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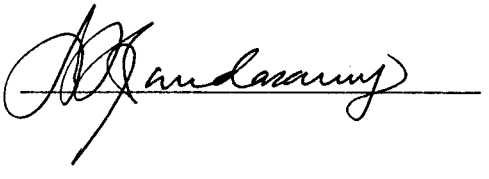

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13. ABSTRACT ***(Maximum 200 Words)***

Breast cancer is the second leading cause of cancer-related deaths for women . Estrogen metabolism as well as mitochondrial alterations are associated with cancer. We propose that the mitochondrial metabolism of estrogens play a major role in the induction of cancer. We hypothesize that diethylstilbestrol (DES) is metabolized by the mitochondria to DES quinone which causes DNA alterations associated with cancer. We further propose that Diallyl Sulfide (DAS) will inhibit this metabolism. To test this hypothesis we isolated mitochondria, microsomes, and nuclei from the breast tissue of female ACI rats. UV analysis revealed that all three organelles catalyzed the oxidation and reduction of DES. DAS was shown to inhibit these reactions by as much as 50%. These results were confirmed by HPLC analysis. These results suggest that metabolism of estrogens may play a critical role in estrogen induced breast cancer. DAS and similar chemicals may inhibit the formation of breast cancer by inhibiting estrogen metabolism. This study provides a rationale for more studies on DAS and similar chemicals on the prevention of breast cancer.

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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. Out next goal is to investigate the potential of DES metabolites to produce DNA adducts in both mitochondrial and nuclear DNA. The inhibition of the production of these adducts by diallyl sulfide will also be determined *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 organells.
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 was used to study the mechanism of estrogen-induced breast cancer. It has been demonstrated that estrogen produce 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.at.,* 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 napthoflavone (50 mg/kg *i.p.*) to induce cytochrome p-450. The rats were sacrificed by carbon dioxide exposure. The breast tissue was be 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 μmol/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 Niranjan *et.al.* (1980 & 85).

Determination of the purity of microsomes:

The purity of microsomes was assessed by both morphological and biochemical analyses. Microsomal prepairations 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 oxidase (Whorton & Tzagoloff, 1967). There was less than 2 % microsomal contamination. Bases 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 prepairations were pure.

Oxidation reaction system:

The reaction conditions consists of 10mM phosphate buffer, pH 7.5, 120μM cumene hydroperoxide, 420 μg/ml mitoplasts and 346 μg/ml of microsomes in a final volume of 1.0 ml. Various concentrations (0-120μM) of DES was used to determine the kinetics constants of the reactions. For specific aim 2 various concentrations of DAS (186 μM and 373 μM) 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 μM NADPH, 420μg/ml mitoplasts and 346 μg/ml of microsomes in a final volume of 1.0 ml. Various concentrations (0-60μM) of DES quinone was used to determine the kinetics constants of the reactions. For specific aim 2 various concentrations of DAS (186 μM and 373 μM) 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(10-50ul) of sample was injected into the HPLC. A methanol/water gradient consisting of 36% to 83% methanol was run using a C_{14} 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 (373μM) 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 Km of 35.7 μM and Vmax of 3.45 nmol/mg protein/min.). The kinetic constants of the reactions were determined by using various concentrations of DES (0-120μM) and two concentrations of DAS (186 μM and 373 μM) With

increasing concentrations of DAS the Km remained constant whereas the Vmax 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 absorbence at 312 nm (Fig 3). In the control reactions little DES quinone was reduced. In the reduction reactions that contained DAS (373μM) 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 disappearnce of DES quinone at various concentrations of DES yielded a Km of 50.0 μM and Vmax of 2.0 nmol/mg protein/min. The kinetic constants of the reactions were determined by using various concentrations of DES Quinone (0-60 μM) and two concentrations of DAS (186 μM and 373 μM). With increasing concentrations of DAS the Km remained constant whereas the Vmax 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 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), individual organells, and DNA to determine the adduct forming potential of DES metabolites.
5. The DNA will be extracted and analyzed for adducts by ^{32}P-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

Mitoplast, microsomes, and nuclei were prepared as described previously. DNA (200μg) was added to the oxidation system described in progress for task one to assess the genotoxicity of oxidative DES metabolites (DES quinone) and the prevention of this toxicity by diallyl sulfide. The DNA will be extracted with organic solvents and analyzed for adducts by ^{32}P-postlabeling.

