JRITY CLASSIFICATION OF THIS PAGE

REPORT DOCUMENTAT

AD-A238 790

Form Approved
OMB No. 0704-0188

REPORT SECURITY CLASSIFICATION

SECURITY CLASSIFICATION AUTHORITY

DECLASSIFICATION / DOWNGRADING SCHEDULE

Approved for public release;
distribution unlimited.

ERFORMING ORGANIZATION REPORT NUMBER(S)

5. MONITORING ORGANIZATION REPORT NUMBER(S)

AFOSR-TR- 91 0632

NAME OF PERFORMING ORGANIZATION	6b. OFFICE SYMBOL (If applicable)	7a. NAME OF MONITORING ORGANIZATION
George Washington Univ.		AFOSR/NL

ADDRESS (City, State, and ZIP Code)	7b. ADDRESS (City, State, and ZIP Code)
Office of Sponsored Research 2121 "I" Street, NW Washington DC 20052	Building 410 Bolling AFB Washington DC 20332-6448

NAME OF FUNDING / SPONSORING ORGANIZATION	8b. OFFICE SYMBOL (If applicable)	9. PROCUREMENT INSTRUMENT IDENTIFICATION NUMBER
AFOSR	NL	AFOSR-88-0016

ADDRESS (City, State, and ZIP Code)	10. SOURCE OF FUNDING NUMBERS			
	PROGRAM ELEMENT NO.	PROJECT NO.	TASK NO	WORK UNIT ACCESSION NO
Building 410 Bolling AFB DC 20332-6448	61102F	2312	A5	

TITLE (Include Security Classification)

Free Radical Mechanisms of Xenobiotic Mammalian Cytotoxicities

PERSONAL AUTHOR(S)

Dickens, Benjamin Fisher

TYPE OF REPORT	13b. TIME COVERED	14. DATE OF REPORT (Year, Month, Day)	15. PAGE COUNT
FINAL (YR 3.5)	FROM 11/1/87 to 4/30/91	June 30, 1991	1 appendix

SUPPLEMENTARY NOTATION

COSATI CODES		
FIELD	GROUP	SUB-GROUP

18. SUBJECT TERMS (Continue on reverse if necessary and identify by block number)

Free Radical, IRP volume I and II chemicals, cytotoxicity membrane lipid peroxidation, spin trapping, ESR, cell culture, endothelial cell, smooth muscle cell

ABSTRACT (Continue on reverse if necessary and identify by block number)

ur initial goal was to identify if free radical mechanisms are involved in the cytotoxicity f a number of IRP volume I and II chemicals. We found that a number of these agents act to nhance membrane lipid peroxidation in response to a standard dose of exogenous free radicals sing chlorinated hydrocarbons (carbon tetrachloride, trichloroethylene, dichloroethylene, richloroethane, dichloroethane) as a model for other IRP chemicals, we established conditior o measure lipid peroxidation in cultured smooth muscle and endothelial cells. These agents nduced lipid peroxidation in the presences of physiological levels of iron in these vascular ells by a mechanism that doesn't require cytochrome P-450. Antiradical treatement with eferoxamine and Probucol (but not SOD, catalase, or mannitol) appear to reduce the toxicity f these agents. We have also detected the presences of free radicals in the cultured cells y ESR spin trapping following exposure to iron and chlorinated hydrocarbons. Although this ree radical production does not appear to require biotransformation by cytochrome P-450, it s also not a result of spontanous oxidation of the IRP chemicals. Instead, it appears that

DISTRIBUTION / AVAILABILITY OF ABSTRACT	21 ABSTRACT SECURITY CLASSIFICATION
☒ UNCLASSIFIED/UNLIMITED ☐ SAME AS RPT ☐ DTIC USERS	

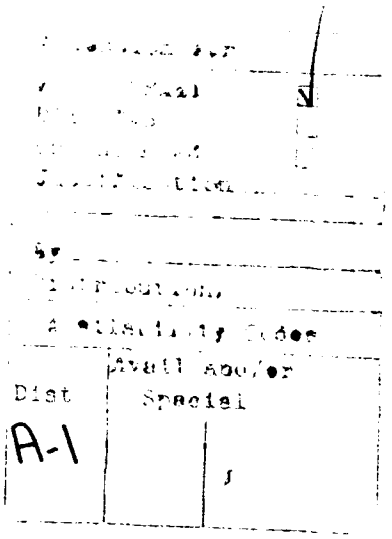
22a. NAME OF RESPONSIBLE INDIVIDUAL	22b. TELEPHONE (Include Area Code)	22c. OFFICE SYMBOL
T. JAN CERVENY, Lt Col, USAF	(202) 767-5021	NL

Form 1473, JUN 86 Previous editions are obsolete

SECURITY CLASSIFICATION OF THIS PAGE

8 JUL 1991

FINAL REPORT FOR AFOSR-88-0016: "FREE RADICAL MECHANISMS OF XENOBIOTIC MAMMALIAN CYTOTOXICITIES"



B. F. Dickens - P.I.
W. B. Weglicki - Co-P.I.

Department of Medicine
Division of Experimental Medicine
The George Washington University Medical Center
Washington, D.C. 20037

Project Duration
November 1, 1987 to April 30, 1991

Submitted, June 30, 1991

Project Objectives and Summary of Results:

It is well known that a frequent mechanism of xenobiotic liver toxicity is biotransformation by cytochrome P_{450}-enzymes to toxic free radical intermediates. The primary objective of this proposal was to determine if free radical production played a role in the vascular toxicity of IRP volume I and II chemicals, and if so, determine the mechanism of the free radical production.

We have established that, at least for a large group of non-aromatic, chlorinated chemicals (including carbon tetrachloride, trichloroethane, trichloroethylene, etc), free radical-induced lipid peroxidation is associated with exposure of cultured vascular endothelial or smooth muscle cell to these agents. Initially, our assumption was that if IRP chemical-associated injury had a free radical component that the mechanism of free radical production would be through a cytochrome-P_{450} associated biotransformation. Our results, however, demonstrate that another mechanism is involved, and that the presence of lipid hydroperoxides (for instance following induction by added iron) is a necessary intermediate in this free radical associated injury. While antioxidants can reduce the extent of cellular lipid peroxidation, the question as to how much of the vascular toxicity of IRP chemicals is directly related to free radical mechanisms remains to be answered.

Major Findings:

A. Three lines of evidence initially suggested that free radical-induced lipid peroxidation may play a role in membrane injury following exposure to selected IRP chemicals:

(1) Isolated Membranes. Many of our initial experiments were performed using isolated biological membranes to rapidly screen the dozens of IRP for free radical-associated injury in a simple system. In these studies, chlorinated and aromatic hydrocarbons enhanced lipid peroxidation induced by an exogenous free radical generating system, while chlorinated aromatic hydrocarbons inhibited it.

(2) Living Cells. Our initial approach with cultured vascular cells was to incubate the cells with both the spin trapping agent methyl nitroso propane (MNP) and IRP chemicals to determine if free radical production occurred. This procedure was hampered by significant problems with cell viability in these studies: the combination of spin trapping agent and IRP chemical was uniformly lethal to the cells. In these studies, we found that carbon tetrachloride, but not other chlorinated halocarbons, gave a reasonably strong MNP-adduct signal. Figure 1 gives the MNP adduct signal for carbon tetrachloride and trichloroethylene, as well as that for paraquat, a xenobiotic with a well known mechanism for the production of free radicals. These studies, however, were severely hampered by the problem with cellular viability, which we eventually overcame using a novel ***ex-vitro*** method (see below)

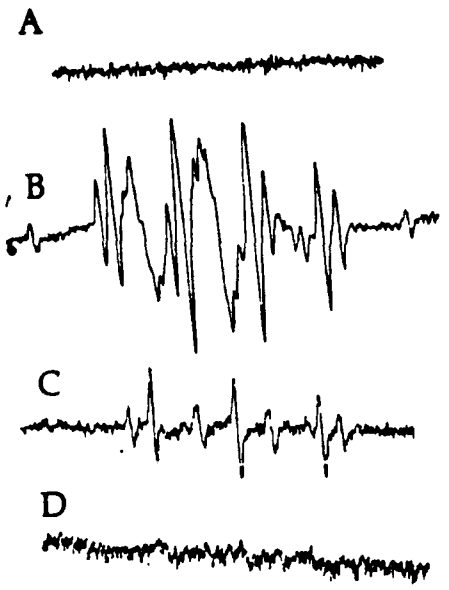


Figure 1. MNP spin adducts from cultured endothelial cells directly exposed to IRP chemicals. A control, B paraquat, C. CCL_4 and D is TCE (Weak Signal).

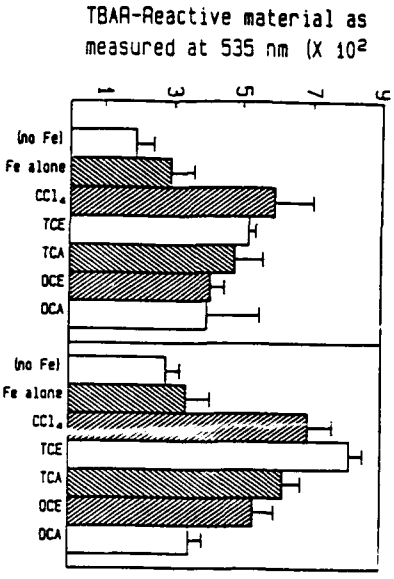


Figure 2. Bar graph showing the lipid peroxidation induced by a variety of halocarbons in cultured cells. Top panel smooth muscle cells, lower panel endothelial cells

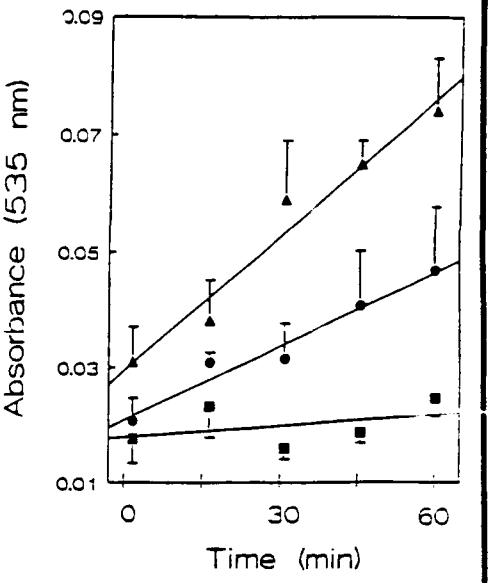


Figure 3. Promoting effect of iron on the lipid peroxidation induced by CCL_4 in cultured cells Top CCl + Fe, Middle CCl_4 alone, bottom, Fe alone

(3) Cultured cells incubated in buffers with trace levels of chelated iron and selected IRP chemicals produced elevated levels of products of lipid peroxidation, indicating enhanced free radical injury (figure 2). This finding was confirmed by the ESR spin trapping of lipid alkoxyl radicals (see C, below) and the discovery of phospholipid hydroperoxides in lipid extracts of cells exposed to selected IRP chemicals (see section G, below)

B. Evidence that cytochrome-P_{450} enzymes are not a major contributing factor in the free radical injury in vascular cells.

