MOLECULAR PHARMACOLOGY, 20:387–394

Quinone-Stimulated Superoxide Formation by Subcellular Fractions, Isolated Hepatocytes, and Other Cells

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Received September 8, 1980; Accepted May 6, 1981

SUMMARY

POWIS, G., B. A. SVINGEN, AND P. APPEL. Quinone-stimulated superoxide formation by subcellular fractions, isolated hepatocytes, and other cells. *Mol. Pharmacol.* **20:** 387–394 (1981).

Quinones can undergo enzymatic one-electron reduction to the semiquinone radical which, in the presence of molecular oxygen, can transfer an electron and form the superoxide anion radical ($O_2^{\cdot-}$). Isolated hepatocytes do not liberate appreciable amounts of $O_2^{\cdot-}$. Simple quinones, such as 2,5-dimethyl-*p*-benzoquinone, stimulate the formation of $O_2^{\cdot-}$ by hepatocytes up to 15 nmoles/min/10^6 cells. Hepatocyte $O_2^{\cdot-}$ formation stimulated by a variety of simple quinones and more complex antitumor quinones is maximal at a quinone one-electron reduction potential (E_7^1) of −70 mV and qualitatively similar to the pattern of $O_2^{\cdot-}$ formation seen with mitochondrial NADH:ubiquinone oxidoreductase and microsomal NADH-cytochrome b_5 reductase. $O_2^{\cdot-}$ production by microsomal NADPH-cytochrome P-450 reductase is maximal at a quinone E_7^1 of −200 mV. Phenobarbital induction, which increases NADPH-cytochrome P-450 reductase, has no effect on $O_2^{\cdot-}$formation by hepatocytes. It is concluded that NADPH-cytochrome P-450 reductase activity is not rate-limiting for quinone-stimulated $O_2^{\cdot-}$ formation by hepatocytes. The sulfonated stilbenes, 4-acetamido-4′-isothiocyano-2,2′-disulfonic acid stilbene and 4,4′-diisothiocyano-2,2′-disulfonic acid stilbene have no marked effect on the formation of $O_2^{\cdot-}$by hepatocytes, suggesting that $O_2^{\cdot-}$ is not transported through anion channels in the plasma membrane. Ethanol has no effect on hepatocyte $O_2^{\cdot-}$ formation, which suggests that intracellular NADH is not rate-limiting. Treatment of hepatocytes with diethyldithiocarbamate, which inhibits cytosolic and mitochondrial superoxide dismutase, increases $O_2^{\cdot-}$ formation by hepatocytes over 2-fold. Feeding rats a copper-deficient diet, which also decreases hepatic cytosolic and mitochondrial superoxide dismutase, has no effect on the quinone-dependent formation of $O_2^{\cdot-}$ by hepatocytes.

INTRODUCTION

Quinones are widely distributed in nature and form an important group of substrates for flavoenzymes. They can undergo either two-electron reduction to the hydroquinone or one-electron reduction to the semiquinone radical (1). The cytotoxic effects of quinones are probably mediated through one-electron reduction to the semiquinone radical (2) or through the formation of the superoxide anion radical by the transfer of an electron from the semiquinone to molecular oxygen (3). Several anticancer drugs of clinical and research interest contain the quinone nucleus (4), and their antitumor activity may be related to their one-electron reduction (2). Tumor cells are deficient in enzymes that normally protect the cell against free radical damage, principally superoxide dismutase (5) and often glutathione peroxidase (6), and catalase (7), and this may explain part of the selective cytotoxicity of the antitumor quinones. However, little is known of factors that govern the formation of free radicals, particularly the superoxide anion radical, by normal cells or tumor cells. The purpose of the present investigation was to study the influence of intracellular enzyme levels and the one-electron reduction potential of quinone substrates upon cellular free radical formation. Isolated rat hepatocytes were chosen for detailed study and a comparison was made with other cell types. Drug-induced free radical formation is difficult to measure even by electron spin resonance spectroscopy under aerobic conditions at levels of drug found under physiological conditions. Most semiquinones react rapidly with molecular oxygen to form superoxide (8), and in the present study superoxide formation has been used as a measure of total free radical formation.

This investigation was supported by Grant CA 25337 from the National Cancer Institute, Department of Health, Education and Welfare, and by a grant from the Eagles National Cancer Fund.

0026-895X/81/020387-08$02.00/0

MATERIALS AND METHODS

Enzyme preparations. Male rats of the Sprague-Dawley strain (Sprague-Dawley, Madison, Wisc.), weighing between 150 and 200 g, were killed by a blow on the head and exsanguinated; the livers were removed and flushed retrogradely through the hepatic vein with 50 ml of 0.9% NaCl solution at 4°. Hepatic microsomes were prepared by ultracentrifugation following homogenization in 0.25 M sucrose as described by Ernster *et al.* (9). The microsomes were washed by suspension in 20 volumes of 0.15 M KCl, then collected by centrifugation before being suspended in 0.15 M KCl at a protein concentration of 10 mg/ml. Rats were induced with three i.p. injections of 80 mg of phenobarbital/kg/day. NADPH-cytochrome P-450 reductase (EC 1.6.2.4) was prepared from hepatic microsomes of phenobarbital-induced rats by the method of Yasukochi and Masters (10). NADH-cytochrome b_5 reductase (EC 1.6.2.2) was prepared from hepatic microsomes of uninduced rats by the method of Spatz and Stritmatter (11). Mitochondria were isolated from the livers of untreated rats, and NADH:ubiquinone oxidoreductase (EC 1.6.5.3) was prepared by the method of Hatefi and Rieske (12). Copper-depleted rats were housed in stainless steel and plastic cages and maintained for 8 weeks on a copper-deficient diet (ICN Nutritional Biochemicals, Cleveland, Ohio) and distilled water.

