Journal of Medicinal and Pharmaceutical Chemistry
VOL. 2 No. 6 (1960)

Chemical Constitution and Anthelmintic Activity—V. Alkoxy- and Chlorophenothiazines

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Anthelmintic activity of substituted derivatives of phenothiazine (I) as tested against mixed infestations of *Syphacia obvelata* and *Aspiculuris tetraptera* in mice[1] appeared to require two factors: the formation of a high proportion of a stable semiquinone radical by a univalent oxidation step, and the possession, in 3-substituted compounds, of a free 7-position. In view of the high activity[1] of 3-methoxy- and 3-chlorophenothiazine, compounds of this type were further investigated.

Preparation of compounds. The compounds used in this work which were not previously known have been described.[2,3] Other compounds had melting points the same as the highest ones recorded in the literature. All samples were of analytical purity.

3-Chlorophenothiazine-5-oxide. A mixture of 3-chlorophenothiazine[4] (6·4 g), 30 per cent hydrogen peroxide (20 ml), ethanol (70 ml), and acetone (50 ml), was refluxed for 1 h. The solution was then evaporated under reduced pressure until solid began to separate. It was then cooled and poured into a large volume of water to yield 6·58 g (97 per cent) of 3-chlorophenothiazine-5-oxide, m.p. 253–255° (from ethanol).

Anal. Calcd. for $C_{12}H_8ClNOS$: C, 57·71; H, 3·23; O, 6·42; S, 12·84. Found: C, 57·68; H, 3·42; O, 7·0; S, 12·66.

3-Chlorophenazothionium chloride. A solution of 3-chlorophenothiazine-5-oxide (777 mg) in glacial acetic acid (20 ml) and concentrated hydrochloric acid (3 ml) rapidly became dark red and was filtered immediately. After 5–10 min standing, the filtrate (*A*) deposited purple crystals which were filtered off after a further 15-min standing. The solid was washed with glacial acetic acid and dry benzene, and dried at room temperature *in*

vacuo over phosphorus pentoxide, affording 444 mg (53·5 per cent) of the salt, m.p. 155–160°.

Anal. Calcd. for $C_{12}H_7Cl_2NS.\frac{1}{2}H_2O$: C, 51·98; H, 2·90; N, 5·05. Found: C, 51·87; H, 2·87; N, 4·84.

The infrared spectrum showed no absorption corresponding to NH bands.

3,7-Dichlorophenothiazine. The filtrate (*A*) from the above experiment was refluxed on the steam bath for 1 h, during which time it became colourless. It was then diluted with water, yielding 345 mg (41·5 per cent) of 3,7-dichlorophenothiazine, which after chromatography on neutral alumina, or recrystallization from ethanol, had m.p. 225–226° (capillary tube), undepressed on admixture with an authentic sample.[3] The melting point was unchanged after sublimation at 170–180°/0·6 mm. The infrared spectrum was identical with that of an authentic sample.

Anthelmintic activity and *oxidation potentials* were determined as described.[1]

Results

Anthelmintic activities and oxidation potentials are shown in Table I. Activities were assessed as outlined previously.[1]

The high activity of 3-methoxyphenothiazine[1] was ascribed to the stability of the semiquinone of this compound. Further examination showed that this substance underwent two successive univalent oxidation steps, both of which lay within the range (550–850 mV) in which optimal activity of substituted phenothiazines had previously been found. The homologous 3-ethoxyphenothiazine (No. 84) showed similar oxidation behaviour and some activity.

The isomeric 1-methoxy and 1-ethoxyphenothiazine (Nos. 85 and 102), however, showed little activity and markedly lower stability on oxidation; these compounds did not give a second electron step, and moreover formed a semiquinone becoming unstable at 50 per cent of the univalent titration step. These findings are in agreement with the known lower stability of *o*- as against *p*-quinonoid systems. As expected, the *m*-substituted 2-methoxyphenothiazine (No. 101) was devoid of activity.

