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Mutagenicity of quinones: Pathways of metabolic activation and detoxification

(benzo[*a*]pyrene quinones/oxygen radicals/NADPH-cytochrome P-450 reductase)

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ABSTRACT **The mutagenicity of various quinones, a class of compounds widely distributed in nature, is demonstrated in the *Salmonella* TA104 tester strain. The metabolic pathways by which four quinones, menadione, benzo[*a*]pyrene 3,6-quinone, 9,10-phenanthrenequinone, and danthron, caused mutagenicity in this test system were investigated in detail as were the detoxification pathways. The two-electron reduction of these quinones by NAD(P)H-quinone oxidoreductase (DT-diaphorase) was not mutagenic, whereas the one-electron reduction, catalyzed by NADPH-cytochrome P-450 reductase, was mutagenic, except for danthron, which was only slightly mutagenic. The mutagenicity of the quinones via this pathway was found to be attributable to the generation of oxygen radicals. The cytochrome P-450 monooxygenase also played a significant role in the detoxification and bioactivation of these quinones. For example, phenanthrenequinone was converted to a nonmutagenic metabolite in a cytochrome P-450-dependent reaction, whereas danthron was converted to a highly mutagenic metabolite. These studies show the complexity of metabolic pathways involved in the mutagenicity of quinones.**

Quinones are widely distributed in nature, and human exposure to them is extensive. The quinones of polycyclic aromatic hydrocarbons are abundant in all burnt organic material, including automobile exhaust, cigarette smoke, and urban air particulates (1–3). Quinones are also found naturally in many of the foods we eat (4–6), and compounds containing the quinone nucleus are widely employed as antitumor agents (7, 8). Despite the magnitude of this human exposure, the pathways by which many quinones are metabolized remain poorly understood and their mutagenicity is largely untested. In a recent study, however, six naturally occurring naphthoquinones, including menadione, were shown to be mutagenic to strain TA2637 with metabolic activation (9), indicating the need for further studies.

Quinones serve as substrates for a wide variety of flavoenzymes, including NADPH-cytochrome P-450 reductase, NAD(P)H-quinone oxidoreductase (DT-diaphorase), NADH-cytochrome b_5 reductase, and NADH-ubiquinone oxidoreductase, and can undergo either a direct two-electron reduction to the hydroquinone or a one-electron reduction to the semiquinone radical (10–13). In the presence of oxygen most semiquinones rapidly autooxidize to form the superoxide anion radical ($O_2^{\cdot-}$) and thus regenerate the quinone (14). This redox cycling can lead to conditions of oxidative stress through the production of $O_2^{\cdot-}$ (15) and has been invoked to explain the cytotoxic and antitumor properties of quinonoid drugs (16). We have recently demonstrated that the xanthine oxidase-dependent superoxide-generating system is mutagenic to the new *Salmonella* tester strain TA104 (unpublished). This finding suggested that the redox cycling of quinones might also be mutagenic, and we have tested this possibility using the TA104 strain, which is sensitive to a wide variety of oxidative mutagens (17). We have also attempted to characterize the pathways by which several different quinones are metabolized and to study the potential mutagenicity of the metabolites and side products formed. We therefore decided to investigate only those quinones that required metabolic activation to exhibit mutagenicity. To limit the scope of this project we also chose not to study quinones that possess reactive leaving groups. Quinones of this type have been shown to interact with DNA via a methide reaction (18–20).

MATERIALS AND METHODS

Materials. Glucose 6-phosphate (Glc-6-*P*), $NADP^+$, deoxycholate, sodium dilauroyl diphosphotidylcholine, dicoumarol, bovine serum albumin, superoxide dismutase (SOD), and thymol-free catalase were obtained from Sigma; NADPH and NADH were from Calbiochem–Behring; and Glc-6-*P* dehydrogenase was from Boehringer-Mannheim (Mannheim, F.R.G.). SKF525A (proadiphen, 2-diethylaminoethyl-2,2-diphenyl valerate) was a gift of Smith Kline & French.

