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II. ERYTHROCYTE MEMBRANE STABILIZATION BY LOCAL ANESTHETICS AND TRANQUILIZERS

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Abstract—1. Low concentrations of the local anesthetics dibucaine HCl and tetracaine HCl protect or stabilize human erythrocytes against hypotonic hemolysis; higher concentrations elicit a detergent hemolysis. Lidocaine HCl and procaine HCl also cause these biphasic effects but at higher extracellular pH.

2. Increasing the extracellular pH enhances the membrane-stabilizing and lytic potencies of these local anesthetics as well as those of the phenothiazine tranquilizers.

3. Decreasing the intracellular pH also enhances the stabilizing and lytic potencies of these tertiary amines.

4. Measurements of the circumferences of the erythrocyte profiles from electron micrographs indicate that $1{\cdot}4 \times 10^{-5}$M prochlorperazine induces a membrane expansion of around 19 per cent. This expansion is compatible with an intramembrane location of the 10^8 molecules per cell that is known to occur (from adsorption studies).

ALTHOUGH it is known that the local anesthetics which are in common clinical use today (dibucaine, lidocaine, etc.) are hemolytic,[1] no mention has ever been made of their antihemolytic effect. The fact that these compounds in very low concentrations stabilize erythrocytes against hypotonic hemolysis forms the basis of this report. The inhibition of hypotonic hemolysis by miscellaneous other anesthetics has been reported (see Ponder[2] for some references). Membrane stabilization of subcellular membrane-bounded organelles has also been reported; tetracaine, cocaine, and lidocaine (Xylocaine) inhibit the spontaneous release of noradrenaline from bovine splenic nerve granules.[3, 4] At high concentrations all these anesthetics, which are surface-active,[5] cause membrane lysis. As a function of the concentration, therefore, these drugs cause a biphasic effect on membranous structures.

The erythrocyte stabilization by local anesthetics, to be reported in this paper, is identical with that caused by the phenothiazine tranquilizers and antihistamines.[6] This similarity is not too surprising, since it has long been known that phenothiazines have a strong local anesthetic potency *in vivo*;[7] unfortunately, very few reports have appeared demonstrating the local anesthetic effect *in vitro*.[8, 9] The local anesthetics and the phenothiazine tranquilizers are tertiary amines, and their water-soluble salts will dissociate in solution into the protonated quaternary amine BH^+, and the accompanying anion or anions. The concentration of BH^+ will depend on the pH of the solution and the pK_a of the compound. If the pK_a is 7·0, the compound will be mostly in the BH^+ form at pH values below 7 and mostly in the free base form, B, at pH values over 7.

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Important questions arise in connection with the phenomenon of membrane stabilization by these tertiary amines. Do the stabilizing drug molecules dissolve into the membrane? A suggestion that this might be the case has been obtained by electron microscopy and will be presented in this paper. Which form of the drug is active in eliciting the effect, the protonated amine BH^+, or the free base B, or both? On the subject of local anesthesia there is an extensive literature on this question and the topic has been reviewed many times (see Ariens *et al.*[10] for references). While many results seem to suggest that the free base B is the active form in producing local anesthesia,[11] there are very convincing experiments that the cationic form of the drug, BH^+, is indeed the active species[9, 12, 13] and that B is important only for penetrating to the site of action.[12–14] Further evidence that the protonated form BH^+ can exert a local anesthetic action of its own comes from the fact that quaternary amines such as N-benzylprocaine and N-benzylcocaine, which are always in the charged form in solution, do cause a local anesthetic action.[15, 16] Quaternary forms of Xylocaine are also potent local anesthetics *in vitro* on Ranvier node and the nerve–muscle junction (B. Hille and A. Steinbach, personal communications).

The experiments reported in this paper are compatible with but do not prove the idea that the protonated form of the local anesthetics and the phenothiazine tranquilizers can cause membrane stabilization. The experiments rely heavily on a rationale provided by Clowes *et al.*[17] and Krahl *et al.*[18] in their work on the inhibition of cell division of Arbacia eggs by local anesthetics. These investigators have argued that if the potency of a tertiary amine increases with the extracellular pH, this may indicate one of two possibilities: either B is indeed the active form, or it is the form in which the drug permeates the membrane and, once inside, becomes protonated (depending on the intracellular pH) and then affects the membrane as the cation.

Varying the intracellular pH should help decide on which of these two possibilities is correct. Clowes *et al.* and Krahl *et al.* pointed out that when an equilibrium is reached for the distribution of a tertiary amine between extracellular and intracellular compartments, the approximate concentrations of B and BH^+ will be as follows:

	Extracellular	*Intracellular*
B	$(1 - e)C$	$(1 - e)C$
BH+	eC	$\frac{b}{1-b}(1 - e)C$

where e indicates the protonated fraction at the extracellular pH; b is the intracellular fraction ionized at the pH of the intracellular aqueous phase (e and b are calculated from the pK_a and the pH); and C is the total concentration of the compound as applied outside; $(1 - e)C$ represents the uncharged moiety, B, which is assumed to equilibrate completely and quickly across the membrane. The extracellular concentration of BH^+ will be eC and should not equilibrate across the cell membrane. The assumption that membrane permeability to charged compounds is extremely low and that to uncharged lipid-soluble compounds it is extremely high is warranted by much experimental work (see Schanker *et al.*[19] for references).

According to the above reasoning, *the concentration of* B *is independent of the intracellular* pH. If membrane stabilization-lysis is primarily a result of the action of

B, therefore, varying the intracellular pH should not have any effect on the drugs' potency. Experiments have been carried out to test whether this is so.

