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Binding of phenothiazines to proteins—Measurement of binding based on the inhibition of the hemolytic activity of phenothiazines on sheep red blood cells

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THERE HAVE been several reports that phenothiazine tranquilizers are bound to blood proteins. Salzman and Brodie[1] found that chlorpromazine in dogs was highly bound to certain organs, such as brain, spleen and kidney, whereas a relatively low concentration of this drug was found in plasma, where most of the chlorpromazine was bound to plasma proteins. Mellinger *et al.*[2] reported that in serum of patients receiving oral forms of thioridazine, an accumulation of the drug occurred within 3–4 days, followed by a well maintained blood level. During our studies *in vitro* on protection of blood cells from lysis by hemolytic phenothiazines,[3] it was found that the extent of hemolysis of blood cells was remarkably reduced in the presence of serum. From these observations, it appeared that there was an affinity for binding between phenothiazines and serum proteins, which occurred both in a highly concentrated system, such as blood plasma, and in a less concentrated system, such as dilute aqueous solutions of serum and serum fractions.

An indication of the importance of the interaction of phenothiazines with blood proteins has been obtained by measuring the activity of normally occurring proteins in the blood after the administration of these drugs. It has been found, for example, that some of the phenothiazine derivatives inhibit the cholinesterase activities of normal human serum[4] and the activities of blood phosphatase and cholinesterase[5] in studies *in vivo*, and also inhibit the hemolytic activity of serum complement in studies *in vitro*.[6]

This report presents a simple and rapid method for testing the patterns of binding of phenothiazines by serum and serum fractions. The method is useful in determining which plasma proteins are involved in the interaction and possibly to relate these results to previous reports about the inactivation of enzymes[4,5] and complement[6] by structurally related phenothiazines.

Materials. Sera were prepared by processing blood freshly drawn from healthy human subjects and animals, and were stored at −55° until use. Serum proteins and fractions were obtained from Mann Research Laboratories. The solutions of serum fraction used were made by weight per cent in physiological saline unless otherwise mentioned. Only freshly prepared solutions were used for each experiment. Chlorpromazine and trifluoperazine were supplied by Smith, Kline & French Laboratories; promazine was supplied by Wyeth Laboratories. The chemicals were dissolved in physiological saline and adjusted to pH 7·3 to 7·4 with 1 M Na_2HPO_4. Since phenothiazines are not very soluble in saline and form colloid-like substances upon standing, 5 mM was the highest concentration used, and the solutions were introduced into the test system as quickly as possible. Sheep blood was collected in modified Alsever's solution and processed as described by Plescia *et al.*[7] A suspension of washed cells in physiological saline was standardized spectrophotometrically, to contain 2×10^9 cells/ml as a stock suspension. The concentration of washed sheep cells used in the experiments was usually 5×10^8 cells/ml, and it was used within 48 hr of storage at 4°.

Measurement of binding by inhibition of lysis of sheep erythrocytes. This method detects the binding of hemolytic chemicals to serum proteins in terms of inhibition of the hemolytic activity of these chemicals, using washed sheep erythrocytes as indicator. At first, the minimum molar concentration of each phenothiazine giving complete lysis of a definite amount of sheep cells, in the absence of serum proteins, was determined. Diluted whole serum or individual serum fractions in solution were preincubated with the phenothiazine present in "lysing" concentration. After incubation at 37° for 30 min, a standard amount of sheep blood cells was added to this drug–protein mixture and then incubated at 37° again for 30 min. After addition of cold saline, the reaction mixture was centrifuged to remove intact cells, and the amount of hemoglobin in the supernatant was read at 451 μm spectrophotometrically. The binding of drug, if any, was shown by the inhibition of lysis of cells, and is reported as the per cent reduction in hemolysis as compared to controls containing the same concentration of phenothiazine without added protein.

