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HYDROPEROXIDE-DEPENDENT OXYGENATION OF trans-7,8-DIHYDROXY-7,8-DIHYDRO BENZO[A]PYRENE BY RAM SEMINAL VESICLE MICROSOMES. SOURCE OF THE OXYGEN

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SUMMARY. Incubation of 15-hydroperoxy-5,8,11,13-eicosatetraenoic acid with ram seminal vesicle microsomes (RSVM) triggers the oxygenation of trans-7,8-dihydroxy-7,8-dihydrobenzo[a]pyrene (BP-7,8-diol). The principal oxidation products are 7,8,9,10-tetrahydroxy-7,8,9,10-tetrahydrobenzo[a] pyrenes which are non-enzymatic hydrolysis products of r-7,t-8-dihydroxy-t-9,10-oxy-7,8,9,10-tetrahydrobenzo[a]pyrene. At short incubation times, an additional product is isolated which is identified as r-7,t-8,t-9-trihydroxy-c-10-methoxy-7,8,9,10-tetrahydrobenzo[a]pyrene. This product appears to arise by solvolysis of the extracted diolepoxide during high performance liquid chromatography using methanol-water solvent systems. The incubation of ^{18}O-labeled 15-hydroperoxy-5,8,11,13-eicosatetraenoic acid with BP-7,8-diol and RSVM leads to very little incorporation of ^{18}O into the stable solvolysis products (analyzed by gc-ms of their peracetates). Parallel incubations conducted with ^{16}O-labeled hydroperoxide under an $^{18}O_2$ atmosphere indicate that the principle source of the epoxide oxygen is molecular oxygen.

INTRODUCTION. Peroxidase activity is widely distributed in the plant and animal kingdom (1,2). The interaction of hydrogen peroxide or organic hydroperoxides with peroxidases generates oxidizing agents which trigger the dehydrogenation or oxygenation of suitable cosubstrates (3-8). Hydroperoxide-dependent oxygenations by peroxidases are particularly interesting because they can provide valuable information regarding the mechanism of catalysis. For example, the demonstration that cytochrome P-450 catalyzes the direct transfer of hydroperoxide

1. The abbreviations used are: PGG_1, 15-hydroperoxy-9α,11α-peroxido-13-trans-prostenoic acid; PGG_2, 15-hydroperoxy-9α,11 -peroxido-5-cis-13-trans-prostadienoic acid; PES, prostaglandin endoperoxide synthetase; BP-7,8-diol, trans-7,8-dihydroxy-7,8-dihydrobenzo[a]pyrene; [^{14}C]-BP-7,8-diol, [7-^{14}C]-trans-7,8-dihydroxy-7,8-dihydrobenzo[a]pyrene; RSVM, ram seminal vesicle microsomes; anti-BP-diolepoxide, r-7,t-8-dihydroxy-t-9,10-oxy-7,8,9,10-tetrahydrobenzo[a]pyrene; 15-[^{18}O]OOH-PGE_2, 15-hydroperoxy-11-hydroxy-9-keto-5-cis-13-trans-prostadienoic acid, labeled with ^{18}O in the hydroperoxy group; 15-OOH-20:4, 15-hydroperoxy-5,8,11,13-eicosatetraenoic acid; 15-[^{18}O]OOH-20:4, 15-hydroperoxy-5,8,11,13-eicosatetraenoic acid labeled with ^{18}O in the hydroperoxy group; syn-BP-diolepoxide, r-7,t-8-dihydroxy-c-9,10-oxy-7,8,9,10-tetrahydrobenzo[a]pyrene; HPLC, high performance liquid chromatography; gc-ms, gas chromatography-mass spectrometry; BP, benzo[a]-pyrene; DPBF, 1,3-diphenylisobenzofuran.

