. 1. ATP dependence and time-course of ghost shape change. (A) Tris ghosts. (B) Hepes ghosts. Shape-change activity was assayed in standard Tris medium without ATP (○,□) and with ATP (●,■). Similar results were obtained in three other experiments using different preparations of membranes.

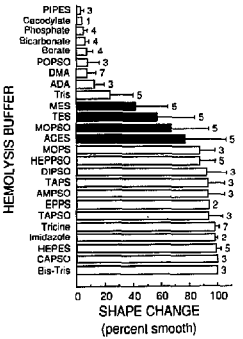

Fig. 2. Effect of hemolysis buffer on ATP-independent shape change. Ghosts were prepared by hemolysis and washing in 10 mM solutions of the indicated test buffers at pH 7.4, 0°C. Bicarbonate buffer was used at 20 mM. Shape-change activity was assayed under standard conditions in Tris buffer. The results show the percentage of smooth forms present after 30 min of incubation without ATP. Error bars show the standard deviation in the percent smoothed; the number of independent experiments on each buffer is shown at the right.

III as defined for intact erythrocytes by Bessis [34] (Fig. 3, rows 1 and 3). DMA ghosts exhibited tight, uniform crenation and initially resembled echinocytes III (Figs. 3A, 3C). At 30°C, in the absence of ATP, DMA ghosts progressed to shapes resembling spheroechinocytes I [34], small, spherical forms studded with a regular array of fine spicules (Fig. 3B). Tricine ghosts were more loosely crenated at 0°C (Figs. 3I, 3K) and smoothed rapidly at 30°C in the absence of ATP to discoid and shallow-cup forms that were stable for at least 30 min (Fig. 3J). Under the same conditions, Mes ghosts yielded a mixture of shapes: smooth forms, which resembled Tricine ghosts at the comparable stage, and 'mature' crenated forms, which resembled the spheroechinocytic stage of DMA ghosts (Fig. 3F). Fig. 3F shows that commitment to smoothing has an all-or-none character – after the initial phase of rapid smoothing, the fraction of Mes ghosts with smooth contours does not increase and intermediate forms are rare. Crenation in the presence of ATP appeared to be lower (Fig. 3, third row). This probably reflects ATP chelation of Mg^{2+} ions, which are effective crenators at low concentrations [35–39], but the possibility that ATP binding to the membrane [40] alters its mechanical properties directly cannot be ruled out. When incubated with ATP at 30°C, DMA ghosts smoothed their contours to disc and cup forms and began to undergo endocytosis (Fig. 3D). Although spontaneous contour smoothing is arre-

