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Formation of Free Radicals from Some Phenothiazine Derivatives as Studied by Electron Spin Resonance

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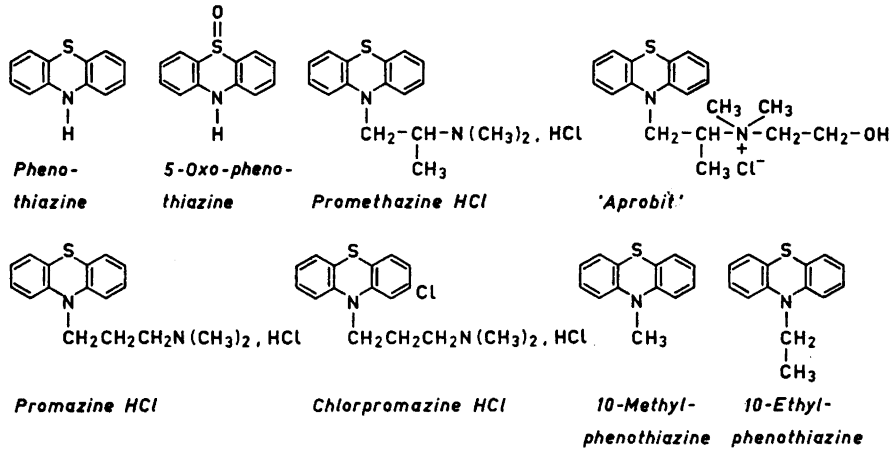
When adsorbed on the cation exchanger Dowex-50 containing a small amount of ferric iron, phenothiazine and phenothiazine derivatives such as promethazine and chlorpromazine are transformed to free radicals. The ESR-spectra of these radicals exhibit a hyperfine structure and a characteristic asymmetry. The radical spectra observed on iron-activated Dowex-50 are compared with those obtained when the phenothiazine substances are dissolved in concentrated sulphuric acid.

Michaelis and collaborators [1] have shown by potentiometric titration that certain phenothiazine dyes, such as thionine, form an intermediate between the oxidized and the reduced state analogous to the semiquinone intermediate observed in the oxidation of hydroquinone. If not dimerized, the configuration of such an intermediate involves an unpaired electron.

Depending on the pharmacological activity of several phenothiazine derivatives, it should be of interest to study the radical forming properties of these compounds. The convenience of electron spin resonance (ESR) has not been used to a great extent for such studies. The only contribution appears to be a paper by Forrest and Forrest [2], who found an ESR signal in the urine of patients treated with chlorpromazine. Free radicals could also be detected by ultraviolet irradiation of a solution of chlorpromazine. No hyperfine structure could be observed in the spectra, neither from the urine, nor from the irradiated solution. The ESR signal exhibited a certain asymmetrical shape and could only be detected in isolated solid state specimens.

In the present paper it will be shown that free radicals are formed from several phenothiazine derivatives when solutions of these compounds are poured on to the cation exchanger Dowex-50 activated with a small amount of ferric iron. The radical spectra exhibit hyperfine lines. Similar radical spectra are obtained, when the phenothiazine substances are dissolved in concentrated sulphuric acid.



EXPERIMENTAL

The ESR-spectrometer was of a type rather similar to that described by Krongelb and Strandberg [3], and was operated at a frequency of about 9 527 Mc/s. It was equipped with a rectangular resonance cavity working in the H_{011} mode. A 100-kc sinusoidal magnetic field was superimposed on the d.c. field of the electromagnet through an internal loop located close to the specimen tube, as described by Ingram [4]. The magnet was a Varian 6″ electromagnet, type V-4007-1, equipped with ring shims. The first derivative of the absorption is recorded against the magnetic field in the ESR spectra shown. The spectra are recorded at room temperature unless otherwise stated. The experiments at liquid air temperature were performed with the aid of a glass Dewar vessel, placed inside the resonance cavity.

The structure formulae of the phenothiazine derivatives investigated are given above.

Phenothiazine. Eastman practical grade was recrystallized from *n*-butanol.

5-Oxophenothiazine was prepared from phenothiazine by oxidation with hydrogen peroxide as described by Pummerer [5].

