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Chemical Constitution and Anthelmintic Activity—IV. Substituted Phenothiazines

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In a preceding paper,[1] examination of a number of tricyclic analogues of phenothiazine (I) showed that only phenothiazine and its close analogue phenoxazine possessed appreciable anthelmintic activity against mixed infestations of *Syphacia obvelata* and *Aspiculuris tetraptera* in mice. In view of this result, a number of substituted phenothiazines (Table I) were examined to investigate the variation of activity with substitution within this series.

The inactivity of phenothiazine sulphoxide and sulphone suggested the importance of the availability of free electron pairs in the sulphur atom; moreover, phenothiazine is known[2] to undergo oxidation, forming a semiquinone radical, and has been shown[3] to have marked antioxidant properties. Accordingly, the oxidation potentials of the 25 substituted phenothiazines under investigation were estimated to examine the possible correlation between anthelmintic activity and oxidation potential. The findings are discussed below.

Results of a spectroscopic examination of one compound which gave an anomalous result are also included.

Materials and Methods

Preparation of compounds. The synthesis of compounds used in this work which were not previously known has been described.[4] Other compounds had the highest melting points recorded in the literature. All samples were of analytical purity.

Table I. Anthelmintic activity and oxidation potential of substituted phenothiazines at 2 g/kg

Laboratory reference no.	Compound	Anthelmintic activity	Oxidation potential[a] (mV)
7	Methylene blue	0[b]	355[b]
75	Thionine	0	378[b]
46	3-Aminophenothiazine	0	451[b]
71	3,7-Dimethoxyphenothiazine	0	475
70	1,2,8,9-Dibenzophenothiazine	0	544
37	3,4,6,7-Dibenzophenothiazine	0	548
69	3-Methoxyphenothiazine	+	590
33	3,7-Dimethylphenothiazine	0	590[c]
27	3,4-Benzophenothiazine	0	628
36	1,2-Benzophenothiazine	±	633
32	3-Methylphenothiazine	±	651
82	3-Phenylphenothiazine	0	679
1	Phenothiazine	+	696[d]
81	3-Fluorophenothiazine	±	722
62	3-Chlorophenothiazine	+	763
78	3-Bromophenothiazine	±	766
83	3-Iodophenothiazine	0	758
5	Phenothiazine sulphoxide	0[i]	800
26	10-Methylphenothiazine	0	846[e]
79	3-Nitrophenothiazine	0	900[f]
19	Phenothiazine sulphone	0[i]	> 900[g]
28	10-Carbethoxyphenothiazine sulphone	0	> 900[g]
52	10-Formylphenothiazine	±	980[f]
4	10-Acetylphenothiazine	0	960[f]
73	10-Benzoylphenothiazine	0	> 920[f]

[a] At 20° in 80% v/v acetic acid (pH approximately 2).
[b] Reference 8.
[c] Recorded[2] as 626 mV in 90% acetic acid at 30°.
[d] Quoted[2] as 701 mV in 90% acetic acid.
[e] Given[2] as 829 mV in 80% and 882 mV in 90% acetic acid, respectively.
[f] Approximate values only; potentials became unstable between 25% and 50% of the univalent titration step, and values were obtained by extrapolation.
[g] Approximate values only; equilibrium was attained very slowly in these compounds.
[h] Tested at 0·5 g/kg.
[i] Tested at 1 g/kg.

Anthelmintic activity. The method of biological assay using mixed infestations of *S. obvelata* and *A. tetraptera* in mice has been previously described.[1] Activity was assessed as

$$\frac{\text{parasites in faeces}}{\text{total parasites found}} \times 100$$

and the signs used in Table I have the following significance:

+ +	high activity (> 90 per cent)
+	marked activity (70–90 per cent)
±	doubtful activity (10–70 per cent)
0	negligible activity (< 10 per cent)

Oxidation potentials. Oxidation potentials were estimated by potentiometric titration of a 2×10^{-4} molar solution with bromine at 20° using 80 per cent (v/v) acetic acid (pH approximately 2) as solvent throughout and employing the technique described by Michaelis.[5]

Spectrophotometric examination of gut fluid extracts. Rats which had been fasted for 3 h were dosed (1 g/kg) with the compound to be examined. Six hours later the animals were killed, the intestinal contents collected and centrifuged, and the supernatant fluid extracted with 'spectroscopically pure' hexane. Absorption spectra were obtained using an extract of the intestinal fluid of control animals as a blank solution; a Unicam SP500 spectrophotometer was used.

