A Fluorimetric and Electron Spin Resonance Study of the Oxygenation of Benzo[*a*]pyrene; an Interpretation of the Enzymic Oxygenation

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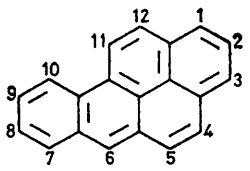
The oxygenation of benzo[*a*]pyrene (BP) was examined by treatment with hydrogen peroxide, Fenton's reagent, peroxy-acids, 9,10-diphenylanthracene peroxide (which generates singlet oxygen), and molecular oxygen. Although these reagents were considered to act in different ways, the same oxidation products (BP-3-ol, BP-6-ol, BP-6-oxyl radical, BP-diones, *etc.*) were produced in the first three systems. The amounts of products, however, depended upon the individual reagents used and also on the reaction conditions. The results are discussed in terms of chemical reactivities, and explain why BP-3-ol but not BP-6-ol is a major metabolite. BP-6-ol, which has not been detected directly as a product of the enzymatic reaction, was formed in the model systems. A mechanism involving direct oxygenation rather than an intermediate arene oxide is postulated.

MODEL systems for enzymic oxygenation have been studied extensively.[1] Various kinds of system such as Fenton's reagent [hydrogen peroxide and iron(II)] the Udenfriend system [aqueous ascorbate, iron(II), and oxygen, usually containing ethylenediaminetetra-acetic acid], peroxy-acids, low oxidation state metal ion and oxygen systems, *etc.* have been used to simulate the



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enzymic hydroxylation of aromatic drugs.[2] However, oxygenation of the carcinogenic benzo[*a*]pyrene (BP) (1) has never been achieved by chemical models.[3] On the other hand, efforts have been made to correlate the nature of the metabolites with mutagenicity[4] or carcinogenicity.[5] At present it is not certain which is the proximate form of BP for carcinogenesis (hydroxy-BPs,[6] BP oxides,[5] BP-6-oxyl radical,[7] 7,8-dihydro-BP-7,8-diol 9,10-oxide,[4a] *etc.*). It is necessary to clarify the pathways of metabolic activation of BP, and the mechanism of mono-oxygenase action whereby the various metabolites are produced.

Studies on model systems for the enzymic oxygenation of BP are expected to give valuable information in this respect. In the present paper, fluorimetric and e.s.r. studies on the products from several chemical systems are reported and the mechanism of oxygenation is discussed in relation to that of the mono-oxygenase action.

EXPERIMENTAL

An Aminco-Bowman spectrophotofluorimeter with a xenon lamp was used for the measurements of fluorescence (excitation at 396 nm) and excitation (emission at 520 nm) spectra of hydroxybenzo[*a*]pyrenes in alkaline solution. U.v. and near-u.v. absorption spectra were measured with a Hitachi EPS-3T spectrometer. E.s.r. spectra were measured with a JES-3BX spectrometer [100 kHz field modulation at room temperature (*ca.* 25 °C)]. Hyperfine splittings were measured after the sample was degassed (to 10^{-2} Torr) by a freeze–pump–thaw technique.

Products were separated and characterized by t.l.c. on silica gel, eluted with benzene containing a small amount of ethanol.

Materials.—BP (Nutritional Biochem. Co.) was purified by elution through an activated alumina column with benz-

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ene in the dark. BP-6-ol was prepared by the method of Fieser and Hershberg,[8] and purified under nitrogen; it was stable to refluxing in benzene under nitrogen, but was unstable under molecular oxygen or in u.v. light, being easily oxidized even in the solid state to BP-6-oxyl radical, BP-diones, *etc.* The free radical was also produced by treatment with chloranil in benzene solution. BP-3-ol was a gift from Dr. H. V. Gelboin. The fluorescence spectra of standard samples (BP-3-ol and BP-6-ol) were measured for solutions in N-sodium hydroxide (2 ml), obtained by shaking with solutions in n-hexane (6.2 μM; 500 μl). 9,10-Diphenylanthracene peroxide (DPAP) was prepared as described in ref. 9.