In the chloro-substituted phenothiazines, 3-chlorophenothiazine (No. 62), possessing a stable semiquinone, was highly active,

while the isomeric 2-chloro compound (No. 103), markedly less stable on oxidation, showed behaviour similar to that of 2-methoxyphenothiazine and was inactive. When both the 3- and 7-positions were occupied, as in 3,7-dichlorophenothiazine (No. 106), activity was lost, but the introduction of the second chlorine into the 2-position, as in 2,7-dichlorophenothiazine (No. 105)

Table I. Anthelmintic activity and oxidation potentials of alkoxy- and chlorophenothiazines at 2 g/kg

Laboratory reference no.	Derivative of phenothiazine	Anthelmintic activity	Oxidation potential (mV)[a]	
			E_1	E_2
69	3-Methoxy	+	590	736
84	3-Ethoxy	±	580	729
85	1-Methoxy	0	698	—
102	1-Ethoxy	0	692	—
101	2-Methoxy	0	[b]	—
62	3-Chloro	+	765	—
103	2-Chloro	0	776	—
106	3,7-Dichloro	0	[b]	
105	2,7-Dichloro	±	[b]	
110	2,8-Dichloro	0	[b]	
104	2-Chloro-7-methoxy	++	662	751
112	4-Chloro-7-methoxy	++	668	756
71	3,7-Dimethoxy	0	474	620
33	3,7-Dimethyl	0	590	804

[a] At 20° in 80% (v/v) acetic acid (pH approximately 2).
[b] Potentials not measured.

resulted in the retention of activity. Introduction of two chlorine atoms into the non-activating *m*-positions, as in 2,8-dichlorophenothiazine (No. 110) did not confer activity.

On the other hand, when the *p*- (i.e. 7-) position is occupied by a methoxy substituent, introduction of a chlorine atom into either the 2- or the 4-position (Nos. 104 and 112) gave compounds possessing extremely stable semiquinones, which again underwent two successive univalent oxidation steps (see Table I and Fig. 1), where E_1 is the normal potential of the lower, and E_2 that of the higher, univalent steps of oxidation. The index potential E

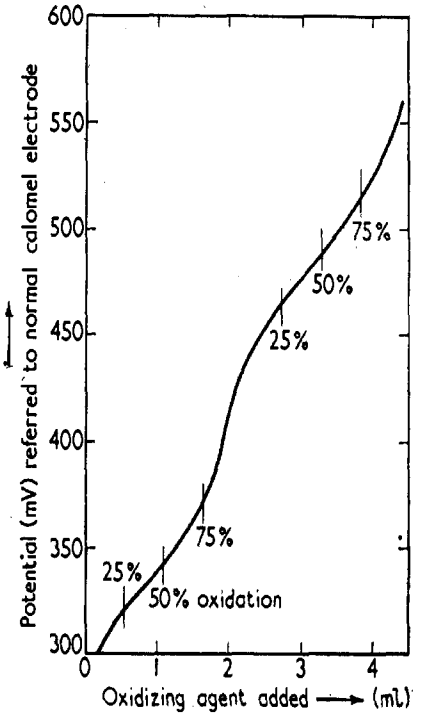


Fig. 1. Oxidative titration of 3-methoxyphenothiazine at 20°

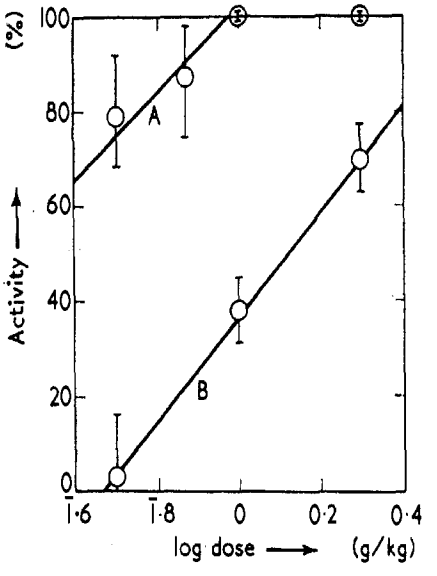


Fig. 2. The relation between log dose (g/kg) and anthelmintic activity $\left(\frac{\text{parasites in faeces}}{\text{total parasites}} \times 100\right)$ of 2-chloro-7-methoxyphenothiazine (A) and phenothiazine (B). (I) = Standard Error

was in every case close to the theoretical value of 28 mV required for a univalent step. Both 3,7-dimethoxy- (No. 71) and 3,7-dimethylphenothiazine (No. 33) showed similar behaviour, but were devoid of anthelmintic activity.

