EQUILIBRIUM AND KINETIC EFFECTS OF DRUGS ON THE SHAPES OF HUMAN ERYTHROCYTES

MICHAEL P. SHEETZ and S. J. SINGER. From the Department of Biology, University of California at San Diego, La Jolla, California 92039. Dr. Sheetz's present address is the Department of Physiology, University of Connecticut School of Medicine, Farmington, Connecticut 06032.

In a previous paper (7) we have proposed and experimentally investigated the hypothesis that biological membranes behave as bilayer couples. This hypothesis states that the two halves of a membrane bilayer, by virtue of differences in their composition of proteins and lipids, may respond differently to a given perturbation; in particular, they may expand or contract differently in the plane of the membrane. If the membrane forms a closed surface, and the two halves remain coupled to one another throughout such changes in their relative surface areas, the shape of the membrane would have to change. This hypothesis was used to provide a molecular explanation of the effects of a wide variety of small molecule organic compounds on the shapes of human erythrocytes. There is a large group of compounds which cause the erythrocyte to develop crenations (3, 1) (see Fig. 1 A, below). They do so increasingly with increasing concentration until at large enough concentrations they cause the cell to form a sphere and eventually to lyse. Most of these crenators are amphipathic, anionic molecules, and include some important compounds such as all the mitochondrial oxidative-phosphorylation uncouplers, free fatty acids, barbiturates, phloretin and phlorizin, bilirubin, and salicylates. There is another large group of compounds which cause the erythrocyte to form cups or invaginations. These shape changes are intensified as the concentration of such a cup-former is increased, and as with crenators, at large enough concentrations the cells form spheres and eventually lyse (3). Cup-formers are almost all amphipathic cations and include important drugs such as the local anesthetics, phenothiazine tranquillizers, antihistamines, colchicine and vinblastine, and reserpine.

At the lower concentrations in which these compounds induce crenations and cup-formation, they also protect the erythrocyte against hypotonic hemolysis (for review, see reference 6). This has been attributed to expansion of the membrane induced by the binding of the drug, with little or no change in the cell volume; thus, it takes a larger volume increase to burst the membrane of the drug-treated than of the untreated erythrocyte. In this paper, our attention is confined to drug effects in these low concentration ranges.

The mechanism we proposed (7) to account for these effects in terms of the bilayer couple hypothesis is as follows. (A mechanical treatment of the bending of bilayers has led Evans [4] to suggest independently that the drug-induced crenation of intact erythrocytes is due to an expansion of outer vs. inner halves of the membrane.) In these low concentration ranges, the amphipathic molecules are nonspecifically bound to the erythrocyte membrane by intercalation of their hydrophobic portions into the lipid regions of the mosaic membrane (8) with their ionic groups in the hydrophilic surfaces. (To a first approximation, the membrane proteins are assumed not to be involved in these drug effects.) At equilibrium, the anionic compounds are preferentially bound to the outer half of the lipid bilayer and the cationic ones to the inner half. The anionic compounds therefore expand the area of the outer surface relative to the inner surface of the membrane, and cause the cell to crenate; the cationic compounds do the opposite, and cause the cell to form cup shapes. This
discrimination between anionic and cationic drugs is attributed to electrostatic interaction with the negatively charged lipid, phosphatidylserine, which is localized to the inner half of the erythrocyte bilayer (9, 5). We further suggested that drugs whose rates of diffusion across the membrane (flip rates) were very slow would be crenators of the intact erythrocyte whatever their net charge, since they would perforce be concentrated into the outer half of the bilayer. Thus, while chlorpromazine is a well-known cup-former of the erythrocyte, we showed that its close analogue, methochlorpromazine, is a crenator (7). As a tertiary amine, chlorpromazine can flip rapidly across the membrane in the neutral form and become charged by binding a proton on the opposite side. As a quaternary amine, however, methochlorpromazine cannot be discharged and the molecule therefore cannot rapidly traverse the membrane.

One crucial feature of our explanation of these drug-induced shape changes of erythrocytes that distinguishes it from other explanations that have previously been offered (6, 2) is the membrane sidedness with which these drugs are presumed to act. This feature suggests some specific experimental tests of our proposal which are the subject of this paper. These tests involve examining the shapes of intact erythrocytes treated with amphipathic drugs under nonequilibrium as compared to equilibrium conditions. The results are entirely consistent with the mechanism we have proposed.

