

BIOLOGICAL MEMBRANES AS BILAYER COUPLES

III. Compensatory Shape Changes Induced in Membranes

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ABSTRACT

We have previously proposed the hypothesis that asymmetric membranes behave like bilayer couples: the two layers of the bilayer membrane can respond differently to a particular perturbation. Such a perturbation, for example, can result in the expansion of one layer relative to the other, thereby producing a curvature of that membrane. In experiments with erythrocytes and lymphocytes, we now demonstrate that different membrane perturbations which have opposite effects on membrane curvature can compensate and neutralize one another, as expected from the bilayer couple hypothesis. This provides a rational basis, for example, for understanding the effects of amphipathic drugs on a variety of cellular phenomena which involve shape changes of membranes.

In two recent papers (14, 15) we have proposed and experimentally tested the hypothesis that biological membranes behave like bilayer couples; that is, the two halves of the bilayer membrane, if they differ in their protein and lipid constituents, can respond differently to membrane perturbations. For example, the two half-layers of the membrane may expand or contract differently in the plane of the membrane; if the membrane forms a closed surface, a change in the relative surface areas of the two halves would lead to changes in the curvature of the membrane. This hypothesis was then applied to the interaction of amphipathic drugs with intact human erythrocytes. A wide range of drugs, most of them negatively charged under physiological conditions, are known to cause erythrocytes to crenate; another broad spectrum of drugs, all positively charged, cause the erythrocyte to assume an invaginated or cup shape (3). It was proposed (14) that in the concentration ranges in which they induce these

shape changes, all of these drugs bind to the membrane by intercalating their hydrophobic portions into the lipid bilayer with their ionic heads in the membrane surfaces; and that drugs which are crenators bind preferentially into and expand the outer half-layer of the membrane, while the cup-formers bind preferentially into and expand the inner half-layer, thus producing the respective shape changes observed. The different equilibrium binding of anionic crenators and cationic cup-formers to the lipid in the two half-layers of the erythrocyte membrane was attributed to electrostatic interaction with the negatively charged lipid phosphatidylserine which is largely confined to the inner half-layer (1, 5, 23). 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 half-layers of the membrane.

In principle, any of a wide variety of membrane

perturbations, acting on either the lipids or the proteins of the membrane, might result in a change in the relative surface areas of the two half-layers of the membrane, with a resultant change in membrane shape. In other words, by quite different molecular mechanisms, similar area and shape changes might be induced in membranes. As a corollary, if any two independent membrane perturbations have opposite effects on the ratios of outer and inner surface areas, they might compensate for one another and neutralize the shape changes each alone induces. This proposal is subjected to the following experimental tests in this paper.

(a) It is known (3) that when a compound that is a crenator and one that is a cup-former are simultaneously added to intact erythrocytes, the cells retain their normal biconcave disk shape. This would constitute a compensatory shape change of the type described above, if it could be established that both compounds are simultaneously bound in the cell membrane. We therefore have carried out experiments with intact erythrocytes to which oleate (a crenator) and chlorpromazine (a cup-former) were added, and used the extent of protection against hypotonic hemolysis (13) as a criterion for the simultaneous binding of the two compounds (see Discussion).

(b) The incubation of erythrocytes in unsupplemented serum or in buffered saline for 24 h at 37°C is known to markedly deplete the intracellular ATP and to crenate the cells (16). First we have provided additional evidence that the molecular mechanism of this crenation is different from that of drug-induced crenation, and then we have shown that the crenated cells can be reversibly returned to the normal biconcave disk shape by the addition of the cup-former chlorpromazine.

(c) Morphological evidence was sought to determine whether mouse splenic lymphocytes undergo drug-induced shape changes comparable to those undergone by erythrocytes.

(d) Experiments were carried out on the "capping" and endocytosis induced in mouse splenic B lymphocytes when these cells are treated with antibodies directed to their surface Ig receptor molecules (9, 18, 20). The rationale for our experiments was as follows. It is known (9, 18, 20) that 2,4-dinitrophenol (DNP) is an inhibitor of the anti-Ig antibody-induced capping and endocytosis of these lymphocytes. This inhibition has previously been attributed to the fact that DNP is an uncoupler of mitochondrial oxidative phosphoryl-

ation, and therefore to an energy requirement for the processes of capping and endocytosis. However, DNP is also an effective crenator of erythrocytes. It therefore seemed possible that the effect of DNP in inhibiting these lymphocyte surface phenomena was not due to its uncoupling activity, but rather to its direct effect on the lymphocyte plasma membrane. The geometry of the shape change in endocytosis suggests, according to the bilayer couple hypothesis, that the change involves an increase in the surface area of the inner half relative to the outer half of the lymphocyte membrane. As a crenator, DNP might exert a compensatory effect and increase the surface area of the outer half relative to the inner, and thus inhibit the endocytosis. One test of this proposal would be to compare 2,4,6-trinitrophenol (TNP) and DNP as inhibitors of these lymphocyte surface phenomena, since TNP is incapable of uncoupling oxidative phosphorylation in intact mitochondria (21, 6) but, on the other hand, is as effective an erythrocyte crenator as DNP. We have indeed found that TNP is at least as effective as DNP in inhibiting antibody-induced capping and endocytosis of lymphocytes. All of these experiments have yielded results which are entirely consistent with the bilayer couple hypothesis, and therefore suggest its more general usefulness.

