Br. J. Pharmac. (1983), **79**, 481–487

Effects of modification of the membranes of intact erythrocytes on the anti-haemolytic action of chlorpromazine

G.V.R. Born & G.M. Housley

Department of Pharmacology, University of London King's College, Strand, London WC2R 2LS

1 Fresh human erythrocytes were washed and incubated at 37°C in physiological saline buffered with Tris, containing calcium and either neuraminidase or trypsin or both enzymes together.

2 Each enzyme alone, as well as both together, released similar amounts of sialic acids without haemolysis, apparently therefore from the same accessible sites on the surface of the cells.

3 Exposure to neuraminidase or/and trypsin did not affect the osmotic fragility of red cells in hypotonic saline.

4 The anti-haemolytic effect of low concentrations of chlorpromazine or thioridazine was diminished by previous exposure of red cells to neuraminidase and/or trypsin. The diminution in the anti-haemolytic effect of chlorpromazine was proportional to the release of sialic acids.

5 The observations suggest that positively charged drugs such as chlorpromazine interact strongly with the negatively charged sialic acid moieties of glycoproteins on cell surfaces, removal of which increases the drugs' access to and disruption of the lipid bilayer in cell membranes.

Introduction

The mechanism(s) by which chlorpromazine and related phenothiazines act as tranquillizers is still unknown (see Hoffmeister & Stille, 1980). One effect of these drugs at low concentrations is a decrease and at high concentrations an increase of the lysis of red blood cells when they are exposed to excessive osmotic or mechanical forces (see Seeman, 1972). The 'stabilizing effect' of low concentrations is also produced on the plasma membranes of other cells as well as on the membranes of intracellular organelles such as mitochondria. Similar effects are produced by a variety of other agents which are not tranquillizers (Seeman, 1972), although mostly at much higher concentrations. In view of this non-specificity, the relationship of the membrane-stabilizing to the tranquillizing effect of the phenothiazines, if any, remains uncertain.

Some time ago (Born, Bergquist & Arfors, 1976; Born & Wehmeier, 1979) it was shown that low concentrations of chlorpromazine inhibit haemostatic platelet aggregation in artificial blood vessels. When fine polyethylene tubes were perfused with heparinized blood at 37°C and punctured, the openings were sealed off within about 2 min by haemostatic plugs of platelets, just as in living blood vessels (Didisheim, Pavlovsky & Kobayashi, 1972). This 'artificial bleeding time' was prolonged when the blood contained chlorpromazine in low concentrations which had no effect on *in vitro* platelet aggregation but which increased the resistance of red blood cells to hypotonic haemolysis. This suggested the possibility that the activation of platelets was inhibited by an action of chlorpromazine on the accompanying red cells. Further experimental analysis has indicated that a direct action on the platelets cannot yet be excluded.

These observations concentrated our interest on the mechanism(s) by which chlorpromazine and similar agents produce their 'stabilizing' effects on biological membranes. The explanation of these effects has remained uncertain. Their attribution to the concentration of the drugs in the lipid bilayer of cell membranes (Burgen & Metcalfe, 1970) has been challenged by observations indicating that there is no such concentration and that the drugs act through micelle formation with one or more components of the cells on their outer surface (Conrad & Singer, 1981).

This paper demonstrates that modification of the intact erythrocyte membrane by certain enzymes changes the antihaemolytic action of chlorpromazine and a related agent. The enzymes used were

neuraminidase which specifically removes terminal sialic acid residues from membrane glycoprotein and trypsin which removes these sialic acids together with some peptide fragments (Cook, Heard & Seaman, 1961).

Some of the results have been communicated to the Physiological Society (Born & Housley, 1981).

