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SHAPE CHANGES OF HUMAN ERYTHROCYTES INDUCED BY VARIOUS AMPHIPATHIC DRUGS ACTING ON THE MEMBRANE OF THE INTACT CELLS

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Abstract—Most of the amphipathic drugs acting on nerves (general and local anesthetics, neuroleptics, antipyretics-analgesics, non-steroidal anti-inflammatory agents, etc.) and some other drugs (anti-histaminic agents, plant alkaloids, etc.) were found to induce instantly one of two typical types of shape changes of human erythrocytes when added into the cell suspension. It was confirmed that, generally, drugs with an anionic polar group induced membrane externalization (crenation), and those with a cationic group induced membrane internalization (invagination). The drugs in the former group are antagonistic against the shape-transforming effects of the drugs in the latter group, and vice versa. Drugs with a neutral polar group or with a quarternary ammonium group induced a complex type of shape change, suggesting the intramembraneous translocation of these drugs.

It is already well established that mature human erythrocytes undergo one of two types of membrane transformation, and a resulting shape change of the cells under *in vitro* action of amphipathic compounds [1, 2]. One transformation is membrane externalization (crenation, exvagination), ultimately leading to the formation of smooth spheres, and the other is membrane internalization (invagination), ultimately leading to spherical cells with invaginated vesicles in the interior [3]. These phenomena are shown schematically in Fig. 1.

Of many amphipathic compounds, those with an anionic polar group were reported to induce crenation and those with a cationic polar group invagination [1, 2]. Behaviour of those with a neutral polar group has not yet been elucidated in detail. As to those with a quarternary ammonium group, which are said to be rather membrane-impermeable, alkylammonium chlorides were reported by Deuticke [1] to induce invagination, while methochlorpromazine and two other drugs were proved by Sheetz and Singer [2] to induce crenation.

The present study tests various kinds of drugs as to their ability to induce such shape changes, revealing a possible relationship between their effects on the erythrocyte membrane and their pharmacological activities and also the mechanism of the membrane transformation.

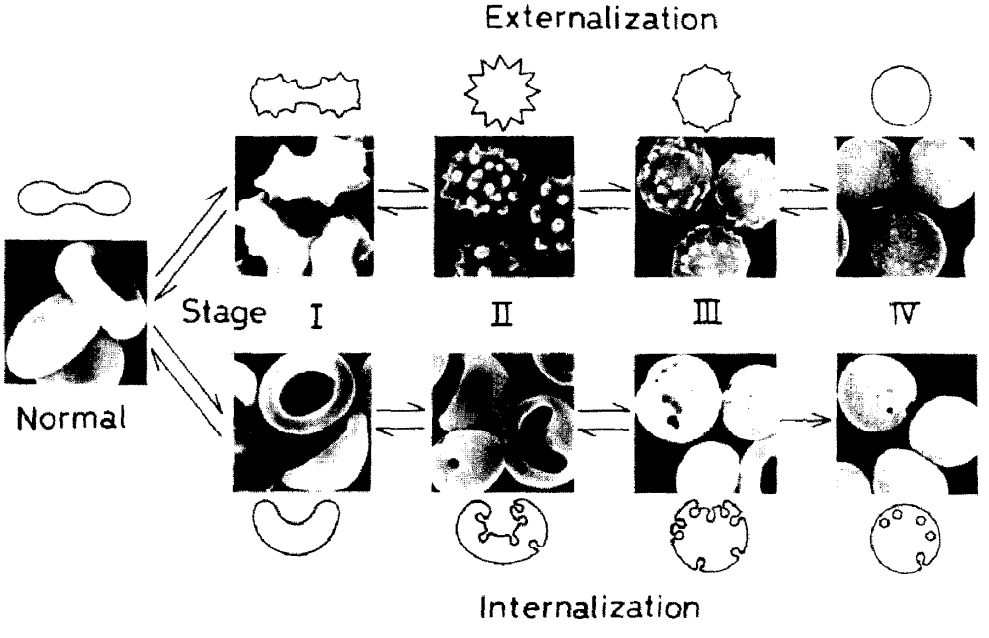


Fig. 1. Two typical types of shape changes of human erythrocytes induced by chemical compounds added to the suspending medium.

MATERIALS AND METHODS

Erythrocytes. Human erythrocytes from freshly drawn ACD-blood, kindly supplied by the Kyoto Branch, Japan Red Cross Blood Center, were washed three times with isotonic saline containing 10 mM phosphate buffer solution (PBS), pH 7.4, and resuspended in PBS.

