REFERENCES

1. D. X. FREEDMAN, J. D. BARCHAS and R. L. SCHOENBRUN, *Fed. Proc.* **21**, 337 (1962).
2. J. MEAD and K. FINGER, *Biochem. Pharmacol.* **6**, 52 (1961).
3. D. BOGDANSKI, A. PLETSCHER, B. BRODIE and S. UDENFRIEND, *J. Pharmacol. exp. Ther.* **117**, 82 (1956).
4. J. CROUT, C. CREVELING and S. UDENFRIEND, *J. Pharmacol. exp. Ther.* **132**, 269 (1961).
5. A. AMIN, T. CRAWFORD and J. GADDUM, *J. Physiol. (Lond.)* **126**, 596 (1954).
6. D. X. FREEDMAN, *J. Pharmacol. exp. Ther.* **134**, 160 (1961).
7. D. X. FREEDMAN and N. J. GIARMAN, *Ann. N.Y. Acad. Sci.* **96**, 98 (Jane. 1962).
8. D. X. FREEDMAN, *Amer. J. Psychiat.* **119**, 843 (1963).
9. D. X. FREEDMAN, *Proc. Third World Cong. of Psychiat.* **1**, 653 (1961).
10. E. W. MAYNERT and G. I. KLINGMAN, *J. Pharmacol. exp. Ther.* **135**, 285 (1962).
11. R. LEVI and E W. MAYNERT, *Fed. Proc.* **21**, 336 (1962).
12. J. C. TOWNE and J. O. SHERMAN, *Proc. Soc. exp. Biol. (N.Y.)* **103**, 721 (1960).
13. J. LEDUC, *Acta physiol. scand.* **53**. 6, Supp. 183 (1961).
14. P. GORDON, *Nature (Lond.)* **191**, 183 (1961)
15. A. GILGEN, R. P. MAICKEL, O. NIKODIJEVIC and B B BRODIE, *Life Sci.* no. 12, 709 (1962).
16. D. X. FREEDMAN, G. K. AGHAJANIAN, E. M. ORNITZ and B. S. ROSNER, *Science* **127**, 1173 (1958).

Further effects of chlorpromazine on the human erythrocyte membrane

(*Received* 3 *March* 1963; *accepted* 28 *May* 1963)

IN PREVIOUS publications[1–3] the authors have demonstrated the ability of phenothiazine derivatives to affect the osmotic permeability of erythrocytes. Further, the alterations in permeability described were obtained with low drug concentrations and correlated well with the clinical potency of these compounds as tranquilizers. The evidence now presented is intended to implicate passive osmotic permeability in the mechanism of action for the phenothiazines.

Erythrocytes were exposed to hypotonic saline, and after centrifugation the supernatants were removed and analyzed for the percentage of hemolyzed cells as previously described.[3] The remaining erythrocytes were analyzed for intracellular ions by a modification of the method of Post and Jolly[4] in the following manner. Cells were suspended in 110 mM magnesium chloride plus sufficient magnesium hydroxide to bring the pH of the wash fluid to 7·4, and then centrifuged at 3000 rev/min in the International clinical centrifuge for 10 min. After discarding the supernatant, the cells were resuspended in fresh magnesium wash fluid and again spun down. The same procedure was repeated a third time. After the final supernatant was decanted, a hemolytic solution consisting of 0·2% (v/v) concentrated ammonium hydroxide reagent plus 0·02% (v/v) Triton X-100 was added to the cells to make a final volume of 10 ml in a volumetric flask. A portion of the hemolysate was analyzed for sodium and potassium with the Baird atomic flame photometer, model KY.[5] A separate portion of hemolysate was added to an equal amount of cyanmethemoglobin reagent (Ortho Pharmaceutical Co., Raritan, N.J.). The concentration of cyanmethemoglobin was then determined by recording the optical density at 540 mμ with the Beckman DU spectrophotometer. The hemoglobin concentration was then read from a standard curve constructed by comparing readings of the unknown with those of known cyanmethemoglobin standards (Ortho). Results were expressed as mEq ion/5 mmoles hemoglobin. This method of expression was chosen because changes in erythrocyte volume which may take place during the experiments do not introduce error into the determinations. Similarly, a correction for interstitial fluid is not required, and the figures obtained are comparable to those presented by the usual method of expression—i.e. milliequivalents of ion per liter of cells. It should

be pointed out that the experiments of Parpart have proven that partial hypotonic hemolysis is an all-or-none process.[6] As a result, the remaining unhemolyzed erythrocytes have the same concentration of hemoglobin as cells suspended in isotonic saline. In similar experiments the authors have established that this is also true for cells suspended in hypotonic saline in the presence of chlorpromazine.

The described analyses were performed in the presence and absence of $2{\cdot}5 \times 10^{-5}$ M chlorpromazine, on erythrocytes suspended in isotonic NaCl solution and in a hypotonic sodium chloride solution resulting in 30 per cent hemolysis. Very little potassium was lost from the cells during the washing procedure described above. A flame photometric analysis performed on these washings indicated that they contained less than 2 per cent of the total intracellular potassium.

TABLE 1. EFFECT OF CHLORPROMAZINE ON THE SODIUM AND POTASSIUM CONTENT OF HUMAN ERYTHROCYTES EXPOSED TO HYPOTONIC STRESS*

	Normal cells	Cells in hypotonic saline	Cells in hypotonic saline with $2{\cdot}5 \times 10^{-5}$ M chlorpromazine
Per cent hemolysis	0	$30{\cdot}0 \pm 6{\cdot}6$	$11{\cdot}7 \pm 4{\cdot}7$
Na^+ mEq per 5 mmoles Hb	11 ± 1 $P < 0{\cdot}001$	41 ± 12	24 ± 6 $P < 0{\cdot}02$
K^+ mEq per 5 mmoles Hb	127 ± 7 $P < 0{\cdot}001$	93 ± 11	109 ± 8 $P < 0{\cdot}02$

* All figures indicate the mean and standard deviation obtained from six separate experiments. Values for Na^+ and K^+ represent the intracellular ionic concentrations of normal cells and of cells remaining after exposure to partially hemolytic saline solutions with and without the presence of $2{\cdot}5 \times 10^{-5}$ M chlorpromazine.