MATERIALS AND METHODS

Erythrocytes

The collection of normal adult human erythrocytes, the procedure used in hemolysis protection experiments, and the preparation of the erythrocytes for scanning electron microscopy in an ETEC Model R1 instrument were all performed as described previously (14). For the depletion experiments, washed erythrocytes at 50–70% hematocrit were incubated in isotonic buffered saline (146 mM NaCl, 20 mM Tris, pH 7.4, containing 100 µg/ml each of penicillin and streptomycin) for 24 h at 37°C. The viscosity of this suspension was followed in a low-shear Zimm-Crothers floating-bob viscometer (22), and was found to increase sharply upon depletion, as previously reported (7). An aliquot of these cells was fixed for microscope examination by the addition of 1 vol of cells to 10 vol of 1% glutaraldehyde in 140 mM NaCl, 10 mM sodium phosphate, pH 7.4. Another aliquot was removed, diluted to 3% hematocrit and treated with 300 µM chlorpromazine in the isotonic buffer for 10 min at 37°C. Part of this aliquot was fixed for microscopy; the rest was washed once by centrifugation in drug-free buffer, and then was fixed. To the remainder of the depleted cells at 50–70% hematocrit was added enough dry adenosine to give a 5 mM solution and the mixture

was further incubated at 37°C. In 2 h, the viscosity of the suspension had returned nearly to normal, and a portion of these repleted cells was fixed and examined microscopically.

Mouse Splenic Lymphocytes

These cells were prepared from freshly killed Swiss-Webster mice by standard procedures. For the experiments on the effects of drugs on lymphocyte morphology, the cells were suspended at a density of about 5×10^7 per ml, in a buffer containing 146 mM NaCl and 20 mM Tris, pH 7.4, and were then treated with 5×10^{-5} M chlorpromazine at room temperature. An aliquot of this suspension was removed after 10 min and fixed with a chlorpromazine-containing glutaraldehyde solution (1) for examination in the scanning electron microscope. To the remaining unfixed suspension, 10 mM TNP in the isotonic NaCl-Tris buffer was added, and, after 10 min, was fixed for electron microscopy.

For experiments on antibody-induced lymphocyte capping and endocytosis, the following procedure was used. First, rabbit antibodies directed to mouse Ig were bound to the lymphocyte surface at 0°C, under which conditions no capping or endocytosis occurs. To 1×10^7 cells in 1.0 ml of Hanks' balanced salt medium containing 0.5% bovine serum albumin was added 0.1 ml (500 µg) of the rabbit antibody at 0°C, and the mixture was incubated at 0°C for 30 min. To one aliquot of cells was added, instead, an equivalent amount of normal rabbit γ -globulin. The cells were washed twice by centrifugation with ice-cold medium and resuspended. Second, portions of the antibody-coated cells were subjected to one or another treatment: 0.1-ml aliquots of the cell suspension were either (a) left at 0°C for another 20 min; (b) brought to 37°C for 20 min; (c) treated with 10 mM NaN₃ (an inhibitor of capping and endocytosis) and brought to 37°C for 20 min; or (d) treated with a drug and brought to 37°C for 20 min. Third, fluorescein-labeled goat antibodies directed to rabbit IgG were added to these cells. All samples described above were washed twice by centrifugation with ice-cold medium containing 10 mM NaN₃, resuspended in 0.2 ml of that same medium, and mixed with 10 µl of the fluorescent goat antibodies for 20 min at 0°C. After two washes with the NaN₃-containing medium, the cells were fixed with 2% formaldehyde in 0.1 M sodium phosphate buffer, pH 7.4. After another washing, the cells were examined by fluorescence microscopy. The fraction of fluorescent cells in the population was determined, and the fluorescence was characterized as strong or weak, and as diffuse, patchy or capped.

Experiments were also carried out to test the reversibility of the effects of drugs on the lymphocyte capping and endocytosis. Lymphocytes were first treated with a drug at a particular concentration for 20 min at 37°C (pretreatment). The cells were then washed by centrifugation twice for various lengths of time in fresh medium at 0°C. The cells were then subjected successively to the

rabbit anti-mouse Ig antibody treatment at 0°C; to a further incubation with or without the drug at 37°C; and to the fluorescein-labeled goat anti-rabbit IgG antibodies; all as described above.

Reagents

DNP and TNP were recrystallized four times before use. Fluorescein isothiocyanate (10% on celite) was obtained from Calbiochem, La Jolla, Calif. Oleate (99% pure) was purchased from Mallinckrodt Inc., St. Louis, Mo. Chlorpromazine-HCl was the generous gift of Smith, Kline, and French Laboratories, Philadelphia, Pa. The preparation of the antibodies and the fluorescein isothiocyanate conjugation were carried out by standard procedures. Bovine serum albumin (Miles-Pentex Fraction V, Miles Laboratories, Inc., Elkhart, Ind.) was defatted by the method of Chen (2).

