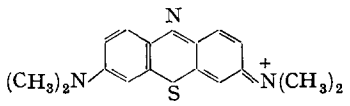
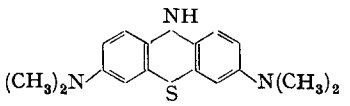
The oxido-reductive properties of organic dyes of biological importance

We have shown in a recent publication[1] that the oxidation-reduction mechanism of the respiratory enzymes, pyridine nucleotide enzymes or flavoproteins, may be related to the energies of the molecular electronic orbitals of the oxidized and reduced forms of the associated coenzymes: DPN^+ or TPN^+, FMN or FAD. In each case a particularly low-lying empty orbital is associated with the oxidized form and a particularly high-lying filled orbital is associated with the reduced form. The oxidized form must thus have a great tendancy to accept electrons and the reduced form to give them up, the oxidation-reduction being accompanied by the appropriate instantaneous redistribution of these orbitals.

Moreover it has been shown that the reduced form of the riboflavin-type coenzymes ($FMNH_2$) possesses a very unusual characteristic, which has not been observed previously in any other existing compound, namely that its highest filled molecular orbital is not only a very high-lying one but in fact an antibonding one, of the type of orbital which is generally occupied by electrons only in the excited states of molecules. The result signifies that the occupation of this orbital in the ground state of the molecule represents a fundamentally unstable arrangement and that $FMNH_2$ will thus have an exceptionally strong natural tendancy to expel the electrons located at that orbital. This particular findings accounts thus not only for the outstanding electron-donor properties of this substance but also for its autoxidability. (In the non-autoxidable DPNH, the highest filled molecular orbital, although located very high, is still a bonding one.)



MeB^+



MeBH

The ability of some non-biological substances and in particular of *methylene blue* to act as electron carriers in a way similar to that of the coenzymes incited us to carry out similar calculations for this compound. The energies of the highest occupied molecular electronic orbital (h.o.m.e.o.) and of the lowest empty molecular electronic orbital (l.e.m.e.o.) in MeB^+ and MeBH are reproduced in Table I where we have tabulated also for comparison the same data for the oxidized and reduced forms of the respiratory coenzymes.

The relatively small absolute value of the coefficient of the energy of the l.e.m.e.o. in MeB^+, which is of the same order of magnitude as that of DPN^+ or FMN, indicates high electroaffinity and accounts thus for the well known electron-acceptor properties of this substance. As to MeBH, this compound manifests the same exceptional property as $FMNH_2$: its h.o.m.e.o. is antibonding, the sign of its coefficient k being negative as is usual only for orbitals occupied in the excited states of molecules. This corresponds to an unstable arrangement, the reduced compound having a natural tendancy to give up the electrons located at this orbital. The easy autoxidability of

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Abbreviations: DPN^+, DPNH, oxidized and reduced diphosphopyridine nucleotide; TPN^+, triphosphopyridine nucleotide; FAD, flavin–adenine dinucleotide; FMN, $FMNH_2$, oxidized and reduced flavin mononucleotide; MeB^+, MeBH, oxidized and reduced methylene blue; h.o.m.e.o., highest occupied molecular electronic orbital; l.e.m.e.o., lowest empty molecular electronic orbital.

TABLE I

ENERGIES OF MOLECULAR ORBITALS

The energies of the molecular electronic orbitals are of the general form $E_i = \alpha + K_i\beta$, whose α and β are respectively, the coulomb and the exchange integrals of the molecular-orbital method (for details, see ref. 2). The numbers given refer to K_i. In general, positive values of K_i correspond to bonding orbitals, occupied by electrons in the ground state of the molecules and negative values of K_i to antibonding orbitals, occupied only in the excited states.

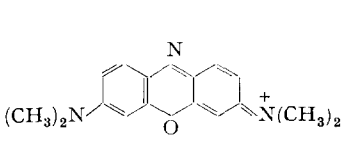
	MeB+	*MeBH*	*DPN*+	*DPNH*	*FMN*	$FMNH_2$
Energy of the l.e.m.e.o.	—0.354	—1.000	—0.356	—0.915	—0.343	—0.979
Energy of the h.o.m.e.o.	0.398	—0.232	1.032	0.298	0.496	—0.105

MeBH is a direct result of this tendency. From the viewpoint of electronic-energy levels, methylene blue and riboflavin are thus strikingly similar, even in their exceptional properties. This theoretical result explains in particular the classical observation by SZENT-GYÖRGYI[3] that methylene blue can act alternatively as hydrogen acceptor and donor in respiration.