We initially assumed that the free radical injury process was mediated through biotransformation of the halocarbons to a free radical intermediate, similar to what happens in the liver. However, several lines of evidence began to suggest that cytochrome-P_{450} enzymes do not play a major role in the free radical injury to vascular endothelial or smooth muscle cells:

- inhibitors of cytochrome-P_{450} enzymes failed to prevent the observed IRP-associated lipid peroxidation in cultured cells.
- in isolated membrane systems where peroxidation induced by these agents could still be observed, we found that trace amounts of iron had to be added (at iron levels which, by themselves, failed to produce measurable lipid peroxidation)
- likewise, acute exposure of cultured endothelial and smooth muscle cells to chlorinated hydrocarbons in the absences of transition metals failed to initiate lipid peroxidation (see figure 3).

- The addition of iron increase the toxicity of halogenated hydrocarbons, while probucol (an lipid-lowering drug with anti-oxidant properties, α-tocopherol, and deferrioxamine (an iron chelator) tend to reduce it.

C. Ex-Vitro Spin Trapping Studies using a Variety of IRP Chemicals

The addition of both spin trapping agents (which are toxic nitrone compounds) and IRP chemicals to cultured cells proved to be too toxic for the cells. We developed an ***ex-vitro*** spin trapping protocol to investigate the production of free radicals following exposure to IRP chemicals without the combined spin trap - IRP chemical toxicity. In this procedure, cultured cells were harvested and then exposed to the IRP chemical plus a trace level of iron chelated to ADP. Following the exposure, the cell suspension was passed though a filter, and the cell-free filtrate collected in a test tube containing the spin trapping agent α-phenyl N-*tert*-butyl nitrone (PBN). These experiments resulted in the detection of spin adduct in cells exposed to a host of chlorinated aliphatic hydrocarbons. The adduct signal, when extracted into toluene, had splitting constants (α_N =2.0 , α_H =13.5) consistent with an alkoxyl adduct of PBN (PBN-OR). We tentatively identified these radicals as being derived from lipid hydroperoxides, a fact confirmed in later studies (see section H, below). The finding that an alkoxyl radical was produced, confirmed our hypothesis that these agents could promote lipid peroxidation in vascular cells.

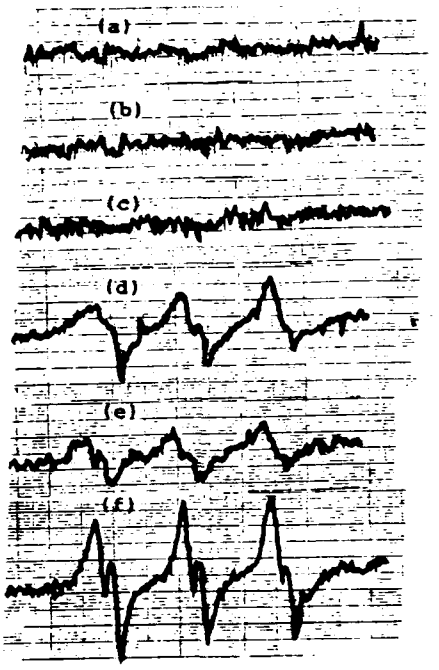


Figure 4. Spin trapped Alkoxyl Radicals from cells using the ex-vitro spin trapping method

D. Spin Trapping in Organic Solvents

While iron, or other transition metals, are well-known requirement for many redox reactions, the need to add exogenous iron to isolated membranes or cultured cells to detect free radical production or lipid peroxidation was still unexpected. One possibility was that the added iron was directly interacting with the IRP chemicals to produce a free radical component. However, spin trapping studies with PBN + iron + halocarbon failed to produce spin adducts. This suggested that the added iron did not directly "transform" the halocarbon into a free radical intermediate, but iron was playing a in the IRP-asscociated lipid peroxidation in isolated membranes and cultured cells (section B, above).

The production of an alkoxyl adduct in the extracellular filtrate of cells exposed to IRP chemicals and our assumption that the requirement for added iron was to initiate lipid peroxidation which was then enhanced by the halocarbons led us to one of two conclusions concerning the origin of these alkoxyl radicals. Either the halocarbons were being transformed through interaction with the lipid hydroperoxide into a lipid peroxidation-promoting halocarbon radical, or these lipophillic agents can act as a non-radical promoter of the "auto-catalyzed" chained of lipid peroxidation.

We tested these two hypotheses using ESR techniques in a mixture of IRP-solvents, PBN, and cumene hydroperoxide (the cumene hydroperoxide was added as to model a lipid hydroperoxide). Incubation of cumene hydroperoxide with PBN in solvents without the addition of cumene hydroperoxide failed to provide an ESR signal. When cumene hydroperoxide was added to the mixture (see table 1), spin adducts were produced with many of the chlorinated halocarbons. As can be seen in this table, agents that were good promoters of lipid peroxidation in our systems, were either highly reactive or reactive with cumene hydroperoxide. While, with the notable exceptions of chlorinated benzene's and n-butyl phthalate, those which failed to give lipid peroxidation in our systems also failed to give rise to PBN adducts when mixed with cumene hydroperoxides *in vitro*. This similarities in response suggest a common mechanism between enhanced lipid peroxidation in cellular models and free radical production in this purely chemical system.

TABLE 1: PBN ESR SPIN ADDUCT PRODUCED
IN THE PRESENCES OF CUMENE HYDROPEROXIDE*

STRONGLY REACTIVE	REACTIVE	NOT REACTIVE
[illegible]	[illegible]	Acetone
[illegible]	[illegible]	Hexane
[illegible]	methylene chloride	ethylene glycol
[illegible]		Benzene
1,3-dichloropropane	m-dichlorobenzene	o-chlorophenol
Dichloroethane	Chlorobenzene	Xylene
Chloroform	n-butyl phthalate	di-methylphenol
		Toluene
		monoethyl ether

**Gray shaded chemical are good promoters of lipid peroxidation in cultured cells and/or the isolated membrane system. The underlined chemicals are weak promoters of lipid peroxidation in these system: we have been unable to promote lipid peroxidation in either system with those agents with no underline or shading.*

E. Since the hyperfine splitting constants varied greatly with the IRP chemical used, our initial assumption was that the cumene hydroperoxide was reacting with the various organic solvent to produce a variety of halocarbon adducts. Two types of control experiments to confirm this assumption, however, led to a different conclusion:

- when highly purified ^{13}C-solvents were used to document that the signals were coming from the IRP chemicals, we obtained the same ESR-spectra observed without the use of the heavy isotope (figure 5), and
- when the samples were dried under N_2 and resuspended in toluene, the PBN-adducts from each IRP sample appeared identical to that of an alkoxyl adduct.

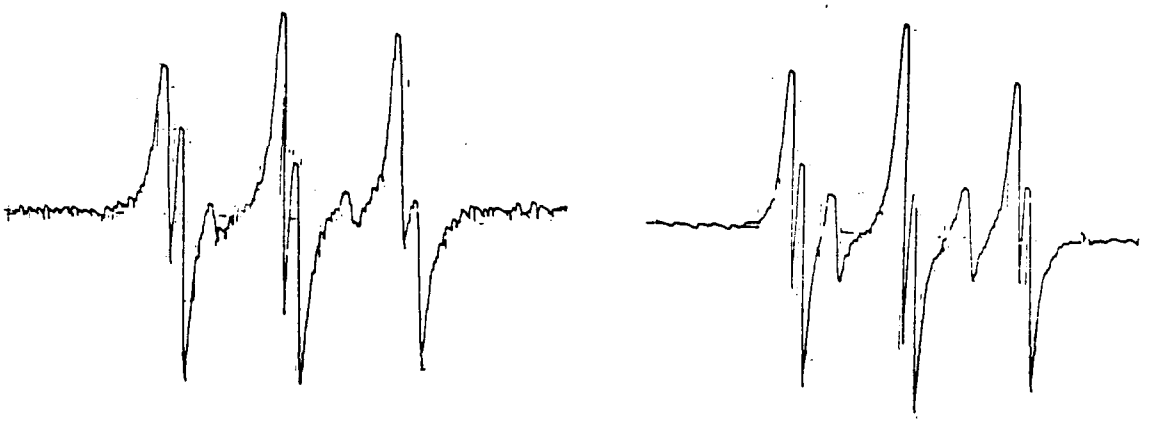


Figure 5. PBN Adduct signals trapped in the presences of cumene hydroperoxide plus $CHCL_3$ left panel, or $13CHCl_3$ (right panel). The adducts have identical hyperfine splitting indicating that they must arise from the cumene hydroperoxide and not the chloroform.

If the ESR adduct signal was arising from the individual halogenated compounds, then using ^{13}C-compounds should have provided increase splitting of the adduct signal, and the spectral differences between the hyperfine splitting constants should have remained even after toluene extraction. These findings demonstrated, for the first time, that halogenated compounds are capable of interacting with hydroperoxides (or with trace amounts LOO· contaminating the hydroperoxide sample) to give enhanced alkoxyl radical (or LOO·) production. This finding has additional applications beyond studies of IRP chemical toxicities. For instance, since chloroform was capable of converting hydroperoxides to free radical intermediates, experimental design involving EPR spin trapping studies of naturally-occuring lipids should avoid lipid extracts using the normal chloroform-methanol procedures. Extracting cellular lipid into chloroform can (apparently) result in greatly elevated levels of "free radicals" (lipid hydroperoxides can be "detoxified" by one of two different glutathione systems without going through free radical intermediates). These problems can be overcome using toluene or other solvents which do not assist in conversion of hydroperoxides to alkoxyl radicals.