Materials. Thymol was obtained from the Fisher Scientific Co. The following firms and laboratories graciously donated drug samples: Smith, Kline & French: trifluoperazine diHCl (Stelazine) and prochlorperazine ethane disulfonate (Compazine). CIBA Pharmaceutical Co.: Nupercaine (dibucaine HCl or cinchocaine HCl). Winthrop Laboratories: tetracaine HCl (Pontocaine) and procaine HCl (Novocain). Astra Pharmaceutical Products, Inc., lidocaine HCl (Xylocaine).

METHODS

The procedure for testing the effects of various drugs on hypotonic hemolysis of human erythrocytes has been described.[6] All experiments were done in triplicate at least, as in the earlier experiments,[6] and the results agreed within 5 per cent. Modifications of the procedure will be mentioned at the appropriate places in Results.

Electron microscopy. The procedure used to examine the effect of the drugs on the ultrastructure of the erythrocytes was as follows. The erythrocytes were treated and suspended in the final drug-test solutions in the same way as for the hemoglobin release experiments. The cells were centrifuged after the 5-min test period, the clear supernatant removed, and 2 ml of 2% glutaraldehyde[20] (in 0·1 M sodium cacodylate buffered to approximately pH 7·2 with 0·1 M HCl) was gently layered over the thin erythrocyte pellet at the bottom of the tube. After 12-min fixation, the glutaraldehyde was removed and 1 ml Palade's fixative[21] was added (2% osmium tetroxide in 0·05 M veronal-acetate-HCl buffer, pH 7·3; no sucrose). During this fixation the pellet was dislodged from the bottom of the tube. After 2 hr (although 30 min is sufficient) the osmium fixative was removed and replaced by a fresh solution of osmium fixative containing 0·5% uranyl acetate (which had been prepared immediately before using, warmed gently under tap-water and filtered before using—excessive warming caused precipitation). This uranyl wash procedure is a slight modification of that used by Farquhar and Palade.[22] After 1·5 hr the uranyl solution was removed (it is preferable to keep the exposure to uranyl acetate shorter than 1 hr, however, to prevent cytoplasmic extraction and uranyl precipitation) and replaced with 100% ethanol. (Gradual dehydration by graded concentrations of ethanol did not result in any difference in structure compared to the procedure of switching directly to 100% ethanol.) The dehydrated pellets were embedded in Epon 812 and sectioned (silver sections) with a diamond knife. The Epon sections were picked up on 150- and 300-mesh carbon-coated, Formvar-covered grids, stained on a drop of 6% uranyl acetate, washed, then stained on a drop of 0·4% lead citrate (in 0·1 N NaOH) for 1 min, washed again, and examined in both the RCA-EMU 3F and Siemens Elmiskop I electron microscopes.

RESULTS

Erythrocyte stabilization by local anesthetics. The stabilizing and lytic properties of Nupercaine HCl, tetracaine HCl, Xylocaine HCl, and procaine HCl are shown in Fig. 1. The protection by Xylocaine and procaine at pH 7·0 is probably osmotic in nature because the concentrations required are greater than those required for protection by NaCl or sucrose (equiosmolar). Lysis of erythrocytes by Xylocaine and procaine in pH 7·0 does not occur at concentrations higher than 0·3 M (see also

Ref. 1) but this high concentration range for these two drugs was not extensively investigated and, therefore, the precise values have been omitted from Fig. 1.

Extracellular pH *variations on erythrocytes.* It will be convenient to refer to C_{50} and C_{ms} as the drug concentrations that cause 50 per cent and maximal stabilizations

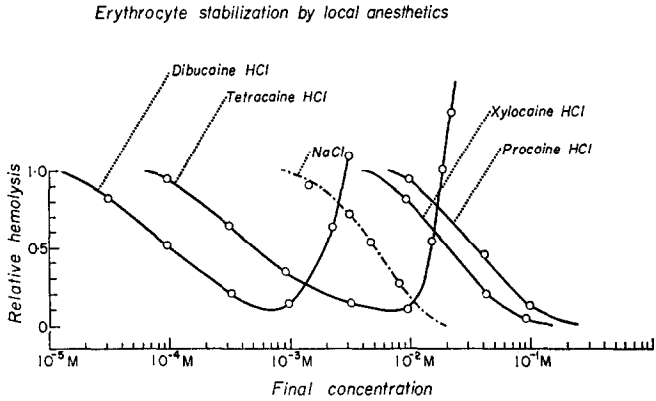


FIG. 1. The stabilizing and lytic effects of various local anesthetics on human erythrocytes. Stabilization of the erythrocytes against hypotonic hemolysis is caused by dibucaine HCl and tetracaine HCl. Stabilization by Xylocaine HCl and procaine HCl occurred at concentrations higher than equiosmolar concentrations of NaCl or sucrose and may represent an osmotic protection of the erythrocyte. High concentrations of the anesthetics caused hemolysis (see text). A relative hemolysis of 1·0 indicates an absolute degree of 60 per cent hemolysis; the erythrocyte concentration was $2{\cdot}4 \times 10^7$ cells/ml final.

respectively. In order to determine which form of the drug (the uncharged free base B, or the protonated amine BH^+) was responsible for membrane stabilization, it was necessary to determine the pH dependency of C_{50}.

The result of an extensive series of experiments involving extracellular pH variations is shown on the right side of Fig. 2. The procedure adopted was as follows. A stock suspension of erythrocytes was made in the usual manner.[6] The extracellular pH of this stock suspension was 7·0 (10 mM sodium phosphate buffer). Aliquots of 0·1 ml from this stock were pipetted into 1·5 ml of hypotonic NaCl test solution. These hypotonic test solutions contained either trifluoperazine diHCl or thymol or Nupercaine HCl at pH 6·0, 7·0, or 8·0, with 10 mM sodium phosphate buffer. The drug concentration range and the final drug concentrations tested (in the hypotonic solutions) are shown in Fig. 2. Since erythrocytes are more fragile when placed in a solution of pH 6, it was necessary to raise the NaCl concentration slightly so that the final amount of hemolysis would be the same as that occurring in the pH 7·0 solution; it was further necessary to reduce the NaCl concentration for testing in pH 8·0, since the cells are less fragile under these conditons. The final solutions used for testing were 77·8 mM NaCl at pH 6·0, 66·6 mM NaCl at pH 7·0, and 57·8 mM NaCl at pH 8·0; the amount of hemolysis under these conditions was around 60 $\pm$ 5 per cent for all the pH values.

