MOLECULAR PHARMACOLOGY, 18, 588–593

Superoxide Production by Purified Hamster Hepatic Nuclei

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Received April 2, 1980; Accepted June 3, 1980

SUMMARY

PATTON, S. E., G. M. ROSEN AND E. J. RAUCKMAN. Superoxide production by purified hamster hepatic nuclei. *Mol. Pharmacol.* 18: 588–593 (1980).

We have found that hamster hepatic nuclei produce substantial quantities of superoxide in the presence of NADPH. We have determined that these nuclei contain copper–zinc superoxide dismutase which can be almost entirely removed by extensive washing. We have suggested that this nuclear superoxide dismutase is in equilibrium with cytoplasmic superoxide dismutase. Finally, we have presented evidence which demonstrates that the flavoprotein FAD-monooxygenase is responsible for most of the nuclear superoxide production.

INTRODUCTION

Chemical carcinogens are generally assumed to exert their effect by covalently binding to cellular DNA and thus causing a subsequent phenotypic alteration resulting in cancer (1). These reactive chemical carcinogens arise via the *in vivo* biotransformation of nonmutagenic precursors, commonly known as procarcinogens to their "active" carcinogenic state. It is now generally accepted that strong electrophiles are the ultimate carcinogens (2), but the extent to which electrophilic free radical species participate in carcinogenesis remains unclear. For example, it is known that a nitroxyl free radical is generated during the metabolism of *N*-hydroxy-2-acetylaminofluorene, but there is only circumstantial evidence implicating this free radical in carcinogenesis (3).

The most extensively studied chemical carcinogens are the polycyclic aromatic hydrocarbons. Jerina *et al.* (4) have proposed a unifying concept which attempts to explain and predict the carcinogenicity of these compounds (5–7). This theory states that an epoxide on a saturated, angular benzene ring that forms part of the "bay region" of the hydrocarbon is a prime candidate to be the ultimate carcinogenic metabolite of the hydrocarbon. This theory has gained wide acceptance based on evidence that benzo[*a*]anthracene 3,4-diol-1,2-epoxide acts as the ultimate carcinogen of benzo[*a*]anthracene (8). However, alternative mechanisms of carcinogenicity involving free radicals cannot be excluded. For example, Nagata *et al.* (9) have presented evidence suggesting that the oxy radical of benzo[*a*]pyrene is formed by various tissue preparations and that this species binds to DNA. Lesko *et al.* (10) have suggested that reactive oxygen species, generated by benzo[*a*]pyrene quinone-mediated one-electron reactions, cause strand scission. Cerutti *et al.* (11) have carried this discussion further by suggesting that the existence of strand scission caused by the indirect action of benzo[*a*]pyrene metabolites may be mediated by hydroxyl radical. Michelson (12) has demonstrated that the effects of carcinogenic hydrocarbons on mammalian cells are greatly stimulated and amplified in the presence of a system which produces superoxide. He further states that in the presence of additional superoxide, hydrocarbons, e.g., anthracene, which are not normally regarded as carcinogenic, show most of the same effects as do the potent carcinogens benzo[*a*]pyrene and 3-methylcholanthrene. Based on this information, he concluded that the morphological effects of carcinogenic hydrocarbons are mediated by the endogenous production of superoxide within the cell. If this were the case, any xenobiotic which promotes excessive production of superoxide would predispose one to cancer. Mason *et al.* (13, 14) have recently shown that some known mutagens, such as nitrofurantoin, greatly stimulate superoxide production. Thus, it is reasonable to hypothesize that superoxide has an involvement in carcinogenesis. This pernicious species could act either by directly activating procarcinogens or by indirectly promoting the generation of carcinogens via the formation of some other radical intermediate, e.g., hydroxyl radical.

Many cellular enzymes are known to produce superoxide; however, superoxide dismutase[1] rapidly removes most of this toxic substance. If one is considering potential superoxide-mediated chromosomal damage, special

This investigation was supported by United States Public Health Service Grants GM 25188 and GM 25753 from the National Institutes of Health.

