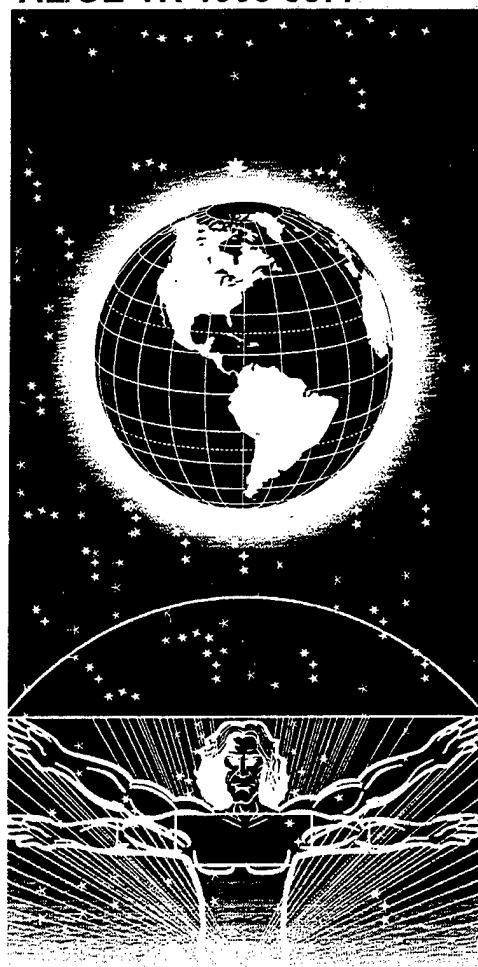




**UNITED STATES AIR FORCE  
ARMSTRONG LABORATORY**

---

**FREE RADICALS IN PRECISION  
CUT BOVINE TESTICLE SLICES:  
EFFECTS OF 1,3,5 TRINITROBENZENE  
MEASURED BY ELECTRON  
PARAMAGNETIC RESONANCE**

**Linda Steel-Goodwin**

OCCUPATIONAL AND ENVIRONMENTAL HEALTH  
DIRECTORATE TOXICOLOGY DIVISION  
ARMSTRONG LABORATORY  
WRIGHT-PATTERSON AFB, OH 45433-7400

**John F. Wyman**

MANTECH ENVIRONMENTAL TECHNOLOGY, INC.  
DAYTON, OH 45437

**Alasdair J. Carmichael**

ARMED FORCES RADIOBIOLOGY RESEARCH INSTITUTE  
BETHESDA, MD 20991

**November 1996**

**Occupational and Environmental Health  
Directorate  
Toxicology Division  
2856 G Street  
Wright-Patterson AFB OH 45433-7400**

Approved for public release; distribution is unlimited.

## NOTICES

When US Government drawings, specifications or other data are used for any purpose other than a definitely related Government procurement operation, the Government thereby incurs no responsibility nor any obligation whatsoever, and the fact that the Government may have formulated, furnished, or in any way supplied the said drawings, specifications, or other data is not to be regarded by implication or otherwise, as in any manner licensing the holder or any other person or corporation, or conveying any rights or permission to manufacture, use, or sell any patented invention that may in any way be related thereto.

Please do not request copies of this report from the Air Force Armstrong Laboratory. Additional copies may be purchased from:

National Technical Information Service  
5285 Port Royal Road  
Springfield, Virginia 22161

Federal Government agencies and their contractors registered with the Defense Technical Information Center should direct requests for copies of this report to:

Defense Technical Information Service  
8725 John J. Kingman Rd., Ste 0944  
Ft. Belvoir, Virginia 22060-6218

## DISCLAIMER

This Technical Report is published as received and has not been edited by the Technical Editing Staff of the Air Force Armstrong Laboratory.

## TECHNICAL REVIEW AND APPROVAL

**AL/OE-TR-1996-0077**

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**



**STEPHEN R. CHANNEL**, Maj, USAF, BSC  
Branch Chief, Operational Toxicology Branch  
Air Force Armstrong Laboratory

| REPORT DOCUMENTATION PAGE                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                          |                                                                 |                                                                | Form Approved<br>OMB No. 0704-0188                                                 |                                                                              |
|--------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|-----------------------------------------------------------------|----------------------------------------------------------------|------------------------------------------------------------------------------------|------------------------------------------------------------------------------|
| Public reporting burden for this collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing the collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden, to Washington Headquarters Services, Directorate for Information Operations and Reports, 1215 Jefferson Davis Highway, Suite 1204, Arlington, VA 22202-4302, and to the Office of Management and Budget, Paperwork Reduction Project (0704-0188), Washington, DC 20503.                                                                                                                                                                                         |                                                                 |                                                                |                                                                                    |                                                                              |
| 1. AGENCY USE ONLY (Leave blank)                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                   |                                                                 | 2. REPORT DATE<br>April 1996                                   |                                                                                    | 3. REPORT TYPE AND DATES COVERED<br>Interim Report - January 1994-March 1996 |
| 4. TITLE AND SUBTITLE<br>Free Radicals in Precision Cut Bovine Testicle Slices: Effects of 1,3,5 Trinitrobenzene Measured by Electron Paramagnetic Resonance                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                       |                                                                 |                                                                | 5. FUNDING NUMBERS<br>Contract<br>PE 61102F<br>PR 2312<br>TA 2312A2<br>WU 2312A202 |                                                                              |
| 6. AUTHOR(S)<br>L. Steel-Goodwin, J.F. Wyman, and A.J. Carmichael                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                  |                                                                 |                                                                |                                                                                    |                                                                              |
| 7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)<br>Armstrong Laboratory, Occupational and Environmental Health Directorate<br>Toxicology Division, Human Systems Center<br>Air Force Materiel Command<br>Wright-Patterson AFB, OH 45433-7400                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                    |                                                                 |                                                                | 8. PERFORMING ORGANIZATION<br>REPORT NUMBER                                        |                                                                              |
| 9. SPONSORING/MONITORING AGENCY NAME(S) AND ADDRESS(ES)<br>Armstrong Laboratory, Occupational and Environmental Health Directorate<br>Toxicology Division, Human Systems Center<br>Air Force Materiel Command<br>Wright-Patterson AFB, OH 45433-7400                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                               |                                                                 |                                                                | 10. SPONSORING/MONITORING<br>AGENCY REPORT NUMBER<br><br>AL/OE-TR-1996-0077        |                                                                              |
| 11. SUPPLEMENTARY NOTES                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                            |                                                                 |                                                                |                                                                                    |                                                                              |
| 12a. DISTRIBUTION AVAILABILITY STATEMENT<br><br>Approved for public release; distribution is unlimited.                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                            |                                                                 |                                                                | 12b. DISTRIBUTION CODE                                                             |                                                                              |
| 13. ABSTRACT (Maximum 200 words)<br>Precision cut tissue slices were prepared from bovine testes and incubated at 37 degrees C in Wymouth's media supplemented with the spin trap N-tert-butyl-a-nitron or dimethyl sulfoxide. Slices were exposed for 0 to 1h with 0-1000 uM 1,3,5 trinitrobenzene (TNB). Radicals were detected in testis after glutathione depletion with diethylmaleate in control, TNB-treated and superoxide-treated slices. The hyperfine coupling constant of the TNB-induced PBN-radical adducts was aN=1.629 mT and aHB=0.353 mT. Analysis of variance showed more radicals were trapped in the TNB-treated slices 100 uM-1000uM when compared to controls (P<0.0001). First order regression analysis of the dose response curve was y=0.04x+23(r2=0.66, n=30). This data shows the <i>in vitro</i> slice technique can be used to measure radicals induced by TNB in bovine testicles. |                                                                 |                                                                |                                                                                    |                                                                              |
| 14. SUBJECT TERMS<br>1,3,5 Trinitrobenzene (TNB)      testis      tissue slice<br>free radicals      superoxide      EPR/spin trapping                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                             |                                                                 |                                                                | 15. NUMBER OF PAGES<br>34                                                          |                                                                              |
|                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                    |                                                                 |                                                                | 16. PRICE CODE                                                                     |                                                                              |
| 17. SECURITY CLASSIFICATION<br>OF REPORT<br><br>UNCLASSIFIED                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                       | 18. SECURITY CLASSIFICATION<br>OF THIS PAGE<br><br>UNCLASSIFIED | 19. SECURITY CLASSIFICATION<br>OF ABSTRACT<br><br>UNCLASSIFIED | 20. LIMITATION OF<br>ABSTRACT<br><br>UL                                            |                                                                              |

## PREFACE

This is one of a series of technical reports describing the results of the electron paramagnetic resonance laboratory data conducted at the Occupational and Environmental Health Directorate, Toxicology Division and at the Armed Forces Radiobiology Research Institute, Bethesda MD. The samples were prepared by and the spectra generated by Dr. John F. Wyman when he was supported by the US Army and the Department of the Air Force Contract No 33615-90-C-0532 from 1994-1996. Collaborative interpretation of the spectra was sponsored by the Air Force Office of Scientific Research Environmental Initiative Program work unit 2312A202 and AFRRRI Work Unit 04630. Lt Col Terry A. Childress served as Contract Technical Monitor for the US Air Force, Armstrong Laboratory, Toxicology Division.

