MOLECULAR PHARMACOLOGY, 22:239–242

Microsomal Reduction of Gentian Violet

Evidence for Cytochrome P-450-Catalyzed Free Radical Formation

WILLARD G. HARRELSON, JR., AND RONALD P. MASON

Laboratory of Environmental Biophysics, National Institute of Environmental Health Sciences, Research Triangle Park, North Carolina 27709

Received March 1, 1982; Accepted April 27, 1982

SUMMARY

The triarylmethane dye, gentian violet, is shown to undergo a one-electron reduction by the cytochrome P-450 monooxygenase system to produce a carbon-centered free radical as demonstrated by direct electron spin resonance techniques. The formation of this species is inhibited by carbon monoxide and metyrapone, suggesting the involvement of cytochrome P-450. Either NADPH or NADH can serve as a source of reducing equivalents for the production of this free radical. Related triarylmethane dyes are also shown to be reduced by the monooxygenase system to form free radicals.

The substituted triarylmethane dyes are brightly colored compounds used as biological stains, as fabric and food dyes, and in cosmetics (Fig. 1). Ingested benzyl violet 4B causes squamous cell carcinomas and mammary carcinomas in rats (1). The best-known of these dyes is gentian violet (crystal violet), which is used extensively in human and veterinary medicine. It is given internally to children and adults for the treatment of pinworm and fungus infections and is applied topically to the umbilical stump of the newborn (2). Gentian violet appears to be mutagenic, but some of the results are conflicting (3). This dye induces chromosome damage in Chinese hamster cells, but not *in vivo* (4). Gentian violet and a number of related triarylmethane dyes bind to adjacent adenine-thymine pairs in DNA (5). These observations raise questions about the potential carcinogenicity of these compounds (6). Despite the fact that we have frequent contact with triarylmethane dyes, little is known about their metabolism or about the mechanism of their toxicity or carcinogenicity.

Free radicals produced metabolically are important in the toxicity of a number of xenobiotic compounds. Either the free radical metabolites of xenobiotics themselves, or superoxide, which can form as a consequence of the free radical metabolite formation, can initiate processes which lead to tissue damage (7). The enzymatic reduction of gentian violet and the other triarylmethane dyes to a free radical metabolite must be considered, because gentian violet can be reduced (either photochemically or electrochemically) to the tri-(*p*-dimethylaminophenyl) methyl free radical (8).

The report that the 9000 × *g* supernatant fraction from liver homogenate can convert gentian violet to a colorless compound (4) and the knowledge that the leuko form of the dye is the two-electron reduction product of gentian violet led to a search for its one-electron reduction product, the tri-(*p*-dimethylaminophenyl)methyl free radical.

Other compounds, such as carbon tetrachloride, are reduced by hepatic microsomal incubations forming free radical intermediates (7, 9, 10). In this communication, we present evidence that the cytochrome P-450-dependent monooxygenase system present in the microsomal fraction from rat liver can enzymatically reduce gentian violet and a number of related compounds to triarylmethyl free radicals.

Crystal violet (gentian violet, C.I. 42555) was 97% pure as obtained from Aldrich Chemical Company (Milwaukee, Wisc.). Hepatic microsomes were prepared from untreated Sprague-Dawley rats as described (11) and kept on ice until use. ESR spectra were recorded with a Varian E-109 spectrometer equipped with an *E*-238 TM_{110} cavity. ESR measurements were made with a modified Gilford rapid sampler, so that different incubations could be introduced without moving the flat cell. The *g*-values of the triarylmethyl free radical metabolites were determined relative to the *g*-value (2.005590) of Fremy's salt in aqueous sodium carbonate (12). A capillary tube containing this secondary *g*-value standard was attached to an aqueous flat cell containing the microsomal incubation, and the unknown *g*-value was calculated as described (13).

When gentian violet was metabolized under a nitrogen atmosphere by rat hepatic microsomes supplemented with NADPH, a single-line ESR spectrum was obtained (Fig. 2A). Under the conditions employed, the amplitude of the ESR spectrum increased for approximately 30 min after the addition of NADPH; a slow decrease in the free radical concentration was then observed.

