lar sedimentation pattern. These two sets of experiments suggest that sodium salicylate, while reducing the RNA synthesizing ability of BHK 21 cells, affects the synthesis of all species of RNA to a similar degree. A marked decrease also was observed in the growth rate of these cells when exposed to sodium salicylate (100–200 μg/ml); this decrease could be the result of diminished RNA synthesis. Paine and Nagington (7) reported that similar doses of sodium salicylate decreased the growth rate of human embryonic kidney, lung, and heart cells but not of embryonic skin fibroblasts.

The observed decrease in RNA synthesis as well as the decrease in macromolecular synthesis by cells of different origins in the presence of salicylate (2) lends support to the hypothesis that salicylate generally interferes with cellular growth (1, 7, 8). However, the effect of sodium salicylate on the function of the specific cell type may be variable. Data presented in this paper suggest that sodium salicylate (200 μg/ml) does not affect mitochondrial activity of BHK 21 cells, since there is no increase in the ^{14}C-carbon dioxide evolved from 6-^{14}C-glucose.

Sodium salicylate (300 μg/ml) minimally increased both C-1- and C-6-derived carbon dioxide production by human polymorphonuclear cells but did not affect phagocytosis and bacterial killing, a phenomenon greatly dependent on hexose monophosphate shunt activity (9). The same concentration of sodium salicylate uncoupled oxidative phosphorylation of the lymphocytes and markedly impaired synthesis of DNA and RNA (1) as well as immunoglobulins (10). Therefore, it is speculated that the function of cell types more dependent on mitochondrial activity may be more susceptible to the inhibitory effects of sodium salicylate.

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Antihemolytic and Antiproteolytic Properties of Substituted Thiosemicarbazidophenothiazines and Thiazolidonylphenothiazines

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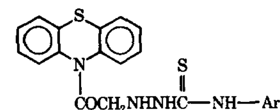
Abstract □ Antihemolytic and antiproteolytic properties of several 10-(1-acetyl-4-arylthiosemicarbazido)phenothiazines and their corresponding cyclized 10-(2-arylimino-3-acetylamino-4-thiazolidonyl)phenothiazines were investigated. *In vitro* protection of hypoosmotic hemolysis of human red blood cells by substituted thiosemicarbazidophenothiazines and substituted thiazolidonylphenothiazines was concentration dependent; the degree of protection ranged from 19 to 32 and 26 to 42%, respectively, at a final concentration of 0.1 m*M*. All phenothiazines exhibited antiproteolytic activity. The *in vitro* inhibition of trypsin-induced hydrolysis of bovine serum albumin by these phenothiazines was concentration dependent and competitive in nature; the degree of inhibition ranged from 30 to 50 and 32 to 79% for substituted thiosemicarbazidophenothiazines and substituted thiazolidonylphenothiazines, respectively, at a concentration of 1 m*M*. Cyclization of substituted thiosemicarbazidophenothiazines into the corresponding cyclized substituted thiazolidonylphenothiazines increased the antihemolytic and antiproteolytic effectiveness of these phenothiazines.

Keyphrases □ Phenothiazines, substituted—antihemolytic and antiproteolytic activities investigated □ Antihemolytic activity—substituted phenothiazines evaluated □ Antiproteolytic activity—substituted phenothiazines evaluated □ Structure–activity relationships—substituted phenothiazines evaluated for antihemolytic and antiproteolytic activities

It has been shown that the membrane-stabilizing effects of phenothiazine derivatives are reflected by delayed spontaneous hemolysis of red blood cells (1, 2). Phenothiazine derivatives also have been reported to exhibit anti-inflammatory effectiveness against formaldehyde-induced arthritis (3, 4). Recent studies indicated correlation between the antiproteolytic activity of amyrin acetates (5) and their anti-inflammatory activity (6). Furthermore, numerous psychotropic agents have been proposed to affect the physicochemical properties of cell membranes, accounting for the basis of their mechanism of action (7).

