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Reconstruction of Erythrocyte Shape during Modified Morphological Response

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Abstract—Changes in erythrocyte shape during morphological response modified by benzalkonium chloride (BzA) were studied in sucrose solutions. Fixation of the cells with glutaraldehyde- and formaldehyde-containing fixatives at some time points is usually inadequate to maintain the current cell shape. Considering the reconstruction of erythrocyte shape, which takes into account the mode of fixative action, we showed that the echinocyte-forming activity of BzA depends on the concentration of this surfactant. It can induce a direct spherostomatocyte–spheroechinocyte transition without altering the near-spherical shape of the cells. On the other hand, the reverse spheroechinocyte–spherostomatocyte transition was always accompanied by some flattening of the cells, although in some instances discoidal shape was not achieved. The data point to asymmetric shape transitions of erythrocytes in sucrose solution, which contradicts the continuum and bilayer-couple models of shape regulation. It seems that the nonuniform structure of native erythrocyte membrane plays a more important role in morphological transitions of these cells than suggested earlier.

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Under physiological conditions, normal human erythrocytes are biconcave discs. A variety of agents can reversibly modify this shape while maintaining constant area and volume [1-4]. One group of agents or conditions, including anionic amphipaths, high salt, high pH, ATP depletion, cholesterol enrichment, and proximity to a glass surface induces a series of crenated shapes, called *echinocytes*, characterized by spiculated surface. Another group of agents or conditions, including cationic amphipaths, low salt, low pH, and cholesterol depletion induces concave shapes called *stomatocytes*. These changes in cell shape are universal in the sense that neither the type nor emergence of the shape depends on which distinct causative agent or condition is used. Hoffman [5] and Sheetz and Singer [6] proposed a mechanism explaining these changes in erythrocyte shape; it is based on membrane imbalance and small area difference ΔA_0 between the two layers of the cell membrane. Thus, any factor expanding the outer monolayer relative to the inner one (increasing ΔA_0) results in formation of convex structures on the cell surface (e.g. echinocytic spicules); conversely, an expansion of the inner monolayer (decreasing ΔA_0) favors concavities (e.g. stomatocytic shapes). Another recent model postulates that the erythrocyte shape is determined by bending rigidity of the lipid bilayer and stretch and shear elasticity of the membrane skeleton [7, 8]. This so-called bilayer-couple theory (BCT) explains the universality of the main, stomatocyte–discocyte–echinocyte or reverse transition by postulating that all shape-changing agents (physical or chemical) act solely or mainly through their effect on ΔA_0. This theory can reproduce various stages of echinocytosis or stomatocytosis by simple varying the relative areas of lipid bilayer and predicts other forms beyond this main sequence, which are observed under certain conditions [7]. The BCT was successfully applied for qualitative and quantitative assessment of erythrocyte shape under the action of various exogenous stimuli [9-15]. Recently, we characterized a morphological response (MR) that spontaneously occurs upon the transfer of ery-

Abbreviations: BzA, benzalkonium chloride; MR, morphological response.

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throcytes into non-electrolytic low-chloride medium containing sucrose [15, 16]. The mechanism of this phenomenon is not clear, but good reproducibility of this simple model allows it to be used for examining the theoretical conceptions on regulatory mechanisms of erythrocyte shape under non-physiological conditions. Since the cationic surfactant benzalkonium chloride can embed in the erythrocyte membrane and modify erythrocyte shape in saline [17], it can be used as a modifier of the cell membrane surface state. This surfactant has a partition coefficient of the order of 1000 [17]; moreover, its effect on the erythrocyte shape was not previously characterized. In connection with this, our goal was to characterize erythrocyte morphology at different stages of MR modified by benzalkonium chloride and to determine how closely its effect follows the bilayer-couple theory.