The *g*-value of the gentian violet free radical metabolite was 2.0028 with an estimated experimental error of

0026-895X/82/050239-04$02.00/0

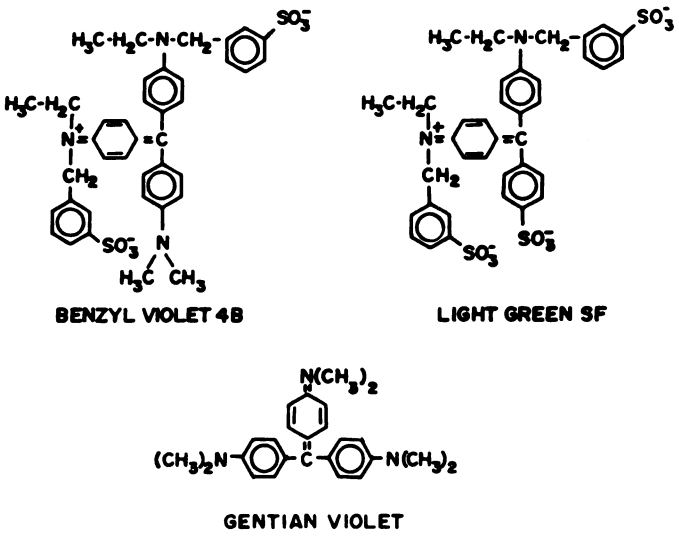

FIG. 1. *Structures of gentian violet and the related triarylmethane dyes, benzyl violet 4B, and light green SF yellowish*

±0.0002 (Table 1). This *g*-value is in close agreement with the reported value of the tri-(*p*-dimethylaminophenyl)methyl radical, $g = 2.00277$ (8). The free radical's broad linewidth of 10 G is primarily the result of inhomogeneous broadening due to the unresolved hyperfine structure of the numerous nuclei (8). The linewidth may be further broadened by the radical's immobilization in the microsomal membrane. When generated electrochemically, this free radical will precipitate from aqueous solution to give a strongly exchange-narrowed spectrum with a linewidth of only 1 G (8), but we have observed only the broader spectrum characteristic of the disaggregated free radical.

Elimination of the NADPH-generating system, microsomes, or gentian violet from the incubation resulted in no ESR spectrum (Fig. 2). Incubations containing heat-denatured microsomes (70° for 30 min) also failed to give an ESR spectrum (Fig. 2D). The free radical could not be detected in the presence of air. NADH can replace NADPH in these incubations, but the activity is about 50% lower. Care was taken to keep the incubations in the dark, because triphenylmethane dyes can be photochemically reduced in the presence of a mild reducing agent such as the ascorbate or oxalate anion (8). In fact, we observed that NADPH will support the photochemical reduction of gentian violet to the tri-(*p*-dimethylaminophenyl)methyl free radical (data not shown).

The effects of metyrapone and carbon monoxide were investigated to determine the role of cytochrome P-450 in the reduction of gentian violet. The inhibition of the free radical formation by a 100% CO atmosphere (Fig. 2B) or 5 mM metyrapone (Fig. 2C) was about 50%. Although cytochrome P-450 is apparently responsible for at least one-half of the reduction of gentian violet to its carbon-centered radical metabolite, other NADPH-dependent enzymes, such as NADPH-cytochrome P-450 reductase, may also reduce gentian violet.

In addition to gentian violet, both light green SF yellowish and benzyl violet 4B can be reduced to triarylmethyl free radicals by microsomal incubations. Deter-