Hepatocytes and other cell types. Hepatocytes were prepared by the method of Stewart and Inaba (13). Exclusion of trypan blue was used to assess cell viability, which was typically 80–90%. Murine leukemia P-388 cells were grown in the ascitic form in male BDF_1 mice for 7 days following the i.p. injection of 10^6 cells. The murine embryonal epithelial cell lines C3H/10T½ and AKR and 3-methylcholanthrene-transformed AKR cells (kindly provided by Dr. Harold Moses, Department of Cell Biology, Mayo Clinic) were grown in culture and harvested during the logarithmic phase of growth.

Incubations. Superoxide formation was measured as the reduction of acetylated cytochrome *c* at 550 nm, $\epsilon_{550} = 19.6\ \text{mM}^{-1}\ \text{m}^{-1}$ (14), with an incubation mixture containing Tris-HCl buffer, pH 7.4 (300 μmoles), $MgCl_2$ (15 μmoles), EDTA (0.3 μmole), acetylated cytochrome *c* (0.18 μmole), and microsomal protein (0.3 mg), NADPH-cytochrome P-450 reductase (40 μg), NADH-cytochrome b_5 reductase (40 μg), or NADH:ubiquinone oxidoreductase (250 μg), all in a final volume of 3 ml at 37°. The quinones, dissolved in 10 μl of dimethyl sulfoxide, were added 1 min prior to the addition of cofactor to give a concentration of 10^{-4} M. This quinone concentration was in excess of the K_m of quinones for microsomal flavoprotein reductases, around 25 μM (15).[1] This amount of dimethyl sulfoxide had no effect upon superoxide formation compared with drug added in the absence of solvent. NADPH or NADH (3 μmoles dissolved in 10 μl of water) was added to start the reaction. Superoxide formation was taken as the difference in the rate of acetylated cytochrome *c* reduction in the absence and presence of superoxide dismutase (33–167 μg/ml). The rate of reduction of acetylated cytochrome *c* by purified NADPH-cytochrome P-450 reductase in the absence of added quinone was less than 1% that of native cytochrome *c*. NADPH and NADH oxidation was measured at 340 nm in the absence of acetylated cytochrome *c*. Cellular superoxide formation was measured by suspending washed cells at around 10^6 cells/ml in Dulbecco's phosphate-buffered saline containing 10 mM glucose and 60 μM acetylated cytochrome *c* (with no added cofactor), and continually gassing with air or oxygen at 37°. Superoxide formation was taken as the difference in the rate of acetylated cytochrome *c* reduction in the absence and presence of superoxide dismutase. Quinones were added dissolved in 10 μl dimethyl sulfoxide (this amount of dimethyl sulfoxide had no effect on cellular metabolism as measured by oxygen utilization). Utilization of cellular oxygen was measured at 37°, in the presence and absence of 60 μM acetylated cytochrome *c*, using a Clark oxygen electrode (Yellow Springs Instrument Company, Yellow Springs, Ohio). Superoxide dismutase activity was measured by the method of Salin and McCord (16), after sonication of the mitochondrial fraction to release membrane-bound enzyme, and related to a standard preparation of bovine blood superoxide dismutase.

Drugs and chemicals. NADH, NADPH, horse heart cytochrome *c*, and superoxide dismutase were obtained from Sigma Chemical Company (St. Louis, Mo.). Acetylated cytochrome *c* was prepared as described by Azzi *et al.* (14). Quinones were obtained from Aldrich Chemical Company (Milwaukee, Wisc.) or Eastman Kodak (Rochester, N. Y.). Antitumor quinones were supplied by the Drug Synthesis and Chemistry Branch, Division of Drug Treatment, National Cancer Institute (Bethesda, Md.). The quinones used, together with their single-electron reduction potentials under IUPAC standard conditions, were *p*-benzoquinone, +99 mV (17); 2-methyl-*p*-benzoquinone, +23 mV (17); 1,4-naphthoquinone-2-sulfonate, −60 mV (18); 2,5-dimethyl-*p*-benzoquinone, −67 mV (17); 2,3,5-trimethyl-*p*-benzoquinone, −165 mV (17); 2-methyl-1,4-naphthoquinone, −200 mV (17); 2,3,5,6-tetramethyl-*p*-benzoquinone, −240 mV (17, 19); indigodisulfonate, −247 mV (19); adrenochrome, −253 mV (20); adriamycin, −292 mV (20); anthracenedione, −348 mV (20); and 9,10-anthraquinone-2-sulfonate, −375 mV (18).

