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## ELECTRON SPIN RESONANCE IN PHOTODYNAMIC DYES

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MANY organic compounds sensitize biological systems to visible light, a phenomenon known as 'photodynamic action'<sup>1</sup>. The photon energy is absorbed by the photosensitizer and presumably transferred by an unknown mechanism to an organic substrate, which is then destructively oxidized by molecular oxygen. Both light-induced and natural (dark) electron spin resonance signals in organic dyes and photosensitizers have been described<sup>2-11</sup>. The formation of unusual molecules by visible light, especially those with unpaired electrons, may be an important factor in the mechanism of photodynamic action. The properties of the dark signals are also pertinent, for the accepted chemical structures of the compounds investigated contain no unpaired electrons. There is some evidence<sup>12,13</sup>, however, that dark signals in several cyanine, triarylmethane and phthalocyanine dyes are caused by impurities probably on the surfaces of crystals.

We have investigated the dark signals in polycrystalline methylene blue, neutral red and acriflavin (acid and neutral), in a single methylene blue crystal and in methylene blue adsorbed on activated alumina, incorporated in gelatin films and taken up on 'Amberlite IR-120' ion exchange resin. The effect of visible light on methylene blue was also examined.

The first derivative of electron spin resonance absorption curves was obtained using a simple spectrometer<sup>14,15</sup>. All but one dye were obtained from Allied Chemical Corp. Some methylene blue was obtained from the Hartman Leddon Co. and some from Matheson, Coleman and Bell. Pure 'certified' grade commercial dye was used in the initial examinations on the effects of temperature. Dye recrystallized several times in the dark from aqueous alcoholic solution was used to investigate the effects of temperature, the absorbed state, and light on the signals. An apparently single crystal of methylene blue was grown

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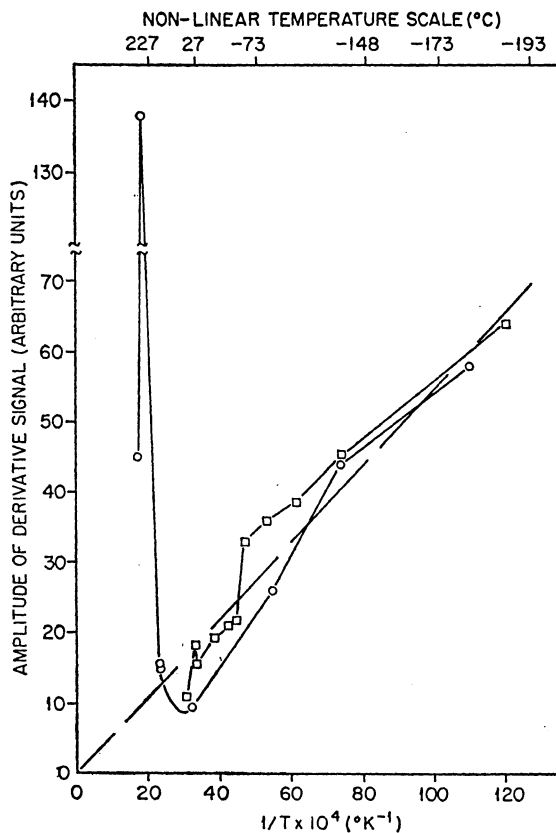


Fig. 1. Plot of polycrystalline methylene blue signal amplitudes for two samples arbitrarily normalized versus reciprocal of absolute temperature ( $1/T$ ). The dashed lines give the  $1/T$  dependence predicted by the Curie law

from aqueous solution. For work on the adsorption of methylene blue on 'Amberlite IR-120' ion exchange resin, the resin was purified by washings with sodium hydroxide, hydrochloric acid, and distilled water. Thin sheets of methylene blue incorporated in gelatin were obtained by dissolving the dye in gelatin solution and evaporating it to a film in air. The sheets were illuminated inside the electron spin resonance cavity with light from a 1,000-W tungsten filament lamp.

