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TRANSFORMATION AND RESTORATION OF BICONCAVE SHAPE OF HUMAN ERYTHROCYTES INDUCED BY AMPHIPHILIC AGENTS AND CHANGES OF IONIC ENVIRONMENT

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SUMMARY

1. The biconcave discoid shape of normal human erythrocytes can be altered reversibly by a great number of amphiphilic agents. Crenated cells are induced, as a rule, by anionic or non-ionized compounds; cup-like cells by cationic substances. Formation of crenated and cup cells probably is induced by interaction of the transforming agents with sites charged oppositely within the membrane.

2. Compounds inducing crenation or cup formation can be regarded as true antagonists, since either group of compounds regularily brings about complete reversal of shape transformations induced by the other one.

3. Suspending erythrocytes in isotonic solutions of non-penetrating anions leads to the occurrence of cup cells, the extent of transformation becoming more pronounced with lowering of extracellular pH. Restoration of normal shape under these conditions is accomplished by addition of penetrating anions. The concentrations of these anions necessary to produce 90% normalisation increase with fall of pH. This type of cup formation supposedly is induced by the increase above a critical value of the pH difference between erythrocyte membrane and adjacent media.

4. The results point to a great lability of the interactions of structural elements of the erythrocyte membrane responsible for the maintenance of biconcave shape. They suggest this shape to result from an equilibrium between opposite forces directed towards formation of crenated or cup cells, respectively.

INTRODUCTION

The problem of how mammalian red blood cells maintain the shape of biconcave discs has been the object of many speculations ever since it was discovered that this ability is a property of the membrane itself[1]. During the last years, evidence has accumulated that ATP[2,3] and probably calcium[4] are involved in the maintenance of the normal shape. In addition, a nonhomogeneous arrangement of structural elements, especially an accumulation of cholesterol around the periphery of the biconcave disc[5], has been suggested to play an important role. The shape of the

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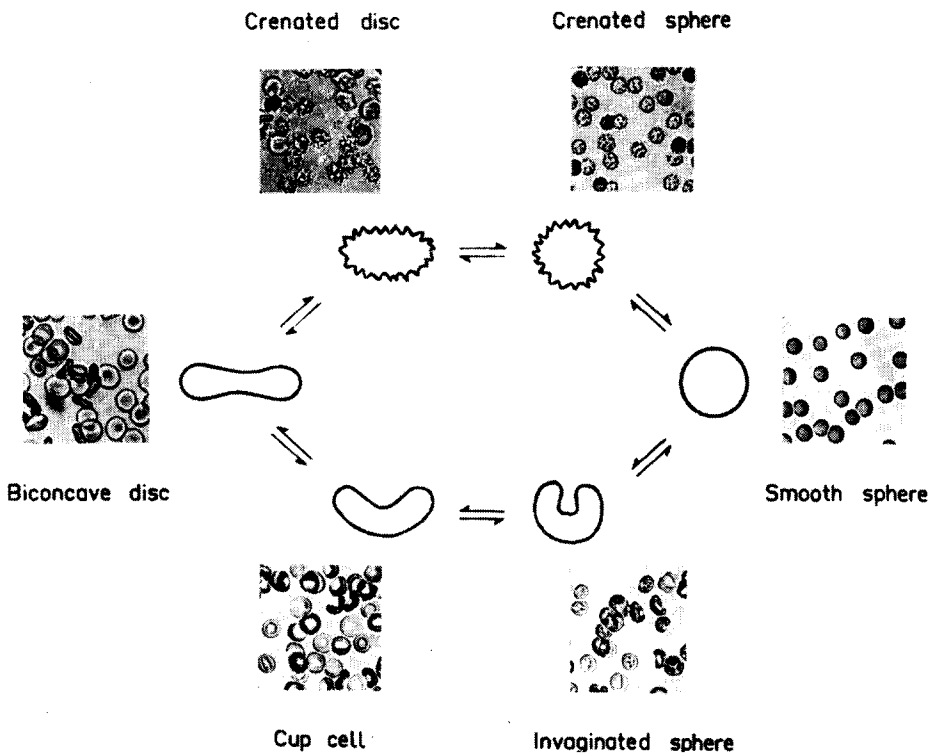

Fig. 1. Principal types of shape transformations of human erythrocytes.

erythrocyte seems to be the result of a delicate equilibrium between divergent forces. Disturbance of this equilibrium principally gives rise to the transformation of biconcave erythrocytes into either crenated or cup-shaped cells, both intermediates in the process of sphering (*cf.* Fig. 1)[6]. The present paper deals with some new observations on the formation of crenated or cup cells induced by various agents and by changes of pH or anion composition of the suspension medium, respectively.