F. HPLC Separation of Phospholipids from IRP-treated Cells: Detection of a Novel Lipid Peak

In an effort to determine the effect of acute or long-term culture in the presences of chlorinated halocarbons, we have investigated the effect of such exposure on the phospholipid composition of cultured cells. While no consistent change between different tested agents could be detected in the usual phospholipid composition, the phospholipid HPLC profiles showed the occurrence of a new lipid peak which migrated between phosphatidylethanolamine (PE) and phosphatidylcholine (PC). Figure 6 shows HPLC tracing with and without the novel lipid peak (Identified by the black arrow).

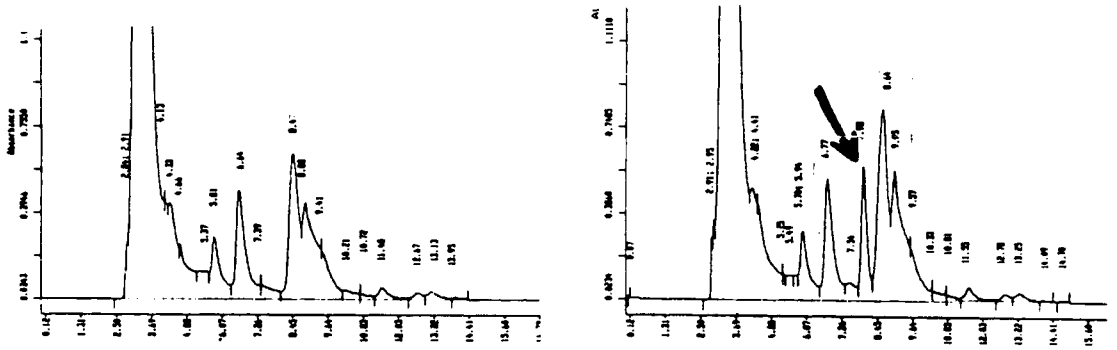


Figure 6 HPLC tracing of phospholipid extracts from smooth muscle cells with and without exposure to TCE. The novel lipid peak (black arrow) was found in about 50% of the cells exposed to , as well as in cells exposed to TCE number of the other halogenated hydrocarbons. The height of the new lipid in this tracing in about twice that normally observed.

G. Identification of the Novel Lipid Peak in IRP-treated Vascular Cells

In my last progress report, I speculated that the new lipid peak might be phosphatidylcholine hydroperoxide (PC-OOH). This working hypothesis was based upon the following results, reported earlier:

- pooled lipid extracts in which the peak was found tested positive for the presences of hydroperoxide by the colorimetric procedure of Aust,
- the unknown lipid could be removed from other lipids in the extract following acetone-precipation, and the lipid was stable in acetone until water was added, at which time an alkoxyl radical adduct was produced and the lipid quickly disappeared from subsequent HPLC tracings,
- the addition of the iron chelator deferrioxamine prevented the loss of lipid and production of alkoxyl signal upon the addition of water, and,
- that other researchers had reported finding PC-OOH in the liver of Carbon Tetrachloride-intoxicated rats.

Since the alkoxyl radical signal we detected in the ex-vitro spin trapping experiments would have to arise from a lipid hydroperoxide, we further investigated the nature of this lipid. The new data, reported below, conclusively confirms the identity of this new lipid peak as a lipid hydroperoxide, however, it has been proven not to be PC-OOH. The various evidence supporting these conclusions are summarized below.

(1) Further Proof that the lipid is a hydroperoxide:

- We modified a fluorescence assay for hydroperoxides based on the conversion of 2,7-dichlorofluorescin to the fluorescent 2,7-dichlorofluorescein. This conversion is produced by through oxidation with peroxides or hydroperoxides. In these experiments, we isolated the new lipid peak through acetone precipitation, resuspended the peak in ethanol, and performed the assay in a spectrofluorometer using 2,7-dichlorofluoresin. The addition of the ethanolic solution of the unknown lipid peak gave an extremely strong fluorescent signal. Since the acetone-lipid extract had been taken completely to dryness prior to resuspension in ethanol, this signal could not be a result of contaminating hydrogen peroxide.

- Spin Trapping Experiments: A lipid hydroperoxide will react with cobalt (II) acetylacetonate through normal Fenton chemistry to produce an alkoxyl radical. We took an aliquot of the lipid extracted in the above assay, and resuspended it in nitrogen-gassed benzene (to remove all oxygen). PBN was added to the solution and it was scanned for free radical production (none was present). Then we added cobalt (II) acetylacetonate. A strong alkoxyl adduct signal was detected. The production of an alkoxyl adduct signal in oxygen-free organic buffers through the interaction with cobalt demonstrates conclusively that a hydroperoxide must be present. It is worthwhile to note that this adduct is exactly the same one formed in our ***ex-vitro*** spin trapping studies.

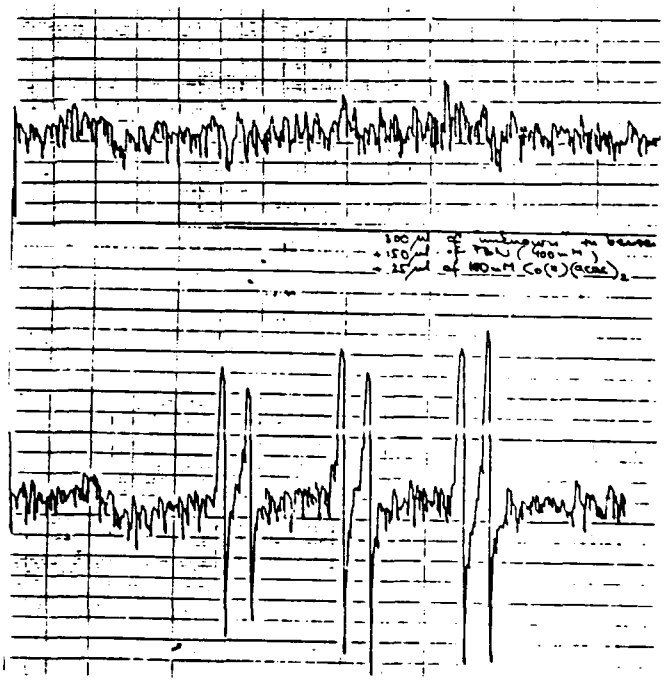


Figure 7. Alkoxyl radical adduct produced in N2-gassed benzene by "unknown" lipid. Top panel, PBN + benzene + unknown lipid, bottom panel 2 minutes after the addition of "unknown" lipid.

(2) Evidence that the new lipid is not PC-OOH.

- I synthesized authentic PC-OOH using soybean lipoxygenase and highly purified PC. Prior to incubation with soybean lipoxygenase, the HPLC tracing

showed only a single peak, identified as the substrate, phosphatidylcholine. When the substrate was incubated with soybean lipoxygenase, two new lipid peaks were produced, both of which eluted **after** the original PC peak. These late eluting peaks were identified lipid hydroperoxides by spin trapping, fluorescent, and colometric techniques. Since, only highly purified PC was available as substrate, they were identified as isomers of PC-OOH. Since the hydroperoxide we detected in our experiments with lipid extracts elutes **prior** to PC, it can not be PC-OOH.

(3) Evidence that the new lipid is PE-OOH. Circumstantial evidence suggest that the new lipid peak is phosphatidylethanolamine with a fatty acyl hydroperoxide in the two position. First, our evidence (see 1 above) clearly demonstrates that the new lipid peak contains a hydroperoxide moiety. Second, neutral lipid hydroperoxides, including prostanoids, leukotrienes, and arachidonate hydroperoxide, all elute with the void volume in our HPLC system: thus, the hydroperoxide must be attached to a polar (phospholipid) molecule. Third one isomer of PC-OOH elutes midway between PC and lyso-PC, while the second isomer elutes immediately prior or coinciding with lyso-PC. Thus, if a hydroperoxide acyl side chain effects PE in a similar manner, PE-OOH would elute between PE and lyso-PE in our chromatography: which is exactly where it elutes. Fourth, while attempts to prepare PE-OOH using soybean lipoxygenase have not been completely successful, two of six attempts resulted in a broad tailing of the PE peak such that much of the tail of the peak was in the region where our "unknown" lipid elutes.

The demonstration that incubation with IRP chemicals can result in extractable lipid hydroperoxides, provides direct support that these agents result in lipid peroxidation. Further, these lipid hydroperoxides in all likelihood, give rise to the alkoxyl adduct signal we detect in our ex-vitro spin trapping method, which at once validates the ex-vitro method, and shows the source of the radical adduct formed. The reason why PC-OOH was isolated from liver extracts of rats and PE-OOH was found in our vascular cellular model is not yet clear. However, it may have something to do with the site of production of the free radicals.

H. Use of Meridian ACAS 570 for Detection of Phospholipid hydroperoxides (and/or H_2O_2) in Cultured Vascular Cells

Our data suggest that, in general, halogenated IRP chemicals can interact with hydroperoxides within biological membranes, but the mechanism of such reactions is unclear. In a recent report, ***phosphatidylcholine hydroperoxide*** has been shown to accumulate in livers of rats intoxicated with CCl_4, and this accumulation is inhibited by α-tocopherol{3108}. In this progress report, I have shown that carbon tetrachloride and similar IRP chemicals induce the production of a different phospholipid hydroperoxide, presumably PE-OOH, in cultured vascular cells. We hypothesized that we should be able to follow the production of lipid hydroperoxides in individual living cultured cells using the oxidation of 2,7-dichlorofluorescin to the fluorescent molecule, 2.7-dichlorofluoroscein by lipid hydroperoxides. These experiments are performed using the Meridian ACAS 570 interactive laser cytometer located within the Department of Biochemistry, here at GWU.

- Initial experiments were performed to determine the sensitivity of the ACAS 570 to detecting hydroperoxides and/or peroxides. To do this, cells were prelabelled with 2,7-DCFDA for 30 minutes, then exposed to various concentrations of H_2O_2 and the extent of cellular fluorescence quantified using the video image digitization on the ACAS 570. We found that the dose response curve differed between endothelial and smooth muscle cells because of high levels of endogenous catalase in endothelial cells. Thus, we have standardized our dose response curve using smooth muscle cells and H_2O_2. This procedure can sensitively measure picomolar levels of H2O2 peroxide, at least in smooth muscle cells (or in azide-poisoned endothelial cells). In these experiments, the fluorescence is relatively uniform throughout the cytoplasm, presumably because H2O2 is equally distributed in the cell. Also, fluorescence was essentially equal in all cells.

