Benzo[a]pyrenedione/Benzo[a]pyrenediol Oxidation–Reduction Couples and the Generation of Reactive Reduced Molecular Oxygen†

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ABSTRACT: The ability of the isomeric quinone metabolites of benzo[a]pyrene, benzo[a]pyrene-6,12-dione, benzo[a]-pyrene-1,6-dione, and benzo[a]pyrene-3,6-dione to undergo reversible, univalent oxidation–reduction cycles involving the corresponding benzo[a]pyrenediols and intermediate semiquinone radicals has been characterized. Under anaerobic conditions, all three benzo[a]pyrenediones are easily reduced to benzo[a]pyrenediols, even by mild biological agents such as NAD(P)H, cysteamine, and glutathione. The benzo-[a]pyrenediols, in turn, are very rapidly autoxidized to the benzo[a]pyrenediones when exposed to air. Substantial amounts of hydrogen peroxide are produced during these autoxidations, and other reactive reduced oxygen species, such as the superoxide and hydroxyl radicals, are probably formed transiently as well. The benzo[a]pyrenediol–benzo[a]pyrenedione interconversions proceed by one-electron steps; the corresponding semiquinone radicals can be monitored by electron spin resonance spectroscopy as intermediates during these reactions carried out at high pH. Benzo[a]pyrenediones induce DNA strand scission when incubated with bacteriophage T7 DNA. This damage is modified by conditions which indicate that reduced oxygen species propagate the free-radical reactions responsible for the strand scission. Benzo[a]pyrenediones are electron-acceptor substrates for NADH dehydrogenase from *Clostridium kluyveri*. Catalytic amounts of these benzo[a]pyrene metabolites, together with this respiratory enzyme, function as cyclic oxidation–reduction couples which link NADH and molecular oxygen in the continuous production of hydrogen peroxide. These data, together with preliminary results with cells in culture, indicate that benzo[a]pyrenediones are potentially harmful metabolites of benzo[a]pyrene, acting by processes which lead to their regeneration rather than depletion; nucleic acid and cell damage is probably produced by the reactive reduced oxygen species resulting from such regenerative oxidation–reduction cycles.

Previously, we reported that a major metabolite of carcinogenic benzo[a]pyrene, B[a]P,[1] is 6-hydroxybenzo[a]pyrene, 6-OH-B[a]P, which is rapidly autoxidized by a one-electron mechanism to the ubiquitous quinone metabolites, B[a]P-6,12-dione, B[a]P-1,6-dione, and B[a]P-3,6-dione (Lesko et al., 1975; Lorentzen et al., 1975). We also reported some properties of this reactive phenol, 6-OH-B[a]P, which can bind to, and cause strand breakage in, nucleic acids as well as injure and morphologically transform Syrian hamster embryonic fibroblasts (Ts'o et al., 1974; Schechtman et al., 1974). Intermediate species produced in the free-radical reactions leading from 6-OH-B[a]P to the B[a]P-diones may be responsible for some of these effects. However, chemical and biological properties of the B[a]P-dione products have not been adequately studied to decide this issue.

In contrast to 6-OH-B[a]P, the B[a]P-diones are apparently stable because they are easily isolated from B[a]P metabolizing systems. Functionally, they can be categorized as extended quinones. Although overall structurally different, many of their chemical properties are comparable to those of the benzoquinones, a class of compounds known for considerable chemical reactivity (Bruce, 1974). A salient feature of quinones in general is their ability to form oxidation–reduction couples with their hydroquinones and semiquinone radicals, species which are all interconvertible by reversible one-electron redox steps.

Here we present evidence that B[a]P-diones are not inactive B[a]P metabolites. They participate with their corresponding hydroquinones (B[a]P-diols) and semiquinone radicals in one-electron step redox cycles; these cycles are coupled with molecular oxygen to form reactive reduced oxygen species, such as superoxide radical and hydrogen peroxide. Furthermore, evidence is presented that these cycles can operate under physiological conditions, can be aided by cellular respiratory enzymes, and may be responsible for injury to nucleic acids and cells in culture.

Experimental Procedures

Materials

Diaphorase type II-L (NADH dehydrogenase, EC 1.6.99.-) from *Cl. kluyveri* was purchsed from Sigma Chemical Co., St. Louis, Mo. Superoxide dismutase (1.15.1.1) from beef erythrocytes was purchased from Miles Laboratories, Elkhart, Indiana.

Benzo[a]pyrenediones. The three B[a]P-diones and [^{3}H]B[a]P-diones were prepared by the autoxidation of 6-OH-B[a]P or 6-OH-[^{3}H]B[a]P (randomly labeled) and purified extensively by column and thick-layer chromatography on alumina. Detailed procedures for the preparation of B[a]-P-diones and their precursor, 6-OH-B[a]P, have been reported in an earlier publication (Lorentzen et al., 1975).

Bacteriophage DNA. Bacteriophage T7 DNA was obtained

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[1] Abbreviations used are: B[a]P, benzo[a]pyrene; 6-OH-B[a]P, 6-hydroxybenzo[a]pyrene; NADH, nicotinamide adenine dinucleotide, reduced form; NADPH, nicotinamide adenine dinucleotide phosphate, reduced form; EDTA, (ethylenedinitrilo)tetraacetic acid; ESR, electron spin resonance.

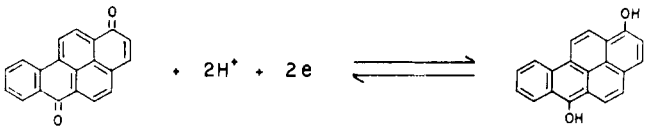

FIGURE 1: B[*a*]P-dione/B[*a*]P-diol half-reaction, using the 1,6 isomer as an example.

by the repeated extraction of purified T7 bacteriophage with freshly zinc dust distilled, buffered phenol. The procedures used for growth and purification of the bacteriophage were those described by Englund (1972). DNA obtained by this method was homogeneous and intact.