Danthron (1,8-dihydroxyanthraquinone) was from Aldrich. Menadione (2-methyl-1,4-naphthoquinone) was from Sigma. The benzo[*a*]pyrene quinones were a gift of the National Cancer Institute. The diesel exhaust quinones, 9,10-phenanthrenequinone and a mixture of 1,6- and 1,8-pyrene quinones (1), were the gift of Dennis Scheutzle of Ford Motor Company.

Enzyme Preparations. S9 liver homogenate was prepared from polychlorobiphenyl-induced rats as described (21). For the mutagenicity tests with S9 the standard enzyme preparation (50 μl of S9 diluted to 0.5 ml per plate) was used (21). For some of these assays (Table 1) NADH was substituted for $NADP^+$/Glc-6-*P* in the S9 enzyme preparation. In these tests NADH was added at twice the molar level of $NADP^+$ and Glc-6-*P* because the oxidation of each Glc-6-*P* molecule normally produces two NADPH.

Purified NADPH-cytochrome P-450 reductase (22) was the generous gift of Bettie Sue Masters. Incubations with P-450 reductase contained 50 μg of sodium dilauroyl diphosphatidylcholine, 25 μg of deoxycholate, 2 μmol of NADPH, 2 μmol of Glc-6-*P*, 1 unit of Glc-6-*P* dehydrogenase, and 2.5 μg of purified P-450 reductase diluted to 0.3 ml with 0.2 M KPO_4 (pH 7.6). The P-450 reductase was added last to prevent its precipitation from solution. This preparation was made just prior to use and kept on ice.

Mutagenicity Assays. All mutagenicity assays were performed with the *Salmonella* tester strain TA104 using a liquid preincubation procedure (21). The quinones were dissolved in dimethyl sulfoxide and added to sterile 13 × 100 mm capped culture tubes, followed by addition of 0.2 M $NaPO_4$

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Abbreviations: DT-diaphorase, NAD(P)H-quinone oxidoreductase; SOD, superoxide dismutase; Glc-6-*P*, glucose 6-phosphate.

Table 1. Effect of dicoumarol, NADPH, and NADH on S9-mediated quinone mutagenicity

Quinone	Concentration, μM	His$^+$ revertants per plate		
		NADPH	NADPH and dicoumarol	NADH
Menadione	60	124 ± 20	211 ± 26	15 ± 6
Benzo[*a*]pyrene 1,6-quinone	30	577 ± 113	582 ± 104	317 ± 55
Benzo[*a*]pyrene 3,6-quinone	30	890 ± 140	866 ± 127	315 ± 47
Benzo[*a*]pyrene 6,12-quinone	90	182 ± 14	201 ± 18	120 ± 9
Pyrene 1,6- + 1,8-quinones	60	1171 ± 212	1204 ± 195	458 ± 53
9,10-Phenanthrenequinone	60	50 ± 6	48 ± 9	18 ± 4
Danthron	60	748 ± 94	740 ± 101	395 ± 33

Quinone concentrations were chosen near the high end of the linear dose–response region. All assays contained S9 and were performed by using the preincubation method (see text). The NADPH S9 preparation contained $NADP^+$ at 4 mM and Glc-6-*P* at 4 mM. The production of NADPH is dependent on endogenous soluble enzymes present in the S9. The NADH S9 preparation contained NADH at 8 mM. Dicoumarol was used at 30 μM. Data points represent the mean ± SD of the number of induced revertants in at least three experiments.

(pH 7.4) (S9 assays) or 0.2 M KPO_4 (pH 7.6) (P-450 reductase assays), with or without SOD, catalase, SKF525A, or dicoumarol and 0.1 ml of an overnight culture of the bacterial strain. The enzyme preparation was added last, bringing the total volume to 0.5 ml for the P-450 reductase assays and 1.0 ml for the S9 assays. All preincubation mixtures were brought to 0.1% bovine serum albumin to help stabilize soluble enzymes and to prevent the addition of SOD or catalase (or both) from altering the total protein concentration. The tubes were incubated with shaking for 30 min at 30°C. Two milliliters of molten top agar containing histidine and biotin were then added, the mixture was plated on minimal glucose, and the plates were scored after 48 hr.