Consider first the results with the nondissociating compound, thymol. The biphasic stabilization–lysis curves were virtually identical for pH 6, 7, and 8. The C_{ms} concentrations were all $1{\cdot}8 \times 10^{-3}$M while the C_{50} values (3×10^{-4}M) were also almost all

the same, differing by about 5 per cent. It should be mentioned here that all these concentrations of thymol are below the solubility of thymol (which is given as 0·102 g per 100 ml or 6·8 × 10^{-3}M in the International Critical Tables and 5·7 × 10^{-3}M by Ferguson[23]).

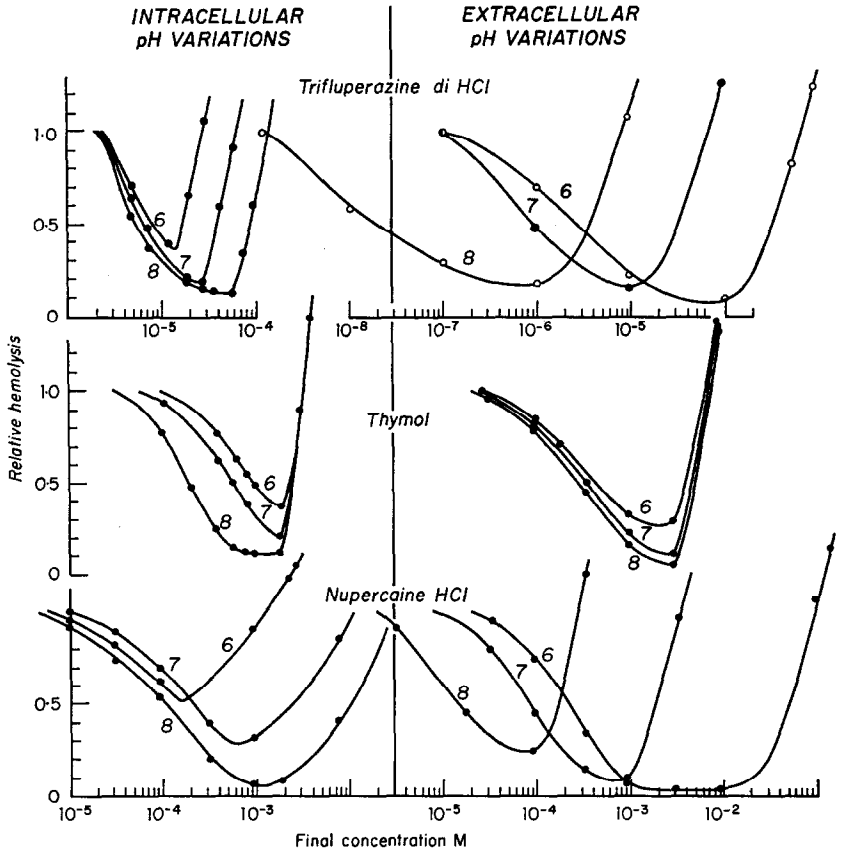


FIG. 2. The effect of extracellular and intracellular pH variations in erythrocytes on the stabilizing potency of trifluoperazine diHCl, thymol, and Nupercaine HCl (dibucaine HCl). See text for explanation and discussion.

The stabilizing and lytic potencies of both the anesthetic and tranquilizer tertiary amines, however, were very sensitive to the external pH and all manifested a similar pH dependency. The potency for both stabilization and lysis was reduced at pH 6 while greatly enhanced at pH 8. Lysis by Nupercaine and by trifluoperazine at pH 6 occurs at high concentrations; for Nupercaine it is above 0·1 M and for trifluoperazine it is above 2 × 10^{-4}M. The pK_a of trifluoperazine is around 7·2 in butanol/water (Swintosky, personal communication) and is 6·4 in aqueous solution,[24] while that for Nupercaine is around 8·54.[25] Very similar results have been obtained for chlorpromazine HCl which has a pK_a of 7·43 (Swintosky, personal communication) as determined by Reese *et al.*,[26] using the Butler method.[27] Differences in ionic strength cannot explain these effects, since they are opposite to what would be

expected if ionic strength played a role.[6] In fact, moreover, it happened to turn out that the ionic strengths of the pH 7 and the pH 8 solutions were identical at 0·091 each. The different contributions to the ionic strength by the NaCl were offset by those of the sodium phosphate buffer ions; ionic strength, therefore, was not responsible for the marked shifts in potency.

These results, considered alone, suggest that the B form plays a role in both stabilization and lysis. As explained in the opening paragraphs, if membrane stabilization–lysis is a result of the action of B alone, varying the intracellular pH should not have any effect on the C_{50}. The next section describes the results of experiments done to test this point.