Apparatus

Ultraviolet and visible spectra were measured on Cary, Models 14 and 15, recording spectrophotometers. Electron spin resonance was measured on a JEOL, Model JES-ME-1X, ESR spectrometer. Ultracentrifuge runs were made on a Beckman, Model E, Analytical Ultracentrifuge.

Methods

Reduction of B[a]P-diones to B[a]P-diols. The B[*a*]P-dione was dissolved in distilled, nitrogen-sparged benzene at 10^{-3} to 10^{-5} M. A solution of sodium dithionite ($Na_2S_2O_4$) (10^{-2} M) was prepared by adding the salt to nitrogen-sparged water. These two immiscible solutions were shaken vigorously, resulting in immediate production of the hydroquinone (B[*a*]P-diol) in the benzene layer accompanied by a dramatic color change. In a glove bag filled with nitrogen, the benzene layer was separated and dried with sodium sulfate. When the B[*a*]P-diol was desired in a different solvent, the benzene was evaporated under vacuum, the vacuum broken with nitrogen, and the solid B[*a*]P-diol redissolved in the appropriate nitrogen-sparged solvent. Even in benzene, any exposure to the atmosphere results in some autoxidation of the B[*a*]P-diol to B[*a*]P-dione, which is easily detected by absorption spectroscopy. In general, we found it desirable to prepare the B[*a*]P-diol immediately prior to each experiment.

The B[*a*]P-diones were also reduced to B[*a*]P-diols directly in nitrogen-sparged 95% ethanol or 1:1 (v/v) 95% ethanol–0.01 M sodium phosphate, pH 7. Reducing agents, usually in excess, were added directly to the B[*a*]P-dione solution, and reduction to the B[*a*]P-diol was measured by absorption spectroscopy. Reducing agents used were sodium dithionite, sodium borohydride, NADH, NADPH, cysteamine, and glutathione. This method was inferior to the two-phase method for preparative purposes. It required a more difficult separation of the B[*a*]P-diol from reducing agent and the high lability of the B[*a*]P-diols in polar solvents frequently led to partially autoxidized products.

Autoxidation of B[a]P-diols. The B[*a*]P-diol was dissolved in nitrogen-sparged 95% ethanol to which an equal volume of nitrogen-sparged 0.01 M sodium phosphate (pH 7) was added. Absorption spectra were measured and air was introduced by pipet or by swirling the solution in air. The reaction immediately went to completion, as was obvious by the accompanying color change. For all three B[*a*]P-diols the absorption spectrum immediately after autoxidation was identical to that of the corresponding B[*a*]P-dione, whose properties have been previously described (Lorentzen et al., 1975).

Assay for Hydrogen Peroxide. The spectrophotometric method used for the quantitative detection of hydrogen peroxide involved the measurement of oxidation of the dye precursor, *o*-dianisidine, by the specific horseradish peroxidase–H_2O_2 complex. It has previously been described in detail (Lorentzen et al., 1975).

Reaction of B[a]P-diones with DNA. T7 bacteriophage DNA was diluted to 6×10^{-4} M nucleoside in 0.01 M sodium phosphate, pH 7.0. To this solution was added 95% ethanol, dropwise with gentle mixing, until the solution was 2:1 (v/v) buffer–ethanol. Under low light intensity, the B[*a*]P-dione was dissolved in an appropriate amount of 95% ethanol and likewise added to the DNA solution, dropwise with gentle mixing, to make the final solution 1:1 (v/v) 0.01 M sodium phosphate (pH 7)–95% ethanol. This solution was incubated at 37 or 22 °C in the dark for the desired period of time. The B[*a*]P-dione was then removed from the DNA by extraction (four to seven times) with an equal volume of freshly zinc dust distilled phenol which had been nitrogen-flushed and buffered with 0.5 M sodium phosphate, pH 7. The remaining phenol was removed by exhastive dialysis vs. 0.01 M phosphate, pH 7.0. In assaying for binding of [^{3}H]B(a)P-diones and 6-OH-[^{3}H]B[*a*]P to DNA, the DNA solution was extensively dialyzed prior to extraction to remove potential water-soluble radioactivity. Control DNA was handled identically to treated DNA, except that 95% ethanol replaced B[*a*]P-dione in 95% ethanol.

Assay for DNA Single-Strand Scission. Sedimentation measurements were made in a Beckman, Model E, Analytical Ultracentrifuge equipped with ultraviolet optics. The analytical zone method described in detail by Studier (1965) was employed for all measurements. In this method, a small amount of DNA was layered onto 0.9 M NaCl–0.1 M NaOH and the sedimentation coefficient was obtained by measuring the rate of sedimentation of the band of DNA through the bulk solution. Molecular weight of the single-stranded DNA was obtained by the relationship.

$$s^0_{20,w} = 0.0528(\text{mol wt})^{0.400}$$

Control T7 DNA had $s^0_{20,w}$ = 36–37, which corresponds to a single-strand molecular weight of 1.3×10^7, indicating essentially no degradation of DNA was produced under the reaction conditions. The mean number of DNA single-strand scissions, p, compared to control DNA was calculated using the following relationship

$$\frac{(\text{mol wt})}{(\text{mol wt})_{\text{control}}} = \frac{2[e^{-p} + (p - 1)]}{p^2}$$