The authors wish to thank Mr. Larry Landes and Landes Meats Inc. for their donation of bull testicles and Dr J Lipscomb for his advice on glutathione reactions. The animals used in this study were handled in accordance with the principles stated in 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.

## TABLE OF CONTENTS

| SECTION                      | PAGE |
|------------------------------|------|
| PREFACE .....                | iii  |
| TABLE OF CONTENTS.....       | iv   |
| LIST OF FIGURES .....        | v    |
| ABBREVIATIONS .....          | vi   |
| 1 INTRODUCTION .....         | 1    |
| 2 MATERIALS AND METHODS..... | 4    |
| Chemicals.....               | 4    |
| Tissue preparation.....      | 4    |
| Statistical analysis.....    | 5    |
| 3 RESULTS & DISCUSSION ..... | 6    |
| 4 REFERENCES .....           | 14   |
| 5 APPENDIX A.....            | 17   |
| 6 APPENDIX B .....           | 22   |

## LIST OF FIGURES

| FIGURE                                                                                                                                                               | PAGE |
|----------------------------------------------------------------------------------------------------------------------------------------------------------------------|------|
| 1     A. Spectrum of control testis slice.<br>B. Spectrum of TNB treated testis slice.<br>C. Control media no testis .....                                           | 6    |
| 2     A. Spectrum of TNB-induced radicals in testis slices<br>B. Computer simulated spectrum of A<br>C. Control media of TNB and no testis.....                      | 7    |
| 3     Possible reaction of (a) DMSO and (b) PBN with decomposition<br>Product of TNB.....                                                                            | 8    |
| 4     A. Spectrum of testes slices incubated in DMSO<br>B. Spectrum of testes slices incubated in TNB in DMSO<br>C. Spectrum of control media with DMSO .....        | 9    |
| 5     A. Spectra of control slices exposed to superoxide with and without DEM<br>B. Spectra of TBN treated slices exposed to superoxide with and without<br>DEM..... | 10   |
| 6     EPR intensity of slices after incubation for 1 h in 0-1 mM TNB .....                                                                                           | 12   |

## ABBREVIATIONS

|         |                                              |
|---------|----------------------------------------------|
| C       | Centigrade                                   |
| DEM     | Diethylmaleate                               |
| DMSO    | Dimethyl sulfoxide                           |
| EPR     | Electron Paramagnetic Resonance spectrometer |
| g       | Gram                                         |
| h       | Hour                                         |
| K       | Kelvin                                       |
| L       | Liter                                        |
| mg      | Milligram                                    |
| mm      | millimeter                                   |
| PBN     | N-tert-butyl- $\alpha$ -nitron               |
| T       | Tesla                                        |
| TNB     | Trinitrobenzene                              |
| $\mu$ l | microliter                                   |

## SECTION 1

### INTRODUCTION

1, 3, 5 Trinitrobenzene (TNB) has been identified as a contaminant along with other munitions in soil and ground water at numerous U.S. Army installations (Walsh and Jenkins, 1992). TNB is a nitroaromatic produced as a biproduct of the manufacture of trinitrotoluene. There are a number of studies reporting damage to the testes in experimental animals by nitroaromatics (Foster, 1992). For example, in experimental animals nitrobenzene (Bond et al, 1981), dinitrobenzene (Cody et al., 1981) and trinitrotoluene (Levine et al., 1981) cause testicular damage. Rats exposed to TNB in diet had depleted sperm counts and degeneration of seminiferous tubules (Kinkead et al, 1995). We found no reports that TNB is a human reproductive toxicant, but there are reports that male workers exposed daily to organic solvents such as benzene, toluene or dinitrotoluene and toluene diamine have oligoasthenoteratozoospermia (Radike, 1985). Oligoasthenoteratozoospermia is a condition of reduced sperm count and those sperm present are immobile and abnormal looking.

*In vivo* rat studies (Kinkead et al., 1995) provided limited information on the mechanism of TNB toxicity, so a precision cut testis slice model was developed. Because of the relatively low amount of intertubular connective tissue, slices from rat testes could not be prepared (Wyman et al., 1996). Bull and dog testes have sufficient intertubular connective tissue to allow preparation of proper slices. The evaluation of TNB toxicity was performed using bull testes because this tissue was easily obtained through a local meat processing company.

Using this *in vitro* slice model, histology preparations of bull testes slices exposed to TNB showed increased necrosis of the germinal epithelium compared to control slices (Wyman et al., 1996) which was a finding consistent with that observed in previous *in vivo* exposures (Kinkead, et al., 1995)

In the presence of diethyl maleate (DEM), testicular slices also had increased lipid peroxidation as measured by TBARS assay. DEM is known to deplete endogenous levels of the antioxidant, glutathione. TNB, in the presence of DEM, blocked the increase in lipid peroxidation in testicular slices (Wyman, et al., 1996).

Lipid peroxidation is a chain reaction which results from the abstraction of a hydrogen atom from a methylene group by a reactive species (Gutteridge, 1995a). Examples of species which can abstract the hydrogen and initiate lipid peroxidation are the hydroxyl radical, alkoxyl radical and peroxy radical (Gutteridge, 1995b). In the testis slice study TBARS depression was dose-dependent (Wyman et al., 1996) and suggested TNB or its decomposition products altered free radical levels or even generated radicals which did not have enough energy to abstract a hydrogen atom necessary for lipid peroxidation.

To test this hypothesis we used electron paramagnetic resonance (EPR) to detect free radicals. It was reasonable to expect that radical adducts generated by testicular slices may be trapped and detected by EPR using spin traps. A number of spin traps have been used to detect radicals in biological slices (Steel-Goodwin et al 1992, Myers et al 1994 and Steel-Goodwin et al 1995). This technique involves the use of nitroso compounds (Lagercratz, 1971) and nitrones (Janzen, 1971) which react with short-lived

radicals to produce more stable adducts (Buettner, 1982). The spectral information contained in the adduct is often less than that of the parent radical (Joshi and Yang, 1981).

Free radical chemistry of the testis was studied to elucidate TNB toxicity effects because: *one*, nitroaromatic compounds have been reported to act through free radical pathways (Romero, et al., 1995); *two*, cells in the seminiferous tubules are very sensitive to free radical damage and are protected by a blood-testis barrier; *three*, the seminiferous tubules have very low levels of catalase (Burhley and Ellis, 1973) which reacts very rapidly with hydrogen peroxide and is important when glutathione peroxidase or reduced glutathione levels are unavailable to remove hydrogen peroxide (Rice Evans et al., 1991) and *four*, unique histones are present in the testes which may act as free radical scavengers (Zenick and Clegg, 1986). Other nitrocompounds have been reported to have cytotoxic effects in testes because their reduction catalyses the formation of superoxide radicals (Decompo et al 1981). Superoxide radicals cannot initiate lipid peroxidation and neither can hydrogen peroxide (Gutteridge, 1995b) and this could explain the lipid peroxidation effects observed in this study (Wyman et al., 1996). The possibility of TNB generated radicals was investigated using EPR/spin trapping.