RESULTS

Enzymatic and Microsomal Superoxide Formation

The ability of quinones to stimulate the formation of superoxide by three flavoprotein enzymes is shown in Fig. 1. The rate of superoxide formation in the absence of added quinone was low, 4 nmoles/min/mg for NADPH-cytochrome P-450 reductase, 22 nmoles/min/mg for NADH-cytochrome b_5 reductase, and 57 nmoles/min/mg for NADH:ubiquinone oxidoreductase. Superoxide formation is plotted against single-electron reduction potentials (E_7^1) determined under standard-state conditions. Although other factors, apart from single-electron reduction potential, may play a role in the reduction of the quinones, this is a convenient way to express the results and clearly shows a different pattern of substrate specificity for superoxide formation by the three enzymes. Quinone one-electron reduction is slower with more electronegative quinones and is probably dependent upon the E_7^1 for each flavoprotein (15). At

[1] G. Powis, B. A. Svingen, and P. Appel, unpublished observations.

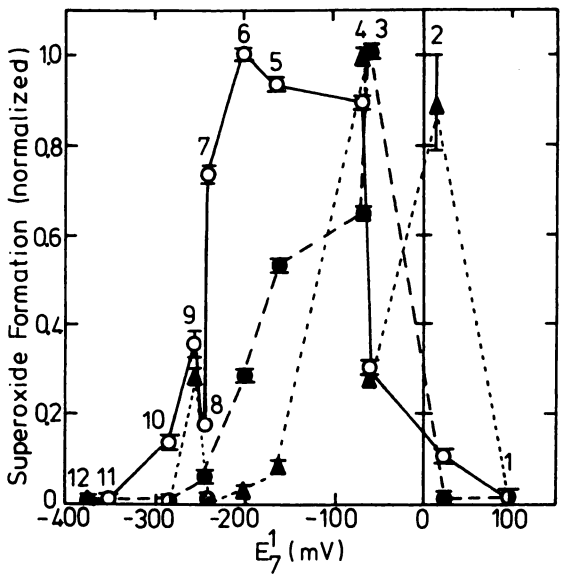

FIG. 1. *Superoxide formation by purified* (○) *NADPH-cytochrome P-450 reductase,* (●) *purified NADH-cytochrome b_5 reductase, and* (▲) *NADH:ubiquinone oxidoreductase*

Results are expressed as a fraction of the maximal rate of quinone-stimulated superoxide formation for each enzyme: 15,270 nmoles/min/mg for NADPH-cytochrome P-450 reductase, 1,603 nmoles/min/mg for NADH-cytochrome b_5 reductase, and 1,454 nmoles/min/mg for NADH:ubiquinone oxidoreductase. Each *point* is the mean of three observations; *bars* indicate standard error of the mean. Quinones were at 10^{-4} M. *1*, *p*-benzoquinone; *2*, 2-methyl-*p*-benzoquinone; *3*, 1,4-naphthoquinone-2-sulfonate; *4*, 2,5-dimethyl-*p*-benzoquinone; *5*, 2,3,5-trimethyl-*p*-benzoquinone; *6*, 2-methyl-2,4-naphthoquinone; *7*, 2,3,5,6-tetramethyl-*p*-benzoquinone; *8*, indigodisulfonate; *9*, adrenochrome; *10*, adriamycin; *11*, anthracenedione; and *12*, 9,10-anthraquinone-2-sulfonate.

higher values of quinone E_7^1, above E_7^1 of the $O_2/O_2^{\cdot-}$ couple, superoxide formation would be expected to decline as electron transfer from the semiquinone to oxygen becomes electrochemically unfavorable. Maximal superoxide formation was seen at a lower E_7^1, below −200 mV, for NADPH-cytochrome P-450 reductase than with NADH-cytochrome b_5 reductase and NADH:ubiquinone oxidoreductase.

There was a linear relationship between superoxide formation by purified NADPH-cytochrome P-450 reductase and the hepatic microsomal fraction in the presence of NADPH (Fig. 2). Hepatic microsomal quinone-dependent superoxide formation did not appear to involve cytochrome P-450 and was not inhibited by 80% carbon monoxide in oxygen or 0.5 mM β-diethylaminoethyl diphenylpropylacetate, both of which are inhibitors of microsomal cytochrome P-450 (results not shown). There was also a linear relationship between superoxide formation by NADH-cytochrome b_5 reductase and the microsomal fraction in the presence of NADH if 1,4-naphthoquinone-2-sulfonate (quinone 3) was omitted (Fig. 2). The low rate of superoxide formation by 1,4-naphthoquinone-2-sulfonate with purified NADH-cytochrome b_5 reductase is also seen in Fig. 1. Superoxide formation did not necessarily represent the total quinone metabolism, and the theoretical 2:1 ratio of superoxide formation to NAD(P)H oxidation was not seen with all quinones (Table 1). In general, at lower rates of metabolism more NAD(P)H was oxidized than superoxide formed. *p*-Benzoquinone, which has a relatively high one-electron reduction potential, was rapidly metabolized by NADH-cytochrome b_5 reductase, but little superoxide formation was seen.