Biochemical Assays. The contribution of DT-diaphorase to the NADPH-dependent reduction of the different quinones by rat liver S9 was determined as the dicoumarol-inhibitable rate of quinone-stimulated NADPH oxidation (10). The change in absorbance was followed at 340 nm, and an extinction coefficient of 6.22 mM^{-1} cm^{-1} was used. Each reaction mixture contained 10 μl of S9, 2.5 mM NADPH, 0.08% Triton X-100, and 30 μM quinone brought to a final volume of 1 ml with 50 mM Tris·HCl (pH 7.5). Dicoumarol was used at 30 μM and addition of NADPH was used to start the reaction.

Superoxide anion ($O_2^{\cdot-}$) production was measured as the reduction of succinylated cytochrome *c* with the wavelength pair of 550–557 nm and an extinction coefficient of 21 mM^{-1} cm^{-1} (23, 24). Each reaction mixture contained 50 μg of dilauroyl phosphatidylcholine, 25 μg of deoxycholate, 1 μmol of NADPH, 2 μmol of Glc-6-*P*, 1 unit of Glc-6-*P* dehydrogenase, 0.5 mg of succinylated cytochrome *c*, 0.25 μg of NADPH-cytochrome P-450 reductase, and 30 μM quinone in dimethyl sulfoxide brought to a final volume of 1.0 ml with 0.2 M KPO_4 (pH 7.6). All reactions were initiated by addition of the quinone. The specificity of the reaction for $O_2^{\cdot-}$ was tested by the addition of 30 units of SOD.

RESULTS

Effect of Dicoumarol on S9-Mediated Quinone Mutagenicity: Role of the Two-Electron Reduction Pathway. The mutagenicity of different quinones representing a variety of chemical classes was tested in the presence and absence of dicoumarol (30 μM) by using liver S9 as the bioactivation system and the *Salmonella* tester strain TA104. Dicoumarol is a potent inhibitor of DT-diaphorase (10), which catalyzes the direct two-electron reduction of quinones to hydroquinones (11, 13, 15). By selectively inhibiting DT-diaphorase, it was thus possible to assess the contribution of this pathway to the mutagenic activity of the different quinones. The data shown in Table 1 reveal that the addition of dicoumarol had no significant effect on the mutagenicity of any of the quinones except menadione. Menadione was approximately twice as mutagenic when dicoumarol was present. Dicoumarol alone showed no significant mutagenicity in this system.

All of the quinones tested that showed mutagenicity in this system were more mutagenic in the presence of NADPH than NADH, indicating a prominent role for NADPH-cytochrome P-450 reductase in mediating quinone mutagenicity (Table 1). Of the quinones tested, 1,6- and 1,8-pyrene quinones were the most mutagenic. Both benzo[*a*]pyrene 1,6-quinone and benzo[*a*]pyrene 3,6-quinone were also highly mutagenic but benzo[*a*]pyrene 6,12-quinone was a relatively weak mutagen in this system, as were menadione and phenanthrenequinone (Table 1). Danthron was also mutagenic (Table 1), in agreement with previous findings with other tester strains (25).

Four of the quinones—namely, menadione, danthron, phenanthrenequinone, and benzo[*a*]pyrene 3,6-quinone—were selected for further study. Menadione is a naphthoquinone that is readily reduced via NADPH-cytochrome P-450 reductase to its semiquinone radical (15). Danthron is an anthracene quinone of much greater stability but structurally related to the anthraquinone antibiotics, such as Adriamycin, which are used in cancer chemotherapy (16, 25). Phenanthrenequinone is present in diesel exhaust and urban air particles (1, 3) and benzo[*a*]pyrene 3,6-quinone is prevalent in much combusted material, including automobile exhaust (1, 2). The relative ability of these four quinones to serve as substrates for the two-electron pathway catalyzed by DT-diaphorase was determined by measuring their respective rates of dicoumarol-inhibitable NADPH oxidation in the liver S9 preparation (Table 2). Menadione is known to be an