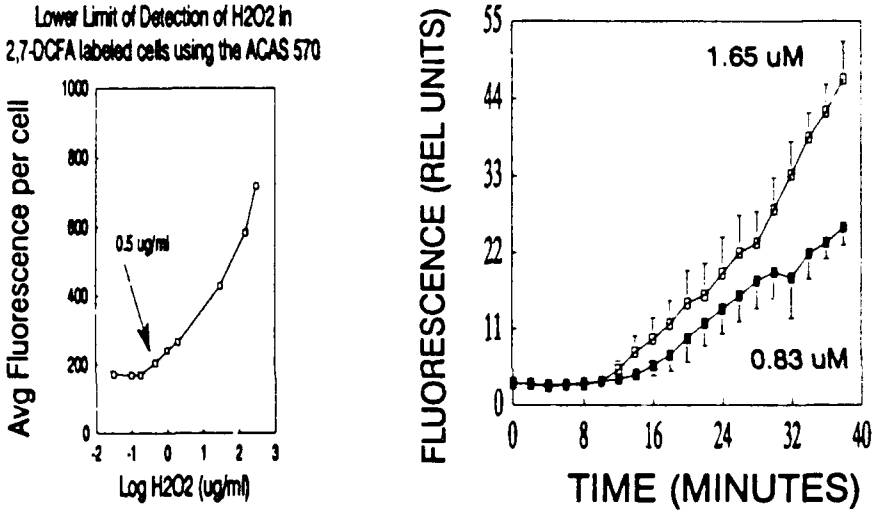


Figure 8 & 9 2,7-Dichlorofluroresecin fluorescence in cultured smooth muscle cells exposed to either H2O2 (left figure) or one of two dose of dihydroxyfumerate + Fe-ADP (right figure). The increased fluorescence in these panels are due to the oxidation of 2,7-Dichlorofluoresecin by peroxides (fig 8) or lipid hydroperoxides (figure 9).

- The fluorescent assay was then used to verify that lipid hydroperoxide production could be followed in living cells. Cultured cells became fluorescent when cumene hydroperoxide was added to cells labelled with 2,7-DCFDA. Further, when lipid peroxidation was initiated in the cells by the addition of a known free radical generating system, dihydroxyfumerate, Fe-ADP, we could follow lipid peroxidation in these cells. This procedure was characterized by a lag phase of around 8 to 12 minutes before the fluorescence increased, followed by a nearly linear increase in fluorescence (Fig). Highly significant differences were found in the rate of fluorescence increase and the maximal increase depending upon the dose of radical generating system. Also, there is a trend towards shorter lag times with higher dose of free radical generating system. We interpret the lag time as the length of time for endogenous antioxidant defenses to be overwhelmed and the onset of lipid peroxidation initiated. As was the case with direct exposure to H_2O_2, the fluorescence was evenly distributed in cytoplasm of individual cells, suggesting uniform injury to all membrane systems within the cell. However, fluorescence varied greatly from cell to cell: lag times before onset was as short as 4 minutes in some cells,

and a few cells failed to increase fluorescence at all during the time of exposure, presumably due to differences in the anti-oxidant capacities of the individual cells.

- The uniform patterns observed with H2O2 and exogenous hydroxyl radical generating systems are not observed in cells exposed to a variety of environmental stresses which are expected to promote lipid peroxidation. In cases were the stress promotes lipid peroxidation, the cells show marked areas of greater hydroperoxide (or peroxide) production. In smooth muscle cells, these area's are peri-nuclear in location; in endothelial cells they are frequently localized in a ring around the center of the cell. The reason for these localized area of greater fluorescence is unknown at the present time. The environmental stress that has proven to be the most reliable in the onset of hypoxia. During the first 20 minutes or so of hypoxia, the fluorescence in 2,7-DCFDA labelled cells increase. During reoxygenation, there is frequently a burst of fluorescence, but often the background fluorescences increases tremendously as the cells become "leaky." Experiments with IRP chemicals have meet with mixed results. As was the case for reoxygenated hypoxic cells, the addition of IRP chemical tends to result in elevated background fluorescence. Additionally, if the cells are left on the stage too long, they tend to become hypoxia or nutrient starved and the cellular fluorescence increases without the addition of IRP chemicals and in these cases many of the chlorinated hydrocarbons actually decrease the fluorescence. A final problem is that the level of ethanol we have been using as a vehicle to deliver the hydrophobic IRP chemicals promotes promote an increase in fluorescence (lipid peroxidation) without the need for the IRP chemicals. Experiments are planned to pursue this potentially exciting finding. Planned experimental design modifications to try and improve the reproducibility of this technique includes altering the method of IRP chemical delivery, performing these experiments in cells preloaded with iron. Two recent reports suggest that by 4 to 5 passages, mammalian vascular cells loss much of their releasable iron, and that they become much more resistant to a whole host of oxidant stress. Pre-loading cultured cells with iron (as an 8-hydroquilone chelate:) restored the cells to the normal (primary isolate) level of resistance against oxidant stress. Therefore, we will load the cells with iron prior to exposing to the IRP chemicals.

I. Anti-oxidative Properties of Harmane and Beta-carboline Alkaloids. A number of anti-oxidant agents prevented the iron-assisted IRP-associated lipid peroxidation in cells and membranes. While Dr. Tse was working on this project, she discovered that Harmane, a beta-carboline alkaloids with a wide variety of pharmacological activity, could also reduce this lipid peroxidation. Since harmane is a derivative of tryptamine, which also has anti-oxidant properties, she performed a series of experiments to investigate the anti-oxidant properties of a number of beta-carboline alkaloids. The result of this soon to be published study [Tse SYE, Mak IT, Dickens BF: Antioxidant properties of harmone and beta-carboline alkaloids. *Biochem. Pharmacol.* 1991, in press] demonstrates the structural components of the molecules responsible for the anti-oxidant properties of these therapeutic alkaloids and suggest why precursors of beta-carbolines, such as tryptophan and tryptamines,

possess anti-oxidant properties. (See attached manuscript).

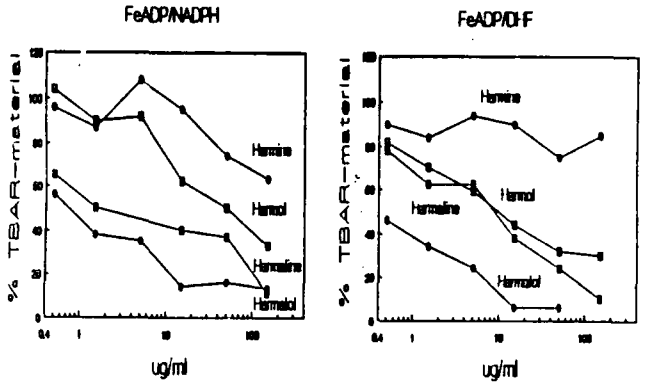


Figure 10. Antioxidant Effects of beta-carbolines against and enzymatic (FeADP/NADPH) and non-enzymatic (FeADP/DHF) free radical generating systems.

Methodology Developed or Enhanced for this Proposal

Ex-vitro Spin Trapping.

Fluorescence Assay of lipid hydroperoxide production using the ACAS 570 and 2,7-dichlorofluorescein fluorescence (see section). While 2,7-dichlorofluorescein fluorescences has been used for some time to separate cells on a cell sorters, the only application of this technique to living cells is one application note from the Meridian company. To date, no published manuscript using this procedure has appears in an edited journal, although Dr. Mark Entman of Dallas Tx, has an article submitted to *Science* and I am preparing a method paper for *J. Mol. Cell. Cardiol.*

Assay for Lipid Hydroperoxides: We have expanded the usefulness of the 2,7-DCF assay to measuring products of lipid peroxidation in the test tube. The initial application of this procedure was to determine if the "unknown" peak we observed in our HPLC tracing was a lipid hydroperoxide. Our laboratory is know using this assay to detect and quantify hydroperoxides in coronary effluents following ischemia. This assay has proven to be highly sensitive and the results are in excellent agreement with the work of others using requiring highly sophisticated equipment.

HPLC detection of PE-OOH and PC-OOH. The HPLC separations obtained using showed that we can detect PC-OOH and presumably PE-OOH in lipid extracts using a normal phase HPLC and UV detection.

Toluene Extractions for Lipid radicals. We have shown conclusively that artifact problems can frequently arise in studies in which PBN is added to chloroform lipid extracts. This

problem can be completely avoided if the lipids are suspended in a solvent such as toluene or benzene.

Published or In Press Manuscripts and Abstracts

Tse SYE, Mak IT, Weglicki WB, Dickens BF: Chlorinated aliphatic hydrocarbons promote lipid peroxidation in vascular cells J. Toxicol. Environ. Health 1990: 390:217-226.

Dickens BF, Tse SYE, Mak IT, Weglicki WB: Pro-oxidant effect of chlorinated hydrocarbons on cultured vascular cells. FASEB J. 1989;4:4714 (abstract)

Tse SYE, Mak IT, Weglicki WB, Dickens, BF: Chlorinated hydrocarbons enhance lipid peroxidation in cultured endothelial cells and smooth muscle cells, J. Mol. Cell. Cardiol. 1988:20(S3):S-36 (abstract)

Tse SYE, Mak IT, Dickens BF: Antioxidant properties of harmone and beta-carboline alkaloids. Biochem. Pharmacol. 1991 (manuscript in press).

Summary and Future Directions

This research has demonstrated that IRP-associated free radical injury - as measured by lipid hydroperoxide formation, ESR-detectable lipid radicals, the production of malondialdehyde, the increased toxicity in the presence of iron, and the protective effect (on lipid peroxidation) of antioxidant agents - is not limited to the liver, but also occurs in vascular cells as well. Unlike the liver, where most of the injury is undoubtedly due to the biotransformation of large amounts of the added chemicals to free radical intermediates, the injury process in the vascular cells appear to be related to the effect of the IRP chemicals on enhancing the autocatalyzed chain of lipid peroxidation, perhaps through a direct effect on lipid hydroperoxide molecules. Normally one of two things happens to a phospholipid acyl hydroperoxide, the acyl group can be cleaved by a phospholipase A_2 and the freed acyl hydroperoxide detoxified by the glutathione-glutathione transferase system, or it can be activated to form an alkoxyl radical, initiating another turn of the autocatalyzed chain of lipid peroxidation. Our data is consistent with the hypothesis that, at least for the halogenated hydrocarbons, lipophillic IRP chemicals swing the balance in favor the second of these two options.