Superoxide Formation by Whole Cells

Hepatocytes. Isolated hepatocytes by themselves do not liberate appreciable amounts of superoxide into the incubation medium as measured by the reduction of acetylated cytochrome *c*. When quinones were added to the medium there was an almost immediate increase in the reduction of acetylated cytochrome *c* that was inhibited by superoxide dismutase, showing it to be due to the formation of superoxide (Fig. 3). Cell-free medium from cells incubated for 10 min with 10^{-4} M quinone showed no increased ability to reduce acetylated cytochrome *c* compared with cell-free medium from incubations in the absence of quinone (results not shown). This

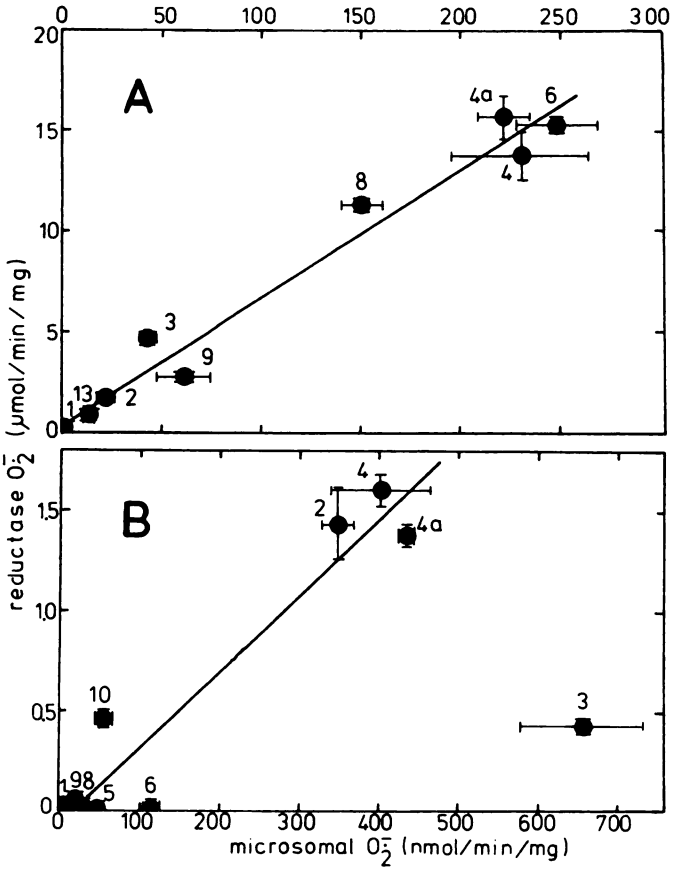

FIG. 2. *Quinone-dependent superoxide formation by hepatic microsomal fraction and purified flavoproteins*

Quinones were at 10^{-4} M and are defined as in Fig. 1. In addition, *4a* is 2,3-dimethyl-*p*-benzoquinone. *Lines* represent computer-derived regression analysis.

A. Microsomes with NADPH as the cofactor compared with NADPH-cytochrome P-450 reductase ($r = 0.97$, $p < 0.05$).

B. Microsomes with NADH as the cofactor compared with NADH-cytochrome b_5 reductase, excluding quinone 3 ($r = 0.91$, $p < 0.05$).

Each *point* is the mean of three observations for each preparation; *bars* represent standard error of the mean. Endogenous rates of microsomal superoxide formation were: NADPH, 6.0 nmoles/min/mg; NADH, 2.7 nmoles/min/mg.

TABLE 1

Quinone-dependent microsomal superoxide formation and cofactor oxidation

Metabolism was determined as described in the text. E_7^1 is the one-electron reduction potential. The values in parentheses are the ratios of superoxide formation to reduced pyridine nucleotide oxidation.

	E_7^1	NADPH as cofactor		NADH as cofactor	
		O_2^-·formed	NADPH oxidized	O_2^-·formed	NADH oxidized
		nmoles/min/mg	*nmoles/min/mg*	*nmoles/min/mg*	*nmoles/min/mg*
Control	—	6	38 (0.2)	1	10 (0.1)
p-Benzoquinone	+99	1	11 (0.1)	49	451 (0.1)
2-Methyl-*p*-benzoquinone	+23	27	52 (0.5)	350	650 (1.9)
1,4-Naphthoquinone-2-sulfonate	−60	48	22 (2.2)	659	398 (1.7)
2-Methyl-1,4-naphthoquinone	−200	253	101 (2.5)	116	79 (1.5)
2,3,5,6-Tetramethyl-*p*-benzoquinone	−240	166	74 (2.2)	26	43 (0.6)

finding suggests that stable products of quinone metabolism capable of reducing acetylated cytochrome *c* are not released into the incubation medium. Changes in hepatocyte superoxide formation and oxygen consumption produced by some of the quinones are shown in Table 2. Acetylated cytochrome *c* had no significant effect upon oxygen consumption. Unlike superoxide formation, which was maximal with 2,5-dimethyl-*p*-benzoquinone, oxygen consumption was depressed by the more electropositive quinones and increased with increasing electronegativity of the quinone. The pattern and extent of superoxide formation by hepatocytes with a variety of quinones are shown in Fig. 4. Values for superoxide formation were similar whether the hepatocytes came from uninduced or phenobarbital-induced rats. Pretreating the hepatocytes with 10^{-5} M diethyldithiocarbamate, an inhibitor of superoxide dismutase (21), decreased levels of cytosolic and mitochondrial superoxide dismutase in the hepatocytes (Table 3) and resulted in more than a 2-fold increase in the rate of superoxide formation (Fig. 5). Diethyldithiocarbamate itself had no effect on the ability of superoxide to reduce acetylated cytochrome *c*. Hepatocytes from rats maintained on a copper-deficient diet for 8 weeks also exhibited reduced levels of cytosolic and mitochondrial superoxide dismutase (Table 3) but had levels of quinone-stimulated superoxide formation similar to those of control hepatocytes.

