SPECTROSCOPIC STUDIES OF THE REACTION OF HYDROXYLATED PROMAZINES AND RELATED COMPOUNDS WITH SULPHURIC ACID

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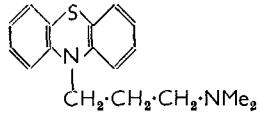
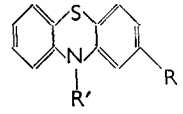
The absorption spectra of the products from the reaction of various hydroxy- and methoxy-phenothiazine derivatives with sulphuric acid are described, and their application to problems of identification of metabolites of promazine and other phenothiazine drugs is discussed. A discussion of the nature of the reaction products is included.

Elucidation of the structure of the metabolites of phenothiazine drugs in man and animals has been complicated by the difficulty of synthesising suitable reference compounds, and by the lack of effective analytical methods for comparing unknown metabolites with standard compounds. It is further complicated by the small amount and impure condition of isolated metabolites.

In dog and man, promazine (I) is oxidised at the sulphur atom and demethylated at the tertiary nitrogen atom of the dimethylaminopropyl side chain (Walkenstein and Seifter, 1959).



(I)



(II)

In man the most important route of metabolism is by hydroxylation in the nucleus of the molecule and subsequent conjugation with glucuronic acid through the hydroxyl group introduced (Beckett and Bolt, unpublished results). No satisfactory method has yet been described for the determination of the position of hydroxylation and it has not yet been possible to synthesise all the ring hydroxylated promazines.

Phenothiazine derivatives react with concentrated sulphuric acid to give products with absorption patterns different from those of the original compounds in both the visible and ultra-violet regions of the spectrum. Dubost and Pascal (1953, 1955) showed that chlorpromazine and its sulphoxide give red colours with concentrated sulphuric acid and used the reaction in a colorimetric assay of these compounds. Rieder (1960) studied the products of this reaction from a number of phenothiazine derivatives and related the position of the main absorption band in the visible region of the spectrum to the nature of the substituent R in II. Street (1962) recorded the absorption maxima of a number of compounds of this type in both the ultra-violet and visible regions of the spectrum before and after treatment with sulphuric acid, and based a quantitative assay as well as a method of qualitative identification, on the intensity and position of the peaks in the region 270 to 300 mμ. Beckett, Beavan

and Robinson (1963) reported on the different absorption maxima in the visible region of the spectrum for the various derivatives in concentrated sulphuric acid solution, and used the extinction value for a semiquantitative assay in studies of the metabolism of chlorpromazine.

Because of the necessity to identify a promazine glucuronide of unknown structure, we have studied further the differences in spectral characteristics of various hydroxy-phenothiazines after treatment with sulphuric acid. It has been found that sufficient differences exist between the spectra produced from the reference compounds examined, to enable the position of ring hydroxylation of promazine metabolites to be determined.

Experimental

Materials

1-Hydroxypromazine hydrochloride (m.p. 210–211°), 4-hydroxypromazine (m.p. 166–167°), 3-methoxypromazine hydrochloride (m.p. 144–145°), and 3-hydroxyphenothiazine (m.p. 177·5–178·5°) were obtained from Dr. H. S. Posner; 2-hydroxypromazine (m.p. 176–178°) and 2-methoxyphenothiazine (m.p. 179–181°) from May and Baker Ltd.; 2-methoxypromazine maleate (m.p. 141–145°) from Smith, Kline and French Ltd.

Infra-red spectra of these compounds were consistent with their stated structures.

General Method of Reaction with Sulphuric Acid

The compound under examination (approx. 100 μg.) was dissolved in the minimum quantity of 70 per cent ethanol and added to 4 ml. of a solution of equal parts by volume of concentrated sulphuric acid and 70 per cent ethanol, to give a final concentration of approximately 25 μg./ml. The absorption measurements were made 15 min. after the phenothiazine compound was added to the ethanol-sulphuric acid mixture. Measurement in the visible region of the spectrum was made at the above concentration, but for ultra-violet measurements the solution was diluted with four times its own volume of the ethanol-sulphuric acid mixture.