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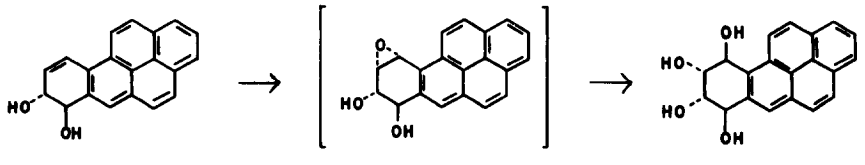
oxygen from cumene hydroperoxide to cyclohexane suggests the intermediacy of a ferryl-oxo derivative of the heme which delivers an oxygen atom to the substrate (9-11).

Hydroperoxide-dependent oxygenations of aromatic hydrocarbons (12-14), aromatic amines (15), and sulfides (16) by a peroxidase in ram seminal vesicle microsomes (RSVM) have been described recently. The natural hydroperoxide substrates appear to be PGG_1[1], PGG_2, and hydroperoxy fatty acids generated from polyunsaturated fatty acids by the action of prostaglandin endoperoxide synthetase (PES), which is present in high levels in this tissue (14,16). The peroxidase component in crude tissue extracts has not been identified but it has been found that the hydroperoxidase component of purified and reconstituted PES exhibits cooxidation activity (16). The physiological role of these peroxidatic oxygenations is unknown, but our finding that BP-7,8,-diol is converted to a highly mutagenic derivative by RSVM during prostaglandin endoperoxide biosynthesis suggests a possible pathological role in carcinogen activation (17).

Investigations in our laboratory and that of Eling have shown that the major product of BP-7,8-diol metabolism by RSVM is a 7,8,9,10-tetrahydroxy-7,8,9,10-tetrahydrobenzo[a]pyrene, (12,13). This compound is the principal non-enzymatic hydrolysis product of the anti-BP-diolepoxide. It has, therefore, been suggested that RSVM oxidize BP-7,8-diol to the strongly mutagenic diolepoxide which hydrolyzes to the stable and non-mutagenic tetraol (18-21) (eq. 1). The most straightforward mechanism which one could suggest to explain the hydroperoxide-dependent epoxidation of BP-7,8-diol by RSVM would involve an enzyme-mediated transfer of the hydroperoxide oxygen into the isolated double bond of the diol. Ample literature precedents support such a transfer and 1 is the major product of the non-enzymatic oxygenation of BP-7,8-diol by m-chloroperoxybenzoic acid (22). In addition, Egan et al have recently demonstrated that microsomal and purified (hemoglobin reconstituted) PES preparations catalyze the transfer of ^{18}O from 15-[^{18}O]OOH-PGE_2 to sulindac sulfide (23). We have, therefore, carried out an investigation of the source of the oxygen incorporated into BP-7,8-diol by RSVM, the results of which are mechanistically provocative. During the course of our studies, we have identified an additional metabolite of BP-7,8-diol which provides further support for the intermediacy of a diolepoxide.



BP-7,8-DIOL

Equation 1

MATERIALS AND METHODS. 15-OOH-20:4 was prepared according to Funk et al (24). 15-[^{18}O]OOH-20:4 was prepared and analyzed for ^{18}O content as described previously (25). BP-7,8-diol, [^{14}C]-BP-7,8-diol, and BP-diolepoxides were obtained through the Standard Chemical Carcinogen Reference Repository of the National Cancer Institute. $^{18}O_2$ was obtained from Bio Rad. Tetraol standards were synthesized from the diolepoxides using literature procedures (20).

Preparation of peracetates - The compound to be derivatized was transferred to a test tube in an organic solvent and the solvent evaporated in a stream of argon. 1 ml of a solution of pyridine:acetic anhydride - 1:1 (v/v) was added and the capped test tube was heated to 60°. TLC analysis on silica gel (chloroform/ethanol - 7:3) indicated that the reaction was complete in 90 min. The reaction mixture was taken to dryness in a stream of argon and the residue was dissolved in carbon disulfide for gas chromatographic analysis.