MATERIALS AND METHODS

Fresh human erythrocytes were kindly provided by the Clinical Laboratory of the S. P. Grigoriev Institute of Medical Radiology, Ukrainian Academy of Medical Sciences. Blood was taken into vacuum tubes containing EDTA as anticoagulant. Following spontaneous sedimentation, the pellet (0.225 ml) was washed with saline (150 mM NaCl) and isotonic HBS solution (5 mM Hepes, pH 7.4, containing 150 mM NaCl). Then the pellet was resuspended in HBS to the final volume of 1 ml and used as the stock suspension.

Morphological response of the cells suspended in a standard sucrose medium (0.3 M) without buffer was monitored using an SA-01 two-beam shape-meter/aggregometer which allows, besides measuring optical density or light transmission, assessment of light fluctuations carrying information on cell shape [18]. The shape index (*SI*) was calculated following the previously described protocol [18] from the equation: $SI = k \cdot D$, where k is a constant depending on amplification factor and meter calibration and D is the root-mean-square value of light fluctuations in the interval of 1 sec. The calibration factor k allows formation of *SI* scale reflecting the erythrocyte discoidal–spherical shape factor (1.00 for discs and 0.06 for spheres). Erythrocytes (7-9 µl of stock suspension) were placed into a cylindrical (diameter 10 mm) glass cell containing 2 ml of HBS so that the initial optical density was 0.30 ± 0.01, which corresponds to cell concentration of $6 \cdot 10^6$/ml. The cell suspension was stirred on a magnetic stirrer at 600 rpm. Morphological changes were monitored using an equally dense cell suspension in medium containing sucrose (Merck, Germany), pH 5.8-6.2. In other experiments additional chemicals (0.5-10 µl of stock solutions) were added directly to the cell to the desired final concentration [15].

To fix the erythrocyte shape, 0.2 ml of the following fixatives were added to 2 ml of cell suspension: FRa (5% glutaraldehyde, 20 mM phosphate buffer, pH 6.4, 150 mM NaCl); FRb (4% formaldehyde, 50 mM phosphate buffer, pH 7.4); FRab (mixture of equal volumes of FRa and FRb); FR5 (5% glutaraldehyde, 20 mM phosphate buffer, pH 5.7, 0.3 M sucrose). Following incubation in a fixative for several hours, the samples were centrifuged, and the pellets were examined under a microscope and photographed at ×800 magnification. The stock solution of benzalkonium chloride (BzA; Fluka, Germany) was prepared with concentration of 0.4 mg/ml. All experiments were carried out at room temperature (22-24°C).

RESULTS ANS DISCUSSION

In some cases, slight changes in *SI* were observed following fixation of erythrocytes in HBS with different fixatives, thus indicating an effect of fixation on cell shape depending on cell donor and fixative type. Nonetheless, in all cases the cells were generally fixed with predominance of discs and small amount of other shapes, thus indicating adequate fixing of erythrocyte with used fixatives in physiological solution. In sucrose solution the cell shape altered dynamically demonstrating triphasic MR in which the first phase is echinocytic, the second discoidal, and the third stomatocytic [15, 16]. In all cases *SI* increased following fixation at the stomatocytic phase 3; the erythrocyte shape was stomatocytic when fixatives FRa, FRab, and FR5 were used, whereas it was echinocytic when FRb was used. Hence, it appears that fixative FRb (formaldehyde) in sucrose medium is inadequate, because it mainly transforms the cells into echinocytes. So we did not use this fixative in further experiments. The fixative FRab that also contains formaldehyde elevated *SI* to a greater degree than the others, but in this case the cells retained stomatocytic shape, although their stomatocytosis degree was less than that observed after fixation with the other reagents containing glutaraldehyde only (data not shown).