While a number of anti-oxidant agents blocked lipid peroxidation induced by the halocarbons, the only agent which effectively reduced loss of cellular viability by the IRP chemicals was deferrioxamine: and this reduction was only in comparisons with cells to

which iron had been previously added to enhance the toxicity. Thus, the question of the role of free radical in the ultimate toxicity of these agents remains open. Two recent papers one presented at FASEB 1991 by Peter Ward (FASEB J, 1991 5(4 pt I):A3009) and the other by G. Balla et al (Iron Loading of endothelial cells augments oxidant damage. *J. Lab. Clin. Med.*, 1990; 116:546-554) provides a possible clue as to why cells we see virtually no protection in viability in our model using anti-oxidant drugs (other than an iron chelator). They both found that following culture, iron had to be added back to cells to augment cellular oxidant damage: and they were working with cells only several passages into culture. Our studies have all ready suggested that augmenting cellular levels of iron with such agents as Fe-ADP greatly enhance the both the loss of cell viability induced by IRP chemicals and the extent of lipid peroxidation. We have initiated studies to restore the iron content of cultured cells using iron-chelated with 8-hydroxyquinoline. We expect that these iron loaded cultured cells will produce more lipid peroxidation in response to IRP chemicals, lose viability at lower levels of exposure due to the toxic effect of the increased lipid peroxidation, and anti-oxidant agents which reduce lipid peroxidation will provide at least modest increases in cellular viability.

APPENDIX ONE
IN PRESS, BIOCHEM. PHARMACOL

Anti-oxidative Properties of Harmane and Beta-carboline Alkaloids

Susanna Y.H. Tse *, I-Tong Mak and Benjamin F. Dickens

Division of Experimental Medicine

Department of Medicine

George Washington University

Washington D.C. 20037.

* To whom correspondence should be addressed :

Department of Neurology

Georgetown University

3800, Reservoir Road, N.W.

Washington D.C. 20007.

Short title : Anti-oxidative Actions of Harmala Alkaloids

Keywords : free radicals, lipid peroxidation, harmane, harmine, harmalol, harmine, tryptamine, 5-hydroxytryptamine, 5-methoxytryptamine, 5-hydroxytryptophan, harmala alkaloids, superoxide, malondialdehyde, dihydroxyfumarate

Abbreviations : *BHT* - butylated hydroxytoluene; *ADP* - adenosine diphosphate; *NADPH* - reduced nicotinamide adenine dinucleotide phosphate; *DHF* - dihydroxyfumarate; *TBA* - thiobarbituric acid; *MOPS* - 4-morpholinepropanesulfonic acid; *MPTP* - 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine; MPP^+ - 1-methyl-4-phenyl-pyridinium ion

ABSTRACT

Beta-carbolines alkaloids are derived as a result of condensation between indoleamine (e.g. tryptamine) and short chain carboxylic acid (e.g. pyruvic acid) or aldehyde (e.g. acetaldehyde), a reaction that occurs readily at room temperature. These compounds have been found endogenously in human and animal tissues and may be formed as a byproduct of secondary metabolism, while their endogenous functions are not well understood. Indoles and tryptophan derivatives have anti-oxidative actions by scavenging free radicals and forming resonance stabilized indolyl radicals. Harmane and related compounds exhibited concentration dependent inhibition of lipid peroxidation (measured as thiobarbiturate reactive products) in a hepatic microsomal preparation incubated with either enzymatic dependent (Fe^{+3}ADP/NADPH) or non-enzymatic dependent (Fe^{+3}ADP/dihydroxyfumarate) oxygen radical producing systems. Alkaloids with hydroxyl substitution and partially desaturated pyridyl ring were found to have the highest anti-oxidative potencies. Substitution of a hydroxyl group by a methoxyl group at the 6 position resulted in a decrease of greater than 10-fold in the anti-oxidative activities. Harmane showed high efficacy in an enzymatic system but low efficacy in a non-enzymatic system. The anti-oxidative effects of harmane in the former system may be attributed to its ability to inhibit oxidative enzymes in the microsomal system. These results suggest that beta-carbolines may also serve as endogenous anti-oxidants.

INTRODUCTION

Harmane (1-methyl-beta-carboline), first isolated in Peganum harmala, and related alkaloids are widely distributed in medicinal plants. Harmala alkaloids have been used in hallucinogenic preparations of South American and African tribes (1). These alkaloids have a wide spectrum of pharmacological actions, including monoamine oxidase inhibition (2), binding to benzodiazepine receptors (3), convulsive or anticonvulsive actions (4), tremorigenesis (5), anxiolytic and behavioral effects (6). Beta-carbolines structurally related to harmala alkaloids have also been found endogenously in mammalian tissues, including the central nervous system, liver, platelet, and in plasma and urine (7-8). The metabolic pathway leading to the formation of beta-carbolines is via Pictet-Spengler condensation between an indolamine, for example tryptamine, and aldehydes, for example acetaldehyde. Such reaction between tryptamine and acetaldehyde occurs non-enzymatically at physiological pH and form 1-methyl,1-carboxyl-tetrahydro-beta-carboline. Once formed, 1-methyl, 1-carboxyl-tetrahydro-beta-carboline is further metabolized, via ring hydroxylation (at the 5, 6, and 7 position) (9), decarboxylation (10), methylation and dehydrogenation (11) to form a series of beta-carboline alkaloids.

Indole precursors of beta-carbolines, e.g. tryptophan and

tryptamines, are known to have anti-oxidative activities (12,13), possibly by scavenging reactive oxygen radicals and forming a stable indolyl radical at the pyrrole ring (14). The indole nucleus in beta-carboline may possess similar anti-oxidant properties. In this study, we have investigated the anti-oxidative potential of a series of tryptamine and beta-carboline derivatives. Structural modification (hydroxyl or methoxyl substitution) and degrees of saturation at the pyridyl ring were found to be important determinants in the anti-oxidative efficacy.

METHODS

Rat Hepatic Microsomes

Male Sprague-Dawley rats (250-300 g body weight, Charles Rivers, MA) was sacrificed by decapitation. Rat liver was excised immediately after sacrifice. The tissue was homogenized in 9 volumes of pH 7.2 buffer (0.25 M sucrose, 0.01 M MOPS and 0.001 M EDTA). The homogenate was centrifuged at 4 ° (10,000 x g, 10 min.). The supernatant was separated, and centrifuged again at 4 oC (100,000 x g, 60 min.). The pellet was washed and suspended in potassium phosphate-sucrose buffer (KSP buffer, pH 7.2, 0.12 M KCl, 0.01 M K_2HPO_4/KH_2PO_4 and 0.05 M sucrose). The suspension was centrifuged (4 °, 100,000 x g, 60 min.). The supernatant was discarded and the pellet was suspended in 10 ml of KSP buffer and

kept at -70 °.

Lipid Peroxidation

Lipid peroxidation was estimated using the thiobarbiturate method. Liver microsomes were resuspended in 500 ul of KSP buffer (final concentration 1 mg protein/ml). Lipid peroxidation was initiated by either one of the following systems :

(i) FeADP-NADPH enzymatic dependent system - stock solution of Fe^{+3}ADP was made up with 100 uM of $FeCl_3$, chelated by 1 mM of ADP. Dilution was made to reach nominal concentration of 6.25 uM of $FeCl_3$. NADPH was added as a cofactor to the incubation mixture (final concentration, 0.2 mM).

(ii) FeADP-dihydroxyfumarate (DHF) non-enzymatic system - dihydroxyfumaric acid undergoes spontaneous auto-oxidation to generate oxygen free radicals. The rate of oxygen radicals production is enhanced in the presence of Fe^{+3}. Incubation mixture consisted of 50 uM of dihydroxyfumarate was added with FeADP (6.25 uM of Fe^{+3}).

Tryptamines or beta-carbolines (hydrochloric salt) were dissolved in KSP buffer. Each compound was added to incubation mixtures to reach final concentrations of 0.5 to 150 ug/ml. All the chemicals and cofactors were added at the beginning of the

experiment. The microsome suspension was incubated for 30 minutes at 37 °. At the end of the incubation, the mixture was acidified by 50 ul of 5 % trichloroacetic acid. 500 ul of 0.5 % thiobarbituric acid (TBA) was added. The mixture was heated at 80 ° for 20 minutes. After rapid cooling in ice, 500 ul of ice cold trichloroacetic acid (70 %) was added to stop further color development. The mixture was centrifuged (10 minutes, 1000 x g) and the supernatant absorbance was measured at 535 nm (Beckmann DU-8 Spectrophotometer). Control incubation mixtures contained only the microsomal suspension and free radical producing systems. Results were expressed as percentage inhibition of lipid peroxidation (absorbance) compared to the control incubation. IC 50 (concentration of 50 % inhibition) was measured from the concentration response curves and converted to micromolar concentrations.

All reagents, tryptamine and beta-carboline analogs were obtained from Sigma Chemical Co. (St. Louis, MI). Structural correlations in the tryptamine and beta-carboline series studied were illustrated in Figure 1.

RESULTS

Harmalol, harmaline, harmol and harmine (0.5 - 150 ug/ml) inhibited lipid peroxidation in both the enzymatic (FeADP-NADPH) (Figure 2A) and non-enzymatic (FeADP-DHF) systems (Figure 2B).

Structural modification by substituting the hydroxyl group with methoxyl group (harmalol to harmaline; harmol to harmine) resulted in 3-15 fold decrease in anti-oxidant efficacies, particularly in the FeADP-DHF system (Figure 2B). Dehydrogenation of the pyridyl ring (e.g. harmalol to harmol; harmaline to harmine) also drastically reduce the antioxidant effects. Of all the beta-carbolines studied, harmalol was found to have the highest antioxidant efficacy, whereas harmine showed little if any antioxidative effects in the concentration range tested.

The antioxidative potency of each compound tested was expressed as IC 50 (concentration at 50% inhibition of lipid peroxidation in the control samples). The IC 50 values of all the compounds tested were listed in Table 1. Butylated hydroxytoluene (BHT), a phenolic antioxidant was used as a positive control. Most beta-carbolines and tryptamines showed similar relative potencies in both the enzymatic and the non-enzymatic systems, with the exception of harmane. Harmane was a weak anti-oxidant in the FeADP-DHF system, while it has high efficacy in the FeADP-NADPH system (Figure 3). The inhibitory effects of harmane in the FeADP-NADPH system may be related to inhibition of oxidative enzymes rather than direct free radical scavenging. The potency ratios and rank order of potencies in the carboline group is listed in Table 2 (A&B). In the FeADP/NADPH system, harmane was similar in potency to harmaline

(potency ratio of harmaline to harmane 1.4, 95% confidence limits, 0.3-7.2). Wwhereas in the FeADP/DHF system, harmane was an ineffective anti-oxidant and was below harmaline and harmol in the potency rank order.