10-Methyl- and *10-ethylphenothiazine* were prepared as described by Gilman *et al.*[6]

10-(2-Dimethylamino-1-propyl)-phenothiazine hydrochloride (promethazine hydrochloride, "Lergigan") was obtained from AB Recip, Stockholm.

10-(3-Dimethylamino-1-propyl)-phenothiazine hydrochloride (promazine hydrochloride, "Centracil") was obtained from AB Astra, Södertälje.

2-Chloro-10-(3-dimethylamino-1-propyl)-phenothiazine hydrochloride (Chlorpromazine hydrochloride) was obtained from AB Leo, Hälsingborg.

[1-(10-Phenothiazinylmethyl)-ethyl]-2-hydroxyethyl-dimethylammoniumchloride ("Aprobit") was obtained from Ab Recip, Stockholm.

The last four substances were drug preparations, and were used without further purification.

Dowex-50 × 4, 50–100 mesh, was purified according to the procedure described by Moore and Stein [7].

Concentrated sulphuric acid-d_2 (D_2SO_4) was delivered by Merck & Co. Ltd, Montreal.

The experiments with the cation exchanger were performed as follows. The specimen tube was filled with the moist ion exchanger. The solution of the substance to be studied was then poured into the tube. After removing air bubbles by shaking, the specimen was ready for recording in the spectrometer. In other experiments, the supernatant solution was removed on a Büchner funnel after equilibration, and the resin washed several times with the solvent before recording the ESR-spectrum. The concentration was 0.05 M both in the experiments on Dowex-50 and that with sulphuric acid, unless otherwise stated. The solvent used is stated in the figure captions.

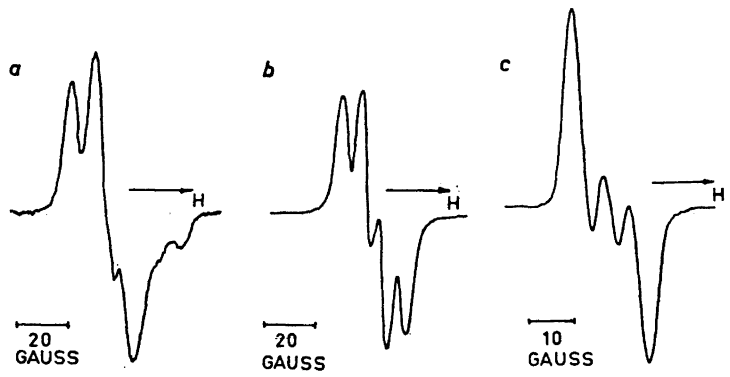


Fig. 1. Phenothiazine: *a*) on Dowex-50. Solvent: benzene. The resin was separated from the supernatant and washed with the solvent before the spectrum was recorded; *b*) in conc. H_2SO_4; *c*) in conc. D_2SO_4.

RESULTS

A. Radicals produced on activated Dowex-50 H^+. When a solution of the phenothiazine derivatives mentioned above is poured on the strongly acid, sulphonated cation exchanger Dowex-50 in the hydrogen form (H^+), ESR-spectra with a hyperfine structure are obtained which indicate the presence of free radicals. In the majority of the substances investigated, the radical concentration reached the maximum value a few minutes after the solution had been in contact with the resin. In order to obtain radicals from promethazine hydrochloride and "Aprobit", however, it was necessary to remove the solvent to some extent from the resin by drying *in vacuo* for about half an hour after the resin had been separated from the solution and washed by the solvent.

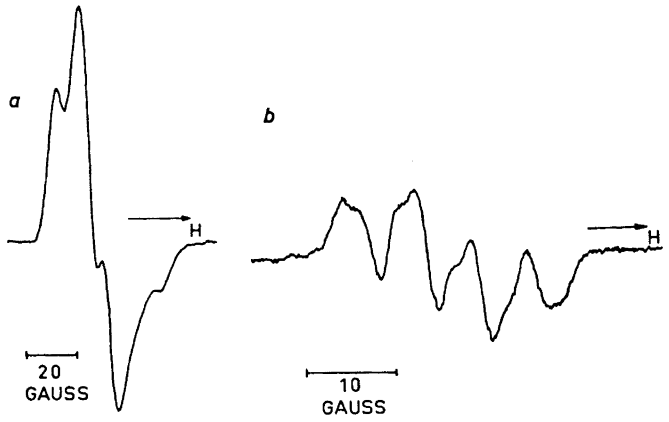


Fig. 2. 5-Oxo-phenothiazine: *a*) on Dowex-50. Solvent: ethanol. The resin was separated from the supernatant and washed with the solvent before the spectrum was recorded; *b*) in conc. H_2SO_4.