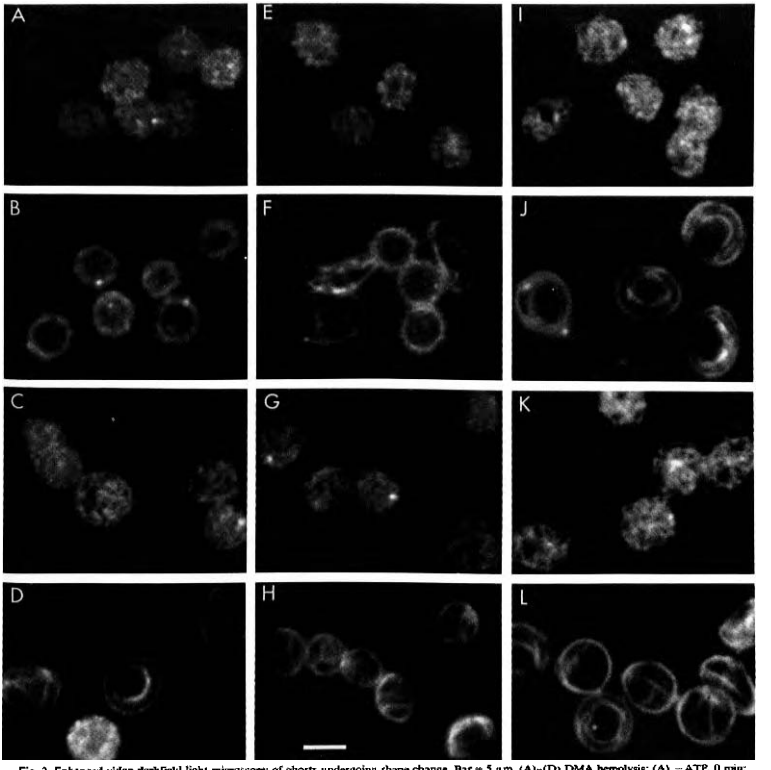

Fig. 3. Enhanced video darkfield light microscopy of ghosts undergoing shape change. Bar = 5 μm. (A)–(D) DMA hemolysis: (A) – ATP, 0 min; (B) – ATP, 30 min; (C) + ATP, 0 min; (D) + ATP, 30 min. (E)–(H) Mes hemolysis: (E) – ATP, 0 min; (F) – ATP, 30 min; (G) + ATP, 0 min; (H) + ATP, 30 min. (I)–(L) Tricine hemolysis: (I) – ATP, 0 min; (J) – ATP, 30 min; (K) + ATP, 0 min; (L) + ATP, 30 min.

sted at the discoid and shallow-cup stages, endocytosis of Mes and Tricine ghosts in the presence of ATP was extensive (Figs. 3H, 3L), as shown most clearly for Mes ghosts (Fig. 3H).

Based on the structure and pK_a values (Table I) of the buffers that compose each of the groups defined above, there is strong correlation between the ionic strength at hemolysis and the development of a requirement for ATP in shape change: Group 3 consists of 12 buffers that promoted > 77% ATP-independent smoothing of crenated membranes; 10 of the 11 synthetic buffers in this group are zwitterionic compounds, most of which were introduced by Good and associates (Refs. 41, 42, and Table I). They contain both positive and negative groups. The positive charges are borne by secondary or tertiary amino groups, while the negative

TABLE I

Systematic names and pK_a values for synthetic buffers

Buffer	Systematic name (Chemical Abstracts)	Manufacturer's name	pK_a (20 °C) [a]	pK_a (0 °C) [b]
Aces	2-[(2-amino-2-oxoethyl)amino]-ethanesulfonic acid	*N*-(2-acetamido)-2-aminoethanesulfonic acid	6.88	7.32 [d]
Ada	[(carbamoylmethyl)imino]diacetic acid	*N*-(2-acetamido)-2-iminodiacetic acid	6.62	6.85 [d]
Ampso	2-hydroxy-3-[(2-hydroxy-1,1-dimethyl-ethyl)amino]-1-propanesulfonic acid	3-[dimethyl(hydroxymethyl)methylamino]-2-hydroxypropanesulfonic acid	9.10 [c]	9.66
Bistris	2-[bis(2-hydroxyethyl)amino]-2-(hydroxymethyl)propane-1,3-diol	bis(2-hydroxyethyl)-iminotris(hydroxymethyl)methane	6.36 [c]	6.66
Capso	3-(cyclohexylamino)-2-hydroxy-1-propanesulfonic acid	3-(cyclohexylamino)-2-hydroxy-1-propanesulfonic acid	9.43 [c]	10.69
Dipso	3-[bis(2-hydroxyethyl)amino]-2-hydroxypropanesulfonic acid	3-[*N*-bis(hydroxyethyl)amino]-2-hydroxypropanesulfonic acid	7.60	7.90
Epps	4-(2-hydroxyethyl)-1-piperazinepropanesulfonic acid	*N*-2-hydroxyethylpiperazine-*N*′-4-propanesulfonic acid	8.00	8.22
Hepes	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid	*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid	7.55	7.85 [d]
Heppso	2-hydroxy-4-(2-hydroxyethyl)-1-piperazinepropanesulfonic acid	*N*-hydroxyethylpiperazine-*N*′-2-hydroxypropanesulfonic acid	7.90	8.10
Mes	4-morpholineethanesulfonic acid	2-(*N*-morpholino)ethanesulfonic acid	6.15	6.38 [d]
Mops	4-morpholinepropanesulfonic acid	3-(*N*-morpholino)propanesulfonic acid	7.01 [c]	7.23
Mopso	2-hydroxy-4-morpholinepropanesulfonic acid	3-(*N*-morpholino)-2-hydroxypropanesulfonic acid	6.95	7.25
Pipes	1,4-piperazinediethanesulfonic acid	piperazine-*N*,*N*′-bis(2-ethanesulfonic acid)	6.82	7.02 [d]
Popso	1,4-piperazinedi-(2-hydroxypropanesulfonic acid)	piperazine-*N*,*N*′-bis(2-hydroxypropanesulfonic acid)	7.85	8.11
Taps	3-{[2-hydroxy-1,1-bis(hydroxymethyl)-ethyl]amino}-1-propanesulfonic acid	3-[*N*-tris-(hydroxymethyl)methylamino]-propanesulfonic acid	8.11 [c]	8.89
Tapso	2-hydroxy-3-{[2-hydroxy-1,1-bis(hydroxymethyl)-ethyl]amino}-1-propanesulfonic acid	3-[*N*-tris(hydroxymethyl)methylamino]-2-hydroxypropanesulfonic acid	7.70	8.06
Tes	2-{[2-hydroxy-1,1-bis(hydroxymethyl)-ethyl]amino}ethanesulfonic acid	*N*-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid	7.50	7.92 [d]
Tricine	*N*-[2-hydroxy-1,1-bis(hydroxymethyl)-ethyl]glycine	*N*-[tris(hydroxymethyl)methyl]glycine	8.15	8.60 [d]
Tris	2-amino-2-hydroxymethylpropane-1,3-diol	tris(hydroxymethyl)aminomethane	8.30	8.90 [d]