[1] The second-order rate constant for the dismutation of superoxide by superoxide dismutase is 2×10^9 M^{-1} s^{-1}. See: Klug, D., J. Raboni and I. Fridovich. A direct demonstration of the catalytic action of superoxide dismutase through the use of pulse radiolysis. *J. Biol. Chem.* 247: 4839–4842 (1972).

0026-895X/80/060588-08$02.00/0

attention must be shown toward enzymes which produce this free radical at or near the genetic material. Rogan *et al.* (15–17) and Bresnick *et al.* (18) have reported that cytochrome *P*-450 is found in rat hepatic nuclei and that this nuclear enzyme can mediate a wide variety of biotransformations, just as it can in the endoplasmic reticulum.

Microsomal NADPH–cytochrome *P*-450 reductase has been shown to produce superoxide (19). In addition, we have found that the microsomal flavoprotein, FAD-monooxygenase,[2] also generates this reactive species (20, 21). Recently, we have demonstrated that this flavoprotein is a constituent of the hamster hepatic nuclear envelope and that this nuclear enzyme catalyzes the oxygenation of both nitrogen- and sulfur-containing compounds (22). The question remains—Is superoxide produced at the nuclear level, and if so, what enzyme(s) is responsible for the formation of this radical species?

In this report, we present evidence demonstrating that superoxide is produced by hamster hepatic nuclear preparations. Second, nuclear FAD-monooxygenase was found to be responsible for a large portion of the superoxide produced. Finally, these nuclear preparations were also shown to contain superoxide dismutase.

MATERIALS AND METHODS

General comments. Superoxide dismutase was generously given to us by Dr. Irwin Fridovich, Department of Biochemistry, Duke University. Diethylenetriaminepentaacetic acid (DETAPAC), hypoxanthine, and cytochrome *c* were purchased from Sigma Chemical Company. Chelex 100 was obtained from Bio-Rad. The spin traps DMPO, OXANOH, OXANO, and TEMPO were prepared according to literature procedures (21, 23).

Unless otherwise indicated all phosphate buffers used were passed through a Chelex-100 column according to the method of Poyer and McCay (24), in order to remove trace amounts of multivalent cation impurities, such as iron.

Isolation of nuclei. Male Syrian golden hamsters (70–110 g) obtained from Gibco Animal Resources Laboratories were used in all experiments. Liver nuclei were prepared by a modification of the method of Bresnick *et al.* (18) as follows: The animals were sacrificed, and then their livers were perfused through the hepatic portal vein with 50–70 ml of ice-cold buffer consisting of 50 mM Tris, 0.25 M sucrose, 25 mM KCl, and 5 mM $MgCl_2$ (TSKM) at pH 8.3. Their livers were rapidly excised, the gall bladders were removed, and, after chilling in TSKM buffer, the livers were forced through a wire-mesh tea strainer with a Teflon pestle. They were then ground with one slow pass by hand of a Teflon pestle in a loose-fitting glass homogenizer. The homogenates were diluted to about 30 ml with TSKM buffer and centrifuged at 120*g* for 8 min to pellet the whole cells, erythrocytes, and clumps of cells. The supernatants were centrifuged at 2500*g* to pellet the nuclei. The nuclear pellets were resuspended in 30 ml each of 2.3 M sucrose containing 3 mM $CaCl_2$ and centrifuged at 35,000*g* for 50 min. The pellicles, consisting of the last remaining red blood cells, whole cells, and clumps were discarded together with the clear yellow supernatants. The buff-colored nuclear pellets were washed several times by resuspension in 20 ml of 1 M sucrose containing 1 mM $CaCl_2$ and centrifuged at 2500*g* for 10 min. The nuclei required for enzyme activity measurements were resuspended in TSKM buffer that had been passed through Bio-Rad Chelex-100 resin before $MgCl_2$ was added and the pH was adjusted to pH 8.3.

