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# EFFECTS OF CHLORPROMAZINE ON BIOLOGICAL MEMBRANES—II.

## CHLORPROMAZINE-INDUCED CHANGES IN HUMAN ERYTHROCYTES

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**Abstract**—Different techniques were employed to study the effects of chlorpromazine on the permeability of human erythrocytes to various compounds. Preliminary experiments indicated that the drug reduced the rate of hemolysis in the presence of isotonic urea or glycerol. In protecting human red blood cells against hypotonic hemolysis, chlorpromazine and trifluoperazine were found to be active at concentrations as low as $7{\cdot}5 \times 10^{-6}$ M and $10^{-6}$ M, respectively. Chlorpromazine sulfoxide, a weak tranquilizer, gave no protection even at concentrations as high as $10^{-3}$ M. These effects correlated well with the clinical potency of the compounds tested and also varied directly with the amounts of each drug adsorbed by the erythrocytes.

RECENTLY it has been shown[1, 2] that concentrations of chlorpromazine as low as $10^{-6}$ M prevented the swelling *in vitro* of subcellular membranes in rat liver and brain mitochondria presumably by effecting a change in membrane permeability in these organelles. The evidence presented included spectrophotometric[1, 2] data as well as changes in liver mitochondrial pellet water and sucrose.[2] Other instances of cell-membrane changes induced by phenothiazine drugs have been reported by various workers. Both promethazine and chlorpromazine were reported to inhibit the water uptake of frog muscle suspended in distilled water.[3, 4] Halpern *et al.*[5] observed that these compounds could reduce the occurrence of spontaneous hemolysis in stored blood. In 1959, Chaplin *et al.*[6] indicated that a phenothiazine derivative reduced the degree of hemolysis of erythrocytes exposed to hypotonic saline. Schales[7] showed that promethazine prevented the increase in osmotic fragility appearing during blood storage. Various workers[8–10] have found recently that chlorpromazine and other psychotropic drugs can prevent the uptake of $^3$H-norepinephrine by various tissues including brain. Similar results have been reported for 5-hydroxytryptamine uptake.[11] Finally, Freeman and Spirtes[12] reported in 1962 that permeability effects of phenothiazine drugs on the mammalian erythrocyte membrane can be correlated with the clinical therapeutic activity of these compounds. The present experiments describe further permeability effects of various phenothiazine derivatives at the cellular membrane level.

## METHODS

### *Measurement of the effect of chlorpromazine on erythrocyte hemolysis*

To measure the effect of chlorpromazine on the hemolysis time of human erythrocytes in urea and glycerol, the photometric method as described by Freeman and

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Spirtes[12] and originated by Jacobs[13] was employed. This method consists of observing the time for appearance of the image of a tungsten filament lamp viewed with the eye through a test tube containing a suspension of hemolyzing erythrocytes. Control values were obtained by recording the hemolysis time in 0·3 M glycerol alone and in 0·3 M urea alone. Drugs to be studied were added in various concentrations to these solutions.

*Measurement of the effect of chlorpromazine on the turbidity of erythrocyte suspensions in hypotonic saline*

Human blood was drawn and immediately placed in an Erlenmeyer flask kept in ice and containing 0·1 ml of heparin. Of this blood, 0·008 to 0·01 ml was transferred to a test tube containing 10 ml of a 0·5% NaCl solution at 25°. The final optical density was always adjusted to 0·270 to 0·280 at 500 mμ by varying the amount of blood added. The mixture contained either the desired concentration of drug to be studied or no drug, in the case of the control. Such a simple control was found to be suitable because no pH changes due to the drug took place at the concentrations tested; similarly the very small increase in osmotic pressure due to the added drug effected no measurable erythrocyte change. The tubes were inverted several times to insure proper mixing and allowed to stand for 30 min. At the end of this period they were again inverted several times, and samples of the suspensions placed in Beckman cuvets with a 1-cm light path. Optical density measurements were made in the Beckman DU spectrophotometer at 500 mμ, a minimal absorbance wave length for hemoglobin, in order to best reflect the turbidity of the cell suspension under study.

*Measurement of the effect of chlorpromazine on the release of hemoglobin from erythrocytes suspended in hypotonic saline*

The effect of various phenothiazine derivatives on the per cent hemolysis of human erythrocytes suspended in hypotonic saline was investigated by the method of Hunter.[14] One-tenth ml of blood was incubated for 30 min in the solution to be studied. The remaining cells were then centrifuged down and the oxyhemoglobin peak at 540 mμ was spectrophotometrically determined on the supernatant. This O.D. value was divided by that representing a totally hemolyzed cell suspension. The quotient was then multiplied by 100, thus yielding the percentage of hemolyzed cells. In contrast to the turbidity experiments, in which a fixed amount of saline was used and the amount of blood varied to obtain a uniform control optical density, for this study the hypotonicity of the saline was adjusted, depending on the individual sample, to obtain a control hemolysis value of about 40%, and 0·1 ml of blood added in all tests. As in the turbidimetric method described above, controls containing no drug were prepared at the same time as the drug studies. Separate experiments were performed as further controls to determine the osmotic effect of the various concentrations of drugs used.