[a] Values from Refs. 41, 42 and 54.
[b] Calculated from values at 20 or 37 °C using temperature coefficients in Refs. 41, 43 and 54.
[c] Values at 37 °C from Ref. 43.
[d] Values from Ref. 42.

charges are provided by sulfonic or carboxylic acid termini. With the exception of Mops (pK_a 7.23), these buffers (e.g., Tricine, Hepes, Taps and Heppso) have pK_a values ≥ 7.8, so that $\geq 74\%$ of the buffer molecules are in the zwitterionic form at hemolysis pH 7.4. Zwitterions do not contribute to ionic strength because they do not bear a net charge; hence, all the synthetic buffers in Group 3 have low ionic strength at 10 mM (0.00001–0.006). Bistris (pK_a 6.66, 0 °C) and imidazole (pK_a 7.38, 0 °C; calculated from Ref. 43) are weak bases, so that the predominant form at hemolysis was uncharged and the concentration of chloride was low (ionic strength 0.0015 and 0.0049, respectively).

With the exception of Tris, which is cationic, the buffers in Group 1, those yielding < 25% ATP-independent shape change, are anionic. Four are inorganic anions – phosphate, bicarbonate, cacodylate and borate. The remaining four members of this group – DMA, Ada, Pipes and Popso – are dicarboxylic or disulfonic acids. Ada, Pipes and Popso have a zwitterionic configuration, but the overall charge is negative due to the presence of a second acidic group. Because they bear a net negative charge, the Group 1 buffers have higher ionic strengths (0.0105–0.0288). Tris falls into the same group because it was used below its pK_a and required the addition of chloride at > 9 mM (ionic strength > 0.009). Group 2 contains the only four buffers that yielded intermediate levels of ATP-independent shape change: Mes, Tes, Mopso and Aces. These are synthetic zwitterionic compounds, and, with the exception of Tes (pK_a 7.92, ionic strength 0.0023), they have pK_a values ≤ 7.32, and ionic strengths ranging from 0.0055 to 0.0091 at hemolysis pH 7.4.