Methods

Preparation of red cells

Blood freshly removed from antecubital veins of apparently healthy volunteers was mixed with 0.1 vol. of 3.2% (w/v) tri-sodium citrate as anti-coagulant and centrifuged at 1200 *g* for 5 min. The supernatant plasma and buffy coat were removed and the packed red cells were washed three times in five times their volume of 0.154 M NaCl solution. The washed cells were resuspended at a concentration of 20% v/v in saline buffered with 10 mM Tris at pH 7.4, containing 5 mM calcium and either 0.2 units neuraminidase (*Clostridium perfringens* from Sigma, type V) or 0.5 mg trypsin (bovine, from Worthington, 3 times recrystallized) per ml packed cells or both enzymes together. These suspensions were incubated at 37°C for 1 h with intermittent gentle shaking and then centrifuged at 1200 *g* for 5 min. In samples of the supernatant, total sialic acids were determined fluorimetrically (Hess & Rolde, 1964) and values corrected for non-specific release (0.015 ± 0.007 μmol/ml packed cells).

Measurement of osmotic fragility

The red cells were washed three more times and suspended at a concentration of 0.55% v/v in 10 mM Tris-buffered saline at pH 7.0. After 15 min at room temperature, samples of the suspension were diluted with 10 mM Tris buffer containing from 0–0.154 M NaCl. After 15 min the samples were centrifuged at 550 *g* for 5 min and the optical densities of the supernatants were determined in a spectrophotometer at 543 nm (Pye Unicam SP6-550) to quantify free haemoglobin. Plots of optical density versus NaCl concentrations provided osmotic fragility curves. From these, the concentration of NaCl was determined at which there was 50% haemolysis. In this concentration of saline, buffered with Tris, the effects of drugs on the osmotic fragility of erythrocytes was determined. Samples of red cells not exposed, i.e. control, and those exposed to enzymes were resuspended in isotonic Tris-buffered saline

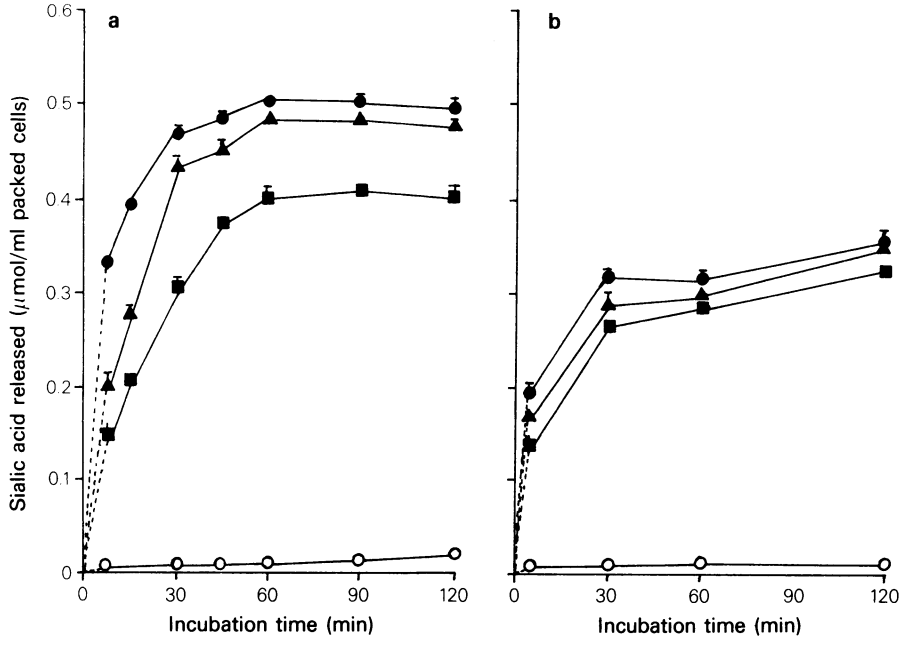

Figure 1 Release of sialic acids from human red cells; for conditions see text. (a) Neuraminidase at concentrations increasing from zero (○) to 0.1 (■), 0.2 (▲) or 0.4 (●) units/ml packed cells and (b) trypsin at concentrations increasing from zero (○) to 0.1 (■), 0.5 (▲) or 1.0 (●) mg/ml packed cells. Each point is the mean of three determinations using the red cells from one individual; vertical bars show the range.

containing different concentrations of chlorpromazine from 1–100 μM. After 15 min at room temperature, samples of each suspension were added to that concentration of buffered saline (0.057 ± 0.0004 M, $n = 10$) which had produced 50% haemolysis in a series of test tubes containing chlorpromazine at the same concentration as before.