## SECTION 2

### MATERIALS & METHODS

**Chemicals:** 1, 3, 5-Trinitrobenzene (TNB) was obtained from Dr. Gunda Reddy, Army Biomedical Research and Development Laboratory, Ft. Detrick, Frederick, MD. All other chemicals were research grade obtained from Sigma or Aldrich.

**Sample Analysis:** Bovine testicles were donated by Landes Meats, Inc., Dayton, OH.

The tissue was prepared and sliced as described previously (Wyman et al 1996) and placed in 1.7 ml incubation medium. The incubation medium ( $\text{pH } 7.4 \pm 0.1$ ) was Waymouth's supplemented with 10% fetal calf serum (Gibco), gentamycin (84mg/ml), L-glutamine (3.5 mg/ml) and sodium bicarbonate (2.4 mg/ml). A saturated solution of potassium superoxide (Aldrich Chemical Co) was prepared in 250  $\mu\text{l}$  dimethylsulfoxide (DMSO, Aldrich Chemical Co) and 5  $\mu\text{l}$  added in experiments involving superoxide. TNB was dissolved in DMSO and added to the media to give final concentrations of 0, 50, 100, 500, or 1000  $\mu\text{M}$  as described previously (Wyman et al 1996). The spin trap, N-tert-butyl- $\alpha$ -phenyl nitron (PBN, Aldrich Chemical Co) was weighed and dissolved in 500  $\mu\text{l}$  DMSO just prior to addition to the incubation media so that the final concentration of PBN in the incubate was 10 mM. All incubations were carried out in media supplemented with diethylmaleate (DEM, Wyman et al., 1996) unless otherwise stated. The slices were incubated in glass scintillation vials and throughout the experiment they were protected from ultraviolet and fluorescent light sources. At the end of the incubation period 0-60 min, the testes slice was homogenized in the media, frozen in liquid nitrogen (77K) and lyophilized. The lyophilized media was rehydrated in 100 $\mu\text{l}$  double distilled water,

vortexed and 20  $\mu$ l was drawn into a glass capillary tube, sealed, and placed in the cavity of an EMS EPR Analyzer (Bruker Instruments, Billerica, MS). The EPR instrument was calibrated with pitch (Bruker, Instruments, MS) and operated, unless otherwise noted, at a magnetic field set at 3.475 mT, modulation amplitude 0.16mT, time constant 1310.72 ms, scan time 167.77 ms, scan range 0.6mT and temperature 25°C. Hyperfine values were determined by direct measurement from the spectra and computer simulated.

**Statistical Analysis:** Analysis of variance was performed using Design Ease® and regression analysis was determined by Sigma Stat® and Sigma Plot® statistical programs.

## SECTION 4

### RESULTS & DISCUSSION

In the control bull testes there were radical adducts detected when the slices were incubated with DMSO alone and with the PBN spin trap. This suggests either the radicals detected are the result of cell damage or that the slices produce radicals as part of their normal metabolism. Cell viability data and TBARS analysis support the latter explanation (Wyman et al, 1996). Figure 1A shows the typical spectrum of PBN radical adducts in lyophilized slices following 10 min incubation. In the presence of TNB, radicals were also detected, Figure 1B. Control media gave only a broad signal, Figure 1C.

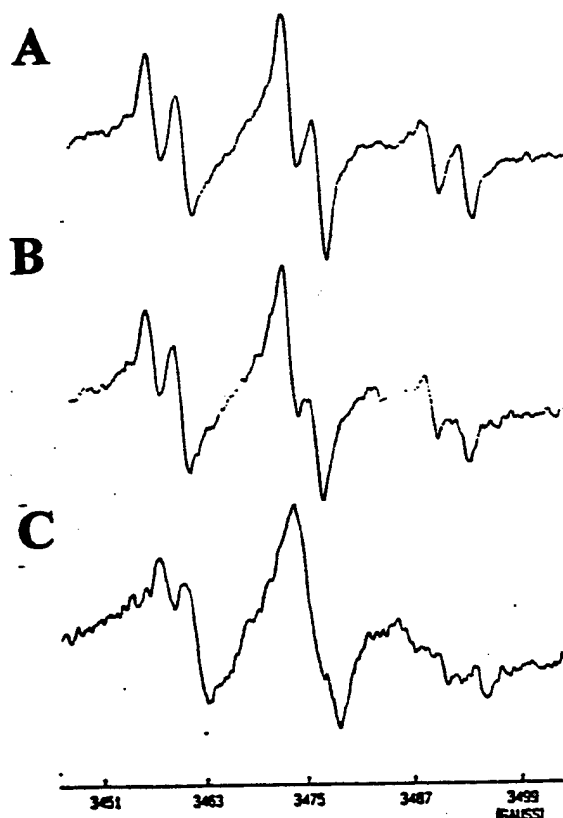
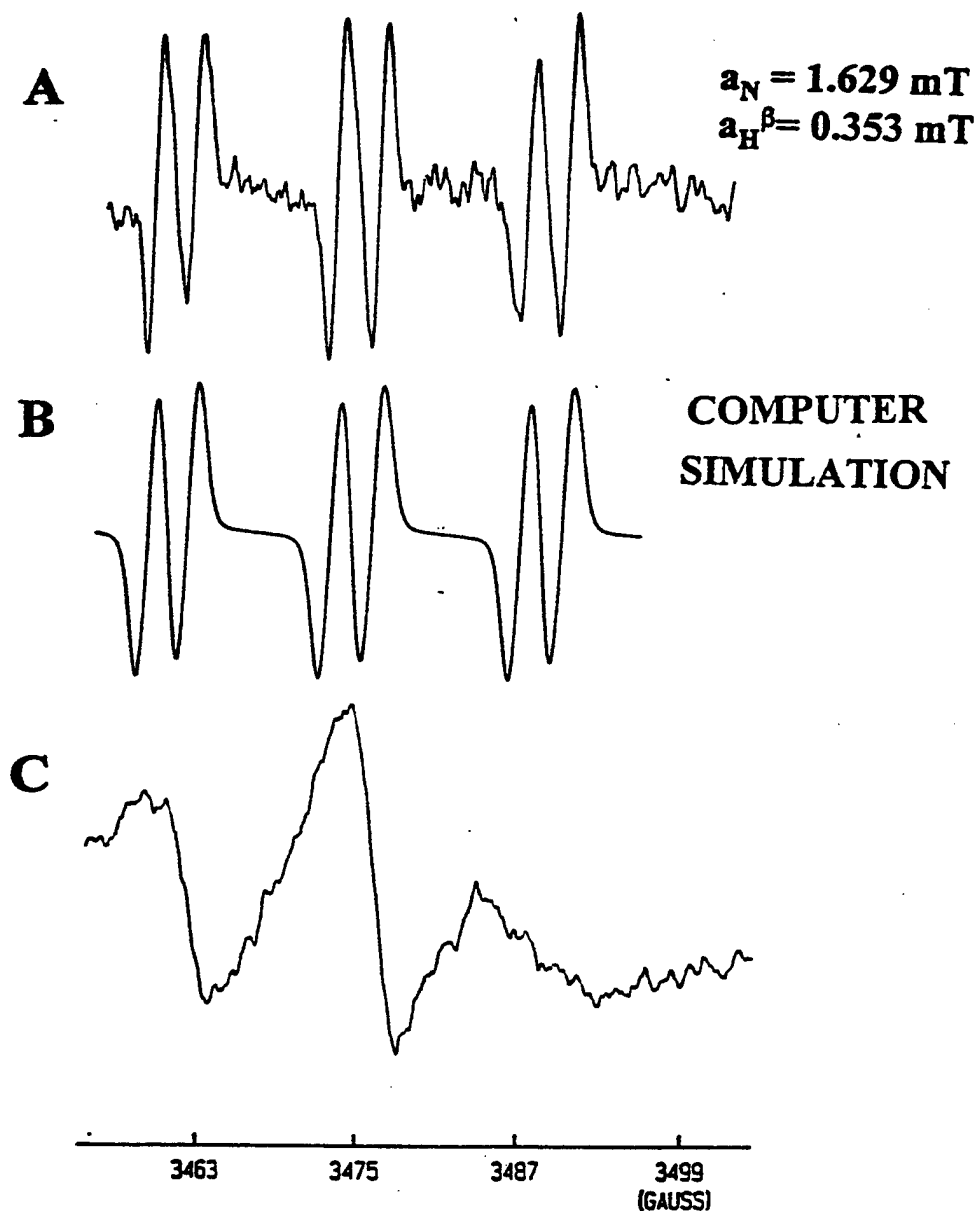