The high activity of 2-chloro-7-methoxyphenothiazine in relation to phenothiazine is shown in Fig. 2. Phenothiazine-5-oxide and phenothiazin-3-one have shown, both in our hands and in those of other workers,[5, 26] variable activity often approaching that of phenothiazine. Phenothiazin-3-one also had considerable toxicity to mice, as did 3-hydroxyphenothiazine which very rapidly underwent oxidation to the quinonimine.

Discussion

It was concluded previously[1] that the requirements for anthelmintic activity in phenothiazines were: (*a*) the formation of a high proportion of a stable semiquinone radical by a univalent oxidation step; and (*b*) possession (in 3-substituted compounds) of a free 7-position.

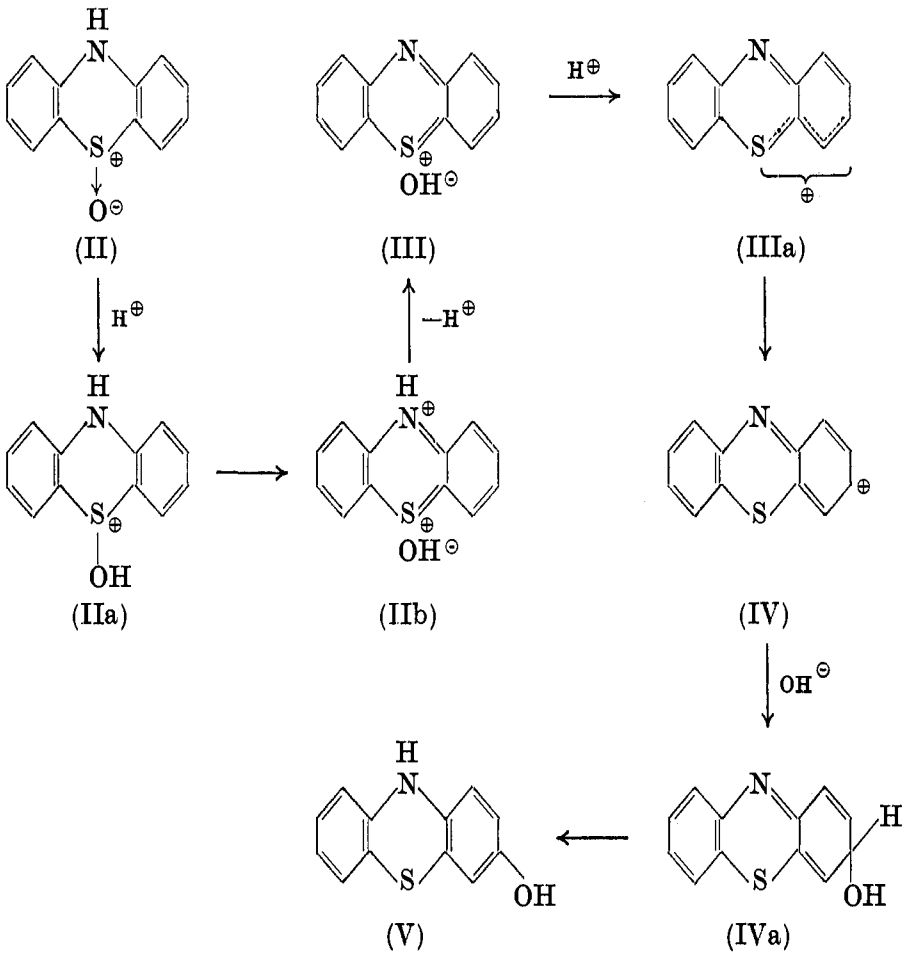
The first oxidation product of phenothiazine is known to be[6] phenothiazine-5-oxide (II), in tautomeric equilibrium, by protonation and deprotonation, *via* (IIa) and (IIb), with a quinonoid phenazothionium compound (III), as shown by the isolation of (III) in the form of phenazothionium salts[7, 8] possessing differing anions, e.g. Cl^- and ClO_4^-.