In sucrose medium many substances can modify the standard MR by influencing its different phases [15, 16, 19]. The effect of BzA is shown in Fig. 1. One can see that the result depends on BzA concentration and MR phase. Changes in *SI* clearly suggest the shape-modifying effect of BzA. The erythrocyte shape differs from discoidal when $SI < 1$, but its distinct type remains unclear. For instance, the second broad peak in Fig. 1 (*SI*1, curve *1*) appearing after addition of BzA can characterize both echinocytic and stomatocytic shapes. At higher BzA concentration *SI* is low at phase 1 and after addition at the MR maximum (Fig. 1, *SI*2), and after addition at phase 3 a small transitional peak appears. To determine what shapes appear following BzA addition in different MR phases, we fixed erythrocytes at the maximum of the second peak and at other temporal points as well. The data

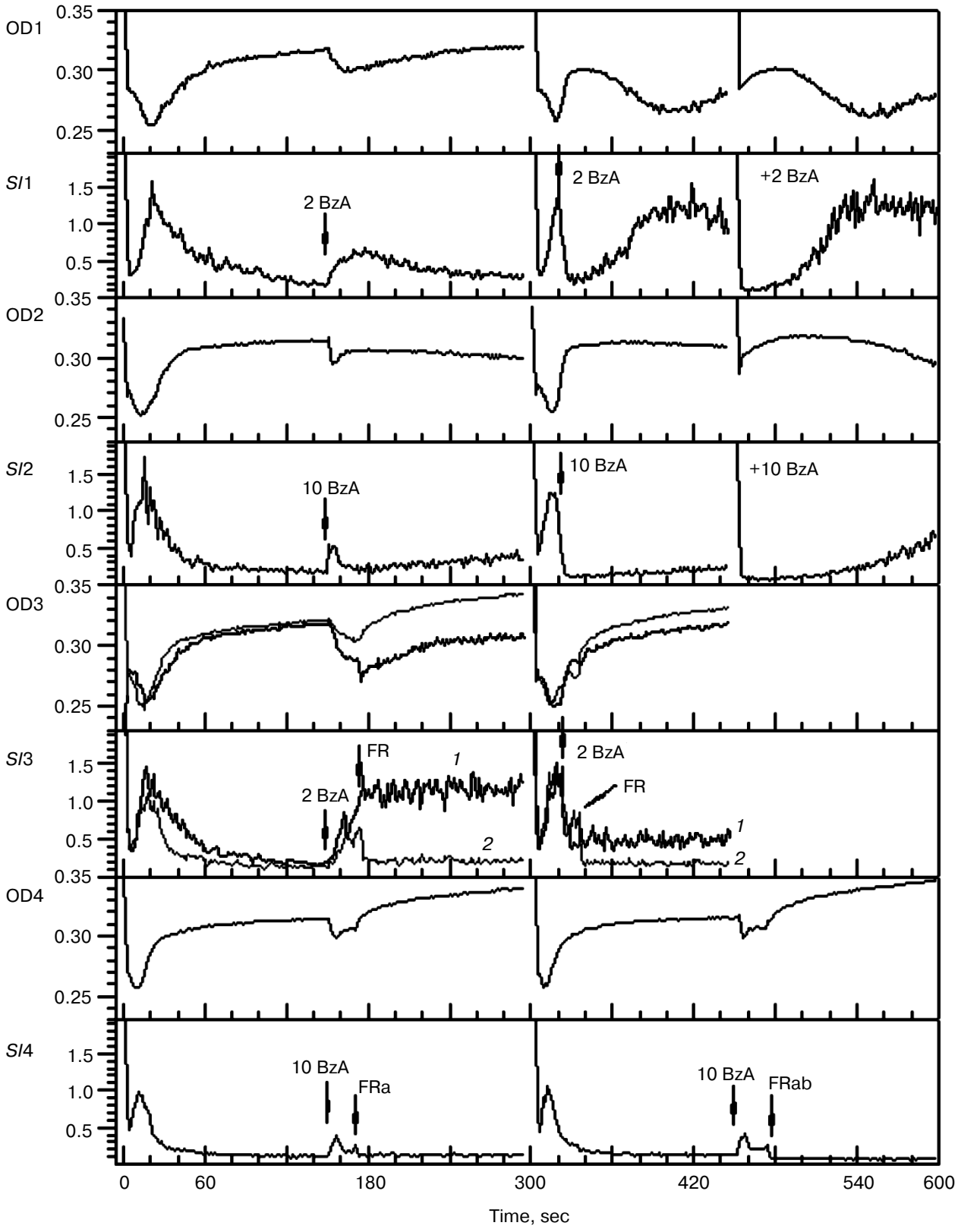

Fig. 1. Changes in optical density (OD) and *SI* of erythrocytes during MR and under the influence of BzA and fixatives at different MR stages. Arrows and their designations specify the type and volume (μl) of added reagent. Designations with symbol "+" indicate that this volume of BzA was added before the cells. FR on *SI*3 specifies addition of 0.2 ml of FRa for curves *1* and FRab for curves *2*.

