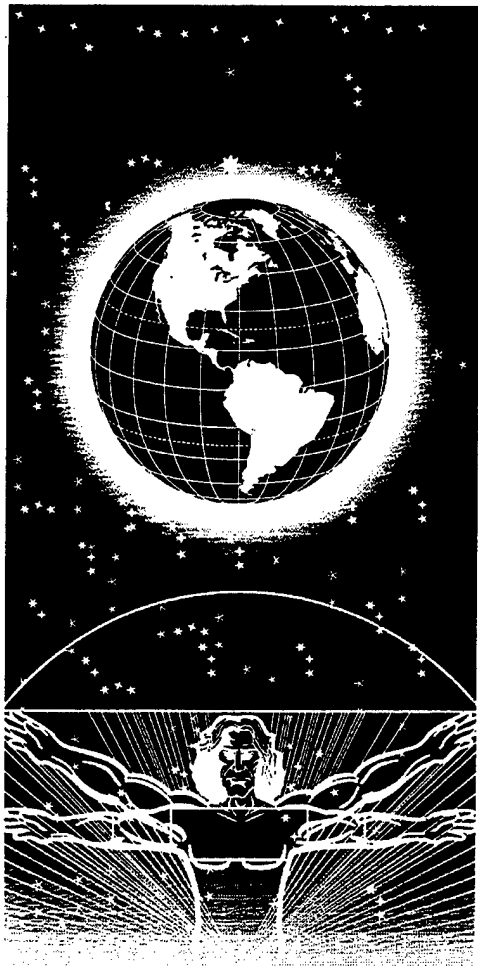




**UNITED STATES AIR FORCE
ARMSTRONG LABORATORY**

**EFFICIENCY OF PBN TO TRAP
3-CAR IN B6C3F1 MOUSE LIVER
SLICES: AN EPR STUDY**

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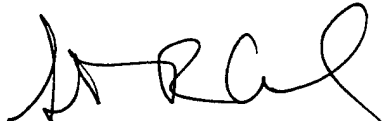
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The animal use described in this study was conducted in accordance with the principles stated in the "Guide for the Care and Use of Laboratory Animals", National Research Council, 1996, and the Animal Welfare Act of 1966, as amended.

This report has been reviewed by the Office of Public Affairs (PA) and is releasable to the National Technical Information Service (NTIS). At NTIS, it will be available to the general public, including foreign nations.

This technical report has been reviewed and is approved for publication.

FOR THE DIRECTOR



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13. ABSTRACT (Maximum 200 words) Several active Air Force bases and bases targeted for closure have measurable quantities of the halocarbon trichloroethylene (TCE) in the soil and ground water. TCE causes liver tumors in B6C3F1 mice. As part of the process to develop an environmental health effects criteria for base clean up a study of the effects of TCE induced free radicals in liver slices had been performed. Radicals of TCE can be identified by electron paramagnetic resonance (EPR) using the spin trap N-tert-butyl- α phenyl nitron (PBN). To quantitate the radicals detected a stable spin label 2,2,5,5-tetramethyl-1-pyrrolidinyloxy-3-carboxamide (3-CAR) is used as a standard. The trapping efficiency of PBN to detect 3-CAR was studied. Known amounts 3-CAR was added to non-TCE treated liver slices of B6C3F1 mice. These liver slices were lyophilized with and without 10 mM PBN. Using the assumption that the double integration of the 3-CAR spectrum in lyophilized is proportional to the number of radicals present and the double integration of the spectrum of PBN-3-CAR in lyophilized liver is proportional to the number of radicals trapped, the efficiency of PBN to trap 3-CAR was determined to be 33%.				
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PREFACE

This is the one of a series of technical reports generated from the experimental laboratory programs conducted in the Electron Paramagnetic Resonance laboratory for the Pharmacodynamic Group, Armstrong Laboratory, Toxicology Division. The research described in this report began in July 1995 and was completed in August 1995. It was sponsored by the Air Force Office of Scientific Research, Bolling AFB, Washington DC, Apprenticeship Program. Mouse liver was obtained from CPT Clay Miller under a program sponsored by AFOSR Environmental Initiative Program Work Unit #2312A202. Lt Col Terry A. Childress served as Contract Technical Monitor for the U.S. Air Force, Armstrong Laboratory, Toxicology Division.

