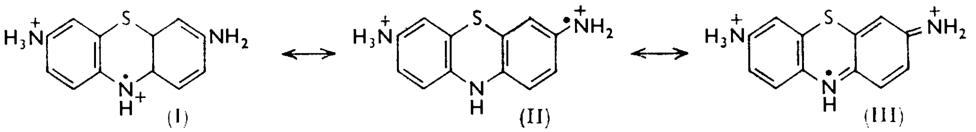
384. *Electron-spin Resonance Observations on the Reduction of Methylene Blue and Related Compounds.*

By P. B. Ayscough and C. Thomson.

Methylene Blue and other thiazine dyes can be reduced by *X*-irradiation in strongly acid solutions containing ethanol or benzoic acid to the same paramagnetic intermediates as are obtained by chemical reduction. Electron-spin resonance spectra have been used to establish the identity of these semiquinones and to provide information regarding the mechanism of the radiolytic reduction.

The use of electron-spin resonance methods to examine the intermediate compounds formed during the oxidation or reduction of organic compounds has yielded much interesting information regarding the electronic structure of free radicals and the mechanism of their formation and destruction. When the proton hyperfine structure is fully resolved, as in the case of the benzosemiquinones,[1] these methods are capable of considerable refinement and permit precise evaluation of the effect of substituent groups on the electron distribution within the semiquinone.

Reduction of thiazine and oxazine dyes has been shown by potentiometric and spectroscopic methods to proceed by a mechanism involving an intermediate semiquinone ion which is stable in strongly acid solution.[2] There has been considerable speculation regarding the structure of this species. Michaelis and his collaborators,[2] for instance, have suggested that the semiquinone is doubly protonated and that its stability may be attributed to the existence of a number of " equivalent " resonance forms such as (I), (II), and (III).


(I) (II) (III)

This implies that the orbital of the unpaired electron extends to the side amino-groups, which should therefore contribute to the hyperfine structure observed in the electron-spin resonance spectra.

There has also been considerable interest in the radiolysis of aqueous solutions of thiazine dyes, especially Methylene Blue,[4] and it has been shown recently that stable solutions of the semiquinone of Methylene Blue can be prepared by the use of visible light [5] or ionising radiation.[6] We have therefore examined the electron-spin resonance spectra of a number of thiazines and oxazines, reduced by chemical means or by *X*-irradiation to the semiquinone, in order to establish the structure of the paramagnetic intermediate. Some further observations on the radiolysis of strongly acid solutions of Methylene Blue containing radical scavengers permit some speculation on the mechanism of the radiolytic reduction.

Experimental

The thionine and Methylene Blue were commercial samples, used without further purification. The other substituted thiazines were kindly provided by the Department of Colour Chemistry. Their purity is not known. Sulphuric acid, ethanol, and benzoic acid were of

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[1] Venkataraman and Fraenkel, *J. Amer. Chem. Soc.*, 1955, **77**, 2707; *J. Chem. Phys.*, 1955, **23**, 588; Venkataraman, Segal, and Fraenkel, *ibid.*, 1959, **30**, 1006.

[2] Michaelis, Schubert, Reber, Kuch, and Granick, *J. Amer. Chem. Soc.*, 1938, **60**, 1678.

[3] Michaelis, Schubert, and Granick, *J. Amer. Chem. Soc.*, 1940, **62**, 204; Michaelis, Granick, and Schubert, *ibid.*, 1941, **63**, 351.

[4] Stern, *Discuss. Faraday Soc.*, 1952, **12**, 227.

[5] Koizumi and Obata, *Bull. Chem. Soc. Japan*, 1958, **31**, 823.

[6] Swallow, *J.*, 1957, 1553.

" AnalaR " quality. Dideuteriosulphuric acid was prepared from sulphur trioxide and 95% deuterium oxide.

The electron spin resonance spectrometer is the Varian model V-4500, operating at about 9400 Mc./sec. The electromagnet was made by Mullard Ltd. It has pole faces 10 in. in diameter and an electronically stabilised power supply capable of stabilising the field to within 1 part in 10^5. The homogeneity of the field in the central inch is better than 0·030 gauss. Sample tubes used in this work were made of soda-glass capillary tubing, of 1 mm. internal diameter, about 3 cm. long, with a small bulb blown on one end. Irradiations were carried out with the solution in the bulb, so that the part of the tube which entered the cavity did not receive enough irradiation to produce a significant electron-spin resonance signal. Wider tubing could not be used with these solutions without overloading the cavity and reducing the sensitivity.