Results

Table I shows the anthelmintic activities and oxidation potentials of 25 phenothiazines. With the exception of 10-formylphenothiazine, activity appears to be confined to compounds possessing oxidation potentials in the region of 550–850 mV. Only three compounds showed marked activity; these were further examined in a larger number of animals, and their anthelmintic activities, together with the standard deviation and standard error, are given in Table II. The possibility that the activity of 10-formylphenothiazine was due to hydrolysis *in vivo* to phenothiazine was next examined.

Table II. Anthelmintic activity of the most active phenothiazine compounds at 2 g/kg

Compound	Laboratory reference no.	Anthelmintic activity %	Standard deviation	Standard error
3-Methoxy-phenothiazine	69	72	±28	±6·5
Phenothiazine	1	70	±28	±7
3-Chloro-phenothiazine	62	70	±21	±5·5

Hydrolysis of N-*formylphenothiazine in intestinal contents.* A hexane solution of phenothiazine showed a sharp maximum at 254 mμ (Fig. 1) similar to that obtained in *n*-heptane,[6] and extracts

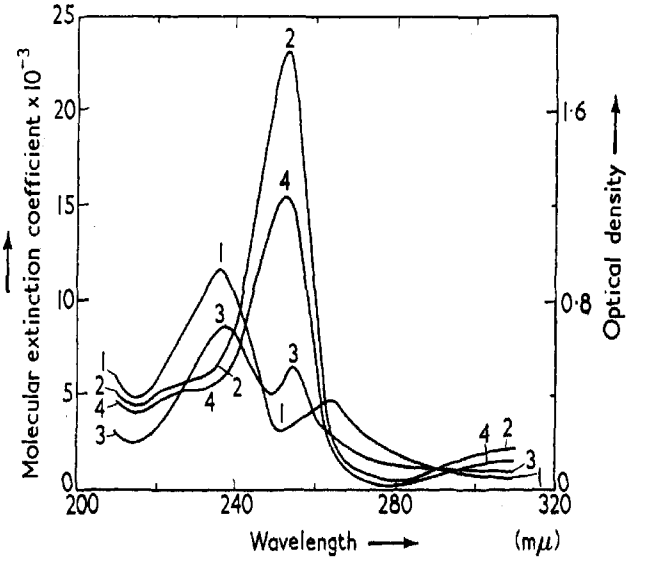


Fig. 1. Extinction curves of *N*-formylphenothiazine (curve 1) and phenothiazine (curve 2) in hexane, and optical density of hexane extracts of intestinal fluid after dosing with *N*-formylphenothiazine (curve 3) and phenothiazine (curve 4)

of intestinal contents of rats which had been dosed with phenothiazine gave similar curves, indicating that within the sensitivity of the method the compound was not changed in its passage through the contents of the small intestine. This result was in agreement with the findings of Esserman.[7]

Pure *N*-formylphenothiazine in hexane showed absorption

maxima at 235 and 263 mμ (Fig. 1). The absorption spectrum of extracts from intestinal contents after dosing rats with *N*-formylphenothiazine showed a broad peak at 239 mμ and a secondary peak at 254 mμ (Fig. 1). The peak at 254 mμ suggests that partial hydrolysis to phenothiazine had, in fact, occurred in the alimentary canal.

Discussion

The compounds possessing the greatest activities (Table II) were found to give oxidative titration curves of high stability

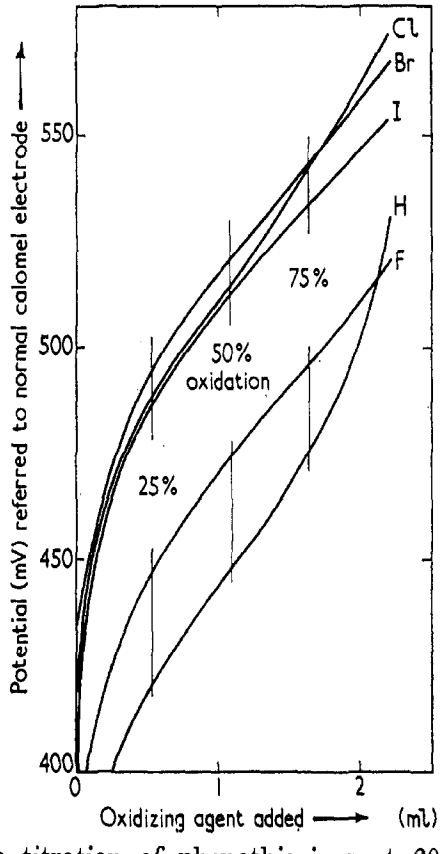


Fig. 2. Oxidative titration of phenothiazines at 20°. Phenothiazine: curve 'H'. 3-Chlorophenothiazine: curve 'Cl'. 3-Bromophenothiazine: curve 'Br'. 3-Iodophenothiazine: curve 'I'. 3-Fluorophenothiazine: curve 'F'

and symmetry corresponding to a single-electron oxidation step (Fig. 2). While 3-chlorophenothiazine showed a stable titration curve, the related fluoro, bromo, and iodo compounds were all markedly less stable in their oxidation behaviour (Fig. 2).

