Trichloroacetic Acid-Induced Binding of Phenylthiourea to Erythrocytes

ALAN B. COMBS▲ and SHRI N. GIRI

Abstract □ Trichloroacetic acid precipitation causes the binding of phenylthiourea to the erythrocytes of a number of vertebrates. In rats, mice, and dogs, Scatchard plot presentations of the binding data indicate that there may be two sets of receptors involved in the binding. The total binding capabilities of the sets of receptors are of the same order of magnitude, but one set is of much greater affinity than the other. The induced binding of phenylthiourea to erythrocytes is probably related to acidity because a neutral precipitating agent did not cause binding.

Keyphrases □ Phenylthiourea binding to erythrocytes—induced by trichloroacetic acid □ Erythrocytes—trichloroacetic acid-induced binding of phenylthiourea □ Trichloroacetic acid—induced binding of phenylthiourea to erythrocytes □ Binding of phenylthiourea to erythrocytes—effect of trichloroacetic acid

The edema-producing thioureas, phenylthiourea and α-naphthylthiourea, exhibit a number of pharmacologically interesting properties. Rats are among the animals most susceptible to the acute toxic effects (1), which include pulmonary edema, pleural effusion (2, 3), and disturbances in carbohydrate metabolism (4–6).

In this laboratory, thiourea and phenylthiourea, after intraperitoneal injection in rats, have been shown to have apparent volumes of plasma distribution greater than the body size, an indication either of storage of the drug or of binding to some physiological component (7). Williams and Kay (8) showed that thiourea appears to be taken up by rat erythrocytes (red blood cells). Giri *et al.* (9, 10) reported that thiourea is not released from rat red blood cells by trichloroacetic acid precipitation, a method frequently used to facilitate drug recovery from biological samples (7, 11). Heating of the rat red blood cell samples, however, did cause the release of thiourea. The trichloroacetic acid-associated binding of thiourea to rat red blood cells was of sufficient magnitude to be a possible explanation for the large apparent volumes of distribution.

The present paper reports the extension of the binding study to phenylthiourea. Because of the large apparent volumes of distribution shown for phenylthiourea, it was of interest to determine if a binding phenomenon similar to that reported for thiourea occurred with phenylthiourea. In addition, since rats are uniquely susceptible to the toxic effects of the thioureas, the possibility that this susceptibility might be related to species differences in the binding of phenylthiourea to red blood cells was explored by measuring the binding in a number of species. The quantitative nature of the binding was determined using binding isotherms and Scatchard plots of the data (11). Finally, preliminary equilibrium dialysis studies with phenylthiourea and rat red blood cells gave evidence of only slight binding in comparison to the binding observed after trichloroacetic acid precipitation of the red blood cells. This indication that the binding might be trichloroacetic acid induced was investigated.

EXPERIMENTAL

Chemicals—The chemicals included Folin-Ciocalteu phenol reagent[1], a carbonate–tartrate solution consisting of 10% sodium carbonate and 0.1% potassium sodium tartrate (0.4 H_2O), practical grade phenylthiourea[2] recrystallized twice from hot 95% ethanol, freshly prepared heparin[3] containing no preservatives, 50% trichloroacetic acid, 1.32 and 5.3 *N* sodium hydroxide, freshly prepared 0.3 *N* barium hydroxide, and 0.3 *N* zinc sulfate. All aqueous reagents were prepared in deionized water.

Sources of Blood—Blood from male Sprague–Dawley rats, weighing over 150 g., was collected by decapitation and pooled. Freshly prepared heparin was used in all cases to prevent clotting. Ten-milliliter samples of blood from each of five mongrel dogs were obtained by venipuncture and pooled. Blood from approximately 150 male Swiss–Webster mice was collected by cardiac puncture after ether anesthesia.

Characterization of Binding—Samples of pooled blood were centrifuged at 4000 r.p.m. for 10 min. and the plasma was removed by aspiration and discarded. The erythrocytes (red blood cells) were rinsed with saline and centrifuged, and the rinse solution was discarded. Then a known volume of saline was added to the packed red blood cells and the suspension was decanted into a graduated cylinder. The volume of packed red blood cells was determined and enough additional saline was added to the cylinder so that 4 ml. of the final dilution contained 1 ml. of packed red blood cells.

Triplicate samples of the red blood cell suspension were incubated with varying amounts of phenylthiourea dissolved in saline. Table I shows the typical incubation scheme used; the items are listed in the order of addition to 25-ml. screw-capped tubes. After the final addition and mixing, the samples were incubated over ice with shaking for 30 min. An additional run at 37° was performed with the mouse red blood cells. After incubation, 2.5 ml. of cold 50% trichloroacetic acid was added to each sample. The samples were then mixed and stored over ice for 30 min. before removing the supernates.