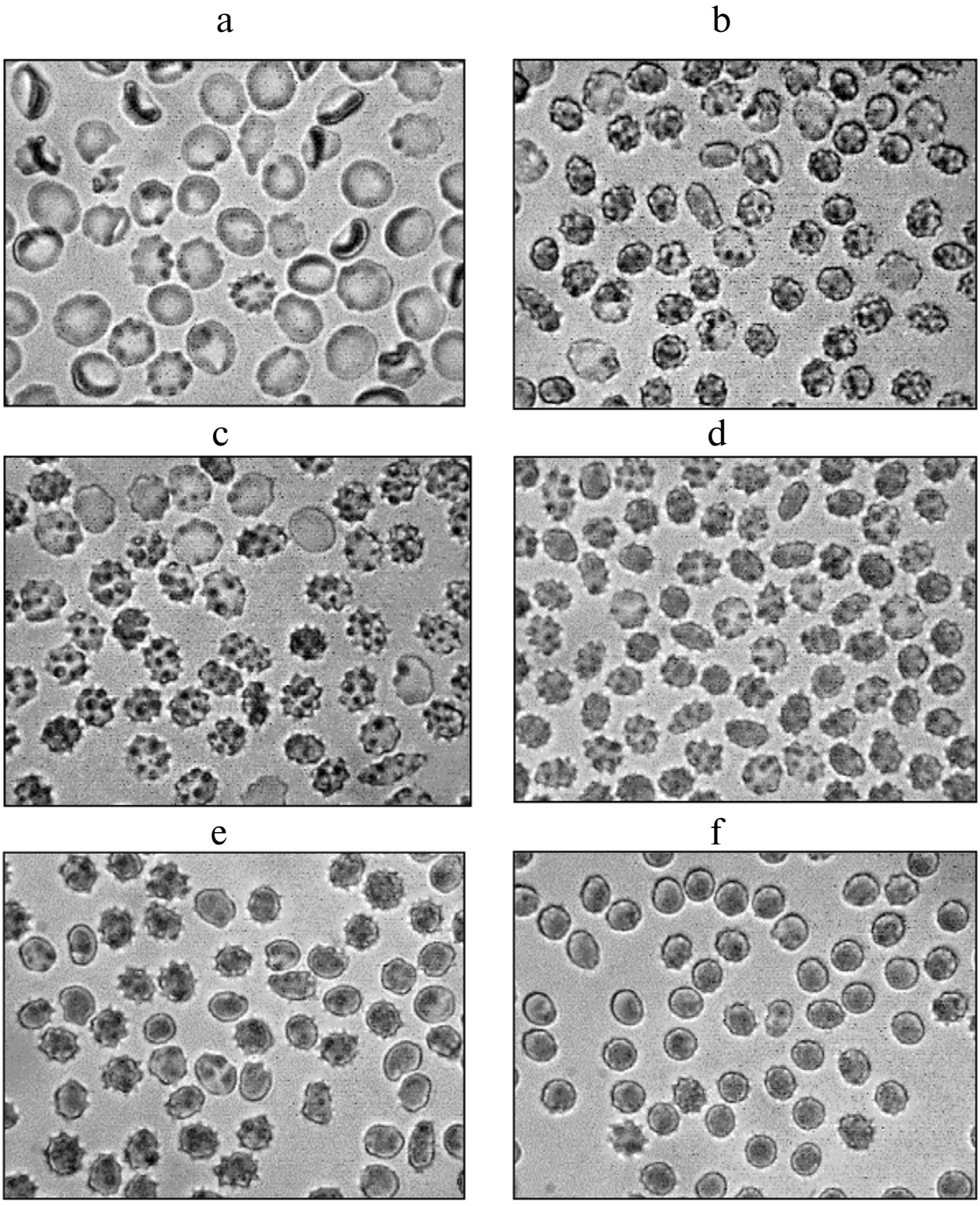

Fig. 2. Shape of erythrocytes after fixation at various MR temporal points corresponding to the data presented in Fig. 1. a) *SI*3, FRa, curve *1*; b) *SI*3, FRb, curve *2*, upon fixation at the maximum of the second *SI* peak following BzA addition 150 sec after addition of the cells (first half of *SI*3); c) *SI*3, FRa, curve *1*; d) *SI*3, FRab, curve *2*, upon fixation following BzA addition 20 sec after addition of the cells (second half of *SI*3); e) *SI*4, FRa; f) *SI*4, FRab.

shown in Fig. 1 (*SI*3 and *SI*4) are indicative of opposite effects of FRa and FRab added at this maximum. The first reagent increases *SI*, whereas the second decreases it. In another case, FRa does not alter *SI*, whereas FRab decreases it again (Fig. 1, *SI*3, curve *2*). Both fixatives decreased *SI* when added to erythrocytes treated with a large amount of BzA (Fig. 1, *SI*4). These data taken together point to a significant effect of the fixation itself on the current erythrocyte shape in sucrose medium containing BzA. Moreover, the shapes appearing after fixation with different reagents can fundamentally differ from each other, as shown in Fig. 2. This suggests that the method of fixation does not allow determination of cell shape at a particular MR stage. Another method of direct visualization of erythrocyte shape by microscopy of erythrocytes distributed in a thin layer or a drop of liquid on a glass slide is complicated by a near-surface effect of the glass [20], which strongly affects the BzA-modified erythrocyte in sucrose medium. So we attempted to reconstruct the cell shape considering the following basic arguments and facts. We can compare the predicted variations of cell shape under certain conditions with the observed data given the effect of the fixatives.

Our data together with others [15, 16] are indicative of stomatocytic erythrocyte shape at phase 3. So, we fixed erythrocytes following their incubation in sucrose medium for 30 min with different BzA concentrations.