METHODS

Experiments were carried out on fresh, washed human red cells, suspended either in saline media or in mixtures of saline and plasma (9:1). For analyzing the shape of the cells, wet smears were examined microscopically between slide and coverslip. These were siliconized prior to use in experiments with red blood cells suspended in pure saline media. General silicone coating of slides proved to be unfavorable, since silicone has a slight tendency to induce cup formation (see below). Each examination of shape was repeated at least three times on different aliquots of a sample.

RESULTS AND DISCUSSION

Influence of amphiphilic substances on cell shape

Normal biconcave red blood cells, suspended in Locke solution, can be converted into crenated discs or crenated spheres not only by fatty acids, alkylsulfonates or bile

TABLE I

SUBSTANCES INDUCING SHAPE TRANSFORMATIONS OF HUMAN RED BLOOD CELLS

Nomenclature of substances according to ref. 8.

Crenated forms		*Cup forms*	
Fatty acids	Dioxopyrazolidines	Alkylammonium chlorides	Hexobendine
Alkylsulfonates	Phenylbutazone	Phenothiazines	Reserpine
Dihydroxybenzenes	Phenopyrazone	Local anesthetics	Prenylamine
Substituted benzoates	Indomethazin	Cinchocaine	Verapamil
Salicylate	Furosemide	Tetracaine	Papaverine
Gentisate	Barbiturates	Procaine	Primaquine[10]
Ethacrynic acid	Phloretine	Antihistamines	Chloroquine
2,4-Dinitrophenol	Phlorrhidzine	Pheniramine	Benzydamine
Saponine	Bilirubine[9]	Brompheniramine	
Lysolethicine	Tannic acid	Bampine	
Bile acids	Thiosemicarbazone	Propranolol	
Alkylpyridinium chlorides	Dipyridamole		

acids[1], but also by many other compounds (Table I)[6,7]. Another group of substances, in contrast, induces the formation of cup cells or invaginated spheres. Both types of transformation are completely reversible upon removal of the transforming agent. Generally, the extent of transformation is unequal at low degrees and becomes more uniform as transformation proceeds towards the crenated or the invaginated sphere and finally the smooth sphere.

The transforming agents are not related to each other chemically. Their only common property is an amphiphilic molecular structure. Each of the two groups (Table I), however, is characterized by an additional feature: substances inducing crenation as a rule are anionic or non-ionized, whereas the cup-forming agents found as yet are cationic without any exception.

Further studies resulted in the interesting finding that crenated cells regularily regain normal biconcave shape upon addition of suitable concentrations of cup-forming agents (Table II). Cup cells, on the other hand, at all stages of transformation, can be reverted into biconcave discs by substances which bring about crenation. This reversal of shape changes, recently reported for the special case of bile or fatty acids and chlorpromazine[11], is certainly different from that described by PONDER[1]. It is indicative of an actual antagonism between agents inducing cup formation or crenation. This antagonism suggests, that the biconcave shape of erythrocytes physiologically is the result of an equilibrium within the membrane of opposite forces directed towards the formation of crenated or cup cells, respectively. Both these states for thermodynamic reasons seem to be more stable than the biconcave one.

Mechanisms and sites of action of the transforming agents are not yet clear. Most probably these compounds are adsorbed by the outer layers or even penetrate into the interior of the membrane in a reaction comparable to the process of film penetration. In consequence they might bring about changes of interfacial tensions or conformational changes of membrane lipoproteins. Crenation presumably is induced by an interaction of anionic agents with cationic groups, while the cationic, cup-forming substances might influence anionic sites, probably interfering with calcium binding[12], as some of them are supposed to do in other membranes[13–15]. If one agrees that interfacial tension is low at the concavities of the erythrocyte[5], cup formation

might be explained for geometrical reasons by an elevation of interfacial tension in that region. This assumption, of course, would be somewhat difficult to reconcile with the finding that cup forms have an increased surface area[16].

Since cup formation is antagonized by agents producing crenation, it may be supposed that the latter compounds either act on a different region of the membrane or bring about reversal of cup formation by influencing the same elements of the membrane in an opposite way, thus abolishing the effects of substances inducing cup formation. At any rate this antagonism may be of some importance for the interpretation of the effects of cholesterol on red blood cell shape[5,17].