The results of these studies are summarized in Table 1; $2{\cdot}5 \times 10^{-5}$ M chlorpromazine brought the 30 per cent control hemolysis down to 12 per cent, indicating that water movements across the erythrocyte membrane had been affected. These data confirm evidence previously presented.[3] More important is the fact that, compared to normal cells, the erythrocytes remaining after the hypotonic treatment gained in sodium content and lost in potassium content, the ratio of gain to loss being 1:1. Previous workers have established that the exchange of ions along their concentration gradient occurs via passive diffusion.[7] Chlorpromazine inhibited this passive ionic leakage in both directions without disturbing the ratio of the ion exchange. If the cations in each vertical column are added, it will be seen that approximately the same total is found in the cells under all conditions even though the over-all movement of Na^+ and K^+ is inhibited by chlorpromazine. The effect of the drug on these cations cannot, therefore, account for its ability to influence water movement across the cell membrane.

Although the authors feel that the action of chlorpromazine on ion movement is purely a passive phenomenon, an alternative explanation for the results might be a possible activation or protection of the existing active transport mechanisms in the erythrocyte membrane. Greig and Gibbons[8] found, however, that concentrations as high as 5×10^{-4} M chlorpromazine were necessary to influence active transport in red cells and, further, that this effect was one of inhibition rather than protection of the transport mechanisms.

It is therefore likely, because of previously presented[1-3] and present evidence, that chlorpromazine inhibits the passive movement of water and ions in erythrocytes exposed to hypotonic stress. In

addition, the ability of the drug to alter water movement appears not to be related to its action on the passive leakage of ions.

Department of Pharmacology,
Hahnemann Medical College,
Philadelphia, U.S.A.

A. R. FREEMAN
M. A. SPIRTES

REFERENCES

1. A. R. FREEMAN and M. A. SPIRTES, *Biol. Bull*, **123**, 495 (1962).
2. A. R. FREEMAN and M. A. SPIRTES, *Biochem Pharmacol.* **11**, 161 (1962).
3. A. R. FREEMAN and M. A. SPIRTES, *Biochem. Pharmacol.* **12**, 47 (1963).
4. R. L. POST and P. C. JOLLY, *Biochim. biophys. Acta* **25**, 118 (1957).
5. A. R. FREEMAN and M. A. SPIRTES, *Clin. chim. Acta* In press.
6. A. K. PARPART, *Biol. Bull.* **61**, 500 (1931).
7. E. PONDER: *Red Cell Structure and Its Breakdown*, p. 60. Springer, Vienna (1955).
8. M. E. GREIG and A. J. GIBBONS, *Science* **123**, 939 (1956).

A note on the purification procedure used to isolate canine liver lipase for kinetic studies with BCME*

(*Received* 6 *June* 1963; *accepted* 11 *June* 1963)

IT HAS been previously reported (1) that the kinetics of tributyrin hydrolysis observed with crude aqueous extracts of acetone powders of dog liver were different from the kinetics observed with a partially purified preparation of the same enzyme. This difference was due to a binding phenomenon between tributyrin and an unknown component in the crude extract which rendered a constant concentration of tributyrin unavailable to the enzyme. Upon partial purification this component was removed, thereby resulting in a change in kinetics. The purification procedure used to partially purify the canine liver lipase for these studies follows.

METHODS

Enzyme assays using the tributyrin "clearing" method have been previously described;[1] in this an enzyme unit is defined as that amount of enzyme inducing an optical density change of 0·001/min under the conditions of assay. Protein concentrations were measured with the following micro biuret method of Dr. V. W. Rodwell. To a 2-ml sample containing 0·2 to 2·0 mg of protein is added 1 ml of biuret reagent. The contents of the tube are mixed, and readings are made in a Beckman DU spectrophotometer set at 540 Mμ against a reagent blank. Results are expressed in terms of a standard of crystalline bovine serum albumin (Armour).

Step 1, *extraction.* Five grams of dog liver actone powder prepared as described[1] were extracted with 50 ml of cold water for 10 min in ice. The mixture was then centrifuged and the supernatant fluid was decanted and saved. The residue was re-extracted with 25 ml of cold water as before, followed by centrifugation. The second extract was combined with the first. This is designated the "crude extract" in Table 1.

Step 2, *lead precipitation.* (Note that all volumes of reagents added refer to the volume of enzyme solution at the beginning of the step.) To the crude extract add 0·02 volume of 1 M $KHCO_3$. Then add 0·15 volume of 0·1 M lead acetate solution by drops with thorough mixing. The precipitate that forms is centrifuged down and discarded. To the supernatant fluid is added 0·06 volume of 0·25 M sodium ethylene diamine tetraacetic acid. This is called "lead fraction I."

Step 3, *first ammonium sulfate precipitation.* To EPb_1 add 0·56 volume of cold saturated ammonium sulfate solution. Centrifuge and discard precipitate. To the supernatant fluid add the same volume of ammonium sulfate solution. The precipitate is collected by centrifugation and made up to 0·5 volume in water. This fraction is designated "ammonium sulfate fraction I."

* Butyl carbamic acid methyl ester.