

AD _____

Award Number: DAMD17-98-1-8081

TITLE: Inhibition of Mitochondrial Estrogen Metabolism as a
Possible Mechanism of Breast Cancer Prevention

PRINCIPAL INVESTIGATOR: Dr. Ronald Thomas

CONTRACTING ORGANIZATION: Florida Agricultural and Mechanical
University
Tallahassee, Florida 32307

REPORT DATE: July 2001

TYPE OF REPORT: Final

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;
Distribution Unlimited

The views, opinions and/or findings contained in this report are those of the author(s) and should not be construed as an official Department of the Army position, policy or decision unless so designated by other documentation.

20020910 088

REPORT DOCUMENTATION PAGEForm Approved
OMB No. 074-0188

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 this 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**

July 2001

3. REPORT TYPE AND DATES COVERED

Final (1 Jul 98 - 30 Jun 01)

4. TITLE AND SUBTITLE

Inhibition of Mitochondrial Estrogen Metabolism as a Possible Mechanism of Breast Cancer Prevention

5. FUNDING NUMBERS

DAMD17-98-1-8081

6. AUTHOR(S)

Dr. Ronald Thomas

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)Florida Agricultural and Mechanical University
Tallahassee, Florida 32307E-Mail: ronald.thomas@famu.edu**8. PERFORMING ORGANIZATION
REPORT NUMBER****9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)**U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012**10. SPONSORING / MONITORING
AGENCY REPORT NUMBER****11. SUPPLEMENTARY NOTES****12a. DISTRIBUTION / AVAILABILITY STATEMENT**

Approved for Public Release; Distribution Unlimited

12b. DISTRIBUTION CODE**13. ABSTRACT (Maximum 200 Words)**

This project addresses the possible role of mitochondrial estrogen metabolism and its inhibition in mammary carcinogenesis of female ACI rats. Diethylstilbestrol (DES) have been shown to cause cancer in several animals species whereas diallyl sulfide (DAS) inhibits cancer. We hypothesis that DES is metabolized by the mitochondria to reactive metabolites producing DNA adducts. We further propose that DAS will prevent this metabolism thus prevent the production of DNA adducts. The results of this study demonstrates that organelles (mitochondria, microsomes, and nuclei) catalyze the oxidation and reduction of DES and diallyl sulfide inhibits this metabolism in a noncompetitive fashion. Furthermore, DAS inhibits the formation of mitochondrial DNA adducts in both *in vitro* and *in vivo*. The results of this study will help elucidate the mechanism of estrogen induced breast cancer. This data will provide a foundation for the further investigation of the chemopreventive properties of DAS and structurally similar compounds.

14. SUBJECT TERMS**15. NUMBER OF PAGES**

19

16. PRICE CODE**17. SECURITY CLASSIFICATION
OF REPORT**

Unclassified

**18. SECURITY CLASSIFICATION
OF THIS PAGE**

Unclassified

**19. SECURITY CLASSIFICATION
OF ABSTRACT**

Unclassified

20. LIMITATION OF ABSTRACT

Unlimited

NSN 7540-01-280-5500

Standard Form 298 (Rev. 2-89)
Prescribed by ANSI Std. Z39-18
298-102

Table of Contents

Cover.....	1
SF 298.....	2
Table of Contents.....	3
Introduction.....	4
Body.....	4
Key Research Accomplishments.....	10
Reportable Outcomes.....	10
Conclusions.....	10
References.....	12
Appendices.....	14

INTRODUCTION

This project addresses the role of mitochondrial estrogen metabolism and the inhibition of estrogen metabolism in mammary carcinogenesis. Female ACI rats serves as our animal model and Diethylstilbestrol (DES) as our model estrogen. Diallyl sulfide, a component of garlic which has been shown to inhibit the induction of cancer in several animal species (Wargovich , 1987; Wargovich *et.al.*, 1988; Wattenburg *et.al.*, 1989; Sparnins *et.al.*, 1988; & Hays *et.al.*, 1987), was tested for its ability to prevent DES metabolism. We have investigated the capabilities of the various subcellular fractions (microsomes, mitochondria, and nuclei) to metabolize DES and the ability of DAS to inhibit this metabolism. We have also investigated the ability of DES to produce DNA adducts in mitochondrial DNA and the ability of diallyl sulfide to inhibit adduct formation *in vitro* as well as *in vivo*. The findings of this study will provide us with a rationale to develop diallyl sulfide and structurally related compounds as chemopreventive agents.

BODY

We propose that diethylstilbestrol (DES) enters into redox reactions in the presence of mitochondrial enzymes. The reactive species generated during redox-cycling of DES are postulated to bind to mitochondrial DNA which may cause mitochondrial genetic instability. This genetic instability is proposed to play an important role in the induction of breast cancer. We further propose that diallyl sulfide will inhibit the redox-cycling of DES. This inhibition may lead to the inhibition of estrogen-induced carcinogenesis. To test this hypothesis we have identified the following specific aims:

1. We will demonstrate the oxidation and reduction of DES by breast mitoplasts.
2. We will demonstrate the inhibition of oxidation and/or reduction of DES by diallyl sulfide.
3. We will demonstrate that DES metabolites generated by breast mitoplasts are genotoxic *in vitro* and *in vivo*.
4. We will demonstrate that DAS will inhibit the *in vitro* and *in vivo* genotoxicity of DES.