*Measurement of drug uptake by erythrocyte suspensions in isotonic NaCl*

The uptake of various phenothiazine drugs by human erythrocytes was studied spectrophotometrically. Complete ultraviolet spectra were first obtained for chlorpromazine, trifluoperazine, and chlorpromazine sulfoxide in the Beckman DU spectrophotometer. Each drug was added to an aqueous solution of 0·9% NaCl to a final concentration of $2{\cdot}5 \times 10^{-5}$ M. Absorption spectra for each of the three compounds were plotted at 5 mμ increments from 230 to 415 mμ. Both chlorpromazine

and trifluoperazine were found to have identical curves which agreed in all details with those published by Salzman and Brodie.[15] An absorption peak at 255 mμ was chosen to measure drug uptake by these two compounds, and 275 mμ was selected to determine chlorpromazine sulfoxide uptake. Since these experiments were carried out after the addition of whole blood to 0·9% NaCl, the chlorpromazine and chlorpromazine sulfoxide curves were repeated with the drugs dissolved in a supernatant obtained from a suspension of 0·1 ml of whole blood in 10 ml of 0·9% saline. They were identical with those obtained in 0·9% saline alone. Experiments in drug uptake were performed by adding, in a test tube, 0·1 ml of whole blood to 10 ml of 0·9% NaCl containing a drug at a concentration of $2{\cdot}5 \times 10^{-5}$ M. The tube was inverted ten times, allowed to stand 15 min, centrifuged at 3000 rpm for 10 min in an International clinical centrifuge, and the supernatant removed for spectrophotometric analysis at the specific wave lengths previously mentioned. The resultant optical densities were compared with those obtained from a $2{\cdot}5 \times 10^{-5}$ M solution of drug made up in a drugless supernatant prepared as described. Per cent uptake by cells was then calculated from the difference between the two values.

Chlorpromazine hydrochloride, chlorpromazine sulfoxide hydrochloride, and trifluoperazine hydrochloride were furnished through the courtesy of Smith, Kline & French Laboratories, Philadelphia, Pa. Sodium chloride, urea, and glycerol of reagent grade were obtained commercially.

## RESULTS

Table 1 shows the results of a preliminary experiment done on human blood from one individual. It is evident that chlorpromazine has a marked effect on the time for

TABLE 1. EFFECT OF CHLORPROMAZINE ON THE HEMOLYSIS TIME OF HUMAN RED BLOOD CELLS IN UREA AND GLYCEROL

| | Molar concentration of chlorpromazine added | | | | | |
|---|---|---|---|---|---|---|
| | 0 | $1 \times 10^{-3}$ | $5 \times 10^{-4}$ | $1 \times 10^{-4}$ | $5 \times 10^{-5}$ | $1 \times 10^{-5}$ |
| 0·3 M urea | 3 | 19 | 7 | 4 | 3 | 3 |
| 0·3 M glycerol | 35 | 220 | 217 | 165 | 70 | 56 |

Each figure represents the average of two experiments done on one sample of human blood. Values indicate total hemolysis time in seconds. Control figures are those obtained when urea or glycerol alone was used.

total hemolysis of human erythrocytes in 0·3 M urea and in 0·3 M glycerol as compared with the control figure for urea and glycerol alone, since a definite retardation of this time period is noted for the urea system in the presence of $5 \times 10^{-4}$ M chlorpromazine and for the glycerol system at drug concentrations as low as $10^{-5}$ M. A control experiment in which the drug was replaced with amounts of NaCl equal in osmolarity to the drug concentrations revealed no differences in hemolysis times from those for urea or glycerol alone. In addition, no pH changes could be observed in the system upon the addition of drugs in any of the concentrations shown in Table 1.

Because these and previously reported[12] results strongly indicated that phenothiazine derivatives affected the permeability of the erythrocyte membrane in a manner similar to that found for mitochondrial membranes,[1, 2] a system was sought that

could more effectively quantitate the actions of drugs of varying tranquilizing potency. For this purpose, the drug-caused alteration of erythrocyte response to hypotonic stress was chosen. Since no osmotic or pH effects due to the drugs were observed in preliminary experiments at the concentrations employed, the control values shown in Table 2 and Fig. 1 were obtained simply with suspensions of erythrocytes in NaCl solutions in the absence of drugs.