Chemicals. The drugs used were kindly supplied by the following companies: benzydamine hydrochloride, mepirizol, sulfadiazine and sulfisomidine (Daiichi Pharmaceutical Co., Tokyo, Japan); diphenhydramine hydrochloride and isoproterenol (Dainippon Pharmaceutical Co., Osaka, Japan); ibufenac, ibuprofen and flurbiprofen (Kakenyaku Kako Co., Tokyo); carbutamide, tolbutamide, thonzylamine hydrochloride, γ-amino-β-hydroxybutyric acid and γ-hydroxybutylic acid (Ono Pharmaceutical Co., Osaka); chlorpromazine hydrochloride, chlorpromazine methyliodide (methochlorpromazine), diethazine hydrochloride, promethazine hydrochloride, perazine dimaleate, prochlorperazine dimaleate, trifluoperazine dihydrochloride, perphenazine dihydrochloride, fluphenazine dihydrochloride and benzydamine hydrochloride (Yoshitomi Pharmaceutical Co., Osaka). Cepharanthine was a gift from Prof. Emer. M. Tomita of our college. All others were the purest of the commercially available products.

The chemical to be tested was dissolved in PBS. The total osmolarity of the solution was adjusted to isotonic by reducing the amount of NaCl in PBS, and the pH to 7.4 by adding HCl or NaOH. The solution previously warmed at 37° was added to the erythrocyte suspension (also prewarmed) to make a final hematocrit of 5–10 per cent. The mixture was incubated at 37° for the time described in each experiment.

Solubilization of *n*-pentanol was aided by sonication for 1 hr.

Morphological observation of the erythrocytes. To 0.5 ml of the drug-treated or non treated (control) erythrocyte suspension was added 2 ml of 0.9% glutaraldehyde solution in 0.1 M phosphate buffer, pH 7.4, and the mixture was allowed to stand for 2 hr at room temperature for fixation. The fixed erythrocytes were washed, air dried and coated with carbon and gold. The preparation was then observed under the scanning electron microscope, type JSM-35, of the Japan Electron Optic Laboratory Co, Tokyo. The accelerating voltage was 20 kV, and magnification of the photographs was 1700. In order to express erythrocyte shape semi-quantitatively, we designed morphological indices. A biconcave disc shape was given a score of 0 and the transformed shape of stages I, II, III and IV in Fig. 1 were given scores of 1, 2, 3 and 4 respectively. Externalization or internalization were expressed by a plus or minus sign respectively. Multiplication of the score corresponding to the stage of the transformed cells by the ratio of transformed cells to total cells and addition of the values gave the morphological index of a given erythrocyte specimen.

RESULTS

Drugs tested in this experiment, and the concentrations necessary to induce stages I–III of externalization or internalization are listed in Tables 1–3. In Tables 1 and 2, only the concentration inducing stage I is noted for some drugs because of the insolubility of the drugs at higher concentrations.

To the crenator group of drugs belong barbiturate anesthetics, acidic anti-inflammatory drugs, aromatic

Table 1. Drugs to induce membrane externalization of human erythrocytes*

Category and name	Concentration range (mM) to give stages I–III of the externalization†
Barbiturate anesthetics	
Hexobarbital	2–
Veronal	40–
Anti-inflammatory agents	
Ibufenac (4-isobutylphenyl acetic acid)	4–10
Ibuprofen (2-4′-isobutylphenyl propionic acid)	4–10
Flurbiprofen (2-[2-fluoro-4-biphenylyl]-propionic acid)	0.1–1.0
Phenylbutazone	0.5–10
Indomethacin	0.2–3.0
Flufenamic acid	0.05–2.0
Antipyretics-analgesics	
Acetanilide	20–
N-acetyl-*p*-aminophenol	20–
Sodium salicylate	20–
Salicylamide	20–
Mepirizol	10–
Oral anti-diabetic agent	
Tolbutamide	20–

* Erythrocytes in 10 per cent suspension were incubated with respective drug at 37° for 30 min.

† The drugs on which no upper limit of the effective concentration range is given, are those which could not induce the advanced stage (stage III) of the shape change, owing to their low solubility.

Table 2. Drugs to induce membrane internalization of human erythrocytes*

Category and name	Concentration range (mM) to give stages I–III of the internalization
Phenothiazine neuroleptics	(see Table 3)
Local anesthetics	
Dibucaine	0.2–2.0
Tetracaine	1.0–7.5
Procaine	50–
Anti-histaminic agents	
Diphenhydramine	1.0–4.0
Chlorpheniramine	0.25–1.5
Thonzylamine	5.0–15
Anti-inflammatory agent	
Benzydamine	0.1–0.5
Anti-malarial agent	
Primaquine	0.2–1.0
Others	
Cepharanthine	0.1–0.8
Colchicine	5.0–25
Quinine	1.25–7.5
Atropine	10–20

* See the footnotes to Table 1.

antipyretics-analgesics and an oral anti-diabetic agent. Most of them are amphipathic compounds with an acidic group.