Reaction Conditions.—Oxygenation reactions were carried out in the dark at room temperature unless otherwise indicated. Mixing of samples in tubes was carried out automatically.

Iron(II)–Hydrogen Peroxide (Fenton's Reagent).—System (A). Hydrogen peroxide (30%; 10 μl) was added to BP (7 μmol) dissolved in benzene (500 μl). The mixture was stirred with iron(II) sulphate (18 μmol) for 5 min. A yellow colour developed. The e.s.r. spectrum of the benzene phase was measured, then, the same benzene phase was shaken with N-sodium hydroxide (2 ml) to extract the hydroxylation products.[10,11] The colour changed from yellow to brown. After filtration the fluorescence and excitation spectra of the anions in the water phase were measured.

System (B). A mixture of iron(II) sulphate (50 μmol) in water (5 ml) and BP (7 μmol) in acetone (1 ml) was treated with hydrogen peroxide (100 μl) for 5 min. Then the products were extracted with n-hexane (3.5 ml). The n-hexane phase was washed with a little water; it showed no e.s.r. spectrum since the BP-6-oxyl radical was quenched by acetone. It was then shaken with N-sodium hydroxide (2 ml) and the fluorescence measurement was carried out on the aqueous phase.

Hydrogen Peroxide.—Hydrogen peroxide (10 μl) and BP (7 μmol) in benzene (500 μl) without iron(II) were stirred for 20 h. Products were studied as described for Fenton's system (A).

Trifluoroacetic or Acetic Acid–Hydrogen Peroxide.—BP (7 μmol) and trifluoroacetic acid (18 μmol) in benzene (500 μl) were shaken for 5 min with hydrogen peroxide (10 μl). The e.s.r. and the fluorescence measurements were carried out as the case of Fenton's system (A). The amounts of products were very small when acetic acid was used instead of trifluoroacetic acid.

Reactions with Molecular Oxygen.—In order to check the reactivity of molecular oxygen in the ground state, BP (70 μmol) dissolved in benzene (5 ml) was stirred for 20 h. In a separate experiment DPAP (111 μmol) (known to generate singlet oxygen [9]) and BP (70 μmol) in benzene (5 ml) were refluxed in a dried apparatus. The e.s.r. and fluorescence spectra of each sample were measured as described above.

Enzymic System.—A mixture (total volume 1 ml), containing microsomal protein of rat liver (0.4 mg), Tris chloride buffer (50mM), and potassium chloride (0.15M) (pH 7.5) with NADPH (0.8mM) and BP (0.1mM) was incubated for 10 min at 37 °C in the dark. The fluorimetric measurement was carried out in alkaline solution after extraction with n-hexane from the reaction mixture.[11]

RESULTS AND DISCUSSION

The excitation spectrum [Figure 1(a)] obtained from oxidation with Fenton's system (A) is nearly the same as that of authentic BP-6-ol [Figure 2(b)] (peaks at 437 and 470 nm). The yield is estimated from the fluorescence intensity as 2 nmol. The formation of BP-6-ol was confirmed by detecting its oxidation product (the oxyl

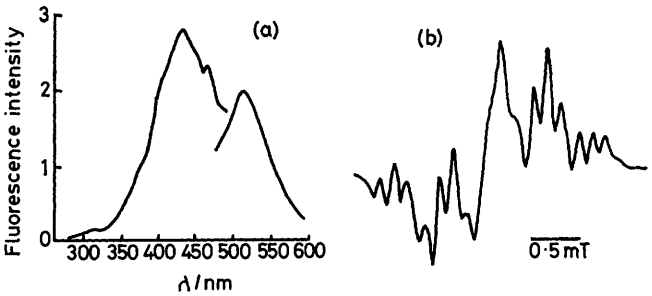

FIGURE 1 (a) Excitation and fluorescence spectra in alkaline solution, and (b) e.s.r. spectrum of products obtained by treating BP in benzene with Fenton's reagent