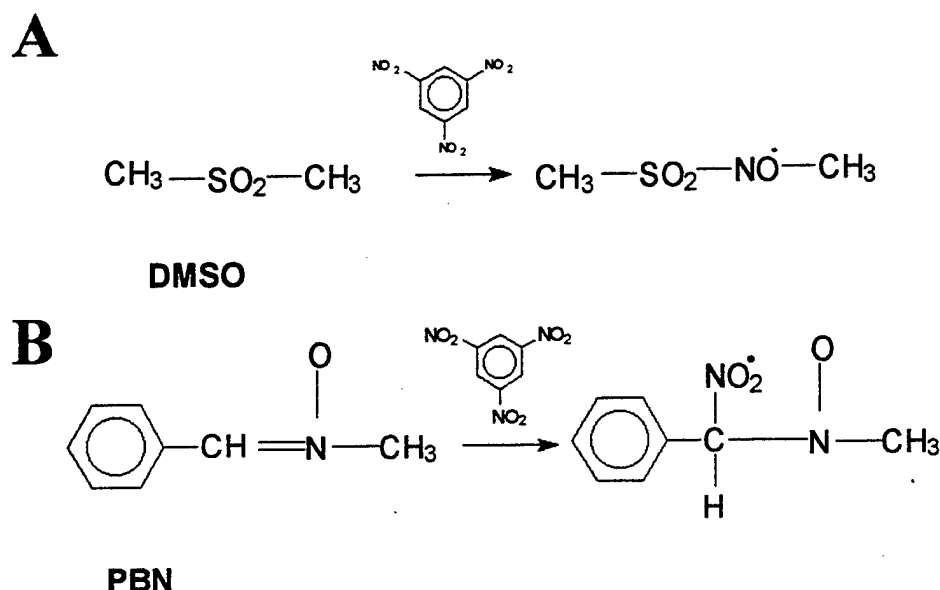
Figure 1. *A. Spectrum of control testis slice. B. Spectrum of TNB-treated testis slice*  
*C. Control media no testis*

The radicals induced by TNB are shown in Figure 2A. When this spectrum was computer simulated the hyperfine coupling constants were  $a_N = 1.629 \text{ mT}$  and  $a_H^\beta = 0.353 \text{ mT}$ , Figure 2B. TNB alone without slices had a broad signal, Figure 2C, which was similar to media alone, Figure 1C.



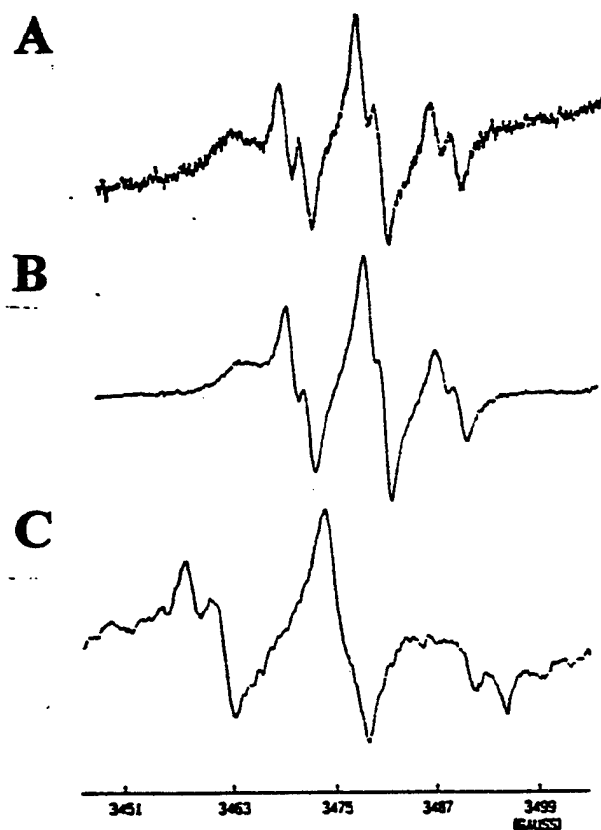
**Figure 2** A Spectrum of TNB induced radicals in testis slices B. Computer simulated spectrum of A. C Spectrum of media with TNB and no testis.

Trinitrobenzene (TNB) is a nitroaromatic which we hypothesised was metabolised similar to dinitrobenzenes and would produce an EPR signal in DMSO, the solvent used to dissolve TNB and the spin trap, PBN. Possible radical adducts are shown in Figure 3.



**Figure 3** *Reactions of DMSO and PBN with decomposition products of TNB.*

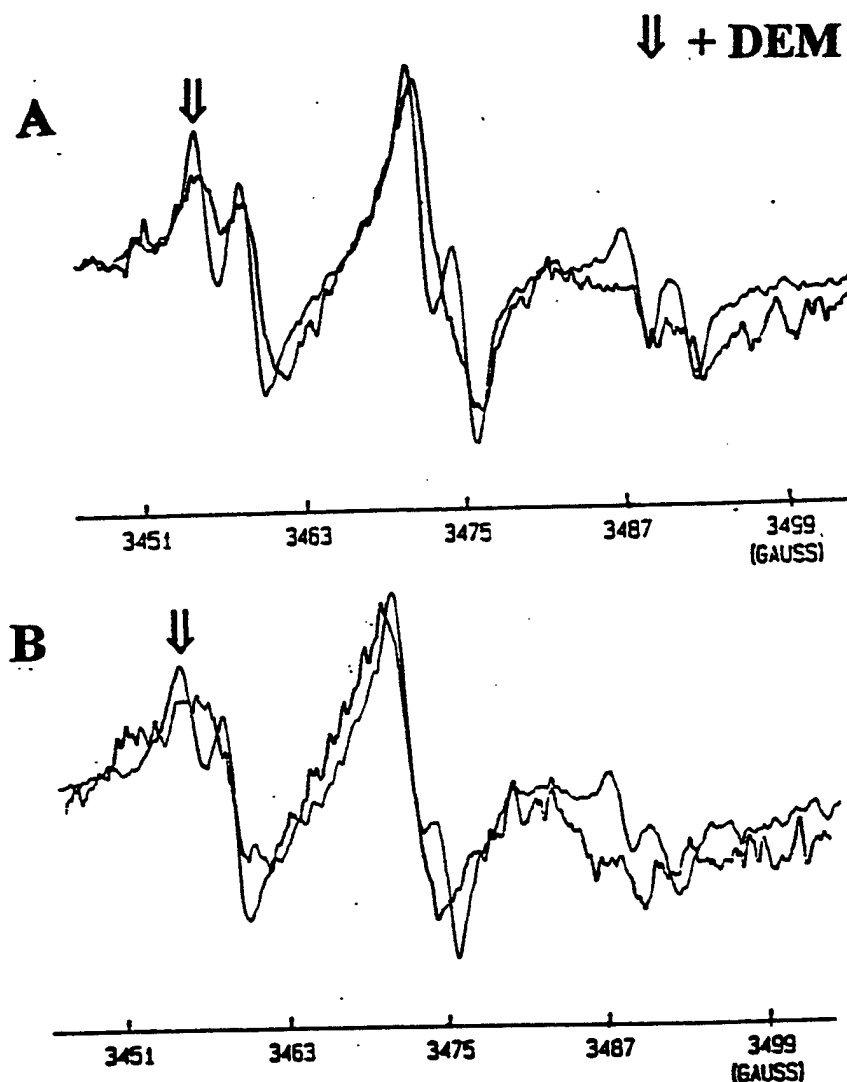
Experimentally radicals were detected in DMSO alone, as well as with PBN, Figures 1& 2. DMSO is a classical solvent for studying the EPR signal of nitrobenzene (Rice Evans et al., 1991) and recently DMSO has been used to trap peroxynitrite-induced radicals formed by reaction of nitric oxide with superoxide (Carmichael and Steel-Goodwin, 1994). Figure 4 shows the radicals detected in DMSO in the presence of slices alone, Figure 4A and with TNB, Figure 4B. Similar spectra were observed in both the control and TNB-treated slices. Control media had a broad signal, Figure 4C.