The animals used in this study were cared for under the *Guide for the Care and Use of Laboratory Animals*, prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Resources, National Research Council, Department of Health and Human Services, National Institute of Health Publication #86-23, 1985, and the Animal Welfare Act of 1966, as amended.

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ABBREVIATIONS

ANOVA	Analysis of variance analysis
3-CAR	2,2,5,5,-tetramethyl-1-pyrrolidinyl oxy-3-carboxamide
dB	decibel
EPR	electron paramagnetic resonance
G	Gauss
mM	millimole/liter
ms	millisecond
mW	milliwatt
PBN	N-tert-butyl-α phenyl nitron
TCE	trichloroethylene

INTRODUCTION

Understanding free radical reactions is important to the military. The main objective of this project is to study free radicals which can be detected in lyophilized tissue. A free radical is an atom, molecule or compound with one or more unpaired electrons (Pryor, 1976). As free radicals will attempt to gain an electron from other compounds in order to pair with their odd electrons, free radicals are classified as highly reactive. Free radicals were first postulated by Fenton in 1893. Following this early work, free radical studies expanded with the recognition that food spoilage is an oxygen free radical process.

The Armed Forces have an interest in oxidative degradation of foods because they need to be able to store food for long periods of time (Pryor 1984, Simic et al 1980). Stored food is used in space missions and by special forces deployed for limited periods. After recognition of the importance of free radicals in radiation injury, the US Army began studying ways to prevent free radical reactions (Vladimirov 1974). Radioprotection is currently studied at the Armed Forces Institute of Radiobiology (Steel-Goodwin et al 1993 & 1994) which trains medical personnel on the effects of nuclear weapons (Walker et al 1989). More recently the US Army has studied radicals produced by chemical weapons and pest control agents (Arroyo et al 1993, 1994, & 1995).

Studies of free radicals are also of interest to the US Air Force for the biological effects of trichloroethylene, TCE, (Steel-Goodwin et al 1994). TCE is the best solution

available to remove grease from aircraft without damaging the metal parts. However as TCE contains chlorine it is a potential environmental hazard. The US Navy also investigates free radical chemistry. Two examples relevant to USAF toxicology studies involve the work of Rivera et al 1992 for the US Navy Medical Research Institute studies on the free radical effects of paint constituents used to coat the hulls of ships and Pace's work in 1994 for the Naval Research Laboratory on the decomposition of the new high energy explosive, ammonium dinitramide.

The liver is the most important organ for metabolism of chemicals in the body. The effects of free radicals on liver can be studied using precision cut liver slices (Barr et al, 1991). Slices can be prepared from the liver of research animals as well as from liver donors. Slices are most like the liver *in vivo* than other *in vitro* techniques because it includes all the populations of cells necessary to study cytotoxicity. The principal technique used to determine free radicals is electron paramagnetic resonance spectroscopy (EPR). EPR is the most sensitive and direct method of measuring free radicals (Mason 1984). In general, the technique measures the effect of a magnetic field on an unpaired electron (free radicals and transition metals). The spinning electron acts as a small magnet. It also interacts with neighboring nuclei. When placed in an external electric field, information is obtained regarding the local environment surrounding the unpaired electron (Bruker 1992). In biological systems free radicals are mostly short-lived and highly reactive species reacting at diffusion controlled rates. For this reason the technique of spin trapping is used for detection of radicals (Buttner 1987).

Spin trapping consists of reacting a short lived radical with a spin trap, usually a nitron or nitroso compound yielding a longer lived nitroxide spin adduct which can be detected by EPR(Mason 1984, Buttner 1987). There are a number of spin traps which can be used to study free radicals at the cellular and sub-cellular level of tissue (Mason 1984, Buttner 1987, Knecht & Mason 1993). There are a number of spin traps available but literature review suggested N-tert-butyl- α phenyl nitron (PBN) should be the trap of choice for liver experiments (Mason 1984, Buttner 1987, Knecht & Mason 1993, Chignell 1984, Rice-Evans et al 1991).