Incubations with 15-[^{18}O]OOH-20:4 - 1.14 mg (4 μmol) [^{14}C]-BP-7,8-diol (purified by HPLC) was added to a freshly prepared suspension of RSVM (250 mg) (25) in 200 ml of 100mM KPO_4 (pH 7.8). Following a 3 min preincubation at 37°, 3.36 mg (10 μmol) 15-[^{18}O]OOH-20:4 was added and the reaction allowed to proceed for 3 min. 1 volume of acetone was added and the products were extracted with 2 x 2 volumes of ethyl acetate. The ethyl acetate was dried over $MgSO_4$ and evaporated under reduced pressure. The residue was dissolved in a small volume of methanol: water - 45:55 and the products were purified by HPLC under the conditions described above for the tetraols. Each product was further purified by HPLC and stored at -80° prior to derivatization and mass spectral analysis.

Incubations with 15-OOH-20:4 under an $^{18}O_2$ atmosphere - Freshly prepared RSVM (250 mg protein) were placed in a chilled round bottom flask attached through a sidearm to a 500 ml reaction vessel containing 200 ml of 100mM KPO_4 (pH 7.8). The vessel was fitted with a 24/40 female joint attached to a manifold containing three 14/20 male joints mounted on opposite sides with respect to each other. At the top of the manifold was a sealing constriction and a fitting for attachment to the vacuum system which was maintained at 10^{-7} Torr. Two of the joints on the manifold were connected to separate degassed methanol solutions of [^{14}C]-BP-7,8-diol and 15-OOH-20:4 through vacuum stopcocks. The third joint on the manifold was connected through a vacuum stopcock to an empty flask which was later used for the reisolation of the $^{18}O_2$. The buffer in the main reaction vessel was degassed by repeated freeze-pump-thaw cycles and the RSVM suspension in the sidearm was lyophilized during the last cycle. After the completion of degassing, 25 ml of $^{18}O_2$ (99.15 atom % excess) was transferred into the vessel via the vacuum system. The entire apparatus was then flame-sealed and removed from the vacuum line. The buffer was warmed to 37° and the RSVM and [^{14}C]-BP7,8-diol were added. The final incubation mixture contained 1% methanol. 3 min later the 15-OOH-20:4 was added and the reaction allowed to proceed for 3 min. The incubation was terminated by transfer of the remaining $^{18}O_2$ into the empty flask attached to the manifold sidearm by immersion in liquid N_2. The main vessel was cooled to 0° and 200 ml of argon-saturated acetone was added to it immediately after venting the entire system. The solution in the vessel was extracted with ethyl acetate and worked up exactly as described above.

High resolution mass spectrometry of the recovered $^{18}O_2$ indicated that it was 98.1 atom % excess ^{18}O. High resolution was necessary to separate the peak due to $^{16}O_2$ from the peak due to a small amount of methanol transferred during O_2 condensatio

Analytical methods - Gas chromatography was performed on a Varian 3700 equipped with a flame ionization detector. A 0.32 x 50 cm column of 5% OV-101 on Chromosorb W-HP was operated isothermally at 250° at a He flow rate of 37 ml/min. The injection port and the detector were maintained at 300° and 320°, respectively.

Gas chromatography-mass spectrometry was performed on a Hewlett-Packard 5985 operated at the Michigan State University Mass Spectrometry Facility. A 0.25 x 18 in column of 1% SP-2100 was operated at a He flow rate of 35 ml/min. The temperature was programmed from 220 to 280° over 8 min. Mass spectra were recorded at an ionizing voltage of 70 eV and a probe temperature of 220°.