TABLE 2. EFFECT OF CHLORPROMAZINE ON THE TURBIDITY OF A SUSPENSION OF HUMAN ERYTHROCYTES IN 0·5% NaCl

| Drug | Optical density × 1000 at 500 mμ | | |
|---|---|---|---|
| | Molar concentration of drug added | | |
| | $1 \times 10^{-4}$ | $1 \times 10^{-5}$ | $5 \times 10^{-6}$ |
| Control | | 276 ± 6 (24) | |
| Chlorpromazine | 311 ± 15 (6)<br>P < 0·001 | 311 ± 12 (6)<br>P < 0·001 | 280 ± 5 (6)<br>P > 0·1 |
| Chlorpromazine sulfoxide | 279 ± 5 (6)<br>P > 0·2 | 279 ± 6 (6)<br>P > 0·2 | |

The mean and standard deviation are presented in each case. P values were determined from Student's *t* test for small samples. The figures in parentheses indicate the number of individual experiments performed.

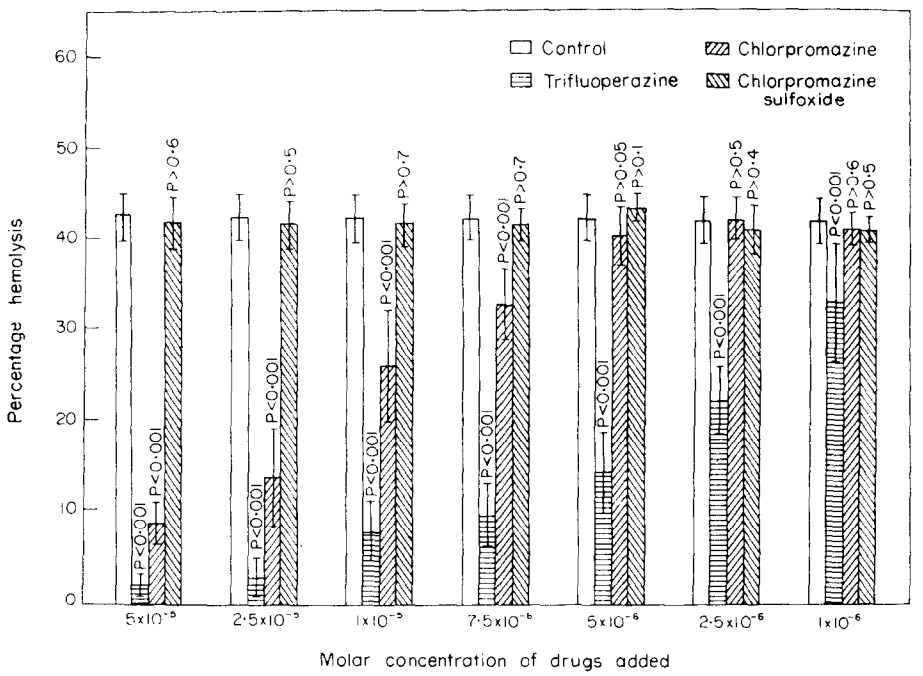


FIG. 1. Effect of some phenothiazine derivatives on the hypotonic hemolysis of human red blood cells *in vitro*. The mean and standard deviation are presented for each drug. P values were calculated from Student's *t* test for small samples. Six individual samples of blood were studied for each drug. Control figures with each drug study were pooled and one over-all control value calculated; 0·1 ml of blood was used in all experiments and the percentage saline suspension fluid varied to obtain a control hemolysis of 40%.

Table 2 shows that a suspension of erythrocytes in 0·5% NaCl has a greater turbidity in the presence of chlorpromazine than in its absence. This effect was significant at a concentration as low as $10^{-5}$ M, whereas chlorpromazine sulfoxide was inactive even at $10^{-4}$ M. The increased turbidity in the presence of chlorpromazine was interpreted to mean that fewer cells were hemolyzed under these conditions. This interpretation was supported by per cent hemolysis measurements performed along with the optical density studies. Hemolysis occurring in the control experiments without added drug averaged 15%. The addition of chlorpromazine at concentrations down to $10^{-5}$ M gave complete protection—i.e. 0% hemolysis—thus explaining why the O.D. readings at $10^{-4}$ and $10^{-5}$ M drug were identical. Obviously, the turbidimetric data are complicated by the presence of varying amounts of released hemoglobin. Although the error caused by the absorption of dissolved hemoglobin is small because readings were taken at 500 mμ, a more quantitative experiment was performed in which the actual percentage of hemolyzed cells could be determined. These studies, presented in Fig. 1, include a third phenothiazine derivative, trifluoperazine, which is more effective clinically than chlorpromazine as a tranquilizing agent. The order of clinical potency is as follows: trifluoperazine > chlorpromazine > chlorpromazine sulfoxide. Because whole blood was used in all the erythrocyte data presented, control experiments were performed in which cells washed three times in 0·9% NaCl were used. The results were identical with those in which whole blood was used.