Table 2. Relative rates of DT-diaphorase-catalyzed reduction of quinones with S9 measured as dicoumarol-inhibitable NADPH oxidation

Quinone	Concentration, μM	NADPH oxidation, nmol/min		
		S9	S9 and dicoumarol	Dicoumarol-inhibitable rate (DT-diaphorase activity)
Menadione	30	76.3	6.4	69.9
Benzo[*a*]pyrene 3,6-quinone	30	9.9	5.9	4.0
9,10-Phenanthrene-quinone	30	41.9	9.7	32.2
Danthron	30	5.8	3.2	2.6

Values represent the mean of duplicate experiments.

excellent substrate for DT-diaphorase (10, 13, 15), but, interestingly, phenanthrenequinone also appeared to serve as a good substrate for this enzyme (Table 2). On the other hand, both benzo[*a*]pyrene 3,6-quinone (26) and danthron were relatively poor substrates.

Ability of NADPH-Cytochrome P-450 Reductase to Catalyze the Mutagenicity of the Different Quinones. To further test the role of NADPH-cytochrome P-450 reductase in catalyzing the mutagenic activity of the above quinones and to elucidate the role, if any, of other enzymes in the S9 preparation involved in their metabolism, we replaced the S9 preparation with purified P-450 reductase in the test system. The results in Fig. 1 show that phenanthrenequinone, menadione, and benzo[*a*]pyrene 3,6-quinone were all highly mutagenic when the purified reductase was used as the bioactivation system, whereas danthron was only slightly mutagenic. These results are virtually the reverse of those seen when the liver S9 preparation was used (compare Table 1 and Fig. 1).

To test the possible role of oxygen radicals in the reductase-mediated mutagenicity of the above quinones to TA104, we measured the mutagenicity of each quinone in the presence of SOD and catalase. All four quinones showed similar patterns, with SOD inhibiting mutagenesis partly, catalase inhibiting to a greater extent than SOD, and the two together inhibiting the reductase-mediated mutagenicity almost completely (Fig. 1). Based on these findings we predicted that there should be a close correlation between the relative rates of $O_2^{\cdot-}$ production and the mutagenicity of these quinones with NADPH-cytochrome P-450 reductase. By measuring the SOD-inhibitable reduction of succinylated cytochrome *c*, we were able to quantitate relative rates of $O_2^{\cdot-}$ formation

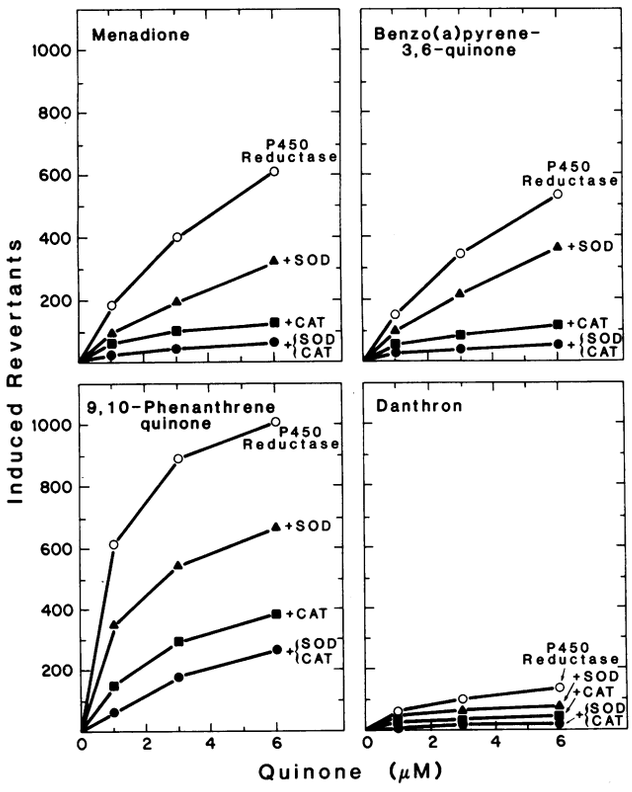

FIG. 1. Relative mutagenicity of quinones with purified NADPH-cytochrome P-450 reductase and the effect of SOD and catalase. All assays contained 2.5 μg of the P-450 reductase and were performed by using the preincubation method (see text). SOD and catalase were used at 25 units each per plate. The mutagenicity of the different quinones is shown with P-450 reductase alone (○) and also in the presence of SOD (▲), catalase (CAT) (■), or SOD and catalase together (●).