Statement of Work (Task 1): To demonstrate the oxidation and reduction of DES catalyzed by various organelles (mitoplasts, nuclei, and microsomes) and the inhibition of this metabolism by DAS.

1. Organelles (mitoplasts, nuclei, and microsomes) will be isolated by differential centrifugation.
2. Ten rats will be needed to isolate adequate amount of organelles form breast tissue.
3. The rats will be dosed with beta naphthoflavone to induce cytochrome p-450s that metabolize estrogen and sacrificed via carbon dioxide exposure.
4. *In vitro* oxidation reactions will be conducted with DES, cumen hydroperoxide (oxidation cofactor), and individual organells.

5. The oxidation products will be analyzed by UV absorption and HPLC analysis.
6. *In vitro* reduction reactions will be conducted with DES quinone, NADH (reduction cofactor), and individual organelles.
7. The reduction products will be analyzed by UV absorption and HPLC analysis.
8. In parallel experiments various concentrations of DAS will be added to determine its inhibitory effects on DES metabolism.

Progress for Task 1

Animal and chemical model:

Female ACI rats were used to study the mechanism of estrogen-induced breast cancer. It has been demonstrated that estrogen produces breast cancer in ACI rats (Shull *et.al.*, 1997). The administration of DES during pregnancy increases the risk of developing breast and endometrial cancer in humans and animals (Colton *et.al.*, 1993).

Specific Aim 1 & 2: Redox cycling of DES and its inhibition by DAS.

Female ACI rats (10) were treated for four days with a daily dose of beta naphthoflavone (50 mg/kg *i.p.*) to induce cytochrome p-450. The rats were sacrificed by carbon dioxide exposure. The breast tissue was removed, weighed, and homogenized in 1:10 wt/vol (0.25 M sucrose, 1.0 mM EDTA, 2.5 uM PMSF). Microsomes, mitochondria, and nuclei from the breast tissue were isolated by differential centrifugation. The mitochondria were treated with a 1.6% digitonin solution and centrifuged to collect pure mitoplast (mitochondria without outer membrane). The removal of the outer membrane of the mitochondria makes the enzymes more accessible to the substrate and reduces the chances of cytosolic contamination. The organelles were used to catalyze the oxidation and reduction of DES. The oxidation and reduction products generated by the organelles were analyzed by UV absorption and HPLC analysis.

Determination of the purity of mitoplasts:

The purity of mitoplasts was assessed by both morphological and biochemical analyses. Mitoplasts were stained with eosin and hematoxylin. Phase contrast microscopy did not reveal any cellular contamination. The determination of cytochrome C oxidase (Whorton & Tzagoloff, 1967), an enzymatic marker of mitochondria, showed 120-125 $\mu\text{mol/mg protein/min}$ specific activity. Microsomal contamination was assessed by measuring the activity of glucose 6-phosphatase, an enzymatic marker of endoplasmic reticulum (Baginski *et.al.*, 1974). The activity of glucose 6-phosphatase in mitoplasts was less than 1% of that found in microsomes (5.0 pmol/mg protein/min in mitoplasts vs 735 pmol/mg protein/min in microsomes). This is in agreement with the report of Niranjana *et.al.* (1980 & 85).

Determination of the purity of microsomes:

The purity of microsomes was assessed by both morphological and biochemical analyses. Microsomal preparations were stained with eosin and hematoxylin. Phase contrast microscopy did not reveal any nuclear contamination. Mitochondrial contamination was assessed by measuring the cytochrome C oxidase activity (Whorton & Tzagoloff, 1967). There was less than 2 % microsomal contamination. Based on enzyme analysis.

Determination of the purity of nuclei:

The purity of the nuclei was assessed by morphological and biochemical analyses. We stained the nuclei preparations with eosin and hematoxylin. Phase contrast microscopy revealed the presence of intact nuclei. There was little cytochrome C oxidase and glucose 6-phosphatase activity which indicated that the nuclei preparations were pure.

Oxidation reaction system:

The reaction conditions consists of 10mM phosphate buffer, pH 7.5, 120 μ M cumene hydroperoxide, 420 μ g/ml mitoplasts and 346 μ g/ml of microsomes in a final volume of 1.0 ml. Various concentrations (0-120 μ M) of DES was used to determine the kinetics constants of the reactions. For specific aim 2 various concentrations of DAS (186 μ M and 373 μ M) was added to test the inhibition of DAS on the oxidation of DES.

No cumene hydroperoxide (oxidation cofactor) will be used in control reactions.

Reduction Reaction System:

The reaction conditions consists of 10mM phosphate buffer, pH 7.5, 50 μ M NADPH, 420 μ g/ml mitoplasts and 346 μ g/ml of microsomes in a final volume of 1.0 ml. Various concentrations (0-60 μ M) of DES quinone was used to determine the kinetics constants of the reactions. For specific aim 2 various concentrations of DAS (186 μ M and 373 μ M) was added to test the inhibition of DAS on the reduction of DES quinone.