**Figure 4** *A Spectrum of testis slices incubated in DMSO. B. Spectrum of testis slices incubated with TNB in DMSO. C. Spectrum of control media.*

To determine if the cytotoxic effects of TNB in testis slices was because TNB-reduction catalyzes the formation of superoxide radicals (Decampo et al, 1981), experiments were repeated with and without dimethylmaleate (DEM) in the incubate in the

presence of superoxide and the results were compared with TNB-exposed samples, treated similarly, Figure 5.

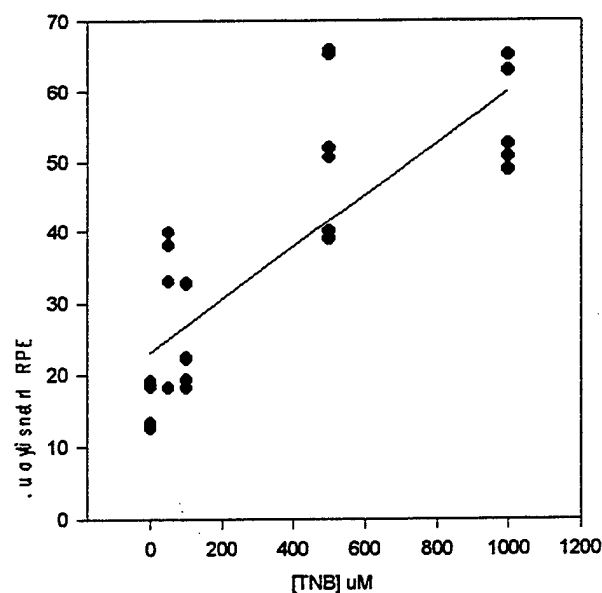


**Figure 5** *A The spectra of control slices exposed to superoxide with and without DEM. B The spectra of TNB treated slices exposed to superoxide with and without DEM.*

Slices of testis incubated without DEM had a broad spectrum, Figure 5A even though superoxide was added. The typical PBN-radical adducts shown in Figure 1A were

detected in the presence of DEM and superoxide, Figure 5A. The TNB-treated slices, Figure 5B were similar to the controls, Figure 5A. In the presence of DEM and superoxide, a triplet of doublets was detected. With no DEM in the incubate the signal was similar to control TNB media, Figure 2C. DEM is a commonly used reagent for tissue glutathione depletion. DEM conjugates with glutathione and this raises the baseline of TBARS (Wyman unpublished data). DEM presence in the incubate probably alters availability of NADPH, an electron donor. Well resolved EPR spectra are known for many of the dinitrobenzenes where superoxide reacts to yield corresponding nitrophenols (Poupko and Rosenthal, 1973), but we found no literature on the effects of superoxide with TNB. The present experimental data does suggest testes slices incubated *in vitro* for 0-60 min with TNB releases free radicals, Figures 1, 2, 4 & 5.

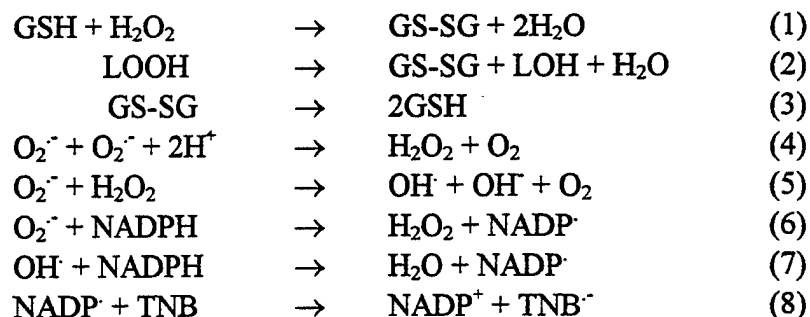
EPR can be used to determine the quantity of radicals by measurement of the intensity of the EPR signal. The EPR intensity measured in the testes slices after incubation for 60 min is shown in Figure 6. By analysis of variance there was more radicals detected in slices with TNB compared to controls ( $r^2 = 0.73$ ,  $F = 27.47$ ,  $P < 0.0001$ ), Appendix A. Linear regression analysis of the plot shown in Figure 6 is given in Appendix B. Further studies are necessary to measure the actual number of radicals detected with known radical standards, such as stable spin labels which have been used successfully for measuring TCE radicals (Steel-Goodwin et al 1994).



**Figure 6.** *EPR intensity of slices after incubation for 1h in 0-1 mM TNB.*

Nitroaromatics are believed to be metabolized by nitroreductases by a mechanism which uses NADPH as the electron donor and molecular oxygen which leads to production of the superoxide radical and regeneration of the nitrocompound (Decompo et al 1981, Mason & Josephy 1985, Biaglow et al 1986, Mason 1990, Romero et al 1995). DEM depletes glutathione in the testis slices and raises the TBARS baseline so it is reasonable to suggest that hydroxyl radicals are generated in the slices and these are involved in the TNB-induced testicular damage. Equations 1-8 shown below are the possible events. Cells are equipped with NADPH-dependent glutathione reductase to reduce the oxidized glutathione, Equation 3. Although superoxide dismutase is the major antioxidant enzyme in the testis, Equation 4, NADPH is also important in reducing

superoxide to hydrogen peroxide, Equation 6. NADPH is also important in nitroreductase activity, Equation 8.



Further studies are necessary to elucidate the mechanism of DEM action in the generation of radicals in the slices especially the role of the unique histones present in the testis. These histones can act as substrates for cysteine. The presence of TNB in the incubate upsets the delicate balance between oxygen and nitrogen centered radicals in the seminiferous tubules. Oxygen centered radicals are necessary for cell division in the germ cells and their levels are normally under strict control by nitrogen centered radicals. Elevation of oxygen centered radicals may explain the pathological damage to both the germ cells and sertoli cells in the slices incubated with TNB (Wyman et al 1996). The decomposition products of TNB have still to be identified, as well as the radical species which is induced by TNB and trapped by DMSO and PBN, Figure 3. However, the role of free radicals in TNB toxicity is particularly timely and this study in the testis compliments new information on the role of cysteine residues in hemoglobin to carry the free radical NO (Jai et al 1996) and the clinical reports that nitroaromatics and pharmaceuticals with similar chemical structure to TNB cause methemoglobinemia even when topically applied (Dinneen et al 1994).

## REFERENCES

- Biaglow, J.E, M.E. Varnes, L. Roizen-Torle, E.P. Clark, E.R. Epp, M. B. Astor, E. J. Hall 1986 Biochemistry of reduction of nitro heterocycles. *Biochem Pharmacol* 35: 77-90.
- Bond, J.A., Chism, J.P., Rickert, D.E. and Popp, J.A. Induction of hepatic testicular lesions in Fischer F-344 rats by single oral doses of nitrobenzene. *Fundam. Appl. Toxicol.* 1, p389, 1981.
- Buettner, G.R. 1982 The spin trapping of superoxide and hydroxyl radicals . In *Superoxide Dismutase* Ed L.W. Oberley Vol II Chapter 4 pp 63-81. CRC Press, Inc Boca Raton, FL.
- Burhley, L.E. and Ellis, L.C. 173 *Fertil. Steril.* 24: 956-961
- Carmichael, A.J. and L. Steel-Goodwin 1994 Decomposition of peroxynitrite studied by EPR/spin trapping *J Free Rad in Biology and Medicine* 2 3: Q5.
- Cody, T.E., Witherup, S., Hastings, L., Stemmer, K., and Christain, R.T. 1,3-Dinitrobenzene: toxic effects in vivo and in vitro. *J. Toxicol. Environ. Health* 7, 829, 1981.
- Decompo, R., R.P. Mason, C. Mottley, and R.P.A. Mugiz 1981 Generation of free radicals induced by nifurtimox in mammalian tissues *J Biol Chem* 256 21: 10930-10933.
- Dinneen, S.F., D.N. Mohr and V.F. Fairbanks 1994. Methemoglobinemia from topically applied anaesthetic spray. *Mayo Clin Proc* 69: 886-888.
- Foster, P.M.D. 1992 The sertoli cell. In *Reversibility in Testicular Toxicity Assessment* Chapter 2 Ed. A.R. Scialli and E.D.Clegg CRC Press Inc, Boca Raton, FL.
- Gutteridge, J.M.C. 1995a Lipid peroxidation and antioxidants as biomarkers of tissue damage. *Clin Chem* 41, 12B, 1819-1828.
- Gutteridge, J.M.C. 1995b Lipid peroxidation: some problems and concepts. In Halliwell B, ed. *Oxygen radicals and tissue injury*, Bethesda MD: FASEB, 1988: 9-19.
- Jai, L, S. Stamler 1996 *Nature* 380, 221
- Janzen, E.G. 1971 Spin trapping *Acc Chem Res* 4, 31.