RESULTS

Combined Effects of Oleate and Chlorpromazine on Intact Erythrocytes

Oleate is a well known crenator of erythrocytes and chlorpromazine a cup-former (3). In the same concentration range in which each compound changes the cell shape, each affords the erythrocyte protection against hypotonic hemolysis (Fig. 1). Mixtures containing oleate at the constant con-

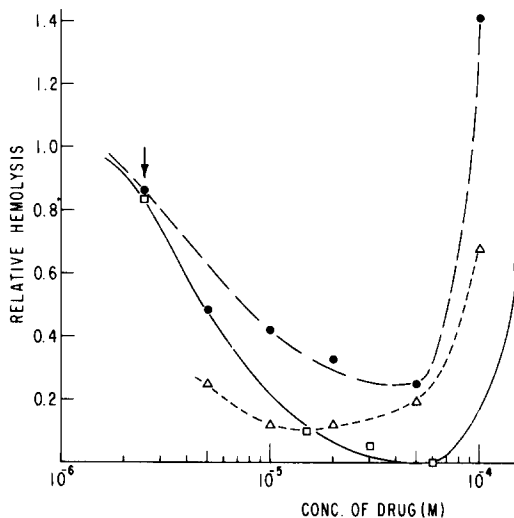


FIGURE 1 The protection against hypotonic hemolysis afforded human erythrocytes by the binding of different concentrations of oleate (●), chlorpromazine (□), and mixtures containing 2.5×10^{-6} M oleate and the indicated concentrations of chlorpromazine (△). The arrow indicates the hemolysis protection afforded by 2.5×10^{-6} M oleate itself. See text for further details.

centration of 2.5×10^{-6} M and different concentrations of chlorpromazine were tested for their effects on erythrocyte shape and on protection against hypotonic hemolysis. In such mixtures, the cells were crenated at chlorpromazine concentrations less than 1×10^{-5} M and cupped at higher concentrations (not shown). At 2.5×10^{-6} M oleate and 1×10^{-5} M chlorpromazine, the erythrocytes appeared to have their normal biconcave disk shape. In mixtures containing 2.5×10^{-6} M oleate and less than 2×10^{-5} M chlorpromazine, the hemolysis protection was substantially greater than with either compound alone at its concentration in the mixture (Fig. 1).

Combined Effects of ATP Depletion and Chlorpromazine on the Shape of Intact Erythrocytes

The well known crenation of erythrocytes that occurs from storage of human erythrocytes in un-

supplemented serum or in buffered saline for 24 h at 37°C is shown in Fig. 2 *b*. If these cells are then treated with 5 mM adenosine at 37°C , their normal biconcave disk shape is restored within 2 h (Fig. 2 *c*). If the crenated ATP-depleted cells are treated with 20 mg/ml of defatted bovine serum albumin, there is no change in cell shape (Fig. 3 *a, b*). It has been suggested (16) that the crenation that occurs upon ATP depletion is due to the formation of lysolecithin and its incorporation into the membrane. Lysolecithin by itself is a known erythrocyte crenator, as is shown in Fig. 3 *c*. (The cells here are so heavily crenated as to be nearly spherical in shape [3].) However, as has already been shown (8), lysolecithin-crenated cells are readily returned to normal shape (Fig. 3 *d*) by the same bovine serum albumin treatment used above. The inability of bovine serum albumin to affect the crenation of ATP-depleted cells therefore demonstrates that the crenation cannot be

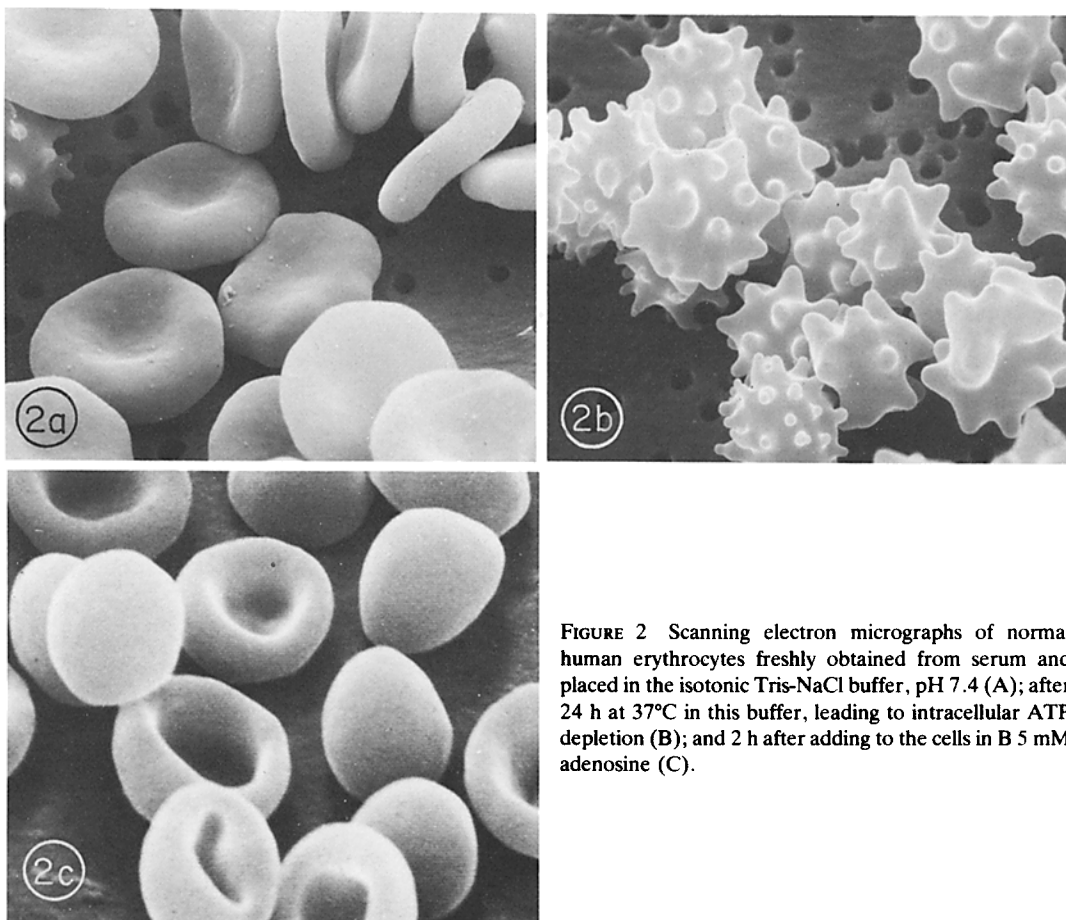


FIGURE 2 Scanning electron micrographs of normal human erythrocytes freshly obtained from serum and placed in the isotonic Tris-NaCl buffer, pH 7.4 (A); after 24 h at 37°C in this buffer, leading to intracellular ATP depletion (B); and 2 h after adding to the cells in 5 mM adenosine (C).