Results

Reduction of B[a]-P-diones to B[a]P-diols. Under anaerobic conditions, the three metabolic B[*a*]P-diones are reversibly reduced to their respective hydroquinones, B[*a*]P-6,12-diol, B[*a*]P-1,6-diol, and B[*a*]P-3,6-diol. An example is shown in Figure 1. The absorption spectra and extinction coefficients of these three diols in benzene are shown in Figure 2. Due to their extreme lability, the B[*a*]P-diols have not previously been characterized as such, but only as dicarboxylic acid esters such as diacetates (Fieser and Hershberg, 1939; Cho and Harvey, 1976). For this reason also, the B[*a*]P-diols have not been detected as B[*a*]P metabolites, although, significantly, Falk et al. (1962) isolated glucuronic acid conjugates of all three B[*a*]P-diols from the hepatobiliary system of rats after B[*a*]P treatment. For preparative purposes, the reduction of the B[*a*]P-diones to B[*a*]P-diols was conveniently performed by a two-phase method (see methods) in which the B[*a*]P-dione in nitrogen-flushed benzene is shaken with a nitrogen-flushed aqueous solution of a benzene-insoluble reducing agent, sodium dithionite ($Na_2S_2O_4$). This method very rapidly reduces the B[*a*]P-dione, allows efficient removal of

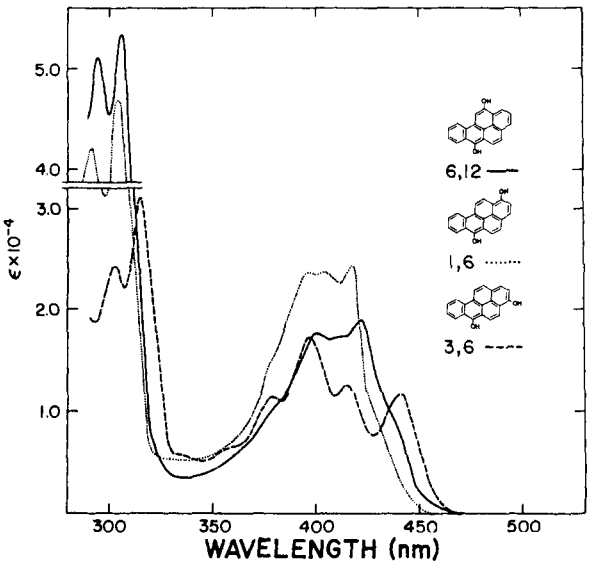

FIGURE 2: Absorption spectra and extinction coefficients of B[*a*]P-diols in nitrogen-sparged benzene; B[*a*]P-6,12-diol (—), B[*a*]P-1,6-diol (· · ·), B[*a*]P-3,6-diol (- - -); wavelength is in nanometers.

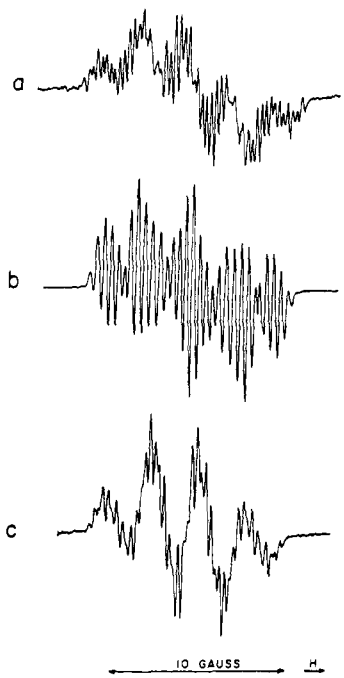

FIGURE 3: ESR spectra of B[*a*]P-semiquinone radical anions obtained from B[*a*]P-diols (5×10^{-4} M) in 1:1 (v/v) 95% ethanol–1 M NaOH by careful exposure to air: (a) B[*a*]P-6,12-semiquinone radical, modulation amplitude 0.10 G, power 3 mW, $g = 2.0043$; (b) B[*a*]P-1,6-semiquinone radical, modulation amplitude 0.20 G, power 1 mW, $g = 2.0041$; (c) B[*a*]P-3,6-semiquinone radical, modulation amplitude 0.05 G, power 2 mW, $g = 2.0043$.

the redox agent which is located solely in the aqueous layer, and affords the pure B[*a*]P-diol in benzene, a solvent in which it is more stable than in polar solvents. The benzene can be removed by evaporation under reduced pressure to give solid B[*a*]P-diol without oxidation, provided exposure to air is diligently avoided.

Reductions of the B[*a*]P-diones to B[*a*]P-diols were accomplished in polar solvents also, but isolation was not usually attempted. The reductions were conveniently monitored by absorption spectroscopy and could be observed visually as well. In aqueous buffer (pH 7)–ethanol solutions, well flushed with nitrogen, all three B[*a*]P-diones were reduced to B[*a*]P-diols rapidly by many reducing agents, including relatively mild biological agents such as NADH, NADPH, cysteamine, and glutathione. This suggests relatively low redox potentials for the B[*a*]P-diones, a point which will be discussed later in more detail.

Autoxidation of B[a]P-diols to B[a]P-diones. All three B[*a*]P-diols are very sensitive to molecular oxygen. Thus, they are autoxidized by exposure to air to yield the respective B[*a*]P-diones. The properties of the B[*a*]P-diones are well known and have been reported in a previous publication (Lorentzen et al., 1975). They were the only final products of B[*a*]P-diol autoxidation detected by spectroscopic and chromatographic methods. In general, we found that pure solutions of unautoxidized B[*a*]P-diols in aqueous ethanol were difficult to obtain, even though the handling of the compounds was carried out in a N_2-filled glove bag using N_2-flushed solvents. The B[*a*]P-diols were readily autoxidized in all solvents examined, including benzene. They can be most easily maintained in benzene because they are less sensitive in this solvent to the traces of oxygen not removed by the procedures used.