RESULTS AND DISCUSSION. The major purpose of this investigation was to determine the source of the oxygen atom incorporated into BP-7,8-diol during its hydroperoxide-

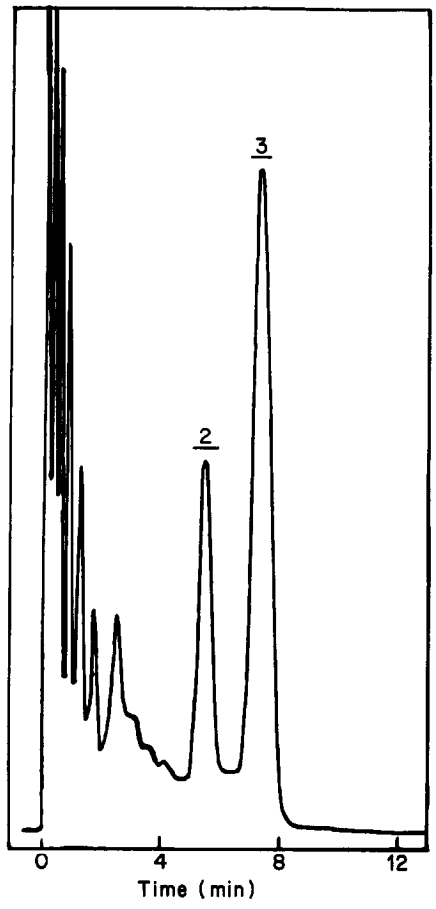


Figure 1. Gas Chromatography of the Peracetyl derivatives of 2 and 3. Conditions described under Materials and Methods.

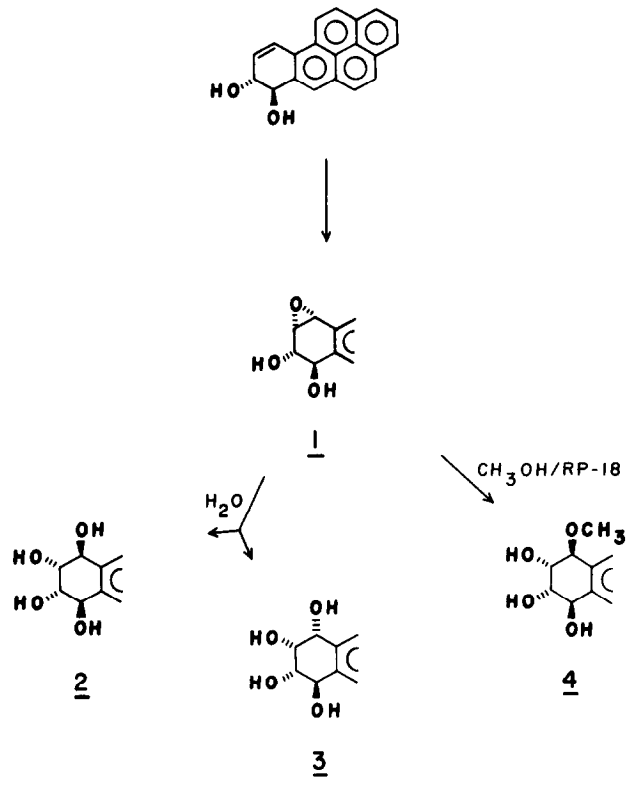
dependent metabolism by RSVM. In order to do this, we had to devise a method for th analysis of the isotopic composition of the stable and isolable tetraol metabolites. Direct probe mass spectrometry would have been ideal for this since the molecular io in the spectra of 2 and 3 are the base peaks (26). Unfortunately, the amounts of purified 2, isolated following repeated HPLC, were too small for analysis by this sample introduction technique. Since 2 is the major BP-7,8-diol metabolite formed by RSVM, it would have been impossible to acquire any information on other possible metabolites. This problem was solved by our finding that the peracetate derivatives of 2 and 3 chromatograph cleanly on non-polar silicone columns at 260° (40 ml He/min) (Figure 1). When analyzed by combined gc-ms, the peracetates exhibit relatively abundant molecular ions at m/e 488 (10-15% of base peak) which are suitable for isotopic analysis by selected ion monitoring. Fragment ions are seen at m/e 428 and 368 corresponding to the loss of one and two molecules of

acetic acid. The peracetate derivatives are easily prepared and appear to be ideally suited for use in structural determinations of benzo ring metabolites of BP.