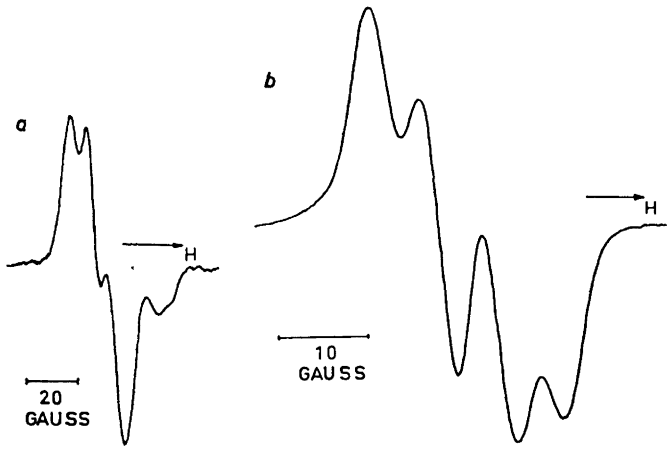


Fig. 3. Promethazine·HCl: *a*) on Dowex-50. Solvent: water. The resin was separated from the supernatant, washed with the solvent and dried for half an hour *in vacuo* before the spectrum was recorded; *b*) in conc. H_2SO_4.

The radicals formed are firmly attached to the resin-phase since the ESR-spectra remained unchanged after the resin had been separated from the solution and washed several times with the solvent.

In order to obtain free radicals, a small amount of ferric (III) iron must be present on the ion exchanger prior to absorption of the phenothiazine derivative. If the ion exchanger is washed with 4 N HCl until the wash-water is free from ferric iron, as indicated by a negative colour-reaction with alkali rhodanide, no ESR signal will be obtained. The resin can be wholly reactivated subsequent to equilibration against a 10^{-4} M $FeCl_3$ solution, followed by washing with H_2O.

With increased concentration of the ferric iron on the resin, the ESR-spectrum gradually loses the hyperfine structure, and at higher concentration a single line only is seen. When the iron concentration is rather high, as occurs after equilibration against a 0.1 M $FeCl_3$ solution, no hyperfine pattern can be observed. In this case the radicals formed are not attached to the resin, as evidenced by the nonappearance of an ESR signal subsequent to separation of the phases and after washing the resin with the solvent.

ESR-spectra similar to those registered on Dowex-50 when activated with ferric iron, can be observed when ceric (Ce^{4+}) ions are present instead of ferric iron. After treating the resin containing phenothiazine radicals with a solution of ascorbic acid in H_2O, the ESR signal is completely abolished.

Specimens of the cation exchanger with the adsorbed radicals could be stored in sealed tubes for several weeks at room temperature without any change in the ESR-spectrum.

When the resin is dried even at room temperature, unpurified preparations of Dowex-50 sometimes give rise to a single narrow ESR line without any phenothiazine substance adsorbed. This signal, which is especially pro-