for the four different quinones (Table 3). In direct agreement with their relative mutagenicities, phenanthrenequinone was the most potent $O_2^{\cdot-}$ generator, being almost twice as potent as menadione, which in turn was far more potent than danthron. Interestingly, the benzo[*a*]pyrene 3,6-quinone-stimulated reduction of succinylated cytochrome *c* catalyzed by the reductase was quite appreciable, being slightly more than half that observed with menadione, which could be in agreement with their relative mutagenicity. However, in contrast to menadione, SOD addition had little or no effect on the rate of cytochrome *c* reduction.

Role of the Cytochrome P-450 Monooxygenase in the Detoxification and Bioactivation of the Different Quinones. The S9 and P-450 reductase enzyme preparations gave very different patterns of mutagenesis for the above quinones. Having ruled out the two-electron reduction pathway as the cause of this variation, we realized that there must be another pathway(s) leading to mutagenic activation of some quinones—notably, danthron—and the detoxification of others. To investigate the possibility that this NADPH-dependent pathway involved the cytochrome P-450 monooxygenase system, we studied the effect of adding the cytochrome P-450 inhibitor SKF525A (proadiphen) (27) to the S9 preparation. The effect of adding SOD and catalase to the S9 preparation was also examined. The results (Fig. 2) demonstrate that each of these four quinones is metabolized quite differently by enzymes present in the S9 preparation.

The mutagenicity of menadione was not significantly affected by the addition of SKF525A, but it was largely inhibited by SOD and catalase (Fig. 2). Both SKF525A and SOD/catalase inhibited the mutagenic activity of benzo[*a*]pyrene 3,6-quinone to some extent (Fig. 2). The mutagenicity of phenanthrenequinone was greatly increased by the addition of SKF525A (Fig. 2). This increase in mutagenicity could be inhibited by SOD and catalase (Fig. 2). Finally, we found that the mutagenicity of danthron was largely inhibitable by SKF525A, but it was not significantly affected by SOD and catalase (Fig. 2).

DISCUSSION

A systematic analysis of the metabolism of four quinones—namely, menadione, phenanthrenequinone, danthron, and benzo[*a*]pyrene 3,6-quinone—to mutagenic metabolites by rat liver S9 preparation and purified hepatic NADPH-cytochrome P-450 reductase has been performed by using the new *Salmonella* TA104 tester strain. Each of the quinones studied required metabolic activation to become mutagenic. None of the quinones tested showed decreased mutagenicity when DT-diaphorase was selectively inhibited with dicoumarol, thus indicating that the two-electron reduction pathway does not contribute to the mutagenic activation of these four quinones. This is in contrast to quinones possessing reactive leaving groups that have been shown to be converted

Table 3. Relative rates of NADPH-cytochrome P-450 reductase-catalyzed production of $O_2^{\cdot-}$ with quinones measured by reduction of succinylated cytochrome *c*

Quinone	Succinylated cytochrome *c* reduced, μmol/min per mg of protein		
	No addition	With SOD	SOD-inhibitable rate
Menadione	35.8	1.0	34.8
Benzo[*a*]pyrene 3,6-quinone	19.1	16.0	3.1
9,10-Phenanthrenequinone	91.4	5.5	85.9
Danthron	3.4	1.5	1.9

Values represent the mean of duplicate experiments. All quinones were added to a final concentration of 30 μM.