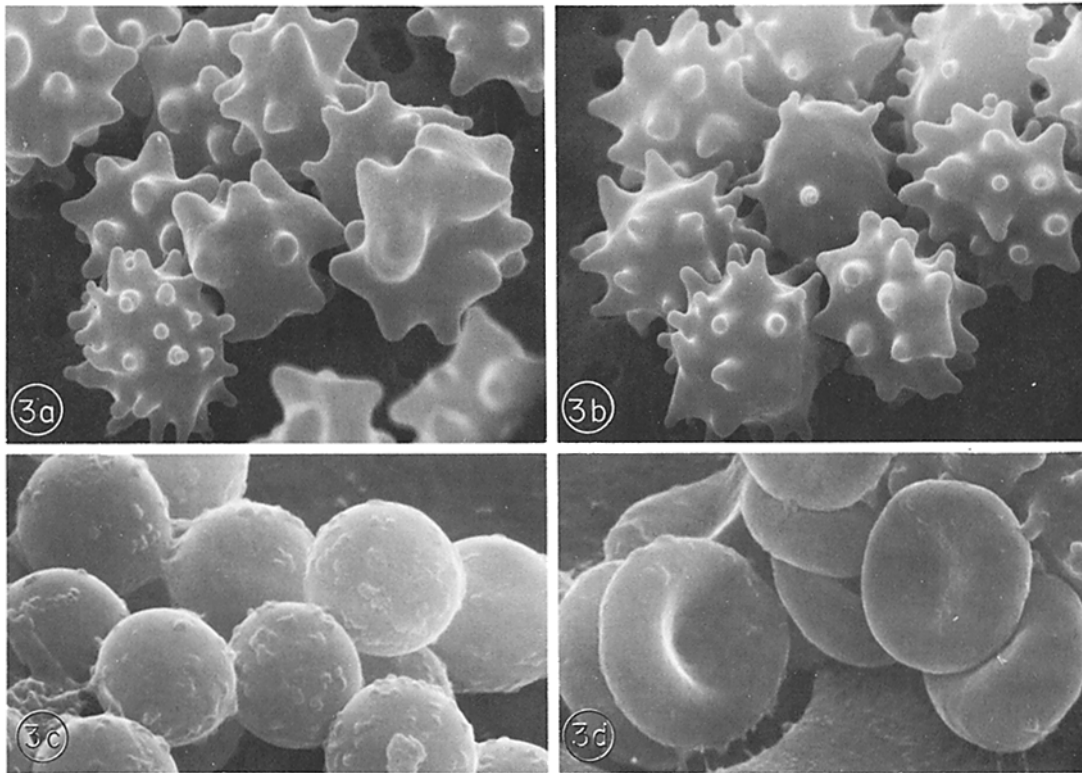


FIGURE 3 Scanning electron micrographs of normal human erythrocytes in isotonic Tris-NaCl buffer: (A) after 24-h ATP-depletion; (B) cells in A treated with 20 mg/ml defatted bovine serum albumin for 10 min at 4°C; (C) fresh cells treated with 50 μg/ml lysolecithin for 1 min at 4°C; and (D) cells in C treated with 20 mg/ml defatted bovine serum albumin for 10 min at 4°C.

attributed simply to the production of lysolecithin. Crenated ATP-depleted cells, however, are restored to nearly normal shape by treatment with 3×10^{-4} M chlorpromazine (Fig. 4 b); if these cells are then washed in drug-free isotonic buffer to remove the chlorpromazine, they return to their former crenated state (Fig. 4 c).

TNP as an Erythrocyte Crenator

At the same concentration, TNP is somewhat more effective than DNP as a crenator of erythrocytes (Fig. 5). That is, on the average the crenations are somewhat more numerous and of smaller radius in TNP- than in DNP-treated cells (14).

The Effects of Drugs on Lymphocyte Morphology

We sought to determine whether splenic lymphocytes change shape in response to the addition of drugs which are crenators or cup-formers of erythrocytes. This was made difficult, however, by

the abundance of microvilli which are normally present in irregular array on lymphocyte surfaces (Fig. 6 a). The ease with which these microvilli are mechanically sheared from the cell surface also complicates the problem. Given these difficulties, we were unable to determine whether any significant change in cell surface morphology was produced by the addition of an erythrocyte crenator (e.g., 10 mM TNP). However, there was a significant reduction in numbers of microvilli and a smoothing of the lymphocyte surface in 5×10^{-5} M chlorpromazine, the erythrocyte cup-former (Fig. 6 b). Furthermore, the addition of 10 mM TNP to the cells containing the chlorpromazine resulted in the reappearance of numerous microvilli (Fig. 6 c).