RESULTS

Reduction of Thionine and Methylene Blue by Titanous Chloride.—Solutions of thionine and Methylene Blue ($\sim 1{\cdot}6 \times 10^{-3}$M) in concentrated sulphuric acid (about 23N) were titrated with titanous chloride in concentrated sulphuric acid in an atmosphere of nitrogen. For examination of the electron-spin resonance spectra, half the amount of titanous chloride needed for complete reduction to the leuco-form was added to the sample of dye and placed in a capillary tube of the same size as those used for the radiolysis experiments. The solutions at this stage were pale green and both showed a well-resolved electron-spin resonance spectrum consisting

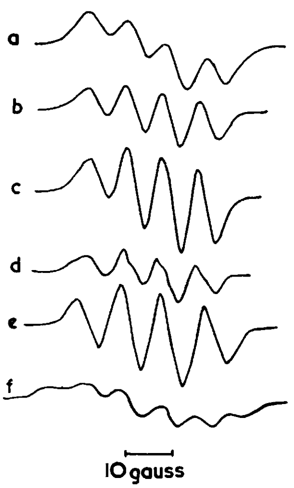


FIG. 1.

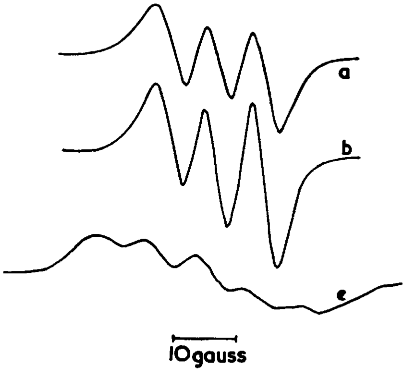
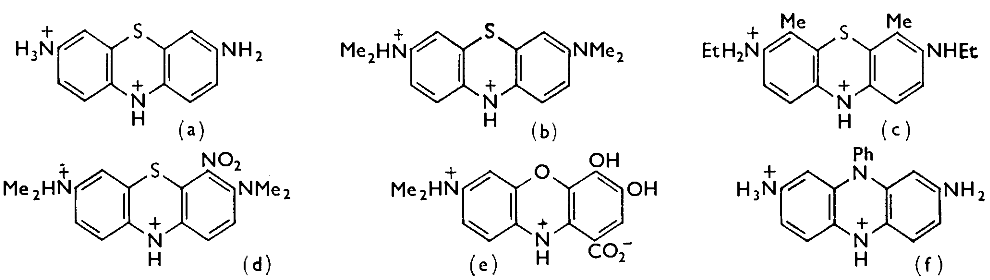


FIG. 2.

FIGS. 1 & 2. Electron-spin resonance of *X*-irradiated thiazines, etc., (Fig. 1) in 23N-H_2SO_4 and (Fig. 2) 23N-D_2SO_4.

(a) Thionine; (b) Methylene Blue; (c) New Methylene Blue; (d) Methylene Green; (e) Phenosafranine; (f) gallocyanine.



of four approximately equally spaced lines (see Fig. 1). A solution of Methylene Blue in ~11N-sulphuric acid showed a similar spectrum, much reduced in size, while a solution in water, treated in the same way, gave no spectrum.

Radiolysis of Solutions of Thionine, Methylene Blue, and Related Compounds.—The electron-spin resonance spectra of five thiazines, one oxazine, and one phenazine in sulphuric acid were examined after a period of *X*-irradiation varying from 5 to 40 min. at a dose rate of about 10^{17} ev ml.$^{-1}$ min.$^{-1}$. The solutions, $1 \cdot 6 \times 10^{-3}$M in dye and 23N in sulphuric acid, were deaerated before irradiation. All solutions contained ethanol, at a concentration of ~0·1M. The results of these experiments are shown in the Table and Fig. 1.

Electron-spin resonance spectra of *X*-irradiated solutions of various dyes in 23N-H_2SO_4 or -D_2SO_4.

