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I. ERYTHROCYTE MEMBRANE STABILIZATION BY TRANQUILIZERS AND ANTIHISTAMINES

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Abstract—1. Human erythrocytes are protected or stabilized against hypotonic and mechanical hemolysis in the presence of low concentrations of many phenothiazines, reserpine, and haloperidol. At high concentrations all these surfactants cause lysis.

2. The erythrocyte stabilization by these compounds is long-lasting and depends on the concentration of erythrocytes.

3. The stabilizing potency correlates aproximately with the clinical potency of the phenothiazine.

4. Adsorption studies indicate that at maximal stabilization there is about 65 Å^2 of erythrocyte membrane associated with one molecule of promethazine, 100 Å^2 for chlorpromazine HCl, 140 Å^2 for trifluoperazine diHCl, and 180 Å^2 for fluphenazine diHCl. Since the area of the phenothiazine ring is about 50 Å^2, these values represent 90 to 25 per cent involvement of the membrane, if no adsorption to hemoglobin occurs.

5. The membrane stabilization is rapidly reversible. Lowering the extracellular drug concentration or photo-oxidizing the adsorbed drug causes the membrane to return to its original condition of fragility.

6. The prevention of hemolysis is also associated with the prevention of K^+ release; this distinguishes membrane stabilization from pro-lysis wherein K^+ is released.

7. Replacing isotonic sucrose by isotonic NaCl potentiates the lytic effect of phenothiazines.

8. The decrease in osmotic fragility (which corresponds to between one third and one half an atmosphere of pressure) may be explained possibly by an expansion of the cell membrane.

THE STUDIES described in this paper were undertaken as part of an investigation of the nature of protection or "stabilization" of the erythrocyte membrane by low concentrations of various compounds. It is known that a variety of surface-active compounds (alcohols, anesthetics, fatty acids, steroids, detergents, and lipid-soluble vitamins) will at low concentrations cause a stabilization of many types of membranes. Since high concentrations cause lysis, a biphasic effect is observed, therefore, as a function of drug concentration.

The tranquilizers and antihistamines, which are surface-active,[1–4] are no exception to this general finding. Erythrocytes are stabilized against hypotonic hemolysis by low concentrations of RP 3300 (2-dimethylaminopropyl, 10-phenothiazine),[1–5] trifluoperazine and chlorpromazine,[6–8] and diphenhydramine.[9] Subcellular membrane-bounded organelles are also stabilized by low concentrations of phenothiazines, other

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tranquilizers, and antihistamines. The spontaneous release of lysosomal acid phosphatase is inhibited by chlorpromazine[10] and by promethazine and diphenhydramine;[9] mitochondrial swelling is inhibited by chlorpromazine,[11, 12] promethazine,[12, 13] and diphenhydramine and pyribenzamine;[13] the spontaneous release of adrenaline from adrenal medulla chromaffin granules is inhibited by trifluoperazine, chlorpromazine, triflupromazine, and reserpine;[14] and the spontaneous release of noradrenaline from bovine splenic nerve granules is inhibited by reserpine.[15] At concentrations higher than 10^{-4} M or 10^{-3} M all these surface-active drugs behave as potent lysins. This has been observed as a direct hemolysis,[1, 16] a complete release of lysosomal enzymes,[17] a reduction in the packed volume of the cells,[18, 19] or a gross anatomical disruption of the cells.[20]

In selecting the erythrocyte as a model for studying the mechanism of membrane stabilization in general there are several advantages; the membrane area of the erythrocytes can be quite accurately estimated, and the erythrocyte is free of intracellular membranes and organelles. Presumably, therefore, any effect of a drug on osmotic hemolysis might be more justifiably interpreted as an effect on the membrane, although it is necessary to exclude effects on the intracellular matrix. Some of the problems and questions which prompted the experiments reported in this paper are as follows. Since only four phenothiazines, RP 3300,[1, 5, 21] promethazine,[21, 22] chlorpromazine,[6–8, 22] and trifluoperazine,[6–8] had been tested on the erythrocyte, it was necessary to know whether the other phenothiazines as well as the other types of tranquilizers, such as reserpine and haloperidol, would cause similar effects. It was important to know whether variations in the concentration of erythrocytes would affect the amount of erythrocyte stabilization against hypotonic hemolysis by a certain concentration of drug and, also, whether erythrocyte stabilization by phenothiazines was reversible.

Materials. The following firms and laboratories graciously donated drug samples: Smith, Kline & French: chlorpromazine HCl (Thorazine), prochlorperazine ethane disulfonate (Compazine), trifluoperazine diHCl (Stelazine), and chlorpromazine sulfoxide HCl. Wyeth: promethazine HCl (Phenergan). Squibb Institute for Medical Research: fluphenazine diHCl (Prolixin). Riker Laboratories Inc.: butyrylperazine dimaleate. Sandoz Pharmaceuticals, Inc.: thioridazine HCl (Mellaril). McNeil Laboratories, Inc.: haloperidol. CIBA Pharmaceutical Co.: reserpine phosphate (Serpasil). Warner-Lambert Research Institute, Research affiliate of Warner-Chilcott Laboratories: ethopropazine HCl (Parsidol). Research Laboratorium Dr. C. Janssen: haloperidol.

METHODS

Preparation of erythrocyte suspension. Human blood (1·5 ml) was drawn by venipuncture and gently placed in a test tube containing 0·075 ml sodium heparin, 1000 units/ml. An aliquot of 0·925 ml of the heparinized blood was then centrifuged for 5 min in a 15-ml conical glass centrifuge tube in a swinging-bucket centrifuge at 1500 *g*. The plasma was carefully removed, including the buffy coat of leukocytes, and then 154 mM NaCl in 10 mM sodium phosphate buffer, pH 7, was added to make a total volume of 12·5 ml. The erythrocytes were resuspended by gentle repeated inversion of the test tube. The concentration of this stock erythrocyte suspension was usually around 400×10^6 cells/ml. The erythrocyte concentrations were obtained

by means of a Spencer bright-line hemacytometer. The experiments to be reported in this paper were carried out primarily on the blood of one person (fasting blood was always used). Although identical results were obtained with the bloods of three other volunteers, it was convenient to use the blood of the same person for each experiment, because the erythrocyte osmotic fragility of every volunteer was different and required a completely different set of freshly prepared test solutions to conduct the experiment. For example, the normal range in adults for 50 per cent erythrocyte hemolysis is between 68 and 75 mM NaCl.[23]