FAD-Monooxygenase activity. FAD-monooxygenase activity of isolated nuclei was determined by measuring the rate of *N,N*-dimethylaniline-dependent NADPH oxidation with a Perkin–Elmer 557 spectrophotometer in the double wavelength mode (λ_{obs} 340, λ_{ref} 420). The assay was run at 37°C, pH 8.3, with a saturating concentration of NADPH (0.1 mM) in 100 mM Tricine buffer containing 24 mM *n*-octylamine and 1 mM diethylenetriaminepentaacetic acid (Sigma). Emulgen 913 detergent (0.5%, Kao-Atlas Ltd., Tokyo) was added to prevent clumping of the nuclei.

Determination of cytochrome P-450 activity. Cytochrome *P*-450 activity was measured by following the rate of reduction of a stable nitroxyl radical, 2,2,6,6-tetramethylpiperidinoxyl (TEMPO), a substrate specific for the ferrous form of this hemoprotein (25). The kinetic studies were conducted using an electron paramagnetic resonance spectrometer (Varian Associates Model E-9) to measure the decrease in the height of the central peak of the nitroxide triplet as a function of time. In a typical experiment, the reaction medium contained 10 μM nitroxide, 250 μM NADPH, 0.1 ml of nuclear suspension, and sufficient buffer (0.1 M phosphate with 1 mM diethylenetriaminepentaacetic acid adjusted to pH 7.4) to bring the final volume to 0.5 ml.

Determination of superoxide production. Superoxide production was measured by following the oxidation of the hydroxylamine 2-ethyl-1-hydroxy-2,4,4-trimethyl-3-oxazolidine (OXANOH) (21) using electron paramagnetic resonance spectrometry. The increase in the height of the central peak of the nitroxide triplet was determined as a function of time. Reaction mixtures were the same as described for the cytochrome *P*-450 activity determination, substituting 100 μM OXANOH in place of TEMPO and conducting the experiments at pH 8.3 and 7.4.

Spin trapping of superoxide and hydroxyl radical. The spin trapping of superoxide was undertaken using the spin probe, 5,5-dimethyl-1-pyrrolidine-*N*-oxide (DMPO). In a typical experiment, the reaction mixture contained 0.1 M DMPO, 250 μM NADPH, 0.1 ml of nuclear suspension, and sufficient buffer (0.1 M phosphate with 1 mM DETAPAC, adjusted to pH 8.3) to bring the final volume to 0.3 ml. When examining whether hydroxyl radical is produced, DMSO (0.5%) was added to the above reaction mixture. See Fig. 1 for epr spectra and instrumental settings.

Determination of superoxide dismutase activity. Superoxide dismutase (SOD) activity in nuclei was assayed by a modification of the method of McCord and Fridovich

[2] This flavoprotein in older literature was referred to as the microsomal mixed-function amine oxidase or as the microsomal *N*-oxidase. The substrates for this monooxygenase are, however, not restricted to amines, and a trivial name based on the nature of the prosthetic group avoids problems posed by broad substrate specificity.