The cation of (III) can, however, undergo a facile acid-catalysed rearrangement *via* a transition stage (IIIa) to the mesomeric form (IV), resulting in the shift of the anionic group to the free 3-position to give a pseudo-base (IVa), the energy of the resulting *p*-quinonoid system (IVa) being less than that of the initial *o*-quinonoid structure (III). Stabilization will finally occur by tautomerism, the proton migrating to give 3-hydroxyphenothiazine (V) as end-product.

Thus phenothiazine-5-oxide was converted, even in the presence of acetic acid only,[9] into (V). Moreover, it was found [9, 10] that when the 3- and 7-positions were both blocked, this rearrangement did not take place. Similarly, 10-methyl- and 10-ethylphenothiazine-5-oxide were transformed[11, 12] by the action of hydrochloric acid into the 3-chloro-10-alkylphenothiazines, and under

the same conditions 3-chloro-10-methylphenothiazine-5-oxide gave 3,7-dichloro-10-methylphenothiazine,[11] and 3-nitrophenothiazine-5-oxide gave 7-chloro-3-nitrophenothiazine.[13]

We have now found that 3-chlorophenothiazine-5-oxide similarly gave, on treatment with hydrochloric acid, a purple intermediate shown by analysis to be 3-chlorophenazothionium



chloride, which on further reaction afforded 3,7-dichlorophenothiazine.

These facts are in accord with Page and Smiles' observation[10] that 10-methylphenazothionium chloride was isolated from the reaction of 10-methylphenothiazine-5-oxide with hydrochloric acid, which afforded 3-chloro-10-methylphenothiazine as the stable end-product, and with Schmalz and Burger's postulated[11] mechanism of protonation of the sulphoxide to give first a sulphonium base (IIa), which is then converted to a sulphonium ion

(IIb), followed by further rearrangement of the latter in the manner shown (III→ IV) to give the products described above.

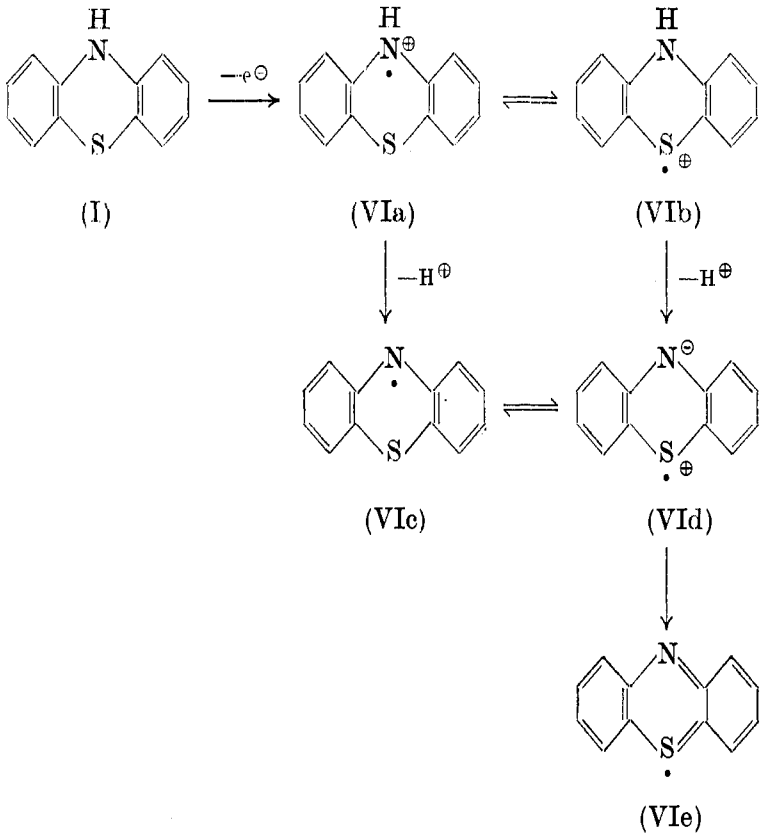
The necessity for a free 3-position for anthelmintic activity suggests that such a rearrangement may operate as part of the mode of action of phenothiazine anthelmintics. That phenothiazine also forms the sulphoxide *in vivo* is shown by several observations[14,15] reporting the isolation of (II) from the blood of phenothiazine-dosed calves exhibiting symptoms of photosensitization and keratitis. With normal doses of phenothiazine, the phenothiazine-5-oxide, which is the oxidation product in the alimentary tract, is transformed into 3-hydroxyphenothiazine, or its sulphate ester, which is then rapidly excreted. With large doses or in young animals, however, the sulphoxide passes into the systemic circulation, setting up the toxic symptoms noted. We have observed that the surface of samples of pure colourless phenothiazine-5-oxide assumes, on keeping in light and air, the purple coloration characteristic of phenothiazin-3-one, while phenothiazine and the 5,5-dioxide are stable under the same conditions.