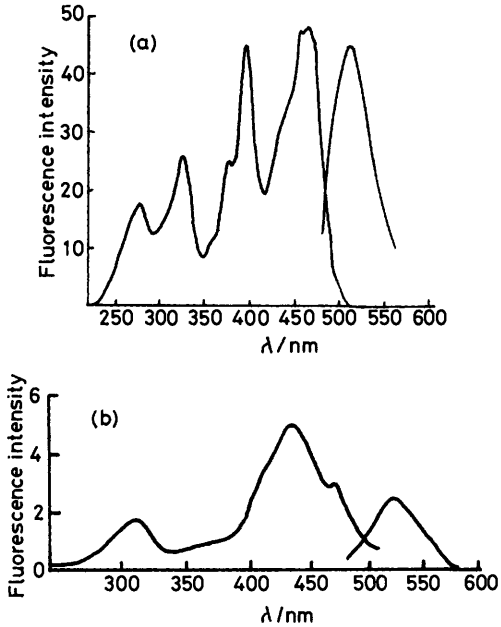

FIGURE 2 Excitation and fluorescence spectra of (a) BP-3-ol and (b) BP-6-ol in alkaline solution

radical) by e.s.r. The observed spectrum [Figure 1(b)] is identical with that formed from authentic BP-6-ol with chloranil or cerium(IV) sulphate (Figure 3), and different from that of the semiquinone radical produced by reduction of BP-dione in the same solvent.

The products formed depend on the solvents used and the conditions of the reaction. For example, when BP was dissolved in acetone [Fenton's system (B)] the results were different from those obtained when benzene was used. When the reagents were simply mixed, the

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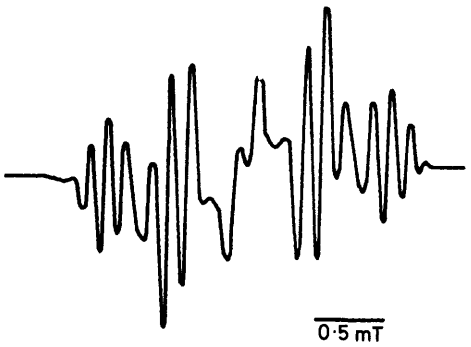

FIGURE 3 E.s.r. spectrum of BP-6-oxyl radical in benzene obtained by oxidation of BP-6-ol

excitation spectrum of the products was nearly the same as that of BP-6-ol, as in the case of Fenton's system (A), where the yield was low (5 nmol). However when aqueous iron(II) sulphate was added dropwise to a solution of BP in acetone and hydrogen peroxide, the excitation spectrum was different. Comparison with spectra of standard samples showed that one of the main products was BP-3-ol (Figure 5) (peaks at 326, 380, 398, and 467 nm in the excitation curve and 518 nm in the fluorescence spectrum). The peaks around 440 nm may correspond to other BP-ols produced simultaneously in lower yields. The lack of a peak at 279 nm may be due to the presence of other hydroxylated derivatives.[11] This spectrum is very similar to that obtained from an enzymic system, in which BP-3-ol has been shown to be a major metabolite.[6,11] Fluorescence intensities provide the yields under the two sets of conditions (3.7 and 2 nmol respectively).