The effect of thioridazine was determined in the same way.

Results

Time courses of release of sialic acids

Sigma neuraminidase and Worthington trypsin were assessed in order to determine the amount of sialic acids removed from the surface of red cells with the least damage as indicated by haemolysis.

The rate of release of sialic acids was greatest for neuraminidase (Figure 1a). After incubation for 2 h a little more sialic acid was released, with slight haemolysis. In all subsequent experiments, neuraminidase was used at a concentration of 0.2 units/ml packed cells.

With trypsin, the rate of release of sialic acids was similar to neuraminidase although the amount removed was slightly less. Experiments in which the supernatant from trypsinized cells were analysed by h.p.l.c. showed that the optimal concentration for the release of surface glycopeptides was between 0.1 and 0.5 mg/ml packed cells; higher concentrations caused variable haemolysis. Trypsin at 0.5 mg/ml released all accessible sialic acids without haemolysis and was the concentration used in all further experiments (Figure 1b).

The amounts of sialic acid released by neuraminidase or by trypsin were similar: 0.436 ± 0.02 and 0.362 ± 0.03 μmol/ml packed cells respectively. The two enzymes added together released only a little more than neuraminidase alone (0.5 ± 0.03 μmol/ml packed cells), indicating that the sialic acids were released by both enzymes from almost the same sites. On the assumption (Cook *et al.*, 1961) that sialic acids accessible to either of these enzymes are on the outer surface of the red cell membrane, neuraminidase alone removed about 85%, trypsin alone about 71% and neuraminidase plus trypsin about 98% accessible sialic acids.

Effect of enzymes on the osmotic fragility of red cells

When human erythrocytes had been exposed to either or both of these enzymes with the consequent release of outer sialic acids and/or glycopeptides, the osmotic fragility of the cells was unaffected; the osmotic fragility curves were the same at and around 50% haemolysis (Figure 2).

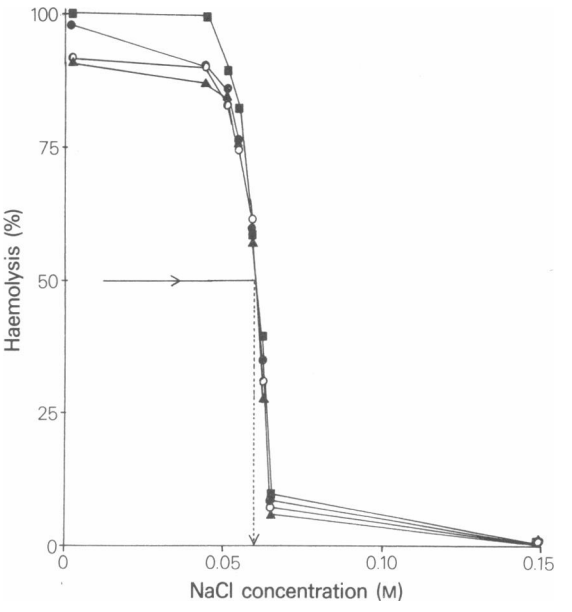

Figure 2 Osmotic fragility of human red cells. Effect of NaCl concentration (M) on the haemolysis of control cells (○) or of cells exposed to neuraminidase (●), trypsin (■), or neuraminidase plus trypsin (▲). The arrow indicates the concentration of NaCl which would produce 50% haemolysis.

Effects of enzymes on the antihaemolytic action of phenothiazines

When control erythrocytes were resuspended in hypotonic salines containing chlorpromazine, concentrations of the drug ranging from about 1 to 30 μM diminished haemolysis progressively; at higher concentrations this effect was reversed and with more than 100 μM haemolysis increased greatly, in agreement with earlier observations (Seeman, 1972). After exposure of erythrocytes to neuraminidase the antihaemolytic effect of chlorpromazine was decreased; more after exposure to trypsin; and most after exposure to neuraminidase plus trypsin (Figure 3a). After cells were exposed to either trypsin alone or trypsin plus neuraminidase haemolysis began to increase at much lower concentrations viz. around 10 μM, chlorpromazine.