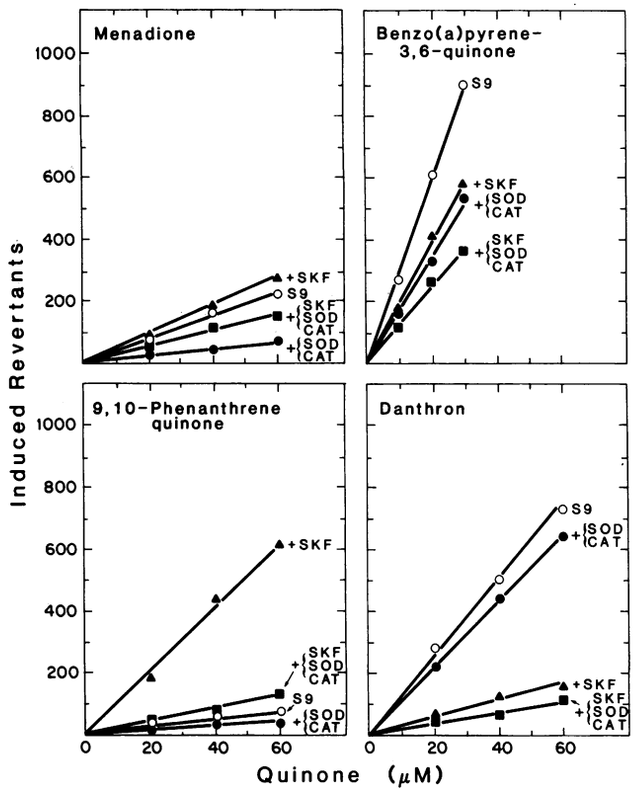

FIG. 2. Effect of SKF525A and SOD/catalase on the mutagenicity of quinones with S9. Quinone concentrations were chosen that gave linear dose–response curves. All assays contained S9 and dicoumarol (30 μM) and were performed by using the preincubation method (see text). SOD and catalase were used at 25 units each per plate. SKF525A was used at 0.2 mM. The mutagenicity of the different quinones is shown with S9 alone (○) and also in the presence of SKF525A (SKF) (▲), SOD plus catalase (CAT) alone (●), or in combination (■).

to mutagens by DT-diaphorase (18–20). In the case of the naphthoquinone menadione, the mutagenicity was increased by the inhibition of DT-diaphorase. This finding supports the contention that this enzyme serves a protective role against menadione cytotoxicity (13, 15) and is consistent with the observation that menadione is an exceptionally good substrate for DT-diaphorase (10). Thus, DT-diaphorase is essentially a detoxification pathway for some quinones (Fig. 3, pathway B).

Using purified NADPH-cytochrome P-450 reductase, it was shown that these four quinones are activated to mutagens by a one-electron reduction pathway (Fig. 3, pathway A). In all cases this mutagenesis was inhibited by SOD and catalase. This finding indicates that the one-electron reduction of these quinones to semiquinones is mutagenic via the formation of $O_2^{\cdot-}$ and, subsequently, H_2O_2. The conclusion that it is the oxygen radicals rather than the quinones themselves or their semiquinone metabolites that are mutagenic is further supported by the correlation found between superoxide production and mutagenicity.

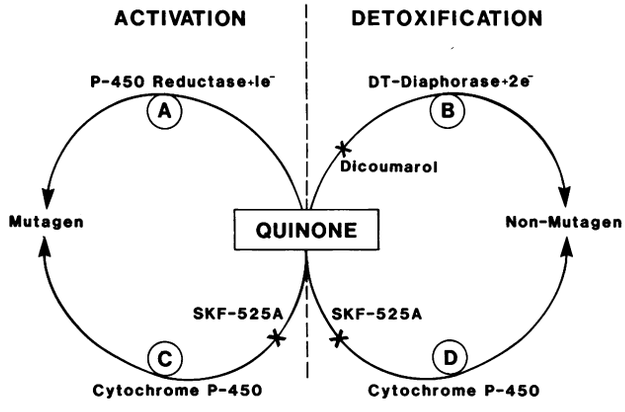

FIG. 3. Schematic illustration of pathways involved in quinone mutagenesis.