MATERIALS AND METHODS
The reagents and general procedures for preparation of erythrocytes for scanning electron microscopy were as previously described (7).

RESULTS AND DISCUSSION
In the experiments with methochlorpromazine, intact erythrocytes were treated with the drug at 0.5 mM concentration in an isotonic Tris-NaCl buffer, pH 7.4, for 10 min at 0°C; an aliquot was removed and fixed, and when examined was found to be strongly crenated (Fig. 1 A) as previously shown (7). The remainder of the unfixed suspension was brought to 37°C, and, after various times of incubation, aliquots were removed for fixation and examination. The cells became less crenated with time. After incubation for 1 h 45 min, the cells were about equally divided between lightly crenated and lightly cupped shapes (Fig. 1 B). Past
this time, the cells became progressively more cupped. If the cells in Fig. 1 B, before fixation, were washed once at 0°C by centrifugation in drug-free isotonic buffer and then were rapidly fixed, they all were strongly cupped (Fig. 1 C).

In other experiments not shown, intact erythrocytes crenated by treatment with 1 mM 2,4,6-trinitrophenol (TNP), or cupped with $3 \times 10^{-3} \text{M}$ chlorpromazine retained their respective shapes over a 2-h period under the same conditions.

In further kinetic experiments, erythrocytes were treated with 10 mM TNP in the isotonic buffer for 30 min at 37°C. The cells, when fixed and examined, were strongly crenated (Fig. 2 A). If these TNP-treated erythrocytes, not fixed, were subjected to one wash in TNP-free buffer at 0°C, and were then fixed and examined, they were all cupped (Fig. 2 B). When the cell suspension in Fig. 2 B, before fixation and without further treatment, was simply warmed to 37°C for 5 min and then fixed, the cells were now crenated (Fig. 2 C), although less strongly than initially (Fig. 2 A). Control erythrocytes subjected to all of these manipulations, but with no TNP, showed the usual normal biconcave disk shape (Fig. 2 D).

We interpret these results as follows. Methochlorpromazine, because of the positive charge on its quaternary nitrogen atom, cannot rapidly flip across the erythrocyte membrane. If intact erythrocytes are examined shortly after the addition of the drug (Fig. 1 A), the cells are crenated because the drug is concentrated in the outer half of the lipid bilayer, thus causing the surface area of the outer half to expand relative to the inner, and producing the observed shape change. But this is a nonequilibrium state. With time at 37°C, the methochlorpromazine molecules flip from the outer to the inner half of the membrane where they are preferentially bound at equilibrium (7). The drug in the outer half-layer is always in equilibrium with the drug in the external solution, and its concentration therefore remains constant. The

![Figure 2](image-url)
concentration of drug in the inner half-layer, however, gradually increases with time (as it also does in the cytoplasm). When there are about equal concentrations of the drug in both half-layers of the membrane, the shape of the cell is nearly normal (actually, probably because of the heterogeneity of the erythrocyte population, the cells are equally divided between lightly crenated and lightly cupped (Fig. 1 B)). If at this stage the cells are rapidly washed in drug-free buffer at 0°C, the drug in the outer half-layer is largely removed, without the drug in the inner half having time to re-equilibrate; in this nonequilibrium state, the inner half layer is now expanded relative to the outer half, and the cells are all strongly cupped (Fig. 1 C).

The fact that neither TNP-crenated cells nor chlorpromazine-cupped cells change their shapes with time under the conditions of these experiments indicates that one cannot account for the time-dependent shape changes observed in the methochlorpromazine experiments by some general shape-repair mechanism. (However, if a glucose-phosphate buffer is used in these experiments rather than the Tris-NaCl, there seems to be a significant but slow return to normal shape of drug-induced crenated and cupped erythrocytes, suggesting that under these conditions a shape-repair mechanism may be operative.)