In fact, the antibonding character of the h.o.m.e.o. of MeBH is even much more pronounced than that of $FMNH_2$. Correspondingly, the autoxidability of MeBH is greater than that of $FMNH_2$ and in practice the rate of oxidation of flavoproteins by oxygen, which is slow, may be greatly increased by addition of methylene blue. The dye serves as a carrier between the flavoprotein and oxygen.

The question may be raised whether other related compounds may be expected to possess the same type of properties and in particular the unusual property of an antibonding highest filled orbital in their reduced form. The problem is under investigation. The unusual property quoted appears to be dependent on the presence of heteroatoms, possessing lone pair of electrons, at suitable positions of an aromatic skeleton. These heteroatoms must nevertheless possess a relatively low electronegativity.

Thus, similar calculations have been carried out for the oxygenated analogue of methylene blue, in which the sulphur atom is replaced by oxygen:



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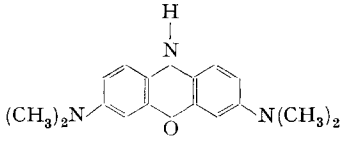
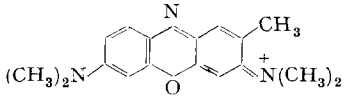


II



III

It was found that in (I), the energy coefficient of the h.o.m.e.o. equals 0.764 and that of the l.e.m.e.o. —0.082; in (II) the energy coefficient of the h.o.m.e.o. equals 0.096 and that of the l.e.m.e.o. —1,128. (I) should thus be an excellent electron acceptor and (II) a very good donor. Nevertheless, (II) lacks the exceptional property

of an antibonding h.o.m.e.o. It may be useful to quote in connection with this result that, unlike methylene blue, this compound probably does not autoxidize: thus capri blue (III), whose structure is very similar to the one that we have calculated does not reoxidize spontaneously, or does so only very slowly[4].

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Université de Paris, Laboratoire de Chimie Théorique, B. PULLMAN
13 rue Pierre Curie, Paris (France) A. PULLMAN

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[4] A. SZENT-GYÖRGYI, private communication.

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Extraction et propriétés de la nitrate-réductase d'*Aerobacter aerogenes*

Dans le cadre des recherches que nous avons entreprises[1,2] sur la réduction bactérienne de l'azote minéral à partir de l'hydrogène, nous avons étudié la réduction du nitrate par *Aerobacter aerogenes* (Souche L III-1), à l'aide de suspensions et d'extraits de cet organisme cultivé sur milieu liquide peptone-glucose, en l'absence de nitrate et en anaérobiose (cultures massives et profondes, non-agitées). Sous atmosphère d'H_2, les suspensions non-proliférantes provenant de ces cultures réduisent le nitrate en nitrite sans phase de latence, à condition d'opérer en présence d'une quantité catalytique de benzyl-viologène qui joue le rôle de transporteur artificiel d'électrons, mais elles sont par contre dépourvues de toute activité en l'absence de cet indicateur d'oxydo-réduction. Ce comportement, qui avait déjà été signalé par LASCELLES ET STILL[3] pour une autre souche du même organisme, montre que les cellules ainsi cultivées possèdent une hydrogénase et une nitrate-réductase, mais sont dépourvues de transporteur naturel capable de coupler physiologiquement les deux enzymes considérés.

Les extraits ont été préparés suivant la technique décrite par NICHOLAS ET NASON[4] et le nitrite formé a été dosé colorimétriquement, après précipitation des protéines par l'acétone. Pour mesurer les consommations d'H_2, on a employé la méthode respirométrique de Warburg, dans les conditions suivantes: température, 37°; pH 7.0 (tampon phosphates), systèmes de 3 ml, contenant 100 μg de benzyl-viologène et 50 μmoles de KNO_3. La réduction et l'oxydation des pyridine-nucléotides ont été estimées spectrophotométriquement à 340 mμ, à la température du laboratoire.

1. En présence d'un grand excès de KNO_3 (50 μmoles), la consommation d'H_2 par les extraits est linéaire en fonction du temps (Fig. 1, courbe 3). Lorsque, par contre, la concentration initiale du substrat est faible (3 μmoles), l'activité passe successivement par deux phases linéaires (courbe 2), dont la première correspond à la conversion quantitative du nitrate en nitrite:

$$H_2 + NO_3^- = H_2O + NO_2^-$$

Le second segment est parallèle à la droite obtenue si on remplace dans les systèmes le nitrate par 3 μmoles de nitrite (courbe 1) et correspond à la réduction de NO_2^- en ammoniaque par le processus non-enzymatique que nous avons décrit dans