Protection and lysis of erythrocytes as determined by turbidity. The time course of erythrocyte hemolysis was followed by densitometry. One ml of a concentrated erythrocyte suspension, 800×10^6 erythrocytes/ml, was pipetted into a 12×75 mm matched glass test tube and the tube put into the well of a Coleman Junior spectrophotometer-nephelometer. The instrument was employed as a densitometer whereby the transmitted light was measured. The Coleman red glass filter (655 mμ) was used. For turbidity measurements of erythrocyte suspensions it is preferable to measure the transmitted light than the scattered light, as shown by Love.[24] The output of the meter was shunted out to a Varian ink-chart recorder, with a mercury cell and a 50 kΩ variable resistor to back off the output voltage and adjust the baseline; 1·5 ml of 10 mM sodium phosphate buffer, pH 7, with or without drug, was then quickly injected into the test tube cuvette by means of a syringe. Mixing was accomplished by means of the turbulent injection. The level of turbidity was an index of the number of intact cells remaining, the turbidity decreasing as the hemolysis increased. These turbidimetric experiments were done in duplicate.

Protection and lysis of erythrocytes as determined by release of hemoglobin. The majority of the experiments reported in this paper were carried out by measuring colorimetrically the amount of hemoglobin released from the erythrocytes. The experiments were carried out as follows: 0·1 ml of the stock erythrocyte suspension was added to 1·5 ml of the test solution in a glass test tube, 12×75 mm, and immediately mixed twice briefly with a Vortex mixer. The test solution was usually composed of 68·5 mM NaCl or 66·5 mM NaCl in 10 mM sodium phosphate buffer, pH 7, containing a drug at a concentration between 10^{-10} and 10^{-2} M. The mixture remained at room temperature (21° to 24°) for 5 min, at which time the erythrocytes were centrifuged for 45 sec at 1500 *g* in a swinging-bucket centrifuge. The hemoglobin content of the clear supernatant was measured by recording the optical density at 543 mμ in a Beckman DU spectrophotometer. All experiments were done in duplicate or triplicate.

Drug adsorption experiments. The adsorption of drug on to human erythrocytes was determined as follows. The procedure was arranged so that both adsorption and erythrocyte stabilization could be studied simultaneously. Tube A contained 1·5 ml of the test drug solution and received a 0·1-ml aliquot of the stock erythrocyte suspension. Tube B contained 1·5 ml of 154 mM NaCl in pH 7 buffer and received 0·1 ml of the stock erythrocyte suspension. Tube C contained 1·5 ml of buffer only and received 0·1 ml of the erythrocytes. Tube D contained 1·5 ml of 68·5 mM NaCl in buffer and received 0·1 ml of the erythrocyte suspension. Tube E contained 1·5 ml of the drug test solution and received an aliquot of 0·1 ml of 154 mM NaCl without erythrocytes. The tubes used for these adsorption experiments were cellulose nitrate tubes, $\frac{5}{16}$ in. $\times$ $1\frac{15}{16}$ in. with a total capacity of about 1·8 ml. The

mixtures were inverted three times; Parafilm was used to stopper the openings. Since adsorption occurs on to the Parafilm and tube, all experimental and control tubes were treated the same way. The tubes then remained at room temperature for 5 min, after which they were centrifuged in a Lourdes angle-head centrifuge for 20 min at 13,000 *g* at room temperature. The supernatants were removed with disposable Pasteur pipettes, and their optical densities at 543 mμ and at 250 mμ or 256 mμ were taken. The wavelength 250 mμ was used for promethazine HCl and chlorpromazine HCl; 256 mμ was employed for fluphenazine diHCl and trifluoperazine diHCl. The u.v. reading of tube A was usually lower than that of tube E because the erythrocytes had adsorbed many of the drug molecules. The purpose of tubes B, C, and D was to correct the u.v. absorption for interfering amounts of hemoglobin and residual plasma. In tube B there was no hemoglobin release at all, and all the u.v. absorption was related to the amount of residual plasma. Tubes C and D had varying amounts of hemoglobin absorbing the u.v. light in addition to the plasma absorption. One could then construct a hemoglobin and plasma correction line by plotting the optical densities for B, C, and D on a graph where the ordinate was absorption at 543 mμ and the abscissa was absorption at 250 mμ. The points invariably fell on a straight line, demonstrating that the u.v. absorption by hemoglobin was proportional to its absorption at 250 or 265 mμ, with allowance made for the baseline absorption due to plasma. The difference, therefore, between the optical densities of tubes A and E represented the amount of drug adsorbed by the erythrocytes, provided certain corrections are made. The difference (A–E) was then corrected for the u.v. contribution from plasma and hemoglobin by means of the correction line. Knowing the drug concentration with and without adsorption and knowing the erythrocyte concentration, we could calculate the number of drug molecules per cell. All adsorption experiments were done in sextuplicate.

Potassium measurements. Potassium, released from erythrocytes, was measured by means of a flame photometer with lithium as an internal standard.

RESULTS

Duration of erythrocyte stabilization. In addition to the fact that phenothiazines protect erythrocytes from hypotonic hemolysis,[1, 6–8] it was found by Freeman and Spirtes[7] that these compounds retard the rate of hemolysis. The time course of hypotonic hemolysis in the presence of these drugs was studied, therefore, by means of densitometry. The results of such an experiment are shown in Fig. 1. At time zero an aliquot of 1·5 ml of 10 mM sodium phosphate buffer, pH 7, with or without prochlorperazine ethane disulfonate, was injected with a syringe into the densitometer cuvette containing 1 ml of a stock erythrocyte suspension in 154 mM NaCl, pH 7. Mixing was rapid because of the turbulence, and the cells immediately started to swell and lyse. It is seen in Fig. 1 that final concentrations of prochlorperazine of 6×10^{-6} M and 6×10^{-5} M markedly reduced the final degree of hemolysis. At higher concentrations, however, it is seen that there was first of all a transient protection, lasting about 60 sec, followed by a hemolysis. These results were quite reproducible and changed only slightly even after the main stock of erythrocytes had been suspended in 154 mM NaCl for 4 hr (dashed lines of Fig. 1).