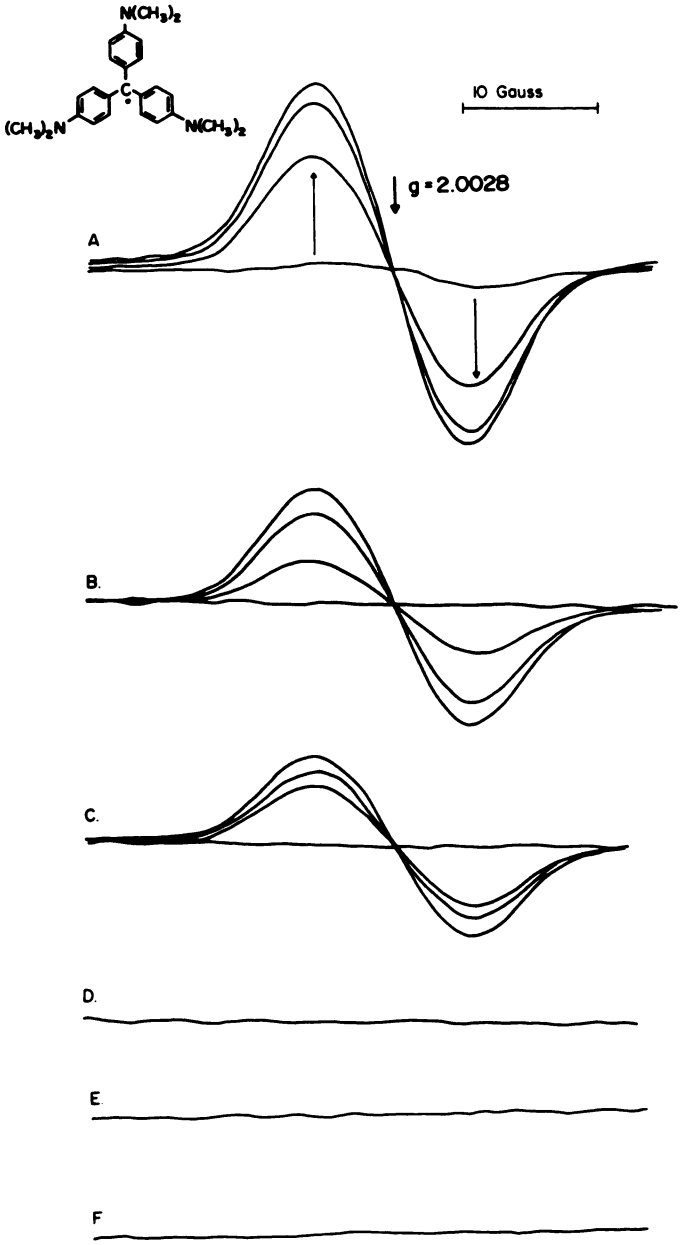

FIG. 2. *ESR spectra obtained from microsomal incubations*

A, ESR spectrum obtained from a microsomal incubation containing 0.5 mM gentian violet; an NADPH-generating system consisting of 1 mM NADPH, 5.5 mM glucose-6-phosphate, and glucose-6-phosphate dehydrogenase (1.0 unit/ml); and rat hepatic microsomal protein (2 mg/ml) prepared as described (7), in 100 mM potassium phosphate buffer (pH 7.7). The oxygen was displaced by purging with nitrogen, and the reaction was initiated with NADPH. The ESR spectra were recorded with a Varian E-109 spectrophotometer equipped with an *E*-238 TM_{110} cavity. The nominal microwave power was 20 mW, and the modulation amplitude was 4.0 G. The scan time was 1 min. Scans were started at four time points (0, 10, 20, and 30 min after addition of NADPH). B, Identical with A, except that the incubation mixture was purged with carbon monoxide before addition of NADPH. C, Same as A, but containing 5 mM metyrapone. D, Same as A, but after 30 min with heat-denatured microsomal protein. E, Identical with A, but after 30 min with the NADPH-generating system omitted. F, Identical with A, but after 30 min with an oxygen atmosphere. Kinetic measurements were made by using a modified Gilford rapid sampler, so that the signal amplitudes of different incubation mixtures could be monitored without moving the flat cell.

TABLE 1

g-Values and linewidths of the triarylmethyl free radicals in microsomal incubations

The *g*-values of the triarylmethane dye free radical metabolites were determined relative to the *g*-value of Fremy's salt in aqueous sodium carbonate ($g = 2.005590$).

Free radical	*g*-Value	Linewidth (G)
Gentian violet	2.0028	10.0
Light green SF yellowish	2.0028	5.5
Benzyl violet 4B	2.0028	5.1

minations of *g*-values and linewidth measurements have been made for these other dye radicals and are shown in Table 1. Gentian violet (500 μM) gave the greatest signal intensity. This result may reflect either gentian violet's greater hydrophobicity, which might result in increased accessibility to cytochrome P-450, or a more positive reduction potential for gentian violet.

The formation of a free radical metabolite of the related dye erythrosine B by the action of horseradish peroxidase and H_2O_2 has been proposed (14). Such a species must be a one-electron oxidation product of erythrosine B, possibly a *p*-substituted phenoxy radical and, as such, is clearly a species distinct from the carbon-centered radicals of triarylmethane dyes, which are the products of one-electron reduction. Apparently, this free radical forms (at least in part) by a one-electron transfer from reduced cytochrome P-450 (Fig. 3), as indicated by its CO and metyrapone sensitivity.

No free radical is detected in the presence of air. At least two explanations are possible for this phenomenon. First, the gentian violet radical metabolite may be produced under aerobic conditions, but subsequently react with oxygen. Either the formation of a triarylmethyl peroxy radical (17), which is presumably too reactive to detect, or the air oxidation of the tri-(*p*-dimethylaminophenyl)methyl radical (to form superoxide and regenerate gentian violet) could account for the absence of an ESR spectrum under aerobic conditions. Second, since rat hepatic microsomes, in the presence of NADPH, will reduce molecular oxygen to superoxide (18–20), it is conceivable that gentian violet and oxygen are reduced at the same site(s). If oxygen is the better electron acceptor, it could prevent the reduction of gentian violet by competitive inhibition.