These effects were explored further by performing

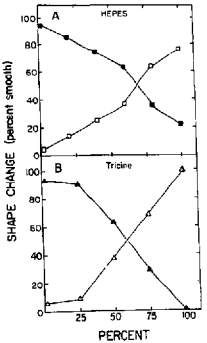

Fig. 4. Dose dependence of the hemolysis buffer effect. (A) Pipes/Hepes mixtures. (B) Tris/Tricine mixtures. Hepes (10 mM, ionic strength 0.0026) was substituted for Pipes (10 mM, ionic strength 0.0241), or Tricine (10 mM, ionic strength 0.0006) for Tris (10 mM, ionic strength 0.0097), at 0–100 mol%. All mixtures were 10 mM, pH 7.4. After washing in hemolysis buffer, the ghosts were assayed for shape change in Tris medium. Values for spontaneous contour smoothing (□, △) and ATP-dependent shape change (■, ▲) represent percentage conversion at 30 min (A) or 20 min (B).

hemolysis in mixtures of buffers. A dose-dependent effect was demonstrated when hemolysis was carried out in mixtures of Pipes and Hepes in which the total concentration of buffer was maintained at 10 mM (Fig. 4A). Reducing the ionic strength by increasing the proportion of Hepes resulted in a roughly linear increase in the capacity for ATP-independent contour smoothing by the resulting ghosts. A similar dose dependence was disclosed by hemolysis in mixtures of Tris and Tricine (Fig. 4B). As the proportion of low-ionic-strength buffer in the hemolysis mixture increased, the initial rate of ATP-independent conversion to smooth forms increased, as well as the plateau approached after 20–30 min (Fig. 4 and data not shown). These results also demonstrate all-or-none responses, in that the proportion of the two subpopulations shifted systematically without the appearance of intermediate forms.

Reversal of hemolysis buffer effects

As described previously [19] and illustrated above, exposure to Group 3 buffers at hemolysis has a marked and persistent effect on ghost shape change behavior. We have also observed that exposure to the low-ionic-strength buffers at later stages can convert ghosts to ATP-independent behavior. Thus, Fig. 5 shows the concentration dependence of the Tricine effect on DMA ghosts. The figure illustrates the results of three separate experiments in which ghosts prepared by hemolysis and two washings in DMA buffer were incubated in mixtures of 10 mM buffer in which Tricine was substituted for DMA at 0–100%. As the proportion of Tricine in the buffer increased, and the ionic strength dropped from 0.0213 to 0.0006, the percentage of spontaneous contour smoothing increased gradually. Exposure to Tricine alone (ionic strength 0.0006) resulted in more marked increases (to 29–95% ATP-independent smoothing). The conversion achieved under these conditions was variable, possible reflecting the amount of DMA carried over with the packed ghosts.

Table II shows the extent to which shape-change behavior determined at hemolysis could be altered by subsequent washing using various buffers and schedules. After hemolysis and washing in Tris or DMA, a single washing with Hepes significantly increased the proportion of ghosts that could smooth their contours without ATP. Two washings with Tricine or Hepes converted the entire population. By contrast, Tricine and Hepes ghosts were highly resistant to attempts to impart the requirement for ATP by washing in DMA or Tris buffer. However, some Hepes ghosts were converted by multiple washings in Tris; the proportion exhibiting ATP dependence increased from 13% with one washing to 27% with four washings.

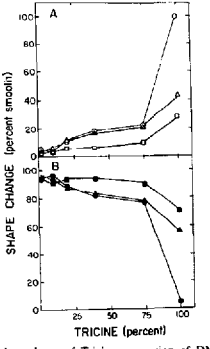

Fig. 5. Dose dependence of Tricine conversion of DMA ghosts to ATP-independent shape-change behavior. (A) ATP-independent contour smoothing. (B) ATP-dependent shape change. Erythrocytes were hemolyzed and washed in 10 mM DMA (ionic strength 0.0213), then incubated on ice for 30 min in mixtures of DMA and Tricine (pH 7.4) with Tricine (ionic strength 0.0006) at 0–100 mol% of the 10 mM buffer. Shape-change assays in Tris medium yielded the indicated percentage conversion to smooth forms. Results from three separate experiments are distinguished by different symbols.