Intracellular pH *variations on erythrocytes.* For the experiments involving intracellular pH variations the erythrocytes were converted to methemoglobin erythrocytes by means of a brief exposure to sodium nitrite. In order to monitor pH changes within the erythrocyte the method of Keilin and Mann[28] was used. The stock suspension of erythrocytes was exposed to 25 mM sodium nitrite in 154 mM NaCl for 4 min, during which time the suspension turned chocolate brown as the hemoglobin was oxidized to methemoglobin. The work of Austin and Drabkin[29] has shown that the spectral properties of methemoglobin vary with pH. A spectral shift with pH also exists for methemoglobin within the erythrocyte, as shown by the results in Fig. 3. To obtain these data, methemoglobin cells were suspended in buffer solutions of different pH and the optical density compared against the optical density of erythrocytes in pH 6. The solutions used were 10 mM sodium phosphate buffer pH 6, 7, and 8; the solution for pH 10 was 10 mM NaOH, all in 154 mM NaCl. Figure 3 shows that the higher the pH the higher the optical density at 577 mμ; the optical density at 630 mμ, however, rose less steeply. Included in Fig. 3 are modifications of the data of Austin and Drabkin[29] on the pH dependency of methemoglobin in free solution. Even to the naked eye the color of the methemoglobin cells can be seen to change with pH; at pH 6 the cells were very brown, at pH 10 they were strongly tinged with red, and at pH 7 and 8 they showed intermediate hues.

The spectral shifts recorded in Fig. 3 are the steady-state values, the measurements having been taken at least after 15 or 20 min after the erythrocytes had been added to the solutions of different pH. With the methemoglobin cells it was also possible to study transient intracellular pH changes, and these results are shown in Fig. 4. Section A shows the change in optical density at 577 mμ when methemoglobin cells, originally in pH 7, were resuspended in pH 8. The O.D. was measured with the cells in pH 7 as a blank; the gaps in the record indicate the moments when zero adjustments were made. Parts B and C show typical records of the O.D. at 577 mμ of the methemoglobin cells, originally in pH 8 or 6, when resuspended in pH 7; the O.D. of cells in pH 7 was again used as a zero blank.

The procedure employed for the intracellular pH variations in the presence of the drugs was as follows. Venous blood (1·5 ml) was mixed with heparin as usual, and 0·925 ml of this mixture was centrifuged. After aspirating the plasma and buffy coat, the cells were resuspended in 15 ml of 25 mM $NaNO_2$ in 154 mM NaCl for 4 min. The nitrite was then washed out with two 15-ml washes of 154 mM NaCl in pH 7·0 buffer. The cells were recentrifuged and finally suspended in 154 mM NaCl, pH 6, 7, and 8, for at least 15 min before proceeding. These latter three suspensions were the methemoglobin cell stocks used in the following experiments. Aliquots of 0·1 ml

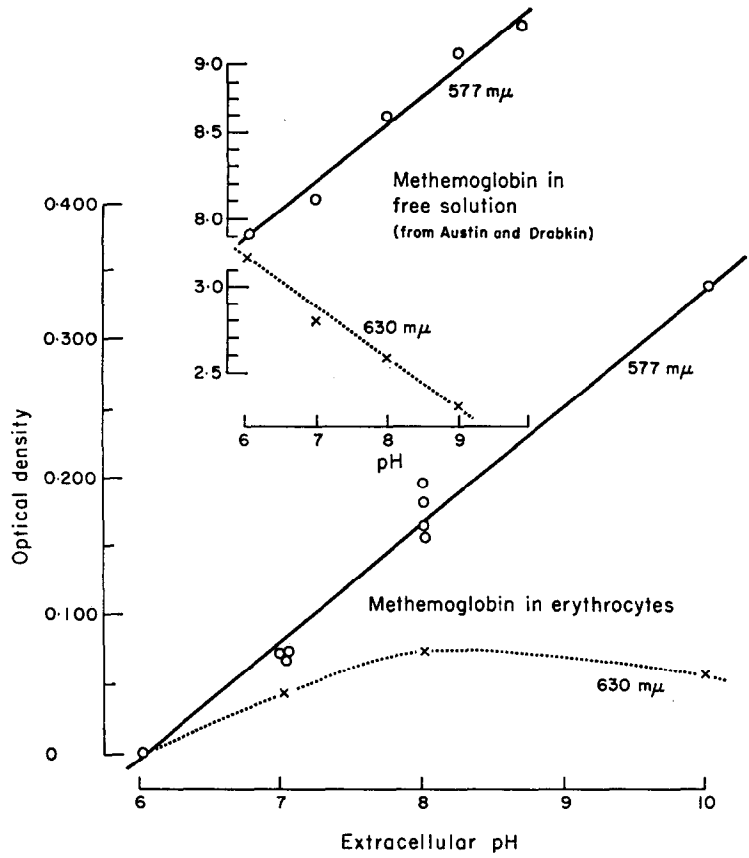


FIG. 3. The changes in spectral properties of methemoglobin that occur in free solution (adapted from Austin and Drabkin[29]) and within the erythrocyte when the pH is changed. The ordinate for the data of Austin and Drabkin is the extinction coefficient at 577 mμ and 630 mμ.

methemoglobin cells from these stock suspensions were added to 1·5 ml hypotonic NaCl test solutions, all of which were buffered at pH 7. These hypotonic test solutions contained thymol, trifluoperazine diHCl, or Nupercaine HCl in varying dilutions. After only 2-min hemolysis had occurred, the cells were centrifuged at 1500 *g* for 45 sec and the released methemoglobin measured spectrophotometrically at 540 mμ (the 540-mμ absorption peak is associated with a slightly higher extinction coefficient for methemoglobin than the 577-mμ peak). The time course of hemolysis under these conditions was about the same as normal erythrocyte hemolysis,[6] and hypotonic hemolysis was 90 per cent complete at around 90 sec. Because of these transient conditons, it is impossible to say what the intracellular pH was in any of these experiments; all that can be said is that the direction of the pH shift was known.