The results obtained in the kinetic experiments with TNP can be interpreted in a manner similar to that for the methochlorpromazine experiments. TNP, an anionic amphipathic compound, is an erythrocyte crenator (Fig. 2 A) because at equilibrium there is a larger concentration bound to the outer half-layer than to the inner. At equilibrium, there is, however, a significant concentration of TNP in the inner half-layer. If the cells are rapidly washed at 0°C, the TNP in the outer half-layer is largely removed, but that in the inner layer does not have time to reequilibrate. In this nonequilibrium state, the inner half-layer is therefore now expanded relative to the outer half, and the cell is cupped (Fig. 2 B). If these washed cells are now warmed to 37°C without any further treatment, the TNP in the inner half comes to its equilibrium distribution in the membrane, with a larger concentration in the outer than in the inner halves, and the cells become crenated (Fig. 2 C). At this stage, however, the cells are less highly crenated than originally (Fig. 2 A) (that is, they have fewer crenations and those they have are of larger radius) because after the washing there is now less total TNP in the cell membranes than originally, and the ratio of outer to inner surface areas is accordingly less than it was originally.

These results are therefore entirely consistent with, and strongly support, the explanation of drug-induced erythrocyte shape changes that is derived from the bilayer couple hypothesis. We have been unable to explain the results by any other proposed mechanism (6, 2).

Further evidence concerning these proposals could be obtained from a study of the uptake of radioactivity labeled methochlorpromazine and trinitrophenol by intact erythrocytes and ghosts. Such reagents are being prepared. If this mechanism of erythrocyte shape changes is accepted, an important corollary is that observations of shape changes as a function of time may be effectively used to determine the flip-rates of different amphipathic drugs in the erythrocyte membrane. This will be the subject of a later communication.

SUMMARY
We have previously proposed that if the two half-layers of a membrane are different in their protein and lipid compositions, they may respond differently to some membrane perturbation (the bilayer couple hypothesis). This hypothesis has been applied to explain the changes in shape of human erythrocytes that are produced by a variety of amphipathic compounds. These compounds are presumed to intercalate by their hydrophobic ends into the lipid portions of the membrane; if the compounds are anions, the binding is preferentially to the outer half of the bilayer, if cations, to the inner half. It is proposed that such preferential binding causes an expansion of one half-layer relative to the other, with a corresponding change in cell shape. The predicted sidedness of these shape changes is now demonstrated in experiments with methochlorpromazine and 2,4,6-trinitrophenol. Under appropriate nonequilibrium or equilibrium or equilibrium conditions, both of these compounds are shown to be either crenators or cup-formers of the intact erythrocyte, depending upon which side of the membrane they are concentrated in. These results therefore strongly support the bilayer couple hypothesis.

Ms. Polly Matzinger carried out some of the preliminary
experiments with methchlorpromazine that were extended in this study.

Michael P. Sheetz was a Dernham Junior Fellow of the American Cancer Society, 1972–1974. This work was supported by United States Public Health Service grants A1-06659 and GM-15971.

Received for publication 17 November 1975, and in revised form 12 February 1976.

REFERENCES

1. BESSIS, M. 1973. Living Blood Cells and Their Ultrastructure. Springer-Verlag, New York. 146–155.
2. BIERI, V. G., D. F. H. WALLACH, and P. S. LIN. 1974. Focal erythrocyte membrane perturbations caused by nitroxide lipid analogues. Proc. Natl. Acad. Sci. U.S.A. 71:4797–4801.
3. DEUTICKE, B. 1968. Transformation and restoration of biconcave shape of human erythrocytes induced by amphiphilic agents and changes of ionic environment. Biochim. Biophys. Acta. 163:494–500.
4. EVANS, E. A. 1974. Bending resistance and chemically induced moments in membrane bilayers. Biophys. J. 14:923–931.
5. GORDESKY, S. E., G. V. MARINETTI, and R. J. LOVE. 1975. The reaction of chemical probes with the erythrocyte membrane. J. Membr. Biol. 20:111–132.
6. SEEMAN, P. 1972. The membrane actions of anesthetics and tranquilizers. Pharmacol. Rev. 24:583–655.
7. SHEETZ, M. P., and S. J. SINGER. 1974. Biological membranes as bilayer couples. A molecular mechanism of drug-erythrocyte interactions. Proc. Natl. Acad. Sci. U.S.A. 71:4457–4461.
8. SINGER, S. J., and G. L. NICOLSON. 1972. The fluid mosaic model of the structure of cell membranes. Science (Wash. D. C.). 175:720–731.
9. ZWAAL, R. F. A., B. ROELOFSEN, and C. M. COLLEY. 1973. Localization of red cell membrane constituents. Biochim. Biophys. Acta. 300:159–182.