Antibody-Induced Capping and Endocytosis of Lymphocytes

By the technique described in the Methods section, the occurrence of antibody-induced endocytosis

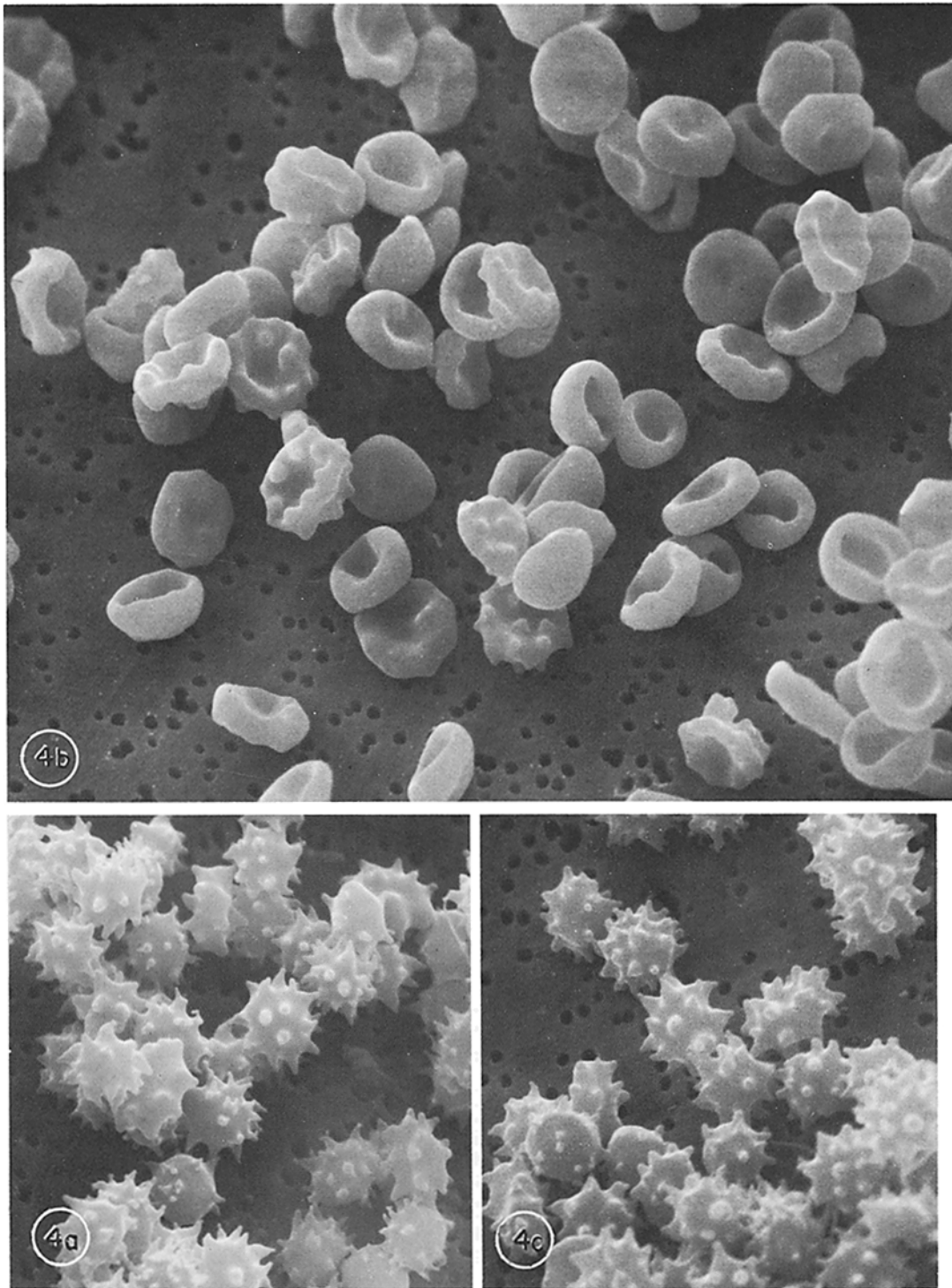


FIGURE 4 Scanning electron micrographs of normal human erythrocytes in isotonic Tris-NaCl buffer: (A) after 24-h ATP-depletion; (B) cells in A treated with 3×10^{-4} M chlorpromazine for 1 min at 4°C; (C) cells in B after a 100-fold dilution into buffer.