The invaginators include phenothiazine neuroleptics (Table 3), local anesthetics, anti-histaminic drugs, a basic anti-inflammatory drug, primaquine, and some other complex natural compounds such as biscoclaurine alkaloid cepharanthine, colchicine, quinine and atropine (Table 2). Except for the last mentioned group, all of these drugs belong to the amphipathic amines, mostly tertiary amines.

As indicated in Table 3, all the phenothiazine neuroleptics induced membrane internalization at relatively low concentrations. The intensity of their shape-transforming effect varied considerably with their side-chain structure, as is true with their clinical effect as neuroleptics, expressed in the table by the routine clinical doses employed.

Table 4. Drugs which induce no change in the shape of human erythrocytes*

Methionine	Thiamine
Glutamic acid	Pyridoxine
γ-Aminobutyric acid (GABA)	Folic acid
γ-Amino-β-hydroxybutyric acid (GABOB)	Anthranilic acid
γ-Hydroxybutyric acid	
	Nicotinic acid
Choline	Nicotinamide
Carnitine	Isoniazid
Tetraethylammonium bromide	Iproniazid
Hexamethonium	
	Epinephrine
Bromvalerylurea	Isoproterenol
Metformin (1,1-dimethylbiguanide)	
Moroxydine (4-morpholinecarboximidoyl guanidine)	Sulfisomidine
	Sulfadiazine
	Carbutamide
Glucuronic acid	

* Erythrocytes in 10% suspension were incubated with drug, in up to 40 mM concentration, at 37° for 30 min.

In contrast to the above-mentioned amphipathic compounds, many water-soluble drugs, mostly natural compounds, did not induce any such change upon incubation for 30 min in a concentration up to 40 mM (Table 4).

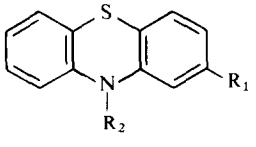
The mode of induction of shape changes by these drugs was investigated in detail with some representative drugs as follows. Figure 2 shows the time dependence of the drug effects. Chlorpromazine induced internalization immediately upon addition to the erythrocyte suspension, and no further progress of the shape change was observed upon incubation for 60 min. Externalization by flurbiprofen was also independent of incubation time. But, the externalization by flufenamic acid, once induced, was reduced slightly

Table 3. Phenothiazine neuroleptics to induce membrane internalization of human erythrocytes*

Name	Side chain† R_1	Side chain† R_2	Drug concentration (μM) to give stages I–III of the internalization	Clinical doses for oral use as neuroleptics (mg/day)
Chlorpromazine	—Cl	$-CH_2-CH_2-CH_2-N(CH_3)_2$	50–100	150–400
Promethazine	—H	$-CH_2-CH(CH_3)-N(CH_3)_2$	100–500	
Diethazine	—H	$-CH_2-CH_2-N(C_2H_5)_2$	200–500	400–1000
Perazine	—H	$-CH_2-CH_2-CH_2-N\langle\rangle N-CH_3$	50–200	250–500
Prochlorperazine	—Cl	$-CH_2-CH_2-CH_2-N\langle\rangle N-CH_3$	20–50	40–80
Trifluoperazine	$-CF_3$	$-CH_2-CH_2-CH_2-N\langle\rangle N-CH_3$	10–50	10–25
Perphenazine	—Cl	$-CH_2-CH_2-CH_2-N\langle\rangle N-CH_2-CH_2-OH$	10–50	20–50
Fluphenazine	$-CF_3$	$-CH_2-CH_2-CH_2-N\langle\rangle N-CH_2-CH_2-OH$	10–50	3–6

* Erythrocytes in 5.0 per cent suspension were incubated with each drug at 37° for 30 min.

† General structure of phenothiazine neuroleptics:



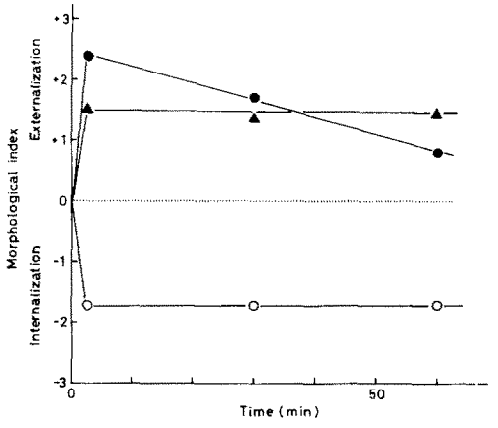


Fig. 2. Time course of drug effects on erythrocyte shape, as expressed by the morphological indices of the treated cells. Erythrocytes in 10% suspension were incubated at 37° with 0.1 mM chlorpromazine (—○—), 0.5 mM flufenamic acid (—●—) and 0.3 mM flurbiprofen (—▲—).

upon incubation. The other anti-inflammatory drugs tested, ibufenac, ibuprofen, phenylbutazone and indomethacin, also belong to this latter group.