Analysis of Phenylthiourea—A colorimetric method for phenylthiourea, based upon its reaction with the Folin-Ciocalteu phenol reagent, was used for analysis (12). The trichloroacetic acid supernates were neutralized with one-tenth of their volume of 5.3 *N* sodium hydroxide. Each supernate and its appropriate blank were diluted 5–50-fold with deionized water, depending upon the amount of phenylthiourea originally present. A 3.0-ml. aliquot of each diluted supernate was placed in a 25 × 150-mm. test tube. To this tube were added 1.0 ml. of 1.32 *N* sodium hydroxide and 1.0 ml. of the carbonate–tartrate solution. Then 1.0 ml. of the phenol reagent (diluted 1:1 with deionized water) was added rapidly while the contents of the test tube were mixed on an electric mixer. Absorbance was measured at 750 nm.[4] after 1.5 hr.

Calculations—The phenylthiourea recoveries from each triplicate sample were averaged. The amounts not recovered were assumed to be bound. The limited solubility of phenylthiourea prevented addition of concentrations greater than 2 mg./ml. to the incubation

[1] Fisher Scientific Co., Fair Lawn, N. J.
[2] J. T. Baker Chemical Co.
[3] Sigma.
[4] Gilford photometer with Beckman monochromator.

Table I—Typical Incubation System for Analysis of Trichloroacetic Acid Associated Binding of Phenylthiourea[a]

Saline, ml.	Phenylthiourea Solution[b], ml.	Milliliters of Red Blood Cell Dilution	Milliliters of Packed Red Blood Cells
6.0	0.0[c]	4.0	1.0
5.0	1.0	4.0	1.0
3.0	3.0	4.0	1.0
0.0	6.0	4.0	1.0
2.0	6.0	2.0	0.5
3.0	6.0	1.0	0.25
3.5	6.0	0.5	0.125
4.0	6.0[d]	0.0	0.0
10.0	0.0[e]	0.0	0.0

[a] The first three columns show the order of addition to 25-ml. screw-capped tubes, making 10.0-ml. final volume. Each sample was in triplicate. [b] The phenylthiourea was dissolved in isotonic saline; the concentrations used were either 1.90 or 1.00 mg./ml. [c] Blank used to estimate color reactions from sources other than phenylthiourea. [d,e] Internal standard and its blank, respectively.

systems. *Relative* increases in phenylthiourea were achieved by decreasing the amount of red blood cells incubated (Table I). The amounts of phenylthiourea incubated with the decreased volumes of packed red blood cells were converted to the value:

$$\frac{\text{micromoles of phenylthiourea incubated per milliliter of solution}}{\text{milliliter of packed red blood cells}}$$

Then the binding isotherm functions: micromoles of phenylthiourea bound per milliliter of packed red blood cells *versus:*

$$\frac{\text{micromoles of unbound phenylthiourea per milliliter of solution}}{\text{milliliter of unpacked red blood cells}}$$

were calculated.

Equilibrium Dialysis—One-centimeter diameter dialysis tubing was cut into 20-cm. strips, and the strips were boiled for 0.5 hr. in deionized water. They were rinsed, flushed with deionized water, and stored under refrigeration until needed. Duplicate 10-ml. total volume dialysis systems, containing the equivalent of 1 ml. of packed rat red blood cells inside the tubing and varying amounts of phenylthiourea in saline outside the bag, were incubated with shaking for 18 hr. at 3–5° in a cold room. After incubation, the solutions outside the dialysis bags were analyzed. The amounts of phenylthiourea not recovered were assumed to be bound.

Red Blood Cell Sedimentation—Rat red blood cells were used in an incubation similar to that shown in Table I. After incubation, the red blood cell suspensions were centrifuged for 10 min. at 2000 r.p.m. in a refrigerated centrifuge at 0°. A 1-ml. aliquot of supernate from each sample was analyzed for phenylthiourea. The cells were then resuspended and precipitated with trichloroacetic acid. The trichloroacetic acid supernates were neutralized and analyzed for phenylthiourea.

Nonacidic Precipitating Agent—An incubation similar to that shown in Table I was performed with dog red blood cells. Triplicate samples of cells were precipitated by a neutral Somogyi (13) method. Then 1.0 ml. of each cell suspension was added to 10.0-ml. samples of freshly prepared 0.3 *N* barium hydroxide and mixed; 10.0 ml. of 0.3 *N* zinc sulfate was added to each with mixing. The resulting precipitate was centrifuged and the supernate was analyzed for phenylthiourea. The remainders of each original red blood cell suspension were precipitated with trichloroacetic acid, and the supernates were analyzed.