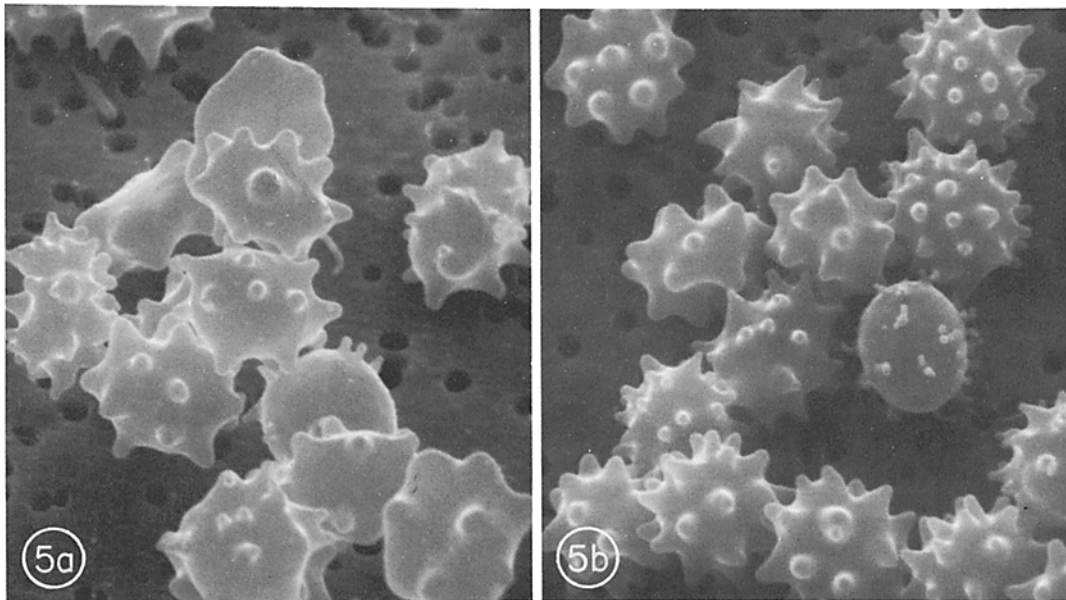


FIGURE 5 Scanning electron micrographs of fresh normal human erythrocytes in isotonic Tris-NaCl buffer after treatment for 1 min at 4°C with: (A) 1 mM 2,4-dinitrophenol; and (B) 1 mM 2,4,6-trinitrophenol.

tosis was scored in the following manner (9, 18). If the rabbit antibodies to mouse Ig had induced extensive endocytosis in the second step, the rabbit antibodies would then be unavailable at the surface of the B lymphocyte for labeling with the fluorescent-tagged goat anti-rabbit IgG antibodies, and the population of cells would be largely unstained. If, however, extensive endocytosis had not occurred, the rabbit antibodies would be present at the cell surface, and the B lymphocytes (representing about 50% of the spleen cell population) would be strongly stained.

The data collected in Table I show that when lymphocytes were incubated for 20 min at 0°C with rabbit anti-mouse Ig antibody, 45–50% of the cells were strongly fluorescent, with the fluorescence exhibiting a patchy distribution over the entire cell surface; if the incubation was done at 37°C, only 8–10% of cells were weakly fluorescent (corresponding to the fraction of cells in the population that were found to stain with trypan blue); if 10 mM NaN_3 or 5–10 mM DNP was present during the 37°C incubation, the fluorescence remained extensive, strong, and patchy. All of these results are as described before (9, 18, 20). The new findings are that 5–10 mM TNP also largely prevented capping and endocytosis, and that even 1 mM TNP was significantly inhibitory. The results in Table II indicate that a prior treat-

ment of lymphocytes with 10 mM DNP, followed by washing of the cells for only 10 min at 0°C, had no effect on the subsequent antibody-induced endocytosis or its inhibition by a second treatment with 10 mM DNP. The effects of pretreatment of the lymphocytes with 10 mM TNP could not be similarly reversed even by a longer washing procedure, but if 5 mM TNP was used in the pretreatment and washing was continued for 60 min at 0°C, significant but partial reversion was achieved.

DISCUSSION

The crux of this investigation is the hypothesis (14, 15) that because the two half-layers of biological membranes are different in their composition, they may undergo different expansion or contraction in response to a perturbation; if the membrane forms a closed surface, the membrane would have to change its curvature. Perturbations which have similar effects on the ratios of outer and inner membrane surface areas should be synergistic, whereas perturbations which have opposite effects should compensate one another. Several diverse experiments are reported which appear to bear out this suggestion.

Normal Drug-Erythrocyte Interactions

As an example of such a compensatory effect, when an erythrocyte crenator such as oleate and a