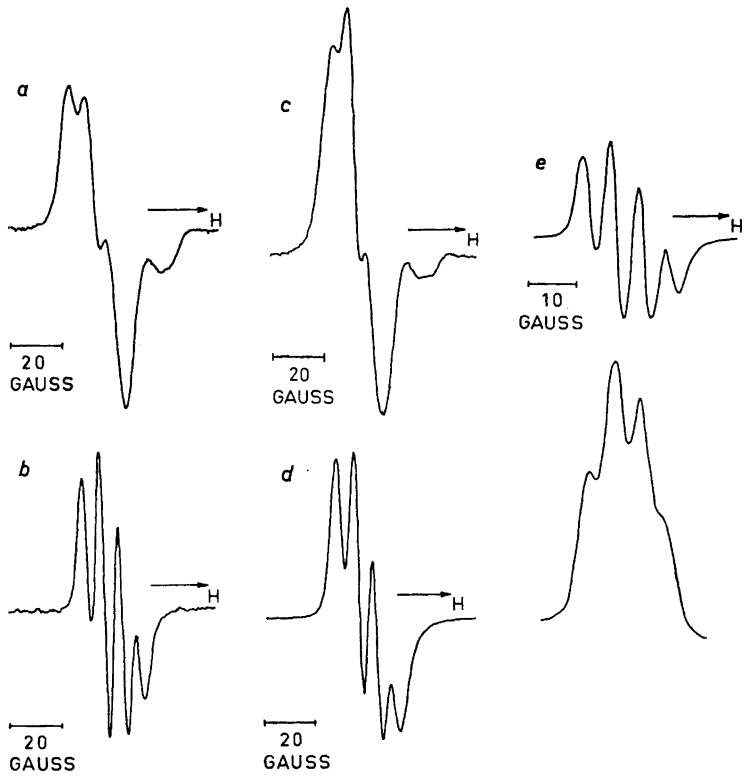


Fig. 4. Aprobit: *a*) on Dowex-50. Solvent: water. The resin was separated from the supernatant, washed with the solvent and dried for half an hour *in vacuo* before the spectrum was recorded; *b*) in conc. H_2SO_4; *c*) in conc. H_2SO_4, 77°K; *d*) in conc. D_2SO_4; *e*) in conc. H_2SO_4. Observed derivative tracing and its integrated absorption.

nounced when the resin is in the sodium form, greatly diminishes after treatment according to Moore and Stein [7]. Such a "false" signal seems in no case to have interfered in the experiments described above as indicated by control experiments.

The spectra of the radicals formed on activated Dowex-50 H^+ exhibit hyperfine splittings superimposed on a rather broad line. The extreme width of this line is about 60 gauss. A characteristic feature of the spectra is their asymmetrical shape. Introduction of a very small crystal of diphenylpicrylhydrazyl (DPPH) in the centre of a specimen tube, filled with dry cation exchanger containing adsorbed radicals, shows that the ESR signal due to DPPH is located approximately in the centre of the spectrum of the phenothiazine substance.

Phenothiazine (Fig. 1a), 5-oxophenothiazine (Fig. 2a), promethazine hydrochloride (Fig. 3a) and "Aprobit" (Fig. 4a) give rise to a four-line spectrum. Promazine hydrochloride (Fig. 5a) exhibits three lines, and chlorpromazine

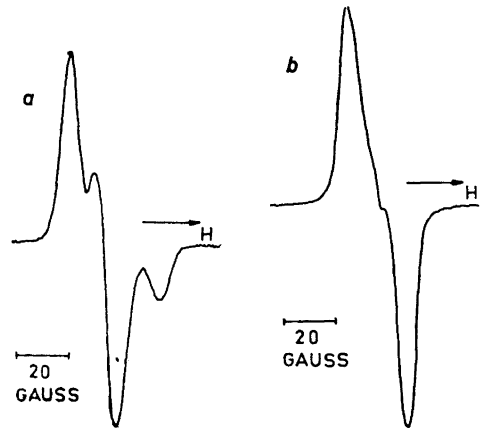


Fig. 5. Promazine · HCl: *a*) on Dowex-50. Solvent: water. The spectrum was recorded without separation of the phases; *b*) in conc. H_2SO_4.

hydrochloride (Fig. 6a) and 10-methylphenothiazine (Fig. 7a) exhibit a number of very incompletely resolved hyperfine lines.