Spin labeling is a technique which makes use of stable nitroxide radicals to label biological components of a cell, allowing them to be monitored by EPR(Rice-Evans et al 1991). A nitroxide spin label can act as a biological marker and yield information on the environment and motion of the component to which it is attached. Subtle changes in environment and motion of the spin labeled component are observed and measured through changes in the nitroxide EPR line shape. It was hypothesized that the spin label 2,2,5,5-tetramethyl -1-pyrrolidinyl oxy-3-carboxamide will have a different EPR line shape when lyophilized with mouse liver slices with and without the nitroxide spin trap PBN.

PBN has been used previously to trap radicals produced by the oxidizing agent ammonium dinitramide(Armengol 1995, Berty 1994, Young 1994). It has also been studied in human and mouse liver slices (Steel-Goodwin et al 1994, Pravec et al 1994, Steel-Goodwin 1995). It is currently used to trap radicals in liver slices exposed to a number of chemical carcinogens. Understanding the efficiency of PBN to trap these

radicals is important for their quantitation. The interaction of 3-CAR with PBN was used to assess the efficiency of this nitroxide to trap a free radical.

METHODS

Chemicals

N-tert-butyl- α phenyl nitron (PBN) and 2,2,5,5,-tetramethyl-1-pyrrolidinyl oxy-3-carboxamide (3-CAR) were obtained from Aldrich and Kodak Chemical respectively.

PBN (1M) was dissolved in DMSO obtained from Sigma Chemical Co, St. Louis MO.

Sample preparation

Ten slices of B6C3F1 mouse liver which were extras not needed from experiments on lipid peroxidation were used in this study. The slices were prepared from B6C3F1 mice as described previously (Steel-Goodwin et al 1994). Each slice was homogenized in preweighed scintillation vials containing solutions supplemented with or without 10mM PBN. 3-CAR was added to the homogenate at concentrations ranging from 0 to 0.5 mM. The homogenate was immediately frozen in liquid nitrogen and lyophilized for 18 h.

EPR analysis

The lyophilized liver homogenate was weighed. A known weight of sample was added to a glass micropipette (Clay Adams, Becton, Dickinson and Company, Parsippany, N.J.) The micropipette was placed in an EPR tube and put in the cavity of a precalibrated EMS104 EPR analyzer. The instrument parameters were: Power 25.06 mW, Sweep width 100 G, Modulation 4.02 G, Sweep Time 10.49 s, Filter time constant 20.48 ms, Receiver Gain 45 dB. The spectra were measured by peak-peak and double integration and compared with and without PBN. The data was normalized to liver weight. From this data the trapping efficiency of the PBN for 3-CAR was determined using the equation:

$$\text{Efficiency} = \frac{\text{EPR Reading 3-CAR}_{\text{PBN}}}{\text{EPR Reading 3-CAR}_{\text{PBN}}} \times 100 \quad \text{Equation 1}$$

Data analysis

The data was analyzed by Analysis of Variance using Design Ease®, computer program.

RESULTS

The EPR spectra of 3-CAR and PBN-3-CAR are shown in Figure 1. When the 3-CAR spin label is in solution the electrons are free to tumble giving a first derivative spectrum shown in, Figure 1A.