Chlorpromazine was found to be maximally antihaemolytic at a concentration of 28 ± 4 μM ($n = 5$) where the corresponding percentage haemolysis was 20.8 ± 8.3.

The action of the enzymes in reversing the effect of chlorpromazine at its maximally antihaemolytic con-

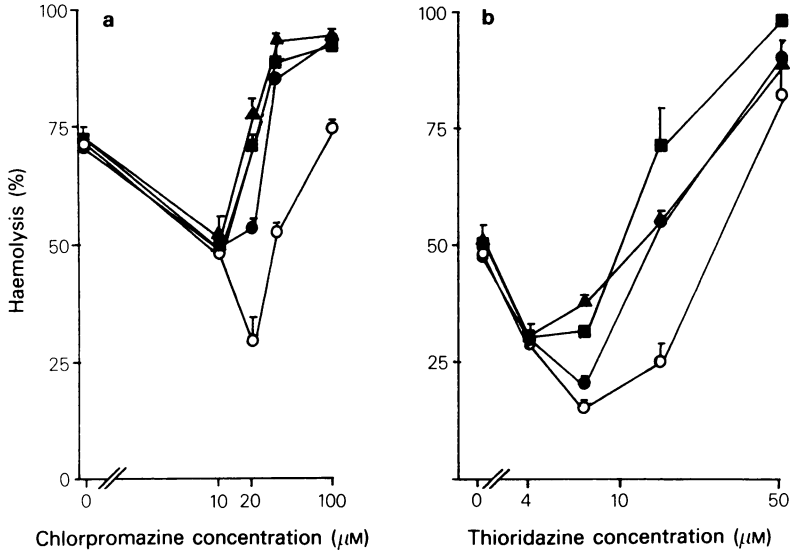

Figure 3 Antihaemolytic effect of chlorpromazine (a) or thioridazine (b) on control red cells (○) and on cells exposed to neuraminidase (●), trypsin (■) or neuraminidase plus trypsin (▲). Each point is the mean of three determinations of haemolysis on red cells from one individual; vertical bars show the range. Red cells were exposed to neuraminidase and trypsin at concentrations of 0.2 units and/or 0.5 mg/ml packed cells, respectively.

centration was expressed as the ratio of haemolysis in the absence of enzyme action to haemolysis after enzyme action. Thus, neuraminidase decreased the anti-haemolytic effect of chlorpromazine about 2.3 times, trypsin about 2.8 times and neuraminidase plus trypsin about 3.1 times.

The effect of thioridazine on control cells and on those which had been exposed to the enzymes was similar to that of chlorpromazine except that thioridazine was maximally antihaemolytic at lower concentrations, i.e. around 7 μM ($n = 3$). Figure 3b shows a typical result using the red cells from one