TABLE II

Reversal of effects induced by hemolysis buffer

Ghosts were prepared from erythrocytes washed in 0.15 M NaCl/20 mM hemolysis buffer/0.25 mM DTE (pH 7.4) by hemolysis and washings as indicated.

Ghost preparation					Shape change in standard Tris medium (% smooth at 30 min)	
Hemolysis	washing					
	1st	2nd	3rd	4th	−ATP	+ATP
DMA [a]	DMA	DMA	DMA [b]	DMA	12	100
DMA [a]	DMA	DMA	Tricine [b]	Tricine	100	100
Tricine [a]	Tricine	Tricine	DMA [b]	DMA	100	100
Tricine [a]	Tricine	Tricine	Tricine [b]	Tricine	100	100
Tris	Tris	Tris	Tris	Tris	8 [d]	90 [d]
Tris	Tris	Tris	Tris	Hepes	24 [d]	98 [d]
Hepes [c]	Hepes	Hepes	Hepes	Tris	89 [d]	100 [d]
Hepes [c]	Hepes	Hepes	Hepes	Hepes	90 [d]	100 [d]
Hepes	Tris	Tris	Tris	Tris	73	100
Hepes	Hepes	Tris	Tris	Tris	80	100
Hepes	Hepes	Hepes	Tris	Tris	85	100
Hepes	Hepes	Hepes	Hepes	Tris	87	100
DMA	DMA	DMA	DMA	Hepes	88	100
DMA	DMA	DMA	Hepes	Hepes	97	100
DMA	DMA	Hepes	Hepes	Hepes	100	100
DMA	Hepes	Hepes	Hepes	Hepes	100	100

[a] Cells were washed with 0.15 M NaCl/20 mM Tris-HCl/0.25 mM DTE (pH 7.4).

[b] Resuspended ghosts were incubated on ice for 30 min before centrifugation.

[c] Packed washed cells were hemolyzed by rapid 1:120 dilution.

[d] Percent smooth at 12 min.

Hemolysis pH effect

A marked pH dependence was revealed when buffers representing each of the three groups were used at 10 mM and at pH values spanning the range pH 6.5–8.5 at intervals of 0.5 pH unit. For Mes, Hepes, Tes, Mops, Bistris and Tricine, spontaneous smoothing was minimal after hemolysis at pH 6.5–7.0 but increased to maximal levels (75–100%) when the same buffers were used at pH 7.5. The spontaneous smoothing activity induced by Mes, Hepes, Tes and Mops fell off sharply at higher pH values. Bistris yielded 100% spontaneous smoothing at pH 7.5–8.5, while Tricine yielded 100% at pH 7.5–8.0, but 76% at pH 8.5. Pipes hemolysis and washing yielded < 16% ATP-independent smoothing over the entire pH range. ATP-dependent shape change was not inhibited by low pH hemolysis – shape change in the presence of ATP was nearly 100% for all the ghost preparations.

As an approach to resolving possible contributions of the buffer chemical structure from pH per se, we examined the results of hypotonic lysis at pH 7.1–7.9 using a series of buffers of varying pK_a values, above, below and within the range of pH tested. These were:

(i) Tris, Taps and Tricine, pK_a values 8.6–8.9 (Figs. 6A, B);

(ii) Popso, DMA and Hepes, pK_a values 7.29–8.11 (Figs. 6C, D); and

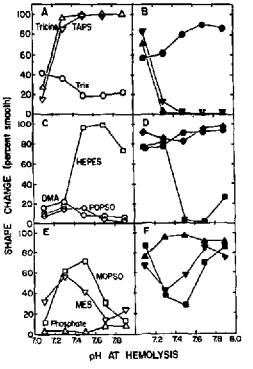

Fig. 6. Effect of hemolysis pH. (A, C, E) Spontaneous contour smoothing. (B, D, F) ATP-dependent component. Hemolysis and washing were performed in 10 mM test buffer at the indicated pH in the range 7.1–7.9. Taps ghosts prepared at pH 7.9 fragmented and were not counted.