Absorption Measurements

Absorption maxima were determined between 200–700 mμ for the untreated compounds and for the reaction products with a Beckman DK2 ratio recording spectrophotometer, using 1 cm. fused silica cells.

Results and Discussion

Discussion of the Method

The intensity of the colour produced in the reaction is largely dependent on the reaction conditions. The significant factors are the concentrations of the sulphuric acid and of the phenothiazine derivative, the temperature reached during the reaction, and the time taken for development of the colour. The influence of temperature increase resulting from mixing sulphuric acid and 70 per cent ethanol is minimised in the method described. Environmental temperature exerts a relatively slight effect on the extinction value. Under all conditions, extinction increases with time after mixing.

These factors have been evaluated in an attempt to devise a quantitative assay (unpublished results).

Using sulphuric acid concentrations between 25 and 65 per cent, v/v as in the method described above, reproducible absorption maxima can be obtained within a period of at least 1 hr. after mixing the reagents. Satisfactory extinctions (0·3–0·7) likewise result and allow the method to be applied to the qualitative identification of the phenothiazine derivatives examined. 70 per cent ethanol was used as a solvent because of its capacity to dissolve both the salts and the free bases of the compounds concerned.

Discussion of the Results

Although the absorption patterns of the pure phenothiazine derivatives examined are similar, sulphuric acid treatment produces sufficiently different spectra to allow the compounds to be distinguished. Fig. 1 shows the absorption spectra of the three hydroxypromazine derivatives examined and Fig. 2 their spectra after treatment.

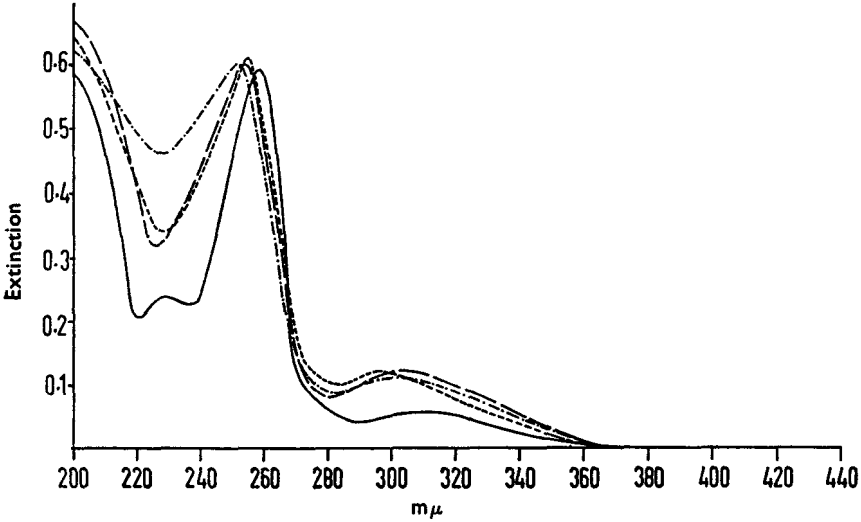


FIG. 1. Absorption spectra of some hydroxy- and methoxy-promazine reference compounds. —, 1-hydroxy-, — · —, 2-hydroxy-, — —, 3-methoxy-, – – – –, 4-hydroxypromazine.

In the case of 3-substituted derivatives (II), 3-hydroxypromazine is apparently not yet available, although the 3-methoxy compound has been synthesised (Posner, personal communication*). The absorption spectra of 2-methoxypromazine and 2-hydroxypromazine after sulphuric acid treatment are almost superimposable (see Table I); by analogy we suggest that the spectra from 3-methoxypromazine and 3-hydroxypromazine would likewise correspond, and that 3-methoxypromazine can therefore be used as a reference standard in determining the position of hydroxylation of promazine metabolites. The spectra of 3-methoxypromazine

* Since submission of the manuscript, 3-hydroxypromazine has become available.

before and after reaction with sulphuric acid are included in Figs. 1 and 2. Spectroscopic characteristics of equivalent sulphides and sulphoxides after treatment are identical.