Influence of anion environment and pH on cell shape

Additional information on the process of formation of cup cells was derived from experiments in which anion composition and pH of the extracellular medium were varied. As could be shown, suspension of red blood cells in isotonic (310 mosM) solutions of sucrose or the Na^+ salts of non-penetrating anions (citrate, *N*-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid)* results in the formation of cup cells. With

TABLE II

REVERSAL OF ONE TYPE OF SHAPE CHANGE OF RED BLOOD CELLS (CUP FORMATION OR CRENATION) BY ADDITION OF SUBSTANCES CAUSING THE OTHER TYPE OF TRANSFORMATION

Nomenclature of substances according to ref. 8.

Crenated form induced by	*Concn. (mM)*	*Normal shape restored by*	*Concn. (mM)*
Dipyridamole	0.05	Tetracaine	0.3
		Chlorpromazine	0.05
		Verapamil	0.25
Dipyridamole	0.003	Phlorrhidzine	0.25
		Hexobendine	0.5
Salicylate	10	Tetracaine	0.5
		Chlorpromazine	0.05
2,4-Dinitrophenol	1	Octylammonium chloride	4.0
Furosemide	10	Chlorpromazine	0.55
Saponine	100 μg/ml	Hexobendine	0.75
Cup form induced by	*Concn. (mM)*	*Normal shape restored by*	*Concn. (mM)*
Verapamil	0.4	Oleate	0.3
Verapamil	0.25	Dipyridamole	0.05
Verapamil	0.1	Dipyridamole	0.01
Chlorpromazine	0.15	Dipyridamole	0.01
Hexobendine	1.0	Dodecylsulfate	0.1
Hexobendine	0.75	Oleate	0.5
Cinchocaine	0.3	Caprylate	12.5
		Octylsulfate	2
		2,4-Dinitrophenole	2
		Dodecylsulfate	0.2
Octylammonium chloride	5	*p*-Nitrobenzoate	15
		Caprylate	12.5
Triton X-100	150 μg/ml	Octylsulfate	1

* HEPES (*cf.*) N. E. Good *et al.*, *Biochemistry*, 5 (1966) 467).

a decrease of pH, transformation proceeds from cup cells to invaginated spheres. Both can be reverted into normal cells by agents inducing formation of crenated cells. Moreover, normalisation of shape under such conditions is accomplished by the addition of isotonic NaCl solution. To quantify this latter effect, chloride concentrations were determined[18] in the extracellular medium of red blood cells, originally transformed into cup cells by suspension in isotonic *N*-hydroxy-ethylpiperazine-*N'*-2-ethanesulfonate solution and then reverted by addition of isotonic NaCl solution until 90% was normal again. From our results it became evident, that with a lowering of pH, extracellular chloride concentrations necessary to restore normal shape increased. The results of three individual experiments are shown in Fig. 2.

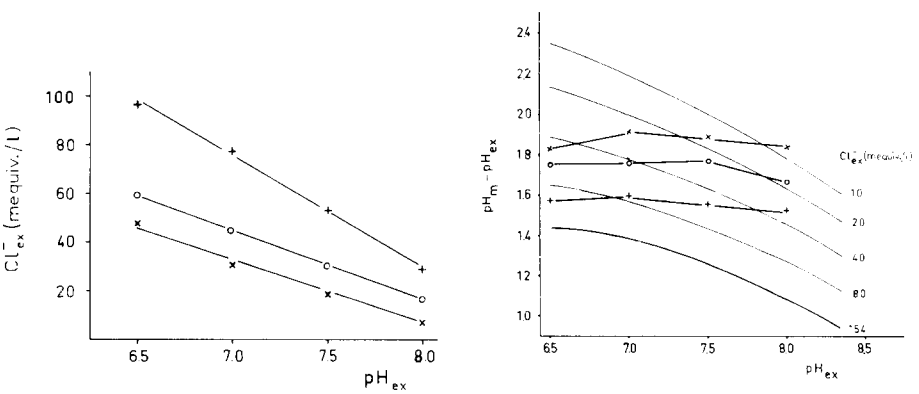

Fig. 2. Extracellular chloride concentrations at 90% reversal of cup cells formed in isotonic *N*-hydroxyethylpiperazine-*N'*-2-ethanesulfonate solution into normal biconcave discs. Data of three individual experiments.