TABLE 1. BINDING OF CHLORPROMAZINE (0·6 mM) BY SERUM FRACTIONS OF DIFFERENT SPECIES

Species	Serum fractions*	Per cent inhibition of hemolysis: Final conc. of serum fraction (%) 0·5	0·25
Human	(a) α-Globulin	90	32
	(b) β-Globulin	100	94
	(c) β-2-Globulin	2	0
	(d) 7S γ-globulin	0	0
	(e) Albumin	10	3
Bovine	(f) α-Globulin	94	60
	(g) γ-Globulin	0	0
	(h) Albumin	5	0
Canine	(i) α-Globulin	36	3
	(j) β-Globulin	9	1
	(k) γ-Globulin	0	0
Chicken	Ovalbumin†	21	3

* Serum fractions were obtained from Mann Research Laboratories with the following designations: (a) fract. IV, Cat. No. 7591; (b) fract. III, Cat. No. 7183; (c) chromat. isolated, Cat. No. 4829; (d) chromat. isolated, Cat. No. 4260; (e) crystallized, 100% pure, Cat. No. 1702; (f) Cohn fract. IV, Cat. No. 7055; (g) fract. II, Cat. No. 3004; (h) crystallized, 100% pure, Cat. No. 2506; (i) Cat. No. 3545; (j) Cat. No. 3189; (k) Cat. No. 2636.

† Ovalbumin, twice crystallized, was obtained from Worthington Biochemical corp.

Optimal conditions for binding. It was found that at constant ionic strength maximal binding occurred within the pH range 7·1–7·4, which was also optimal for the lysis of sheep cells by phenothiazines. Preliminary studies indicated that temperature changes did not greatly affect the extent of binding of chlorpromazine. Since the phenothiazines used are of interest in human therapy, 37° was chosen as the reaction temperature for the hemolysis inhibition studies.

The minimum molar concentration of phenothiazine giving 95–100 per cent lysis of $2{\cdot}5 \times 10^8$ sheep cells in our test system was determined using different concentrations. The results (Fig. 1) showed that the per cent lysis of sheep cells was increased with increased concentration of the chemical. The same concentration of each chemical, however, did not give the same per cent lysis of sheep cells. For example, at a concentration of 0·6 mM, chlorpromazine gave more than 90 per cent lysis, while promazine gave only 20 per cent lysis. The optimal test concentration for binding studies, therefore, had to be individually established for each compound.

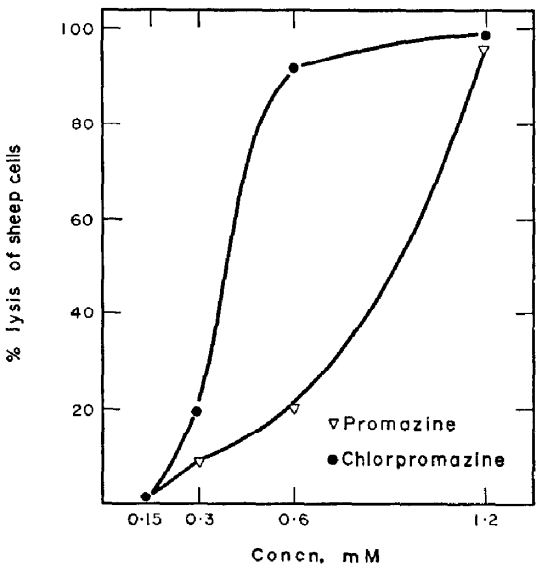

FIG. 1. Lysis of washed sheep cells at different concentrations of phenothiazines.

Comparison of serum proteins of different species on the binding of chlorpromazine. Serum proteins of different species (at a range of dilutions) had different capacities to bind chlorpromazine (final concentration, 0·625 mM). It was interesting to find that human serum had the greatest binding power. The relative effectiveness of the five sera tested in binding chlorpromazine were in the order of : human (1:12) > pig (1:8) > rabbit (1:8) > sheep (1:6) > guinea pig (1:6). (The ratio in parentheses shows the dilution of serum at which about 50 per cent inhibition of hemolysis was found.)

Binding of chlorpromazine by individual serum components. Fractions of serum proteins were tested to determine which component of serum predominates in the binding of chlorpromazine, as shown in Table 1. In the three species tested, the α-globulin fractions had strong binding power. Albumin exhibited much less binding with chlorpromazine, in contrast to the generally strong affinity of albumins in the binding of chemicals and drugs,[8] including haptens.[9] The β-globulin fraction appears to have more binding power than α-globulins, at least in the human species. Human β-2-globulin and all the γ-globulins had no detectable binding capacity. Chicken ovalbumin was included for comparison with serum proteins and was found to have a binding capacity similar to that of serum albumins.