Tryptamine, 5 hydroxy-tryptamine and 5 methoxy-tryptamine, similar to other tryptophan derivatives, were all found to be potent inhibitors of lipid peroxidation in both the enzymatic (FeADP-NADPH) and the non-enzymatic (FeADP-DHF) systems. 5 hydroxy-tryptophan is a relatively weak anti-oxidant in both systems. Similarly but to a smaller extent, substitution of the hydroxyl group by methoxyl group decreased the anti-oxidative efficacy. The potency ratios and the rank order of potencies in the tryptamine group is listed in Table 3. In the FeADP/NADPH system, 5-hydroxy-tryptamine and 5-methoxy-tryptamine were similar in potencies (potency ratio : 2.4, 95% confidence intervals 0.7-9.1). Nonetheless, both compound were better antioxidants than tryptamine and 5-hydroxy-tryptophan. In the FeADP/DHF system, distictions in the anti-oxidant potencies among 5-hydroxy-tryptamine, 5-methoxy-tryptamine and tryptamine were less clear, although there is small differences in the potencies. These differences were not significant since the 95% confidence intervals of the potency ratios overlapped with unity.

DISCUSSION

The presence of beta-carboline alkaloids in mammalian tissues raise speculations regarding their functions as endogenous benzodiazepine receptor ligands, as mediators of ethanol intoxication, or in the regulation of monoamine oxidases. However, whether these alkaloids serve any physiological functions or whether they are simply byproducts of secondary metabolism is still unknown. We have shown in this study that beta-carbolines have antioxidative actions, and the relative efficacies are highly dependent on structural modification of the beta-carboline ring. Beta-carbolines derived from secondary emtabolism and Pictet-Spengler condensation may serve as endogenous antioxidants in vivo.

Recently, it was suggested that endogenous beta-carbolines may be metabolized to intermediates that resemble MPTP (15). MPTP is metabolized to a proximal toxin MPP^+ via free radical intermediates. In analogy, free radical intermediates may also be involved in the metabolism of beta-carbolines, as the indolyl nucleus in tryptophan and tryptamine is known to form stable free radicals (16). Oxidation of tryptophan and tryptamine, for instance, may form the indolyl radicals which are resonance stabilized. Resonance stabilized free radical species may also be formed during the oxidation of beta-carbolines, and the formation and stability of such resonating radical species may

explain the relative anti-oxidative potencies. Similarly, scavenging of oxygen free radicals by beta-carbolines and tryptamines may also lead to the formation of secondary radical species which are resonance stabilized. Our results indicated that hydroxylation of the ring greatly enhanced the anti-oxidative efficacies, whereas dehydrogenation of the pyridyl ring greatly decreased the efficacies. Phenolic compounds are known to be effective antioxidants (17), due to the formation of stable phenoxy radicals (18). In the case of harmalol, the phenoxy radical formed may be stabilized by dislocation of the lone electron over the beta-carboline ring, which may be further stabilized by the many possible resonance structures (Figure 4A). Whereas in the case of harmaline, formation of the phenoxy radical would be impossible due to methoxy substitution, and further, resonation to the semiquinone forms (as in harmalol) is hindered. Harmaline may still have anti-oxidatant properties due to the stability of the radical formed at the allylic sites of the pyridyl ring. However, in the cases of harmol and harmine, the number of resonance structures at the positions allylic to both the pyrrole and pyridyl nitrogen is not possible after dehydrogenation. Harmol is found to be a more effective anti-oxidant compared to harmine, probably due to the hydroxyl group substitution and the formation of the phenoxy radical (Figure 4B).

Interestingly, tryptamine, 5-hydroxytryptamine and 5-

methoxytryptamine have similar anti-oxidative potencies. In is probable that the stable indolyl radical formed is the major contributing factor in stabilizing the radical intermediates. This may also explain our results that 5-hydroxytryptophan is much less effective as an antioxidant compared to 5-hydroxytryptamine. Substitution of the carboxyl group and its inductive effects may have destabilized the indolyl radical.

REFERENCES

1. Efron DH, Holmstedt B, and Kline NS, *Ethnopharmacologic Search for Psychoactive Drugs*. pp 339-373, Raven Press, N.Y., 1967.

2. Fuller RW, Wong CJ, and Hemrick-Luecke SK, MD 240928 and harmaline : opposite selectivity in antagonism of the inactivation of types A and B monoamine oxidase by pargyline in mice, *Life Sciences*, **38**: 209-412, 1986.

3. Rommelspacher H, Bruning G, Susilo R, Nick M and Hill R, Pharmacology of harmalan (1-methyl-3,4-dihydro-beta-carboline), *Eur. J. Pharmacol.*, **109**: 363-371, 1985.

4. Loew GH, Nienow J, Lawson HA, Toll L and Uyeno ET, Theoretical structure-activity studies of beta-carboline

analogs. *Mol. Pharmacol.*, **28**: 17-31, 1985.

5. Lutes J, Lorden JF, Beales M and Lotmans GA, Tolerance to the tremorogenic effects of harmaline : evidence for altered olivo-cerebellar function. *Neuropharmacol.*, **27**: 849-855, 1988.

6. Barbaccia ML, Ravizza L and Costa E, Maprotiline: an antidepressant with an unusual pharmacological profile. *J. Pharmacol. Exp. Ther.*, **236**: 307-312, 1986.

7. Airaksinen MM and Kari I, Beta-carbolines, psychoactive compounds in the mammalian body. *Medical Biology*, **59**: 21-34, 1981.

8. Beck O and Faull KF, Concentrations of the enantiomers of 5-hydroxymethtryptoline in mammalian urine : implications for *in vivo* biosynthesis. *Biochem. Pharmacol.*, **35**: 2636-2639, 1986.

9. Beck O, Jernstrom B, Martinez M and Repke BD, In vitro study of the aromatic hydroxylation of 1-methyltetrahydro-beta-carboline (methtryptoline) in rat. *Chem.-Biol. Interactions*, **65**: 97-106, 1988.

10. Gynther J, Lapinjoki SP, Airaksinen MM and Peura P,

Decarboxylation of 1,2,3,4-tetrahydro-beta-carboline-1-carboxylic acid in brain homogenate and catalysis by pyridoxal-5'-phosphate. *Biochem. Pharmacol.*, **16**: 2671-2675, 1986.

11. Susilo R and Rommelspacher H, Formation of 1-methyl-beta-carbolines in rats from their possible carboxylic acid precursor. *Naunyn-Schmiedeberg's Arch. Pharmacol.*, **337**: 566-571, 1988.

12. Uemura T, Kanashiro M, Yamano T, Hirai K and Miyazaki N, Isolation, structure, and properties of the beta-carboline formed from 5-hydroxytryptamine by the superoxide anion generating system. *Journal of Neurochemistry*, **51**: 710-717, 1988.

13. Christen S, Peterhans E and Stocker R, Antioxidant activities of some tryptophan metabolites : possible implication for inflammatory diseases. *Proc. Natl. Acad. Sci. USA*, **87**: 2506-2510, 1990.

14. DeFelippis MR, Murthy CP, Faraggi M and Klapper MH, Pulse radiolytic measurement of redox potentials : the tyrosine and tryptophan radicals. *Biochemistry*, **28**: 4847-4853, 1989.

15. Collins MA, Neafsey EJ, Cheng BY, Hurley-Gius K, Ung-Chhun

NA, Pronger DA, Christensen MA and Hurley-Gius D, Endogenous analogs of *N*-methyl-4-phenyl-1,2,3,6-tetrahydropyridine : indoleamine derived tetrahydro-beta-carbolines as potential causative factors in Parkinson's Disease. *Advances in Neurology*, **45**: 179-182.

16. Jovanovic SV and Simic MG, Repair of tryptophan radicals by antioxidants. *Journal Free Rad. Biol. Med.*, **1**: 125-129.

17. Burton GW and Ingold KU, Autoxidation of biological molecules. 1. The antioxidant activity of vitamin E and related chain-breaking phenolic antioxidants in vitro. *J. Am. Chem. Soc.*, **103**: 6472-6477 1981.

18. Valoti M, Sipe Jr. HJ, Sgaragli G and Mason R, Free radical intermediates during peroxidase oxidation of 2-t-butyl-4-methoxyphenol, 2,6-di-t-butyl-4-methylphenol, and related phenol compounds. *Arch. Biochem. Biophys.*, **269**: 423-432.

19. Tallarida RJ and Murray RB, *Manual of Pharamcologic Calculations with Computer Programs, 2 nd edition*. Springer-Verlag, N.Y. 1987.

Figure 1

Structural derivatives of tryptamines and beta-carbolines investigated for anti-oxidative actions.

Figure 2

Beta-carbolines inhibition of lipid peroxidation in hepatic microsomes. (A) enzymatic system with FeADP-NADPH and (B) non-enzymatic system with FeADP-dihydroxyfumarate. Each data point represents three separate dose-response experiments with duplicates. The rank order of potency is :

HARMALOL > HARMALINE > HARMOL >>> HARMINE

Figure 3

Anti-oxidative effects of harmane and harmaline. (A) Harmane was a potent inhibitor of lipid peroxidation in the FeADP-NADPH system but not in the FeADP-DHF system, possibly by inhibition of oxidative enzymes. (B) Harmaline, also known to inhibit monoamine oxidases, showed similar efficacy in the enzymatic system, possibly acted by both enzyme inhibition and direct scavenging.

Figure 4

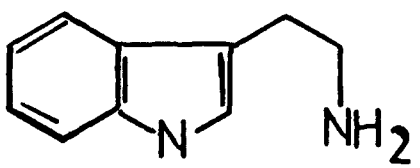
Possible resonance structures of (A) harmalol and (B) harmol radicals. In harmalol, the radical may be stabilized by delocalization over the pyrrole ring, as well as by resonation among the various allylic sites. Harmol can form a radical that may be stabilized by delocalization over the pyrrole and the pyridyl ring. However, the allylic sites are not available. Both harmalol and harmol can form the semi-quinone radical at the 7-hydroxyl position, while this structure is not possible in harmane and the methoxy-derivatives.