B. Radicals produced in concentrated sulphuric acid. When the phenothiazine derivatives concerned in this case are dissolved in concentrated sulphuric acid, radicals with hyperfine splittings are produced. Compared with the corresponding spectra of the radicals formed on Dowex-50, these spectra are of a much more symmetrical shape, even if a certain asymmetry can be recognized also in these spectra. Phenothiazine (Fig. 1b), 5-oxophenothiazine (Fig. 2b), promethazine hydrochloride (Fig. 3b) and "Aprobit" (Fig. 4b) show four lines. Promazine hydrochloride (Fig. 5b) and chlorpromazine hydrochloride (Fig 6b)

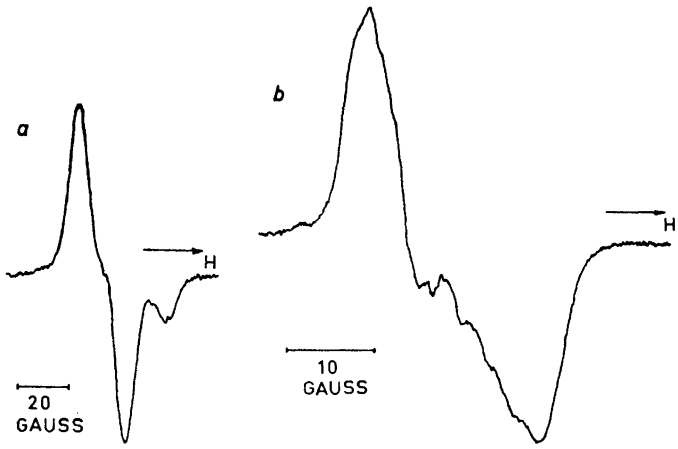


Fig. 6. Chlorpromazine·HCl: *a*) on Dowex-50. Solvent: water. The spectrum was recorded without separation of the phases; *b*) in conc. H_2SO_4.

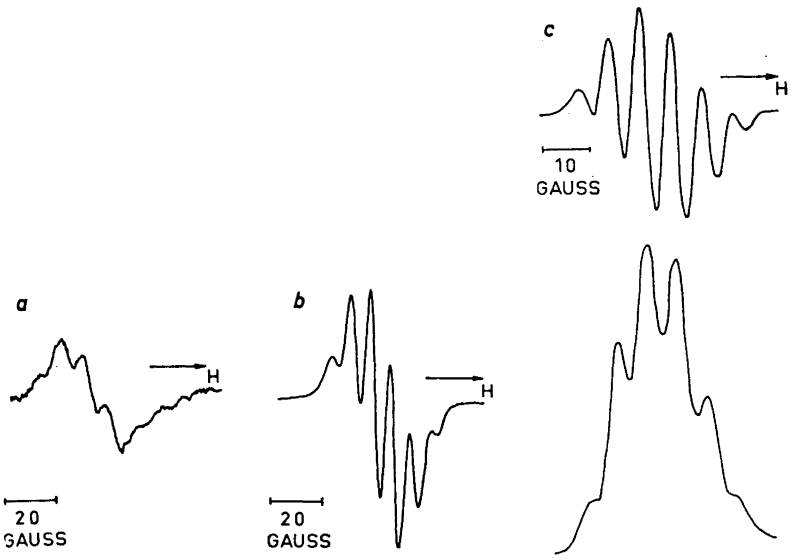


Fig. 7. 10-Methylphenothiazine: *a*) on Dowex-50. 0.1 M solution in benzene. The spectrum was recorded without separation of the phases; *b*) in conc. H_2SO_4; *c*) in conc. D_2SO_4. Observed derivative tracing and its integrated absorption.

exhibit a single line, in the centre of which an incompletely resolved hyperfine pattern can be seen.

10-Methylphenothiazine exhibits six distinct hyperfine lines both in H_2SO_4 (Fig. 7b) and in D_2SO_4 (Fig. 7c). 10-Ethylphenothiazine (Fig. 8) gives rise to an ESR-spectrum rather similar to that obtained from chlorpromazine hydrochloride, *i.e.* a single line, with an incompletely resolved hyperfine pattern in the centre. Further experiments with D_2SO_4 are described in the discussion below.

DISCUSSION

Several aromatic hydrocarbons are known to form free radicals when dissolved in concentrated sulphuric acid. The subject has been reviewed by Wertz [8] and Ingram [4]. The mechanism of the radical formation is most probably the same for the hydrocarbons and the phenothiazines here considered. The hyperfine splittings of the spectra observed when the phenothiazine substances are dissolved in concentrated sulphuric acid, will now be discussed. Consider the unsubstituted phenothiazine. By configurational interaction between the π-orbital of the odd electron and a σ-state of the ring nitrogen, the electronic levels are split into three ($M_I=1$). By further interaction with a σ-state of the hydrogen attached to the ring nitrogen, each of these levels are split in two ($M_I=\frac{1}{2}$). By overlapping of some of the last mentioned levels, four lines are produced as shown in the suggested energy level diagram in Fig. 9.