Our previous study of the metabolism of BP-7,8-diol by RSVM utilized an incubation time of 15 min in an attempt to generate maximal amounts of isolable metabolites (12). Under these conditions, the principal products are 2 and 3 which are identical to authentic standards when compared by HPLC, ultraviolet, and mass spectroscopy (as the peracetates). Since preliminary experiments indicated that significant exchange of $^{18}O_2$ with $^{16}O_2$ occurred under these conditions in our original apparatus (25), we made two modifications in our experimental method. The first was to carry out the incubations in a system which was completely isolated from the atmosphere and into which all compounds were added as degassed solutions through high vacuum stopcocks (see Experimental Procedures). The second modification was to shorten the incubation time to 3 min to minimize the potential for $^{18}O_2$ - $^{16}O_2$ exchange. In fact, a detailed study showed that BP-7,8-diol metabolism by RSVM is complete within 5 min.

When incubations of [^{14}C]-BP-7,8-diol (20 μM, 2.5 μCi/μmol), RSVM (1 mg protein/ml), and 15-OOH-20:4 (50 μM) are carried out for 3 min, another radioactive product, less polar than 2 and 3 is observed in the HPLC profile. Its ultraviolet spectrum exhibits typical pyrene absorptions indicating that it has arisen by metabolism of the isolated double bond in the dihydrobenzo ring of BP-7,8-diol. Treatment of the unknown metabolite with acetic anhydride in pyridine converts it to a derivative which can be analyzed by gc-ms. The mass spectrum exhibits a molecular ion at m/e 460 and prominent peaks at m/e 400 (M^+-HOAC), 340(M^+-2 HOAC), 298(340-ketene), and 282(298-0). The molecular ion is consistent with a 7,8,9,10-tetrahydrobenzo[a]pyrene containing three acetates and one methoxyl group. This suggests that the unknown metabolite is a methyl ether formed by methanolysis of epoxide 1 (Scheme 1). Authentic 4, prepared by solvolysis of 1 in acidic methanol exhibits an identical retention time on HPLC and an identical mass spectrum when analyzed as the triacetate (27). Since only trace amounts of methanol are present in the incubation mixture (1%) and since this product is not seen when the incubations are allowed to proceed for 15 min, it is probable that it arises during the workup. When authentic 1 is injected onto the HPLC column (Lichrosorb RP-18) under identical conditions, the major products are tetraol 2 and methyl ether 4. Tetraols 2 and 3 are not converted to 4 during extraction and chromatography. It seems likely that 4 is formed during chromatography by methanolysis of 1 extracted from the incubation mixture. This provides further evidence for the formation of the diolepoxide, 1, during the hydroperoxide-dependent oxidation of BP-7,8-diol by RSVM.

15-[^{18}O]OOH-20:4 was prepared and analyzed for ^{18}O content as previously described (25). It was incubated with RSVM and BP-7,8-diol under an atmosphere



Scheme 1

of $^{16}O_2$. Parallel incubations were performed using 15-OOH-20:4, RSVM, and BP-7,8-diol under an atmosphere of $^{18}O_2$ in the apparatus described in Materials and Methods. The products from both sets of experiments were independently extracted and purified by repeated HPLC. Each of the isolated products was converted to a peracetate derivative and analyzed by gc-ms as described above. Full spectral analysis of each compound indicated that in those experiments where ^{18}O incorporation was observed only a single atom of ^{18}O was incorporated. Isotopic composition was, therefore, determined by selected ion monitoring of M^+ and $M^+ + 2$ and the ^{18}O content was calculated as atom % excess. The results of these experiments are summarized in Table 1. It is evident that the principal source of oxygen incorporated into BP-7,8-diol during its oxidation by RSVM in the presence of 15-OOH-20:4 is atmospheric oxygen, not hydroperoxide oxygen.