The following conclusions emerged from the results described in Table I: (*a*) apart from phenothiazine, only a few 3-substituted phenothiazines showed activity; (*b*) no 3,7-disubstituted phenothiazines at all possessed any activity, even though the same substituent had exhibited activity in the 3-monosubstituted series; (*c*) substitution at position 10 (ring-nitrogen) gave inactive compounds. It was shown that the apparent activity of *N*-formylphenothiazine was due to partial hydrolysis to the parent compound occurring in the gut of dosed animals.

These findings appeared to indicate that in the active 3-substituted phenothiazines a free 7-position and a hydrogen atom at the ring nitrogen were both necessary for activity. Oxidative titration of phenothiazine gave a typical univalent oxidation step, with an index potential E_i close to the theoretical value of 28·0 mV.[8] The titration curve shows high stability and there is practically no drift evident, even at the end of the titration (Fig. 2).

In the case of methylene blue and thionine, the index potential was found[9] to differ little from that for a bivalent oxidation step ($E_i = 14{\cdot}0$ mV) and any contribution from a semiquinone radical will be very small. The semiquinone formation constant k is given by equation (1):

$$k = S^2/RT \tag{1}$$

(where $R+T \rightleftharpoons 2S$ expresses the equilibrium between totally oxidized, totally reduced, and the semioxidized form); then at 50 per cent oxidation, where $R = T$ and the ratio of semiquinone to total amount a $(= R+T+S)$ will clearly be a maximum,[8] $(S/a)_{\text{max.}}$ will be given by equation (2):

$$(S/a)_{\text{max.}} = \sqrt{k}/(2+\sqrt{k}) \tag{2}$$

Since k is related[8] to the index potential E_i by equation (3):

$$k = \left[10^{E_i/0{\cdot}06} - \frac{3}{10^{E_i/0{\cdot}06}}\right]^2 \tag{3}$$

it follows that if $E_i = 14{\cdot}3$, then $k = 10^{-5}$ and $(S/a)_{\text{max.}} = 0{\cdot}001$, i.e. not more than 0·1 per cent of the semiquinone will be present; while if $E_i = 28$, then $k = 4$ and $(S/a)_{\text{max.}} = 0{\cdot}5$, 50 per cent of the redox system existing in the form of the semiquinone.[16]

Compounds showing oxidation behaviour similar to methylene blue and giving divalent oxidation steps were (Table I): No. 46 ($E_i = 15$), No. 70 ($E_i = 13$), No. 37 ($E_i = 14$), No. 27 ($E_i = 19$) and No. 36 ($E_i = 18$ mV).

Substances resembling phenothiazine in giving a distinct univalent curve, stable to the end of a single-electron step, were: No. 71 ($E_i = 26$), No. 69 ($E_i = 27$), No. 33 ($E_i = 29$), No. 32 ($E_i = 27$), No. 82 ($E_i = 28$) and No. 26 ($E_i = 27$ mV).

A third group was exemplified by 3-iodophenothiazine, where only the first half of the curve showed stable potentials; at 50 per cent oxidation the system became unstable, and a marked downward drift of potential set in, the E_i for 50–75 per cent oxidation being less than that for 25–50 per cent oxidation. This group included No. 81 (E_i, 27 and 22 respectively), No. 83 (E_i, 26 and 22), and No. 78 (E_i, 26 and 23 mV).

No. 79, the 10-acylated phenothiazines (Nos. 52, 4 and 73), and the sulphoxide (No. 5) and sulphone (No. 19) were all markedly unstable past the 50 per cent oxidation step.

It appeared, therefore, that the formation of a high proportion of a stable semiquinone radical by a univalent step, with $E_i = 28$ mV, was an empirical requirement for activity. In view of the rôle of free radicals in cell respiration,[10] it would be tempting simply to regard the semiquinone itself as the active anthelmintic agent, in agreement with the optimum stability of this species in the potential region of 550–850 mV. However, the oxidation potentials of the phenothiazines themselves, even at physiological pH values, would be well above that range of potentials in which metabolic processes function.

