

## **A Note on Some Old and Some Possible New Redox Indicators**

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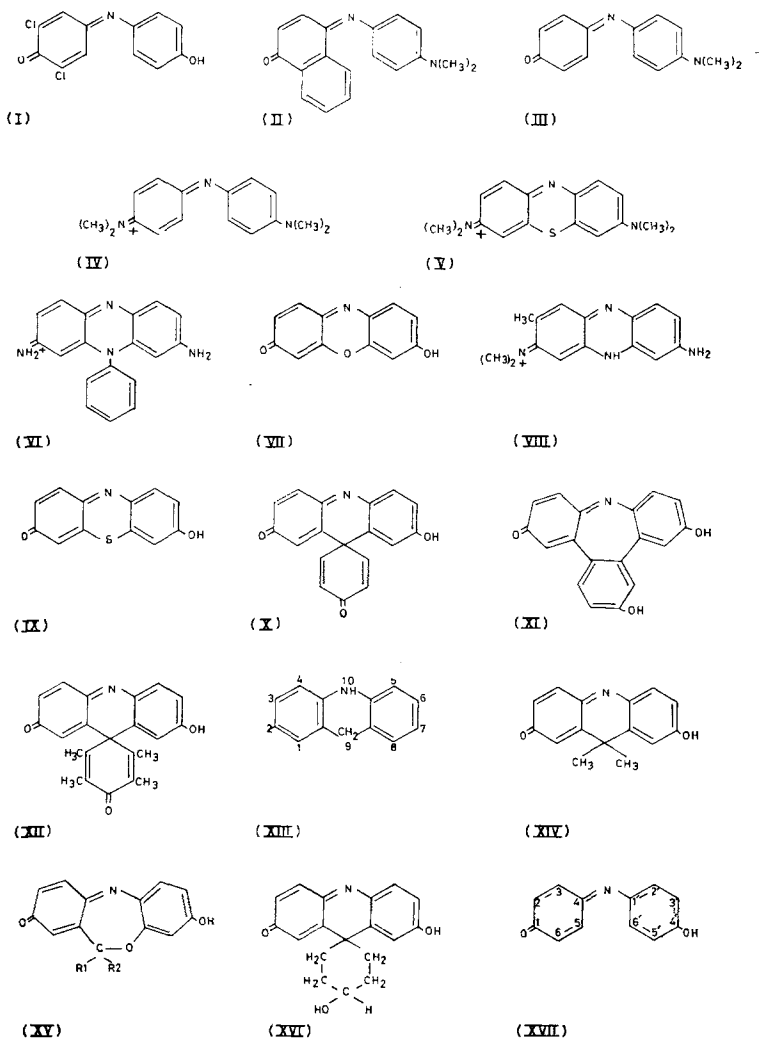
### *Summary*

Starting with the frequently used dye 2,6-dichloroindophenol some of the chemical properties of the quinonimide class of dyes are described. Consideration of the effects of completing a hetero-six-membered ring, as in the azine, thiazine and oxazine classes, on the properties of the compounds is suggested as a lead towards the development of some redox indicators perhaps more desirable than the indophenols. This approach developed from a study of the Liebermann nitroso-reaction for phenols. Some properties of the redox indicators which resulted from that work are described in relation to energy transduction in the chloroplast.

In studies on energy transduction, especially that involving preparations of chloroplasts much use has been made of dichlorophenol indophenol (I). This was one of the indophenol dyes developed by Mansfield Clark and colleagues<sup>1</sup> and was first used to determine photochemical activity with chloroplast preparations by Holt and French<sup>2</sup> in 1948.

The use of indophenols in biology was described by Ehrlich<sup>3</sup> in 1885. The now obsolete term “Indophenol Oxidase” which referred in part to the enzyme cytochrome *c* oxidase (EC 1.9.3.1.) and in part to polyphenol oxidases<sup>4</sup> (*para*, EC 1.10.3.2. and *ortho*, EC 1.10.3.1.) originated in the following way. The “Indophenol reagent” used contained a mixture of unsymmetrical dimethyl paraphenylene diamine and  $\alpha$ -naphthol.<sup>5</sup> These components by the loss of four molecular equivalents of hydrogen formed the insoluble blue vat dye originally known as indophenol (II). This dye was formerly used as an adjunct to indigo or to replace it. But it is destroyed by acids, a property shared by all indophenols and the other quinonimide dyes of a similar type. The dye, when produced from the reagent could be reduced by hydrogen donors produced in living cells. The leuco compound was easily oxidized in presence of air by the oxidase. Three processes would have to be considered when using the reagent, synthesis of the dye (partially an irreversible process), reduction of the dye to the leuco compound

and reoxidation to the dye. The second and third processes only involve two hydrogen equivalents. The use of the reagent could result in a confused interpretation. Lack of colour given could either indicate no activity at all or a very high activity of reducing systems.