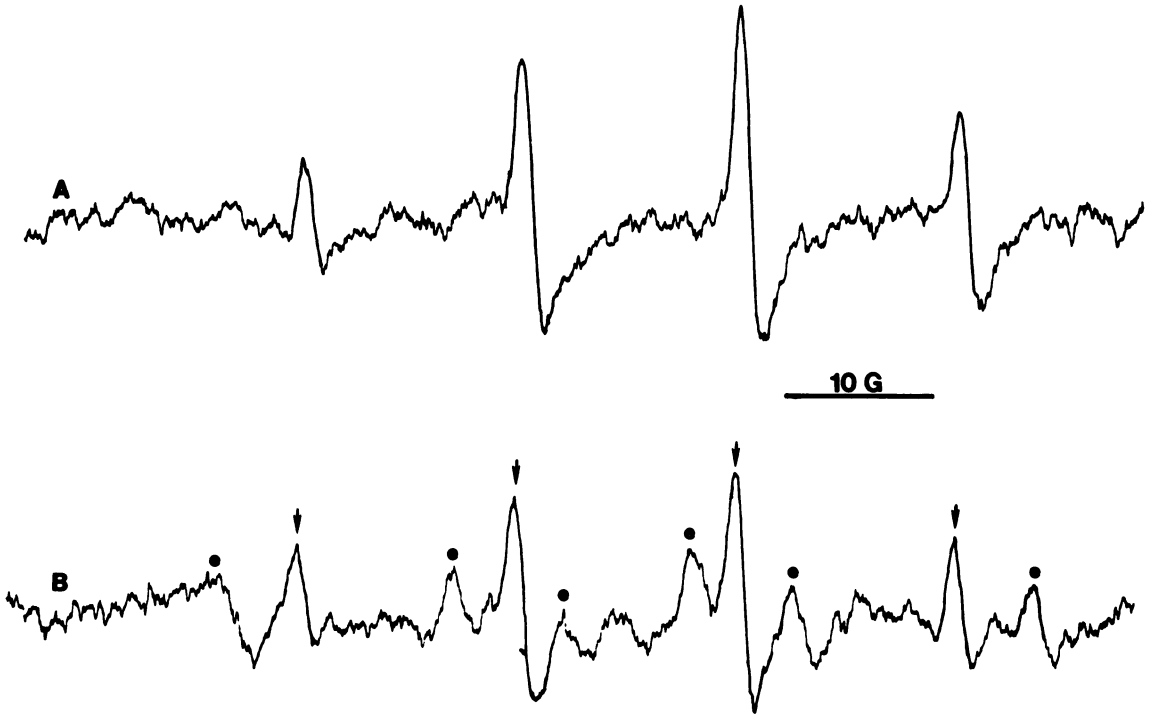


FIG. 1. *Electron spin resonance spectra obtained by reacting purified hamster hepatic nuclei with NADPH: (A) with DMPO alone, $A_N = A_H = 14.9$ G; (B) with DMPO and DMSO (0.5%), $A_N = 15.8$ G and $A_H = 22.8$ G*

From these data and from the rates of spin trapping each of these radicals by DMPO, we have calculated that approximately 80% of the epr spectrum is due to the superoxide adduct of DMPO. The microwave power was 10 mW and the modulation frequency was 100 kHz with an amplitude of 0.63 G. The sweep time was 6.25 G/min and the response time was 10 s.

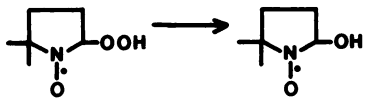
(26). Under saturating conditions of hypoxanthine in Chelexed TSK buffer consisting of 50 mM Tris, 0.25 M sucrose, and 25 mM KCl, at pH 7.8, sufficient xanthine oxidase was added to produce a suitable control cytochrome *c* reduction rate at 25°C, monitored on a Perkin–Elmer 557 spectrophotometer in the double wavelength mode (λ_{obs} 550, λ_{ref} 600). Nuclear SOD activity was then measured by determining the rate of inhibition of cytochrome *c* reduction (note Table 1).

Protein determination. All enzyme measurements were normalized to protein by the method of Bradford (27) using the dye reagent and standard bovine γ-globulin obtained from Bio-Rad Laboratories.

RESULTS AND DISCUSSION

When hamster hepatic nuclei, free of endoplasmic reticulum, were incubated in the presence of NADPH, the generation of superoxide was determined by the oxidation of hydroxylamines to nitroxides mediated by this free radical species (20, 21). Since this conversion can also be mediated by a variety of other one-electron oxidants, such as hydroxyl radical, additional experiments were required to verify that superoxide was responsible for this oxidation. Spin-trapping techniques were then employed to confirm that superoxide was produced by hamster hepatic nuclei.

We have previously provided unequivocal evidence demonstrating that DMPO,[3] a nitrone spin trap, can react with both superoxide and hydroxyl radical (28). The resulting nitroxides exhibit epr spectra explicitly characteristic of each species (28). However, we have shown that the hydroperoxy spin-trapped adduct, DMPO-OOH, is unstable and rapidly decomposes into the hydroxy spin-trapped adduct, DMPO-OH, and a nonradical species.