Solute	Solvent	No. of hyperfine lines	Average separation (Mc/sec.)	Relative peak height *
Thionine	H_2SO_4	4	22	100
Methylene Blue	,,	4	21·5	135
New Methylene Blue	,,	4	21·5	210
Methylene Green	,,	4	21·5	8·5
Gallocyanine	,,	4	25	19 †
Phenosafranine	,,	6	22	6·5
Thionine	D_2SO_4	3	21·5	85
Methylene Blue	,,	3	20·5	120
Phenosafranine	,,	5	22	12·5

* Peak heights are corrected to constant radiation dose, a linear dependence of concentration on dose being assumed. † Decomposes on storage.

It will be observed that the spectra of thionine, Methylene Blue, New Methylene Blue, and Methylene Green are very similar, consisting of four almost equally spaced lines with the average separation slightly but significantly greater in the case of thionine. The spectra obtained from thionine and Methylene Blue are identical with those obtained by chemical reduction. The line separation of the spectrum observed in irradiated gallocyanine is appreciably greater but the pattern is otherwise identical, the four peaks having an intensity ratio close to 1 : 2 : 2 : 1 when observed under low microwave power. Phenosafranine, containing two ring nitrogen atoms, is reduced to a species with a poorly resolved six-line spectrum in which the relative intensities are approximately 1 : 3 : 5 : 5 : 3 : 1.

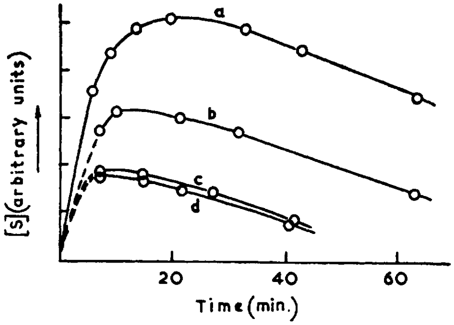


FIG. 3. Variation with time of irradiation in peak heights of semiquinone [S], of Methylene Blue ($1 \cdot 6 \times 10^{-3}$M) in 23N-H_2SO_4 with, as scavengers, (a) 0·1M-ethanol, (b) 0·02 M-benzoic acid, (c) 0·005M-ethanol, and (d) 0·005M-benzoic acid.

When thionine and Methylene Blue were dissolved in dideuteriosulphuric acid and irradiated as before, spectra consisting of three equally intense lines were observed in both cases, and phenosafranine in the deuterated acid gave a spectrum with five lines instead of six (see Fig. 2). The average separation of the lines was unchanged.

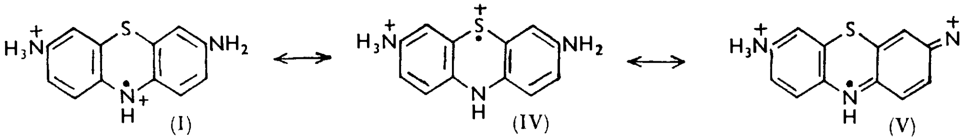
Samples of Methylene Blue irradiated in ~11N-sulphuric acid displayed the same spectrum but reduced in magnitude by a factor of ten. The use of higher concentrations of acid also caused a reduction in the peak size, irradiation in 28N-sulphuric acid reducing the peak size by a half compared with 23N-acid, while no spectra could be observed when 98% acid was used. Thionine showed a similar, though less marked, effect.

No detailed study of the effect of the radiation dose or of the nature or concentration of scavenger was made, though a number of preliminary experiments have demonstrated some significant features of the complex interactions between these factors. Some typical experiments are summarised in Fig. 3 in which the concentration of semiquinone is plotted against time of irradiation for a number of different mixtures. All contained $1{\cdot}6 \times 10^{-3}$M-Methylene Blue in 23N-sulphuric acid and a variable concentration of scavenger (ethanol or benzoic acid). The following features deserve special mention: (i) the maximum concentration of semiquinone is markedly dependent on the concentration of scavenger initially present, though, for low concentrations at least, it is the same for benzoic acid as for ethanol; (ii) the maximum concentration is reached more quickly when the concentration of scavenger is small; (iii) the subsequent decay is approximately linear and independent of the scavenger concentration; (iv) the maximum concentration (about 3×10^{-4}M in the presence of 0·1M-ethanol) is much less than the theoretical value based on Michaelis's value for the formation constant; [3] (v) no signal is observed in the absence of a scavenger.