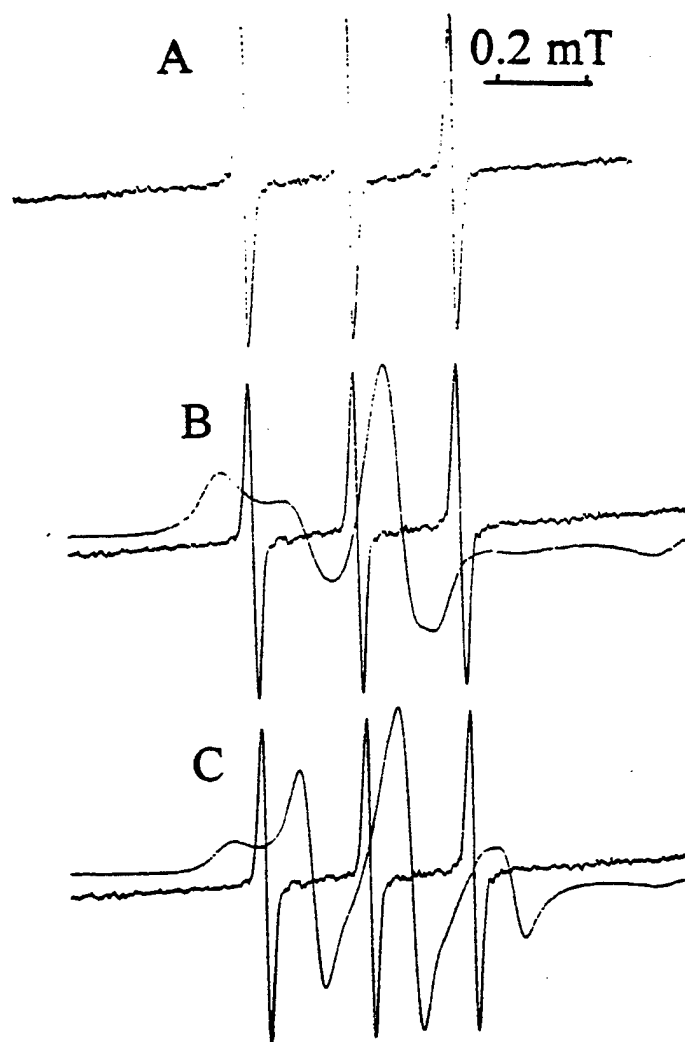


Figure 1. EPR first derivative spectra of 3-CAR. (A) 3-CAR in solution (B) 3-CAR before and after lyophilization (C) 3-CAR before and after lyophilized with liver slice in the presence of 10mM PBN.

However, when added to liver and lyophilized to a powder the free radicals are immobilized. Figure 1B shows the first derivative spectrum of 3-CAR in solution before and after lyophilization. The hyperfine splitting of the 3-CAR spectrum is no longer the same because the electrons are immobilized in the solid and are not freely moving. A spin trap will react with a free radical. When PBN reacts with 3-CAR it gives a spectrum with a difference in the line shape, Figure 1C. Comparison of the spectra of lyophilized liver with 3-CAR $\pm$ 10 mM PBN, Figure 2, show subtle differences in the hyperfine splitting. The difference in these spectra are shown in Figure 2B.