Fig. 3. pH differences between red cell membrane and surrounding medium according to the fixed charge concept. Thin lines: changes of pH difference with extracellular pH at different extracellular chloride concentrations. Thick lines: pH difference at 90% reversal of cup cells into biconcave discs. Data of 3 individual experiments (+, ○, ×). pH_m values were calculated from extracellular chloride concentrations and pH_{ex} values given in Fig. 2 according to ref. 20, assuming the total concentration and the dissociation constant of fixed dissociable groups within the membrane to be 3 M and 10^{-9}, respectively.

In confirmation of earlier observations, formation of cup cells could furthermore be induced by lowering extracellular pH, at normal chloride levels, below 5.8 (ref. 19) and by suspension of red blood cells in hypotonic media[1]. Under these conditions, the normal shape of the cells could also be restored by elevation of extracellular chloride concentrations.

The most likely cause of this type of cup formation seems to be changes of the pH difference between the membrane and the surrounding media. This assumption is based on the hypothesis, strongly supported by recent findings on the kinetics of anion transfer[20,21], that pH values inside the red cell membrane (pH_m) are correlated to extracellular pH (pH_{ex}) by a Donnan equilibrium, the position of which depends on the concentration of positive dissociable fixed charges in the membrane and on the total concentration of penetrating anions[20].

By quantitative evaluation it can be demonstrated that according to this fixed charge hypothesis, pH values should be higher in the membrane than in the medium.

Moreover, this difference should increase when extracellular pH or total anion concentration are lowered (Fig. 3). On the basis of these theoretical considerations, pH values within the membrane after 90% reversal of cup forms were computed from chloride concentrations and pH values given in Fig. 2. From the results of these calculations it became evident, that the hypothetical membrane pH at 90% reversal of cup cells would change with extracellular pH. This finding disproved an explanation, considered at first, namely that red blood cells change their shape principally when pH_m exceeds a critical value.

As could be shown, however, the difference of pH between membrane and medium, at which 90% of cup cells have regained normal shape, is constant and independent of extracellular pH according to the fixed charge hypothesis (Fig. 3). It therefore seems probable, that the red cell can only maintain its biconcave shape, when the pH difference between membrane and medium is kept below a critical value. This explanation of course does not exclude the possibility that not the H^+ gradient *per se*, but some related gradient, like that of electrical potential, is in fact responsible.

The alternative hypothesis that cup formation induced by diminution of extracellular chloride is due to the concomitant changes of the difference between intracellular and extracellular pH was also considered. Quantitative evaluation of measurements of intracellular pH (Fig. 4), however, did not provide results in favor of this theory, except that cup formation in isotonic NaCl solution at pH values below 5.8 might be explained more easily on this basis.

The mechanisms by which changes of the pH difference between membrane and medium influence the shape of red cells still remain unclear. In principle, processes similar to those discussed in connection with the effects of cup-forming agents should be involved.

Our results can be regarded as new evidence for the importance of physicochemical properties of the membrane, especially of labile interactions of structural elements, in the maintenance of the biconcave shape of the red cell. How ATP is involved in this process is not understood. Studies concerning the interference of shape-changing substances and pH gradients with maintenace of shape by ATP are in progress.

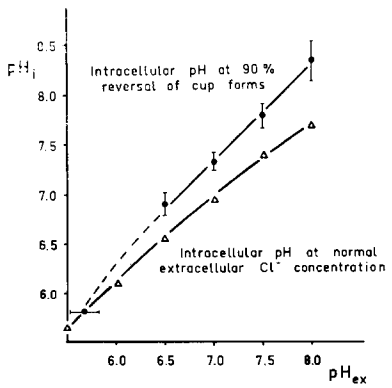

Fig. 4. Intracellular pH as a function of extracellular pH under different conditions. Intracellular pH values were either measured directly after hemolysing sedimented red blood cells by digitonine, or computed from the extracellular pH and the distribution ratio of chloride.

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WATER AND ELECTROLYTE CONTENTS OF RAT RENAL CORTICAL SLICES INCUBATED IN MEDIUM CONTAINING *p*-CHLOROMERCURIBENZOIC ACID OR *p*-CHLOROMERCURIBENZOIC ACID AND OUABAIN

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SUMMARY

1. Slices of rat renal cortex were leached anaerobically at 0.5° and subsequently reincubated at 25° in either oxygenated ordinary medium, oxygenated medium containing 0.2 mM *p*-chloromercuribenzoic acid (PCMB) or oxygenated medium containing 0.2 mM PCMB and 10 mM ouabain.