No further hemolysis occurs after 1·5 min (Fig. 1 and Table 1). Since stabilization

and hemolysis were essentially complete in 5 min, it was decided that it would be easier and more accurate to separate the cells at that time and measure the amount of hemoglobin released.

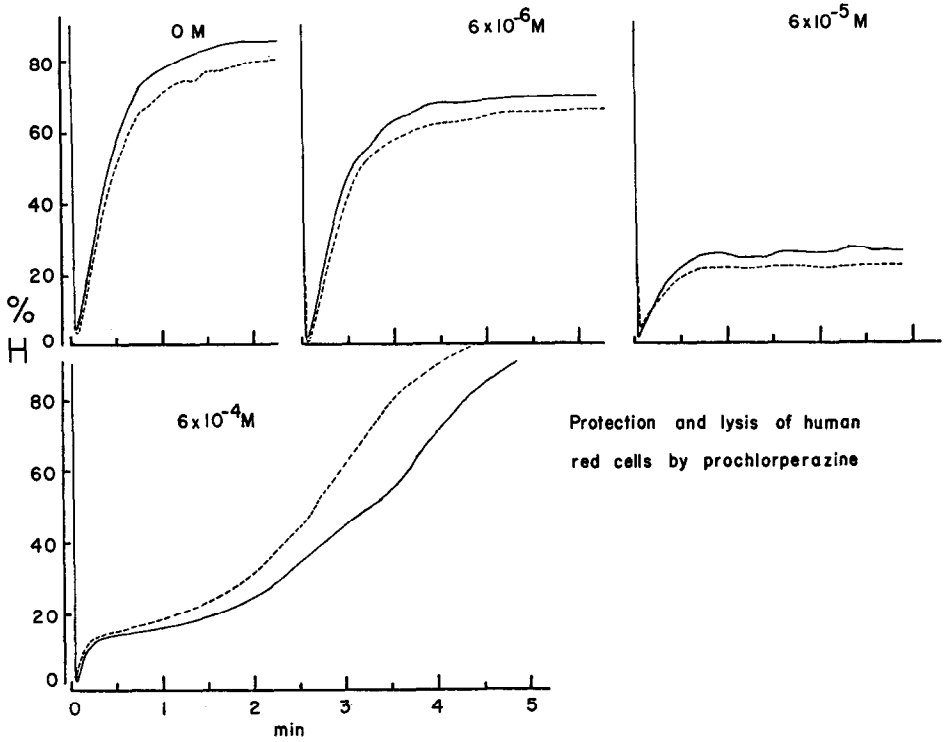

FIG. 1. The protection and lysis of human erythrocytes by prochlorperazine ethane disulfonate by means of densitometry. At zero time an aliquot of buffer with or without drug was injected rapidly into the densitometer cuvette which already contained an aliquot of erythrocytes in buffered saline. The above densitometer tracings indicate the loss of turbidity of the erythrocyte suspension with time. Maximal protection against hyptotonic hemolysis occurred at 6×10^{-5}M; higher concentrations caused direct lysis of the erythrocytes with complete clarity of the suspension developing after 5 min. The dashed lines indicate a repeat of the experiments after the red cells had been suspended in buffered saline for over 4 hr; % H indicates the per cent hemolysis.

The results shown in Fig. 2A compare the data obtained by turbidimetric and by hemoglobin release methods. It is seen that the results are very similar. Rather than plot the per cent hemolysis directly, each value for the per cent hemolysis in the presence of the drug was divided by the per cent hemolysis in the absence of the drug. This gives the relative hemolysis.

Effect of varying the cell density. Figures 2A and 2B show that the degree of protection or hemolysis depends on the concentration of erythrocytes. The relationship between the erythrocyte concentration and the maximal stabilization is not linear, however, and is explicitly shown in Fig. 2B. The independence of the maximal stabilization concentration below 3×10^7 cells/ml means that small

variations in the erythrocyte concentration below this value will not affect the degree of protection. The experiments were arranged, therefore, such that the final concentration of erythrocytes never exceeded $2{\cdot}8 \times 10^7$ cells/ml.

TABLE 1. DURATION OF STABILIZATION BY PROCHLORPERAZINE

Time	Optical density units	
	0 M	10^{-5}M
10 min	0·296	0·025
0·3 hr	0·296	0·020
1·0 hr	0·302	0·024
1·2 hr	0·310	0·031
3 hr	0·310	0·030
20 hr	0·290	0·050
26 hr	0·295	0·052

Duration of stabilization of erythrocytes against hypotonic hemolysis by prochlorperazine ethane disulfonate. Red cells were suspended in 66·7 mM NaCl with and without the drug at time 0 min. The numbers in the columns indicate the optical density of the hemoglobin released after the time indicated on the left. No significant loss of stabilization occurred over the first few hours.

It has been known for some time that the lysis of erythrocytes by hemolysins and detergents depends not only on the concentration of the lysin but also on the concentration of the erythrocytes.[25] The simple expressions derived for hemolysis[25, 26] are equally suitable for membrane stabilization. Because the molecules adsorb onto the erythrocyte, the concentration at maximal stabilization, C_{ms}, will depend on the number of erythrocytes per ml as follows:

$$C_{ms,\,n} = C_{ms,\,0} + \frac{X}{N} \cdot n \qquad (1)$$

where $C_{ms,n}$ is the concentration at maximal stabilization for n cells/ml; $C_{ms,0}$ is the concentration at maximal stabilization for $n = 0$ or a very low cell concentration; X is the number of molecules adsorbed per cell where the cell area is 163×10^8 Å^2; and where N is Avogadro's number. This equation is plotted three times in Fig. 2B for three different values of X, 10^7, 10^8, and 10^9 molecules per cell, and with 7×10^{-6}M as the $C_{ms,0}$ for prochlorperazine. It is seen that the best fit to the experimental data is when X is around 10^8 molecules per cell. This fit agrees with the adsorption findings to be presented later in Table 2.