In 1929 it was shown by David Keilin<sup>6</sup> that a cold suspension of living yeast would oxidize the reagent when sufficiently aerated. At ordinary temperatures there was no reaction and the oxidase had formerly been considered absent from yeast cells. Keilin explained the result in terms of the difference in rates of oxidation and reduction processes, the latter having a higher temperature coefficient. This was an important experiment in the development of knowledge of hydrogen

or electron transport in cellular respiration. Now it happens that the dye produced from the "indophenol reagent" should not properly be called an indophenol. It belongs, according to Mansfield Clark<sup>7</sup> to a class known as *indoanilines* represented by the so called phenol blue (III). When both oxygen atoms in the indophenol are replaced by an amine or imine group the compounds were termed *Indamines* as for example Bindschedler's green (IV). This dye corresponds to methylene blue (V) without the sulphur atom. The sulphur completes the heterocyclic ring connecting two phenyl residues. Methylene blue is comparatively resistant to acids. The other types of quinonimide compounds which do not have the heterocyclic ring are easily decomposed. Phenolindophenol for example gives initially in acid solution quinone, ammonia and probably *p*-aminophenol. It follows that it is not usually practicable to isolate the free indophenols for storing. Mansfield Clark stated that the neutral or alkaline solutions of the indophenols tend to decompose and that these dyes are best stored as their dry alkali salts.

For the detection of biological oxidizing systems the original indophenol reagent was some times used without the  $\alpha$ -naphthol. The coloured product known as "Wursters red" resulted from a partial oxidation of the asymmetrical dimethyl-*p*-phenylene diamine. This gives a quinhydrone or semiquinone type of compound. There is, however, no stable link between two phenylene residues and the system is liable to undergo further reactions which are irreversible in terms of hydrogen transport, just as in the formation of the blue dye in presence of  $\alpha$ -naphthol. In addition the simple quinone imides easily break down in aqueous solution to quinones and ammonia.

Indophenols have the advantage that in general they are soluble near the neutral point and that the leuco compounds are not rapidly oxidized by atmospheric oxygen<sup>8</sup> so that they can be used as indicators of hydrogen transfer in open vessels. They also react rapidly with a variety of hydrogen donors. This property depends on the fact that the oxidation-reduction (O.R.) potentials are in a more oxidizing range than is the case with the other quinonimide dyes such as methylene blue (thiazine), saffranine (VI) (azine) or resorufin (VII) (oxazine). With these dyes, where a quinonimide nitrogen forms part of the heterocyclic six-membered ring, the name of the class is taken from the atom which completes the ring. Thus the parent substance for methylene blue is phenothiazine. The effect on the O.R. potential of the quinonimide dye when the heterocyclic structure is completed is to change the characteristic potentials at pH 7 ( $E_m$ ) towards a more reducing potential. Bindschedler's green (IV)  $E_m$  0.225 V, pH 7, and methylene blue (V)  $E_m$  0.015 V show the effect of the sulphur linkage, and also on the position of the absorption bands which have been displaced towards the violet end of the spectrum. In the azine series the effect of the nitrogen atom is much greater, as neutral red (VIII),

$E_m - 0.252$  V. Resorufin  $E_m - 0.06$  V shows that the effect of the oxygen atom on the O.R. potential is similar to that of sulphur.

With the two symmetrically placed substituents the change of a phenolic to an amino group does not in general cause a marked effect. Thionol (IX) derived from dihydroxy phenothiazine has  $E_m$  recorded = 0.037 V, slightly more positive than methylene blue. Phenol indophenol with  $E_m$  0.228 is very near to Bindschedler's green at pH 7.

The effect of different single additional substituents in the case of indophenols, quinones and indigo sulphonates was studied by Clark and by Fieser and others and it will not be reviewed here. The previous discussion was intended to show the influence of the bivalent substitutions in positions 5 and 6' (using Clark's numbering) of a quinonimide dye which produce the heterocyclic ring.