Our present finding regarding the source of the oxygen introduced into BP-7,8-diol is similar to our earlier results regarding the source of the oxygen introduced into DPBF (25). In the case of DPBF, we suggested that the oxygenation occurs by a free radical chain process initiated by an unidentified oxidant released following the interaction of a hydroperoxide with the peroxidase (25). The experimental evidence for this mechanism includes the source of the oxygen (O_2) and the stoich-

Table I. Identification of the Source of the Oxygen Incorporated into BP-7,8-Diol

Product Analyzed	[^{18}O]-ROOH/$^{16}O_2$ [a,b]	[^{16}O]-ROOH/$^{18}O_2$ [a]
2 - $(OAc)_4$	7	91
3 - $(OAc)_4$	-- [c]	89
4 - $(OAc)_3$	4	90

a) Data expressed as atom % excess [^{18}O] for the incorporation of one atom of [^{18}O], single determination; b) data generated using 15-[^{18}O]OOH-20:4 with 91 atom % excess [^{18}O]; c) amounts of 3 isolated were insufficient for analysis.

iometry of DPBF oxidized to hydroperoxide added which varies up to 3000:1. The stoichiometry of BP-7,8-diol oxidized to hydroperoxide added is less than 0.1:1. However, the true value of this number cannot be determined accurately because there are reducing impurities in the microsomal preparation which effectively compete with BP-7,8-diol for oxidizing equivalents generated from the hydroperoxide. A similar effect has been seen by Egan et al. in the oxidation of sulindac sulfide by RSVM (16). Although no mechanistic information can be gleaned from the stoichiometry data, it should be noted that hydroperoxide-dependent oxygenation of BP-7,8-diol is strongly inhibited by antioxidants. For example, 25 μM butylated hydroxyanisole completely inhibits the metabolism of BP-7,8-diol to mutagenic derivatives and to the tetraol 2.

Although a complete mechanistic description of the hydroperoxide-dependent epoxidation of BP-7,8-diol cannot be made at present, the source of the oxygen and the antioxidant inhibition suggest that the oxidation is radical in nature. Precedents do exist for the epoxidation of hydrocarbons by radical mechanisms, although the yield of epoxide is usually low (28). However, in a few cases, such as those described by Padwa and Brodsky (29) and Hart and Lavrik (30), epoxides are isolated in yields of 65-90% following hydrocarbon autoxidation. Since these processes are undoubtedly free radical in nature, it seems that the general type of oxygenation which our data implies for BP-7,8-diol is precedented, if uncommon.

The peroxidase in RSVM which is responsible for the oxygenation of BP-7,8-diol has not yet been identified. In fact, our earlier work has shown that boiling the RSVM preparation does not completely abolish the oxygenation triggered by 15-OOH-20:4 (12). This suggests that the initiator may be a spurious oxidant released

during the interaction of hydroperoxides with RSVM. Since the intact peroxidase increases hydroperoxide turnover, it may also increase the rate of production of the oxidizing agent. This could account for the higher levels of oxidation seen with intact microsomes relative to boiled microsomes.

Egan et al. have demonstrated that the hydroperoxide oxygen is directly transferred from 15-OOH-PGE_2 to sulindac sulfide (23). We find that the hydroperoxide oxygen is transferred from 15-OOH-20:4 to BP-7,8-diol with very low efficiency. This difference does not appear to be due to a difference in the hydroperoxide utilized since we have shown that PGG_2 (a precursor to 15-OOH-PGE_2) and 15-OOH-20:4 are kinetically and mechanistically equivalent in the RSVM catalyzed oxygenation of DPBF (25). Since the enzyme preparations are nearly identical, a comparison of the study of Egan et al. with our own provides a dramatic confirmation of our earlier speculation that the mechanism of hydroperoxide-dependent oxygenations may depend not only on the nature of the peroxidase but also on the nature of the oxidized substrate (25). One cannot assume that a particular mechanism obtains based solely on precedents established in unrelated systems. Other recent literature reports suggest that such complexity may be general for proteins exhibiting peroxidase activity (31-34).

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