When 10-methylphenothiazine is dissolved in concentrated sulphuric acid, six lines are observed. This spectrum is consistent with the interaction between the π-orbital of the ring system, the ring nitrogen, and the three equivalent hydrogens of the methyl group (see Fig. 9).

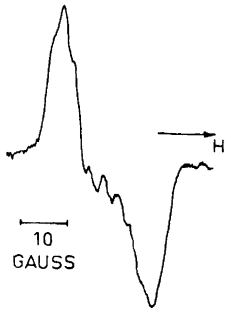


Fig. 8. 10-Ethylphenothiazine in conc. H_2SO_4.

The relative intensities of the four-line spectrum of "Aprobit" in H_2SO_4 (Fig. 4e) and of the six line spectrum of 10-methylphenothiazine in D_2SO_4 (Fig. 7c) obtained by double integration of the derivative curves are shown in Table 1 together with the theoretical intensity distribution consistent with the energy level diagram in Fig. 9. For comparison, the line intensities corresponding to the interaction of the odd electron with three and four equivalent hydrogens respectively (binomial distribution) are also shown. The distributions obtained experimentally seem to support the splitting mechanism suggested in Fig. 9, but the asymmetry of the curves and the incomplete separation make the tracing of the individual lines somewhat ambiguous. The intensity distribution obtained must therefore be considered uncertain.

The interaction of the odd electron with the methyl hydrogens in 10-methylphenothiazine is of a type different from that of the configurational interaction with an excited state, and is due to a spatial overlapping of wave functions with the same symmetry. This mechanism is generally termed hyperconjugation. In order to obtain an equal interaction with all the three methyl hydrogens, it is necessary to assume that the methyl group is rotating with a frequency faster than that of the hyperfine splitting (Ingram *et al.*[9]).

Table 1.

Relative line intensities	Four-line spectrum "Aprobit" in H_2SO_4 Fig. 4e	Six-line spectrum 10-Methylphenothiazine in D_2SO_4 Fig. 7c
Found	1 : 2.2 : 2.0 : 0.8	1 : 4.1 : 7.4 : 6.6 : 3.2 : 1.1
Calculated according to the energy level diagram in Fig. 9	1 : 2 : 2 : 1	1 : 4 : 7 : 7 : 4 : 1
Binomial distribution	1 : 3 : 3 : 1	1 : 5 : 10 : 10 : 5 : 1

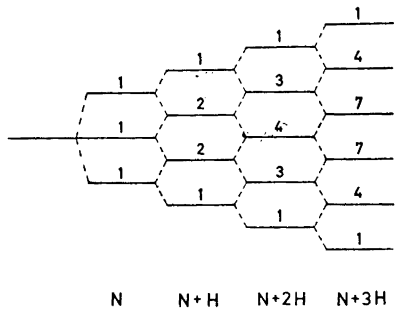


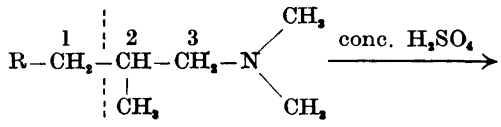
Fig. 9. Energy level diagram of the splitting due to one nitrogen, one nitrogen and one hydrogen, one nitrogen and two hydrogens, one nitrogen and three hydrogens. Only one of the two electronic levels is shown. The figures give the relative intensity distributions.