The biological consequences of this free radical metabolism are unknown, but the known chemistry of the triphenylmethyl free radical suggests that the reactions of the triarylmethyl radical metabolites may be of toxicological significance. Like most carbon-centered free radicals, the triphenylmethyl radical reacts with oxygen to form a very reactive peroxyl free radical (17), which should initiate lipid peroxidation. In addition, the triphenylmethyl free radical can add across conjugated double bonds (21).

The genetic toxicity of gentian violet in the Rosenkranz assay is reported to be decreased in the presence of 9000 × *g* supernatant from liver homogenate (4). The toxicity of gentian violet to *Salmonella typhimurium* is also decreased by liver supernatant, but this effect occurs with thermally inactivated supernatant (22). It is of interest that light enhances the genotoxicity of gentian violet (22), because, in the presence of NADPH, room light photoreduces gentian violet to the same triarylmethyl free radical that is formed by enzymatic reduction. Since the radical can be detected only in the absence of oxygen, anaerobiosis may increase the mutagenicity of the triarylmethane dyes as it does nitro drugs and carcinogens (23).

REFERENCES

1. Iheda, Y., S. Horiuchi, A. Imoto, Y. Kodama, Y. Aida, and K. Kobayashi. Induction of mammary gland and skin tumors in female rats by the feeding of benzyl violet 4B. *Toxicology* **2**:275–284 (1974).
2. Bale, M. S. Management of the umbilicus with crystal violet solutions. *Can. Med. Assoc. J.* **124**:372–373 (1981).
3. Combes, R. D., and R. B. Haveland-Smith. A review of the genotoxicity of food, drug and cosmetic colours and other azo, triphenylmethane and xanthene dyes. *Mutation Res.* **98**:101–248 (1982).
4. Au, W., M. A. Butler, S. E. Bloom, and T. S. Matney. Further study of the genetic toxicity of gentian violet. *Mutation Res.* **66**:103–112 (1979).
5. Muller, W., and F. Gautier. Interactions of heteroaromatic compounds with nucleic acids. A:T-specific non-intercalating DNA ligands. *Eur. J. Biochem.* **54**:385–394 (1975).
6. Arrhenius, E., *et al.* Evaluation of the carcinogenic risk of chemicals to man. *Int. Agency Res. Cancer Monogr.* **16**:153–220 (1978).

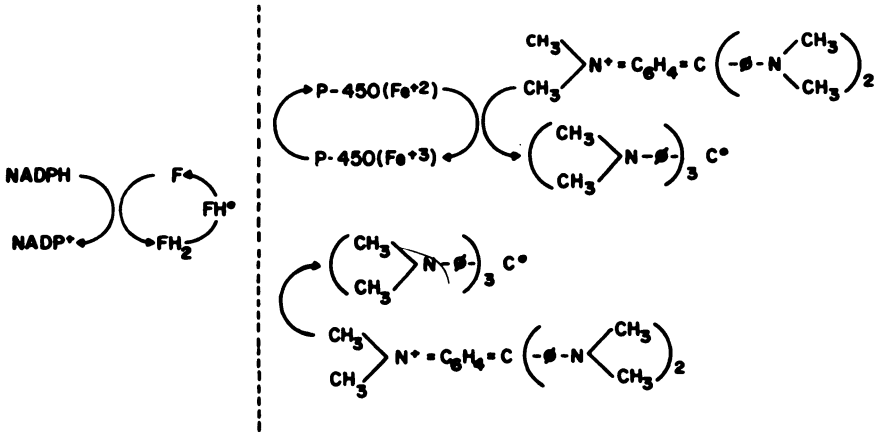

FIG. 3. *Proposed pathway of the cytochrome P-450-catalyzed reduction of gentian violet*

FH, FH•, and F represent the NADPH-cytochrome P-450 reductase flavin oxidation states of the FMN and FAD prosthetic groups. The mechanism of electron donation to cytochrome P-450 is an area of active research (15, 16). Competing reduction of gentian violet by NADPH-cytochrome P-450 reductase, or other cytochrome P-450-independent routes, has not been excluded.