SCHEME I

In addition, DMPO-OH can be directly produced via the spin trapping of hydroxyl radical. Finally, in biological systems where peroxidases routinely decompose hydroperoxides such as DMPO-OOH to alcohols such as DMPO-OH, the observation of the distinctive spectrum of DMPO-OH is not definitive evidence for the generation of hydroxyl radical. Thus, when DMPO was incubated with hamster liver nuclei and NADPH, it was not surprising to observe an epr spectrum characteristic of DMPO-OH (Fig. 1). One method of verifying whether hydroxyl radical has been spin trapped is to utilize the ability of this methodology to distinguish dissimilarities between various radical species. For example, hydroxyl radical reacts with DMSO to produce DMSO radical ($\cdot CH_2S(O)CH_3$), while superoxide does not undergo such

[3] Abbreviations used: DMPO, 5,5-dimethyl-1-pyrroline-*N*-oxide; DMPO-OOH, 5,5-dimethyl-2-hydroperoxypyrrolidinoxyl; DMPO-OH, 5,5-dimethyl-2-hydroxylpyrrolidinoxyl; OXANOH, 2-ethyl-1-hydroxy-2,4,4-trimethyl-3-oxazolidine; OXANO, 2-ethyl-2,4,4-trimethyl-3-oxazolidinoxyl.

TABLE 1

Effect of repeated washings on superoxide dismutase activity in hamster hepatic nuclei[a]

Nuclear preparation	SOD[b] activity (inhibition of cytochrome *c* red'n)	Percentage decrease in SOD activity
	nmol/min/mg protein	
Unwashed nuclei (fresh from 2.3 M sucrose solution)	0.583 ± 0.04	—
Nuclei washed once (with 1 M sucrose solution)	0.145 ± 0.01	75
Nuclei washed twice (with 1 M sucrose solution)	0.063 ± 0.005	84
Nuclei washed thrice (with 1 M sucrose solution)	0.047 ± 0.005	92

[a] The kinetic parameters were determined using liver nuclei from four groups of hamsters. These values are presented as means ± SD.

[b] Superoxide dismutase.

an electron abstraction (29, 30). Once this secondary radical has been produced, it can be spin trapped by DMPO leading to an epr spectrum distinguishable from that of the hydroxyl radical adduct, DMPO-OH. As shown in Fig. 1, when DMSO (0.5%) is added to the mixture of DMPO, hamster hepatic nuclei, and NADPH, two distinct epr spectra are observed: the four-line DMPO-OH and, to a lesser extent, the six-line DMPO-$CH_2S(O)CH_3$. Calculations based on experiments measuring the relative rates of hydroxyl, DMSO radical, and superoxide radical trapping by DMPO[4] allow us to estimate that approximately 80% of the DMPO-OH spectrum shown in Fig. 1 comes from superoxide (via decomposition of DMPO-OOH) and the remainder may be attributed to hydroxyl radical. Upon the addition of superoxide dismutase (1 μg/ml) to the hamster hepatic nuclei–NADPH–DMPO mixture, the concentration of the DMPO-OH spin adduct is approximately 20% of control. The contribution of hydroxyl radical oxidation of the hydroxylamine, OXANOH, to the corresponding nitroxide, OXANO (Fig. 2), is minimal. We arrive at this conclusion based on the determination that the rate of spin trapping superoxide by DMPO is 10^{-3} times that for the reaction of superoxide with OXANOH.[5] However, the rate of hydroxyl radical trapping by DMPO is equivalent to that observed for OXANOH oxidation.[6] Another mitigatory factor in nuclear superoxide production and measurement, which must be considered if quantitative