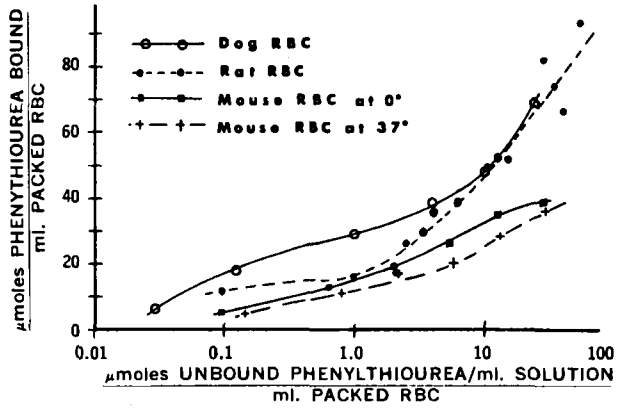

Figure 1—*Isotherm presentation of the binding data. Pooled red blood cells (RBC) were incubated for 30 min. and then precipitated with trichloroacetic acid as described in the text. Each point represents the average of triplicate samples.*

Table II—Dialysis Data[a]

Theoretical Concentration of Phenylthiourea, μmoles/ml.[b]	Measured Concentration of Phenylthiourea, μmoles/ml.	Phenylthiourea Bound by Red Blood Cells, %	Phenylthiourea Bound if Precipitated by Trichloroacetic Acid[c], Approximate %
3.93	3.03	23.0	60
1.97	1.55	21.5	85
1.31	1.20	8.0	90
0.66	0.57	13.0	100

[a] Duplicate 10.0-ml. total volume dialysis systems containing the equivalent of 1 ml. of packed rat red blood cells inside the dialysis bag and varying amounts of phenylthiourea outside were incubated with shaking for 18 hr. at 3–5° in a cold room. [b] The theoretical equilibrium concentration of the added phenylthiourea if no binding occurred. [c] These quantities were extrapolated from the rat red blood cell binding isotherm (Fig. 1).

RESULTS

Characterization of Binding—Phenylthiourea was bound to the red blood cells of rats, dogs, and mice after trichloroacetic acid precipitation. The results in binding isotherm form are presented in Fig. 1. Internal standard recoveries varied from 94 to 101%. A small amount of human and White Leghorn rooster blood was available. These samples also bound phenylthiourea after trichloroacetic acid precipitation.

Equilibrium Dialysis—The results of the dialysis experiment are shown in Table II. The recoveries were erratic but were always much greater than the amount that would be expected after trichloroacetic acid precipitation was extrapolated from the rat red blood cell binding isotherm (Fig. 1).

Red Blood Cell Sedimentation—The results shown in Table III indicate that little phenylthiourea was bound by centrifuged, unprecipitated red blood cells and that much more was bound after trichloroacetic acid precipitation.

Nonacidic Precipitation—The results presented in Table IV show that much more phenylthiourea was bound after trichloroacetic acid precipitation than after the neutral Somogyi precipitation.

Table III—Red Blood Cell Sedimentation Data[a]

Theoretical Concentration of Phenylthiourea, μmoles/ml.[b]	Measured Concentration of Phenylthiourea in Supernate before Trichloroacetic Acid, μmoles/ml.	Measured Concentration of Phenylthiourea in Supernate after Trichloroacetic Acid, μmoles/ml.	Percent Bound before Trichloroacetic Acid	Percent Bound after Trichloroacetic Acid
1.25	1.19	0.000	4.8	100.0
3.74	3.52	0.475	5.8	87.3
7.48	7.33	3.17	2.0	57.6

[a] Triplicate 10.0-ml. total volume incubation systems containing 1 ml. of packed rat red blood cells, saline, and varying amounts of phenylthiourea were incubated for 30 min. over ice. After incubation the suspensions were centrifuged and 1.0 ml. of each supernate was analyzed. Then the red blood cells were resuspended and precipitated with trichloroacetic acid, and the supernate was again analyzed. [b] The theoretical equilibrium concentration of the added phenylthiourea if no binding occurred.

Table IV—Nonacidic Precipitation Data[a]

Theoretical Concentration of Phenylthiourea, μmoles/ml.[b]	Measured Concentration of Phenylthiourea in Supernate after Neutral Somogyi Precipitation, μmoles/ml.	Measured Concentration of Phenylthiourea in Supernate after Trichloroacetic Acid, μmoles/ml.	Percent Bound after Neutral Somogyi Precipitation	Percent Bound after Trichloroacetic Acid
3.95	3.54	0.93	10.4	76.4
7.90	7.46	4.10	5.6	48.1
15.80	14.91	11.22	5.6	29.0

[a] Triplicate 10.0-ml. total volume incubation systems containing 1 ml. of packed dog red blood cells, saline, and varying amounts of phenylthiourea were incubated for 30 min. over ice. After incubation, a 1.0-ml. aliquot from each cell suspension was precipitated with 10.0 ml. of 0.3 *N* barium hydroxide followed by 10.0 ml. of 0.3 *N* zinc sulfate. The resulting supernate was analyzed for phenylthiourea. The remainder of each original cell suspension was precipitated with trichloroacetic acid, and the supernates were analyzed for phenylthiourea. [b] The theoretical equilibrium concentration of the added phenylthiourea if no binding occurred.