C_{50} and the NaCl *concentration of the hypotonic test solution.* It will be convenient to refer to C_{ms} and C_{50} as the drug concentrations which confer maximal stabilization respectively. It was necessary to determine to what extent C_{ms} and C_{50} depended on the osmotic gradient across the erythrocyte membrane. Two levels of control per cent hemolysis, 67 and 30 per cent, were obtained by adjusting the final NaCl concentration to 64·2 and 68·5 mM respectively. The C_{ms} remained at 10^{-5}M prochlorperazine in both cases. The osmotic gradient, while not influencing the C_{ms}, did, however, change

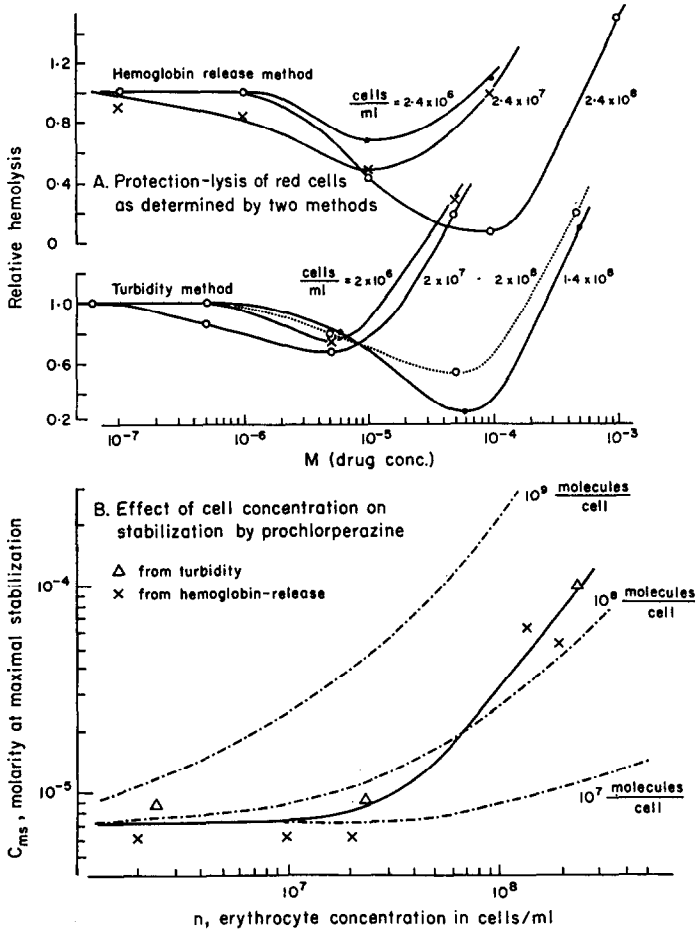

FIG. 2. Effect of varying the cell density. A. Comparison of data on the protection–lysis of erythrocytes as determined by two methods, hemoglobin release and turbidity. Higher concentrations of erythrocytes require higher concentrations of prochlorperazine ethane disulfonate to cause stabilization against hypotonic hemolysis. The ordinate represents the amount of hemolysis relative to control, which has no drug.

B. The relation between C_{ms}, the concentration of the drug at maximal stabilization, and n, the erythrocyte concentration in cells/ml. The triangular experimental points have been obtained from the turbidity experiments; the crosses come from the hemoglobin release experiments. The dashed lines represent the relation predicted by equation (1) for $X = 10^9$, 10^8, or 10^7 molecules adsorbed per cell. The best fit to the experimental data occurs with 10^8 molecules per cell. Independent confirmation of this value comes from adsorption experiments (Fig. 5 and Table 2).

the C_{50} slightly. Because of this small shift in C_{50}, the control hemolysis for most of the experiments was kept the same, between 65 and 75 per cent; this was done by adjusting the final NaCl concentration (usually to around 68·5 mM NaCl or 0·400 g per 100 ml NaCl).

The effect of repeated washings on erythrocyte stabilization. Three stock suspensions of erythrocytes were prepared. One suspension contained unwashed erythrocytes, prepared as outlined in Methods, a second batch of erythrocytes was washed three

times, and the third set of cells was washed six times. The wash solution used was 154 mM NaCl in 10 mM sodium phosphate buffer, pH 7, the same solution that the cells were finally suspended in. Each washing volume was 35 times the volume of the erythrocytes. Aliquots of the three stock suspensions were tested for stabilization–lysis by prochlorperazine. The results revealed that there was little effect of repeated

TABLE 2. PHENOTHIAZINE ADSORPTION ON TO ERYTHROCYTES

	C_{ms} (M)	C_{50} (M)	Å²/molecule at C_{ms}
Promethazine HCl	3×10^{-4}	2×10^{-5}	65
Chlorpromazine HCl	10^{-4}	2×10^{-6}	98
Trifluoperazine diHCl	$3{\cdot}5 \times 10^{-5}$	$9{\cdot}7 \times 10^{-7}$	139
Fluphenazine diHCl	$3{\cdot}5 \times 10^{-5}$	$1{\cdot}5 \times 10^{-6}$	180

The adsorption of the phenothiazine tranquilizer on to erythrocytes was measured concomitantly with the protection of the erythrocytes against osmotic hemolysis. C_{ms} is the concentration of the drug at maximal protection or stabilization; C_{50} is the drug concentration at 50 per cent erythrocyte stabilization. Å²/molecule represents the area of the erythrocyte in square Ångstroms that is associated with a single molecule of the drug at maximal stabilization; this value is obtained, knowing the amount of drug removed from the supernatant by the erythrocytes and the average area of the cell. The results above indicate that the strongest tranquilizer, fluphenazine, is associated with the least number of molecules adsorbed to the cell membrane, thus giving a high value of Å²/molecule; promethazine, weak clinically, requires many more molecules per cell to cause maximal stabilization. A value of around 150 Å²/molecule would correspond to an adsorption of about 10^8 molecules per cell. These values assume low adsorption to hemoglobin.

washing on the C_{50} and the C_{ms}; the C_{ms} was $1{\cdot}4 \times 10^{-5}$ M for 0, 3, and 6 washes; the C_{50} was 4×10^{-6} M for no wash and $3{\cdot}7 \times 10^{-6}$ M for 3 and 6 washes. Because of these results the cells were not washed for any of the other experiments.