No NADPH (reduction cofactor) will be used in control reactions

HPLC analysis:

The oxidation and reduction products from similar reactions as previously described were extracted with water saturated ethyl ether. The reaction mixture was dried under nitrogen and metabolites were reconstituted in methanol. An appropriate amount(10-50 μ l) of sample was injected into the HPLC. A methanol/water gradient consisting of 36% to 83% methanol was run using a C₁₄ reverse phase column from 0 to 30 minutes at a flow rate of 1 ml/min. The U.V. detection was performed at a wavelength of 254nm.

Results for Task 1:**Oxidation of DES:**

Mitoplasts were incubated in the presence of DES and cumene hydroperoxide. DES quinone was detected by U.V. spectroscopy. The UV spectral analysis of the mitochondrial mixture containing DES and cumene hydroperoxide revealed a gradual increase in the absorbance at 312 nm. The spectral pattern was identical to that of synthetic DES quinone (Fig 1). In the control reactions no DES quinone was produced. In oxidation reactions that contained DAS (373 μ M) the production of DES quinone was reduced by 50%. The rate of DES quinone formation in the presence of mitoplasts and cumene hydroperoxide was dependent on the concentration of DES (Fig. 2). A Lineweaver-Burk plot of rate of formation of DES quinone at various concentrations of DES yielded a K_m of 35.7 μ M and V_{max} of 3.45 nmol/mg protein/min.). The kinetic constants of the reactions were determined by using various concentrations of DES (0-120 μ M) and two concentrations of DAS (186 μ M and 373 μ M) With

increasing concentrations of DAS the K_m remained constant whereas the V_{max} decreased (3.45, 2.44, and 1.82 nmol/mg protein/min. respectively. This data indicates that the mitochondria is capable of metabolizing DES to DES quinone and DAS is capable of inhibiting this metabolism.

Based on the kinetic constants the nature of this inhibition seems to be noncompetitive. Results from the oxidation in microsomal reactions were similar to those in mitochondria. *i.e.* The microsomes were capable of oxidizing DES to DES quinone and DAS inhibited this oxidation (Data not shown).

Reduction of DES quinone:

Mitoplasts were incubated in the presence of DES quinone and NADH. The disappearance of DES quinone was detected by UV spectroscopy. The UV spectral analysis of the mitochondrial mixture containing DES quinone and NADH revealed a gradual decrease in the absorbance at 312 nm (Fig 3). In the control reactions little DES quinone was reduced. In the reduction reactions that contained DAS (373 μ M) the amount of DES quinone reduced was decreased by 50%. The rate of DES quinone disappearance in the presence of mitoplasts and NADH was dependent on the concentration of DES quinone (Fig. 4). A Lineweaver-Burk plot of rate of disappearance of DES quinone at various concentrations of DES yielded a K_m of 50.0 μ M and V_{max} of 2.0 nmol/mg protein/min. The kinetic constants of the reactions were determined by using various concentrations of DES Quinone (0-60 μ M) and two concentrations of DAS (186 μ M and 373 μ M). With increasing concentrations of DAS the K_m remained constant whereas the V_{max} decreased (2.0, 1.5, and 1.25 nmol/mg protein/min. respectively). This data indicates that the mitochondria is capable of reducing DES Quinone to DES and DAS is capable of inhibiting this reduction. Based on the kinetic constants, the nature of this inhibition seems to be noncompetitive. Results from the reduction in microsomal reactions were similar to those in the mitochondria. *ie* The microsomes were capable of reducing DES quinone to DES and DAS inhibited this reduction (Data not shown).

The kinetic constants for the mitochondrial and microsomal oxidation and reduction reactions are summarized in Table 1. The kinetic constants were not determined for nuclear oxidation and reduction reactions due to the small amount of nuclei that could be isolated from the breast tissue.

HPLC Analysis:

Oxidation and reduction reactions for all three cellular subfractions (mitochondrial, microsomal, and nuclear) were confirmed by HPLC analysis (Table 2). HPLC analysis revealed that DAS inhibited the oxidation of DES in a dose dependent manner in the mitochondria, microsomes, and nuclei. Similar results were seen in reduction reactions (Table 3).

Statement of Work (Task 2): To demonstrate the *in vitro* production of DNA adducts by DES metabolites and the inhibition of adduct formation by DAS.

1. Organelles (mitoplasts, nuclei, and microsomes) will be isolated by differential centrifugation.
2. Ten rats will be needed to isolate adequate amount of organelle from breast tissue.
3. The rats will be dosed with beta naphthoflavone to induce cytochrome p-450s that metabolize estrogen and sacrificed via carbon dioxide exposure.

4. *In vitro* oxidation reactions will be conducted with DES, cumen hydroperoxide (oxidation cofactor), individual organelles, and DNA to determine the adduct forming potential of DES metabolites.
5. The DNA will be extracted and analyzed for adducts by ³²P-Postlabeling.
6. Parallel experiments will be conducted with the addition of Diallyl sulfide to oxidation reaction mixtures to determine if DAS will inhibit the formation of DNA adducts generated by the metabolism of DES.