Since the osmotic fragility of methemoglobin cells was greater than that of hemoglobin cells, appropriate changes had to be made in the NaCl content of the hypotonic test solutions in order to keep the final per cent hemolysis at the same level. Aliquots of methemoglobin cells from the pH 6 stock suspension were tested in drug solutions containing 78·6 mM NaCl, pH 7; aliquots of methemoglobin cells from the pH 7

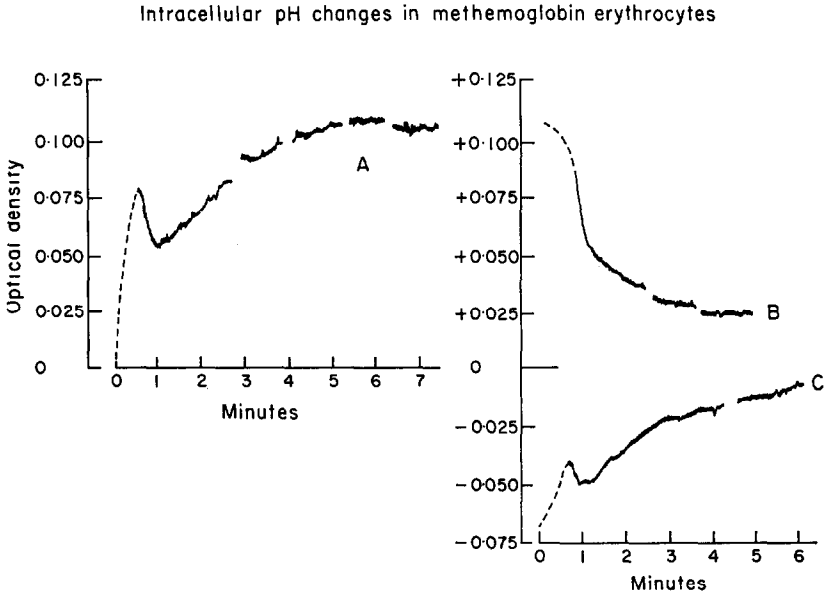


FIG. 4. The variations in intracellular methemoglobin optical density (at 577 mμ). A. When methemoglobin cells, originally in pH 7, are resuspended in pH 8. B. When methemoglobin cells, originally in pH 8, are resuspended in pH 7. C. When cells, originally in pH 6, are resuspended in pH 7. The zero blank for the spectrophotometer was an equal concentration of intact methemoglobin cells suspended in pH 7. The shifts in optical density represent mostly a change in the spectral absorption of methemoglobin; osmotic shifts in cell volume were usually completed within 30 to 45 sec and may possibly be the explanation for the irregular first part of each tracing.

stock suspension were tested in drug solutions containing 71·8 mM NaCl, pH 7; aliquots from the pH 8 suspension were tested in drug solutions containing 68·5 mM NaCl, pH 7.

The results of these experiments are shown on the left side of Fig. 2; each point in Fig. 2 is the average of quadruplicate determinations which agreed to within 8 per cent.

A. *The lytic limbs*

It will be convenient to consider first only the lytic limbs of the results dealing with the intracellular pH variations, and to consider the lytic limb of thymol before the others. It is seen that the lytic phases for pH 6, 7, and 8 are all identical (left side of Fig. 2).

The lysis by the tertiary amines, however, was markedly affected by the intracellular pH changes. Intracellular acidification enhanced the potency of trifluoperazine by lowering the threshold concentration for hemolysis from $2{\cdot}8 \times 10^{-5}$M (at pH 7) to $1{\cdot}2 \times 10^{-5}$M (at pH 6). Intracellular acidification also enhanced the potency of Nupercaine by lowering the threshold concentration for hemolysis from about $5{\cdot}5 \times 10^{-4}$M (at pH 7) to about $1{\cdot}3 \times 10^{-4}$M (at pH 6). Intracellular alkalinization diminished the potency of trifluoperazine by raising the threshold concentration necessary for hemolysis from $2{\cdot}8 \times 10^{-5}$M (at pH 7) to about $5{\cdot}5 \times 10^{-5}$M (at pH 8). Intracellular alkalinization diminished the potency of Nupercaine by raising

the concentration threshold for hemolysis from $5{\cdot}5 \times 10^{-4}$M (at pH 7) to about $1{\cdot}2 \times 10^{-3}$M (at pH 8). The conclusion that can be made from these results is that since intracellular pH variations affect the lytic potency, the B form cannot be entirely responsible for the effect (as outlined in the rationale of Krahl *et al.*[18]) and that BH^+ plays some part in causing hemolysis.

B. *The stabilization limbs*

Consider only the stabilization limbs of the intracellular pH results shown in Fig. 2 on the left side. Again consider the results with thymol first; it is seen that the pH 8 cells were more easily stabilized with thymol than the pH 7 or the pH 6 cells. For example, the C_{50} for thymol at pH 7 was $5{\cdot}5 \times 10^{-4}$M, at pH 6 it was $9{\cdot}5 \times 10^{-4}$M, and at pH 8 it was $1{\cdot}9 \times 10^{-4}$M. It is impossible to say why these C_{50} stabilization values are not the same. All that can be stated with any certainty is that it is not because thymol changes its state of protonation between pH 6 and pH 8.

Now consider the stabilization limbs with Nupercaine (bottom left in Fig. 2). The C_{50} at pH 7 is 2×10^{-4}M. Intracellular acidification enhances the potency of Nupercaine by *lowering* the C_{50} from 2×10^{-4}M (at pH 7) to $1{\cdot}3 \times 10^{-4}$M (at pH 6). It should be noticed that curve 6 is *to the left* of curve 7 in the stabilization zone. Such an enhancement of the Nupercaine potency should not occur if *only* the B form were responsible for stabilization (Krahl *et al.*[18]). Indeed if the Nupercaine B form was acting alone, varying the pH should lead to a shift *relatively* similar to thymol. In fact, however, an intracellular acidification with thymol leads to a *drop* in potency, the C_{50} going from $5{\cdot}5 \times 10^{-4}$ (at pH 7) to $9{\cdot}5 \times 10^{-4}$M (at pH 6).