The data shown in the table demonstrate a progressive increase in number of echinocytes in samples fixed with FRab and diskoids and other flat shapes in samples fixed with FRa as the BzA concentration is increased. This suggests that FRa flattens stomatocytes, that is, lowers the stomatocytosis degree. FRab has primarily the same effect, but it forms echinocytes with further elevation of BzA concentration. Hence, we can deduce that both reagents possess echinocyte-forming activity, especially with FRab. Given this feature, we can interpret the data shown in Fig. 1. Our conclusion completely follows the hypothesis that the second *SI* peak appearing after addition of BzA lies within stomatocytic shapes. That is just the reason why FRa shifts these shapes to more flattened ones (increasing *SI*), whereas FRab, possessing higher echinocyte-forming effect, forms echinocytes (Fig. 2). When added at the maximum of the MR, BzA induces echinocyte formation, and therefore FRa fixes them rather adequately and FRab enhances this echinocytosis (decreasing *SI*). Our conclusion is also supported by the data that fixation with FRa not at the maximum of second peak but much later (500 sec after the maximum) results in far less increase in *SI* and predominance of stomatocytic shapes in samples (data not shown). Increase in concentration of BzA enhances this echinocyte-forming factor, so that the cells rapidly transform into spheroechinocytes that are equally fixed with both fixatives (Fig. 2, e and f). A direct stomatocyte–spheroechinocyte transition is observed without transient discoidal shapes. Although small *SI* and optical density peaks might be interpreted as certain transient states between stomatocyte and spheroechinocyte, the presented photos only show some smooth spheroids with thin spicules. No flattened cells are observed at these fixing points. Hence, spheroechinocyte can be formed directly from spherostomatocyte, i.e. transition between opposite shape classes occurs.