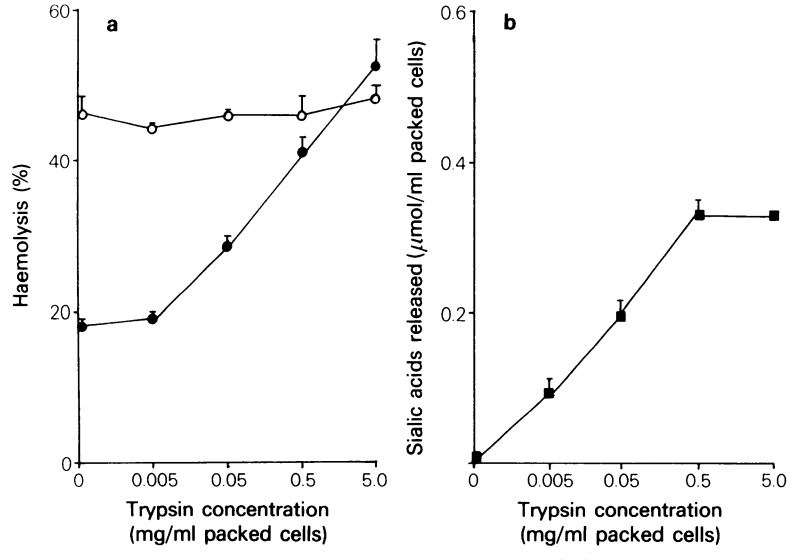

Figure 4 Effect of increasing concentrations of trypsin on (a) haemolysis (%) in the absence (○) and presence (●) of 20 μM chlorpromazine; and (b) release of sialic acids (μmol/ml packed cells). Each point is the mean of three determinations using red cells from one individual; vertical bars show the range.

individual. The increase in haemolysis after exposure to the enzymes was not as great as with chlorpromazine.

Effect of trypsin concentration on the anti-haemolytic action of chlorpromazine

Red cells were incubated as described with trypsin at concentrations from 5 μg to 5 mg/ml packed cells. The antihaemolytic action of chlorpromazine decreased with increasing enzyme concentration up to the maximum tested (5 mg/ml) although the effect was near maximal with one-tenth of that concentration (Figure 4a). The amount of sialic acid released was proportional to the enzyme concentration (Figure 4b) and therefore to the decrease in anti-haemolytic action of chlorpromazine.

Discussion

These results show that the anti-haemolytic effect of chlorpromazine was decreased and its haemolytic effect made manifest at lower concentrations after erythrocytes had been exposed to neuraminidase, to trypsin, or to both enzymes together. Trypsin re-