These observations prompted studies of the antihemolytic and antiproteolytic properties of some substituted thiosemicarbazidophenothiazines and their corresponding cyclized substituted thiazoli-

Table I—Antihemolytic and Antiproteolytic Properties of 10-(1-Acetyl-4-arylthiosemicarbazido)phenothiazines[a]



Compound	Ar	Protection of Hypoosmotic Hemolysis[b], %		Inhibition of Trypsin-Induced Hydrolysis of Bovine Serum Albumin[c], %		
		0.01 mM	0.1 mM	0.1 mM	0.5 mM	1.0 mM
I	C_6H_5	Nil[d]	24 ± 1.2	9 ± 0.7	22 ± 0.9	40 ± 1.0
II	2-$CH_3C_6H_4$	Nil	19 ± 1.0	Nil[d]	20 ± 0.6	36 ± 1.1
III	4-$CH_3C_6H_4$	Nil	20 ± 1.5	24 ± 1.0	27 ± 0.5	47 ± 0.6
IV	2-$OCH_3C_6H_4$	6 ± 1.4	26 ± 0.9	Nil	Nil	30 ± 0.9
V	4-$OCH_3C_6H_4$	Nil	20 ± 1.0	14 ± 0.8	20 ± 1.2	33 ± 0.5
VI	4-ClC_6H_4	11 ± 1.0	32 ± 1.2	17 ± 0.5	31 ± 1.3	42 ± 0.8
VII	4-BrC_6H_4	Nil	29 ± 1.0	18 ± 0.7	28 ± 0.5	45 ± 1.0
VIII	4-IC_6H_4	13 ± 1.1	32 ± 0.9	14 ± 0.6	15 ± 0.8	36 ± 1.2
IX	1-$C_{10}H_7$ (naphthyl)	Nil	20 ± 1.4	12 ± 1.0	40 ± 0.6	50 ± 0.6

[a] The assay procedure and the contents of the reaction mixture are as indicated in the text. Each experiment was done in duplicate, and the values indicate mean values of three separate experiments with ± standard error of the mean. [b] Antihemolytic activity was determined as protection against hypoosmotic hemolysis of human red blood cells by phenothiazine derivatives used at final concentrations of 0.01 and 0.1 mM. [c] The degree of inhibition of the activity of trypsin during hydrolysis of bovine serum albumin by phenothiazine derivatives was determined at the final concentrations of 0.1, 0.5, and 1.0 mM. [d] Represents experiments exhibiting no protection of hypoosmotic hemolysis and no inhibition of trypsin-induced hydrolysis of bovine serum albumin.

donylphenothiazines in an attempt to elucidate the biochemical basis for their anticonvulsant properties (8).

EXPERIMENTAL[1]

Determination of Hypoosmotic Hemolysis—Assay of hypoosmotic hemolysis was carried out by following the method reported earlier (9). Fresh heparinized human blood was oxygenated with a mixture of oxygen (90%) and carbon dioxide (10%) for 60 sec. To 0.1 ml of the whole blood was added 3 ml of a buffer solution (0.425% NaCl; sodium phosphate buffer, 5 mM, pH 7.4; total osmolality of 135 mosmoles/liter), with or without the test compounds. The tubes were shaken gently two or three times and then allowed to stand at room temperature (28–30°) for exactly 5 min. These tubes were then centrifuged for 5 min at 1000×g to separate the cells.

Under these conditions, hemolysis of the human blood cells occurred but in no case exceeded 50%. The absorbance of the supernate was read at 540 nm in a colorimeter. Percent protection was calculated by comparing the absorbance values observed in the presence of the substituted phenothiazines with those observed in the control tubes without the test compounds. The control values were represented as 100% hypoosmotic hemolysis. Absorbance values for the blank containing 0.1 ml of blood in 3 ml of 0.85% NaCl (normal saline) were substracted from the values obtained for both the control and the experimental tubes containing the test compounds.

All substituted phenothiazines were dissolved in the buffered saline solution of pH 7.4 (osmolality of 135 mosmoles/liter) and were used at a final concentration of 0.001–0.1 mM.

Assay of Proteolytic Activity of Trypsin—The antiproteolytic activity of substituted phenothiazines was determined by evaluating their ability to inhibit trypsin-induced hydrolysis of bovine serum albumin. The reaction mixture consisted of 0.05 M tromethamine buffer (pH 8.2), 0.075 mg of crystalline trypsin (1 g was sufficient to hydrolyze 250 g of casein), 0.03 mM bovine serum albumin, and water in a total volume of 1 ml. The substituted phenothiazines were dissolved in dimethylformamide and were used at final concentrations of 0.001–0.1 mM. An equivalent amount of dimethylformamide, added to the control tubes, had no effect on trypsin activity.