Look now at the stabilization limbs with trifluoperazine (top left in Fig. 2) and compare, for example, the pH 8 and pH 7 curves. The C_{50} at pH 8 is $5{\cdot}5 \times 10^{-6}$M and at pH 7 it is 7×10^{-6}M. Intracellular acidification, therefore, apparently diminished the potency by about 20 per cent; this diminution, however, is *relatively smaller* than the diminution that occurs upon intracellular acidification in the presence of the nonionic thymol drug, wherein the C_{50} rises from $1{\cdot}9 \times 10^{-4}$M (at pH 8) to $5{\cdot}5 \times 10^{-4}$M (at pH 7), a 200 per cent drop in potency. In other words, if the nonionic B form of trifluoperazine was *alone* responsible for the effect, we expect a 200 per cent drop in potency going from pH 8 to pH 7, but in fact we only get a 20 per cent drop.

According to the rationale of Krahl *et al*[18] this may be interpreted, in a guarded and cautious manner, that B alone is not completely responsible for the effect. The BH^+ form, therefore, must play some part, however small it may be. Similar considerations pertain to the effects of Nupercaine at pH 8 *relative* to pH 7 as compared to thymol at pH 8 *relative* to pH 7; similar considerations also pertain to the effect of trifluoperazine at pH 7 *relative* to pH 6 as compared to thymol at pH 7 *relative* to pH 6.

Once more it should be pointed out that these effects do not arise from differences in ionic strength or salt concentration. For example, the trifluoperazine pH 8 procedure was done not only with 71·8 mM NaCl but also with 68·5 mM NaCl, and identical results in the relative hemolysis curves were obtained.

Electron microscopy of the stabilized erythrocytes. One possible mechanism for erythrocyte stabilization against hypotonic hemolysis would be a drug-induced expansion of the erythrocyte membrane. It is possible that the active drug molecules penetrate part way into the membrane; for example, the long hydrophobic portion of the drug molecule could penetrate into the membrane while the charged part of

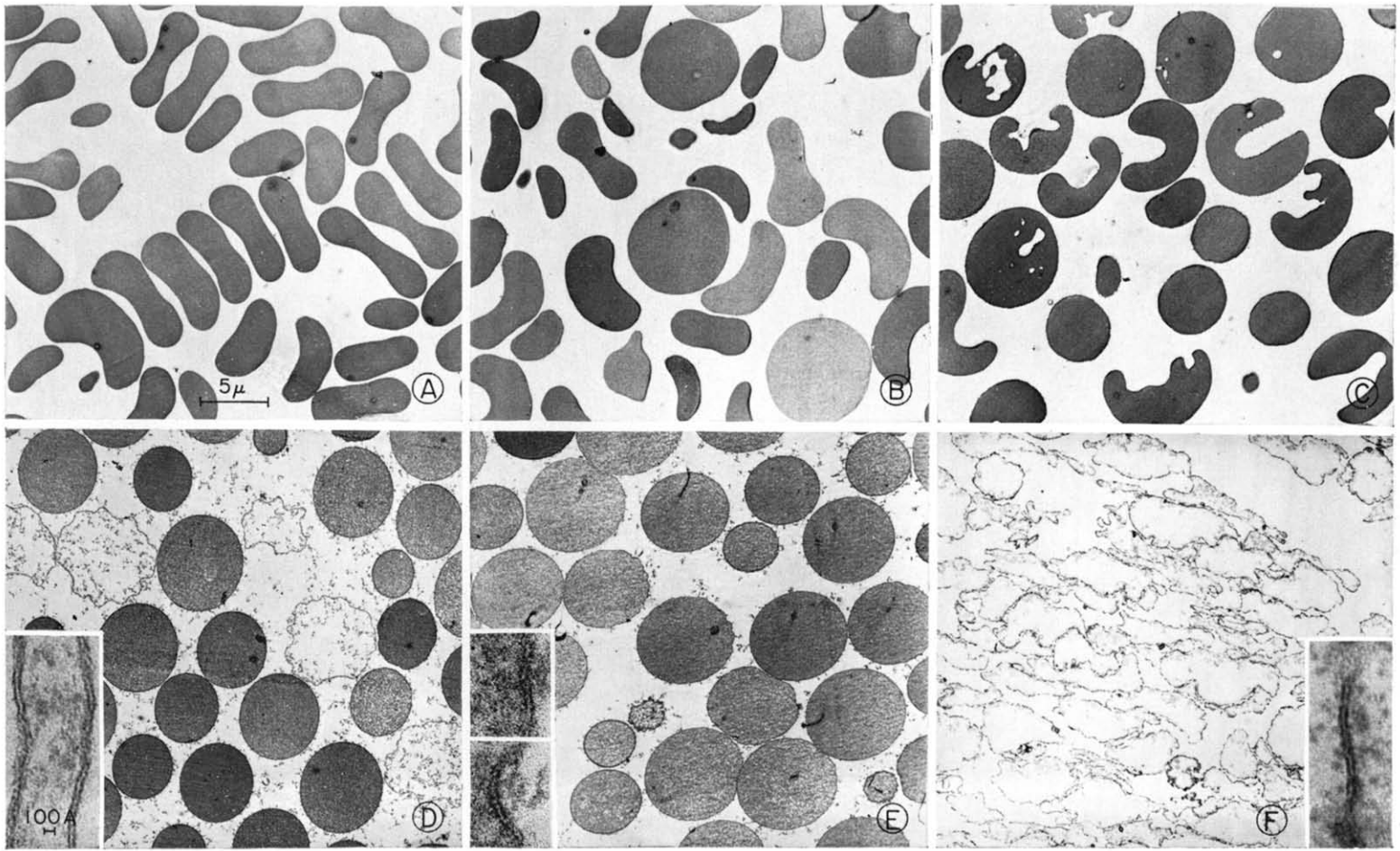
the molecule would remain on the edge of the membrane. This type of penetration by tranquilizers[30] and local anesthetics[31] into lipid and lipoportein monolayers is known to occur. The possibility of a membrane expansion was investigated by means of electron microscopy as follows.