The dependence of the drug effects on drug concentration is shown in Figs. 3 and 4. The effects of crenators, such as flufenamic acid and flurbiprofen, and of invaginators, including chlorpromazine, benzydamine, primaquine and dibucaine, increased with increasing drug concentration to give cells stage III, expressed by the morphological indices of Fig. 3. In Fig. 4 are shown the scanning electron micrographs of the erythrocytes with changed shape, treated with increasing concentrations of flufenamic acid or chlorpromazine.

It was noted that most of the invaginators ultimately gave spherical cells of the internalization stage IV at concentrations higher than those shown in Fig. 3, while most of the crenators, with the exception of flufenamic acid, did not lead to the final stage (stage IV) of the externalization.

The shape transforming-actions of the crenators and of the invaginators are antagonistic to each other (Fig. 5). For example, 0.5 mM flurbiprofen induced crenation to stage II (→2 in Fig. 5), and the further addition of primaquine in 0.2 mM concentration reversed the shape to a normal biconcave disc. Addition of higher concentrations of primaquine even brought about inva

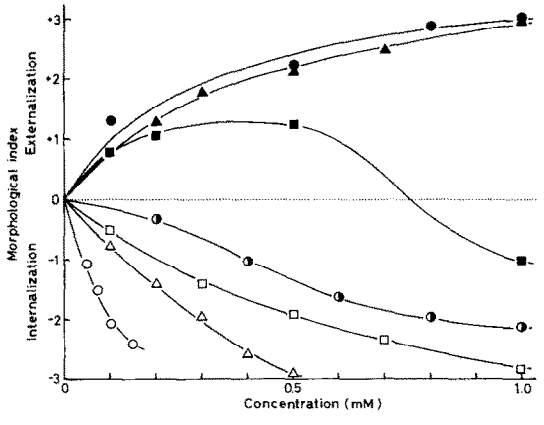


Fig. 3. Dose–response effects of drugs on erythrocyte shape, expressed by the morphological indices of the treated cells. Erythrocytes in 10% suspension were incubated at 37° with chlorpromazine (—○—), benzydamine (—△—), primaquine (—□—), dibucaine (—◑—), flufenamic acid (—●—) and flurbiprofen (—▲—) for 5 min and with methochlorpromazine (—■—) for 30 min.

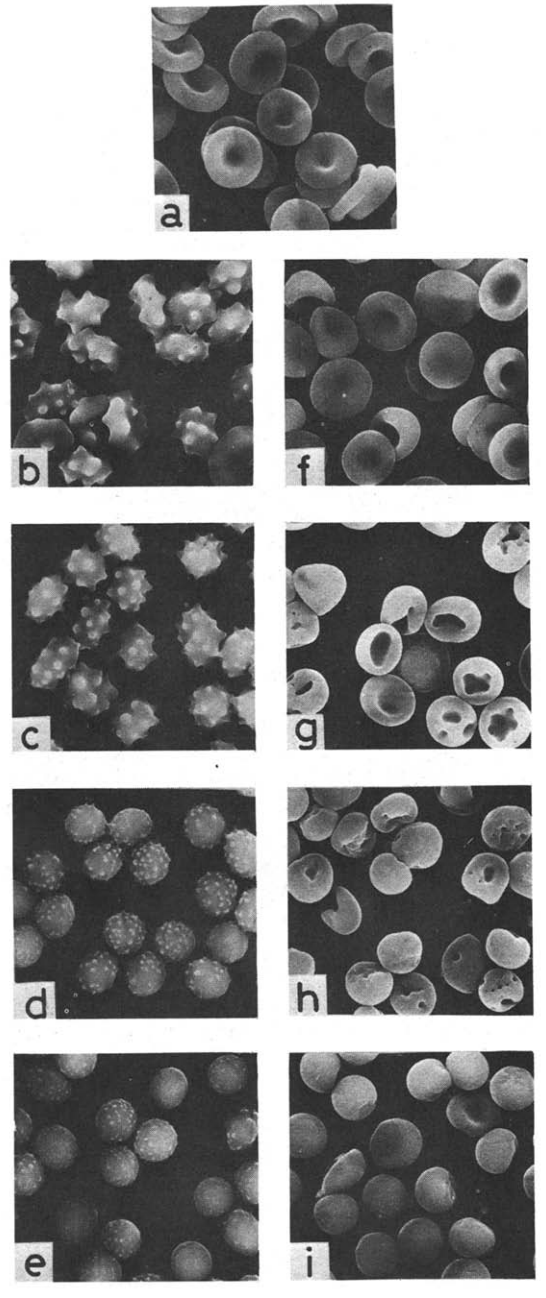


Fig. 4. Dose–response effects of flufenamic acid and chlorpromazine on erythrocyte shape, expressed by scanning electron micrographs of the treated cells. Intact erythrocytes (a) in 10% suspension were incubated at 37° for 5 min with 0.05 mM (b), 1.0 mM (c), 2.0 mM (d) and 3.0 mM flufenamic acid (e) or the cells in 5.0% suspension were similarly incubated with 0.03 mM (f), 0.06 mM (g), 0.12 mM (h) and 0.2 mM chlorpromazine (i).