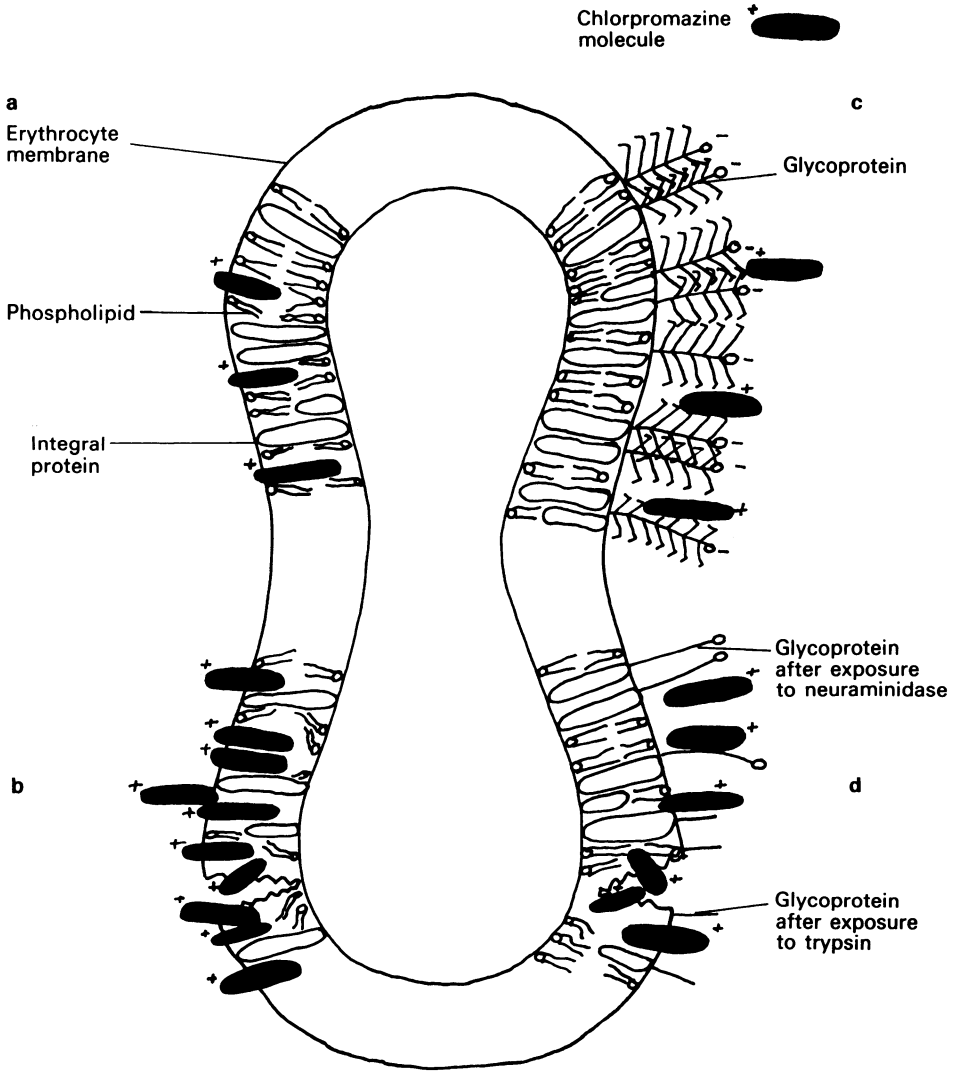

Figure 5 The effects of neuraminidase and trypsin on proposed actions of chlorpromazine on the red cell membrane. (For description see text.)

moved a little less sialic acid than neuraminidase but trypsin with neuraminidase together removed a little more, indicating that most of the sialic acids were released by both enzymes from the same sites, presumably accessible glycoproteins on the outer surface of the cell membranes.

At physiological pH, chlorpromazine is a monovalent cation, and so is thioridazine. Glycoprotein-bound sialic acids are monovalent anions which account for most of the net negative charge excess of erythrocytes as of other cells. It is tempting, therefore, to involve electrostatic interactions between the drug and the cells in explaining our observations. Any explanation has to take account also of the current controversy over the mechanism of action of chlorpromazine on biological membranes, including those of red cells, which has arisen from different conclusions about the distribution of chlorpromazine between cell membranes and medium. From earlier evidence (Seeman, 1972; Burgen & Metcalfe, 1970) it appeared that chlorpromazine became concentrated in the lipid bilayer of membranes, as might be expected of a lipophilic compound, thereby causing expansion and increased fluidity of the membrane. More recently a technique referred to as hygroscopic desorption (Conrad & Singer, 1979) failed to confirm any significant concentration of chlorpromazine in biological membranes which was, however, demonstrable in artificial membranes consisting of pure phospholipids. It was suggested that the action of chlorpromazine involves formation of mixed micelles with components, possibly peptides, on the outer surface of cell membranes. There seems to be no other evidence for this yet.

The two mechanisms may now be considered in conjunction with our observations (Figure 5). If chlorpromazine is concentrated in the lipid bilayer (left side of the figure), the anti-haemolytic or 'stabilizing' effect of low concentrations could be explained by assuming hydrogen bonding between drug molecules and fatty acid chains of the phospholipids (Figure 5a); and the haemolytic or disruptive effect of high concentrations by an excessive accumulation between those fatty acids (Figure 5b).

On the alternative hypothesis that the stabilizing effect depends on interaction(s) outside the lipid bilayer, a primary interaction would presumably be between the cationic drug and the anionic sialoprotein (Figure 5c). Thereby the drug molecules could be positioned for maximizing secondary attraction e.g. by hydrogen bonding with the glycopeptide moieties of the external proteins. By removing the sialic acids, whether by neuraminidase or by trypsin (Figure 5d) this primary interaction is abolished so that even at lower concentrations chlorpromazine penetrates the lipid bilayer sufficiently to cause disorganization underlying haemolysis. This hypothesis would account also for observations (Harris & Saifer, 1962) on ionic bonding between positively charged phenothiazines and negatively charged carboxyl groups of N-acetyl neuraminic acid and on hydrogen bonding between phenothiazines and hydrophobic components of cell membranes, calculated from free energies of absorption (Kwant & Seeman, 1969).