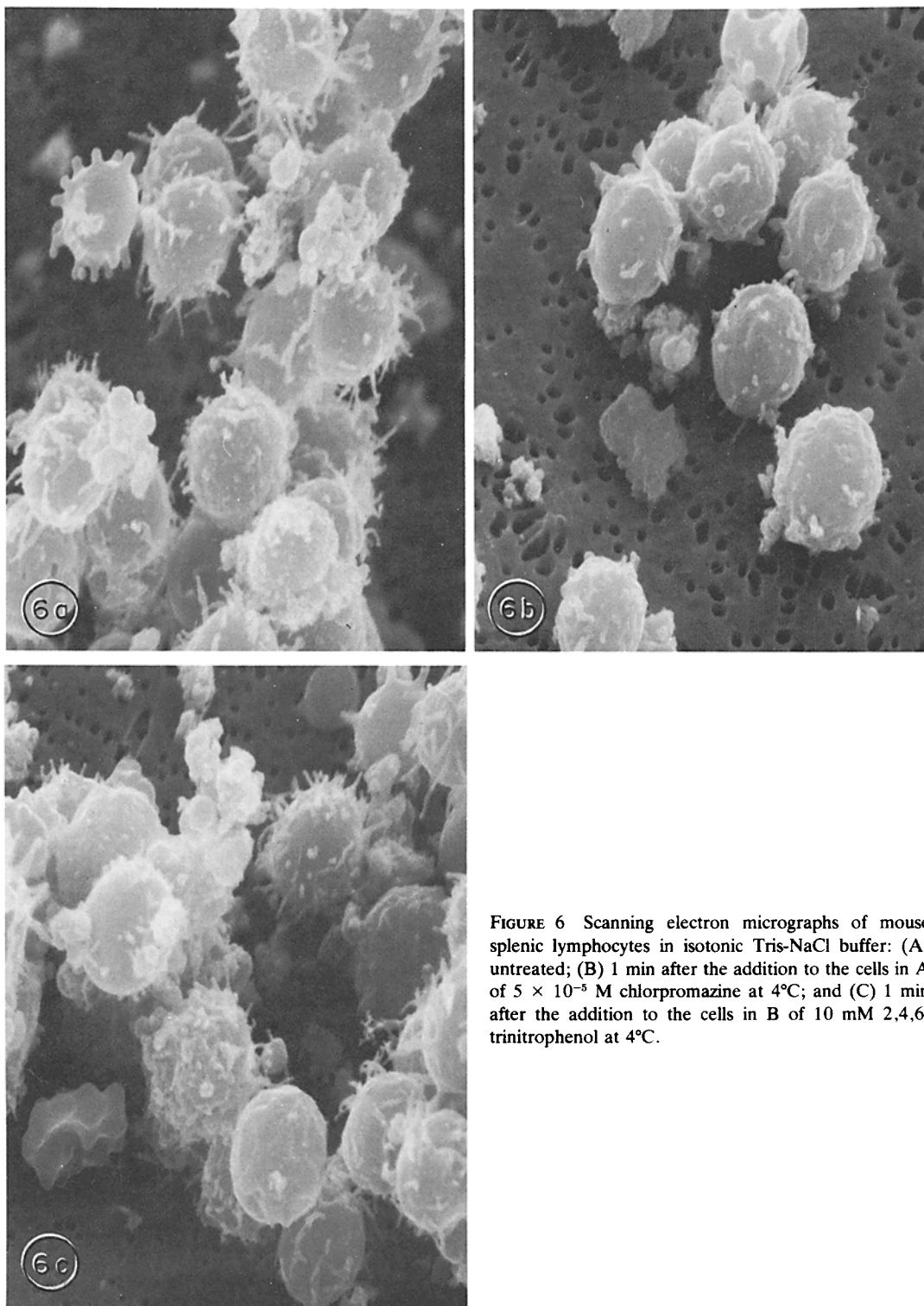


FIGURE 6 Scanning electron micrographs of mouse splenic lymphocytes in isotonic Tris-NaCl buffer: (A) untreated; (B) 1 min after the addition to the cells in A of 5×10^{-5} M chlorpromazine at 4°C ; and (C) 1 min after the addition to the cells in B of 10 mM 2,4,6-trinitrophenol at 4°C .

TABLE I
Effects of Drugs on Antibody-Induced Capping and Endocytosis of Lymphocytes

Drug	Concentration	Fluorescent cells*	Nature of fluorescence
	mM	%	
None	—	43‡ 10; 8	Strong, mostly patches Weak, diffuse
NaN ₃	10	34	Strong, patches
DNP	10	50	Strong, patches
	5	33	Strong, patches
TNP	10	54	Strong, patches
	5	37	Strong, mostly patches
	1	26	Strong, caps and patches

* On the average, 10% of the cells stained with trypan blue; no correction was made for these dead cells.

‡ In this experiment, the cells were kept at 0°C for 20 min in the second stage (Materials and Methods Section); all others were brought to 37° for 20 min in the second stage.

TABLE II
Reversibility of Drug Effects on Lymphocyte Endocytosis

Drug	Pretreatment* concn.	Time of washing	Second treatment* conc.	Fluorescent cells
	mM	min	mM	%
None	—	60	—	12
DNP	10	10	10	49
	10	10	0	17
TNP	10	10	10	54
	10	10	0	46
	10	60	0	46
	5	60	5	50
	5	60	0	28

* For 20 min at 37°C.

cup-former such as chlorpromazine are added together in the right proportions, the erythrocyte recovers its normal biconcave disk shape (3). We have shown (Fig. 1) that such mixtures confer upon erythrocytes greater protection against low ionic strength hemolysis than either compound alone. Hemolysis protection by a drug is interpreted to mean (13) that the drug causes an expansion of the surface area of the membrane without a significant increase in cell volume, thus requiring a larger volume influx (and a longer time) to burst the cell. With this interpretation, the greater hemolysis protection obtained with the mixture of 2.5×10^{-6} M oleate and 1×10^{-5} M

chlorpromazine than with either alone strongly suggests that the two compounds are simultaneously bound when the erythrocyte shape is restored to normal, and hence that the effects of the two compounds on shape cancel each other out.

The Effects of ATP-Depletion on Erythrocyte Shape

It has been suggested that the formation of lysolecithin is responsible for the crenation of erythrocytes upon ATP-depletion. By the results shown in Fig. 3, however, the crenation produced by ATP-depletion and by lysolecithin are readily distinguished upon treatment with bovine serum albumin. Also, ATP-depleted crenated cells are known to be much more rigid than drug-crenated cells (7), again suggesting that although the cells appear similar, a different mechanism for the crenation is involved in the two cases.

In a separate study (Sheetz and Singer, manuscript in preparation) we have obtained evidence that ATP-depletion produces a disaggregation of the spectrin complex (10) which is attached to the inner surface of the erythrocyte membrane (11). We propose that this disaggregation somehow causes an increase in the ratio of the surface areas of the outer vs. inner half-layers of the erythrocyte membrane, leading to the crenation. For our present purposes, the precise details of this mechanism are not important, so long as it is accepted that the crenation occurring upon ATP-depletion is different in mechanism from drug-induced crenation. Under these circumstances, the fact that the cup-forming drug chlorpromazine can restore ATP-depleted crenated cells to nearly normal shape (Fig. 4 b) is therefore a new kind of compensatory shape change, different from that considered above when two shape-compensating drugs are used. Here we suggest that the intercalation of chlorpromazine preferentially into the lipid in the inner half-layer of the bilayer compensates for the opposite area changes that are induced by the ATP-depletion. Further indication of the compensatory nature of the shape change is the fact that washing out the chlorpromazine from the membrane restores the cell to its ATP-depleted crenated state (Fig. 4 c).