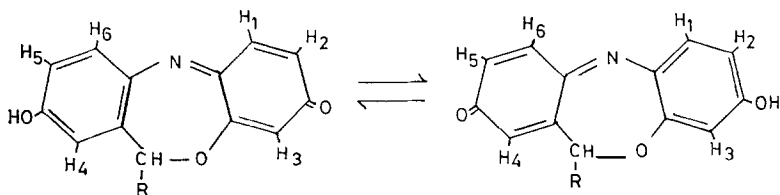
Suggestions for possible new redox indicators resulted from a study of the Liebermann nitroso-reaction with phenol<sup>9</sup> (Hill *et al.*, 1970). It was found that the dye described by Liebermann<sup>10</sup> could not wholly be accounted for in terms of an indophenol. The interpretation given throughout the literature originated from an early publication by Baeyer and Caro<sup>11</sup> who prepared nitrosophenol and obtained indophenol from it, thereby concluding that the dye obtained by Liebermann must also be indophenol. The indophenols in weakly alkaline solutions do not show visible fluorescence; the product produced from ordinary phenol as described by Liebermann shows a marked red fluorescence of the blue alkaline solution. By following the fluorescence together with the characteristic absorption spectrum a stable crystalline substance was obtained. This was found, in agreement with the original work of Liebermann to have 6 carbon atoms more than the indophenol. A bivalent group in positions 5 and 6', giving a more stable molecule and accounting for the fluorescence, was indicated. Nietzki (1888) had already put Liebermann's  $C_{18}$  dye into the oxazine class with resorufin, which is strongly fluorescent, when he wrote one of the first text books on chemistry of dyestuffs.<sup>12</sup> It was concluded that this group was 4 cyclohexanone 2,5-diene (1,1); in fact the compound (X) was produced in small yield when indophenol itself was treated with phenol in sulphuric acid. In this reaction as 4 equivalents of hydrogen have to be removed this results in reduction of the indophenol to the leuco compound. In Liebermann's original method there was a virtual excess of nitrite which acted as the oxidizing agent. If the leuco compound of (X), which is a spirocyclohexane derivative, as treated with aqueous mineral acid reoxidation gives an amorphous product soluble in weak alkali with a violet colour showing no fluorescence. It is concluded that an intermolecular reaction has resulted in the cyclohexane ring becoming aromatic and forming a phenylene residue which then gives a seven-membered heterocyclic ring (XI). The structure (XI) must at present be regarded as provisional until further

evidence has been obtained. That this substance was also present in the original preparations of Liebermann seems certain as he described the colour as being "pure cobalt blue". With the substance giving the red fluorescence the colour of the alkaline solution would be described as pure blue without a reddish tinge apart from the fluorescence. Both these products have more oxidizing O.R. potentials than the corresponding phenolic derivatives in the series thiazines or oxazines. At a neutral pH "Liebermann's blue" is a little more reducing than indophenol itself while "Liebermann's violet" (XI) is a little more oxidizing. When phenol indophenol is treated with durenol (2,3,5,6-tetra methyl phenol) in sulphuric acid a Liebermann's blue (XII) is formed. The methyl groups in the cyclohexane residue would seem to account for the fact that the leuco compound remains unchanged when treated with acid.

The substance (X) is to be regarded as an acridan (XIII) derivative and would therefore belong to a series of dyes discovered by Kehrmann and colleagues<sup>13</sup> (1919) which they called carbazines. This name was used because a carbon atom completed a six-membered heterocyclic ring as the sulphur does in the thiazines. But the parent substance of Kehrmann's carbazines is known as 9,10 dehydroacridine or acridan. These dyes, as phenolic compounds, may be prepared from an indophenol (see (XVII) for numbering) with disubstituted methanol group in position 5 or 6'. The leuco compound of this tertiary alcohol readily forms the acridan ring on warming with mineral acid by the loss of a molecule of water. The 9,9-dimethyl acridan derivative (XIV) was prepared by Goldstein and Kopp (1928)<sup>4</sup> and was termed by them 2-hydroxy-C-dimethyl carbazone (7). The secondary and primary alcohols do not react in this way to give the acridan derivatives.