Thus, when Fenton's reagent reacted with BP in inhomogeneous medium or with insufficient mixing, the main product was BP-6-ol. On the other hand, the main product was BP-3-ol when iron(II) sulphate solution was added dropwise to BP and hydrogen peroxide in acetone. This might be explained as follows. Position 6 of BP is the most chemically reactive,[8,12] and one would therefore expect oxygenation to occur at this position. However, BP-6-ol is unstable in the presence of molecular oxygen and is easily oxidized further to non- or weakly fluorescing products like BP-6-oxyl radical and BP-diones (Figure 7). Thus, in the efficient oxidation procedure [dropwise addition of iron(II)] most of the BP-6-ol was oxidized further, and position 3 of BP was attacked. Then the BP-3-ol, which is more stable, would accumulate. This explanation is supported by the fact that the main fluorescing product was BP-3-ol in the trifluoroperacetic acid system (Figure 8), in which the reaction

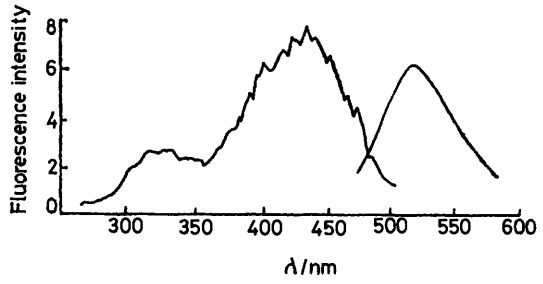

FIGURE 4 Excitation and fluoresence spectra in alkaline solution of the products obtained by treating BP in acetone with Fenton's reagent

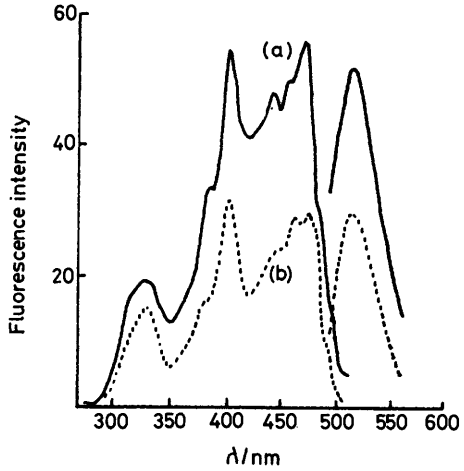

FIGURE 5 Excitation and fluoresence spectra in alkaline solution of the products obtained (a) by treating BP in acetone with Fenton's reagent [system (B)] and (b) by the enzymic reaction

proceeded efficiently, because trifluoroacetic acid is miscible with benzene. From the fluorescence intensity, the formation of 14 nmol of BP-3-ol was estimated. The hyperfine structure of the e.s.r. spectrum was the same as that of BP-6-oxyl radical, showing the BP-6-ol had been oxidized. The e.s.r. intensity is *ca.* 50 times that of the product from Fenton's system (A).

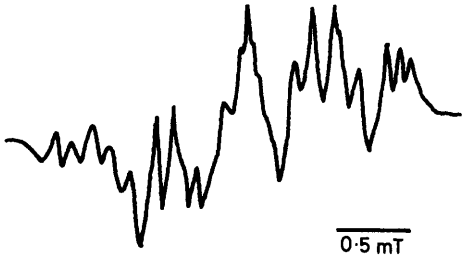

FIGURE 6 E.s.r. spectrum of the product obtained by treating BP with hydrogen peroxide

In trifluoroperacetic acid O–O polarization is considered to occur, as a result of the inductive effect of the trifluoroacetyl group. This was supported by the fact that the reaction was extremely slow when acetic was used instead of trifluoroacetic acid. Oxygenation by this system is thought to proceed by transfer of an oxygen atom in a concerted mechanism.