The rates of these autoxidations are rapid. In polar solvents, the reactions were complete in the period of time it took to introduce air into the solutions and measure spectroscopic changes. Visually, color changes that accompany the conversion of B[*a*]P-diols to B[*a*]P-diones occurred simultaneously with air exposure. More accurate rate measurements have not been performed, but these observations seem to indicate that the half-lives of the B[*a*]P-diols in air saturated aqueous-ethanol solutions at physiological pH are less than 1 s.

Hydrogen Peroxide Formation. The rapid autoxidations of the B[*a*]P-diols in aqueous buffer–ethanol solutions were accompanied by the rapid production of hydrogen peroxide. This was measured by a previously described assay (Lorentzen et al., 1975) specific for hydrogen peroxide. While the hydrogen peroxide production was too rapid for routine kinetic measurements, the final concentration of peroxide after autoxidation was invariably similar to the initial concentration of each B[*a*]P-diol. This provides evidence that the predominant mechanism for these autoxidations involves the overall transfer of two electrons from the B[*a*]P-diol to molecular oxygen,

$$\text{B[}a\text{]P-diol} + O_2 \rightarrow \text{B[}a\text{]P-dione} + H_2O_2$$

Detection of Semiquinone Radicals. During autoxidation of the B[*a*]P-diols at high pH, semiquinone radical anions can be detected as intermediates to the production of B[*a*]P-diones. Careful exposure of synthetically prepared B[*a*]P-diols in aqueous sodium hydroxide (1 M)–ethanol solutions to small amounts of air produced intensely colored solutions. The ESR spectra of these solutions are shown in Figure 3. The ESR spectra produced by these same species were also observed as intermediates in the reduction of B[*a*]P-diones to B[*a*]P-diols by alkaline solutions containing dimethyl sulfoxide or sodium dithionite.

Nagata et al. (1974) previously reported ESR spectra obtained by treating the B[*a*]P-diones with alkaline dimethyl sulfoxide. The hyperfine structures in Figure 3a,b for the 6,12- and the 1,6-semiquinone radicals of B[*a*]P, respectively, are similar to those reported by these workers. Accounting for solvent and modulation differences, both sets of spectra, undoubtedly, are due to the two designated semiquinone radicals. The spectrum they reported as due to the 3,6-semiquinone radical of B[*a*]P is different from our spectrum of this radical

TABLE I: Induction of Single-Strand Scission in T7 DNA by B[*a*]P-diones.[a]

B[*a*]P-dione (Concn) (M)	Incubation Time (h)	Other Treatment	Mean No. of Single-Strand Scissions/Intact Strand
None	21		0
6,12 (3×10^{-4})	21		8
1,6 (3×10^{-4})	21		90
3,6 (3×10^{-4})	21		29
1,6 (1.5×10^{-4})	4		3
1,6 (1.5×10^{-4})	11		8
1,6 (1.5×10^{-4})	22		27
1,6 (1.5×10^{-4})	22	Prior N_2 flush, 3 min	9
1,6 (1.5×10^{-4})	22	3×10^{-6} M $CuSO_4$	53
None	22	3×10^{-6} M $CuSO_4$	0
1,6 (1.5×10^{-4})	22	Superoxide dismutase (10 μg)	4
1,6 (1.5×10^{-4})	22	3×10^{-4} M NADPH	52
None	22	3×10^{-4} M NADPH	2
3,6 (3×10^{-4})	21	3×10^{-4} M EDTA	0.3

[a] In 1:1 (v/v) 95% ethanol-0.01 M sodium phosphate, pH 7 at 37 °C. DNA concentration was 3×10^{-4} nucleotide in all cases.

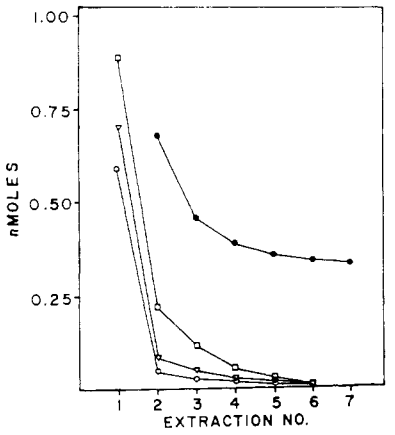

FIGURE 4: Amount of B[*a*]P metabolite that remains associated with T7 DNA after successive extractions with buffered phenol, 6-OH-B[*a*]P (●), B[*a*]P-6,12-dione (○), B[*a*]P-1,6-dione (□), B[*a*]P-3,6-dione (▽). Each metabolite (1.5×10^{-4} M) was incubated with DNA (3×10^{-4} M nucleotide) in 1:1 (v/v) 95% ethanol-0.01 M sodium phosphate, pH 7, for 20 h at 22 °C prior to extraction. The amount of 6-OH-B[*a*]P bound to the DNA after seven extractions corresponds to 1 molecule per 1400 nucleotides.

shown in Figure 3c and rather similar to the spectrum we both report for 1,6-B[*a*]P-semiquinone radical (Figure 3b). The ESR spectrum for the 3,6 isomer in Figure 3c, however, is very similar to one reported by Nagata et al. (1974), which was obtained by oxidation of 3-OH-B[*a*]P with alkaline ceric sulfate. Since these conditions are sufficiently strong to oxidize 3-OH-B[*a*]P to B[*a*]P-3,6-dione with the semiquinone radical detectable as an intermediate, we conclude that their spectrum measured during this reaction as well as the one in Figure 3c are the spectra of 3,6-B[*a*]P-semiquinone radical anion.