It was observed, however, that indophenols prepared from 3-hydroxyphenol carbinols by action of quinone chlorimide in dilute alkali, showed a colour change from blue to purple on keeping the reaction mixture for 3-4 days. This change required the presence of air. The products could be isolated as stable crystalline substances, dissolving in weak alkalis to give brilliant purple solutions. Dr. B. R. Webster and Dr. G. R. Bedford<sup>15</sup> very kindly examined 2 samples of these compounds. The mass spectra suggested molecular ions 16 mass units higher than the originally proposed structures which represented the compounds as a dihydroxy acridine and a dihydroxy methyl acridine. The mass spectrum of the desmethyl compound gave a molecular ion at  $m/e$  227 which measured 227.0584,  $C_{13}H_9NO_3$  requires 227.0582. This was consistent with the structure (XV) proposed from the n.m.r. spectrum. The n.m.r. spectra were interpreted by Dr. Bedford as shown in Table I. Two patterns were shown for the aromatic rings suggesting they were not identical and a  $CH_2$  group in the desmethyl compound and a  $CHCH_3$  group in the methyl compound

TABLE I. The n.m.r. spectra of "desmethyl purple" (D.M.P.) and "methyl purple" (M.P.) in  $D_6$  dimethylsulphoxide ( $d_6$  d.m.s.o.) solution can be described as an equilibrium mixture of forms I and II



The n.m.r. spectra were interpreted as follows:

Compound R =	H	CH <sub>3</sub>
H <sub>1</sub>	7.28 doublet J = 9 Hz	7.29 doublet J = 9 Hz
H <sub>2</sub>	6.55 doublet of doublet J = 9, 2 Hz	6.55 doublet of doublet J = 9, 2 Hz
H <sub>3</sub>	6.05 doublet J = 2 Hz	6.00 doublet J = 2 Hz
H <sub>4</sub>	6.75 doublet J = 2 Hz	6.70 doublet J = 2 Hz
H <sub>5</sub>	6.85 doublet of doublet J = 8, 2 Hz	6.84 doublets of doublets J = 8, 2 Hz
H <sub>6</sub>	7.47 doublet J = 8 Hz	7.46 doublet J = 8 Hz
CH	4.97 singlet	5.13 quartet J = 7 Hz
R	4.97 singlet	1.52 doublet J = 7 Hz

whereas a =CH and a =CCH<sub>3</sub> would be expected on the originally proposed structures. These chemical details are given here because the structure (XV) represents a possibly new and easily accessible type of quinonimide dye. Corbett<sup>6</sup> (1970) in a study of structure and spectra of the indoaniline dyes had shown the effect of the asymmetry as regards the two aromatic rings, as shown in (III). While solubility properties of these methylene oxy compounds are influenced by the nature of the substituents R<sub>1</sub>R<sub>2</sub> on the methylene carbon the visible absorption spectra are very little affected. The compound R<sub>1</sub> = H and R<sub>2</sub> = CH<sub>3</sub> has been found by me to be very useful as an indicator of hydrogen transport with chloroplast preparations. The region of absorption is sufficiently removed from the red end of the spectrum at pH 7 and above so that it does not, in contrast to (I) obscure the red absorption band of chlorophyll.

The compound (XVI) was prepared from (X) by reduction of the cyclohexanonediene residue to the 4'-hydroxycyclohexane with AlNi alloy in alkaline solution. (XVI) has a very reduced fluorescence compared with (X). The absorption spectrum is similar to (XIV) but this has a strong red fluorescence in its alkaline solution. It is thought that the lack of fluorescence shown by XVI may be due to "boat" and "chair" forms in the cyclohexane part. Dr. Norman Good<sup>17</sup> observed a ten-fold increase in fluorescence when the alkaline salt was

present in glycerol. The compound (XII) in spite of the 4 methyl groups has a more marked fluorescence than (X). Both the compounds (XII) and (XVI) are reduced by chloroplast preparations in light. They do not obscure the red absorption band of chlorophyll to an appreciable extent when used in sufficient concentration for direct measurement of oxygen. The reduction of (XVI) is found closely to resemble that with an equivalent amount of ferricyanide.<sup>18</sup> This is shown in Fig. 1. The effect of adding  $\text{NH}_3$  as an uncoupler also is