(iii) Mopso, Mes and phosphate, pK_a values 6.38–7.25 (Figs. 6E, F).

As illustrated in Fig. 6A, when erythrocytes were hemolyzed in Tris-HCl at pH 7.1, 42% of the Tris ghosts converted to smooth discs in the absence of ATP, but, as the pH at hemolysis increased, the population of membranes that changed shape without ATP decreased to 10% at pH 7.7. In contrast, when erythrocytes were hemolyzed in 10 mM Taps or Tricine at pH 7.1, only 16–28% of the membranes spontaneously smoothed their contours, whilst at higher hemolysis pH, the fraction that smoothed without ATP exceeded 80%. Since the pK_a values of Taps and Tricine are > 7.9, the zwitterionic form predominated and the ionic strength was low (≤ 0.0044) at all the hemolysis conditions tested.

A strikingly different pattern of pH dependence was found for buffers with low pK_a values (Figs. 6E, F). A peak of 72% ATP-independent shape change was observed for Mopso ghosts produced by hemolysis at pH 7.5, but this fell to 13% when the pH at hemolysis was increased by 0.4 pH units. Similarly, as the hemolysis pH was raised from pH 7.1 to 7.3, spontaneous smoothing of Mes ghosts increased to a peak value of 57%; further increases in pH produced populations of ghosts with decreased ATP independence. In the pH range studied, the ionic strength of MES ranged from 0.0084 to 0.0097, and of Mopso ranged from 0.0042 to 0.0082 (0 °C). Hemolysis in phosphate buffer produced membranes that exhibited the full repertoire of shape change in the presence of ATP, with a low background of ATP-independent contour smoothing.

Finally, Figs. 6C, D demonstrate the pH dependence of the effects of Hepes, DMA and Popso. When erythrocytes were hemolyzed in Hepes buffer at pH 7.5 or 7.7, > 95% of the resulting ghosts transformed rapidly to smooth forms without ATP. However, increasing the hemolysis pH to 7.9 reduced the yield of ghosts with spontaneous smoothing activity to 74%. Since Hepes has a pK_a of 7.85, this coincided with a drop in the zwitterionic species and an increase in the ionic strength. The pH at hemolysis exerted a negligible effect on shape change with DMA or Popso, which have intermediate pK_a values but are persistently anionic.

The biphasic pH dependence observed with several buffers appears to reflect the interplay between pH effects on the membrane and the balance of anionic and zwitterionic forms in the buffer that determines its ionic strength. Thus, in the case of zwitterionic buffers with pK_a values below 7.3, increased susceptibility of the membranes above pH 7.1 was offset by increasing the ionic strength in the critical pH range.

Variability of Tris ghosts

Considerable variability was observed in the extent of ATP-independent contour smoothing in ghosts made by hemolysis in Tris buffer. Fig. 7 illustrates the variation in the pH profile obtained in six separate experiments. ATP-independent shape change in these samples was relatively high, with peaks at pH 7.5–8.0. In one experiment, treatment of the Tris hemolysis buffer with activated charcoal reduced ATP-independent contour smoothing in pH 7.4 Tris ghosts from 66% to < 20%. We also observed that the high background of ATP-independent shape change in Tris ghosts was reduced to < 10% when erythrocytes were hemolyzed in Tris buffer made with specially processed (low endotoxin) cell culture grade water. (In the case of Bistris, a cationic buffer with a much lower ionic strength, use of this water did not reduce the high level of spontaneous smoothing (data not shown)). A high level of spontaneous contour smoothing in Tris ghosts appeared to be correlated with the presence of traces of endotoxins in the laboratory deionized water used to make up the buffer. However, addition of *E. coli* lipopolysaccharide (an endotoxin standard) to 2.5–40 pg/ml hemolysis buffer in cell culture grade water did not replicate the effect observed with laboratory water, leaving the significance of endotoxin contamination uncertain. overall, the variability in Tris buffer is still not understood.