Erythrocyte stabilization by various tranquilizers and antihistamines. The compounds that were tested for their ability to produce stabilization-lysis were reserpine phosphate, butyrylperazine dimaleate, prochlorperazine ethane disulfonate, fluphenazine diHCl, thioridazine HCl, triflupromazine HCl, chlorpromazine HCl, ethopropazine HCl, promethazine HCl, and haloperidol. (Because of the aqueous insolubility of haloperidol, this drug was dissolved in high concentration in ethanol; 0·05-ml aliquots were added to the 1·5-ml hypotonic test solutions about 20 sec before adding the erythrocytes. Independent experiments revealed that the low final concentration of ethanol did not affect the release of hemoglobin.)

The biphasic effect was produced by all these compounds. A sample of the results is in Fig. 3, except that for ethopropazine HCl which will be found in Fig. 4. The values of C_{50} for these compounds range between 10^{-7} and 10^{-5} M. There appears to be only a crude correlation between the C_{ms} values and the clinical antipsychotic potency of the tranquilizers. The approximately 10-fold difference in clinical potency[2] between chlorpromazine and the piperizinated phenothiazines is matched by a 10-fold reduction in the amount of drug required to confer maximal protection on the hypotonic hemolysis. Chlorpromazine sulfoxide (CPZ-SO), completely ineffective in psychosis, confers protection at 10^{-2} M (Fig. 3). This high concentration may protect the erythrocytes by raising the osmotic pressure of the external solution. The results

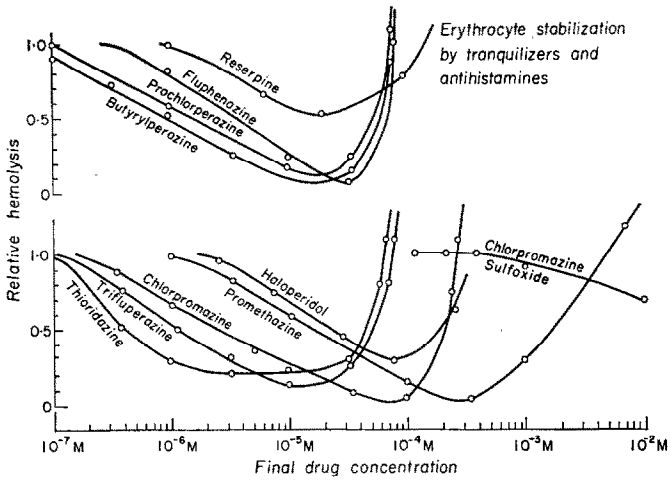

FIG. 3. The stabilizing and lytic effects of various tranquilizers and antihistamines on human erythrocytes. Stabilization of the erythrocytes against hypotonic hemolysis is caused by butyrylperazine dimaleate, prochlorperazine ethane disulfonate, fluphenazine diHCl, reserpine phosphate, thioridazine HCl, trifluoperazine diHCl, chlorpromazine HCl, promethazine HCl, and haloperidol. High concentrations of all the compounds caused direct lysis. Stabilization by chlorpromazine sulfoxide occurred at concentrations higher than equiosmolar concentrations of NaCl or sucrose. All the compounds were dissolved in aqueous solution except haloperidol which was added to the final aqueous solution from concentrated stock ethanolic solutions. The final concentration of the erythrocytes was $2{\cdot}4 \times 10^7$ cells/ml. A relative hemolysis of 1·0 indicates an absolute degree of hemolysis of around 40 per cent.

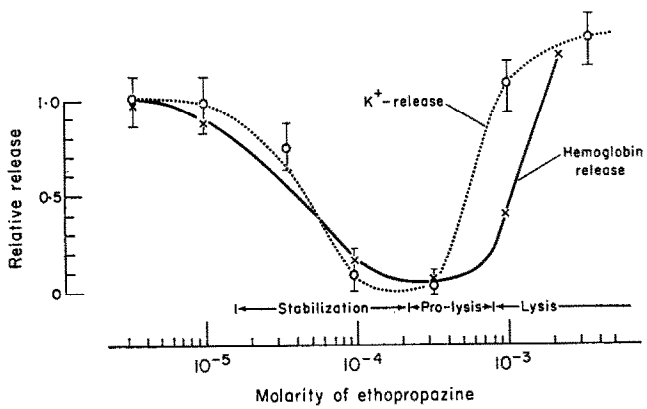

FIG. 4. The relationship between stabilization of the erythrocyte membrane and the phenomenon of pro-lysis.[33] Ethopropazine HCl causes a stabilization of the erythrocyte membrane against hypotonic disruption, as shown by both the retention of hemoglobin and K^+. At higher concentrations the K^+ is released far more readily than the hemoglobin (pro-lysis). A relative hemolysis of 1·0 here represents an absolute hemolysis of about 65 per cent. The vertical bars represent standard deviations.

of Freeman and Spirtes,[6] however, do suggest that the protective effect of chlorpromazine sulfoxide is more than just an osmotic protection. The C_{ms} for haloperidol is around 7×10^{-5} M, which is practically identical with that for chlorpromazine. Pharmacological studies of animals indicate, however, that haloperidol is more potent than chlorpromazine by a factor of about 2·5 to 170 on a molar basis[27] while, clinically, haloperidol is more potent by about 25-fold.[27]

Mechanical strengthening of erythrocyte membranes by phenothiazines. The cells are also mechanically stronger. With the atomization method of Andreasen,[28] the mechanical fragility of erythrocytes in the presence of a stabilizer was tested. Erythrocytes were suspended in 104 mM NaCl in 10 mM sodium phosphate buffer, pH 7, containing $9{\cdot}4 \times 10^{-6}$ M prochlorperazine ethane disulfonate. These cells were then passed through an all-glass atomizer at 0·14 atmosphere air pressure. The unbroken cells were then centrifuged and the released hemoglobin measured. Without the drug the average optical density of the hemoglobin released in the atomization procedure was $0{\cdot}171 \pm 0{\cdot}008$ (mean $\pm$ S.D. of eighteen determinations); in the presence of $9{\cdot}4 \times 10^{-6}$ M prochlorperazine, the average optical density was $0{\cdot}153 \pm 0{\cdot}005$. There is significant difference (P less than 0·001; Student's t test) between these two values. Both the osmotic and mechanical fragilities, therefore, are reduced by drug stabilization.

The adsorption of phenothiazines by erythrocytes. It is known that phenothiazines adsorb to brain tissue,[29] to erythrocytes,[7] and to mitochondria.[30] It was possible to determine both the protection and the amount of drug adsorption on to the erythrocyte simultaneously, as explained in Methods.