The Effects of Drugs on Lymphocyte Morphology

The discussion to this point has been confined to adult human erythrocytes and the shape changes

they undergo. There is more information about the erythrocyte membrane than any other at the present time, and the asymmetrical distributions of its proteins and phospholipids in the two halves of the membrane are well documented (17). How general are such asymmetrical distributions for membranes other than erythrocyte membranes? That the proteins of many membranes are asymmetrically oriented and distributed is reasonably well established (17), and, in some cases, suggestive evidence has been obtained that the distribution of their phospholipids is also asymmetric (19). As an indirect approach to the latter problem, we may ask whether lymphocytes, for example, change shape in an analogous fashion to erythrocytes upon treatment with specific amphipathic drugs. If so, and if our explanation of these drug-induced erythrocyte shape changes is accepted, such results would suggest that the zwitterionic and anionic phospholipids in the lymphocyte plasma membrane are organized as they are in the erythrocyte membrane. Because lymphocytes are normally covered with irregular masses of numerous microvilli, it was difficult to tell whether erythrocyte crenators induced any further crenation of these cells. However, the erythrocyte cup-former chlorpromazine at 5×10^{-5} M produced lymphocytes with a much smoother surface than normal, with many fewer microvilli (Fig. 6 *a* and *b*); the addition of 10 mM TNP to the cells in chlorpromazine induced the reformation of numerous microvilli (Fig. 6 *c*). These changes are analogous to those produced with erythrocytes when treated successively with a cup-former and a crenator as discussed above. These results are therefore consistent with, but do not prove, the proposal that the plasma membrane of the lymphocyte is molecularly asymmetric much like the erythrocyte membrane, and responds to drugs by similar mechanisms (14).

In these lymphocyte experiments, and in others discussed below, TNP is used as an analogue of DNP. The experiments shown in Fig. 5 establish that TNP is indeed an effective crenator of intact erythrocytes in the concentration range of about 1–10 mM, and, if anything, is somewhat more effective than DNP at the same concentration.

The Effects of DNP and TNP on Lymphocyte Surface Phenomena

Our results on the effects of erythrocyte crenators on antibody-induced capping and endocytosis of mouse splenic lymphocytes show that TNP is at least as effective as DNP in inhibiting both the

capping and endocytosis (Table I). This inhibition is more difficult to reverse than with DNP added at the same concentration (Table II), but this may be due to TNP binding more strongly to the membrane, or to its slower flip-rate in the membrane (15). These results vitiate the conclusion that the inhibitory effect of DNP is connected with the uncoupling of mitochondrial oxidative phosphorylation, since TNP is not an uncoupler with intact cells and mitochondria (6, 21). This is not to say that the lymphocyte capping and endocytosis do not require energy; in fact the inhibition of these processes by NaN_3 suggests that they do. The inhibitory effect of DNP, however, can no longer be taken as evidence for such an energy requirement.

One possible explanation for the inhibition by DNP and TNP is that suggested in the introductory paragraph: namely that the lymphocyte plasma membrane, like the erythrocyte membrane, behaves like a bilayer couple, that those compounds that crenate the erythrocyte act by a similar intercalation mechanism on the lymphocyte membrane, and that, in doing so, they induce surface area and shape changes that are opposite to those presumed to be involved in capping and endocytosis; i.e. that the inhibition is due to compensatory shape changes. While this explanation is consistent with the results obtained, it is not established by them, and certain features of the explanation clearly require more direct substantiation. For example, while the geometry of the endocytotic events suggests that an increase in the ratio of inner to outer surface areas of the lymphocyte membrane is involved, there is no independent evidence that such an area change is also involved in capping. The clustering of Ig receptors into a cap by anti-Ig antibodies might in principle lead to such area changes, and to corresponding shape changes, but the lymphocyte plasma membrane is so convoluted in appearance in thin sections examined by transmission electron microscopy that one cannot determine whether capped regions of the membrane are altered in shape. More studies are required to demonstrate how these lymphocyte surface phenomena are affected by a range of compounds that in the appropriate concentration range are erythrocyte crenators and cup-formers.

Our results show that, whatever the mechanism by which they operate, DNP and TNP have a direct effect on the lymphocyte plasma membrane. The mechanisms whereby other drugs (12) affect lymphocyte surface phenomena may also be direct effects on the plasma membrane. Colchi-

cine, for example, is used to implicate microtubules in these phenomena, but, at concentrations of 0.1 mM, colchicine is an effective erythrocyte cup-former (3), indicating that it can interact directly with the membrane.

Finally, the usefulness of the bilayer couple hypothesis, in explaining and predicting the results discussed in this paper, suggests that the hypothesis may be relevant for a variety of cellular phenomena in which membrane shape changes occur. Such shape changes may involve, at least locally, a change in the relative areas of the inner and outer half-layers of the particular bilayer membrane.

This work was supported by U. S. Public Health Service grants AI06659 and GM15971 to S. J. S.

Dr. Sheetz was Dernham Junior Fellow of The American Cancer Society, Inc., 1972-1974. Dr. Painter was U.S. Public Health Service Postdoctoral Fellow, 1971-1973. Papers I and II in this series are references 14 and 15.

Received for publication 1 December 1975, and in revised form 26 February 1976.

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