Joshi, A. and G.C. Yang 1981 Spin trapping of radicals generated in the uv photolysis of alkyl disulfides *J Org Chem* 46: 3736-3738.

Kinkead, E.R., R.E. Wolfe, C.D. Flemming, D.J. Caldwell, C.R. Miller, and G.B. Marit (1995) Reproductive toxicity screen of 1,3,5-trinitrobenzene administered in the diet of Sprague-Dawley rats. *Toxicol. Indust. Health* 11 (3) 309-323.

Lagercratz, C.J. 1971 Spin trapping of some short-lived radicals by the nitroxide method. *J. Phys Chem* 75 3466-3475

Levine, B.S., Furedi, E.M., Gordon, D.E., Lish, P.M., and Barkley, J.J. Subchronic toxicology of trinitrotoluene in Fisher F-344 rats *Toxicology* 32, 253, 1984.

Mason, R.P., Josephy, P.D. 1985 Free radical mechanism of nitroreductase: In: Rickert, D.E. ed. *The toxicity of nitroaromatic compounds*. New York: Hemisphere Publishing Corp pp 121-140.

Mason, R.P. 1990 Redox cycling of radical anion metabolites of toxic chemicals and drugs and the Marcus theory of electron transfer. *Environ. Health Perspect.* 87: 237-243

Myers, Jr., L.S., A.J. Carmichael, and T.C. Pellmar 1994 Radiation chemistry of the hippocampal brain slice 1994 *Adv. Space Res.* Vol 14 (10) 453-456.

Poupko, R and I. Rosenthal 1973 Electron transfer interactions between superoxide ion and organic compounds. *J. Phys Chem* 77: 1722-1724.

Radike, M. Reproductive Toxicology Chapter 16 In *Industrial Toxicology* Ed. Williams, P.L. and J.L. Burson 1985, Van Nostrand Reinhold.

Romero, I.A., T. Lister, H.K. Richards, M.P. Seville, S.P. Wylie, D.E. Ray 1995 Early metabolic changes during m-dinitrobenzene neurotoxicity and the possible role of oxidative stress. *Free Rad. Biol and Med* 18 (2) 311-319.

Rice-Evans, C.A., A.T. Diplock, M.C.R. Simons 1991 In *Techniques in free radical research* Ed R.H. Burdon and P.H. van Knippenburg Elsevier Science Publishing Co., NY.

Steel-Goodwin L, Arroyo CM, Gray BH and Carmichael AJ. EPR detection of nitric oxide dependent spin adducts in mouse jejunum. In *Biology of Nitric Oxide Vol 2 Enzymology, Biochemistry and Immunology*, ED S. Moncada, MA Marletta, JB Hibbs Jr and EA Higgs Portland Press, London pp 80-84, 1992

Steel-Goodwin L, Pravec TL and Carmichael AJ Trichloroethylene: An EPR Study *Free Rad Biol Med* 1994: (2) 3 Q10.

Walsh, M.E. and T.F. Jenkins 1992 Identification of TNT transformation products in soil. Special Report 92-16. U.S. Army Corps of Engineers, Cold Regions Research & Engineering Laboratory.

Wyman, J.F., J.S. Eggers, C.D. Flemming 1996 Preparation of bovine testicular tissue slices: Evaluation of 1,3,5-trinitrobenzene toxicity. Reproduc. Toxicol. (In revision).

Zenick H and E.D. Clegg 1989 Assessment of male reproductive toxicity: a risk assessment approach. In Principles and Methods of Toxicology A.W. Hayes Ed., 2nd ed. pp 275-309 Raven Press, New York.

## SECTION 5

### APPENDIX A

Data was analyzed using the Design Ease® for Windows from Stat-Ease Inc. Minneapolis, MN.

#### 1. Design Layout

| Std | Den Id | Run | Block | TNB<br>su<br>Factor | EPR<br>Response |
|-----|--------|-----|-------|---------------------|-----------------|
| 1   | 1      | 19  | 1     | 1000                | 62.88           |
| 2   | 1      | 23  | 1     | 1000                | 65.14           |
| 3   | 1      | 5   | 1     | 1000                | 48.76           |
| 4   | 1      | 16  | 1     | 1000                | 52.41           |
| 5   | 1      | 13  | 1     | 1000                | 48.90           |
| 6   | 1      | 2   | 1     | 1000                | 50.67           |
| 7   | 2      | 17  | 1     | 500                 | 50.56           |
| 8   | 2      | 30  | 1     | 500                 | 51.86           |
| 9   | 2      | 26  | 1     | 500                 | 38.99           |
| 10  | 2      | 8   | 1     | 500                 | 39.95           |
| 11  | 2      | 15  | 1     | 500                 | 65.33           |
| 12  | 2      | 7   | 1     | 500                 | 65.85           |
| 13  | 3      | 28  | 1     | 100                 | 32.66           |
| 14  | 3      | 27  | 1     | 100                 | 32.79           |
| 15  | 3      | 29  | 1     | 100                 | 22.04           |
| 16  | 3      | 24  | 1     | 100                 | 22.37           |
| 17  | 3      | 20  | 1     | 100                 | 18.15           |
| 18  | 3      | 1   | 1     | 100                 | 19.18           |
| 19  | 4      | 6   | 1     | 50                  | 32.91           |
| 20  | 4      | 21  | 1     | 50                  | 33.04           |
| 21  | 4      | 9   | 1     | 50                  | 38.09           |
| 22  | 4      | 22  | 1     | 50                  | 39.82           |
| 23  | 4      | 14  | 1     | 50                  | 18.12           |
| 24  | 4      | 25  | 1     | 50                  | 18.12           |
| 25  | 5      | 4   | 1     | 0                   | 12.67           |
| 26  | 5      | 11  | 1     | 0                   | 12.48           |
| 27  | 5      | 12  | 1     | 0                   | 12.76           |
| 28  | 5      | 18  | 1     | 0                   | 18.29           |
| 29  | 5      | 10  | 1     | 0                   | 13.34           |
| 30  | 5      | 3   | 1     | 0                   | 19.08           |

#### BRUKER EMS104 ####

DATE : 24-APR-1996  
TIME : 14:19:00  
OPERATOR :  
SAMPLE : s30um  
UNITS :  
COMMENT :

MP : 25.06  
HSL : 100.00  
RMA : 4.02  
RTC : 10.49  
RRC : 20.48  
RPF : 38  
RPH : 0  
HCF : 0  
JNS : 0.30  
SH : 4  
24Apr 96

\*\*\*\*\*

1: 62.88 TESTS  
2: 65.14  
3: 48.76  
4: 52.41  
5: 48.90  
6: 50.67  
7: 50.56  
8: 51.86  
9: 38.99  
10: 39.95  
11: 65.33  
12: 65.85  
13: 32.66  
14: 32.79  
15: 22.04  
16: 22.37  
17: 18.15  
18: 19.18  
19: 32.91  
20: 33.04  
21: 38.09  
22: 39.82  
23: 18.12  
24: 18.12  
25: 12.67  
26: 12.48  
27: 12.76  
28: 18.29  
29: 13.34  
30: 19.08  
31: 10.04 STOPS  
32: 22.07  
33: 45.68  
34: 65.22  
35: 50.75