Our results leave open another possibility, namely that glycoproteins external to the lipid bilayer present a barrier to its penetration by chlorpromazine and compounds of similar structure; the few molecules which do enter the membrane sufficing to stabilize the bilayer by the postulated secondary interactions. This would be in accord with the suggestion (Conrad & Singer, 1981) that if an amount of chlorpromazine undetectable by hygroscopic desorption was dissolved in the membrane interior, the consequences could be significant, particularly if the drug were concentrated in the boundary regions where the lipid acyl chains come up against the hydrophobic surfaces of the integral proteins (Bieri & Wallach, 1975). Higher concentrations of chlorpromazine would disrupt the membrane by its detergent actions on both glycoproteins and lipids.

We thank the Wellcome Foundation Limited and the Vandervell Foundation for Studentships for G.M.H.; the British Heart Foundation and the Fritz Thyssen Foundation of Cologne for generous support; and Professor H.R. Morris and Dr A.T. Etienne of the Department of Biochemistry, Imperial College, London, for glycopeptide analysis.

Chlorpromazine hydrochloride was kindly supplied by May and Baker Ltd, and thioridazine hydrochloride by Sandoz Products Ltd.

References

BIERI, V.G. & WALLACH, D.F.H. (1975). Variations of lipid-protein interactions in erythrocyte ghosts as a function of temperature and pH in physiological and non-physiological ranges. A study using paramagnetic quenching of protein fluorescence by nitroxide lipid analogues. *Biochim. biophys. Acta*, **406**, 415–423.

BORN, G.V.R., BERGQUIST, D. & ARFORS, K.E. (1976). Evidence for inhibition of platelet activation in blood by a drug effect on erythrocytes. *Nature*, **259**, 233–235.

BORN, G.V.R. & HOUSLEY, G.M. (1981). Decrease in the antihaemolytic effect of chlorpromazine by the removal of sialic acids from the surface of erythrocytes. *J. Physiol.*, **325**, 40–41P.

BORN, G.V.R. & WEHMEIER, A. (1979). Inhibition of platelet thrombus formation by chlorpromazine acting to diminish haemodynamically induced haemolysis. *Nature*, **282**, 212–213.

BURGEN, A.S.V. & METCALFE, J.C. (1970). The application

of nuclear magnetic resonance to pharmacological problems. *J. Pharm. Pharmac.*, **22,** 153–169.

CONRAD, M.J. & SINGER, S.J. (1979). Evidence for a large internal pressure in biological membranes. *Proc. natn. Acad. Sci., U.S.A.*, **76,** 5202–5206.

CONRAD, M.J. & SINGER, S.J. (1981). The solubility of amphipathic molecules in biological membranes and lipid bilayers and its implications for membrane structure. *Biochemistry*, **20,** 808–818.

COOK, G.M.W., HEARD, D.H. & SEAMAN, G.V.F. (1961). Sialic acids and the electrokinetic charge of the human erythrocyte. *Nature*, **191,** 44–47.

DIDISHEIM, P., PAVLOVSKY, M. & KOBAYASHI, I. (1972). Factors affecting haemostatic plug formation in an extra-corporeal model. *Ann. N.Y. Acad. Sci.*, **201,** 307–315.

HARRIS, A.F. & SAIFER, A. (1962). In *Cerebral Sphingolipidoses*. ed. Aronson, S.M. & Volk, B.W. New York: Academic Press.

HESS, H.H. & ROLDE, E. (1964). Fluorimetric assay of sialic acid in brain gangliosides. *J. biol. Chem.*, **239,** 3215–3220.

HOFFMEISTER, F. & STILLE, G. (Eds) (1980). In *Psychotropic Agents*, Part 1. Handb. Exp. Pharm. 55/1. Berlin: Springer-Verlag.

KWANT, W.O. & SEEMAN, P. (1969). The membrane concentration of a local anaesthetic (chlorpromazine). *Biochim. biophys. Acta*, **183,** 530–543.

SEEMAN, P. (1972). The membrane actions of anaesthetics and tranquillizers. *Pharmac. Rev.*, **24,** 583–655.

(Received November 10, 1982.
Revised December 16, 1982.)