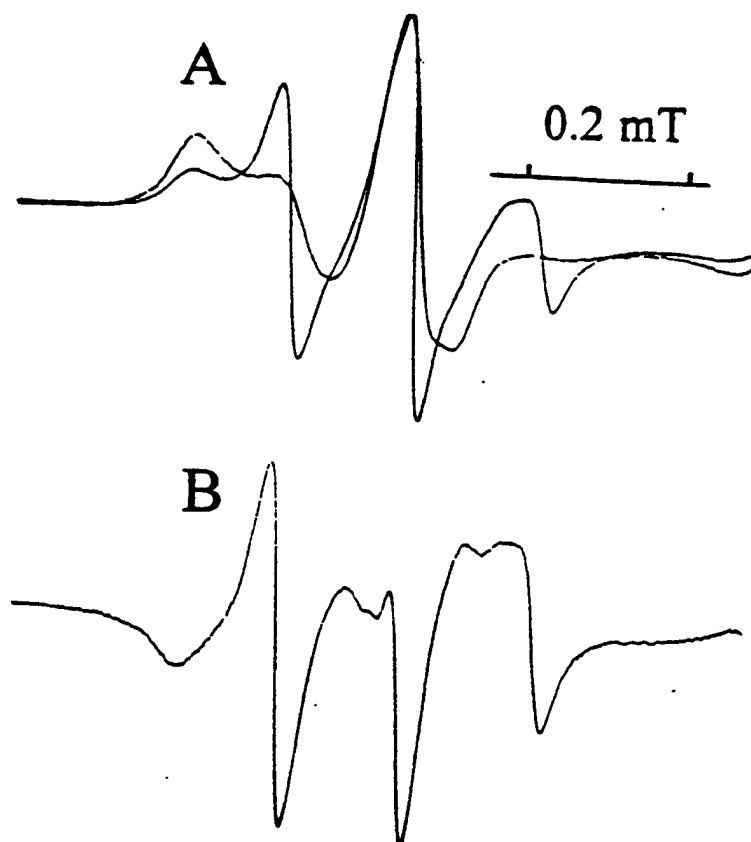


Figure 2 *First derivative spectra of 3-CAR lyophilized with liver. (A) Spectra 3-CAR $\pm$ 10mM PBN (B) Difference spectrum of the first derivative spectra shown in (A).*

The peak-peak values and the double integration of the EPR first derivative spectra of lyophilized 3-CAR were compared with and without 10mM PBN and normalized for liver weight. This data is summarized in Table I.

Treatment Group	[PBN] mM	Double Integration EPR a.u.	Peak Height EPR a.u.
A	0	1016 ± 50	0.60 ± 0.0001
A	10	272 ± 22*	0.23 ± 0.04*
B	0	287 ± 3	0.66 ± 0.009
B	10	272 ± 21	1.09 ± 0.004*
C	0	8374 ± 40	5.25 ± 0.02
C	10	2584 ± 103*	3.75 ± 0.002*
F	0	1597 ± 191	1.07 ± 0.001
F	10	972 ± 11*	0.99 ± 0.005*
G	0	6169 ± 356	3.00 ± 0.008
G	10	1749 ± 120*	1.51 ± 0.02*

Table 1. Mean ± SD of computer analysis of data., * $p < 0.050$

The computer analysis of this data indicated the data was normally distributed and there were significant differences between the groups studied. Figure 3 pictorially shows the results of the computer analysis.