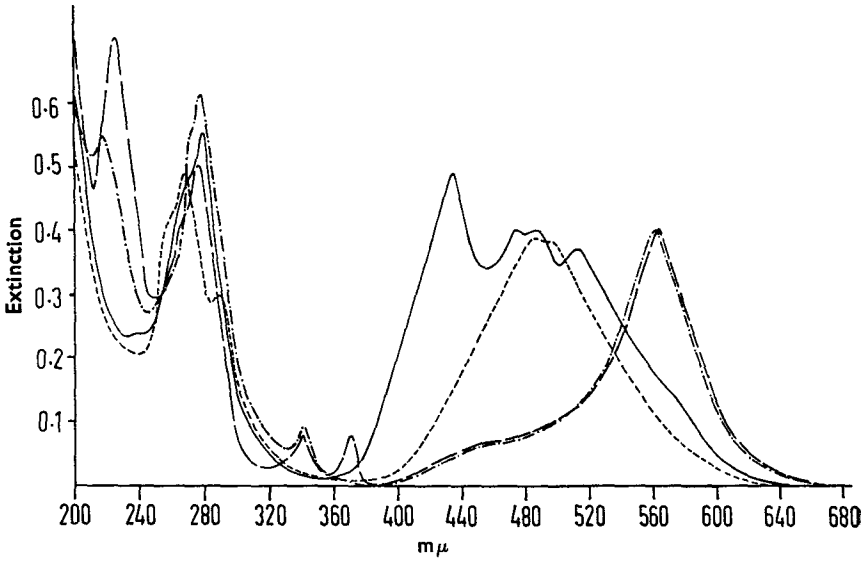


FIG. 2. Absorption spectra of the hydroxy- and methoxy-promazine derivatives of Fig. 1, after treatment with sulphuric acid. Key as in Fig. 1. Dilution (see method) is necessary at about 320 mμ.

The spectra of 2- and 3-substituted derivatives, after sulphuric acid treatment are similar, differing significantly only between 340 and 380 mμ. Further evidence that the peak at approx. 370 mμ is a feature of 3-substituted phenothiazines as opposed to their 2-substituted isomers is provided by the spectra generated from 2-methoxy and 3-hydroxyphenothiazine (Fig. 3). This Figure indicates that the spectral characteristics between 340 and 380 mμ after sulphuric acid treatment are controlled by the position of the ring substituent, and also shows that the nature of R′ in II has no qualititative effect on the spectrum in this region. Confirmation of this difference between 2- and 3-substituted derivatives is important since these are the most likely sites of biochemical hydroxylation.

TABLE I

ABSORPTION MAXIMA OF THE PRODUCTS OF THE REACTION BETWEEN VARIOUS HYDROXY AND METHOXY DERIVATIVES OF PROMAZINE AND 50 PER CENT V/V SULPHURIC ACID IN ETHANOL AT ROOM TEMPERATURE

Compound	λ (mμ)													
1-Hydroxypromazine			(270)	281						477		490		513	
2-Hydroxypromazine	219			278		343			(440)						558
2-Methoxypromazine	219			278		343			(440)						565
2-Methoxyphenothiazine	219			277		343		428							554
3-Methoxypromazine		225		279		342	372								565
3-Hydroxyphenothiazine		221		276		342	369		443						549
4-Hydroxypromazine			(262)	271	292						486		500		

Figures in brackets indicate shoulders on the main peaks.

The absorption maxima of the various compounds mentioned above are summarised in Table I, and a careful comparison of the spectrum of an unknown aglycone after sulphuric acid treatment with this reference data will provide conclusive evidence about the position of mono-hydroxylation of the promazine metabolite. It is also realised that evidence about the structures of aglycones from glucuronides of other phenothiazine drugs can be obtained in this way, provided it is possible to obtain the necessary reference data from the equivalent model metabolites.