Progress for Task 2

In vitro: Mitoplast, microsomes, and nuclei were prepared as described previously. DNA (200µg) was added to the oxidation system as described previously to assess the genotoxicity of DES metabolites (DES quinone) and DAS (100uM) will be added to reaction system to determine if DAS will inhibit the formation of DES quinone adducts.

DNA Isolation:

The mitochondria and nuclei will be isolated by differential centrifugation. The DNA was isolated from purified mitoplast and nuclei. Pure mitoplast and nuclei was suspended in 50 mM Tris, 10 mM EDTA, pH 8.0 containing 1% SDS. After five minutes of gentle shaking, samples are to be treated with RNAase-A (150 ug/ml) and RNAase T1 (20 U/ml) for 30 minutes and incubated with proteinase k (500 ug/ml) for 1 hr at 37°C. The DNA will be extracted once with phenol:chloroform:isoamyl alcohol (25:24:1) and three times with chloroform:isoamyl alcohol (24:1). Two volumes of ice-cold ethanol will be added in aqueous extract, chilled at -80°C for 15 minutes and centrifuged at 11,000 x g. The purity of DNA will be checked by ultraviolet spectroscopy and agarose gel electrophoresis.

³²P-Post Labeling:

Analysis of covalent modification in DNA will be carried out by the ³²P-post labeling technique (Reddy & Randerath, 1989). DNA (10 ug) will be digested with micrococcal nuclease, spleen phosphodiesterase, and nuclease P1 as described previously (Gupta *et al.*, 1982; and Reddy & Randerath, 1989). Enzymatic reactions and AT-³²P will be used to label the digested nucleotides. The unmodified nucleotides will be separated from the adducts on polyetheleneimine(PEI)-cellulose TLC plates using D1, 2.3 M sodium phosphate, pH 5.7. The resolution of ³²P-post labeled adducts will be carried out on polyetheleneimine(PEI)-cellulose TLC plates with D3, 4.2 M lithium formate and 6.8 M urea, pH 3.3; D4, 0.8 M sodium phosphate, 0.5 M Tris and 8.5 M urea, pH 8.2; and D5, 1.7 M phosphate, pH 6.0. The ³²P-labeled adduct spots will be detected by autoradiography using Kodak X-Omat film. The spots containing ³²P-post labeled adducts will be excised from TLC plates, and levels of radioactivity will be determined by Cerenkov counting. The amount of adducts will be calculated according to the procedures described previously (Reddy & Randerath, 1989).

Results Task 2:

³²P Post Labeling analysis revealed that oxidation reaction in the presence of mitoplast and DES produced three major DNA adducts. These adducts had a similar migration pattern as the dGMP reacted with DES quinone. Based on chromatographic mobility we believe that the adducts produced by the mitochondrial oxidation system are dGMP adducts. Furthermore, DNA adducts were produced in the oxidation system containing DAS. (figure 5) However, the total amount of DNA adducts produced in the DAS containing reactions were decreased by 89% compared to the reactions containing no DAS. (Table 3). No DNA adducts were produced in reactions without cumen hydroperoxide (oxidation cofactor). (Data not shown) Therefore we conclude that DAS inhibits the formation of DES induced mitochondrial DNA adducts *in vitro*. We expect similar results in the microsomal and nuclear reactions. These reactions have not been performed as of now.

Statement of Work (Task 3) : To determine the *in vivo* formation of DNA adducts produced by DES and its inhibition by DAS.

1. Five groups of five animals will be used in this study. There will be one control, one group dosed with DES alone, and three groups dosed with DES and three different concentrations of DAS.
2. The animals will be dosed with DAS one hour before DES.
3. The animals will be sacrificed four hours after DES exposure.
4. DNA from the mitochondria and nucleus will be analyzed by ³²P-Postlabeling for DNA adduct formation.

Progress for Task 3:

In vivo: Five groups of ten rats each was dosed with various concentrations of DES and DAS. The treatments are as follows: Group 1(Control) will receive corn oil only by i.p. injection. Group 2 will receive DES (150mg/kg, i.p.); Group 3 will receive DES (150mg/kg i.p.) and + DAS (25mg/kg p.o.); Group 4 will receive DES (150mg/kg, i.p) and .DAS (50mg/kg p.o.); Group 5 will receive DES (150mg/kg, i.p) and. DAS 100mg/kg p.o.). Both mtDNA and nDNA from breast tissue will be isolated from each group and analyzed by ³²P-postlabeling.

Results for task 3:

³²P Post Labeling analysis revealed that DES treatment of 150mg/kg produced two mitochondrial DNA adducts. These adducts were not seen in the control treated rats. (figure 6). Further more no adducts were found in any of the DAS treated rats. We conclude the DAS inhibits the formation of DES induced DNA adducts in the breast tissue *in vivo*. This inhibition is speculated to occur by augmenting the regulation of metabolic enzymes and well as direct metabolic inhibition.