The polycrystalline dye in electron spin resonance sample tubes immersed in a water bath at 18° C was irradiated by exposure to a 200-W tungsten lamp at a

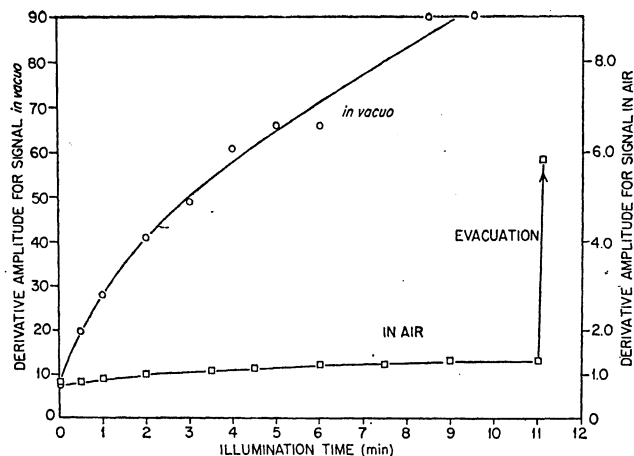


Fig. 2. Illumination of polycrystalline methylene blue in air and *in vacuo*. Note that the scale on the right for methylene blue in air is expanded ten times that on the left for methylene blue *in vacuo*.

distance of 6 cm. The exposure rate, determined by thermocouple measurements, was roughly  $1,500 \text{ ergs mm}^{-2} \text{ sec}^{-1}$  and the effective area of 0.1-g sample about  $50 \text{ mm}^2$ .

In polycrystalline methylene blue, and also in neutral red, a singlet absorption line with a width (peak-to-peak derivative width) of about 18 gauss and a  $g$ -value approximately that of diphenylpicrylhydrazyl (DPPH) was observed. The methylene-blue signal remained after two recrystallizations of the dye. The dark signal in neutral red increased markedly on heating, a result also observed by Lagercrantz and Yhland<sup>5</sup> with methylene blue. A small electron spin resonance absorption was seen in polycrystalline acriflavin (hydrochloric acid and neutral). The signals in methylene blue and neutral red corresponded to unpaired spin concentrations of roughly  $1.2 \times 10^{18}$  and  $2.2 \times 10^{17}$  spins/g respectively, using DPPH as the reference material. The signals did not saturate at the micro-wave power used. The dark signal from the single methylene blue crystal showed no hyperfine splitting, although the amplitude and width of the resonance changed with orientation in the magnetic field. Some of the dark signals, but not those observed in recrystallized methylene blue, may have come from the impurities (roughly 10 per cent) in dyes as commercially prepared.

A normal dark signal appeared when methylene blue was adsorbed on activated alumina and ion exchange resin, or incorporated in gelatin. The purified resin alone showed a small signal. The appearance of the methylene

blue dark signal when absorbed on the resin indicated that the cationic part of the methylene blue molecule was responsible for the signal.

The dark signal in polycrystalline methylene blue, heated or cooled in the electron spin resonance cavity, varied inversely with the absolute temperature from 84° to 423° K (Fig. 1). The non-linearity observed in this range is probably caused by changes in dielectric constant or other complications. Extensive departure from linearity was observed above 425° K, which corresponded to the melting and/or decomposition of the material. The separately measured signal at 121° K saturated at a lower micro-wave power than at 298° K. From the approach to  $1/T$  dependence predicted by the Curie law, we conclude that the signal is not due to conduction band electrons, which should show no temperature dependence.

Visible light induces a signal in methylene blue adsorbed on fused silica in the absence of oxygen. This signal is quickly and reversibly quenched by oxygen<sup>16</sup>. We have found light induction in three other systems—polycrystalline methylene blue, methylene blue adsorbed on activated alumina and methylene blue incorporated in gelatin—and have shown that induction can occur in oxygen. Previously, light induction in oxygen was not observed, probably because of the dilute dye concentrations used.

For methylene blue adsorbed on activated alumina *in vacuo*, 4 h of illumination increased the dark signal amplitude about 25 per cent; re-admittance of dry oxygen reduced the signal to approximately its initial value. Illumination of a sheet of dyed gelatin in which no dark signal was observed elicited a small signal that saturated after about 10-min exposure, and decayed but slowly. Evacuation for about 3 h multiplied the signal six times. Illumination for 13 min more *in vacuo* elicited a further increase of approximately 30 per cent. Exposure to dry oxygen produced no immediate change. Examination of separate controls revealed that no signal was produced by illumination of the alumina and gelatin alone in the *ESR* sample tubes. It was incidentally observed that visible illumination of the ion-exchange resin alone produced an increase in the small dark signal.

Results for the illumination of the dark-recrystallized dye in air and *in vacuo* are shown in Fig. 2. Evacuation of the sample illuminated in air produced about the same increase in signal as evacuation of the non-illuminated sample. The sample illuminated *in vacuo* was later exposed to dry oxygen and no immediate effect noted. It was then kept at 96° C for 40 min in the dark: the signal increased in amplitude and decreased in line width. This heat effect thus overrides any possible annealing-out of the light-induced resonances.