INDOLE

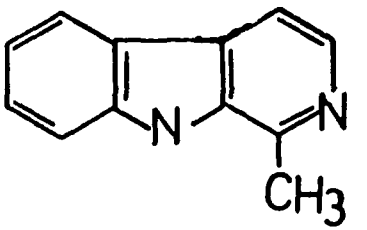
DIHYDRO-β-CARBOLINE

β-CARBOLINE

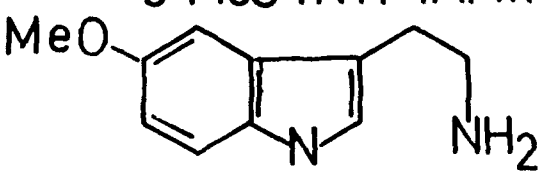
TRYPTAMINE



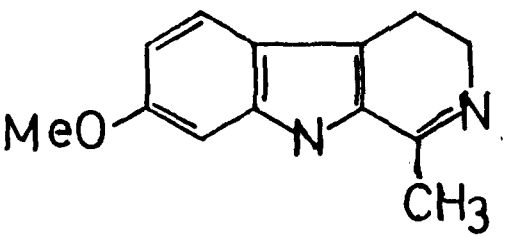
HARMANE



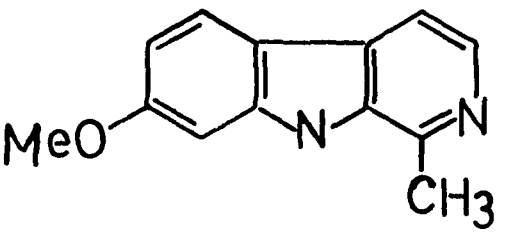
5 MeO TRYPTAMINE



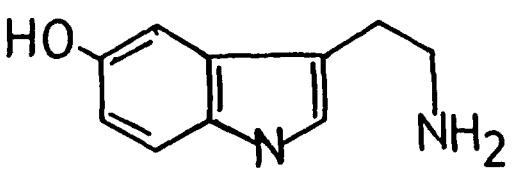
HARMALINE



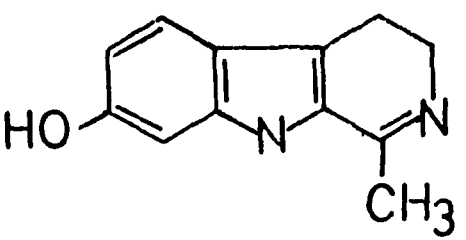
HARMINE



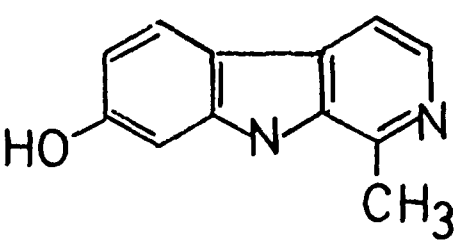
5-OH-TRYPTAMINE



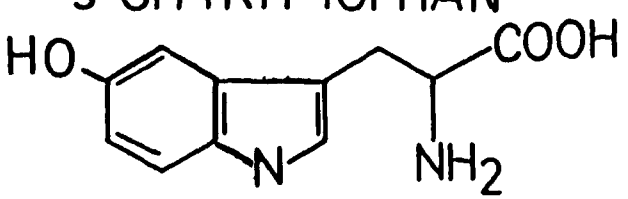
HARMALOL

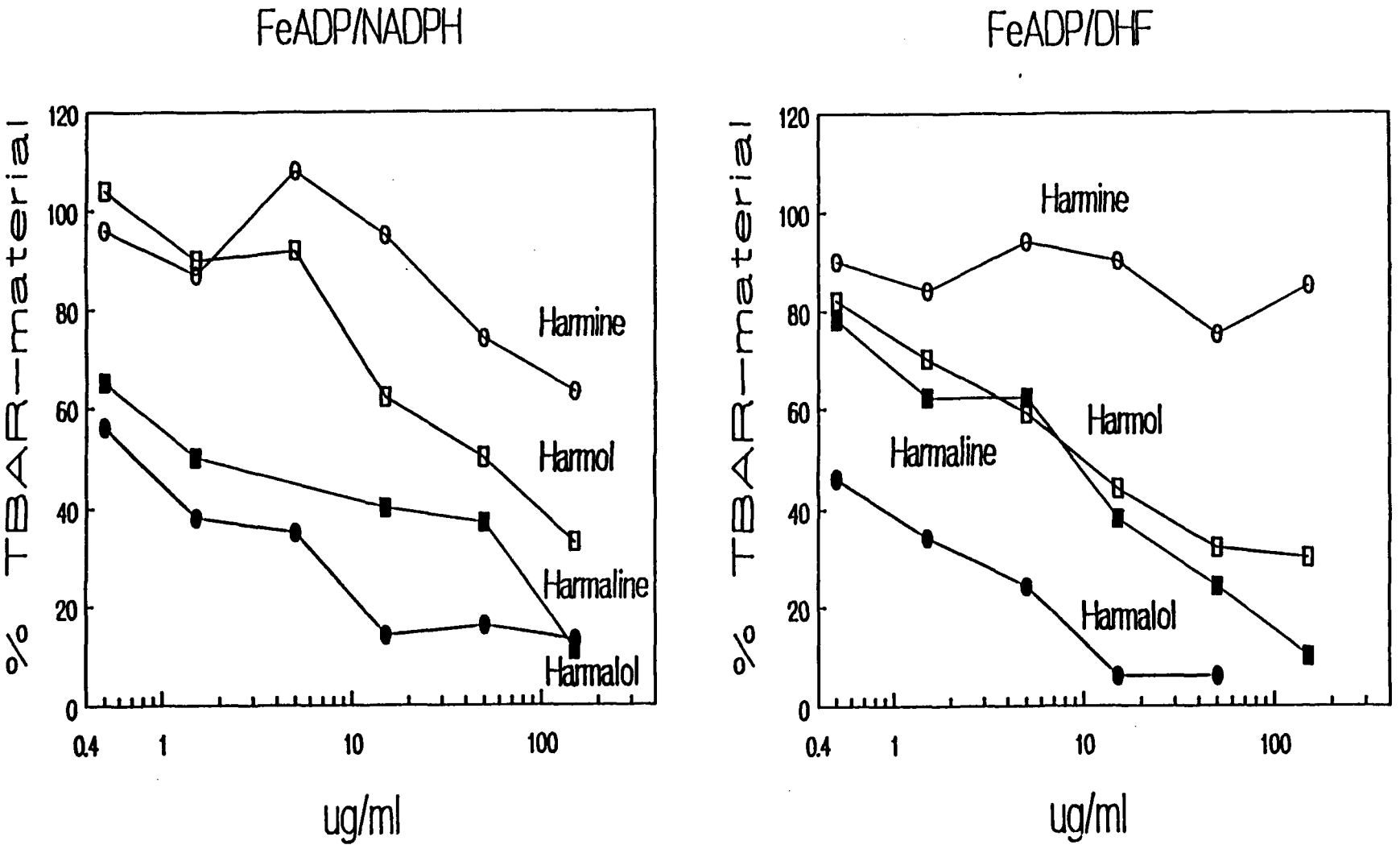


HARMOL

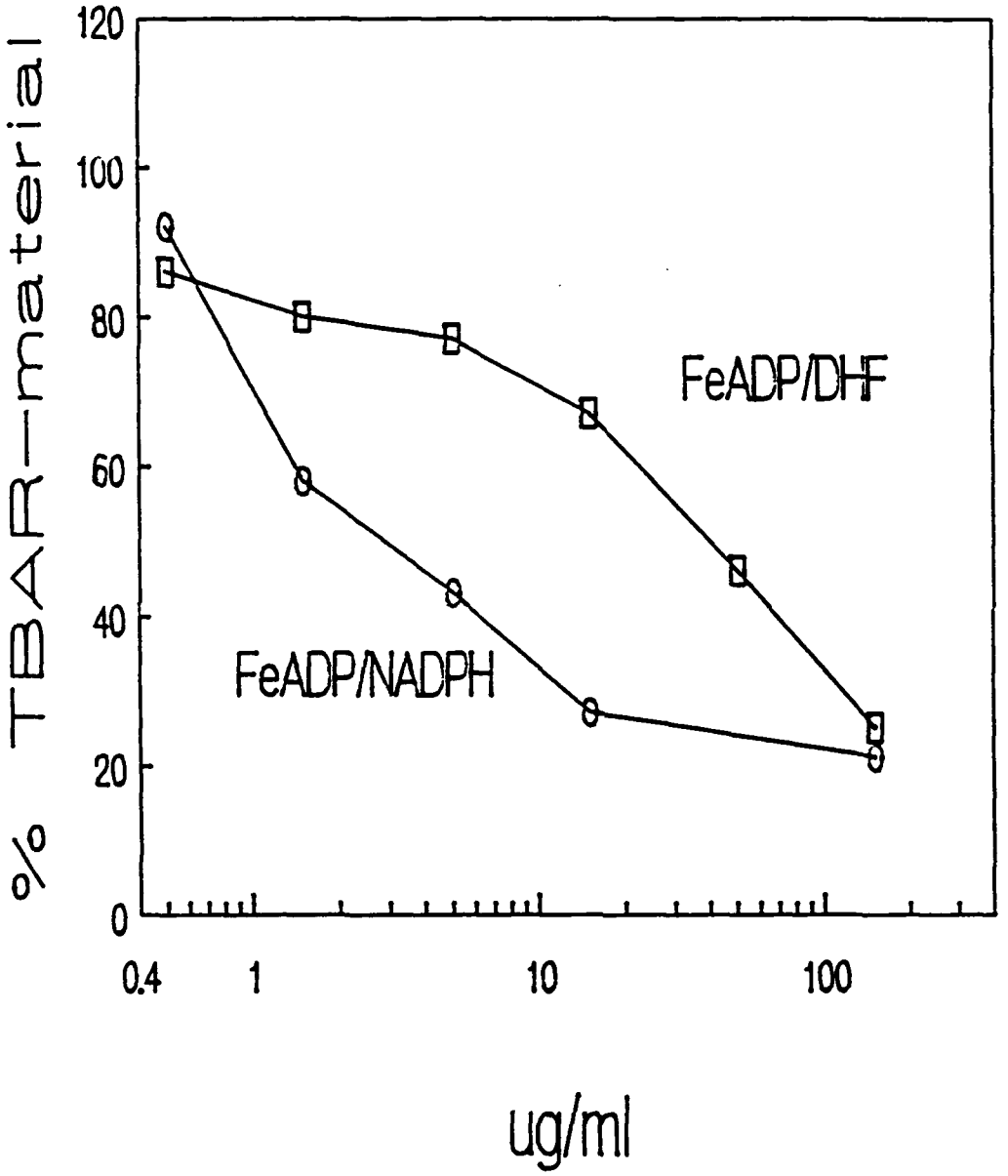


5-OH-TRYPTOPHAN



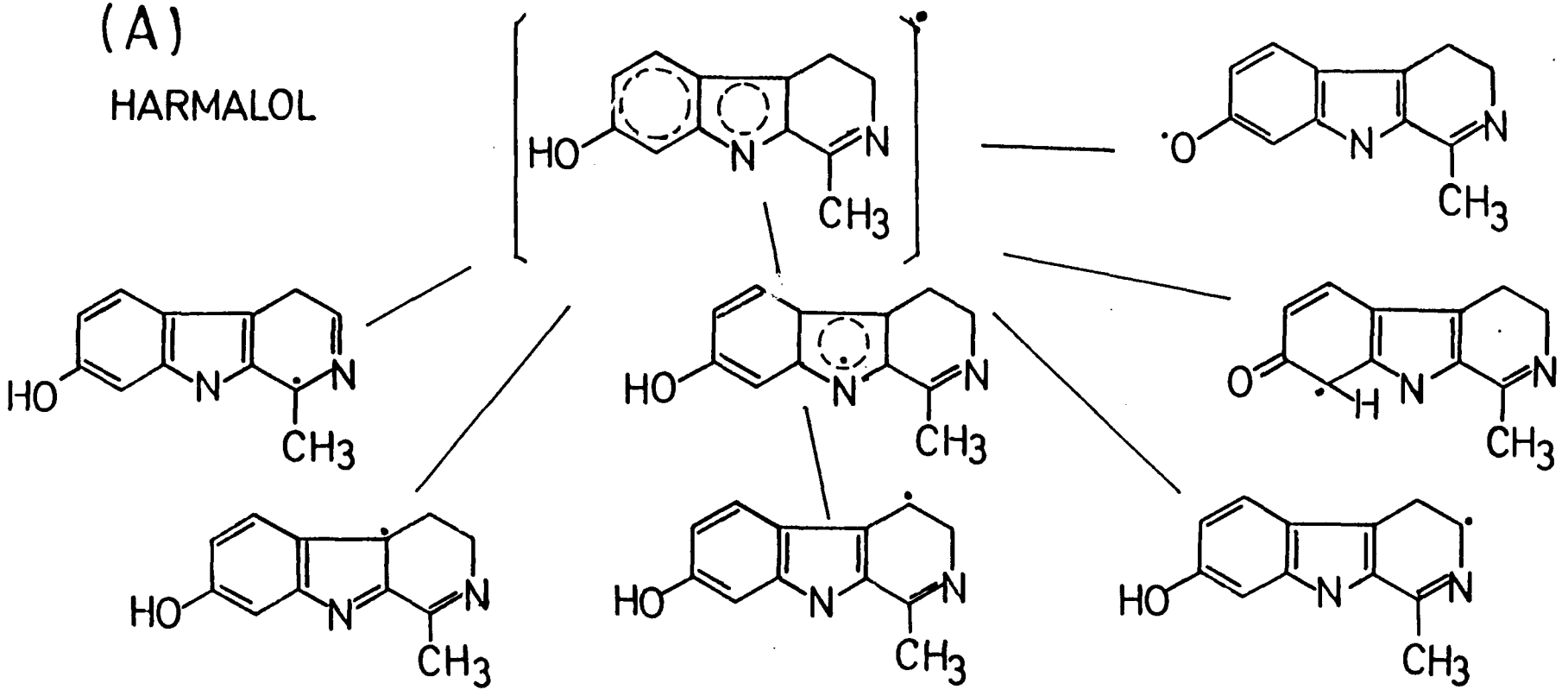

FeADP/NADPH
% TBAR-material
120
100
80
60
40
20
0
0.4
1
10
100
ug/ml
Harmine
Harmol
Harmaline
Harmalol
FeADP/DHF
% TBAR-material
120
100
80
60
40
20
0
0.4
1
10
100
ug/ml
Harmine
Harmol
Harmaline
Harmalol

Harmane


% TBAR-material
120
100
80
60
40
20
0
0.4
1
10
100
FeADP/DHF
FeADP/NADPH
ug/ml

(A)

HARMALOL



(B)

HARMOL

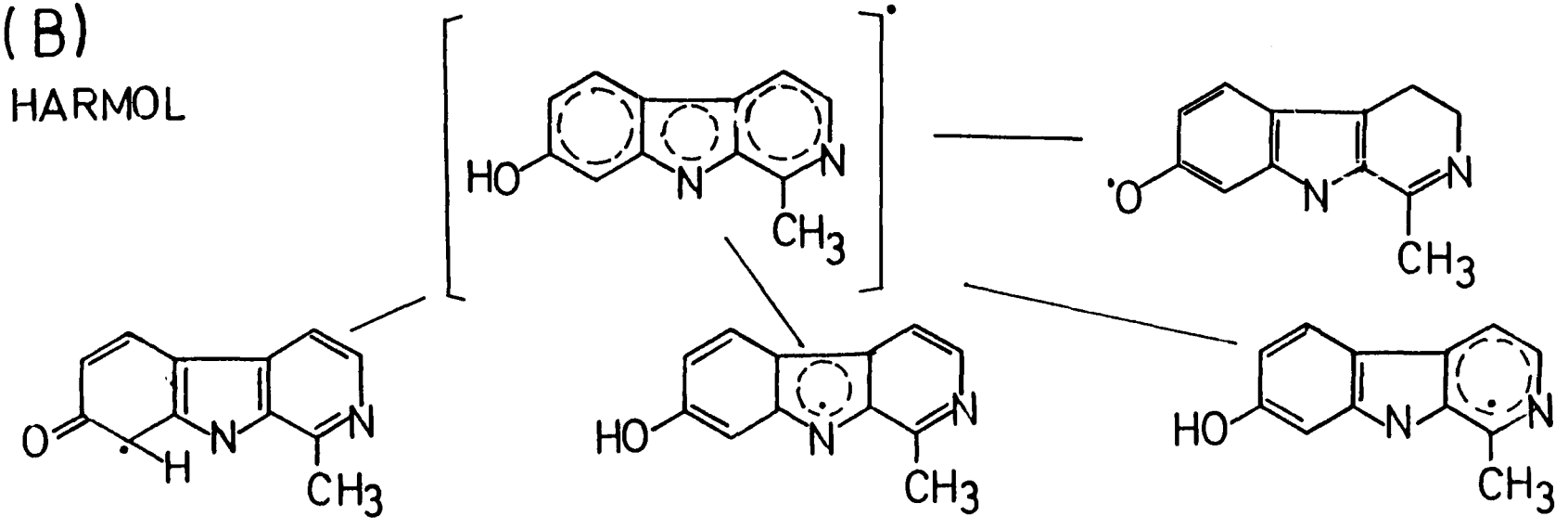


TABLE 1

IC 50 (uM)

Compound	FeADP/NADPH	FeADP/DHF
5-OH-Troptophan	145	159
5-OH-Tryptamine	5.6	2.8
5-MeO-Tryptamine	10.1	4.0
Tryptamine	17.8	4.0
Harmalol	3.2	1.7
Harmaline	10.4	23.9
Harmol	213	38
Harmine	>600	>600
Harmane	11.7	145
BHT	0.54	0.51

Table 1
Anti-oxidative potencies of tryptamines and beta-carbolines. IC50's (concentration at 50% inhibition of lipid peroxidation) were calculated from the dose-response curves.

Table 2

(A) FeADP-NADPH System

	Harmaline	Harmane	Harmol
Harmalol *vs.*	5.1* (1.4-28.1)	5.7* (1.2-50.2)	75.6* (19.5-597.7)
Harmaline *vs.*	-	1.4 (0.3-7.2)	20.1* (6.3-20.6)
Harmane *vs.*	-	-	8.6* (2.7-40.0)

Potency Rank Order :

Harmalol > Harmaline ≥ Harmane > Harmol > Harmine

(B) FeADP-DHF System

	Harmaline	Harmol	Harmane
Harmalol *vs.*	11.0* (4.1-39.4)	34.1* (13.4-115.0)	129.7* (29-1451)