The results of such adsorption–stabilization experiments are shown in Figs. 5A and 5B for the antihistamine promethazine HCl and for fluphenazine diHCl, respectively. The line for relative hemolysis indicates the usual biphasic stabilization–lysis curve. The number of drug molecules adsorbed per cell increased as the concentration of the drug increased. It was impossible to measure how many drug molecules were adsorbed beyond the C_{ms}, since no cell pellets could be obtained at the centrifugal accelerations used (only 13,000 g).

From the number of adsorbed molecules per erythrocyte and the average area of a human erythrocyte (163 μ^2),[25] it was possible to calculate the area of the erythrocyte membrane associated with one molecule, and this is shown in Figs. 5A and 5B. At the concentration of maximal stabilization, C_{ms}, the area was around 65 Å^2/molecule for promethazine and 180 Å^2/molecule for fluphenazine. Other values are in Table 2. These values compare with an area of the phenothiazine ring of about 50 to 60 Å^2 (Ref. 2). All these calculations are tentative and will have to be modified when the relative affinities of phenothiazines for hemoglobin and erythrocyte ghost membrane are determined.

The reversibility of erythrocyte stabilization. The membrane may be stabilized by an irreversible type of chemical reaction or a reversible physicochemical one. Experiments were done, therefore, to see whether erythrocyte stabilization was a reversible phenomenon. The results demonstrate that the phenothiazines will desorb off the membrane with great ease and, in doing so, allow the erythrocyte membrane to return to its original condition of osmotic fragility. The results of such experiments are shown in Fig. 6. Curve X of Fig. 6A shows results with prochlorperazine, obtained in the usual manner; that is, the 0·1-ml aliquot of cells was pipetted directly into 1·5 ml of hypotonic

test solution. Curve Y of Fig. 6A represents results wherein the 0·1-ml aliquot of erythrocytes was first added to the drug solution in 154 mM NaCl for 1 min, then centrifuged and resuspended in 1·5 ml of hypotonic saline. The stabilization in

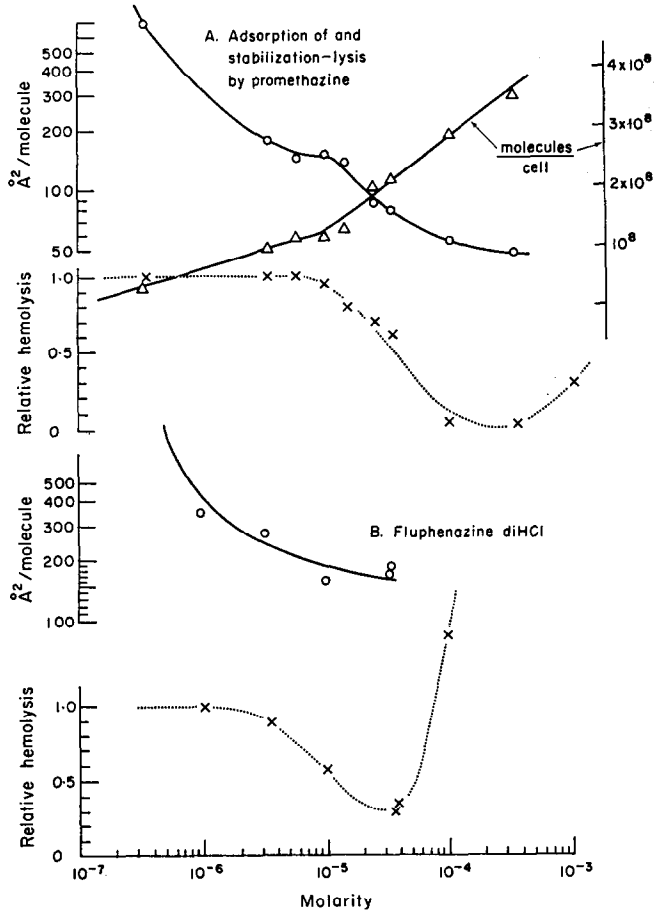

FIG. 5. The adsorption of phenothiazines by erythrocytes. A. Promethazine HCl. B. Fluphenazine diHCl. The phenothiazine stabilization of the erythrocytes against hypotonic hemolysis is indicated by the dashed lines. The amount of phenothiazine adsorbed by the erythrocytes concomitant with stabilization is shown by the line with the triangles (molecules/cell). Dividing the number of molecules/cell by the average erythrocyte area gives the amount of membrane area associated with one stabilizing molecule. It is seen that at maximal stabilization there is about 60 Å²/molecule for promethazine and 170 to 180 Å²/molecule for fluphenazine diHCl. The adsorption of further molecules is associated with direct lysis of the cells.

procedure Y was much less than in X, presumably because of the partial desorption of the drug. A third procedure Z was done as follows: the 0·1-ml aliquot of erythrocytes was mixed with drug in 154 mM NaCl for 1 min, then centrifuged and resuspended in 0 M drug 154 mM NaCl for 1 min, centrifuged again, and finally resuspended in hypotonic test solution. It can be seen that there was no stabilization whatsoever

as a result of this procedure. Although there were probably many molecules still bound and adsorbed to the membrane, it is apparent that the majority desorbed off the membrane.