These views are confirmed by the results of oxidative titrations of phenothiazine. Further addition of oxidant to the semiquinone of (I), i.e. after the removal of one electron, results in a fall of the potential, equilibrium being established only very slowly after each further addition of oxidant. When the quantity of oxidant added corresponds to the removal of a second electron, the potential reached is that obtained as the *starting potential* of phenothiazine-5-oxide (II), and further addition of oxidant then results in an unstable titration curve similar to that obtained by oxidative titration of (II).

The reduced stability, observed[1] in some 3-substituted phenothiazines, of the titration curves beyond 50 per cent of oxidation may thus indicate a preferential removal of a second electron from the semiquinone formed (rather than from the unoxidized phenothiazine molecules present), as shown by the lower values (21–23 mV) of the index potential E_i between 50 and 75 per cent oxidation, e.g. in the case of 3-fluorophenothiazine.[1]

The first step in the oxidation of phenothiazine (I) thus results in the abstraction of one electron to give the semiquinone (VI) which may exist as the mesomeric forms (VIa) and (VIb). These,

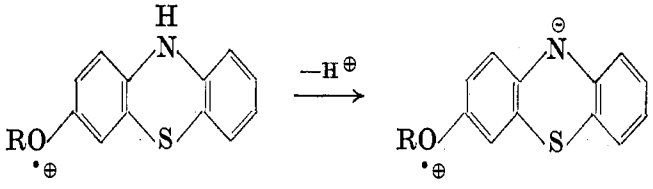
by loss of a proton, give rise to the two limiting resonance forms (VIc) and (VId), the charge separation in (VId) resulting in a bond rearrangement to (VIe) which will thus be a contributor to the total resonance state of (VI). The existence and stability of



(I) (VIa) (VIb) (VIc) (VId) (VIe)

this semiquinone has been demonstrated[16, 17, 19] for (I) and a number of substituted phenothiazines.

The extraordinary stability of the oxidation products of the

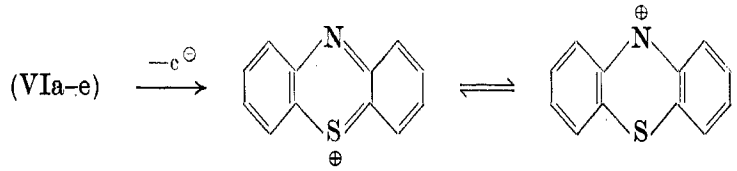


(VIIa) (VIIb)

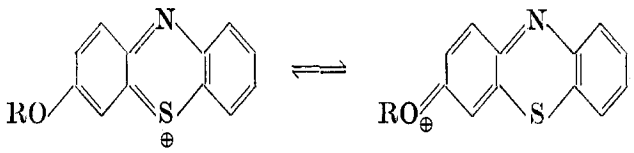
3-alkoxyphenothiazines is accounted for by the additional resonance stabilization of their semiquinones[19] through the existence of further mesomeric forms such as (VIIa) and (by loss of a proton) the charge-separated form (VIIb).

Removal of a second electron from all possible canonical forms of the semiquinone (VI, a–e) can be easily shown to result in only one stable end-product, namely the phenazothionium ion (VIII), stabilized by resonance with forms such as (VIIIa), or, in the case of 3-alkoxy substituted compounds, (IXa).