Impediments:

We were delayed in some of our experiments due to HPLC problems. We had further problems in relation to Tropical storm/Hurricane Alison in the form of computer failure which resulted in the loss of data and freezer failure which resulted in the loss of samples. We had originally proposed to analyze *in vitro* reactions including microsomal, nuclei, and mitoplast. We only reported the results from the mitoplast because microsomal and nuclei samples were lost. However, we do plan to carryout these experiments in the near future. After getting positive results in the *in vitro* studies we proceeded with the *in vivo* studies. We originally proposed to analyze both mitochondrial and nuclear DNA for adducts. We choose to analyze the mitochondrial DNA first because it was the major theme of our project. We have not as of yet analyzed the nuclear DNA. Again we plan to analyze the results form the nuclear DNA and publish these data.

KEY RESEARCH ACCOMPLISHMENTS

- ◆ We have demonstrated the oxidation and reduction of DES by mitochondria, microsomes, and nuclei isolated from breast of female ACI rats.
- ◆ We have demonstrated that diallyl sulfide inhibits both the oxidation and reduction of diethylstilbestrol in all three systems (mitochondria, microsomes, and nuclei) .
- ◆ These results were demonstrated by UV analysis and confirmed by HPLC.
- ◆ Based on the kinetic constants the nature of this inhibition appears to be non competitive.
- ◆ We have demonstrated that the mitochondria metabolizes DES to reactive intermediates that bind to DNA *in vitro* as well as *in vivo*.
- ◆ We have demonstrated that DAS inhibits the mitochondrial production of the DES induced DNA adducts *in vitro* as well as *in vivo*.

REPORTABLE OUTCOMES**Abstract:**

R.D. Thomas, Chemoprevention by the Metabolic Inhibition of Diethylstilbestrol. Presented at the "Era of Hope" Department of Defense Breast Cancer Research Program. June 2,000.

CONCLUSIONS

We have demonstrated that organelles (mitochondria, microsomes, and nuclei) isolated from the breast of female ACI rats catalyze the oxidation and reduction of DES. This redox-cycling has been demonstrated to produce reactive oxygen species such as superoxide radicals and DES quinone. The reactive molecules can cause DNA damage and ultimately mutations that can cause cancer. The demonstration of the redox-cycling of DES by mitoplast and nuclei are of significance in that these are not traditional organelles of metabolic study. However, they contain the most critical macromolecule (DNA) in regards to carcinogenesis. In addition to

demonstrating that these organelles can metabolize DES to reactive intermediates, we have demonstrated that diallyl sulfide inhibits this metabolism in a noncompetitive fashion in all three organelles. This inhibition may help explain the chemopreventive actions of diallyl sulfide. Furthermore, we have shown that DAS inhibits the formation of mitochondrial DNA adducts in both *in vitro* and *in vivo*. This demonstrates that the direct inhibition of DES metabolism plays a role in the inhibition of DNA adduct formation. We have also shown that DAS administered several days prior to DES, inhibits the formation of DES induced DNA adducts. This implies that some process other than the direct inhibition of metabolism is involved in the inhibition of DES induced DNA adducts. We propose that the expression of CYP450 and Glutathione-S-transferase may be augmented by DAS to afford protection from the production of DES induced genotoxicity. The results of this study will help elucidate the mechanism of estrogen induced breast cancer. This data will provide a foundation for the further investigation of the chemopreventive properties of DAS and structurally similar compounds.

REFERENCES

- Baginski, E.S., Foa, P.P., and Zak, B. (1974) Glucose-6-phosphatase. In: *Methods in Enzymatic Analysis* (ed. H. U. Bergmeyer), Academic Press, Inc., New York, vol. 2, p876-880
- Colton, T., Greenburg, E., Noller, K., Resseguie, L., Bennekou, C., Heeren, T., & Zhang, Y. (1993). Breast cancer in mothers prescribed diethylstilbestrol in pregnancy. *JAMA*, 269(16), 2096-2100.
- Hays, M. A., Rushmore, T.H., & Goldberg, M. T. (1987) Inhibition of hepatocarcinogenic responses to 1,2-dimethylhydrazine by diallyl sulfide, a component of garlic oil *Carcinogenesis* 8(8) 1155-1157.
- Niranjan, B.G. and Avadhani, N.G. (1980) Activation of aflatoxin B₁ by a mono-oxygenase system localized in rat liver mitochondria. *J. Biol. Chem.* 255, 6575-6578.
- Niranjan, B.G., Avadhani, N.G., and DiGiovanni, J. (1985) Formation of benzo(a) pyrene metabolites and DNA adducts catalyzed a rat liver mitochondrial mono-oxygenase system. *Biochem. Biophys. Res. Commun.* 131, 935-42.
- Reddy, M.V.; and Randerath, K. (1989) Nuclease P1-Mediated Enhancement of Sensitivity of ³²P-Postlabeling test for Structurally Diverse DNA Adducts *Carcinogenesis* 7(9) 1543-1551.
- Gupta, R., Reddy, M. & Randerath, K. (1982). ³²P-postlabeling analysis of non-radioactive aromatic carcinogen-DNA adducts. *Carcinogenesis* 3, 1081-1092.
- Shull, J.D. Spady, T.J., Snyder M.C., Johansson, S.L., and Pennington, K.L. (1997) Ovary Intact but not ovariectomized female ACI rats treated with 17beta-estradiol rapidly develop mammary carcinoma. *Carcinogenesis* Aug; 18(8):1595-601.
- Sparnins, V., Barany, G. and Wattenberg, L. (1988) Effects of organosulfur compounds from garlic and onions on benzo(a)pyrene-induced neoplasia and glutathione S-transferase activity in the mouse. *Carcinogenesis* 9:131-134.
- Wargovich, M. A. (1987) Diallyl sulfide, a flavor component of garlic (*Allium sativum*), inhibits dimethylhydrazine-induced colon cancer. *Carcinogenesis* 8(3):487-489.
- Wargovich, M. A., Woods, C., Eng, V., Stephens, L., and Gary, K. (1988) Chemoprevention of N-Nitromethyl-benzylamine Induced Esophageal Cancer in Rats by the Naturally Occurring Thioether Diallyl. *Cancer Research*. 48:6872-6875.