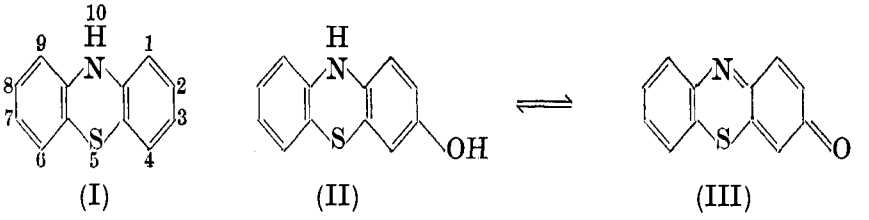
It is known that *in vivo* phenothiazine undergoes oxidation and is excreted as conjugates of the colourless and relatively water-soluble leuco compounds, 3-hydroxyphenothiazine (II) and 3,7-dihydroxyphenothiazine (IV), which are easily and reversibly oxidized in air to their coloured quinonoid forms, phenothiazin-3-one (III) and 7-hydroxyphenothiazin-3-one (V).

Phenothiazin-3-one shows[9] an oxidation potential of 440 mV at pH 2, which brings it into the range of the redox potentials of enzyme systems functioning as hydrogen carriers, e.g. the cytochrome system.

From the very weakly basic nature of the phenothiazine

compounds, it seems clear that the test substances exist as the free bases at any pH found *in vivo*. Activity is apparently not related to lipoid–water partition coefficients.[6]

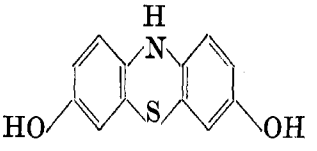
Phenothiazine probably enters the parasite through the cuticle.[11] In contrast to the low water-solubilities (0·01 to 0·2 mg/100 ml) and high lipoid–water partition coefficients (10^3 to 10^4) shown by the compounds in Table I, 3-hydroxyphenothiazine (or its



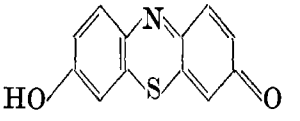
(I)

(II)

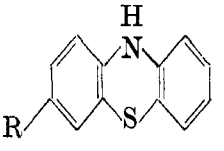
(III)



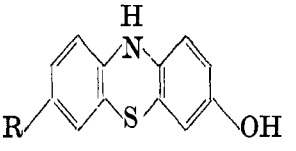
(IV)



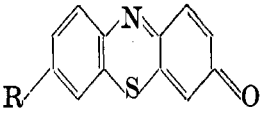
(V)



(VII)



(VI)



(VIII)

conjugate) possesses a much higher water-solubility (53 mg/100 ml) and lower partition coefficient (0·08) than phenothiazine, and rapid excretion of the oxidized material by the host would be expected. Swales and Collier[12] have demonstrated the presence of conjugated 3-hydroxyphenothiazine in the urine within 30 min of dosing, indicating rapid alimentary absorption. It thus appears possible that the presence of the *solid* phenothiazine compound in the host gut contents is a prerequisite not only for a reasonable concentration therein of the readily-excreted oxidation product but also for the attainment *in the parasite* of a toxic concentration of the active substance. It is possible that this active substance is derived from (VI), existing in thermodynamic

equilibrium with the corresponding solid phenothiazine (VII) present in the host.

The function of the active derivative may then be to transfer electrons (possibly with simultaneous detachment of a proton, when the electron transfer would be equivalent to a hydrogen transfer) in some part of the respiratory system of the parasite. Such single electron transfers could be facilitated by the stable semiquinone[8,9] of the redox system between (VI) and the corresponding phenothiazin-3-one (VIII). It is uncertain, however, whether the action of the anthelmintic could be attributed to inhibition within the cytochrome–cytochrome oxidase system, because there is some doubt about the importance of this system in *Ascaris lumbricoides*,[13,17] which show sensitivity to the action of phenothiazine; and Collier and Allenby[14] have found no effect on cytochrome oxidase activity with phenothiazine, phenothiazin-3-one or phenothiazine-5-oxide, though inhibition was shown by 3-hydroxyphenothiazine.[15]

It is interesting that phenoxazine, shown[1] to have the same anthelmintic activity as phenothiazine (83 per cent, standard deviation ± 19), has been reported[2] to undergo a univalent oxidation with a potential of 724 mV in 90 per cent acetic acid.

The most active compounds in this series, 3-methoxy- (No. 69) and 3-chlorophenothiazine (No. 62) were further investigated and the results are reported in the following paper.

Summary. Examination of 25 substituted phenothiazines against *Syphacia obvelata* and *Aspiculuris tetraptera* in mice showed that only those compounds having oxidation potentials in the range of 550 to 850 mV had significant activity. Anthelmintic activity appears to require two factors: (*a*) the formation of a high proportion of a stable semiquinone radical by a univalent oxidation step with $E_{\frac{1}{2}} = 28$ mV, and (*b*) possession (in 3-substituted compounds) of a free 7-position.

The presence of solid phenothiazine in the host gut may be necessary to reach a reasonable concentration of the active substance therein and, thence, a toxic concentration in the parasite.

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