Predominant erythrocyte shape after fixation with FRa and FRab depending on BzA concentration

[BzA], μg/ml	Fixative FRa	Fixative FRab
0	S	S
0.2	S	S
0.4	S	S + E
1	S + D	E + A + S
2	D + A	E + A

Note: Erythrocytes under standard concentration were added into sucrose medium containing the specified BzA concentration, incubated for 30 min, and fixed as described in "Materials and Methods". S, stomatocytes; D, discocytes; E, echinocytes; A, flat acanthocytes. When different forms were present in samples, the predominant form is indicated first.

We also intended to determine whether or not a reverse transition is possible between echinocyte and stomatocyte. We presumed from a similar supposition that any stomatocyte-forming agent should first induce the echinocyte–discocyte transition and, with increase of its effect, possible direct transition into stomatocyte without formation of transient discoidal shape. At present, we know several agents and conditions, such as calcium, Na_2EDTA, and low pH (HCl) [15], possessing stomatocytic effect on MR in non-electrolytic media. These agents have an interesting property. When present in the sucrose medium the cells are added into, they completely inhibit MR phase 1, that is, the cells retain discoidal shape rather than being transformed into echinocytes. Both calcium and HCl affect MR phase 3 insignificantly, whereas Na_2EDTA has a slightly inhibitory effect. If the agents are added to the cells some time after the beginning of the MR, they activate subsequent MR phases, whereas their addition at phase 3 activates it, that is, in this case they demonstrate stomatocyte-forming activity. Since BzA activates MR phase 1 to transform erythrocytes into spheroechinocytes, addition of the specified agents at this phase can be use to verify the given supposition. Indeed, all the reagents at low concentrations resulted in rapid and significant increase in *SI*, suggesting the echinocyte–discoidal transition. This can be interpreted as a compensatory effect of a stomatocytic factor