Other factors affecting quinone-stimulated superoxide formation by control hepatocytes are shown in Table 4. Little reduction of acetylated cytochrome *c* was seen in the presence of superoxide dismutase, suggesting that 2,5-dimethylbenzosemiquinone was not being released by hepatocytes. A similar effect was seen with the other quinones in the presence of superoxide dismutase (results

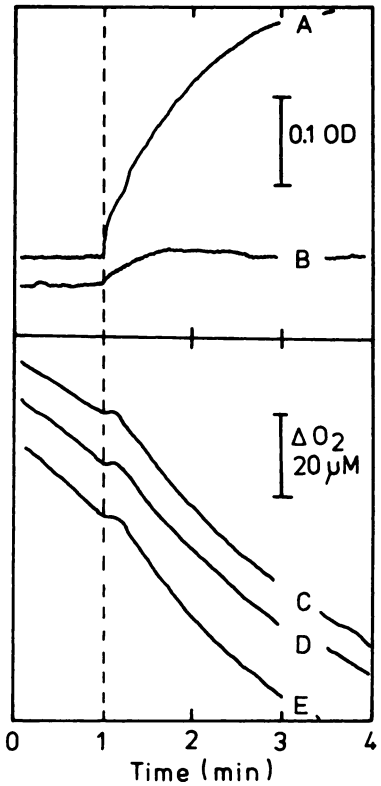

FIG. 3. *Superoxide formation and oxygen utilization by isolated hepatocytes, 0.5 × 10^6 cells/ml at 37°*

2,5-Dimethyl-*p*-benzoquinone, 10^{-4} M, was added to the incubation medium at 1 min (– – –).

Upper panel. Superoxide formation measured as the reduction of acetylated cytochrome *c* (the cuvettes were continually gassed with air). *A*, control hepatocytes; *B*, in the presence of superoxide dismutase, 166 μg/ml.

Lower panel. Oxygen utilization (samples were initially saturated with air at 37°); *C*, control hepatocytes; *D*, in the presence of 60 μM acetylated cytochrome *c*; *E*, in the presence of superoxide dismutase, 166 μg/ml.

TABLE 2

Oxygen consumption and superoxide formation by hepatocytes

Each value is the mean (± standard error of the mean) of three determinations. Acetylated cytochrome *c*, 60 μM, was included in some determinations of oxygen consumption at the same concentration as in the assays for superoxide formation.

	O_2^-·formation	O_2 consumption	
		Basal	+ Acetylated cytochrome *c*
	nmoles/10^6 cells/min	*nmoles/10^6 cells/min*	*nmoles/10^6 cells/min*
Control	0.1 ± 0.0	37.8 ± 5.5	37.8 ± 9.7
p-Benzoquinone	6.3 ± 0.8	23.0 ± 4.7	20.6 ± 1.8
2-Methyl-*p*-benzoquinone	10.8 ± 1.8	26.7 ± 4.0	24.6 ± 3.8
2,5-Dimethyl-*p*-benzoquinone	14.7 ± 0.6	52.4 ± 4.2	53.6 ± 4.0
2-Methyl-1,4-naphthoquinone	4.6 ± 0.6	89.8 ± 6.2	65.2 ± 9.7
Adriamycin	0.2 ± 0.4	71.5 ± 5.2	62.3 ± 8.5

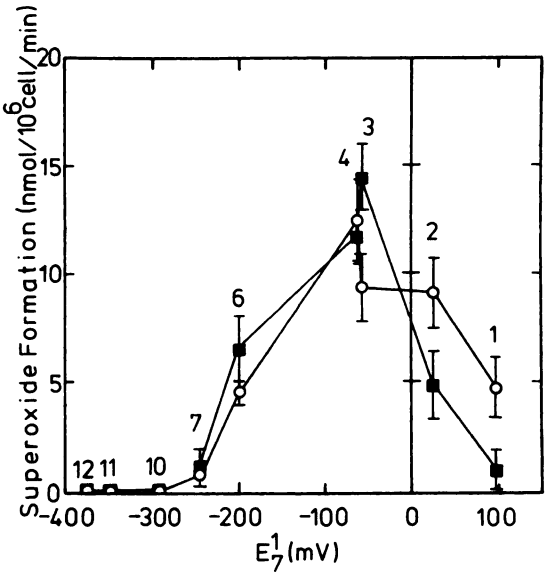

FIG. 4. *Superoxide formation by hepatocytes*

Quinones were present at a concentration of 10^{-4} M and are defined as in Fig. 1. ○, Control hepatocytes (6); ■, hepatocytes from phenobarbital-pretreated rats (3). Values in parentheses are numbers of preparations; *bars* represent standard error of the mean.