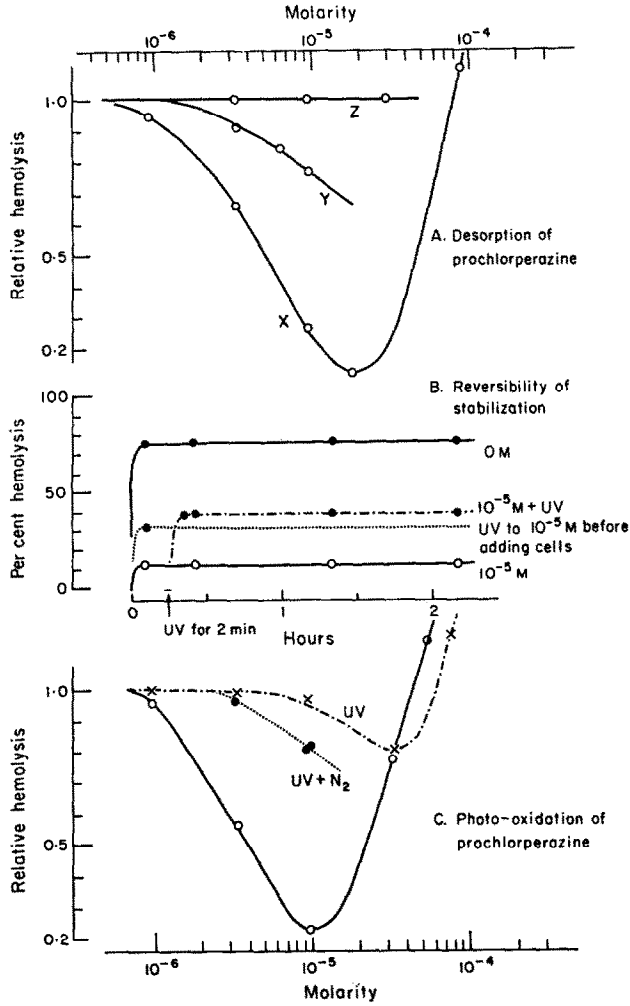

FIG. 6. The reversibility of stabilization. A. Erythrocytes stabilized by prochlorperazine ethane disulfonate revert to their original osmotic fragility when the drug desorbs off the membrane. Curve X shows the stabilization (and lysis) of erythrocytes suspended in hypotonic saline containing the drug; Y represents data wherein the cells were first added to the drug solution in 154 mM NaCl and then resuspended in hypotonic saline without drug. Procedure Z was the same as Y except that the cells were washed for 60 sec before resuspension in hypotonic saline.

B. The reversibility of stabilization is shown by photochemical oxidation of the drug on the cell membrane. When cells, stabilized by 10^{-5}M prochlorperazine, are irradiated by u.v. light for 2 min, there is a very quick loss of the stabilizing potency; this loss is almost identical with the loss of potency occurring when the u.v. irradiates the 10^{-5}M solution before the cells were added.

C. Ultraviolet illumination reduces the stabilizing potency of a phenothiazine. Irradiation of the prochlorperazine solutions before adding the cells increases the concentration required for 20 per cent stabilization from 2×10^{-6} to 2×10^{-5}M. This photo-oxidation is prevented to some extent by bubbling N_2 through the solutions.

Another way to demonstrate reversibility of the stabilization phenomenon is by taking advantage of the photochemical sensitivity of phenothiazines. When a quartz cuvette containing erythrocytes stabilized in hypotonic solution by prochlorperazine was irradiated with a 2-min pulse of u.v. light, the level of stabilization was immediately reduced. The results, shown in Fig. 6B, indicate that the control value of per cent hemolysis was around 75 per cent. The presence of 10^{-5} M prochlorperazine, however, reduced the per cent hemolysis to only about 10 per cent, and this stabilization effect lasted for at least 2·5 hr. A 2-min u.v. light pulse on to the stabilized erythrocytes increased the hemolysis to around 40 per cent. If the u.v. light irradiated the drug solution before the cells were added, about 32 per cent hemolysis occurred. These results indicate that photochemical oxidation of prochlorperazine reduces the stabilizing potency of the compound and also that the membrane stabilization is reversible. The u.v. light emanated from a mercury vapor arc lamp and passed through a heat filter (water in a quartz flask). The u.v. effect was not a direct effect on the cells since, if the u.v. light irradiated the cells before the drug was added, results similar to those in Fig. 6B were obtained.

A further demonstration that u.v. irradiation reduces the potency of the phenothiazines, and that this reduction results partly from photochemical oxidation, is shown by the results of Fig. 6C. Three series of quartz cuvettes containing prochlorperazine were prepared; one series was irradiated by u.v. light for 10 min; another was irradiated in the same way except that N_2 gas bubbled constantly before, during, and after the u.v. irradiation period; and a third series was not irradiated at all. Figure 6C indicates that u.v. irradiation drastically reduced the stabilizing potency, while the bubbling of nitrogen gas somewhat offset this loss.

Effect of ionic strength on lysis. Since it is known that the surface activity of an ionic detergent varies with the ionic strength,[31] it seemed of interest to examine whether the emulsifying potency of these tranquilizer cationic detergents would also be affected by ionic strength. The experiment was carried out in the following way. Erythrocytes were suspended at a final concentration of $2{\cdot}38 \times 10^7$ cells/ml in tubes containing a sucrose–NaCl mixture such that the total osmotic pressure (assumed that the osmotic coefficients of sucrose, Na^+, and Cl^- were each equal to unity) was 328 mOsmoles/l. in each test tube. The test tube solutions also contained trifluoperazine diHCl ranging from $2{\cdot}8 \times 10^{-4}$ M to $9{\cdot}4 \times 10^{-4}$ M (final concentrations). Immediately after the cells were added to the tranquilizer–sucrose–NaCl solution they started to hemolyze, the rate of lysis depending on the concentrations of all three chemical compounds. An arbitrary end point to the hemolytic reaction was taken as the time required for the turbidity of the tube to clear sufficiently so that the word "Kimax" (printed on the glass wall of a pipette situated behind the test tube) could be read. This simple method has been used many times by other investigators; duplicate experiments agreed within 5 per cent.

Figure 7 shows the sort of results that have been obtained by varying the sucrose/NaCl ratio. It is seen that the time interval before complete hemolysis occurred became markedly reduced at higher NaCl concentrations (lower sucrose concentrations). Reducing the trifluoperazine concentration from $5{\cdot}6 \times 10^{-4}$ M to $2{\cdot}8 \times 10^{-4}$ M lengthened the time interval about 8-fold, as indicated by Fig. 7. Reducing the drug concentration still further lengthened the time interval inordinately; that is, 10^{-4} M trifluoperazine diHCl in 328 mOsmolar sucrose–NaCl solution did not cause hemolysis

within the 4-hr observation period (10^{-4} M trifluoperazine diHCl in 66·7 mM NaCl does, however, cause complete lysis within 5 min; Fig. 3).