Table V—Binding Constants and Receptor Capacities[a]

Source of Red Blood Cells	Binding Constants: High Affinity Receptors	Binding Constants: Low Affinity Receptors	Receptor Capacities, μmoles/ml. of Packed Red Blood Cells: High Affinity Receptors	Receptor Capacities, μmoles/ml. of Packed Red Blood Cells: Low Affinity Receptors	Receptor Capacities, μmoles/ml. of Packed Red Blood Cells: Total Capacity
Rat	37.6	0.069	11	87	98
Dog	9.60	0.044	29	79	108
Mouse (red blood cells incubated at 0°)	6.82	0.126	13	34	47
Mouse (red blood cells incubated at 37°)	6.30	0.105	10	38	48

[a] Data were derived from Scatchard plots of the binding data (Fig. 1). Two sets of receptors were assumed, and the binding constants and capabilities were determined by trial-and-error substitution to give the best visual fit to the data.

DISCUSSION

Characterization of Binding—The binding data do not fall into the simple sigmoid curves that would be expected from such log-linear plots (Fig. 1) if a single set of similar receptors were involved (14). For this reason, Scatchard plots (11) were constructed of the data in terms of:

$$\frac{\text{micromoles of phenylthiourea bound per milliliter of packed red blood cells}}{\left[\dfrac{\text{micromoles of bound phenylthiourea per milliliter of solution}}{\text{milliliter of packed red blood cells}}\right]}$$

versus micromoles of phenylthiourea bound per milliliter of packed red blood cells. The regression curves giving the best visual fit to the data, assuming two classes of receptors, were determined by trial-and-error substitution. The resulting binding constants and receptor capacities are shown in Table V. The binding of phenylthiourea in all of the animals tested suggests the ubiquity of the phenomenon, but the data are not sufficient to allow any conclusions about the differential species toxicity shown by phenylthiourea.

The comparison of mouse red blood cells incubated at two different temperatures is shown in Fig. 1 and Table V. The total binding capacities are not appreciably changed by the increase in temperature from 0 to 37°, but the binding constant for each set of receptors is decreased. These findings are consistent with the observation of Giri *et al.* (9) and Giri and Combs (10) that as incubation temperature increased, the binding of thiourea to rat red blood cells decreased. The explanation might be that temperature-dependent structural changes in the receptors decrease their affinity for phenylthiourea.

Mechanism of Binding—Preliminary equilibrium dialysis studies gave rise to the suspicion that the trichloroacetic acid-associated binding might, in fact, be induced by trichloroacetic acid. This was confirmed by the dialysis results (Table II) and red blood cell sedimentation results (Table III). Only a small amount of phenylthiourea was bound after dialysis with intact red blood cells. The same amount of red blood cells would have bound much more phenylthiourea if they had been precipitated with trichloroacetic acid. As shown in Table III, the recovery of phenylthiourea from the supernates of centrifuged red blood cell suspensions was nearly quantitative, while recovery after trichloroacetic acid precipitation of the identical suspensions was much less. All of the evidence indicates that the binding is induced by trichloroacetic acid precipitation rather than being resistant to trichloroacetic acid precipitation.

Acidic protein precipitating agents other than trichloroacetic acid (perchloric acid and tungstic acid) have been shown in this laboratory to be associated with the binding of thiourea to rat red blood cells. After it was established that the binding of phenylthiourea to red blood cells was induced by trichloroacetic acid, it was decided to test the effect of nonacidic precipitation upon the binding. The data in Table IV indicate that much less phenylthiourea is bound after neutral Somogyi precipitation than after trichloroacetic acid precipitation. It seems likely, therefore, that induction of binding is associated with low pH. Because dialysis, red blood cell sedimentation, and neutral Somogyi precipitation each gave some evidence of binding of a lower magnitude than the induced binding, it may be that a low pH causes activation of the receptors responsible for the slight binding shown in these studies. Characterization of the noninduced binding and its possible relationship to the large apparent volumes of distribution found with phenylthiourea in rats is currently under study.

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▲ To whom inquiries should be directed. Present address: College of Pharmacy, University of Texas at Austin, Austin, TX 78712