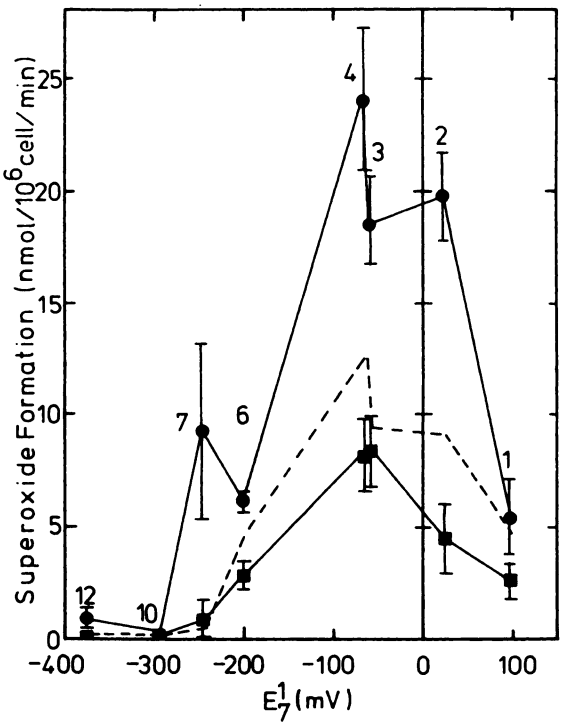

FIG. 5. *Superoxide formation by superoxide dismutase-deficient hepatocytes*

Quinones were present at a concentration of 10^{-4} M as in Fig. 1. ●, Hepatocytes from rats pretreated with 10^{-5} M diethyldithiocarbamate for 30 min (3); ■, hepatocytes from rats maintained on a copper-deficient diet for 8 weeks (3). Values in parentheses are numbers of preparations; *bars* represent standard error of the mean. - - -, Values for untreated hepatocytes from Fig. 4.

not shown). Carbon monoxide and β-diethylaminoethyl diphenylpropylacetate had no effect upon quinone stimulated superoxide formation. 4-Acetamido-4′-isothiocyano-2,2′-disulfonic acid stilbene and 4,4′-diisothiocyano-2,2′-disulfonic acid stilbene are specific inhibitors of anion exchange which have been reported to inhibit the passage of superoxide across vesicles formed by erythrocyte ghosts (22) and to inhibit the release of superoxide by granulocytes (23). At concentrations which almost completely inhibit the transport of superoxide across the red cell membrane, 4-acetamido-4′-isothiocyano-2,2′-disulfonic acid stilbene and 4,4′-diisothiocyano-2,2′-disulfonic acid stilbene had no marked effect upon superoxide formation by hepatocytes. Ethanol, which increases the levels of cytosolic and mitochondrial NADH (24), had no significant effect on superoxide formation. Rotenone, a classical inhibitor of mitochondrial oxidative phosphorylation (25), had a moderately inhibitory effect upon the formation of superoxide by hepatocytes. Antimycin A, also an inhibitor of mitochondrial oxidative phosphorylation (25), had no significant effect. Rotenone and antimycin A had no effect upon quinone-stimulated superoxide formation by NADH:ubiquinone oxidoreductase

TABLE 3

Inhibition of hepatocyte superoxide dismutase by diethyldithiocarbamate (DDC) and copper deficiency

	Superoxide dismutase[a]	
	Mitochondrial	Cytosolic
	μg/mg protein	*μg/mg protein*
Control	1.3 ± 0.3	14.0 ± 1.6
DDC pretreatment[b]	0.3 ± 0.1[c]	3.8 ± 0.9[c]
Copper-deficient diet[d]	0.5 ± 0.2[c]	8.9 ± 1.4[c]

[a] Values are means of three determinations ± standard error of the mean.

[b] Hepatocytes were exposed to 10^{-5} M diethyldithiocarbamate for 30 min at 37°, washed, and assayed for superoxide dismutase activity.

[c] $p < 0.05$.

[d] Hepatocytes were prepared from rats maintained on a copper-deficient diet for 8 weeks.

TABLE 4

Factors affecting quinone-stimulated superoxide formation by hepatocytes

Hepatocyte superoxide formation was stimulated with 10^{-4} M 2,5-dimethyl-*p*-benzoquinone. Drugs and chemicals were added 1 min before the addition of the quinone.

	% Control[a]
Control	100.3 ± 1.9
SOD,[b] 167 μg/ml[c]	2.0 ± 1.2[d]
SKF-525A,[e] 0.1 mM	93.7 ± 8.3
$CO:O_2$ (8:2)	109.3 ± 5.2
SITS,[f] 0.1 mM	93.2 ± 1.1[d]
DIDS,[g] 0.1 mM	89.1 ± 6.3
Ethanol, 30 mM	115.9 ± 9.0
Rotenone, 1 μM	61.8 ± 5.5[d]
Antimycin A, 1 μM	87.7 ± 4.2

[a] Values are means ± standard error of the mean of three preparations.