When human erythrocytes, originally suspended in isotonic saline (154 mM NaCl, pH 7) for 5 min, were fixed with glutaraldehyde and osmium (see Methods), they appeared as biconcave disks (or sectional variations thereof) in the electron microscope (Fig. 5A). When erythrocytes, originally suspended in isotonic saline (154 mM NaCl) and then exposed to hypotonic saline (85·6 mM NaCl) for 5 min, were fixed in glutaraldehyde and osmium, the cells, although still essentially biconcave, were somewhat swollen; this is shown in Fig. 5B. When erythrocytes were exposed for 5 min to an isotonic saline solution (154 mM NaCl) containing a concentration of tranquilizer which is associated with a high degree of membrane stabilization[6] ($1{\cdot}4 \times 10^{-5}$M prochlorperazine ethane disulfonate), the cells became cup-shaped and convoluted (Fig. 5C).

It was possible to measure the circumferences of the erythrocyte profiles in the electron micrographs by applying thin (1/64th inch wide) pressure-sensitive tape (from Chart-pak Inc., Leeds, Mass.) on to the curvilinear edges of the erythrocytes in the micrographs. The tape was then peeled off the photograph, straightened gently, and the length measured on a centimeter ruler; the accuracy of this method is ± 1 mm for a length of about 100 mm, or 1 per cent reproducibility. This procedure was followed for 106 erythrocyte profiles in the series of Fig. 5A, 113 profiles in the series of Fig. 5B, and 136 profiles in the series of Fig. 5C. By allowing for magnification, these profile circumferences were converted to microns. These results are plotted in the histograms of Fig. 6.

The distribution of cell circumferences of cells suspended in 154 mM NaCl (Fig. 5A series) is represented by the dashed line in the top part of Fig. 6. Since the ranges and modes of the circumference profile distributions for cells suspended in 154 mM NaCl (Fig. 5A) and in 85·6 mM NaCl (Fig. 5B) were virtually identical, these two groups of profiles were grouped together to make a larger collective group of 219

FIG. 5. The effect of a membrane stabilizer, prochlorperazine ethane disulfonate, on human erythrocytes, as examined by electron microscopy. A. Cells suspended in 154 mM NaCl, pH 7. B. Cells suspended for 5 min in 85·5 mM NaCl, pH 7; the cells have become swollen by about 10 per cent compared to A. C. Cells suspended for 5 min in 154 mM NaCl, pH 7, containing $1{\cdot}4 \times 10^{-5}$M drug; the cells are cup-shaped and their irregular membrane circumferences have expanded by about 19 per cent on the average (see Fig. 6). The cells of A, B, and C are from the same experiment and were all fixed in glutaraldehyde and osmium. D. Cells suspended for 5 min in 67·5 mM NaCl, pH 7; these cells underwent 42 per cent hemolysis. Five erythrocyte ghosts are seen in this particular field. The inset gives an example of the membrane thickness of a collapsed normal ghost (about 54 Å between the centers of the dense leaflets). E. Cells suspended in 67·5 mM NaCl, pH 7, containing $1{\cdot}3 \times 10^{-5}$M prochlorperazine; under these conditions there was 0 per cent hemolysis and no or very few ghosts were found. The insets are two examples of the cell membrane (57 Å). The cells in D and E are from the same experiment and were fixed in osmium only. F. Higher concentrations of prochlorperazine (10^{-4}M) immediately emulsified the erythrocytes into ghosts and small vesicles; the membrane is extremely undulant and in low magnification gives an almost dotted appearance—the thickness, however, as shown in the inset, is approximately normal (57 Å). The low-magnification photographs were taken with the RCA EMU-3, the high magnifications with the Siemens Elmiskop I microscope.


5μ
A
B
C
100A
D
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F

profiles. The mode of the erythrocyte profiles in this collective group of control erythrocytes measured 18 microns.

The mode of the circumference profiles of the erythrocytes that were suspended in $1{\cdot}4 \times 10^{-5}$M prochlorperazine ethane disulfonate in 154 mM NaCl (Fig. 5C) was 21·5 microns (see Fig. 6), about 3·5 microns longer than the mode of the control cell profiles. This presumably represents an expansion of the erythrocyte membrane (19·5 per cent) associated in some way with the presence of the drug. The circumferences of the "extracellular holes" in the cells in Fig. 5C were not considered; these holes were not numerous and, if accounted for, led to even greater values for the membrane expansion.

A second feature which suggests that an expansion of the membrane must have occurred is the following. Of the 219 control erythrocyte profiles, the widest cell diameter measured was 8·3 microns. If this particular erythrocyte had been sectioned by the diamond knife precisely through the equator of the cell such that the profile was a perfect circle, the circumference of the circle would have been 27 microns. In other words, 27 microns represented the largest cell circumference profile that could have been observed in the control population of cells. Among the cells treated with prochlorperazine there were about 10 per cent, however, which had cell profiles exceeding 27 microns in length and this is indicated in Fig. 6 by the hatched region. It is important to mention that the erythrocytes were photographed at random from ten different fields of many different thin sections (more than five). Thin sections were taken from two randomly chosen zones of each Epon block, and two Epon blocks