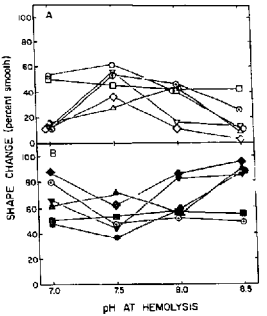

Fig. 7. Variability in the pH dependence of the effect of Tris buffer. (A) Spontaneous contour smoothing. (B) ATP-dependent component. The pH profiles shown were obtained in six separate experiments and are distinguished by different symbols.

Discussion

Fig. 8 is a scatter plot showing the relationship between spontaneous smoothing behavior and ionic strength for all hemolysis conditions represented by results in Figs. 2 and 6, together with pH data not shown. This plot reveals that there is a critical condition near ionic strength 0.01: buffers with higher ionic

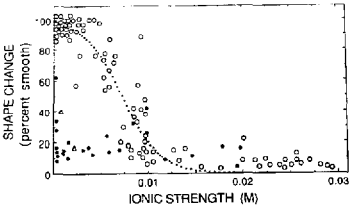

Fig. 8. Relationship of spontaneous smoothing to buffer ionic strength. Results from Figs. 2 and 6 and pH dependence data not shown are plotted against ionic strength calculated from the appropriate pK_a values (Table I and Ref. 55). The contribution of cell washing buffer and cell electrolytes (1/60) to ionic strength at hemolysis is not included. Results for hemolysis pH ≥ 7.3 are indicated by open circles (○), except for two deviant points, representing Epps and Hepes at pH 7.3, which are distinguished by open triangles (△). Small filled circles (●) are the results for hemolysis at pH 6.5–7.1. The curve was generated by fitting the arbitrary sigmoid function $y = 100[1 + e^{-A(x-B)}]^{-1}$ to the open circles. Parameter B (= 0.00724) is the ionic strength yielding 50% shape change; parameter A (= 502.9) is proportional to the slope of the curve at the midpoint and is a measure of the sensitivity to changes in ionic strength.

strengths always generated high proportions of ghosts that required ATP to change shape. Buffers with ionic strength below 0.005 always yielded high spontaneous smoothing, provided that the pH was greater than 7.3. In the ionic strength range 0.002–0.01, we observed a marked divergence in the results with different buffers (Figs. 2 and 8). For example, some of the deviant points in Fig. 8 represent Tes (pH 7.4) (0.00231, 57%); Tes (pH 7.5) (0.00275, 76%); and Mes (pH 7.5) (0.0093, 88%). We have obtained comparable results varying the ionic strength systematically by adding NaCl or adjusting the buffer concentration (Raval, P.J. and Fairbanks, G., unpublished observations). As suggested by Fig. 8, the ionic strength generating half-maximal spontaneous smoothing and the maximal activity approached at very low ionic strength in these experiments is both buffer-dependent and variable. The variability may reflect individual differences in the donor population or experimental variation in the physiological state of the cells at hemolysis. In addition, it is known that many zwitterions have stabilizing effects on supramolecular structures in living systems [44,45], and it seems quite likely that these effects, which vary with the chemical structure of the dipolar ions [46], modulate the response of the membranes to low-ionic-strength shock in the synthetic buffers.

Several earlier studies have examined crenation of ghosts by divalent cations, monovalent salts, and phosphate buffers [35–39]. Of particular interest in the context of our work are reports that ghosts incubated in 10 mM Hepes [37] or phosphate at low ionic strength [39] progressively lost their capacity to crenate. This decay was accelerated by raising the temperature and was retarded by reducing the pH from 8 to 6 [39]. The pH and ionic strength dependence of the acquisition of spontaneous smoothing behavior parallels the conditions that promoted loss of sensitivity to crenating agents in the earlier studies, suggesting that the two phenomena are closely related. Consistent with this is our observation that ghosts that smoothed spontaneously were often less tightly crenated initially (Fig. 3).