Effect of ionic strength on stabilization. To test the effect of ionic strength on the property of membrane stabilization, a series of tubes containing NaCl and sucrose

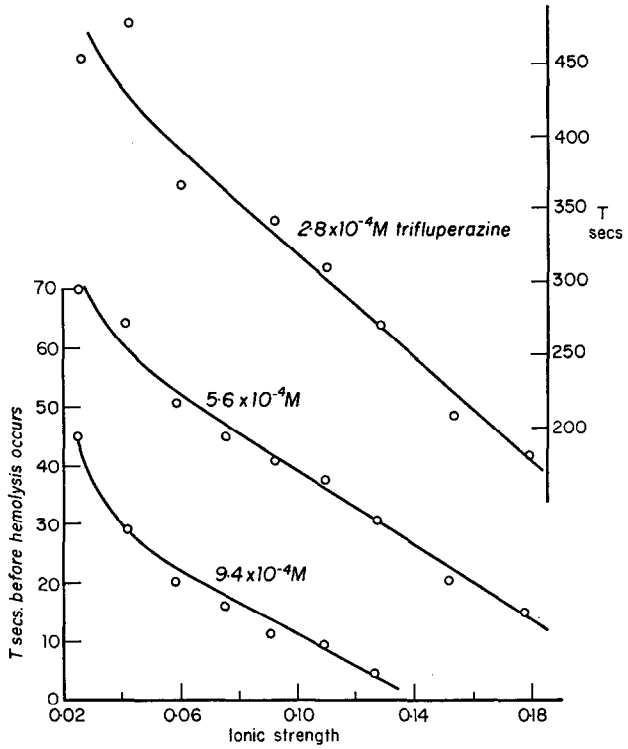

FIG. 7. An increase in NaCl concentration (while keeping the osmolarity approximately constant with sucrose) potentiates the hemolytic activity of trifluoperazine diHCl. Erythrocytes were suspended in solutions of varying NaCl/sucrose ratios, where the total osmolarity was 328 mOsmolar. T represents the number of seconds before trifluoperazine caused around 95 per cent hemolysis.

was prepared such that the total theoretical osmotic pressure was equivalent to 68·5 mM NaCl. The C_{50} for prochlorperazine ethane disulfonate was determined for each NaCl–sucrose combination; when 70·2 mM NaCl was used, the C_{50} was $3{\cdot}5 \times 10^{-6}$ M; when 34·2 mM NaCl + 0·071 M sucrose was used, the C_{50} was about $4{\cdot}2 \times 10^{-6}$ M; and when 0·139 M sucrose was used, the C_{50} was about 5×10^{-6} M. The stabilizing potency, in other words, was only slightly enhanced by the higher salt content. Qualitatively this agrees with the acceleration of hemolysis at higher ionic strength; whether or not there was a true "theoretically equiosmolar" replacement of solute is impossible to say, since the activity coefficients are not known in this system.

DISCUSSION

The results indicate that the entire group of clinically important tranquilizers, including the nonphenothiazines reserpine and haloperidol, cause the biphasic effect

of erythrocyte membrane stabilization and lysis. While all the tranquilizers and presumably all the antihistamines cause membrane stabilization, not all membrane stabilizers will act as tranquilizers.

The adsorption results of Table 2 demonstrate that a higher clinical potency of the compound is associated with a lower number of molecules per cell for maximal stabilization. For example, for promethazine virtually 100 per cent of the membrane was associated with the presence of a molecule, since the value was around 65 $Å^2$/ molecule and the size of the molecule is about 50 or 60 $Å^2$; fluphenazine, the most potent clinically, conferred the same effect with only about 25 per cent involvement of the membrane. It should be pointed out that Freeman and Spirtes[7] also noted a correlation between clinical and cytological potencies. A correlation between the amount of adsorbed drug and the degree of membrane stabilization may not hold for the mitochondrial membrane, as indicated by the work of Spirtes and Guth.[11]

The role of ionic strength. The fact that stabilization and lysis are both reduced by replacing the salt with sucrose agrees with other findings reported in the literature. Weil-Malherbe and Posner[14] found that lower concentrations of triflupromazine HCl were needed to protect chormaffin granules in the presence of isotonic sucrose. Kinsky[32] found that the lytic potency of vitamin A (presumably retinol) on *Bacillus megaterium* protoplasts was considerably reduced by replacing the 0·6 M NaCl solution by 0·6 M sucrose.

Pro-lysis and stabilization. Davson and Danielli[33] found that certain substances in sublytic doses would cause a release of the intracellular potassium from erythrocytes without causing significant losses in hemoglobin; this effect was referred to as *pro-lysis*. The concentration range of pro-lysis is quite small, the minimal pro-lytic and minimal hemolytic concentrations varying from each other only by a factor of around 2. For example, pro-lysis with butanol[34] occurs between 0·25 and 0·4 M, where 0·4 M is the minimal hemolytic concentration. This relatively narrow concentration range should be compared with the 10- to 50-fold range between minimal and maximal stabilization. The concentration range of pro-lysis would probably fit somewhere between the concentration region of stabilization and that for lysis, overlapping both zones somewhat; this is outlined in Fig. 4.

The decrease in erythrocyte osmotic fragility caused by the drugs can be offset by lowering the osmotic pressure of the test solution. For example, in one series of experiments using 64·2 mM NaCl, 67 per cent of the erythrocytes hemolyzed in the absence of any drug, while 34 per cent hemolyzed in the presence of $9·4 \times 10^{-6}$ M prochlorperazine. In the presence of this drug concentration it was necessary to reduce the NaCl concentration to 52 mM before stabilization could be overcome and a hemolysis of around 70 per cent could be obtained. In other words, the osmotic fragility curve (per cent hemolysis vs. NaCl concentration) was shifted by an order of magnitude of around 10 mM NaCl. This corresponds to a shift of about one half an atmosphere osmotic pressure. Judah[9] found that 5×10^{-4} M diphenhydramine shifted the osmotic fragility curve by about 0·04 g per 100 ml or 6·9 mM NaCl; this amounts to about one third an atmosphere of pressure.

One possible explanation that may account for these findings is that the erythrocyte membrane is expanded by the tranquilizers somewhat in the same way that lipid monolayers are penetrated by these compounds.[35] This idea of membrane expansion

is very similar to that considered by Skou[36] on the basis of lipid monolayer penetration by local anesthetics.

Membrane expansion of the erythrocyte would lead to an increase in the surface area/volume ratio of the cell. According to the results of Rand and Burton,[37] cells with higher area/volume ratios should be associated wih a lower osmotic fragility. This mechanism would be reversible, long-lasting, and associated with adsorption of the drug to the membrane.

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