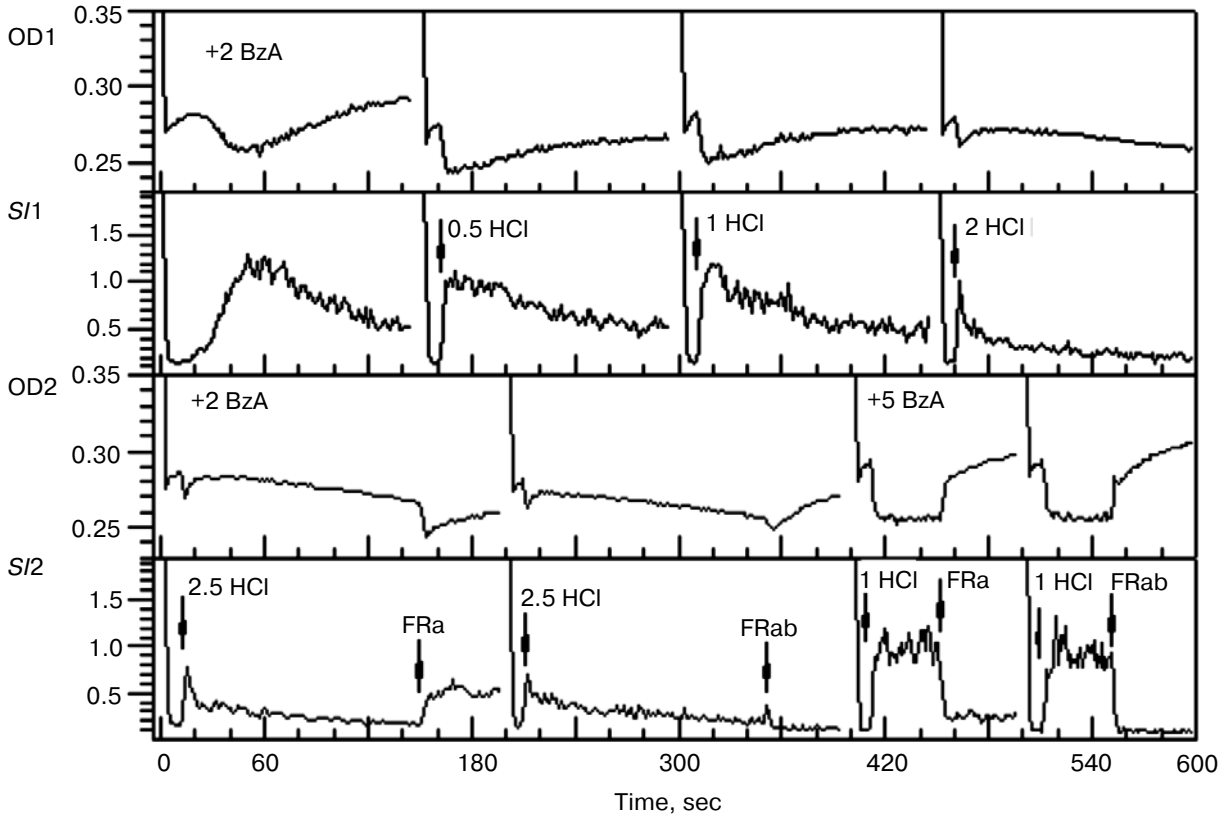

Fig. 3. Effect of BzA and HCl on MR dynamics. Arrows indicate the moment of addition of specified volume (μl) of 0.1 M HCl or 0.2 ml of fixatives into the erythrocyte suspension. Designations with symbol "+" indicate that this volume of BzA was added before the cells.

preventing echinocytosis. This response was maintained with increase in calcium concentration to 10 mM. Both calcium and Na_2EDTA inhibited MR phase 3, so that the cells kept a discoidal shape for a long time. This suggest more complex nature of calcium and Na_2EDTA effects on MR than their simple stomatocytic activity, because in the latter case we should expect a subsequent stimulation of stomatocytic phase 3, which does not actually occur. Hence, in the presence of BzA both these agents only manifest their stomatocytic activity at phase 1, which is expressed as cell flattening, but does not lead to stomatocyte formation. The effect of HCl appears to be closer to the theoretical anticipation. The data presented in Fig. 3 (*SI*1) show that a small concentration of HCl results in stimulation of *SI* restoration followed by its decrease, whereas high HCl concentrations diminish the amplitude of the appearing *SI* peak. These data can be interpreted as a transition between echinocytes and stomatocytes. Figure 3 (*SI*2) shows how the fixatives influence the final erythrocyte shape. As in the previous cases, FRa results in *SI* increase, whereas FRab decreases it.

The observed pattern is characteristic of stomatocyte fixation. Photos of these cells show discocytes and stomatocytes in the first case and echinocytes in the second (data not shown). Thus, HCl actually induces the echinocyte–stomatocyte transition. The possibility of this transition without a transient discoidal shape, as in the case of the reverse stomatocyte–echinocyte transition, cannot be determined clearly from our data. On one hand, the observed *SI* and optical density changes are 2-3-fold less than those that should be observed upon the transition through the discoid shape (compare Fig. 3, OD1 and OD2). However, this might be associated with either fast rate of this process or heterogeneity of the erythrocyte population, resulting in averaging of the effect and understating of *SI* value.