## 2. Analysis of EPR Data

| SOURCE    | SUM OF<br>SQUARES | DF | MEAN<br>SQUARE | F<br>VALUE | PROB > F |
|-----------|-------------------|----|----------------|------------|----------|
| MODEL     | 7363.3671         | 4  | 1840.84        | 27.47      | < 0.0001 |
| RESIDUAL  | 1675.3610         | 25 | 67.01          |            |          |
| COR TOTAL | 9038.7281         | 29 |                |            |          |

|          |         |                |      |
|----------|---------|----------------|------|
| ROOT MSE | 8.1862  | R-SQUARED      | 0.81 |
| DEP MEAN | 35.2403 | ADJ R-SQUARED  | 0.78 |
| C.V. %   | 23.2297 | PRED R-SQUARED | 0.73 |

Predicted Residual Sum of Squares (PRESS) = 2412.52

### TREATMENT MEANS (ADJUSTED, IF NECESSARY)

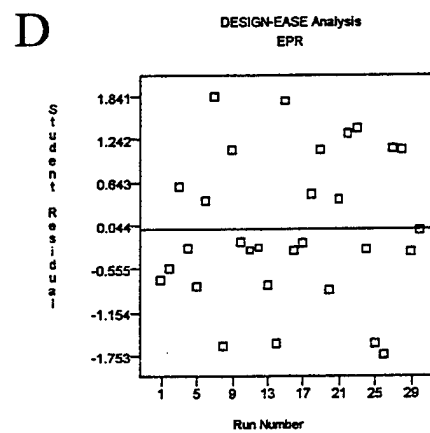
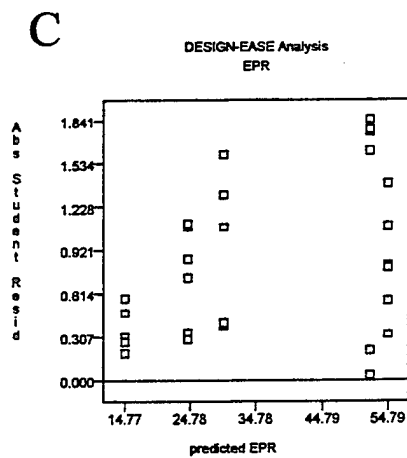
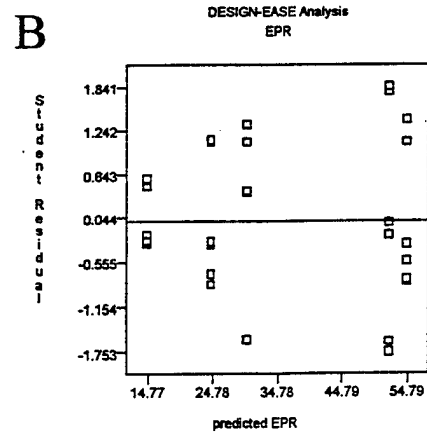
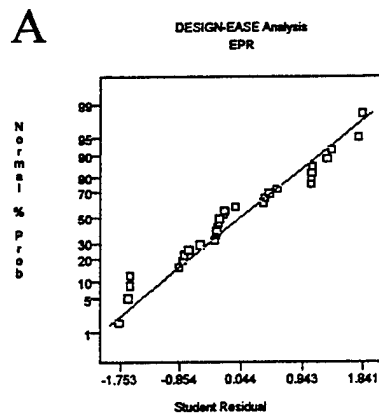
#### ESTIMATED MEAN STANDARD ERROR

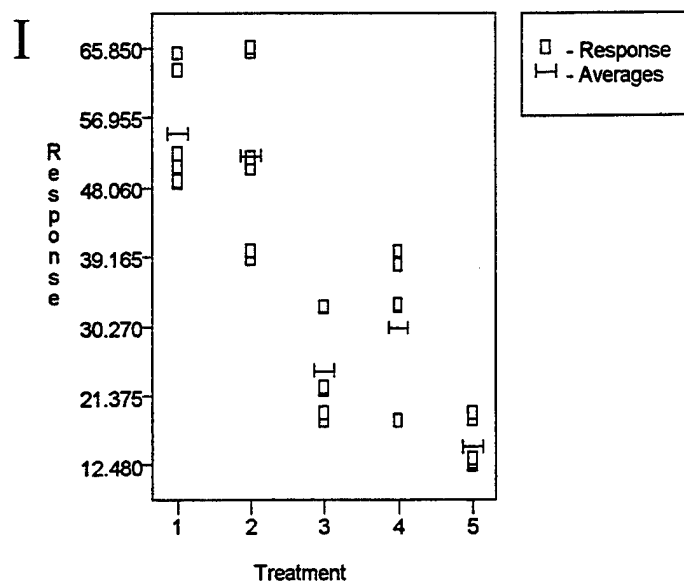
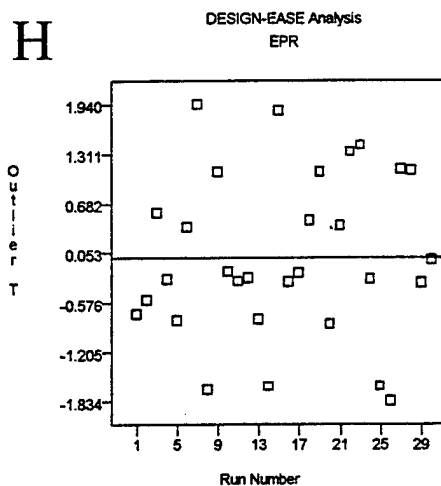
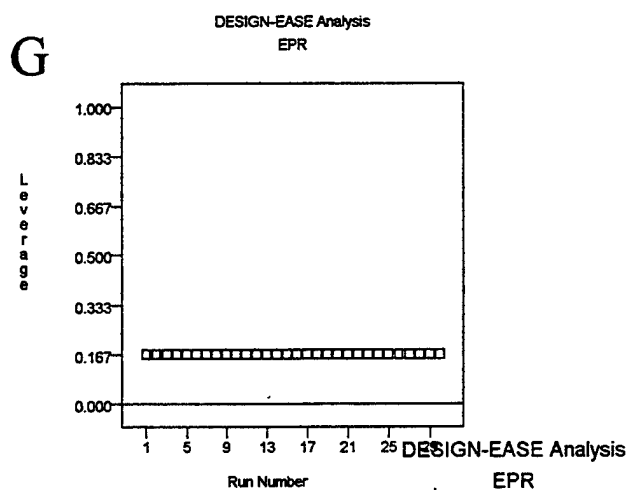
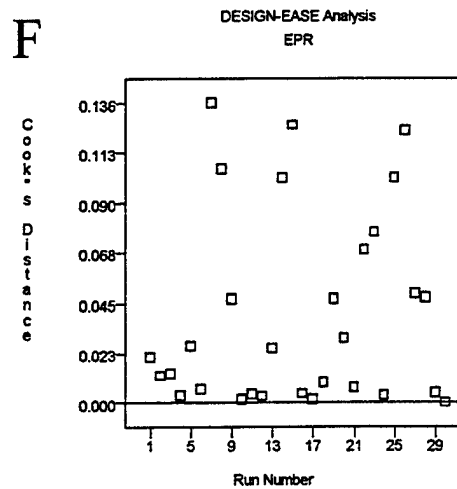
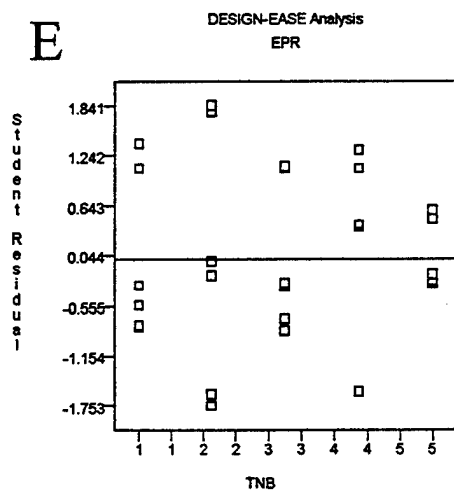
|      |         |        |
|------|---------|--------|
| 1000 | 54.7933 | 3.3420 |
| 500  | 52.0900 | 3.3420 |
| 100  | 24.5317 | 3.3420 |
| 50   | 30.0167 | 3.3420 |
| 0    | 14.7700 | 3.3420 |