DISCUSSION

The striking similarity between the spectra of all the thiazines and the oxazine suggests that the interacting nuclei giving rise to the hyperfine spectra are the same in all. The 1 : 2 : 2 : 1 intensity ratio could arise from interaction with one nitrogen atom and one proton if the proton hyperfine splitting were half the overall nitrogen splitting. Such coincidental superposition has been observed before where the electron interacts with an $=NH^+$ or NH_2^+ group,[7,8] and it seems likely that the nitrogen involved is that in the central ring since the side-chain nitrogens have different numbers of protons attached and one would expect to observe significant differences in the hyperfine spectrum between the various compounds examined. The change from four to three lines when irradiation is carried out in dideuteriosulphuric acid also indicates that only one proton is involved. (Because of the much smaller magnetic moment of deuterium compared with hydrogen the deuteron hyperfine splitting will be less than the line width in these samples and will not be resolved.) Further support for the conclusion that the main interaction of the unpaired electron is with the central nitrogen and a proton attached to it is derived from the spectra observed from phenosafranine. In this case the six lines may be attributed to interaction with two nitrogens in the central ring and one proton. For further confirmation replacement of the single proton by deuterium leaves a spectrum consisting of five lines corresponding to interaction with two equivalent nitrogen atoms, the line separation being similar to that of the unsubstituted phenazine ion.[8] The fact that the hyperfine separation is the same in phenosafranine as in the thiazines suggests that in these semiquinone ions the electron distribution is symmetrical in the central ring, though, of course, the zero nuclear spin of sulphur and oxygen precludes the observation of hyperfine spectra from these atoms.

It is suggested by Michaelis that the semiquinones of thiazines and oxazines are doubly protonated in strongly acid solution. The evidence presented in this paper indicates that at least one acidic proton is attached to the central nitrogen atom and that the unpaired electron has an equal probability of being found on the sulphur (or oxygen) and on the nitrogen. We therefore conclude that the structure of the ions may be represented, in resonance terms, by structures (I), (IV), (V), thionine being taken as the simplest example.



On the assumption that the main hyperfine interaction is with an $=NH^+$ group some further observations may be made regarding the distribution of the unpaired electron within these semiquinone ions: (i) The individual line widths are very similar to those

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[7] Melchior and Maki, *J. Chem. Phys.*, 1961, **34**, 471.

[8] Matsunaga and McDowell, *Proc. Chem. Soc.*, 1960, 175.

of other nitrogen-containing species in solution, *e.g.*, diphenylpicrylhydrazyl,[9] diphenylnitric oxide,[10] etc., and are much greater than those observed for hydrocarbon radicals and ions in solution, *e.g.*, triphenylmethyl,[11] naphthalene anion,[12] etc. This difference is caused, in part at least, by unresolved hyperfine structure, as has been shown by the observation of individual proton hyperfine lines less than 1 Mc./sec. wide in solutions of diphenylpicrylhydrazine in tetrahydrofuran,[13] carefully dried and degassed. A similar explanation probably applies to the systems under discussion, although the necessity of using strongly acid media makes it unlikely that sufficient resolution can be achieved for individual lines to be observed. However, if one assumes that the observed broad lines are in fact envelopes of numerous narrow lines caused by interaction with other hydrogen and nitrogen atoms in the ions an upper limit of about 2 Mc./sec. is set on the splitting caused by side-chain nitrogen atoms which shows that structures such as (II) are unimportant in contributing to the stability of these ions. (ii) Although the unpaired spin density on the side chains appears to be small, differences between the electron distribution on the various ions may be observable as differences in line width. The only ion with a line width differing markedly from the others is that derived from phenosafranine. The increased line width presumably results from interaction with the additional aromatic ring. (iii) Similarly, differences in line separation must reflect changes in the electron density at the central nitrogen atom. The fact that in only one species, that derived from gallocyanine, itself a zwitterion, is the change in line separation significant, indicates again that the orbital of the unpaired elctron is restricted to the central part of the molecule and is little affected by substituent groups. (iv) Since the height of the absorption peaks is a measure of the concentration of the semiquinone ion in equilibrium with the fully reduced and oxidised forms, the figures in Col. 5 of the Table indicate the relative stability of the various ions examined. Unfortunately the complicating effect of the irreversible decomposition on prolonged irradiation, noted earlier and discussed in the subsequent section, means that such a comparison can be only very approximate. The comparative lack of stability of the semiquinone of Methylene Green is, however, very marked, and may perhaps be attributed to the single nitro-group which reduces the symmetry of the doubly protonated ion and decreases the resonance energy.