[b] Superoxide dismutase.

[c] Acetylated cytochrome c reduction in the presence of SOD. All other values are superoxide formation measured in the absence and presence of SOD.

[d] $p < 0.05$.

[e] β-Diethylaminoethyl diphenylpropylacetate.

[f] 4-Acetamido-4′-isothiocyano-2,2′-disulfonic acid stilbene.

[g] 4,4′-diisothiocyano-2,2′-disulfonic acid stilbene.

TABLE 5

Superoxide formation by different cell lines

Superoxide formation was determined as described in the text. All quinones were added at 10^{-4} M and are listed in order of decreasing electron affinity. Murine leukemia P-388 cells were grown for 7 days in the ascitic form in BDF_1 mice. AKR and C3H cells were grown in culture and studied during their exponential phase of growth. Values are means ± standard error of the mean of three observations.

	P-388	C_3H	AKR	Transformed AKR
	nmoles/min/10^6 cells	*nmoles/min/10^6 cells*	*nmoles/min/10^6 cells*	*nmoles/min/10^6 cells*
Control	0.0 ± 0.0	0.0 ± 0.0	0.1 ± 0.0	0.6 ± 0.0
p-Benzoquinone	0.0 ± 0.0	1.3 ± 0.2	1.0 ± 0.0	0.7 ± 0.1
2-Methyl-*p*-benzoquinone	0.9 ± 0.2	8.6 ± 0.4	4.9 ± 0.3	2.0 ± 0.3
1,4-Naphthoquinone-2-sulfonate	0.5 ± 0.2	13.1 ± 0.2	6.6 ± 0.2	5.0 ± 0.7
2,5-Dimethyl-*p*-benzoquinone	0.9 ± 0.2	16.9 ± 5.7	13.4 ± 1.2	10.3 ± 0.5
2-Methyl-1,4-naphthoquinone	0.3 ± 0.1	2.6 ± 0.9	3.8 ± 0.3	2.2 ± 0.6
Indigodisulfonate	1.0 ± 0.0	0.0 ± 0.0	3.5 ± 0.3	7.6 ± 1.6
9,10-Anthraquinone-2-sulfonate	0.0 ± 0.0	0.0 ± 0.0	0.1 ± 0.0	0.4 ± 0.1

(results not shown), and the inhibition seen with hepatocytes probably represents a nonspecific inhibitory effect secondary to an altered energy metabolism.

Other cells (*Table 5*). Murine leukemia P-388 cells exhibited low rates of quinone-stimulated superoxide formation. C3H/10T½, AKR, and chemically transformed AKR murine embryonal cells exhibited rates of quinone-dependent superoxide formation similar to those seen with hepatocytes. In all cases, maximal superoxide formation was seen at E_7^1 around −70 mV.

DISCUSSION

Superoxide anion radicals have previously been shown to traverse the membranes of erythrocytes (22) and granulocytes (23) through anion channels. The sulfonated stilbenes 4-acetamido-4′-isothiocyano-2,2′-disulfonic acid stilbene and 4,4′-diisothiocyano-2,2′-disulfonic acid stilbene at concentrations which inhibit superoxide anion transport in red cells and granulocytes (22, 23) had no marked effect upon superoxide formation by hepatocytes. Superoxide anion radicals have been shown to cross artificial lipid bilayer membranes at temperatures above the lipid phase-transition (26), and it is possible that intracellularly generated superoxide can pass directly through the hepatocyte lipid membrane, which is fluid at 37° (27). Semiquinones may also pass through the hepatocyte membrane, and we cannot say with certainty whether they react with oxygen within the cell or in the external medium. The lack of an effect of sulfonated stilbenes and the fact that superoxide is detected despite high levels of intracellular superoxide dismutase might suggest that the superoxide could be formed outside the cell. On the other hand, semiquinones react rapidly with oxygen and would not be expected to diffuse far in the presence of oxygen.

Ilan *et al.* (17) have reported that 2,5-dimethylbenzoquinone reacts with O_2 with $k = 4.8 \times 10^6$ M^{-1} sec^{-1}. Assuming a semiquinone concentration of 1 μM and an air-saturated medium (~250 μM O_2), the half-life of the semiquinone can be calculated to be 0.6 msec, which would give a mean diffusion distance (20) of 0.7 μm. The situation is further complicated by the fact that the concentration of O_2 in the lipid plasma membrane, through which the semiquinone presumably must pass, is 8 times that in the aqueous medium (28). It has to be considered that superoxide might be formed by enzymes on the extracellular surface of the plasma membrane. This is unlikely since it would have to involve transfer of reducing equivalents across the plasma membrane. In addition, although the plasma membrane fraction from mouse liver exhibits NAD(P)H oxidase activity and adriamycin stimulates NADH oxidation, there is no accompanying superoxide formation (29). Superoxide appearing in the incubation medium probably represents only a fraction of the superoxide formed within the cell. Some superoxide will be destroyed by intracellular superoxide dismutase before it reaches the external environment. Increased oxygen utilization would represent 25% of superoxide formation if the combined action of intracellular superoxide dismutase (Eq. 1) and catalase (Eq. 2) converted all of the superoxide to oxygen and water. Increased oxygen utilization would represent 50% of superoxide formation if superoxide was converted by superoxide dismutase to hydrogen peroxide (Eq. 1) which was then completely destroyed by glutathione peroxidase with the oxidation of reduced glutathione.