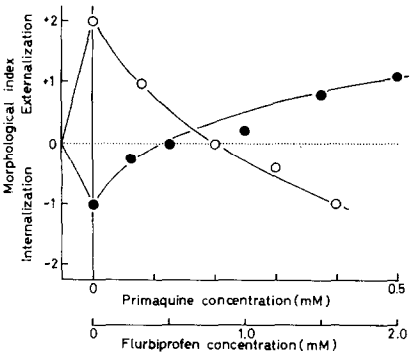


Fig. 5. Example of the antagonism between the effects of membrane-externalizing and -internalizing drugs, expressed by the morphological indices of the treated cells. Erythrocytes in 10% suspension were incubated at 37° for 5 min with a solution of 0.5 mM flurbiprofen and then with various concentrations of primaquine (—○—). They were similarly incubated first with 0.2 mM primaquine and then with various concentrations of flurbiprofen (—●—).

Table 5. Pairs of drugs which exert antagonizing effects on erythrocyte shape *

Drugs to induce externalization	Concn (mM)	Drugs to restore normal shape	Concn (mM)
Lysophosphatidylserine	0.08	Primaquine	1.0
Lysophosphatidylserine	0.08	Decylamine	0.8
Lysophosphatidylserine	0.08	Dibucaine	1.0
Lysophosphatidylcholine	0.02	Chlorpromazine	0.05
Flurbiprofen	0.4	Chlorpromazine	0.05
Flurbiprofen	0.5	Primaquine	0.2
Drugs to induce internalization	Concn (mM)	Drugs to restore normal shape	Concn (mM)
Chlorpromazine	0.05	Flurbiprofen	0.4
Primaquine	0.2	Flurbiprofen	0.5

* Successive treatments of 10% erythrocyte suspension with the drug in the concentration stated in the left column and then with the drug in the right column, gave the normal-shaped cells.

gination of the cells. Conversely, the cells invaginated at stage I (−1 in Fig. 5) by 0.2 mM primaquine were reversed to a normal biconcave disc by 0.5 mM flurbiprofen and further advanced to crenation by higher concentrations of flurbiprofen.

Such an antagonistic effect between a crenator and an invaginator was also observed with other pairs of the drugs, as listed in Table 5.

Shape change of erythrocytes induced by amphipathic compounds with a quarternary ammonium group or by aliphatic alcohols. The effect of methochlorpromazine, a quarternary ammonium salt, on erythrocyte shape is very complex (Fig. 3). Just after the addition of this compound in 0.2 mM concentration to the erythrocyte suspension at 37°, crenation occurred. A higher concentration, 1 mM, brought a more advanced stage of crenation (Fig. 6 a and b). Having once occurred, however, the crenation began to subside gradually when the treated cells were allowed to stand at 37°, and after 60 min the cells were a mixture of the less crenated and invaginated cells (Fig. 6 d and e). This shift from crenation to invagination was enhanced with increasing concentrations of this drug. If such a treatment was performed at 4°, although initial crenation