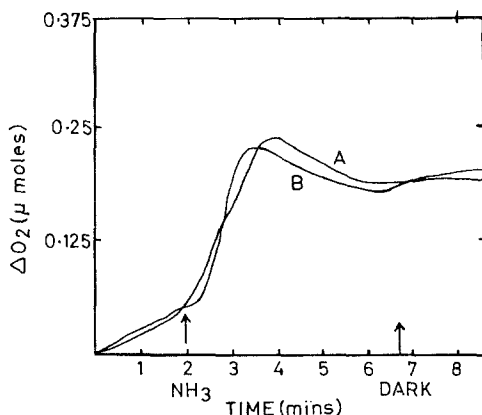


Figure 1. Simultaneous recording of oxygen production in light comparing equivalent amounts of dye and of ferricyanide.<sup>17</sup>

Pea chloroplasts prepared in 0.33 M sorbitol and 50 mM orthophosphate pH 6.5, broken in electrode vessel, assayed in 0.33 M sorbitol and 50 mM Hepes pH 7.6. In 3 ml: 100  $\mu\text{g}$  chlorophyll, equivalent dye (0.5  $\mu\text{moles}$ ) and ferricyanide (1  $\mu\text{mole}$ ). Trace A, ferricyanide, B, dye (XVI),  $\text{NH}_4\text{Cl}$  added to final concentration of 6 mM (Stokes and Walker 1970).

practically identical when (XVI) is compared with ferricyanide. This is in contrast with the uncoupling effect which is shown with 2,6-dichloroindophenol. It was found that there was an appreciable rate of reoxidation of the leuco compound in the dark as there is with the indophenol. This would be of little consequence under the appropriate conditions of chloroplast concentration and light intensity.

The accepted chemical nomenclature for (X) which here was referred to as Liebermann's blue is 7-hydroxy spiro [acridine-9, 1'-cyclohexa-2'5'-diene]-2(9H),4-dione. This does not suggest either a convenient trivial name, nor a convenient initial letter abbreviation. The trivial name "dichroin" was given in 1888 by Brunner and Chuit.<sup>9</sup> This term was used by Nietzki in his textbook in 1888. In 1923 the name appeared in Beilstein<sup>20</sup> as *verbindung*  $\text{C}_{18}\text{H}_{15}\text{O}_3\text{N}$ ,  $\alpha$ -phenyl dichoin but as it had not been actually characterized as a pure compound later editions omit it. Mansfield Clark (1923) refers to the name phenoldichroin as an alternative one for phenol indophenol. There is now a basis for reviving

this trivial name, which obviously refers to the simultaneous appearance of blue and red tints in the alkaline solution. If the term phenol now is used to indicate the origin of the cyclohexane residue then compound (XII) would be "durenol dichroin" and compound (XVI) "reduced dichroin". These can be abbreviated as DC, DDC and RDC. These three compounds have the plane of the cyclohexane residue at right angles to the plane of the quinonimide part. The substance here referred to as "Liebermann's violet" could be termed "dichroin violet" or DCV. If the numbering of the indophenol residue (XVII) according to Clark is considered then 2,6 dichloro dichroin DCDC would be intelligible as would be 2,6 dichloro dichroin sulphonate (3'). It was most convenient to name the purple compounds after the substituents  $R_1R_2$  as methyl purple,  $R_1 = H$ ,  $R_2 = CH_3$ , desmethyl purple,  $R_1 = R_2 = H$ , and dimethyl purple,  $R_1 = R_2 = CH_3$ . But the name methyl purple is already in use for a pH indicator. So the purple compounds might be called simply methyleneoxy indophenols (5,6'), keeping the carbon atom on the left as (XV). They could be indicated by initial letters with the nature of the substituent groups  $R_1$  and  $R_2$  indicated.

From the point of view of the use of the quinonimide dyes as indicators of oxidation-reduction it is a matter for regret that they cannot be described on a uniform basis. For example the numbering of acridine nucleus according to some authors begins next to the carbon atom, while in the oxazines the numbering begins next to the nitrogen atom. The asymmetrical numbering that Mansfield Clark used for the indophenols bears no relation to the other quinonimide dyes. It is the structural relationship in the series that we emphasize here as being of importance. The "bridging group" for indophenol (5,6') has an important influence on the oxidation reduction properties and on the absorption spectra. This effect is apparently minimal when the bridging group, forming the six-membered ring, is of a saturated character; it would then be regarded as potentially incapable of being involved in any tautomeric changes. This is perhaps the simplest generalization concerning the range of properties exhibited by the compounds cited here. The *practical* advantage of a bridging group would be to give a structure more stable than the original indophenol.

#### *Acknowledgements*

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