TABLE 3. PER CENT UPTAKE OF VARIOUS PHENOTHIAZINE DRUGS BY HUMAN ERYTHROCYTES SUSPENDED IN 0·9% SALINE

| | Drug | | |
|---|---|---|---|
| | Trifluoperazine | Chlorpromazine | Chlorpromazine sulfoxide |
| Drug taken up by erythrocytes (%) | 26·2 ± 2·3 | 13·7 ± 2·0 | 1·6 ± 2·5 |

One-tenth ml of whole blood was suspended in 10 ml of 0·9% NaCl in the presence of each drug. All figures represent the mean and standard deviation obtained from six separate experiments. The per cent of drug removed from solutions was calculated from spectrophotometric readings taken on the supernatant at an optimal absorption wave length for each (255 mμ for trifluoperazine and chlorpromazine, 275 mμ for chlorpromazine sulfoxide) before and after contact with erythrocytes.

A statistically significant reduction ($P < 0·001$) of the percentage of cells hemolyzed occurred with chlorpromazine as low as $7·5 \times 10^{-6}$ M, the control hemolysis value at this level being 42% and the drug hemolysis level 33%. Trifluoperazine shows a similar effect at $10^{-6}$ M, thus exhibiting 7·5 times more activity than chlorpromazine in the system. Chlorpromazine sulfoxide was inactive *in vitro* at any of the concentrations shown in the figure and also at concentrations as high as $10^{-3}$ M which are not shown. In this series of experiments then, the minimal effective dose of drug needed for activity, as well as the actual quantitative effect noted, varied inversely with its clinical activity.

Because drug activity might be directly related to affinity for the erythrocyte, the uptake of the three compounds in 0·9% NaCl suspension fluid was measured in the presence of $2{\cdot}5 \times 10^{-5}$ M drug.

Approximately twice as much trifluoperazine is taken up by the cells as chlorpromazine at the concentration studied, and very limited absorption of the sulfoxide takes place in the same 15-min period. Additional experiments indicated that the values presented in Table 3 were essentially unchanged if the drugs were incubated with the cells for longer periods (up to 30 min). Thus, as with the erythrocyte-protection studies listed in Fig. 1, the drug uptake figures correlate well with the order of clinical potency for the phenothiazines.

## DISCUSSION

Davson[16] has classified substances affecting erythrocytes according to their behavior in aqueous solution: Class I contains those substances that are nonhemolytic, and Class II includes those substances that, at any concentration, cause hemolysis. NaCl is given as an example of a Class I compound and urea or glycerol of Class II.

The possibilities have been tested that the phenothiazine drugs reduce the permeability of the erythrocyte membrane to both water and to two Class II hemolytic compounds. That phenothiazine derivatives may inhibit the water permeability of a cell membrane is supported by the evidence presented here that chlorpromazine protects human erythrocytes against hypotonic hemolysis. The efforts of other authors lending support to this hypothesis have already been mentioned.[4, 6, 7, 12] In a previous paper Freeman and Spirtes[12] also demonstrated that chlorpromazine can slow the hemolysis time of human erythrocytes in water. Preliminary evidence now presented proves that this drug also slows the hemolysis time of human erythrocytes in the presence of glycerol or urea (i.e. Class II compounds).

A problem of interpretation arises when a likely mechanism for these permeability changes is sought. One obvious conclusion is that the drug can interact directly with the membrane to cause a specific chemical change resulting in a decrease of permeability to water and certain organic compounds. However, it is also possible that the change in membrane permeability to the same compound is secondary to one of the many effects of the phenothiazines on the mammalian organism. For instance, acute $K^+$ loss alone with a gain in $Na^+$ has been shown to occur in erythrocytes exposed to hypotonic saline solutions.[17]

Data which will be discussed in detail in a future publication indicate that the inhibitory effect of the drug upon water entrance into the erythrocyte cannot be caused by an increased outward movement of $K^+$.

In the past, much evidence has been accumulated indicating that the osmotic passage of water through the erythrocyte membrane is a passive process not requiring metabolic energy.[18] The work presented in this paper seems to favor the premise that chlorpromazine affects the passive movement of water into the red cell via a direct membrane-drug interaction resulting in a specific water permeability change.

Effects of chlorpromazine on erythrocyte oxidative metabolism have been demonstrated[19] so that drug-induced permeability changes secondary to metabolic alterations still cannot be entirely excluded. However, drug concentrations inducing the changes presented in Fig. 1 under aerobic conditions are far below the level reported necessary to affect red cell oxidative metabolism (above $1 \times 10^{-4}$ M). In addition,

preliminary experiments now under way in our laboratory indicate that neither anaerobiosis nor low temperatures affect the ability of chlorpromazine to protect erythrocytes against hypotonic stress.

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