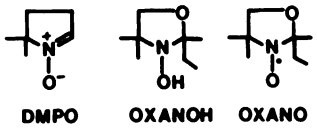


FIG. 2. *Structures of spin traps and spin label used in this investigation*

estimates of superoxide flux are to be made, is the presence of endogenous superoxide dismutase. Michelson (12) has suggested that mammalian cell nuclei contain superoxide dismutase for protection against the putative damaging effects of superoxide on the genome. As shown in Table 1, hamster hepatic nuclei contain superoxide dismutase, most of which can be removed upon washing the nuclei. However, upon the addition of sodium cyanide (2 mM) to the thrice-washed nuclei, we observe a marked increase in the rate of superoxide production. This suggests that nuclear superoxide dismutase is the copper–zinc species and that a definitive amount of this enzyme cannot be removed upon repeated washings. It is our opinion that, *in vivo*, this nuclear superoxide dismutase is in equilibrium with the cytoplasmic enzyme. It is also possible that upon cell disruption, cytoplasmic superoxide dismutase contaminates the nuclei which were maintained SOD-free by "compartmentalization" or that there is a specific "nuclear SOD." We feel that these two latter hypotheses are unlikely.

A question remains as to the source of the hydroxyl radical trapped by DMPO. Since there has never been any evidence to indicate that either the cytochrome *P*-450 system or FAD-monooxygenase can generate such a species, we feel that the origin of this free radical is the iron-catalyzed Haber-Weiss reaction (33).

$$2\,O_2^{\cdot-} + 2H^+ \rightarrow H_2O_2 + O_2$$
$$Fe^{3+} + O_2^{\cdot-} \rightarrow Fe^{2+} + O_2$$
$$H_2O_2 + Fe^{2+} \rightarrow HO^{\cdot} + HO^- + Fe^{3+}$$

$$3\,O_2^{\cdot-} + 2\,H^+ \rightarrow 2\,O_2 + HO^- + HO^{\cdot}$$

Experiments were undertaken to determine the enzyme systems responsible for the generation of nuclear superoxide. Both cytochrome *P*-450 reductase (18) and FAD-monooxygenase (22) have been shown to be present in the nuclear envelope and both are known to produce superoxide in other organelles (19–21). We examined the effect of two different pHs upon the generation of nuclear superoxide. As shown in Table 2, superoxide production at pH 8.3 is considerably greater than at pH 7.4.[7] In addition, it has been reported that FAD-monooxygenase is much more sensitive to heat than is the cytochrome *P*-450 system (31). We found that if hamster hepatic nuclei are warmed to 50°C for 8 s in the presence of BHT (to stabilize the cytochrome *P*-450 system), superoxide production is diminished by 85%, while cytochrome *P*-450 activity is decreased by only 20% (Table 3). From the data presented in Tables 2 and 3, we suggest that FAD-

[4] The rates of spin trapping superoxide and hydroxyl radical by DMPO are 10 and 3.4×10^9 M^{-1} s^{-1}, respectively. Note: Finkelstein, E., G. M. Rosen and E. J. Rauckman. Spin trapping of superoxide and hydroxyl radical: practical aspects. *Arch. Biochem. Biophys.* **200:** 1–16 (1980). The spin trapping of DMSO free radical by DMPO is approximately 10^6 M^{-1} s^{-1} (unpublished results).

[5] The rates of reaction of superoxide, generated from a xanthine–xanthine oxidase system at pH 7.8, with DMPO and OXANOH are 10 and 10^4 M^{-1} s^{-1}, respectively. Note: Finkelstein, E., G. M. Rosen and E. J. Rauckman. Spin trapping: Kinetics of the reaction of superoxide and hydroxyl radical with nitrones. *J. Amer. Chem. Soc.* **102:** 4994–4999 (1980).

[6] The rate of reaction of hydroxyl radical with either DMPO or OXANOH is diffusion limited with a rate constant of approximately 10^9 M^{-1} s^{-1}.

[7] The change in the rate of OXANO formation at pH 7.4 and 8.3 cannot be accounted for by the difference in the rate of superoxide dismutation at these pH values. This is based on the fact that the rate constant of OXANO formation is 10^4 M^{-1} s^{-1} and the concentration of OXANOH used in the reaction mixture is 5 mM.