Wattenberg, L., Sporn, V., and Barany, G. (1989) Inhibition of N-Nitrosodiethylamine Carcinogenesis in Mice by Naturally Occurring Organosulfur Compounds and Monoterpenes. *Cancer Research* 49:2689-2692.

Wharton, D. and Tzagoloff, A. (1967) Cytochrome oxidase from beef heart. *Methods. Enzymol.* 10, 245-250.

APPENDICES

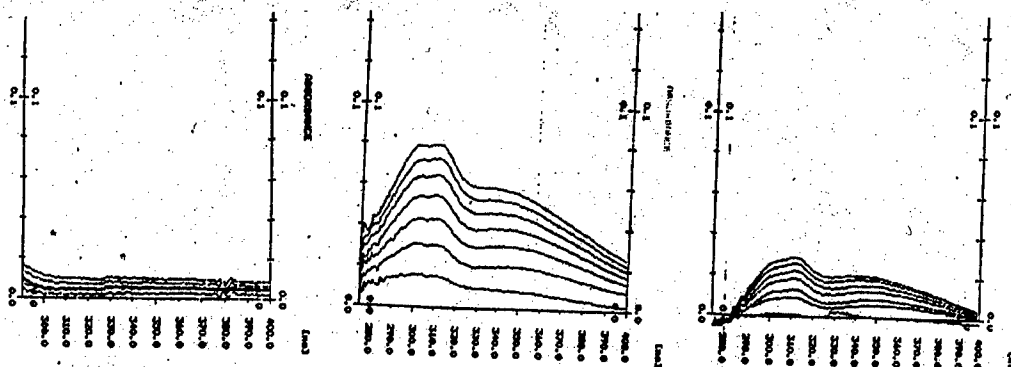


FIG 1. Oxidation of DES to DES Quinone catalyzed by mitoplasts: Panel A represents the control reaction *i.e.* contains no cumen hydroperoxide. Panel B represents the complete reaction. Panel C represents the complete reaction with 373 μ M DAS. The oxidation was monitored by UV spectroscopy. The lowest absorbencies were recorded at time 0. An increase in absorbance was recorded every 30 seconds.

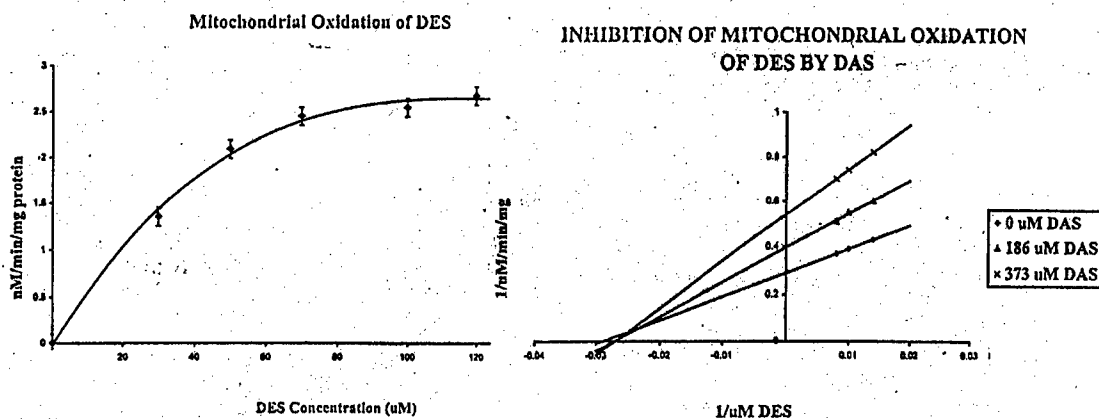


FIG 2. Influence of various concentrations of on the rate of oxidation of DES to DES Quinone by mitoplasts. The reaction mixture consisted of mitoplast (0.42 mg equivalent protein), 120 μ M cumen hydroperoxide, and various concentrations of DES (0-100 μ M) in a final volume of 1 ml of 10mM phosphate buffer pH 7.5. A Lineweaver-Burk plot of rate of formation of DES quinone and its inhibition by DAS revealed a constant k_m with a varying V_{max} . Values represent the means of four experiments.