Oxygen present during illumination interacts with methylene blue molecules to inhibit induction of long-lived resonance centres. If added after irradiation *in vacuo*, oxygen interacts with the abnormal molecules and quenches the apparent signal in several systems. The rate of diffusion of oxygen into the material is important for quenching and may explain the lack of immediate quenching in the gelatin and polycrystalline samples. Similar results were obtained by Kholmogorov and Terenin<sup>21</sup> with several triphenylmethane and xanthene dyes. However, a reversed action of oxygen has been observed for the dyes rose bengal, phloxine, and haematoporphyrin<sup>7,17,21</sup>. A possible explanation of the latter results has been proposed<sup>21</sup>. Some observed signals, especially in unirradiated dyes, may be those of biradicals since molecular weights are several hundred, and the accepted chemical structures have even numbers of electrons.

Our results suggest that the energy of the excited dye is transferred to oxygen, perhaps first in the photodynamic process, at least with methylene blue as the sensitizer. This conclusion can be fitted into the schemes of Schenck, *et al.*<sup>18</sup>, Oster<sup>19</sup>, and Kauzmann<sup>20</sup>. Because we have examined only the dye and oxygen in the solid-state rather than in solution with the oxidizable substrate, conclusions based on our results are tentative.

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<sup>1</sup> Blum, H. F., *Photodynamic Action and Diseases Caused by Light* (Reinhold Publishing Co., New York, 1941).

<sup>2</sup> Chernyakovskii, F. P., Kalmanson, A. E., and Blyumenfeld, L. A., *Optics and Spectroscopy*, **9**, 414 (1960).

<sup>3</sup> Neiman, R., and Kivelson, D., *J. Chem. Phys.*, **35**, 162 (1961).

<sup>4</sup> Moshkovskii, Y., *Dokl. Akad. Nauk S.S.S.R.*, **130**, 1277 (1960).

<sup>5</sup> Lagercrantz, C., and Yhland, M., *Acta Chem. Scand.*, **15**, No. 5, 1204 (1961).

<sup>6</sup> Kholmogorov, V. E., and Terenin, A. N., *Dokl. Akad. Nauk S.S.S.R.*, **137**, 199 (1962).

<sup>7</sup> Castellani, A. (personal communication).

<sup>8</sup> Sidran, M., Colloque A.M.P.E.R.E. Istituto di Fisica, Universita di Pisa, Pisa, Italy, 1960.

<sup>9</sup> Needler, W. C., Griffith, R. L., and West, W., *Nature*, **191**, 902 (1961).

<sup>10</sup> Schenck, G. O., and Wolgast, R., *Naturwiss.*, **24**, 737 (1961).

<sup>11</sup> Lagercrantz, C., and Yhland, M., *Acta Chem. Scand.*, **16**, 508 (1962).

<sup>12</sup> Kholmogorov, V. E., and Glebovskii, D. N., *Optics and Spectroscopy*, **12**, 728 (1962).

<sup>13</sup> La Valley, J. E., Leifer, A., Dougherty, P. H., and Koral, M., *J. Phys. Chem.*, **66**, 2403 (1962).

<sup>14</sup> Kirby-Smith, J. S., and Randolph, M. L., in "Immediate and Low Level Effects of Ionizing Radiations", Special Suppl., *Intern. J. Rad. Biol.*, **115** (1959).

<sup>15</sup> Conger, A. D., and Randolph, M. L., *Rad. Res.*, **11**, 54 (1959).

<sup>16</sup> Simpson, L. P., senior thesis (Princeton University, work done under supervision of Profs. H. F. Blum and J. Turkevitch) 1962.

<sup>17</sup> Smith, D. E., Santamaria, L., and Smaller, B., in *Free Radicals in Biological Systems*, 305 (Academic Press, New York, 1961).

- <sup>18</sup> Schenck, G. O., Kinkel, K. G., and Koch, E., *Naturwiss.*, **41**, 425 (1954).  
Schenck, G. O., *ibid.*, 452.
- <sup>19</sup> Oster, J., *J. Chim. Phys.*, **55**, 899 (1959).
- <sup>20</sup> Blum, H. F., and Kauzmann, E., *J. Gen. Phys.*, **37**, 301 (1954).
- <sup>21</sup> Kholmogorov, V. E., and Terenin, A. N., *Dokl. Akad. Nauk S.S.S.R.*, **146**, 652 (1962).