Short Communications

Electron-Spin-Resonance Studies of the Structure and Formation of Bacterial Diazodiphenoquinone Pigments

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The formation of diazodiphenoquinone pigments during bacterial oxidations of pyridine-diols can be accounted for in terms of a free-radical mechanism similar to that operating for oxidations *in vitro* involving azasemiquinone radicals. Electron-spin-resonance spectra are of great value in pigment structure determinations.

Azaquinonoid structures have recently become accepted for a group of blue bacterial pigments found notably in species of *Arthrobacter* and *Pseudomonas* (Kuhn *et al.*, 1965). Certain members of this group can be produced by bacterial oxidation when using pyridinols as carbon and nitrogen sources, and owe their blue colour to the presence of the diazodiphenoquinone structure (Kuhn *et al.*, 1965; Ensign & Rittenberg, 1965; Knackmuss, 1968).

Much work has recently been devoted to the chemistry of azaquinones in order to gain information on the structures and biogenesis of these natural products (Knackmuss, 1973), and the formation of these blue pigments from pyridinols *in vitro* and *in vivo* must necessarily involve processes of hydroxylation, oxidation and, at some stage, the coupling of two azaquinonoid units.

Corresponding types of reactions occur in the chemistry of benzoquinones, and the technique of e.s.r. (electron-spin-resonance) spectroscopy has proved a useful means of solving some of the structural and mechanistic problems involved by studying the intermediate semiquinone radicals (Ashworth & Dixon, 1972).

The present paper describes the application of e.s.r. spectroscopy to studies of structure and formation of diazodiphenoquinone pigments. The results indicate that the bacterial oxidation may proceed via a free-radical mechanism.

Materials and methods

Pyridine-2,3-diol was obtained from Ralph N. Emanuel Ltd., Wembley, Middx., U.K., and pyridine-2,5-diol was synthesized by the method of Behrman & Pitt (1958). Identical samples of diazodiphenoquinone were obtained (a) by oxidation of pyridine-2,3-diol with potassium bromate in strongly acid solution by

the method of Boyer & Kruger (1957), and (b) by bacterial oxidation of pyridine-2-ol by *Arthrobacter crystallopoietes* as described by Kuhn *et al.* (1965).

The e.s.r. spectra were recorded on a Varian E-3 instrument. Oxidations of pyridinediols were carried out by using both flow and static methods.

The flow method, similar to that described by Dixon & Norman (1963), consisted of a solution of the diol (0.01 M in a suitable solvent, water or aq. alcohol) being mixed with a solution of potassium ferricyanide (0.01 M in 0.1 M-NaOH) just before passage through an aqueous-solution cell situated in the cavity of the spectrometer.

The flow rate and the point of mixing could be varied so that the solution was observed at any time between several seconds and a few milliseconds after mixing.

The static method consisted of mixing the two solutions in a sample tube and transferring the resultant mixture to an aqueous-solution cell in the spectrometer cavity. This method had the advantage of only requiring small amounts of diol, and solid lead dioxide could also be used as an oxidant in this system (approx. 1 mg/ml of diol solution). The reductions were carried out by using the static method. To a few ml of a solution of quinone (0.01 M) in dimethylformamide were added a few drops of alkaline sodium dithionite solution; the mixture was shaken and transferred to the aqueous-solution cell.

Results and discussion

Reduction of the diazodiphenoquinone, obtained from the bacterial and bromate oxidations previously described, gave rise to the e.s.r. spectrum shown in Fig. 1. This spectrum is readily interpreted as that arising from radical (I), in which hyperfine splittings due to two equivalent nitrogen atoms (1:2:3:2:1,

Fig. 1. Structures and hyperfine splitting constants for radicals observed during: (a) reduction of diazodiphenoquinone pigment (with e.s.r. spectrum shown); (b) oxidation of pyridinediols
a, Hyperfine splitting.

$96\mu\text{T}$) and two equivalent protons (1:2:1, $32\mu\text{T}$) are observed. Since this radical is generated by simple electron transfer under the conditions used, the structure of the original quinone suggested by Kuhn *et al.* (1965) is confirmed. Radicals of related structure in the biphenyl series have been observed (Stone & Maki, 1964), in which the odd electron is distributed equally between the two rings.