7. Mason, R. P. Free-radical intermediates in the metabolism of toxic chemicals, in *Free Radicals in Biology* (W. A. Pryor, ed.), Vol. V. Academic Press, New York, 161–222 (1982).
8. Leaver, I. H. Photoreduction of crystal violet in solution and in poly(vinyl-alcohol) films. *Photochem. Photobiol.* **16**:189–196 (1972).
9. Mason, R. P., and C. F. Chignell. Free radicals in pharmacology and toxicology-selected topics. *Pharmacol. Rev.* **33**:189–211 (1981).
10. Wolf, C. R., W. G. Harrelson, W. M. Nastainczyk, R. M. Philpot, B. Kalyanaraman, and R. P. Mason. Metabolism of carbon tetrachloride in hepatic microsomes and reconstituted monooxygenase systems and its relationship to lipid peroxidation. *Mol. Pharmacol.* **18**:553–558 (1980).
11. Lu, A. H. Y., R. Kuntzman, S. West, M. Jacobson, and A. H. Conney. Reconstituted liver microsomal enzyme system that hydroxylates drugs, other foreign compounds, and endogenous substrates. II. Role of the cytochrome P-450 and P-448 fractions in drug and steroid hydroxylations. *J. Biol. Chem.* **247**:1727–1734 (1972).
12. Yonezawa, T., T. Kawamura, M. Ushio, and Y. Nakao. Solvent effects on the *g*-factor of semiquinones. *Bull. Chem. Soc. Japan* **43**:1022–1027 (1970).
13. Mason, R. P., F. J. Peterson, and J. L. Holtzman. The formation of an azo anion free radical metabolite during the microsomal azo reduction of sulfonazo III. *Biochem. Biophys. Res. Commun.* **75**:532–540 (1977).
14. Floyd, R. A. Erythrosin B (red dye no. 3) mediated oxidation-reduction in brain membranes. *Biochem. Biophys. Res. Commun.* **96**:1305–1311 (1980).
15. Iyanagi, T., and R. Makino. Reaction mechanism of hepatic NADPH-cytochrome P-450 reductase, in *Microsomes, Drug Oxidations and Chemical Carcinogenesis* (M. J. Coon, A. H. Conney, R. W. Estabrook, H. V. Gelboin, J. R. Gillette, and P. J. O'Brien, eds.), Vol. I. Academic Press, New York, 407–410 (1980).
16. Vermilion, J. L., D. P. Ballau, V. Massey, and M. J. Coon. Separate roles for FMN and FAD in catalysis by liver microsomal NADPH-cytochrome P-450 reductase. *J. Biol. Chem.* **256**:266–277 (1981).
17. Melamud, E., and B. L. Silver. A rotating peroxy group: the ESR spectrum of ^{17}O-enriched triphenylmethylperoxyl. *J. Magn. Resonance* **14**:112–116 (1974).
18. Aust, S. D., D. L. Roerig, and T. C. Pederson. Evidence for superoxide generation by NADPH-cytochrome *c* reductase of rat liver microsomes. *Biochem. Biophys. Res. Commun.* **47**:1133–1137 (1972).
19. Mishin, V., A. Pokrovsky, and V. V. Lyakhovich. Interactions of some acceptors with superoxide anion radicals formed by the NADPH-specific flavoprotein in rat liver microsomal fractions. *Biochem. J.* **154**:307–310 (1976).
20. Ullrich, V., and H. Kuthan. Autoxidation and uncoupling in microsomal monooxygenations, in *Biochemistry, Biophysics and Regulation of Cytochrome P-450.* (J. Å. Gustafsson, J. Carlstedt-Duke, A. Mode, and J. Rafter, eds.). Elsevier/North-Holland Biomedical Press, New York, 267–272 (1980).
21. Forrester, A. R., J. M. Hay, and R. H. Thompson. *Organic Chemistry of Stable Free Radicals.* Academic Press, New York, 71–72 (1968).
22. Levin, D. E., T. J. Lovely, and E. Klekowski. Light-enhanced genetic toxicity of crystal violet. *Mutation Res.* **103**:283–288 (1982)..
23. McCalla, D. R. Metabolic activation of nitroheterocyclic compounds in bacteria and mammalian cells, in *Short-Term Tests for Chemical Carcinogens* (H. F. Stich and R. H. C. San, eds.). Springer-Verlag, New York, 37–47 (1981).

Send reprint requests to: Dr. Ronald P. Mason, Laboratory of Environmental Biophysics, National Institute of Environmental Health Sciences, P.O. Box 12233, Research Triangle Park, N. C. 27709.