Binding of structurally related phenothiazines by serum proteins of different species. To determine the ability of different proteins to inhibit the hemolytic properties of chemically different phenothiazines, various serum proteins (at 1 per cent final concentration) were incubated with three phenothiazines (at the concentration which just achieved 90 per cent hemolysis of sheep cells): promazine (1·2 mM) chlorpromazine (0·6 mM) and trifluoperazine (0·25 mM). Bovine α-globulin strongly inhibited hemolysis by chlorpromazine and trifluoperazine, but only weakly inhibited promazine. Bovine γ-globulin was ineffective with all three phenothiazines. Both bovine and human albumins showed weak to moderate inhibition of hemolysis by the three phenothiazines. By comparison, chicken ovalbumin strongly inhibited hemolysis by promazine and chlorpromazine, but hardly affected the hemolytic action of trifluoperazine.

The results obtained in this investigation indicate that this method can detect differences in the affinity for binding between serum proteins and various phenothiazines. It is interesting to consider the kind of binding forces which may be operative in this interaction. Chatten and Harris[10] investigated the relationship between the dissociation constant, pK_b (H_2O), and the half neutralization potential of a number of phenothiazines. One would expect that the monohydrochloride salts of phenothiazine derivatives would be almost completely dissociated to form the cationic amine in aqueous solution at pH 7·2 to 7·4, since compounds such as chlorpromazine and trifluoperazine were found to have a pK_b (H_2O) value of around five.

There is reason to suggest, therefore, that the binding involves to some extent the interaction of negatively charged groups of serum proteins with cationic groups of the side chains of phenothiazine derivatives. This view is supported by the finding that phenothiazines were not detectably bound by

γ-globulins. Klotz *et al.*[11] found that organic cations were not bound appreciably by certain proteins, such as serum γ-globulin and trypsin, and postulated that the higher content of hydroxyl groups in these proteins reduced the availability of carboxyl groups for binding.

The possible involvement of hydrophobic forces in the binding of phenothiazines should also be considered. The importance of hydrophobic forces in binding to serum proteins has been shown by Decker anf Foster,[12] who concluded that the binding of dodecyl benzensesulfonate to bovine serum albumin is primarily due to hydrophobic interactions.

The findings are of interest in the light of observations that phenothiazines inhibit serum cholinesterase and phosphatase activities *in vivo*,[4,5] since these two enzymes are located in the α-2-globulin fraction.[13] This coincides with our present finding that human and bovine α-globulins possessed high affinity for binding with phenothiazines *in vitro*.

The effect that substituents at positions 2 and 10 have on the binding of three phenothiazines to various proteins was shown in the last experiment presented. Promazine and chlorpromazine differ only in a chloro-substituent at position 2 in chlorpromazine. This substituent appeared to give chlorpromazine a greater affinity for binding to the serum proteins as compared to promazine. Trifluoperazine has a trifluoromethyl group as substituent, and also a piperazine group in its side chain. The affinity of this compound for various serum proteins was similar to that of chlorpromazine. The presence of the piperazine group in the chain at position 10 markedly enhances therapeutic and hemolytic activities of phenothiazines,[14] but this group does not appear to have as much of an effect on the affinity of the molecule for serum proteins, as detected by this method.

The binding of phenothiazines to serum proteins possibly explains the low hemolytic activity *in vivo* that has been found for these compounds, even when administered to humans in relatively large doses over long periods of time, and should be considered, therefore, as an important factor in protecting erythrocytes from hemolysis under conditions of heavy dosage. This binding probably has other important roles in the functioning of phenothiazines as useful drugs, such as providing a mechanism for transporting them to their active sites, and perhaps delaying their rapid inactivation by enzymes.

The results obtained from the present technique are reproducible with reasonable accuracy, but it can only be regarded as a qualitative test for phenothiazine binding. It would be possible, however, to make this technique quantitative by calibrating the present inhibition of hemolysis in terms of a quantitative measurement of binding, such as measuring the complexes formed by interaction with proteins.[15] Our most recent study *in vitro* on the binding of thioridazine with serum proteins showed that thioridazine can be measured quantitatively even at a low concentration by a spectrophotometric method.[16]

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