The underlying structural basis for spontaneous smoothing is not yet known. Hemolysis in Hepes and other zwitterionic buffers in Group 3 yielded ghosts that remained pink despite repeated washing (Ref. 22 and results not shown). This phenomenon is probably simply another effect of the low ionic strength of the buffers in this group; retention of hemoglobin after hemolysis and washing in phosphate buffers at low ionic strength has been demonstrated by Dodge et al. [47]. In any case, these observations suggested that increased membrane retention of endogenous ATP or other cytoplasmic components, including hemoglobin, might be responsible for contour smoothing without ATP. Our results on conversion of shape-change behavior seem to rule out this possibility. Thus, Tricine ghosts and Hepes ghosts were relatively resistant to conversion to ATP dependence by washing in Tris or DMA (Table II), even though this removed most of the membrane-bound hemoglobin. (But more recent results indicate that > 50% conversion can be achieved by a high-salt wash (Raval, P.J. and Fairbanks, G., unpublished observations).) Conversely, white DMA or Tris ghosts were converted to ATP independence by one or two washings in Hepes or Tricine.

Attention has been focused recently on ATP-dependent aminophospholipid flipping as the basis for the progressive membrane curvature change in ATP-dependent smoothing and endocytosis [26,27,30]. The spontaneous contour smoothing differs from the active process in that it cannot be inhibited by vanadate (Refs. 22, 29 and unpublished observations). However, it is clear that, in principle, the development of this property could involve events in the bilayer independent of the aminophospholipid-flipping enzyme system. Lange et al. [39] have proposed that disturbances in lipid asymmetry due to the hemolytic trauma are responsible for susceptibility to salt-induced crenation. In their view, the effect of incubation at low ionic strength involves partial restoration of the normal lipid distribution, possibly facilitated by dilation of the hemolytic hole [48]. Such changes might be reflected in decreased bilayer resistance to bending or increased tension in the inner lipid monolayer under conditions of shape-change in-

cubation without ATP. However, the physical basis for spontaneous smoothing could equally well reside in the plasticity and ionic strength dependence of the organization of the spectrin-actin network and in its linkage to the inner surface of the membrane [16,49]. Alterations in the pattern of protein associations in the network induced by exposure to low ionic strength would be expected to alter the mechanical properties of the skeleton. In continuum mechanical terms [16], organizational changes leading to increased tension in the skeleton 'gel' when the temperature is raised would favor reduction of positive curvature and smoothing of the composite trilaminar membrane. Lange et al. [50] have found no evidence that ghosts resistant to crenation had expanded skeletons, but the changes in relative areas of the inner and outer membrane surfaces during shape transitions may be too small to detect hy direct observation [6]. Further study of the effects of low-ionic-strength buffers on the dynamics of bilayer and skeleton properties during subsequent shape-change incubations should be helpful in establishing which of the membrane domains is the locus of the hemolysis buffer effect.

There have been various reports of the toxic effects of zwitterionic buffers in cell culture systems [51], including inhibition of the development of spontaneous mechanical activity of arterial and venous smooth muscle [52] and the blockage of chloride channels of *Drosophila* neurons [53]. We have no evidence that such toxicity is involved in the hemolysis buffer effects described here. The apparatus using ATP for curvature change is clearly functional in ghosts produced by all 25 buffers at 10 mM. However, although all the membrane preparations undergo smoothing and invagination when ATP is supplied, there are differences in their hemoglobin content and responses to incubation without ATP. The variability of Tris ghosts in shape-change studies is clearly unacceptable and, despite intimations that this might be related to the presence of organic contaminants in the water, this problem remains unresolved. Ghosts made with Group 1 buffers (e.g., DMA and phosphate) are ATP-dependent in the initial smoothing phase of shape change, which can then be assayed simply by counting. Zwitterionic buffers from Group 2 or 3 should be acceptable if supplemented with salt or applied at higher concentrations to increase the ionic strength at hemolysis.

Acknowledgments

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