It seems probable (see later) that, after sulphuric acid treatment, monoglucuronides of promazine would have the same spectra as their aglycones. Identification of metabolites would thus be facilitated because the glucuronides are more stable than their parent phenothiazine aglycones.

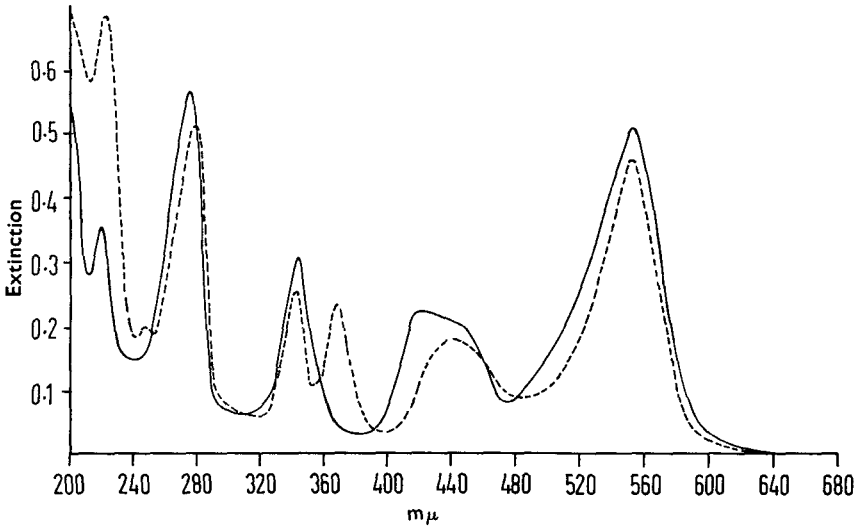
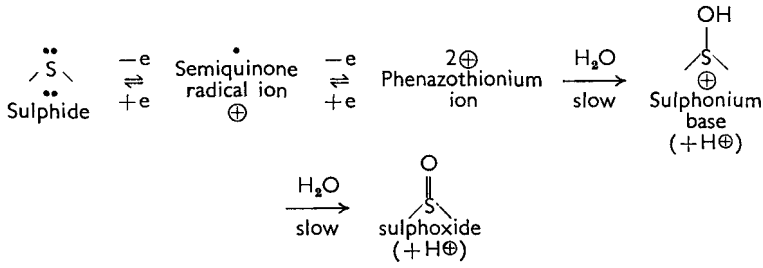


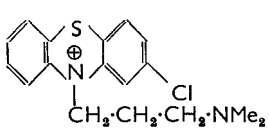
FIG. 3. Absorption spectra of the coloured oxidation products of 2-methoxyphenothiazine (—) and 3-hydroxyphenothiazine (- - -). Dilution as in Fig. 2.

The Nature of the Reaction Products

Reaction sequences have been described to explain the bivalent reversible oxidation of phenothiazine and related compounds (Cymerman Craig, 1960; Granick, Michaelis and Schubert, 1940; Michaelis and Granick, 1941; and Michaelis, Granick and Schubert, 1941). The oxidation involves semiquinone free radicals as intermediates.

Using chlorpromazine as an example, Borg and Cotzias (1962) have applied these theories to phenothiazine derivatives with a side chain on the nitrogen atom of the nucleus and have shown that the first product of oxidation arises by loss of one electron, and is a semiquinone radical ion. Its formula is best represented by III. This product then loses a second electron to form the phenazothionium ion IV. In the presence of water the phenazothionium ion forms a sulphonium base, and then chlorpromazine sulphoxide. The general process is represented as follows—(Borg and Cotzias, 1962).