All substituted phenothiazines were preincubated with trypsin for 10 min prior to the addition of bovine serum albumin, and the reaction mixture was further incubated for 5 min after the addition of bovine serum albumin (5). The reaction was stopped by the addition of 5 ml of 15% (w/v) trichloroacetic acid solution. The acid-soluble products of protein breakdown, obtained after centrifugation, were determined (10) as an index of the enzyme activity. Suitable 0.5-ml aliquots of the acid-soluble supernatant solution were added to 5 ml of a freshly prepared mixture of 8% Na_2CO_3 solution and a solution containing 0.064% $CuSO_4$ and sodium potassium tartrate (0.12%) in equal volumes. The mixture was allowed to stand at room temperature for 10 min, and the absorbance was measured at 750 nm against a reagent blank in a spectrophotometer[2].

RESULTS AND DISCUSSION

The membrane-stabilizing property of phenothiazine derivatives was evaluated by determining the decrease in the degree of hypoosmotic hemolysis of human red blood cells. All 10-(1-acetyl-4-arylthiosemicarbazido)phenothiazines protected hypoosmotic hemolysis when used at a final concentration of 0.1 mM, and the degree of protection ranged from 19 to 32% (Table I). The use of 0.01 mM solutions of these compounds (except IV, VI, and VII) failed to provide protection from hypoosmotic hemolysis; IV, VI, and VII decreased hypoosmotic hemolysis by 6–13%.

In the present study, cyclization of substituted thiosemicarbazidophenothiazines into the corresponding 10-(2-arylimino-3-acetylamino-4-thiazolidonyl)phenothiazines resulted in the increased effectiveness of the cyclized compounds to provide protection against hypoosmotic hemolysis (Table II). The degree of protection observed with substituted thiazolidonylphenothiazines ranged from 26 to 42% at a final concentration of 0.1 mM. Compound VIII was an exception, since a slight decrease in its antihemolytic activity was observed on cyclization into XVII. The antihemolytic activity of these phenothiazines was concentration dependent, and greater protection from hypoosmotic hemolysis was observed with the use of higher concentrations.

The inhibitory effects of substituted thiosemicarbazidophenothiazines and their corresponding cyclized thiazolidonylphenothiazines on the hydrolysis of bovine serum albumin by trypsin are recorded in Tables I and II, respectively. All compounds were used at final concentrations of 0.1, 0.5, and 1.0 mM. The degree of inhibition of trypsin was concentration dependent and increased with a simultaneous increase in the concentration of these phenothiazine derivatives. Such an inhibition of trypsin-induced hydrolysis of bovine serum albumin ranged from 30 to 50% with substituted thiosemicarbazidophenothiazines (Table I) and from 32 to 79% with substituted thiazolidonylphenothiazines (Table II) at a final concentration of 1.0 mM. Thus, as was observed with antihemolytic activity, cyclization of 10-(1-acetyl-4-arylthiosemicarbazido)phenothiazines into the corresponding 10-(2-arylimino-3-acetylamino-4-thiazolidonyl)phenothiazines resulted in increased antiproteolytic activity.

[1] Commercial chemicals were obtained from Sigma Chemical Co., St. Louis, Mo. The syntheses and the physical constants of 10-(1-acetyl-4-arylthiosemicarbazido)phenothiazines and 10-(2-arylimino-3-acetylamino-4-thiazolidonyl)phenothiazines were reported earlier (8).

[2] Hitachi Perkin-Elmer spectrophotometer model 139.

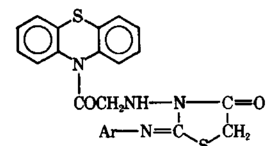


Table II—Antihemolytic and Antiproteolytic Properties of 10-(2-Arylimino-3-acetylamino-4-thiazolidonyl)phenothiazines[a]

Compound	Ar	Protection of Hypoosmotic Hemolysis, %		Inhibition of Trypsin-Induced Hydrolysis of Bovine Serum Albumin, %		
		0.01 mM	0.1 mM	0.1 mM	0.5 mM	1.0 mM
X	C_6H_5	24 ± 1.1	36 ± 1.0	5 ± 0.7	26 ± 0.5	65 ± 1.1
XI	2-$CH_3C_6H_4$	21 ± 1.2	36 ± 1.3	6 ± 0.8	27 ± 0.7	63 ± 0.6
XII	4-$CH_3C_6H_4$	18 ± 0.9	27 ± 1.1	4 ± 1.0	20 ± 1.0	59 ± 0.5
XIII	2-$OCH_3C_6H_4$	25 ± 1.5	37 ± 1.2	20 ± 0.7	33 ± 1.3	45 ± 0.8
XIV	4-$OCH_3C_6H_4$	19 ± 1.3	40 ± 0.9	4 ± 0.4	13 ± 0.7	32 ± 0.6
XV	4-ClC_6H_4	15 ± 1.0	30 ± 1.4	17 ± 0.8	26 ± 0.6	35 ± 0.9
XVI	4-BrC_6H_4	18 ± 0.9	42 ± 0.9	18 ± 0.7	50 ± 0.9	58 ± 0.7
XVII	4-IC_6H_4	12 ± 1.2	29 ± 1.1	24 ± 1.0	57 ± 0.8	79 ± 0.5
XVIII	1-$C_{10}H_7$ (naphthyl)	12 ± 1.1	26 ± 1.5	5 ± 0.8	24 ± 0.5	41 ± 1.0

[a] Assay procedures for the determination of antihemolytic and antiproteolytic properties of phenothiazine derivatives are as indicated in Table I.