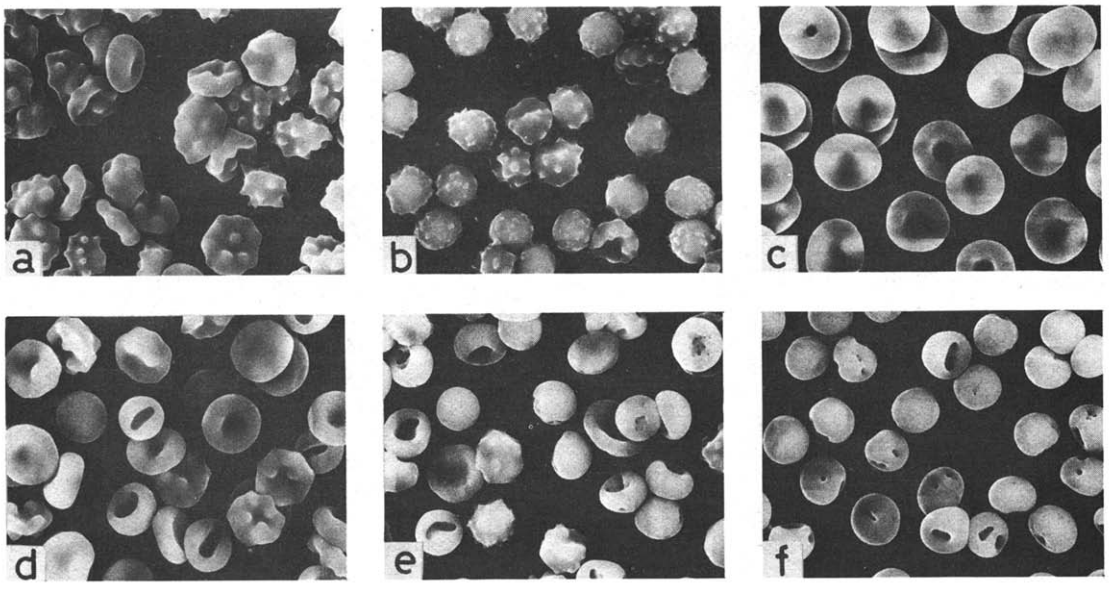


Fig. 6. Effect of methochlorpromazine on erythrocyte shape. Erythrocytes in 5.0% suspension were incubated at 37° with 0.2 mM methochlorpromazine for 1 min (a) and 60 min (d), and with 1.0 mM of the compound for 1 min (b) and 60 min (e). The treated cells in b and e were washed with PBS to give c and f respectively. The cells were observed under a scanning electron microscope. No shape change was detected in the control cells washed with PBS under otherwise the same conditions.

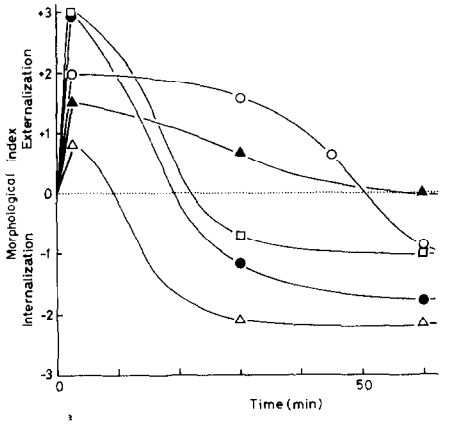


Fig. 7. Time course of the effects of quarternary ammonium compounds to induce biphasic shape change of human erythrocytes. Erythrocytes in 5.0% suspension were incubated at 37° with 0.5 mM (—○—) and 1.0 mM methochlorpromazine (—●—), 50 mM tri-*n*-butyl-propylammonium iodide (—△—), 40 mM benzyldimethylphenylammonium chloride (—▲—) and 0.03 mM zephiramine (---| —).

occurred just as at 37°, no shift from membrane externalization to internalization occurred in the treated cells with the lapse of time.

When the cells treated for 1 min with 1 mM methochlorpromazine were immediately washed with saline, they assumed a normal shape (Fig. 6 b and c). When the cells had been treated with the same concentration of the compound for 60 min and then washed, they took a typical invaginated form (Fig. 6 e and f).

Three other quarternary ammonium compounds, tetradecyldimethylbenzylammonium chloride (zephiramine), benzyldimethylphenylammonium chloride and tri-*n*-butyl-propylammonium iodide, produced a similar effect in a concentration range of 0.01–0.1, 10–40 and 20–50 mM respectively (Fig. 7).

An analogous effect was also observed in the case of some aliphatic alcohols (Figs. 8 and 9). With increasing chain length of the alcohols (a 30-min incubation in 0.2 M concentration of alcohols with carbon numbers from 2 to 4), or increasing concentration (a 30-min incubation in 0.2 to 0.6 M *n* propanol) or increasing time of incubation (0.5 to 5 hr in 1.5 M ethanol), the crenated cells, having once appeared, shifted to invaginated cells via biconcave discs (Fig. 8). Washing crenated cells with saline, which had been produced by treating them for 30 min in 1.5 M ethanol (Fig. 9a) or for 10 min in 0.1 M *n*-pentanol (Fig. 9 c), yielded normal-shaped cells (Fig. 9 b) and cup-shaped cells (Fig. 9 d) respectively.