At neutral pH, no intermediate species during the autoxidation of B[*a*]P-diols to B[*a*]P-diones were detected either by ESR or absorption spectroscopy. It is well known (Bruce, 1974) that semiquinone radicals derived from benzoquinones are much more stable in the anionic form existing at high pH than the un-ionized form existing at physiological pH. This is apparently true for the B[*a*]P-semiquinone radicals also, allowing their facile detection in alkaline medium only.

Interaction of B[a]P-diones with Nucleic Acid. Like many polycyclic aromatic hydrocarbons, including B[*a*]P and its derivatives, the B[*a*]P-diones form physical complexes with DNA. These B[*a*]P-dione–DNA associations are not dissociated by dialysis nor by an electric field during sucrose gradient electrophoresis. Whereas 99.8–99.9% of physically bound parent hydrocarbon, [^{3}H]B[*a*]P, can be removed efficiently from DNA by a procedure involving precipitation of the DNA and washing of the precipitate with ethanol and ether (Ts'o and Lu, 1964), neither the repeated application of this method nor repeated extraction of the DNA solutions with ether, chloroform, or ethyl acetate were sufficient to remove the [^{3}H]-B[*a*]P-diones efficiently and completely from [^{3}H]B[*a*]P-dione–DNA complexes. The nature of this physical association is unknown, although the electron-deficient benzoquinones are known to form π and charge transfer complexes with many kinds of aromatic compounds (Bruce, 1974). We believe these B[*a*]P-dione–DNA associations are not covalent because, as is shown in Figure 4, repeated extraction with buffered phenol completely removed the bound B[*a*]P-diones from T7 bacteriophage DNA. The superiority of phenol as an extractant may lie in its potential for complexing with hydrocarbon derivatives, together with its partial mutual miscibility with aqueous solutions. In contrast to B[*a*]P-diones, 6-OH-[^{3}H]-B[*a*]P cannot be completely removed from T7 DNA by this procedure (Figure 4). Even though 6-OH-B[*a*]P autoxidizes to B[*a*]P-diones during the period of incubation with DNA, a nearly plateau level of residual 6-OH-B[*a*]P-derived material remains bound to the DNA after five to six phenol extractions. As has been reported earlier, this binding is most likely covalent in nature (Ts'o et al., 1974, 1976).

Incubation of B[*a*]P-diones with homogeneous T7 bacteriophage DNA in aqueous buffer–ethanol solutions produced damage in DNA measured as single-strand scission. The reduction in molecular weight was measured by alkaline sedimentation after reaction of the DNA with B[*a*]P-diones at 37 °C. In Table I, this damage is recorded as the mean number of single-strand breaks produced compared to control DNA. The ability to induce strand scission was greatest for B[*a*]P-1,6-dione, followed by B[*a*]P-3,6-dione. B[*a*]P-6,12-dione caused the least strand scission.

As can also be seen in Table I, the degree of strand scission is modified by various conditions. Thus, the reduction in the amount of dissolved oxygen produced by a brief nitrogen flushing of the solution prior to incubation significantly reduced the amount of B[*a*]P-1,6-dione-induced DNA breakage. The addition of superoxide dismutase reduced strand scission

considerably, and the presence of EDTA protected the DNA essentially completely from this damage. On the other hand, the addition of either a transition metal ion salt, $CuSO_4$, or a cellular reducing agent, NADPH, to the incubation solution greatly increased the amount of scission induced by B[*a*]P-1,6-dione over that induced by B[*a*]P-1,6-dione alone. These data point to the involvement of free radicals and oxygen in B[*a*]P-dione induced DNA damage, as will be discussed shortly.

Enzymatic Reduction of B[a]P-diones. B[*a*]P-diones are electron-acceptor substrates for a diaphorase, NADH dehydrogenase from *Cl. kluyveri*. Reduction of the B[*a*]P-diones by NADH is mediated by this enzyme. In air, these reductions were not observed directly because B[*a*]P-diols are immediately autoxidized back to B[*a*]P-diones. Rather, in Figure 5 the kinetics of oxidation of NADH is demonstrated by the decrease in absorbance at 340 nm. The addition of B[*a*]P-3,6-dione, for example, in *catalytic* amounts to the enzyme and NADH initiates the rapid and continuous depletion of NADH to completion. NADH is not oxidized at any appreciable rate by catalytic amounts of B[*a*]P-diones without enzyme, even though, as mentioned earlier, excess NADH can reduce B[*a*]P-diones in the absence of oxygen.

The oxidations of NADH mediated by the B[*a*]P-diones, and shown in Figure 5 for the case of B[*a*]P-3,6-dione, were accompanied by the production of hydrogen peroxide, not equivalent to the catalytic amount of B[*a*]P-dione added, but more nearly equivalent to the larger amount of NADH oxidized. This indicates that molecular oxygen is the ultimate electron acceptor and that both oxygen and B[*a*]P-diones are linked to the NADH oxidation, a notion which will be discussed in more detail.

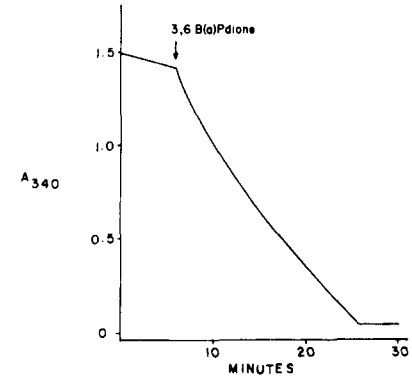

FIGURE 5: Oxidation of NADH (initial 2.4×10^{-4}), measured by decrease in absorbance at 340 nm, with NADH dehydrogenase from *Cl. kluyveri* (2 units) using a catalytic amount of B[*a*]P-3,6-dione (5×10^{-6} M) as an electron acceptor. After 30 min, H_2O_2 concentration was measured 1.9×10^{-4} M.