A further complication in making valid comparisons of stability is the effect of pH on the equilibrium constant, observed by Michaelis *et al.* The formation constant of the semiquinone $k = [S]^2/[R][T]$ where [R], [S], and [T] are the equilibrium concentrations of reduced, half-reduced, and oxidised form, respectively, has a maximum at about 23N-sulphuric acid for thionine and Methylene Blue, but not necessarily for other dyes.

While the structure and stability of the semiquinone ions formed during the radiolytic reduction of thiazines, etc., may thus be readily understood, the mechanism by which they are formed and destroyed is not at all clear. The radiolysis of strongly acid solutions has not been much studied and the nature of the reactive intermediates is not known, although the formation of hydrogen atoms in the radiolysis of aqueous sulphuric acid has been established.[14] In strongly acid media it is likely that H_2^+ becomes increasingly important compared with hydrogen atoms[15] and it seems possible that the reduction of, say, thionine proceeds by transfer of hydrogen from H_2^+ to the ring nitrogen of the dye, rather than by addition of free hydrogen atoms. Alternatively the first stage of the reduction may be the addition of an electron followed by proton transfer from the solvent.

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[9] Hutchison, Pastor, and Kowalsky, *J. Chem. Phys.*, 1952, **20**, 534.

[10] Hoskins, *J. Chem. Phys.*, 1956, **25**, 788.

[11] Chesnut and Sloan, *J. Chem. Phys.*, 1960, **33**, 637; Ayscough, McCann, and Wilson, *Proc. Chem. Soc.*, 1961, 16.

[12] Ward and Weissman, *J. Amer. Chem. Soc.*, 1957, **79**, 2086.

[13] Deguchi, *J. Chem. Phys.*, 1960, **32**, 1584.

[14] Livingston, Zeldes, and Taylor, *Discuss. Faraday Soc.*, 1955, **19**, 166; Livingston and Weinberger, *J. Chem. Phys.*, 1960, **33**, 499.

[15] Rigg and Weiss, *J. Chem. Phys.*, 1952, **20**, 1194; Rigg and Weiss, *J.*, 1952, 4198.

A repetition of either of these processes would result in the formation of the stable leuco-compound.

The hydroxyl radicals, formed by decomposition of the solvent in the primary process, are believed to be responsible for the irreversible decomposition of the solute,[4] presumably by attack at other positions in the molecule, and it is assumed that the ethanol or other scavenger removes hydroxyl radicals and prevents this process, which would otherwise predominate.

Although the results so far obtained do not permit a detailed kinetic analysis of these processes they are compatible with the mechanism outlined above. For instance, the correspondence between the maximum concentration reached and the amount of scavenger initially present suggests competition between the scavenger and the semi-quinone, or one of the species with which it is in equilibrium, for the species causing the irreversible decomposition (presumably hydroxyl radicals). If this is so, both ethanol and benzoic acid must be relatively inefficient scavengers since, even with a sixty-fold excess of ethanol, the maximum concentration attained is substantially less than that calculated from the data quoted by Michaelis for this system. The fact that the subsequent decay is virtually independent of the initial concentration of scavenger may mean that in this part of the process the scavenger has been used up and that we are then observing the sum of the reversible and irreversible reduction processes which would depend primarily on the dose rate.

Koizumi and Obata[5] have discussed the efficiency of various scavengers in this system and conclude that benzoic acid is a better scavenger than ethanol, though their evidence is not convincing. Our results do not indicate any significant difference in the behaviour of these two compounds under our experimental conditions. It seems likely that a careful examination of this system might provide useful information on the behaviour of scavengers in irradiated acids but it is also possible that the scavenger is playing a more direct role in the formation of the semiquinone or that the equilibrium between the various forms of Methylene Blue is being disturbed by the presence of other reactive species in the irradiated system.

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