$$4O_2^{\cdot-} + 4H^+ \rightarrow 2H_2O_2 + 2O_2 \quad (1)$$

$$\frac{2H_2O_2 \rightarrow 2H_2O + O_2}{4O_2^{\cdot-} + 4H^+ \rightarrow 2H_2O + 3O_2} \quad (2)$$

Assuming in both cases that limiting conditions are approached, superoxide released into the external medium would represent 25–50% of the total superoxide formed, calculated from increased oxygen utilization for 2,5-dimethyl-*p*-benzoquinone, although much less for 2-methyl-1,4-naphthoquinone (2–4%) and adriamycin (below 1%).

Diethyldithiocarbamate pretreatment of hepatocytes inhibited the copper-containing cytosolic superoxide dismutase by 73% and the manganese-containing mitochondrial enzyme by 77%. Pretreating hepatocytes with diethyldithiocarbamate produced over a 2-fold increase in quinone-dependent superoxide formation. A copper-deficient diet has been reported to lower the levels of superoxide dismutase in brain and blood (30). The copper-deficient diet used in the present study reduced soluble hepatic superoxide dismutase activity by 36% and, surprisingly, since the diet contained manganese, mitochondrial superoxide dismutase activity by 62%. However, there was no change in quinone-mediated super-

oxide formation by hepatocytes prepared from copper-deficient rats, possibly because in these hepatocytes the remaining superoxide dismutase activity was sufficient to accommodate the increased superoxide formation or because superoxide formation is in some way compromised by the copper-deficient diet.

Experimental conditions are considerably removed from the standard-state conditions at which quinone E_7^1 values are determined. This makes a thermodynamic consideration of the reduction-oxidation equilibria between quinones and oxygen difficult. The change in E_7^1 with concentration can be calculated from

$$\Delta E_7^1 \cong \frac{RT}{nF} \log \frac{[Q']}{[Q^\circ]} \quad (3)$$

where $[Q']$ and $[Q^\circ]$ are the concentrations of quinone under experimental and standard-state conditions. At 10^{-4} M quinone, ΔE_7^1 is −236 mV. For O_2 at 1 M the E_7^1 of the $O_2/O_2^{\cdot-}$ couple is −155 mV (18). Using air-saturated buffer (O_2, 217 μM), ΔE_7^1 is −214 mV. The similarity between the changes in E_7^1 makes it reasonable to compare E_7^1 for quinones under standard-state conditions and E_7^1 for O_2 at 1 M. However, it is clear that quinones considerably more electropositive than the −155 mV of $O_2/O_2^{\cdot-}$ can form appreciable amounts of superoxide. This is particularly the case with hepatocytes, where quinones up to E_7^1 +23 mV increase superoxide formation. A possible explanation is that intracellular quinone concentrations are much lower than the 10^{-4} M added to the incubation medium. This could lower the effective E_7^1 of the quinones even more than that of the $O_2/O_2^{\cdot-}$ couple.

Of the flavoenzymes studied, the pattern of quinone-dependent superoxide formation by hepatocytes with a maximum at an E_7^1 of −70 mV was closest to that seen with NADH:ubiquinone oxidoreductase. Superoxide formation with NADH-cytochrome b_5 reductase, although exhibiting a similar maximum, extended to include the more electronegative quinones down to E_7^1 −200 mV, whereas NADPH-cytochrome P-450 reductase included quinones with an E_7^1 below −200 mV. Phenobarbital pretreatment had no effect on quinone-dependent superoxide formation by hepatocytes. Phenobarbital induction increases hepatic microsomal NADPH-cytochrome P-450 reductase (10) but has no effect on microsomal NADH-cytochrome b_5 reductase (measured as NADH-ferricyanide reductase). The lack of an effect of phenobarbital suggests that NADPH-cytochrome P-450 reductase activity is not rate-limiting in quinone-stimulated superoxide formation by hepatocytes. However, it remains a possibility that the rate of NADPH formation, which is not limiting for mixed-function oxidase activity of isolated hepatocytes, is rate-limiting for quinone-stimulated superoxide formation. Ethanol had no significant effect on quinone-stimulated superoxide formation by hepatocytes. Although the primary effect of ethanol oxidation is to increase cytosolic NADH, the transfer of reducing equivalents across the mitochondrial membrane also increases mitochondrial NADH (24). The lack of an effect of ethanol suggests that the supply of NADH does not limit quinone-stimulated superoxide formation. It is not possible to state with certainty the major site for the intracellular one-electron reduction of quinones and the consequent site for the formation of superoxide. It is possible that the quinones are not reduced directly by flavoenzymes but are reduced by intracellular electron carriers such as endogenous quinones and ascorbate. However, of the three flavoproteins studied, the evidence most strongly favors mitochondrial NADH:ubiquinone oxidoreductase as being a site for quinone-dependent superoxide formation by hepatocytes, and perhaps by other cells.

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