Discussion

Benzo[*a*]pyrenediones are almost invariably found after metabolism of benzo[*a*]pyrene, sometimes accounting for more than 50% (Wang et al., 1974) of the total metabolic yield; Selkirk et al. (1975) reported that they represent a major portion of B[*a*]P metabolism by human liver and lymphocytes. Although several metabolic pathways leading to the B[*a*]P-diones are possible, based on our previous findings the predominant pathway is most likely the initial hydroxylation at position six, followed by autoxidation of the resulting labile phenol, 6-OH-B[*a*]P, by a free radical mechanism to the B[*a*]P-diones (Lorentzen et al., 1975). These B[*a*]P-diones exhibit the chemical properties of quinones in their ability to form highly reversible redox couples with their hydroquinones, the corresponding B[*a*]P-diols.

The potentials of the B[*a*]P-dione–B[*a*]P-diol half-reactions have been measured by Moriconi et al. (1962). Their results indicate that all three B[*a*]P-diones have similar E^0s of about 0.44 V in 95% ethanol, 0.1 M HCl. These are lower E^0s than that of 1,4-benzoquinone (0.71 V) under the same conditions, but comparable to those of naturally occurring quinones, such as ubiquinone (0.54 V) and menadione (vitamin K) (0.42 V) (Clark, 1960). The potentials are pH dependent; a more reliable indication of oxidizing power of the B[*a*]P-diones under physiological conditions is given by $E^{0'}$, which is the midpoint potential for the couple at pH 7 rather than pH 1. Accordingly, $E^{0'}$ is approximately 0.4 V lower than E^0, which indicates that the B[*a*]P-diones have very low potentials at neutral pH. A consequence of this is that neither the reduced (B[*a*]P-diol) nor oxidized (B[*a*]P-dione) forms of the couples are particularly thermodynamically favored under physiological conditions, and the predominance of either could be influenced by mild redox agents. This is supported by our observations that mild biological reducing agents can produce the B[*a*]P-diols from B[*a*]P-diones effectively, but that the presence of air highly favors the predominance of the B[*a*]P-diones. Equally important, our observations show that the couples are highly reversible, and interconversions of B[*a*]P-diones and B[*a*]P-diols under appropriate redox conditions are characterized by low-energy barriers and, hence, are rapid.

The rapid autoxidations of B[*a*]P-diols take place by free-radical mechanisms. This would be expected based on knowledge of other autoxidations, and is supported by the detection of the B[*a*]P semiquinone radical anions during the autoxidations under conditions of high pH. As a consequence, oxygen is reduced univalently to produce, initially, the superoxide anion radical, $O_2^{-}\cdot$, which rapidly dismutates to hydrogen peroxide and oxygen. This is a likely pathway for the formation of the H_2O_2 produced by these autoxidations of B[*a*]P-diols. Whether the most highly reactive reduced oxygen species, the hydroxyl radical, is produced during these autoxidations has not been measured directly. But, much evidence indicates that OH· can be formed by at least two processes, both possible under our experimental conditions. These are the so-called Haber–Weiss reaction (Haber and Weiss, 1934)

$$O_2^{-}\cdot + H_2O_2 \rightarrow OH\cdot + OH^- + O_2$$

and the well-known Fenton reaction of H_2O_2 with a transition metal ion to produce hydroxyl radical.

The notion that reduced oxygen species are responsible for the B[*a*]P-dione-induced DNA strand breakage (Table I) is strongly supported by the observed protection of the DNA by superoxide dismutase. It is well known that peroxides, and peroxide and hydroxyl radical generators, cause strand breakage and other inactivating lesions in DNA (Freese et al., 1967; Rhaese et al., 1968; Massie et al., 1972). Metal ion chelation is the most likely process responsible for the complete protection of DNA by EDTA from B[*a*]P-dione-induced breakage. DNA is normally a rich source of metal ions and the observation that the addition of Cu^{2+} makes DNA more susceptible to B[*a*]P dione-induced damage supports the chelation model. It will be of vital interest to determine the direct involvement of the four reactive oxygen species, i.e., singlet oxygen, superoxide radical, hydrogen peroxide, and hydroxyl radicals, in producing observed DNA damage.

Some compounds with quinone functional groups, such as ubiquinones and the vitamin K's, have natural cellular functions probably associated with electron transport (Thomson,

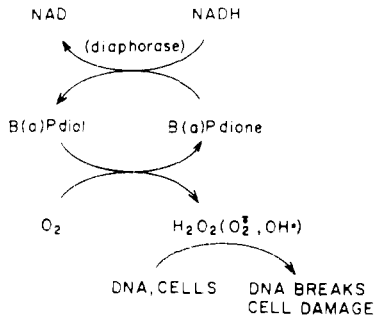

FIGURE 6: A cyclic scheme showing the involement of the B[*a*]P-dione/B[*a*]P-diol redox couples in the production of reactive reduced oxygen species at the expense of cellular reducing power.

1971). It has also been reported that a variety of respiratory enzymes can accept many quinone compounds, in addition to naturally occurring ones, as electron acceptor substrates, effecting the transfer of electrons from a donor to the quinone (Marteus, 1963). The results of the experiment shown in Figure 5 demonstrate that the B[*a*]P-diones, with their extended quinone functional groups, are also capable of being electron-acceptor substrates for the respiratory enzyme, NADH dehydrogenase. The knowledge that B[*a*]P-diols are rapidly autoxidized to B[*a*]P-diones with concomitant production of H_2O_2 strongly implies a role for molecular oxygen in linking the NADH disappearance to peroxide formation. The cyclic scheme in Figure 6 generalizes the data from this experiment and provides a model for most of the results reported in this paper. According to this scheme, the autoxidative recycling of B[*a*]P-dione is responsible for linking NADH and oxygen in the continuous production of reduced oxygen species until reducing power is totally depleted.