Spectra identical with that in Fig. 1 were observed during the oxidation of pyridine-2,3- and -2,5-diols in a static system by using potassium ferricyanide or lead dioxide as oxidant. The radical was detected only in solutions of pH greater than approx. 11.5 and could be observed for several hours under these conditions.

In an attempt to detect precursors of the dimeric radical, the flow method was used and a number of spectra were observed which could be ascribed to

radicals involved in primary and secondary stages of the reaction (see Fig. 1).

Flowing pyridine-2,3-diol against a potassium ferricyanide solution, as described above, gave rise to radical (II) (observation approx. 0.01 s after mixing) and radical (III) (approx. 5 s after mixing). These radicals are similar to those found in the benzosemiquinone series, apart from the presence of a nitrogen splitting in the spectra (Ashworth & Dixon, 1972). The 2,5-diol gave no spectra identifiable with the primary or secondary hydroxylation product, but, with methanol as solvent for the diol, radical (IV) was observed (approx. 5 s after mixing), in which addition of the solvent to the heterocyclic ring had occurred.

Knackmuss (1973) has presented a mechanism for the formation of diazodiphenoquinone pigments from the oxidation of pyridinols *in vitro*. The present e.s.r. results confirm that addition of solvent (water

Scheme 1. Reaction scheme for the formation of diazodiphenoquinone pigment from oxidation of pyridine-2,3-diol

or alcohol) can occur at the C=N bonds of azasemiquinones and that dimerization at some stage leads to the formation of the diazodiphenoquinone structure. The e.s.r. data, however, further show that the oxidation processes involved occur by way of one-electron transfer stages involving the formation of azasemiquinone radicals.

In addition, the dimerization is highly characteristic of a free-radical coupling reaction. Radical (III) (see Fig. 1) has ring-proton splittings close to those of the related semiquinone of trihydroxybenzene (Ashworth & Dixon, 1972), which is known to dimerize through the carbon atom bearing the large odd-electron density (C-5). Since the nitrogen atom in the pyridine ring has only a small effect on the odd-electron density distribution, it is reasonable to assign the large splitting to the 5-position of the azasemiquinone (III).

Dimerization would then lead to the diazodiphenoquinone, which has been shown to be the final oxidation product.

The proposed reaction scheme for the oxidation of pyridine-2,3-diol *in vitro* is given in Scheme 1. The three azasemiquinones involved have all been detected by e.s.r. and were shown in Fig. 1.

The production of diazodiphenoquinone pigments by bacterial oxidation has been shown to involve pyridine-2,3,6-triol as an intermediate (Holmes *et al.*, 1972) and the corresponding mono-oxygenase has been isolated (Holmes & Rittenberg, 1972). The mono-oxygenase reactions are strongly favoured by aeration of the cultures and the bacterial oxidations studied so far appear to be closely parallel in terms of reaction products and stable intermediates that could be isolated to the oxidation processes observed *in vitro*

(Knackmuss, 1973). In view of these similarities, the possibility arises that the bacterial oxidations involve the same types of azasemiquinone radicals reported in the present paper. Moreover, the mono-oxygenase reaction uses atmospheric oxygen in some form, and this oxidant is known to initiate free-radical processes in many areas, notably in the reactions involving benzosemiquinone radicals discussed above. However, since there is no known clear evidence for mono-oxygenases acting by one-electron mechanisms, the possibility exists that the bacterial oxidations are occurring by two-electron steps, and that the radical involved in the dimerization step shown in Scheme 1 arises non-enzymically in a simple autoxidation process.

Attempts to observe e.s.r. signals during bacterial oxidations carried out in the present study have so far been unsuccessful. This may be due to the fact that the oxidation is not sufficiently rapid to allow an appreciable concentration of radical to develop. A second factor may be that the conditions (e.g. pH) used for the bacterial oxidation are not suitable for the presence of observable concentrations of azasemiquinones, an effect found in studies of the related benzosemiquinones. Indeed, although lead dioxide oxidation of the diols gave the same blue pigment at

pH 7.0 and pH 11.5, presumably by the same mechanism, azasemiquinone radicals were observed in the latter case only.

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