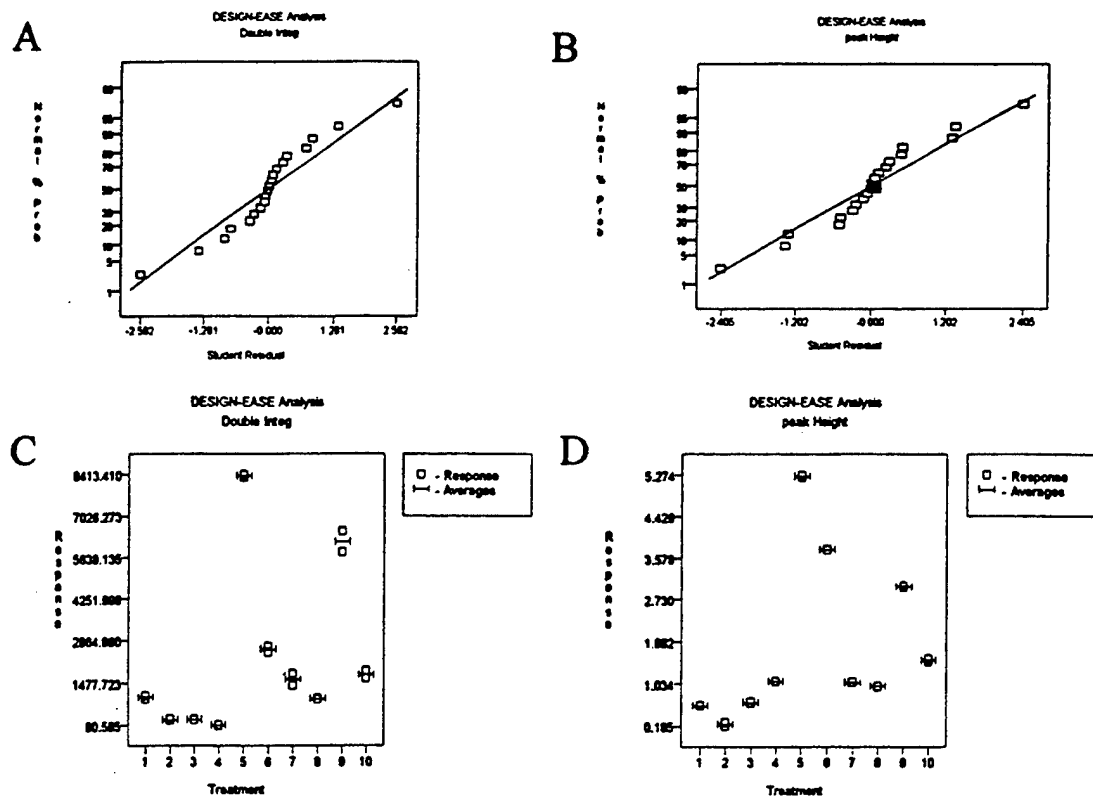


Figure 3 Results of ANOVA (A) Normal probability of double integration results (B) Diagnostic graph of response of double integrations (C) Normal probability of peak height results (D) Diagnostic graph of response of peak heights

Figure 3A is the normal distribution of the double integration of the EPR spectrum and Figure 3B shows the average differences between the groups. Figure 3C is the normal distribution of the peak height of the EPR spectrum and Figure 3D is the average differences between the groups.

Using equation 1, the efficiency of PBN to trap 3-CAR was calculated using the double integration data, Table 2.

Double Integration Data

37.07
36.79
32.04
32.26
33.38
33.05
33.35
33.03
26.14
26.12
29.51
29.48
43.48
43.15
37.58
37.83
30.82
30.52
30.8
30.5
29.97
29.95
33.84
33.84
 33 ± 4

Table 2. *Results of calculations of trapping efficiency using Equation 1*

From this data the efficiency of PBN to trap 3-CAR was $33 \pm 4 \%$.

DISCUSSION

The efficiency of PBN to trap the radiation induced TCE• radical has been calculated to be 20.6% (Steel-Goodwin and Carmichael 1995). This radical has been shown to have the same hyperfine coupling constant as the TCE radical produced in liver slices (Carmicheal and Steel-Goodwin, unpublished data).

The liver slice technique is a useful method to study the metabolism and biological effects of chemicals (Fraga et al 1988). It has been used to study the effects of TCE on mouse and human liver slices (Steel-Goodwin et al 1994, Pravecsek et al 1994) and more recently the effects of oxidizer ammonium dinitramide on human slices (Steel-Goodwin 1995).

For studies involving quantitation it should always be remembered that the radicals trapped are not necessarily all the radicals present in the tissue. Radicals are unpaired electrons which make them very reactive. The amount of radicals trapped depends on a number of assumptions.

First, the trap must be non-toxic. Studies by Mason (1984) suggest that PBN is nontoxic at 10 mM which is the concentration used in these experiments. Second, there is the assumption that the trap is not being metabolized by the tissue. In this experiment the samples were harvested immediately preventing liver metabolism. Third, there has to be sufficient trap to react with the radicals rather than other components of the cell. In this experiment we choose 3-CAR, a stable radical, because studies suggest it is not permeable to hepatocytes (Steel-Goodwin and Dean unpublished data).

The concentration of the 3-CAR $\pm$ PBN is proportional to the peak-peak and double integration of the spectrum and this was used to calculate the trap efficiency. The efficiency of PBN to trap 3-CAR was determined to be $33 \pm 4 \%$. This value is similar to the efficiency of this trap for the lipophilic TCE $\bullet$ radical.

In conclusion, if radicals are quantitated using EPR/spin trapping technique, the efficiency of the trap should be determined for each compound which is being studied. Quantitation of radicals by EPR/spin trapping only detects the radicals trapped. If the efficiency of the trap for the radical is known, then a true estimate of the radicals present in the sample can be determined.

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