Preliminary results in our laboratory show that B[*a*]P-diones induce strand breakage in the DNA of cells in culture; the cytotoxicity of B[*a*]P-diones towards Syrian hamster cells is substantially greater than that of B[*a*]P and is highly dependent on the amount of oxygen in the medium (Lorentzen, 1976; Lorentzen, Caspary, Leavitt and Ts'o, manuscript in preparation). This tends to support the notion that the oxygen-dependent cyclic process depicted in Figure 6 can operate in cells and produce dramatic results. Such a mechanism of action is not unprecedented. For example, there is considerable evidence that the toxicity of the quinoid antibiotic, streptonigrin (a substituted 1,4-benzoquinone), towards bacteria is caused by reduced oxygen species produced by the cycling of streptonigrin between its quinone and semiquinone forms (Gregory and Fridovich, 1973; Fridovich, 1975).

There are similarities between the effects of the B[*a*]P-diones reported here and the effects of high-energy radiation that should be mentioned. Hydroxyl radicals, superoxide radicals, hydrogen peroxide, and organic peroxides are products of ionizing radiation of biological materials in aqueous medium (Kotin and Falk, 1963; Paquot, 1958) and are probably responsible for much of the tissue damage caused by this physical agent. There is also the well-known fact that oxygen has an enhancing effect on radiation-induced tissue alteration, including tumorigenesis (Dettmer et al., 1968).

Even though the identity of the B[*a*]P-3,6-dione as a metabolite of B[*a*]P has been known for over 30 years, very few detailed reports of biological assays of any of the B[*a*]P-diones have been published. Recently, Wislocki et al. (1976) reported that B[*a*]P-diones did not induce significant levels of mutation in *S. typhimurium* and a Chinese hamster V79 cell line, although moderate mutagenicity was observed for the B[*a*]P-dione precursor, 6-OH-B[*a*]P, in both these systems. To our knowledge, the results of only one tumorigenicity study have been reported; in this study, no tumors were induced in ten mice treated subcutaneously with a "crude" sample of B[*a*]P-3,6-dione (Berenblum and Schoental, 1943).

It is important to repeat that the B[*a*]P-diones, and, by implication, 6-OH-B[*a*]P, make up a substantial portion of the metabolic yield of B[*a*]P in mammalian, including human, cells. For these reasons, our current studies are directed toward examining whether the properties of the B[*a*]P-diones reported here have any bearing on the toxic, mutagenic, tumor-initiating, and tumor-promoting capacities of B[*a*]P toward mammalian tissue.

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References

Berenblum, I., and Schoental, R. (1943), *Cancer Res. 3*, 145.

Bruce, J. M. (1974), in Rodd's Chemistry of Carbon Compounds, 2nd ed, Vol III, Coffey, S., Ed., Amsterdam, Netherlands, Elsevier, p 1.

Cho, H., and Harvey, R. (1976), *J. Chem. Soc. Perkin Trans. 1*, 836.

Clark, K. (1960), in Oxidation-Reduction Potentials of Organic Systems, Baltimore, Md., Williams and Wilkins.

Dettmer, C., Kramer, S., Gottleib, S., and Aponte, G. (1968), *J. Natl. Cancer Inst. 41*, 751.

Englund, P. (1972), *J. Mol. Biol. 66*, 209.

Falk, H., Kotin, P., Lee, S., and Nathan, A. (1962), *J. Natl. Cancer Inst. 28*, 699.

Fieser, L., and Hershberg, E. (1939), *J. Am. Chem. Soc. 61*, 1565.

Freese, E. B., Gerson, J., Taber, H., Rhaese, H., and Freese, E. (1967), *Mutat. Res. 4*, 517.

Fridovich, I. (1975), *Annu. Rep. Med. Chem. 10*, 257.

Gregory, E., and Fridovich, I. (1973), *J. Bacteriol. 114*, 1193.

Haber, F., and Weiss, J. (1934), *Proc. R. Soc. London, Ser. A 147*, 332.

Kotin, P., and Falk, H. (1963), *Radiat. Res. Suppl. 3*, 193.

Lesko, S., Caspary, W., Lorentzen, R., and Ts'o, P. (1975), *Biochemistry 14*, 3978.

Lorentzen, R. (1976), *Proc. Am. Assoc. Cancer Res. 17*, 100.

Lorentzen, R., Caspary, W., Lesko, S., and Ts'o, P. (1975), *Biochemistry 14*, 3970.

Marteus, C. (1963), *Enzymes, 2nd Ed. 7*, 517.

Massie, H., Samis, H., and Baird, M. (1972), *Biochim. Biophys. Acta 272*, 539.

Moriconi, E., Rakoczy, B., and O'Connor, W. (1962), *J. Org. Chem. 27*, 2772.

Nagata, C., Tagashira, Y., and Kodama, M. (1974), in Chemical Carcinogenesis, Part A, Ts'o, P., and DiPaolo, J., Ed., New York, N.Y., Marcel Dekker, p 87.

Paquot, C. (1958), in Organic Peroxides in Radiobiology, Haissensky, Ed., London, Pergamon Press, p 3.

Rhaese, H., Freese, E., and Melzer, M. (1968), *Biochim. Biophys. Acta 155*, 491.

Schechtman, L., Lesko, S., Lorentzen, R., Ts'o, P. (1974), *Proc. Am. Assoc. Cancer Res. 15*, 66.

Selkirk, J., Croy, R., Whitlock, J., and Gelboin, H. (1975), *Cancer Res. 35*, 3651.