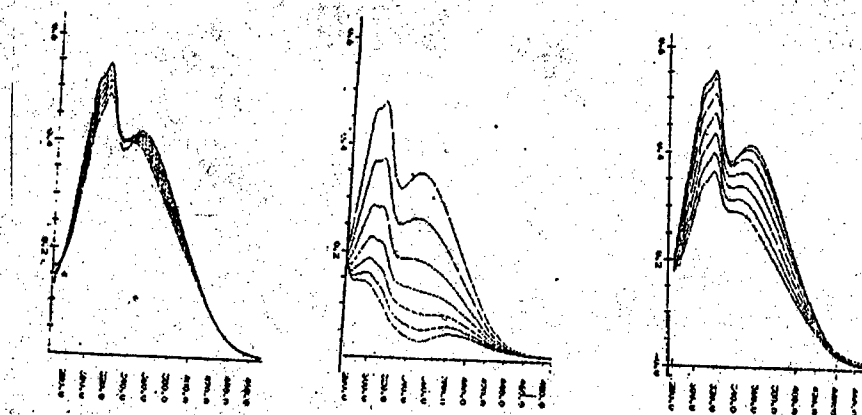


FIG 3. Reduction of DES Quinone to DES catalyzed by mitoplasts: Panel A represents the control reaction *i.e.* contains no NADH. Panel B represents the complete reaction. Panel C represents the complete reaction with 373 μM DAS. The reduction was monitored by U.V. spectroscopy. The highest absorbencies were recorded at time 0. A decrease in absorbance was recorded every 30 seconds.

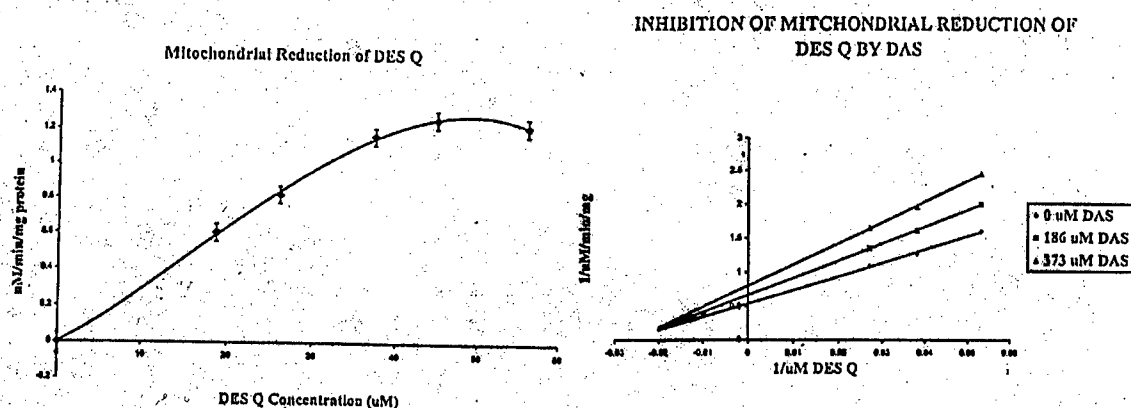


FIG 4. Influence of various concentrations of on the rate of reduction of DES Quinone to DES by mitoplasts. The reaction mixture consisted of mitoplast (0.42 mg equivalent protein), 50 μM NADH, and various concentrations of DES quinone (0-44 μM) in a final volume of 1 ml of 10mM phosphate buffer pH 7.5. A Lineweaver-Burk plot of rate of formation of DES and its inhibition by DAS revealed a constant k_m with a varying V_{max} . Values represent the means of four experiments.

Table1 : KINETIC CONSTANTS OF OXIDATION AND REDUCTION REACTIONS

	Oxidation		Reduction	
	Microsomes	Mitochondria	Microsomes	Mitochondria
K _m	80 μ M	35.7 μ M	100 μ M	50 μ M
V _{max₀} 0 μ M DAS	5.56 p.mol	3.45 p.mol	12 p.mol	2.0 p.mol
V _{max_I} 186 μ M DAS	4.16 p.mol	2.44 p.mol	14 p.mol	1.5 p.mol
V _{max_{II}} 373 μ M DAS	3.33 p.mol	1.82 p.mol	22 p.mol	1.25 p.mol

Table 2: HPLC ANALYSIS OF OXIDATION REACTIONS

Conditions	OXIDATION PRODUCTS (DES Q) pmoles/min/mg protein		
	Microsomes	Mitoplasts	Nuclei
Control (-ChP)	ND	ND	ND
Complete System	3.12 $\pm$ 0.20	2.45 $\pm$ 0.16	0.196 $\pm$ 0.038
+ DAS (186 μ M) (% Inhibition)	2.04 $\pm$ 0.15 35 %	1.66 $\pm$ 0.14 27 %	0.117 $\pm$ 0.014 40 %
+DAS (373 μ M) (% Inhibition)	1.49 $\pm$ 0.12 52 %	1.23 $\pm$ 0.12 50 %	0.078 $\pm$ 0.020 60 %

COMPLETE SYSTEM: 346 μ g/ml microsomes, 420 μ g/ml mitoplast, and 450 μ g/ml nuclei, 120 μ M Cumen Hydroperoxide (ChP), 100 μ M DES, 186 μ M & 373 μ M DAS (Diallyl Sulfide) in 1 ml 10mM potassium phosphate buffer pH 7.5.