In the present study, preincubation of these phenothiazine derivatives for 0, 10, 20, and 30 min (8) prior to the addition of bovine serum albumin in no way altered their antiproteolytic activity. These preincubation studies indicated a rapidly reversible nature of inhibition of trypsin by these phenothiazine derivatives during hydrolysis of bovine serum albumin. This finding was further supported by kinetic studies with 10-[2-(4-iodophenyl)imino-3-acetylamino-4-thiazolidonyl]phenothiazine (XVII); the method reported by Dixon (11) was followed for graphic evaluation of the nature of inhibition and determination of the K_i value for this inhibitor. As is evident from Fig. 1, XVII exhibited a competitive nature of inhibition of trypsin during hydrolysis of bovine serum albumin and possessed a K_i value of 0.12 mM.

These studies failed to provide any relationship between antihemolytic and antiproteolytic properties of phenothiazine derivatives and their chemical structure, so a definite structure–activity relationship was not exhibited (Tables I and II). The protection of hypoosmotic hemolysis indicated that these phenothiazines presumably prevent the various processes in the cell membranes, as reported earlier for other psychotropic drugs (12–14). However, the similarity between increased antihemolytic and antiproteolytic effectiveness observed in this study and a similar increase in anticonvulsant activity observed on cyclization of substituted thiosemicarbazidophenothiazines into the corresponding substituted thiazolidonylphenothiazines (8) provided evidence that antihemolytic and antiproteolytic activities may account for some biochemical effects for the anticonvulsant property of these phenothiazine derivatives.

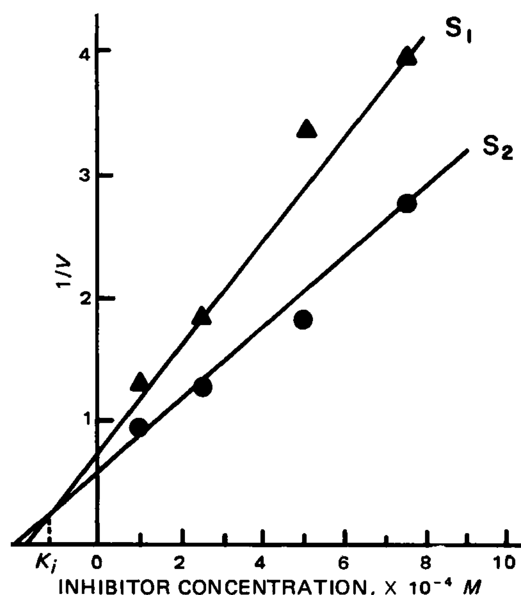


Figure 1—*Competitive inhibition of the activity of trypsin by XVII during hydrolysis of bovine serum albumin, where 1/V represents the reciprocal of the enzyme activity as determined by the amount of the products of protein breakdown using different concentrations of XVII. Assay procedures and the contents of the reaction mixture are as indicated in Table I. In all experiments, trypsin was incubated with XVII for 10 min prior to the addition of bovine serum albumin, which was used at two concentrations of 0.025 mM (S_1, ▲) and 0.033 mM (S_2, ●). The K_1 value (inhibitor constant) for XVII was 0.12 mM during trypsin-induced hydrolysis of bovine serum albumin.*

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Spectrophotometric Determination of Diphenhydramine Hydrochloride Using Dipicrylamine

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Abstract ☐ A spectrophotometric procedure for the determination of diphenhydramine hydrochloride based on the reaction with dipicrylamine was developed. A yellow complex forms and is easily extractable by chloroform at pH 5. The mole ratio of diphenhydramine hydrochloride to dipicrylamine in the complex is 1:3. The absorbance of the complex obeys Beer's law over the concentration range of 3–10 μg of diphenhydramine hydrochloride per ml of chloroform. This procedure can be carried out in the presence of other compounds without interference.