In phenothiazine itself the unsubstituted phenazothionium ion (VIII) appears to tautomerise, at least at room temperatures, under the weakly acidic conditions of the titration, to the stable sulphoxide (II) by the proton exchange (III) → (II), *via* the sulphonium base (IIa), whereas in the 3-alkoxyphenazothionium ion (IX) there exists additional resonance stabilization due to contributing forms such as (IXa). The preferred reaction in the



(VIII) (VIIIa)



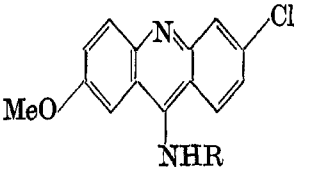
(IX) (IXa)

latter case, in strongly acid medium or at elevated temperatures, is then rearrangement of the type (III) → (IV) resulting in a symmetrical 3-alkoxy-7-hydroxyphenothiazine.

The presence of a chlorine atom in the 2- or 4-position of a 7-alkoxyphenothiazine might be expected, by its inductive effect, to facilitate the latter rearrangement, and to be responsible for the enhanced activity of Nos. 104 and 112 (Table I). It is clear that no such rearrangement can occur if both the 3- and 7-positions are blocked, but that nevertheless both an extremely stable semiquinone and (subsequently) a stable phenazothionium ion should arise if the 3- and 7-positions were occupied by alkoxy groups. This is confirmed by the high stability and the concomitant lack of anthelmintic activity of 3,7-dimethoxy- (No. 71) and 3,7-dimethylphenothiazine (No. 33), the latter exerting its stabilizing influence by hyperconjugation.

As yet there is no definite evidence of how phenothiazine exerts its toxic action on nematode parasites. It has been suggested however[20] that the chemotherapeutic activity of phenothiazine (known to have a paralysing rather than a lethal effect on worms[20]) may be related to paralysis of the muscular system of the parasite. It has been observed[20] that an overdose of phenothiazine will give rise to symptoms of muscular incoordination and paralysis in the host. Phenothiazine derivatives have also been shown[21,22] to affect a number of respiratory enzyme systems both *in vitro* and *in vivo*, and it is possible that phenothiazine, through a free radical intermediate, depresses some respiratory mechanism or interferes with a vital redox system in the parasite.

The same structural features which contribute to a high stability of the initial semiquinone of type (VI) and (VII) would also be expected to assist the stability of a semiquinone formed from the 3-substituted-7-hydroxyphenothiazines (which would themselves have redox potentials closely similar to those of respiratory enzymes), formed by the rearrangement *in vivo* of 3-substituted phenazothionium ions of type (VIII) and (IX).



(X)

It is interesting that the potent antimalarial drug quinacrine (X), a 5-substituted 2-chloro-7-methoxyacridine, possesses the same relative disposition of the chloro and methoxy substituents as 2-chloro-7-methoxyphenothiazine (No. 104), and that the well known oxidation theory of Schönhöfer[25] postulates that antimalarial action is connected with increased susceptibility to oxidation or quinone formation.

Recent work has, in fact, shown[23] that many analogues of quinacrine possess marked anthelmintic activity when tested against *Aspiculuris tetraptera* and *Syphacia obvelata*, and that the aminoacridines form a redox system[24] operating in two univalent steps, with an intermediate free radical of great stability, the nature of which has been related to the bacteriostatic activity within that series. Quinacrine itself is also an anthelmintic.[18]

Summary. An examination of the anthelmintic activity against mixed infestations of *Syphacia obvelata* and *Aspiculuris tetraptera* of 14 alkoxy- and chloro-substituted phenothiazines showed that optimum activity required: (*a*) the formation of a high proportion of a stable semiquinone radical, by a univalent oxidation step, and (*b*) the possession (in 3-substituted compounds) of a free 7-position, in agreement with results previously obtained. The significance of these results is discussed.

Acknowledgements. The authors are indebted to Mr. B. Thompson for technical assistance. This work forms part of the research programme of the McMaster Animal Health Laboratory, C.S.I.R.O. Division of Animal Health and Production, whom one of us (M.E.T.) wishes to thank for the award of an Ian McMaster Scholarship. The authors are grateful to Mr. D. A. Gill and Dr. D. F. Stewart for their continued interest and support.

(*Received 9 April, 1960*)

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