Table 3: HPLC ANALYSIS OF REDUCTION REACTIONS

Conditions	REDUCTION PRODUCTS (DES) pmoles/min/mg protein		
	Microsomes	Mitoplast	Nuclei
Control (-ChP)	ND	ND	ND
Complete System	924 ± 119	1.27 ± 0.19	0.16 ± 0.033
+ DAS (186µM)	895 ± 140	0.99 ± 0.14	0.10 ± 0.014
(% Inhibition)	3.1 %	22 %	38 %
+DAS (373µM)	543 ± 120	0.89 ± 0.12	0.075 ± 0.020
(% Inhibition)	41%	30 %	53 %

COMPLETE SYSTEM: 346 µg/ml microsomes, 420 µg/ml mitoplast, and 450 µg/ml nuclei, 50 µM NADH, 44.8 µM DES, 186 µM & 373 µM DAS (Diallyl Sulfide) and 1 ml 10mM potassium phosphate buffer pH 7.5.

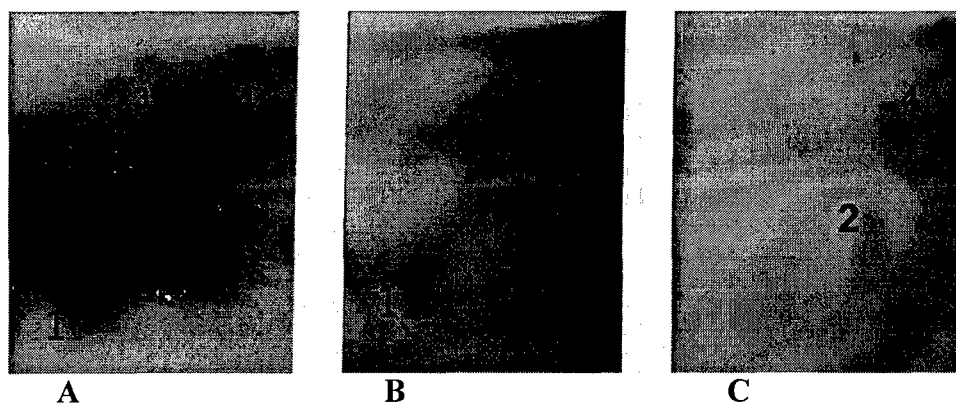


FIG 5. The *in vitro* production of DES induced DNA adducts by mitochondria and their inhibition by Diallyl Sulfides. Panel A is dGMP reacted with DES Q. Panel B represents the DES reacted with mitochondria. Panel C represents DES reacted with DES, DAS, and microsomes.

Table 4. Relative adduct levels for fig 5

Adduct #	DES+dGMP	DES+Mitochondria	DES+DAS+Mito.
1	9.6×10^{-5}	3.4×10^{-7}	-----
2	7.4×10^{-4}	5.1×10^{-6}	6.3×10^{-7}
3	4.1×10^{-6}	-----	-----
4	1.3×10^{-5}	7.1×10^{-7}	8.9×10^{-8}
total		61.5×10^{-7}	7.12×10^{-7}

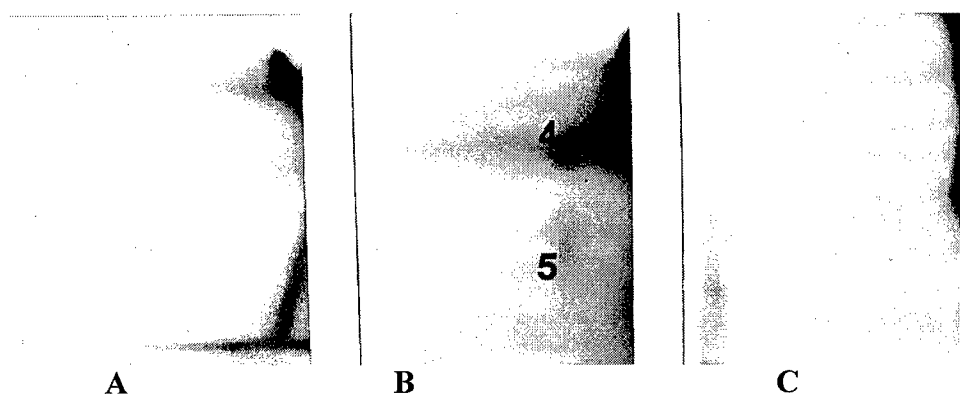


FIG 6 The *in vivo* production of DES induced DNA adducts by mitochondria and their inhibition by Diallyl Sulfides. Panel A represents DNA isolated from rats treated with corn oil (control) . Panel B represents DNA isolated from rats treated with the DES only. Panel C represents DNA isolated from rats treated with DES and DAS

Table 7. Relative adduct levels from fig 6.

Adduct #	Corn oil control	DES only	DES and DAS
4	----	5.4×10^{-8}	----
5	----	3.5×10^{-8}	----