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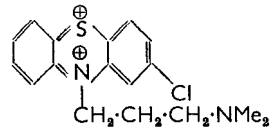


(IV)

Our own unpublished studies with chlorpromazine have shown that the compound is oxidised to its colourless sulphoxide through a coloured intermediate by refluxing with 50 per cent sulphuric acid. Under mild conditions the reaction proceeds only as far as the coloured product. The absorption spectrum of this product is the same as that produced from chlorpromazine by Borg and Cotzias. Free radicals have been demonstrated in solutions of phenothiazine derivatives in strong sulphuric acid (Piette and Forrest, 1962). Hence sulphuric acid acts on chlorpromazine and other phenothiazine compounds under atmospheric conditions by an oxidation process involving bivalent loss of electrons. In the presence of the acid at room temperature the steps involving loss of protons in the above reaction sequence are obviously inhibited. The coloured product is the semiquinone radical ion.

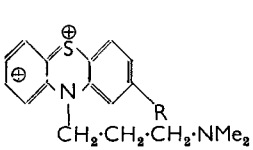
TABLE II

ANODIC HALF-WAVE POTENTIALS OF OXIDATION OF SULPHIDES, AND ABSORPTION MAXIMA OF PHENAZOTHIONIUM IONS, OF COMPOUNDS RELATED TO PROMAZINE. ANODIC HALF-WAVE POTENTIALS ARE QUOTED FROM THE WORK OF EISDORFER AND HAINES (PERSONAL COMMUNICATION) AND ABSORPTION MAXIMA FROM THAT OF STREET (1962), AND RIEDER (1960)

Compound	R =	Anodic $E_{\frac{1}{2}}$	λ max. (mμ) of absorption of oxidation product
2-Methoxypromazine	OMe	+0·459	567
Promazine	H	+0·517	510–512
Chlorpromazine	Cl	+0·578	527–529
Fluopromazine	CF_3	+0·642	500

The ease of oxidative loss of electrons in II will be influenced by the character of R. Anodic half-wave potentials at pH 5 and 25° for loss of the first electron indicate that loss occurs more readily in the series R = OMe > H > Cl > CF_3 as one proceeds from groups which are electron donating to those which are electron attracting. The absorption maxima (Table II) of the sulphuric acid oxidation products however are not in the same sequence as above; wavelength is reduced in the order R=OMe > Cl > H > CF_3, since in the sulphide a +T effect is exerted by a –OMe substituent, and a –I effect is exerted by a –Cl or –CF_3 substituent,

whereas in the oxidation product the –Cl group exerts a +T effect while the effect of other substituents is unchanged. The +T effects from –OMe and –OH substituents are similar and the oxidation products have similar spectra. For this reason it is believed that glucuronides and aglycones would behave similarly.



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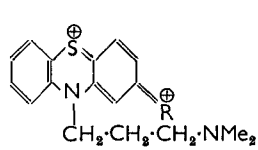

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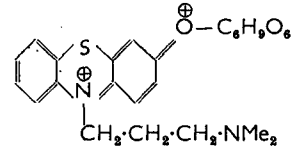

(VII)

In the product from oxidation with sulphuric acid a contribution is made by the phenazothionium ion. From 2-methoxypromazine, 2-hydroxypromazine and chlorpromazine this consists principally of structures IV and VI: from fluopromazine it consists of structures IV and V. As shown by the hydroxypromazines, the position of the hydroxyl group in the phenothiazine nucleus has an influence on the spectrum produced. 2- and 3-Substituted compounds give rise to phenazothionium ions which are *p*-quinones, whereas 1- and 4-substituted compounds give rise to *o*-quinones. In the latter the charge separation is less than in the former, and the wavelength of absorption (Table I) is thus lower. The ions from 1-hydroxypromazine and 3-hydroxypromazine have one end of the quinonoid system at the heterocyclic N atom, and those from 2-hydroxypromazine and 4-hydroxypromazine have the corresponding end of the system at the sulphur atom. Thus electronically the four phenazothionium ions are different, and this is illustrated spectroscopically by their absorption maxima (Table I), although in some of the cases the differences exist only in the fine structure of the spectrum.

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The paper was presented by MR. CURRY.