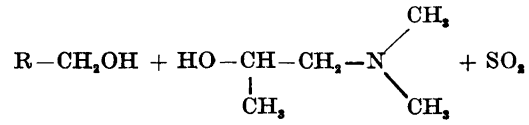
In the ethyl group of 10-ethylphenothiazine there are two different kinds of hydrogen position. Hence, the hyperfine pattern should be the combination of an interaction with two equivalent hydrogens in the methylene group and the ring nitrogen which gives rise to a five-line spectrum, and an interaction with the three methyl hydrogens which will split each of these lines into another four lines, if a non-integral relation between the coupling constants of the two kinds of hydrogen is assumed. Due to the large number of lines and perhaps also the relatively low spin density of the unpaired electron at the hydrogens most distant from the ring nitrogen, such a predicted hyperfine pattern will not be resolved. (Fig. 8). A similar interpretation seems to be valid for the incompletely resolved hyperfine spectra of promazine hydrochloride (Fig. 5b) and chlorpromazine hydrochloride (Fig. 6b), where the unpaired electron interacts with the σ-state of the hydrogens in the amino-alkyl chain. A possible interaction with the nitrogen of the side chain will further complicate the hyperfine pattern.

In none of the experiments described is there any evidence for an interaction of the unpaired electron in the π-orbital of the ring system with the edge protons of the two symmetrical side-rings of the phenothiazine nucleus. This means that the unpaired electron is distributed mainly over the central ring and in the side chain. This behavior is probably connected with the presence of the sulphur atom, which is known to have a localizing effect on the electron distribution. Naturally, such a suggestion does not exclude a further splitting due to the edge protons that will be observed, perhaps, in a highly resolved spectrum.

In order to explain the four-line pattern of promethazine hydrochloride (Fig. 3b) and "Aprobit" (Fig. 4b), it is necessary to assume a cleavage of the amino-alkyl side chain leaving, attached to the ring nitrogen of the phenothiazine nucleus, a group which makes interaction with one hydrogen possible. The presence of a branching in the carbon side chain of promethazine hydrochloride and "Aprobit" contrary to the situation prevailing in the other phenothiazine derivatives investigated, makes such a splitting probable. This assumption is further supported by the fact that there is a vigorous evolution of

SO_2 with foaming, when the promethazine hydrochloride and "Aprobit" are dissolved in concentrated sulphuric acid. Such a reaction is not observed for phenothiazine derivatives which have an unbranched side chain. The side chain is probably split between the carbons 1 and 2 according to the formula





R = phenothiazine nucleus.

It is also necessary to assume a restricted rotation of the hydroxymethyl group left attached to the ring system after the splitting of the carbon side chain, thus allowing interaction with only one of the hydrogens of the hydroxymethyl group. The hypothesis of a splitting of the side chain between carbons 1 and 2 is further strengthened by the fact that when "Aprobit" is dissolved in concentrated deuterium sulphuric acid (D_2SO_4), a four-line spectrum (Fig. 4d) is observed, which appears very similar to that obtained in H_2SO_4 (Fig. 4b). In case of a split between the ring nitrogen and the alkyl chain, the four-line spectrum of "Aprobit" observed in H_2SO_4 (Fig. 4b) had to be due to a radical of a structure very similar to, or identical with, that obtained from unsubstituted phenothiazine, *i.e.* to a radical involving a hydrogen attached to the ring nitrogen. The last mentioned radical structure for "Aprobit" after splitting is not very probable, since unsubstituted phenothiazine exhibits a three-line spectrum when dissolved in D_2SO_4 (Fig. 1c) due to exchange of the hydrogen attached to the ring nitrogen. The deuterium nucleus has a magnetic moment, but this is about three times smaller than that of the proton (*cf.* Kopferman [10], p. 398). It has also a spin of 1 instead of ½ for the proton, and hence, the expected splitting of the nitrogen levels due to the deuterium atom seems to be unresolved under the actual experimental conditions. A four-line spectrum in H_2SO_4 and a three-line spectrum in D_2SO_4 might also be consistent with a radical structure in which a hydroxyl group is left attached to the ring nitrogen. However, it seems to be a general feature of ESR-spectra that no interaction is obtained with the protons of hydroxyl groups (Ingram *et al.*[11]), with the result that a four-line spectrum in H_2SO_4 and a three-line spectrum in D_2SO_4 therefore appear to be wholly consistent with a radical structure with a hydrogen attached to the ring nitrogen as in unsubstituted phenothiazine. Consequently the four-line spectrum both in H_2SO_4 and in D_2SO_4 supports the hypothesis that "Aprobit" is split between carbons 1 and 2 of the side chain, as shown in the formula. In this connection it may be pointed out that there is no evidence for an exchange of the hydrogens of the methyl group

attached to the ring nitrogen, since 10-methylphenothiazine shows a six-line spectrum both in H_2SO_4 (Fig. 7b) and in D_2SO_4 (Fig. 7c).