Keyphrases ☐ Diphenhydramine hydrochloride—spectrophotometric analysis, color complex with dipicrylamine, pharmaceutical formulations ☐ Dipicrylamine—color complex formation for spectrophotometric analysis of diphenhydramine hydrochloride, pharmaceutical formulations ☐ Spectrophotometry—analysis, diphenhydramine hydrochloride, pharmaceutical formulations ☐ Antihistaminic agents—diphenhydramine hydrochloride, spectrophotometric analysis, pharmaceutical formulations

5-(Hydroxymethyl)-2-furaldehyde is a well-known product of the Maillard reaction (1–3). It is frequently present in pharmaceutical preparations, particularly in syrups, and can interfere in the chemical analysis for active components.

USP XVIII (4) described a UV assay for diphenhydramine hydrochloride (I) elixir, in which 5-(hydroxymethyl)-2-furaldehyde interferes with the chemical analysis, the nonaqueous titrimetry for the powder, and also the UV assays for the capsule and injection preparations. The separation of the base on a cation exchanger, followed by quantitative spectrophotometric determination, was reported (5, 6).

A UV method was developed to measure the steam-distillable compound after hydrolysis and oxidation of diphenhydramine (7). An addition compound formation between diphenhydramine and tetrabromophenolphthalein ethyl ether in ethylenedichloride was used (8), as was ion-pair formation between the amine and an indicator dye (9). Nonaqueous titrimetry and salt partition were used in analysis procedures for organic bases (10, 11).

Quantitative TLC also was described (12). A modification of the USP XVIII method was described in which the effect of 5- (hydroxymethyl)-2-furaldehyde was eliminated (13). A column chromatography procedure was reported for the separation of I from a capsule formulation with determination by UV (14). However, these methods lack the simplicity and sensitivity to determine microamounts of I.

The present study describes a direct, simple, and sensitive procedure for the determination of I spectrophotometrically. This method is applicable for powder, syrup, capsule, injection, and elixir dosage forms without interferences. The procedure depends on the formation of a complex between I and dipicrylamine (II) which is extractable by chloroform at pH 5. This method can be carried out successfully in the presence of 5-(hydroxymethyl)-2-furaldehyde and other compounds.

EXPERIMENTAL[1]

Reagents and Chemicals—A 0.001 *M* diphenhydramine hydrochloride (I) aqueous solution (USP reference standard) and a 0.001 *M* dipicrylamine (II) in 0.4% sodium carbonate solution were prepared. All buffers used were of the BP standard.

General Procedure—To a 50-ml separator were added 1-5 ml of 0.001 *M* I, 1 ml of 0.001 *M* II, and 10 ml of pH 5 buffer solution. The mixture was shaken, and the complex formed was extracted with 5, 3, and 2 ml of chloroform by vigorous shaking. The extracts were collected in a 10-ml volumetric flask and then diluted to volume with chloroform. The absorbance of the complex in chloroform was measured at 420 nm *versus* a similarly prepared blank. Any other pharmaceutical preparations should be diluted to contain less than 100 μg/ml of I and then analyzed as described.

RESULTS AND DISCUSSION

A yellow-colored complex with a maximum absorption at 420 nm developed (Fig. 1) when I reacted with II. It was completely extractable by chloroform at pH 5. A calibration curve was plotted for various concentrations of I. Beer's law was followed over the concentration range of 30–100 μg of I/10 ml of chloroform. The molar absorptivity was 1.45×10^4.

The effects of temperature, pH, and the presence of many compounds were studied. A pH of 5 gave optimum results and different temperatures had no effect on complex formation and extraction.

Effect of Other Compounds—To determine the effect of other compounds, a standard solution containing 90 μg of I and the compound in question were placed in a separator and analyzed by the described method. The following compounds did not interfere when added in the indicated amounts: ammonium chloride[2] (10 mg), menthol[2] (100 mg), sucrose[2] (100 mg), sodium citrate[2] (10 mg), saccharin sodium[2] (5 mg), acetaminophen[3] (10 mg), phenylephrine hydrochloride[3] (100 mg), ascorbic acid[3] (10 mg), 5-(hydroxymethyl)-2-furaldehyde[4] (20 mg), orange oil[5] (0.1 ml), cinna-

[1] A Coleman Junior II model 6/20 spectrophotometer with 1-cm glass cells and a Beckman 10 recorder were used. A Beckman H_3-type pH meter was used for pH measurements.
[2] Present with I in syrups.
[3] Components of a diphenhydramine capsule marketed as Flustop.
[4] Frequently present in syrups.
[5] Constituents of I elixir.