Studier, W. F. (1965), *J. Mol. Biol. 11,* 373.
Thomson, R. H. (1971), Naturally Occurring Quinones, New York, N.y., Academic Press.
Ts'o, P., Barrett, J., Caspary, W., Lesko, S., Lorentzen, R., and Schechtman, L. (1976), in Aging, Carcinogenesis, and Radiation Biology, Smith, K., Ed., New York, N.Y., Plenum Press, p 373.
Ts'o, P., Caspary, W., Cohen, B., Leavitt, J., Lesko, S., Lorentzen, R., and Schechtman, L. (1974), in Chemical Carcinogenesis, Part A, Ts'o, P., and DiPaolo, J., Ed., New York, N.Y., Marcel Dekker, p 113.
Ts'o, P., and Lu, P. (1964), *Proc. Natl. Acad. Sci., U.S.A. 51,* 272.
Wang, I., Rasmussen, R., and Crocker, T. (1974), *Life Sci. 15,* 1291.
Wislocki, P., Wood, A. Chang, R., Levin, W., Yagi, H., Hernandez, O., Dansette, P., Jerina, D., and Conney, A. (1976), *Cancer Res. 36,* 3350.

Specificity of the Weak Binding between the Phage SPO1 Transcription-Inhibitory Protein, TF1, and SPO1 DNA†

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ABSTRACT: The interaction of the phage SPO1 protein, transcription factor 1 (TF1), with DNA has been analyzed by membrane filter binding and by sedimentation methods. Substantially specific binding of TF1 to helical SPO1 DNA can be demonstrated by nitrocellulose filter-binding assays at relatively low ionic strength (0.08). However, TF1–DNA complexes dissociate and reequilibrate relatively rapidly and this makes filter-binding assays unsuitable for quantitative measurements of binding equilibra. Accordingly, the sedimentation properties of TF1–DNA complexes have been explored and a short-column centrifugation assay has been elaborated for quantitative measurements. Preferential binding of TF1 to the hydroxymethyluracil-containing SPO1 DNA has also been demonstrated by short-column centrifugation. TF1 binds relatively weakly and somewhat cooperatively to SPO1 DNA at many sites; TF1–DNA complexes dissociate and reequilibrate rapidly. At 20 °C in 0.01 M phosphate, pH 7.5, 0.15 KCl, one molecule of TF1 can bind to approximately every 60 nucleotide pairs of SPO1 DNA.

The SPO1 DNA-binding protein, transcription factor 1 (TF1), is synthesized after SPO1 infection of *Bacillus subtilis* 168M (Wilson and Geiduschek, 1969; Johnson and Geiduschek, 1972). Within 20 min after infection at 37 °C, more than 10^5 molecules have been synthesized in each infected cell (Johnson and Geiduschek, 1972). TF1 selectively inhibits in vitro transcription of native, bihelical SPO1 DNA (and other hmU-containing phage DNA) by bacterial DNA-dependent RNA polymerase (Wilson and Geiduschek, 1969). We have previously described the purification of this protein and some of its molecular properties (Johnson and Geiduschek, 1972; Johnson and Geiduschek, 1974).

In the experiments that we present here, we have explored the interaction of TF1 with DNA. For our first experiments along these lines, we made use of the ability of nitrocellulose membranes to bind proteins and protein–nucleic acid complexes, as first described by Jones and Berg (1966). The properties of these complexes first suggested to us that TF1 and SPO1 DNA must dissociate more readily than do complexes between DNA and RNA polymerase, repressors, unwinding proteins, or histones (Hinkle and Chamberlin, 1970; Gilbert and Muller-Hill, 1967; Riggs et al., 1970; Ptashne, 1967; Alberts and Frey, 1970; Alberts et al., 1972; von Hippel and McGhee, 1972). Accordingly, we examined TF1–DNA complexes and their dissociation by centrifugation analysis. In this paper, we present the results of these explorations and of a more detailed measurement of the equilibrium constant for DNA–TF1 binding.

Materials and Methods

(*1*) *Buffers.* The following buffers are referred to in the text by abbreviation: G: 10 mM glycine (Na), pH 10, 0.1 mM EDTA, 0.1 mM dithiothreitol. GD: buffer G but with 1.1 mM dithiothreitol. PK: 0.01 M phosphate (Na), pH 7.5, 0.15 M KCl. TM: 10 mM Tris-HCl, pH 7.5, 5 mM $MgCl_2$. TCED: 10 mM Tris-cacodylate, pH 6; 0.1 mM EDTA, 0.1 mM dithiothreitol (10 mM Tris-cacodylate is 10 mM Tris base, 10 mM cacodylic acid, adjusted to pH 6 with HCl). TCEDK: TCED with 0.25 M KCl. TED: 100 mM Tris-HCl, pH 7.5, 0.1 mM EDTA, 0.1 mM dithiothreitol. TKE: 100 mM Tris-HCl, pH 7.5, 0.1 M KCl, 0.1 mM EDTA. TKED: TKE with 0.1 mM dithiothreitol.

(*2*) *Unlabeled, ^3H- and ^{35}S-labeled TF1.* TF1 was purified through the phosphocellulose column purification step of our previously described purification method (Johnson and Geiduschek, 1972). Only peak phosphocellulose fractions of constant specific activity (units of TF1/mg of protein) or constant specific radioactivity (units of TF1/cpm) were used in these studies. The molecular-weight homogeneity of TF1 was verified by acrylamide gel electrophoresis (Laemmli,

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[1] Abbreviations used are: DEAE, diethylaminoethyl; EDTA, (ethylenedinitrilo)tetraacetic acid; Tris, 2-amino-2-hydroxymethyl-1,3-propanediol.