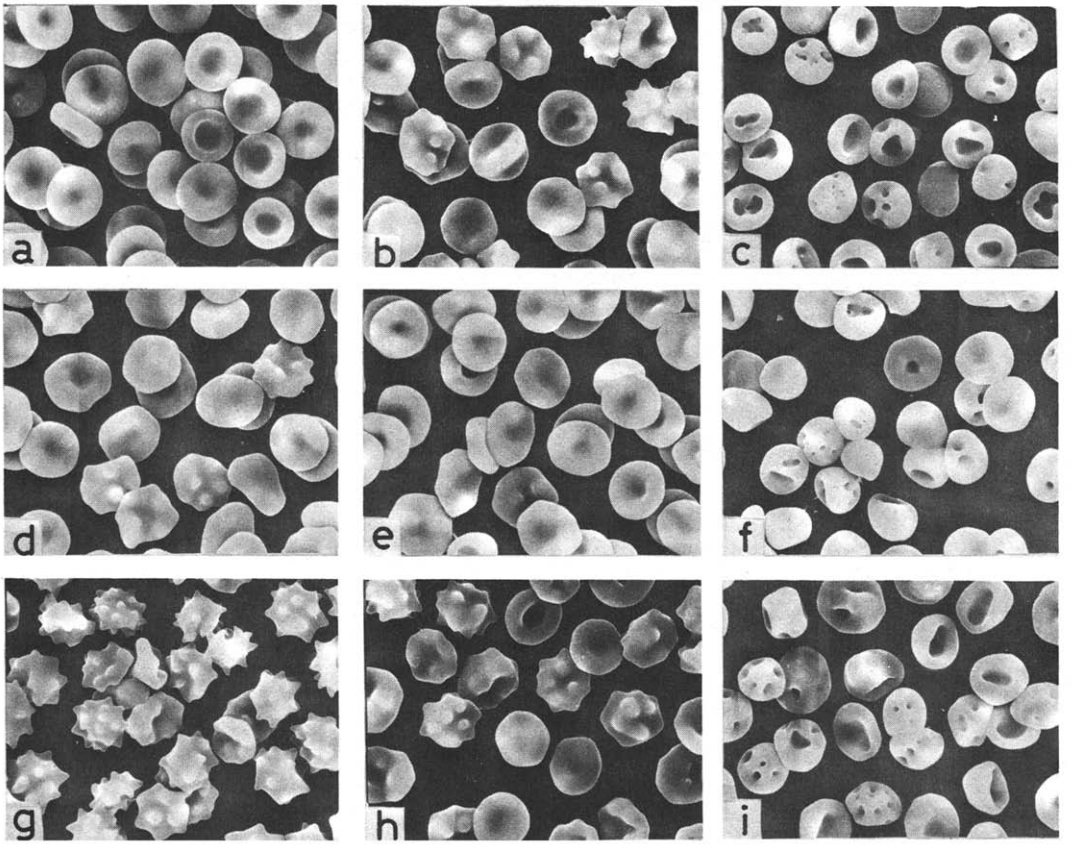


Fig. 8. Effect of aliphatic alcohols on erythrocyte shape. Dependence of the effect on chain length and concentration of the alcohols and on time of treatment. Erythrocytes in 5.0% suspension were incubated at 37° for 30 min in 0.2 M solution of ethanol (a), *n*-propanol (b) and *n*-butanol (c), and in 0.2 M (d), 0.4 M (e) and 0.6 M *n*-propanol (f). They were also incubated at 37° in 1.5 M solution of ethanol for 0.5 hr (g), 1 hr (h) and 5 hr (i). The cells were observed under a scanning electron microscope.

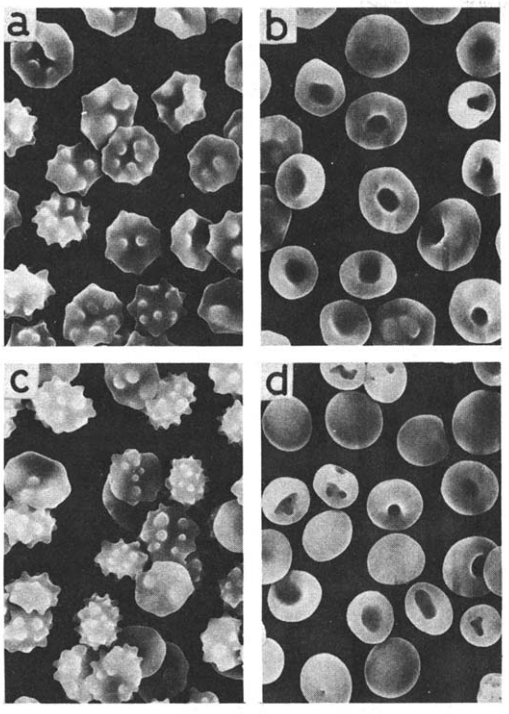


Fig. 9. Effect of saline washes on the shape of the alcohol-treated erythrocytes. Erythrocytes in 5% suspension were incubated at 37° with 1.5 M ethanol for 30 min (a) and these cells were then washed with PBS (b). Erythrocytes were similarly incubated with 0.1 M *n*-pentanol for 10 min (c) and these cells were then washed with PBS (d). They were observed under a scanning electron microscope.

DISCUSSION

Studies of the effects of a large number of drugs has revealed that almost all the amphipathic drugs act on the membrane of the mature human erythrocyte to induce membrane transformation and a resulting shape change of the cells. In contrast, many drugs which have many polar regions in their molecules and are highly water-soluble do not exert such an action on the cells, probably because they cannot be incorporated into the membrane.

Many amphipathic drugs with an anionic group induced crenation, while many amphipathic amines induced invagination. Such a result is an extension of previous work on amphipathic compounds in general [1, 4–7] and is explainable by the hypothesis of Sheetz and Singer [2]. According to them, permeable cationic drugs intercalate mainly into the inner (cytoplasmic) half of the membrane lipid bilayer, because of their interaction with an acidic phosphatidylserine located in this half, and expand it relative to the exterior half of the bilayer, thus inducing invagination. Anionic drugs do the opposite, because of their repulsion by phosphatidylserine, and cause the cell to crenate.