| Treatment | MEAN<br>DIFFERENCE | DF | STANDARD<br>ERROR | t FOR H0<br>COEFFICIENT=0 | PROB >  t |
|-----------|--------------------|----|-------------------|---------------------------|-----------|
| 1 vs 2    | 2.70               | 1  | 4.726             | 0.572                     | 0.5724    |
| 1 vs 3    | 30.26              | 1  | 4.726             | 6.403                     | < 0.0001  |
| 1 vs 4    | 24.78              | 1  | 4.726             | 5.242                     | < 0.0001  |
| 1 vs 5    | 40.02              | 1  | 4.726             | 8.468                     | < 0.0001  |
| 2 vs 3    | 27.56              | 1  | 4.726             | 5.831                     | < 0.0001  |
| 2 vs 4    | 22.07              | 1  | 4.726             | 4.670                     | < 0.0001  |
| 2 vs 5    | 37.32              | 1  | 4.726             | 7.896                     | < 0.0001  |
| 3 vs 4    | -5.48              | 1  | 4.726             | -1.161                    | 0.2568    |
| 3 vs 5    | 9.76               | 1  | 4.726             | 2.065                     | 0.0494    |
| 4 vs 5    | 15.25              | 1  | 4.726             | 3.226                     | 0.0035    |

| OBS<br>ORD | ACTUAL<br>VALUE | PREDICTED<br>VALUE | RESIDUAL | STUDENT<br>LEVER | COOK'S<br>RESID | OUTLIER<br>DIST | T<br>T | VALUE | RUN<br>ORD |
|------------|-----------------|--------------------|----------|------------------|-----------------|-----------------|--------|-------|------------|
| 1          | 62.88           | 54.79              | 8.087    | 0.167            | 1.082           | 0.047           | 1.086  | 19    |            |
| 2          | 65.14           | 54.79              | 10.347   | 0.167            | 1.385           | 0.077           | 1.412  | 23    |            |
| 3          | 48.76           | 54.79              | -6.033   | 0.167            | -0.807          | 0.026           | -0.802 | 5     |            |
| 4          | 52.41           | 54.79              | -2.383   | 0.167            | -0.319          | 0.004           | -0.313 | 16    |            |
| 5          | 48.90           | 54.79              | -5.893   | 0.167            | -0.789          | 0.025           | -0.782 | 13    |            |
| 6          | 50.67           | 54.79              | -4.123   | 0.167            | -0.552          | 0.012           | -0.544 | 2     |            |
| 7          | 50.56           | 52.09              | -1.530   | 0.167            | -0.205          | 0.002           | -0.201 | 17    |            |
| 8          | 51.86           | 52.09              | -0.230   | 0.167            | -0.031          | 0.000           | -0.030 | 30    |            |
| 9          | 38.99           | 52.09              | -13.100  | 0.167            | -1.753          | 0.123           | -1.834 | 26    |            |
| 10         | 39.95           | 52.09              | -12.140  | 0.167            | -1.625          | 0.106           | -1.683 | 8     |            |
| 11         | 65.33           | 52.09              | 13.240   | 0.167            | 1.772           | 0.126           | 1.856  | 15    |            |
| 12         | 65.85           | 52.09              | 13.760   | 0.167            | 1.841           | 0.136           | 1.940  | 7     |            |
| 13         | 32.66           | 24.53              | 8.128    | 0.167            | 1.088           | 0.047           | 1.092  | 28    |            |
| 14         | 32.79           | 24.53              | 8.258    | 0.167            | 1.105           | 0.049           | 1.110  | 27    |            |
| 15         | 22.04           | 24.53              | -2.492   | 0.167            | -0.333          | 0.004           | -0.327 | 29    |            |
| 16         | 22.37           | 24.53              | -2.162   | 0.167            | -0.289          | 0.003           | -0.284 | 24    |            |
| 17         | 18.15           | 24.53              | -6.382   | 0.167            | -0.854          | 0.029           | -0.849 | 20    |            |
| 18         | 19.18           | 24.53              | -5.352   | 0.167            | -0.716          | 0.021           | -0.709 | 1     |            |
| 19         | 32.91           | 30.02              | 2.893    | 0.167            | 0.387           | 0.006           | 0.380  | 6     |            |
| 20         | 33.04           | 30.02              | 3.023    | 0.167            | 0.405           | 0.007           | 0.398  | 21    |            |
| 21         | 38.09           | 30.02              | 8.073    | 0.167            | 1.080           | 0.047           | 1.084  | 9     |            |
| 22         | 39.82           | 30.02              | 9.803    | 0.167            | 1.312           | 0.069           | 1.332  | 22    |            |
| 23         | 18.12           | 30.02              | -11.897  | 0.167            | -1.592          | 0.101           | -1.645 | 14    |            |
| 24         | 18.12           | 30.02              | -11.897  | 0.167            | -1.592          | 0.101           | -1.645 | 25    |            |
| 25         | 12.67           | 14.77              | -2.100   | 0.167            | -0.281          | 0.003           | -0.276 | 4     |            |
| 26         | 12.48           | 14.77              | -2.290   | 0.167            | -0.306          | 0.004           | -0.301 | 11    |            |
| 27         | 12.76           | 14.77              | -2.010   | 0.167            | -0.269          | 0.003           | -0.264 | 12    |            |
| 28         | 18.29           | 14.77              | 3.520    | 0.167            | 0.471           | 0.009           | 0.464  | 18    |            |
| 29         | 13.34           | 14.77              | -1.430   | 0.167            | -0.191          | 0.001           | -0.188 | 10    |            |
| 30         | 19.08           | 14.77              | 4.310    | 0.167            | 0.577           | 0.013           | 0.569  | 3     |            |

### 3. Diagnostic and Interpretation Graphs



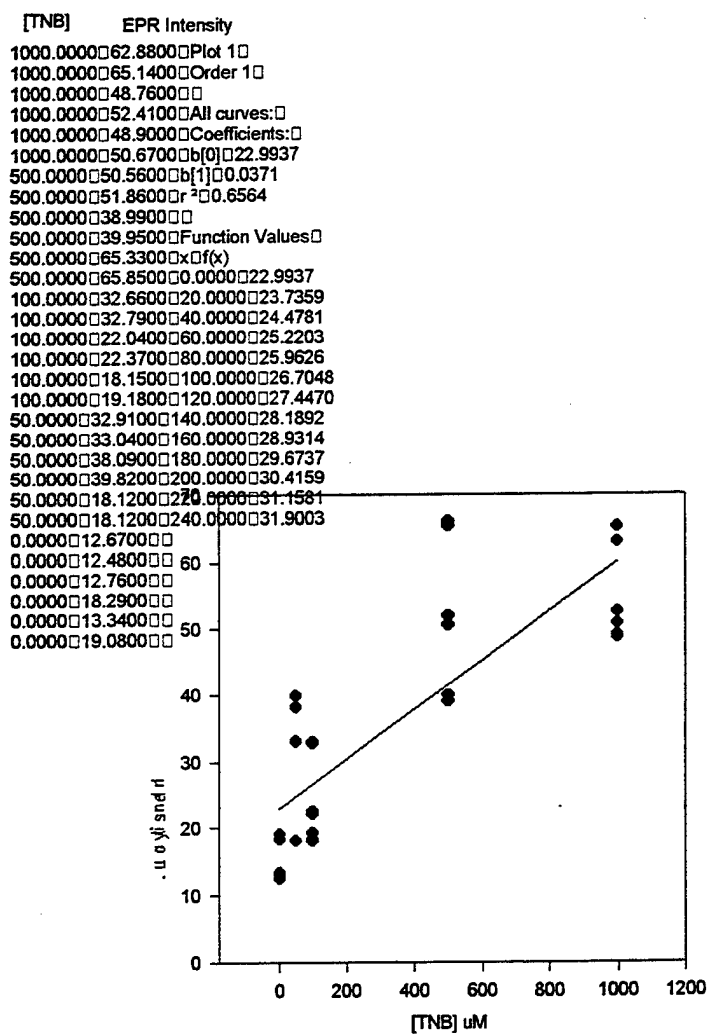


## SECTION 6

### APPENDIX B

#### Regression Analysis of EPR data

##### 1. EPR data of Slices.



2. EPR of spin label standards (0-500 uM) run under the same conditions as the slices.