One possible complication is the case of benzo[*a*]pyrene 3,6-quinone. Table 3 shows that the P-450 reductase-dependent reduction of this quinone leads to the reduction of cytochrome *c* in the absence of significant $O_2^{\cdot-}$ formation. It is plausible that the species reducing cytochrome *c* in this case is benzo[*a*]pyrene 3,6-diol. This could explain the failure of SOD to inhibit the reduction of cytochrome *c* in the presence of benzo[*a*]pyrene 3,6-quinone. However, SOD and, to a greater extent, catalase inhibit the mutagenicity of this quinone in TA104 (Fig. 2), suggesting that its mutagenicity is mediated via oxygen radicals. The oxygen radicals could arise from the autooxidation of benzo[*a*]pyrene 3,6-diol rather than from the semiquinone intermediate reacting with oxygen. This, under the assay conditions employed for $O_2^{\cdot-}$ formation, the benzo[*a*]pyrene 3,6-diol could reduce the cytochrome *c* and be oxidized back to the quinone without generating $O_2^{\cdot-}$, whereas under the mutagenicity assay conditions benzo[*a*]pyrene 3,6-diol could accumulate and autooxidize, generating $O_2^{\cdot-}$ and H_2O_2 in the process (28).

The cytochrome P-450 monooxygenase system also seems to play a significant role in either the detoxification or mutagenic activation of these quinones (Fig. 3, pathway C). The relative contribution of this pathway and the P-450 reductase-dependent pathway (Fig. 3, pathway A) to the observed mutagenicity of the different quinones was therefore evaluated. This was done by adding SKF525A, a potent inhibitor of cytochrome P-450 (27), and/or SOD/catalase to the S9 enzyme preparation. Because the mutagenicity of menadione was inhibited by SOD/catalase, we conclude that it is attributable to the $O_2^{\cdot-}$ and H_2O_2 formed by redox cycling—i.e., pathway A is the prevalent activation mechanism (Fig. 3). With benzo[*a*]pyrene 3,6-quinone, both SKF525A and SOD/catalase inhibit mutagenesis to some extent, and because these inhibitors act in an additive fashion it seems likely that both pathways A and C (Fig. 3) contribute to the mutagenicity of this quinone. The fact that the benzo[*a*]pyrene quinones are mutagenic in this new tester strain raises again the question of the role of the metabolites in benzo[*a*]pyrene carcinogenesis (29).

Phenanthrenequinone exhibited an altogether different pattern of mutagenesis. When the S9 enzyme preparation was used alone, there was a very low level of mutagenicity that was inhibitable by SOD and catalase. The addition of SKF525A greatly increased this mutagenicity, but this increase was still inhibitable by SOD and catalase. These results indicate that this quinone is usually converted to a nonmutagenic (nonredox cycling) metabolite by the cytochrome P-450 monooxygenase system (Fig. 3, pathway D). Inhibition of this enzyme system allows the quinone to enter pathway A (Fig. 3) and act as a substrate for the P-450 reductase, thus leading to the production of oxygen radicals and mutagenesis in TA104. The final quinone tested was danthron. This quinone was found to be a very poor redox cycler but a good substrate for the cytochrome P-450 monooxygenase system. However, unlike phenanthrenequinone, the metabolite of danthron formed by this enzyme system was highly mutagenic in TA104—i.e., pathway C is the prevalent activation mechanism (Fig. 3).

In conceiving this project it was hoped that generalizations might be found concerning the metabolic activation of quinones to mutagens. Instead it was found that the observed level of mutagenesis was dependent on a series of pathways,

each of which acts differently on the four quinones analyzed. Fig. 3 summarizes these findings but does not take into account the fact that the detoxification of quinones may occur via a glutathione S-transferase pathway (30, 31). However, conjugation with reduced glutathione does not prevent certain quinones from redox cycling and generating oxygen radicals (32). The role of glutathione in protecting against quinone mutagenicity therefore requires further study. This work demonstrates that the correlation between mutagenic characteristics and the structure of quinones is complex, depending on both their chemical properties and their ability to interact with cellular enzymes. Quinones are very abundant in nature and continue to show promise as antitumor agents, indicating that their mutagenicity and carcinogenicity are areas worthy of future investigation.

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