In other experiments with different BzA and HCl levels, small peaks on both optical density and *SI* curves were always detected, which are very likely indicative of some deviations of the cell shape from spheroechinocyte during this transition. In our opinion, the most plausible model is the following: a preliminary decrease in echinocytosis degree is required for the echinocyte–stomatocyte transition, but discoidal shape is unessential. In other words, our data suggest the possibility of direct echinocyte–stomatocyte transition, when echinocytosis degree is below a distinct critical value. If the echinocytosis degree has exceeded this value, echinocytes cannot transform into stomatocytes without preliminary flattening. This conclusion is supported by observations that

HCl added at different stages of MR phase 2 in the presence of BzA, once reaching a certain concentration, initiates a direct transition of the cells into stomatocytes without the transient *SI* and optical density peaks. It is likely that this phenomenon can enable determination of threshold *SI* value and morphological characterization of cell shape in this state. The difference between the direct echinocyte–stomatocyte transition and the reverse stomatocyte–echinocyte transition demonstrates that the latter can occur with virtually unchanged near-spherical cell shape. Our data confirm the general view about antagonism between echinocyte- and stomatocyte-inducing stimuli, that is, one agent can countervail the effect of another. This is well observed when the cells – by lapse of time – become less sensitive to the blocking effect of BzA. Their response in this case approximates the control MR, and less acid is necessary for the same response. And otherwise, the level of acid (stomatocyte-inducing agent) should be increased with increase in concentration of BzA to reach the same effect. Thus, by varying the balance between the two agents, one can obtain virtually identical erythrocyte shape transitions.

Our data suggest that fixation of erythrocytes in different MR phases usually leads to modification of their current shape. Only in a few cases the fixation was not accompanied by significant change in *SI*, thus indicating that these shapes were adequately fixed. This is probably because BzA itself causes a considerable increase in sensitivity of the cells to the modifying effect of fixatives, primarily that of FRab. Here we possibly deal with synergism of these two echinocyte-forming agents. This is also supported by the fact that adequate fixation of discoidal shape with FRa at BzA concentration of 0.4 μg/ml (data not shown) turns to inadequate fixation at 1 μg/ml, thus leading to a drastic decrease in *SI* and formation of echinocytes (Fig. 3, *SI*2). Our point of view is that the spherostomatocyte–spheroechinocyte transition follows the bilayer-couple theory. When the area of the outer monolayer increases due to incorporation of BzA molecules, this can result in formation of thin spicules growing on the smooth outer side of the stomatocyte. The growth of these new spicules can be accompanied by disappearance of invaginations, which are initially localized inside of stomatocyte, and thus result in formation of spheroechinocyte. If the spicule growth and disappearance of invagination occur with different rates, this can explain why the erythrocytes are not converted into discocytes. However, an additional supposition is required in this case, that the events affecting spicules and invaginations are independent, that is, are local rather than generalized as implied by the bilayer couple theory. The reverse transition is harder to explain even with the supposition of local character of spicules. To form a stomatocyte, an echinocyte should first reduce spicules, which must lead to cell flattening. Such partial flattening was observed in our experiments. However, it remains uncertain why this process, if initiated, does not proceed further to form discocytes, as occurs in standard MR, both in the absence and presence of BzA. The causative factors resulting in cell transition from distinct echinocyte stage into stomatocyte, bypassing the discoidal shape, remain uncertain and require further investigations.

It is worth noting in conclusion that some of our data cannot be explained by the bilayer-couple theory – both in its initial [6, 11] and more sophisticated forms [7, 8, 21] – which underlies our conception about mechanisms of erythrocyte shape variations. The data on local character of formation of both spicules and erythrocyte membrane invaginations also cannot completely explain our data, so the recent models of this process require reconsideration.

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