Many drugs tested caused shape changes immediately after being added to the erythrocyte suspension, and no further progress of the change could be observed. This is probably because their hydrophobic interaction with the hydrocarbon chains of the membrane lipids is strong enough to permit the immediate incorporation of the drug molecules into the membrane lipid bilayer. Externalization induced by some anti-inflammatory agents (Fig. 2), however, was reduced gradually upon incubation at 37° for 60 min. This phenomenon may indicate that the drug, intercalated into the exterior half of the membrane lipid bilayer, was gradually translocated into the inner half or cytoplasm upon incubation, and that the expansion of the exterior half relative to the inner half was gradually reduced with a resultant retrogradation of the externalization.

When a crenator drug and an invaginator drug are added to an erythrocyte suspension successively, they may be incorporated independently into a separate leaflet of the bilayer and exert an antagonistic effect as a result. Thus, addition of both of them in the appropriate concentrations brought about the formation of normal-shaped cells, in spite of the presence of the drugs in the membrane. The reason why the extracellular concentrations of the crenator and the invaginator required to give antagonistic effects are not equal, is not clear; this might be due to the fact that the ratio of the numbers of the drug molecules to be incorporated per total numbers added varies with each drug, depending on its chemical structure. These antagonistic effects of the drugs on the cell shape can also be explained by the above-mentioned hypothesis of Sheetz and Singer.

It was revealed that, of the cationic amphipathic drugs tested, the potency of chlorpromazine and its derivatives and analogues is roughly proportional to their clinical potency. For example, as shown in Table 3, the halogenated phenothiazine derivatives with a piperadine nucleus in the side chain, including prochlorperazine, trifluoperazine, perphenazine and fluphenazine, which have stronger clinical potency than the halogenated or non-halogenated ones with a simple alkyl side chain, such as chlorpromazine, promethazine and diethazine, caused the invagination at a remarkably lower concentration range than that of the latter group of tranquilizers.

The membrane-transforming effects of amphipathic drugs with a quarternary ammonium group as well as some neutral amphipathic compounds are rather complex. The former compounds were reported to be cup-formers by Deuticke [1] and to be crenators by Sheetz and Singer [2]. The reason for such a discrepancy was clarified by the experimental results of the present study; they behave in either one way or the other depending on the condition of the treatment. As proved with four different compounds of this category, these drugs induced crenation immediately upon addition to the cell suspension, but upon continuation of the treatment, the crenation tended to be reduced and finally was replaced by invagination (Figs. 6 and 7). Such a shift from membrane externalization to internalization occurred faster when a higher concentration of the drugs was employed. A similar phenomenon was also observed in the case of the action of aliphatic alcohols (Fig. 8).

A probable explanation for these complex types of membrane transformation is that the quarternary ammonium compound, which penetrates into the membrane with difficulty, for an unknown reason, first gets into the outer leaflet of the lipid bilayer but then gradually begins to translocate into the inner leaflet by an attraction force between its positive charge and the

negative charge of the phosphatidylserine molecules located in the inner leaflet; the alcohols without any appreciable electric charge may pass the lipid bilayer from the outer to the inner direction, driven only by the concentration gradient between the extra- and intracellular media. The fact that no shift from membrane externalization to internalization occurred when the cells were treated with methochlorpromazine at a lower temperature (4°), which should restrict their intramembraneous movement by decreasing the microviscosity of the lipid bilayer, supports such a hypothesis of the translocation of the drug molecules in the bilayer. Washing experiments with the methochlorpromazine-treated (Fig. 6) and alcohol-treated erythrocytes (Fig. 9) also seem to support such a view. Because saline washes should remove only the drug molecules exposed on the outer leaflet of the membrane bilayer, the drug-treated erythrocytes in which the drugs are located only in the outer leaflet of the bilayer will have the normal shape after saline washes, while those in which the drug molecules are distributed in both leaflets will take an invaginated shape after saline washes, induced by the effect of the residual drug molecules in the inner leaflet.

In the present work, the mode of induction of the membrane transformation by the drugs was considered only from the standpoint of their modifying influence on the membrane lipid bilayer. However, a possibility of participation of the membrane proteins in these phenomena cannot be denied. Actually, it was reported that similar shape changes of the ghost membrane were related to the phosphorylation of the membrane proteins, particularly of spectrin [8, 9]. Studies along this line are now under way in our laboratory.

In any case, studies of the effects of these drugs on the membrane and the cell shape of erythrocytes will give valuable information on the mechanism of the membrane transformation and possibly on the mode of pharmacological action of these drugs on the mammalian cell membranes in general.

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