TABLE 2

Rate of superoxide generation by hamster hepatic nuclei[a]

pH	Rate of nitroxide formation
	nmol/min/mg protein
7.4	0.48 ± 0.15
8.3	0.73 ± 0.15
8.3 + SOD[b] (0.28 μg/ml)	0.48 ± 0.10
8.3 + SOD (0.56 μg/ml)	0.24 ± 0.10

[a] The kinetic parameters were determined using liver nuclei from eight groups of hamsters. These values are presented as means ± SD.
[b] Superoxide dismutase.

TABLE 3

Effect of heat on superoxide generation[a]

	pH	Control rate	Rate after warming to 50°C for 8 s
		nmol/min/mg protein	*nmol/min/mg protein*
Superoxide flux	8.3	0.73 ± 0.09	0.10 ± 0.06
Cytochrome *P*-450 activity	7.4	0.70 ± 0.12	0.56 ± 0.12

[a] The kinetic parameters were determined using liver nuclei from six groups of hamsters. These values are presented as means ± SE. The control rates of superoxide production and cytochrome *P*-450 activity were determined using thrice-washed hamster hepatic nuclei and 0.1 mM BHT to stabilize cytochrome *P*-450. After the nuclei containing 0.1 mM BHT were heated to 50°C for 8 s and immediately cooled in an ice bath, the rates of superoxide production and cytochrome *P*-450 activity were, again, determined. Note the experimental section for additional details.

monooxygenase is responsible for most of the superoxide generated by hamster hepatic nuclei. This is not to say that cytochrome *P*-450 reductase does not play a more significant role in the generation of superoxide in species where the levels of FAD-monooxygenase are considerable less than in the hamster (e.g., rat). However, in man, where it has been shown that there is a relatively high ratio of hepatic FAD-monooxygenase to cytochrome *P*-450 (32), the generation of superoxide by this flavoprotein may be of major importance.

The quantity of superoxide produced per milligram of nuclear protein is quite low when compared to microsomal superoxide generation.[8] However, it must be pointed out that the bulk of nuclear protein is contained in chromatin and nucleoplasm. In fact, the nuclear envelope may be only 3% of the total nuclear protein (33). Since we have previously established that nuclear FAD-monooxygenase is restricted to the nuclear envelope (22), the actual production of superoxide per milligram of nuclear envelope protein is much higher than indicated in Table 2.

In conclusion, we have found that hamster hepatic nuclei produce substantial quantities of superoxide in the presence of NADPH. We have determined that these nuclei contain copper–zinc superoxide dismutase which can be almost entirely removed by extensive washing. We suggest that this nuclear superoxide dismutase is in equilibrium with cytoplasmic superoxide dismutase. Finally, we have presented evidence which suggests that the flavoprotein FAD-monooxygenase is responsible for most of the nuclear superoxide produced. The role of cytochrome *P*-450 reductase in nuclear superoxide production has not been specifically determined, but we feel that in hamster hepatic nuclear preparations this reductase is secondary to FAD-monooxygenase. Nevertheless, it would be naive to assume that cytochrome *P*-450 reductase does not contribute to the general pool of nuclear superoxide.

The role of superoxide in carcinogenesis has not been clearly defined, although many investigators have accumulated data implicating superoxide in the development of neoplasms. For example, Michelson has shown that the effects of carcinogenic hydrocarbons on mammalian cells are greatly stimulated in the presence of superoxide (12). Mutagens, such as nitrofurantoin (14), have also been shown to produce superoxide in microsomal systems. If a genetic mechanism of superoxide-mediated cancer initiation is involved, the production of superoxide at the nuclear level would clearly facilitate this action. Finally, it has recently been shown that rat hepatic nuclei undergo lipid peroxidation (34). Since superoxide and/or hydroxyl radical are the most likely initiators of such a process, this observation by Baird *et al.* (34) supports the experimental data presented herein.

[8] The rate of superoxide production in hamster liver microsomes is 4 nmol/min/mg protein, compared to 0.73 nmol/min/mg protein for the nuclei as shown in Table 2.

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