Harmaline *vs.*	-	2.3* (1.1-5.2)	4.9* (2.0-14.0)
Harmol *vs.*	-	-	2.8* (1.2-7.4)

Potency Rank Order :

Harmalol > Harmaline > Harmol > Harmane > Harmine

Table 2 : Potency ratios and 95% confidence intervals. The potency ratios and the confidence intervals between two compounds were computed from the dose-response curves [19]. The potencies of two compounds are not significantly different if the 95% confidence intervals overlaps with unity. Compounds are ordered and compared in descending order of potencies. In the carboline group, harmine is not included in the calculation as the IC 50 is above 600 uM, and its potency is much smaller than all the other compounds in the carboline group. * $p < 0.05$.

TABLE 3

(A) FeADP-NADPH SYSTEM

	5-Methoxy-Tryptamine	Tryptamine	5-Hydroxy-Tryptophan
5-Hydroxy-Tryptamine *vs.*	2.4 (0.7-9.1)	5.7* (2.0-21.4)	24.9* (6.1-216.1)
5-Methoxy-Tryptamine *vs.*	-	3.6* (1.8-7.5)	9.9* (5.4-20.3)
Tryptamine *vs.*	-	-	2.4* (1.1-5.3)

Potency Rank Order :

5-hydroxytryptamine ≳ 5-methoxytryptamine > tryptamine > 5-hydroxytryptophan

(B) FeADP-DHF SYSTEM

	Tryptamine	5-Methoxy-Tryptamine	5-Hydroxy-Tryptophan
5-Hydroxy-tryptamine *vs.*	1.7 (0.4-7.6)	4.1* (1.5-13.3)	97.4* (21-1174)
Tryptamine *vs.*	-	2.4 (0.8-8.3)	49.7* (11.4-530.6)
5-Methoxy-tryptamine *vs.*	-	-	29.1* (10.2-119.8)

Potency Rank Order :

5-hydroxytryptamine ≳ Tryptamine ≳ 5-methoxytryptamine > 5-hydroxytryptophan

Table 3 : Relative potencies and 95% confidence intervals of the potency ratios in the tryptamine group. The potency ratios and confidence limits were computed from the dose response curves, as described in ref. 19. The potencies between two compounds are not significantly different if the 95% confidence intervals overlaps with unity. * $p < 0.05$.