The main difference between the radical spectra observed in sulphuric acid and those obtained on activated Dowex-50 is the highly asymmetrical shape of the latter. The asymmetry may be due either to the presence of more than one radical species or an anisotropy of the g-value. The last alternative infers that the g-value along the symmetry axis of the radical molecule is not equal to that in an orthogonal direction. As long as the radicals are free to rotate at a frequency of the same order of magnitude as that of the microwave power used, the effect of the g-value anisotropy is blotted out. When the rotation is restricted, however, the actual ESR-spectrum is a superposition of spectra corresponding to a series of randomly orientated symmetry axes.

In radicals containing a sulphur atom, the unpaired electron is known to be incompletely delocalized and the ESR-spectrum to exhibit a g-value anisotropy. Consequently, the presence of a sulphur atom in the phenothiazines suggests that the asymmetrical shape of the radical spectra observed on Dowex-50 depends on a g-value anisotropy combined with a restricted rotation of the radicals when attached to the ion exchanger. Asymmetrical ESR-spectra analogous to those here discussed have been described by Faber and Rogers [12] in an investigation of paramagnetic ions adsorbed on cation exchangers.

In order to decide whether the asymmetry is due to a g-value anisotropy or not, "Aprobit" was dissolved in concentrated H_2SO_4 and the ESR-spectrum recorded at room temperature (Fig. 4b). It was now observed that the symmetrical spectrum changed into an asymmetrical one, which was very similar to that obtained on Dowex-50 at room temperature, when the solution in sulphuric acid was cooled to the temperature of liquid air (Fig. 4c). The rotation of the radical molecules is thus hampered both when fixed to the resin-phase at room temperature and when the solution in sulphuric acid is cooled to a very low temperature. The small asymmetry that can be recognized in the spectra of the sulphuric acid at room temperature indicates a slight degree of restricted rotation even at this temperature due to the viscosity of the solution. From the experiment described it is evident that the asymmetrical shape of the radical spectra observed on Dowex-50 very probably depends on a g-value anisotropy and a restricted rotation, and not to the presence of more than one radical species.

When comparing the radical spectra for each substance observed in sulphuric acid with those on Dowex-50, it is noted that the number of lines is equal for several of the substances. Thus phenothiazine, 5-oxophenothiazine, promethazine hydrochloride, and "Aprobit" exhibit a four-line spectrum both in sulphuric acid and on Dowex-50. This fact implies that the branched chain of promethazine hydrochloride and of "Aprobit" is split not only in sulphuric acid, but also in the redox reaction on Dowex-50. The six hyperfine lines of 10-methylphenothiazine in sulphuric acid (Figs. 7b and 7c) are not resolved on Dowex-50 (Fig. 7a) probably due to the anisotropy effect and the restricted rotation discussed above, which blur the individual lines. When comparing the radical spectra of promazine hydrochloride (Fig. 5b) and chlorpromazine hydrochloride (Fig. 6b) observed in sulphuric acid with those obtained on Dowex-50 (Figs. 5a and 6a), it is expected that the incompletely resolved hyperfine

pattern in sulphuric acid should be further impaired on Dowex-50 due to the anisotropy effect and restricted rotation. Such a state of affairs appears to prevail in the case of chlorpromazine hydrochloride (Figs. 6a and b). However, the three-line spectrum of promazine hydrochloride on Dowex-50 (Fig. 5a) is not expected and the origin is not wholly understod. It may depend on a non-interaction of the three nitrogen levels with the side chain hydrogens due to improper orientation.

From the discussion above it is obvious that the radicals formed by the one-electron oxidation with ferric iron on the cation exchanger are very probably of a structure similar to those found in sulphuric acid.

Free radicals really being formed *in vivo* from phenothiazine derivatives and the possible role of natural ferric-compounds in such a hypothetical reaction are open questions, as are the importance of such radicals for the pharmacological activity of these compounds.

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