FIGURE 7 T.l.c. of oxidized BP-6-ol; *F*, BP-6-ol; *E*, BP-6-oxyl radical; *B*, *C*, and *D* BP-3,6-,-1,6-, and -6,12-diones, respectively; *A*, unidentified product

When BP was treated with air or singlet oxygen, no derivative of BP was detected by e.s.r., fluorimetry, or

[12] A. Windaus and S. Rennhak, *Z. physiol. Chem.*, 1937, **249**, 256.

t.l.c. This shows that molecular oxygen, both in the ground triplet state ($^3\Sigma_g^-$) and in the singlet state ($^1\Delta_g$) is less reactive towards BP. When BP was treated with hydrogen peroxide, the same e.s.r. signal (of the BP-6-oxyl radical) was observed as with Fenton's system

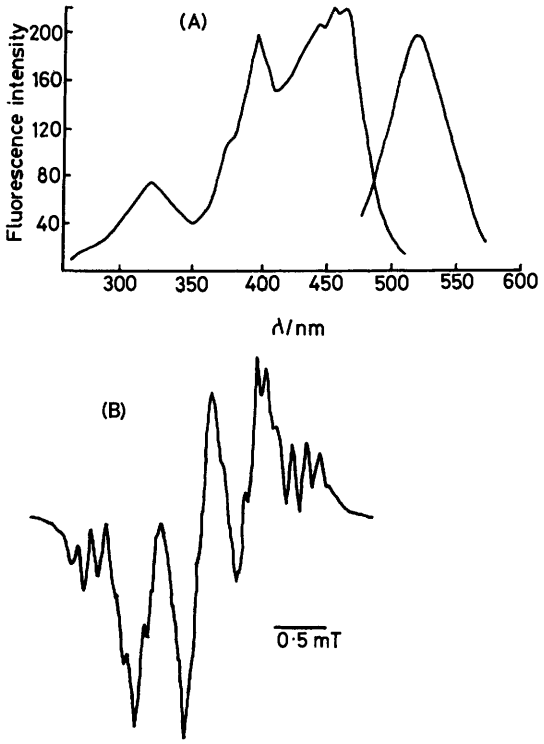

FIGURE 8 (A) Excitation and fluoresence spectra in alkaline solution of the products obtained by treating BP with trifluoroacetic acid and hydrogen peroxide; (B) e.s.r. spectrum of the benzene phase of reaction mixture

(Figure 4). This indicates that even if no iron(II) is added, active oxygen species are generated from hydrogen peroxide, though the yield is low. Free radical species [·OH and $\cdot O_2H$ (hydrogen peroxide or Fenton's reagent [13])] and hydroxy-cation-like species (trifluoroperacetic acid) are reactive and are considered to give the same products from BP, *i.e.* oxygen atoms activated in different ways attack the same positions of BP. This can be explained in terms of chemical indices,[14] which show that delocalization energies, frontier electron densities, *etc.*, are the same for both radical and ionic reactions in alternant hydrocarbons such as BP.

In the case of polycyclic aromatic hydrocarbons, arene oxides [15] have been implicated as obligatory intermediates which give rise to mono-ols, diols, diones, and their conjugates commonly detected as metabolites. However it is considered that hydroxylation by either free radical species or hydroxy-cation-like species at position 6 of BP through NIH shift of some oxides is difficult. From recent observations on the oxygenation of olefins by peroxy-acids a mechanism involving initial transfer of an oxygen atom to a carbon centre has been postulated.[16] Moreover, a mechanism involving direct hydroxylation of aromatic substrates rather than reaction *via* arene oxides has been reported.[17] The mechanism whereby BP-6-ol is formed directly is consistent with these results.

In enzymic reactions the excitation spectrum of the products is similar to that of BP-3-ol but not to that of BP-6-ol. The present study explains this fact in that enzymic reactions must correspond to the stronger oxygenation reaction in the present model systems. Considerable amounts of BP-6-oxyl radical and BP-diones are also produced in enzymic reactions.[6,7]

Although the production of BP-6-ol in the metabolism of BP has been assumed from the detection of conjugates [18,19] and from the observation of BP-6-oxyl radical,[7] it has never been observed directly. Our direct detection of BP-6-ol by fluorimetry in model systems under weak oxygenation conditions strongly suggests that reactive BP-6-ol molecules are formed *in vivo*, and that some of them are the precursors of the above metabolites and are partly converted into BP-diones.

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