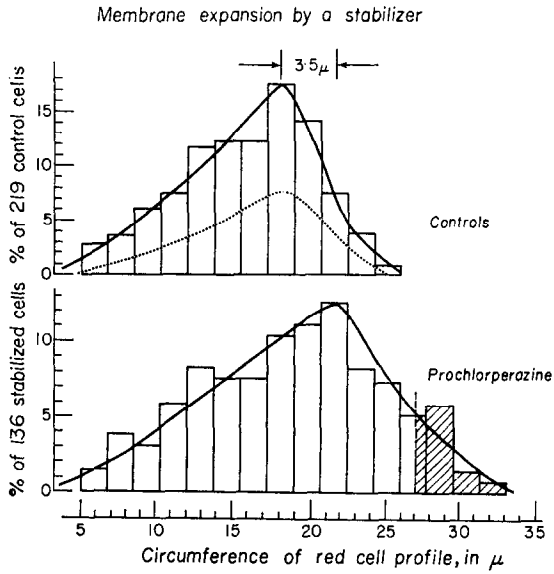

FIG. 6. The circumference profiles of the erythrocytes in Fig. 5 (A, B, and C) were measured with narrow tape, and the distribution of these circumferences is shown above. The top histograms are for the control cells in 154 mM NaCl alone (dashed line) and for both cells in 154 mM and 85·6 mM NaCl combined (solid line); the mode is about 18 μ. In the presence of $1{\cdot}4 \times 10^{-5}$M prochlorperazine (see Fig. 5C) the mode is about 3·5 μ larger. The largest circumference that might have been seen (see text) in the control population is 27 μ; there were many cells in the drug-treated sample which had larger circumferences (hatched area).

were randomly chosen from five Epon blocks prepared for each experiment. The cells, therefore, may be considered to have been randomly photographed. Compression artifact, shown to exist in methacrylate sections,[32] is small in Epon thin sections (Palade and Stoeckenius, personal communications).

Erythrocytes that had been suspended in hypotonic saline alone, and also those that had been suspended in hypotonic saline containing the phenothiazine tranquilizer prochlorperazine, were examined. When erythrocytes were suspended in 67·5 mM NaCl for 5 min, approximately half the erythrocytes hemolyzed and became ghosts; an example of this is seen in Fig. 5D—the amount of hemolysis in this particular experiment was 42 per cent. In the presence of $1{\cdot}3 \times 10^{-5}$M prochlorperazine, however, this hypotonic solution caused no hemolysis, and no or very few ghosts were observed (Fig. 5E). The extracellular dense material appearing in Figs. 5D and 5E presumably represents some residual plasma protein (since these cells are not washed before using[6]) or extracted material.[33] (The control erythrocyte membrane had a value of $53{\cdot}8 \pm 6{\cdot}9$ Å, mean ± S.D., for 171 measurements; the stabilized membrane had a value of $56{\cdot}8 \pm 4{\cdot}4$ Å for 429 measurements.)

Erythrocytes in pro-lysis and lysis. The erythrocytes shown in Fig. 5F had been exposed to 10^{-4}M prochlorperazine ethane disulfonate for 5 min before being fixed by osmium; this tranquilizer surfactant clarified the turbidity within 30 sec, and it was necessary to centrifuge at 10,000 *g* for 10 min to bring down all the erythrocyte ghosts. The membrane under these conditions is extremely undulating and twists about 90° every 1000 Å along the plane of the membrane. This twisting can be made out in the low-magnification photograph as an apparent thickening and thinning every 0·1 micron along the length of the membrane; at higher magnification, however, the membrane can be seen to have approximately the same dimensions as the normal cell membrane (see inset of Fig. 5F).

Erythrocytes have also been fixed in the pro-lytic[6] concentration region, which happens to be 3×10^{-5}M for prochlorperazine under the present experimental conditions. The cells contained very many subsurface cisterns but retained the electron-dense matrix. The width of the pro-lytic cell membrane was $57{\cdot}3 \pm 5{\cdot}1$ Å for 82 measurements.

DISCUSSION

The extracellular pH variations by themselves indicate that the B form of the drug plays a role in stabilization and in lysis. The intracellular pH variations indicate that the B form is not completely responsible for the hemolytic limb of the biphasic curve and that BH^+ does play *some* part. That the BH^+ form also plays some role in stabilization is more difficult to demonstrate, but it is clear from the lower left graphs of Fig. 2 that intracellular pH 6 is more stabilizing than intracellular pH 7 for Nupercaine; it is the reverse for the nonionic thymol drug.

Expansion of the erythrocyte membrane. The histograms of Fig. 6 indicate that prochlorperazine ethane disulfonate at a concentration of $1{\cdot}4 \times 10^{-5}$M caused an expansion of the erythrocyte membrane in isotonic solution (154 mM NaCl, pH 7). The membrane expansion amounts to around 20 per cent (3·5/18). In 1908 Traube[34] observed that amyl alcohol inhibited hypotonic hemolysis; he postulated that the compound caused a "thickening of the lipid membrane." The present results do not

support the idea of an appreciable thickening of the membrane by the phenothiazine stabilizer. Additional measurements of erythrocytes stabilized by butanol also do not reveal appreciable differences from the normal 55 Å thickness (measured between the centers of the two dense lines).

A possible explanation of the erythrocyte stabilization is an increase in the surface area/volume ratio of the cells which could be brought about by (1) an expansion of the membrane, (2) a shrinkage of the cell, or (3) both. This would be in keeping with the hypothesis of Gansslen,[35] who proposed that cells which were more spherical were osmotically more fragile, because they required a smaller amount of swelling before their spherical hemolytic size was attained. Volume changes have been very difficult to measure under the present conditions because of the small hematocrit (0·19 per cent).

It is known that erythrocyte stabilization by the phenothiazine tranquilizers is associated with around 10^8 molecules per cell.[6] The volume of these adsorbed molecules would be of the order of 10^{11} to 2×10^{11} cubic Å. The volume of the erythrocyte membrane is around 10^{12} cubic Å per cell (the membrane is 70 Å wide from edge to edge and is 160 square microns in area[2]). If these molecules occupy space *within* the membrane, therefore, they ought to expand the membrane by 10 to 20 per cent; the experimentally observed membrane expansion of 19·5 per cent (Fig. 6) might arise in this way.

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