DNA Ioslation:

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.

^{32}P post-labeling analysis:

To date we have not completed our ^{32}P Post-labeling analysis. We have completed all of the *in vitro* reactions and are in the process analyzing these reactions by ^{32}P Post-labeling. We anticipate having this work completed by December and continuing with the *in vivo* studies in January.

Impediments:

We were delayed in some of our experiments due to HPLC problems. This delayed our completion of the analysis of some of the DES reduction reactions. These analyses should have been completed last year. Currently we have completed the reduction analyses and are working on analyzing the DNA for DNA adducts by ^{32}P Post-labeling. This process was held up due to the fact that we had to change the type of cellulose sheets used in the TLC aspects of the ^{32}P Post-labeling procedure. The cellulose we used in the past is no longer commercially available. We had to make adjustments in the solvent concentrations to adjust for the migration of the adducts on the new sheets. We have made the needed adjustments and feel confident that we will have the project completed on time. Using existing funds we have hired a graduate student to work part time on the project to insure its timely completion.

KEY RESEARCH ACCOMPLISHM ENTS

- 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.

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 mechanism of the chemopreventive actions of diallyl sulfide. The results of this study will help elucidate the mechanism of estrogen induced breast cancer. This metabolic inhibition may play a role in the chemoprevention of estrogen induced cancer. This data will provide a foundation for the further investigation of the chemopreventive properties of DAS and structurally similar compounds.

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APPENDICES

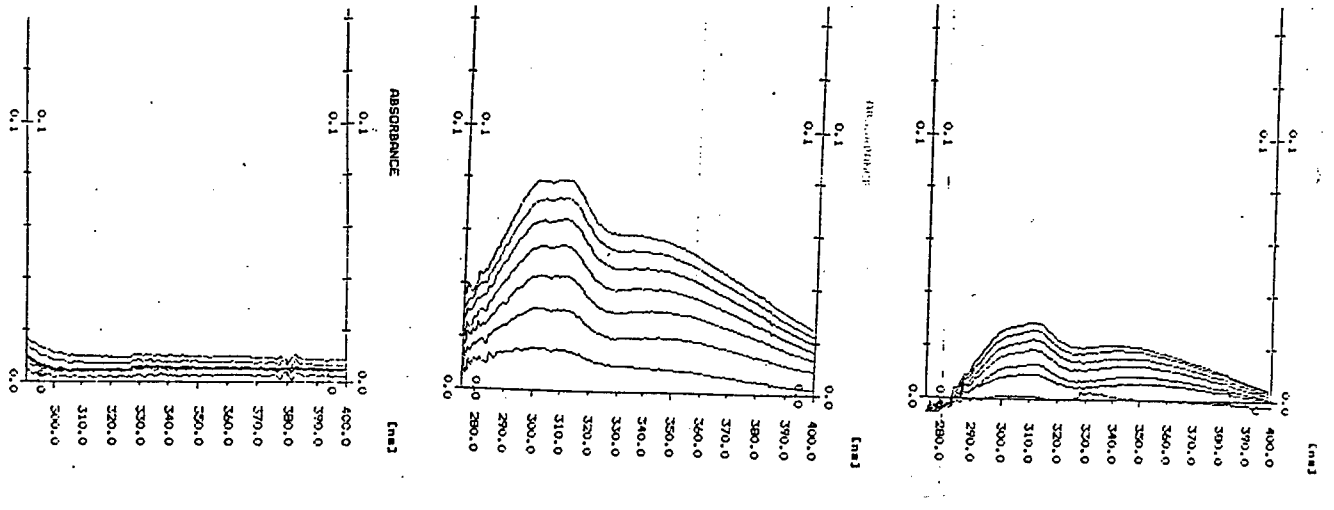

FIG 1. Oxidation of DES to DES Quinone catalyzed by mitoplasts: Panel A represents the control reaction *i.e.* contains no cumen hydroperoxide. Panel B represents the complete reaction. Panel C represents the complete reaction with 373 μM DAS. The oxidation was monitored by UV spectroscopy. The lowest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

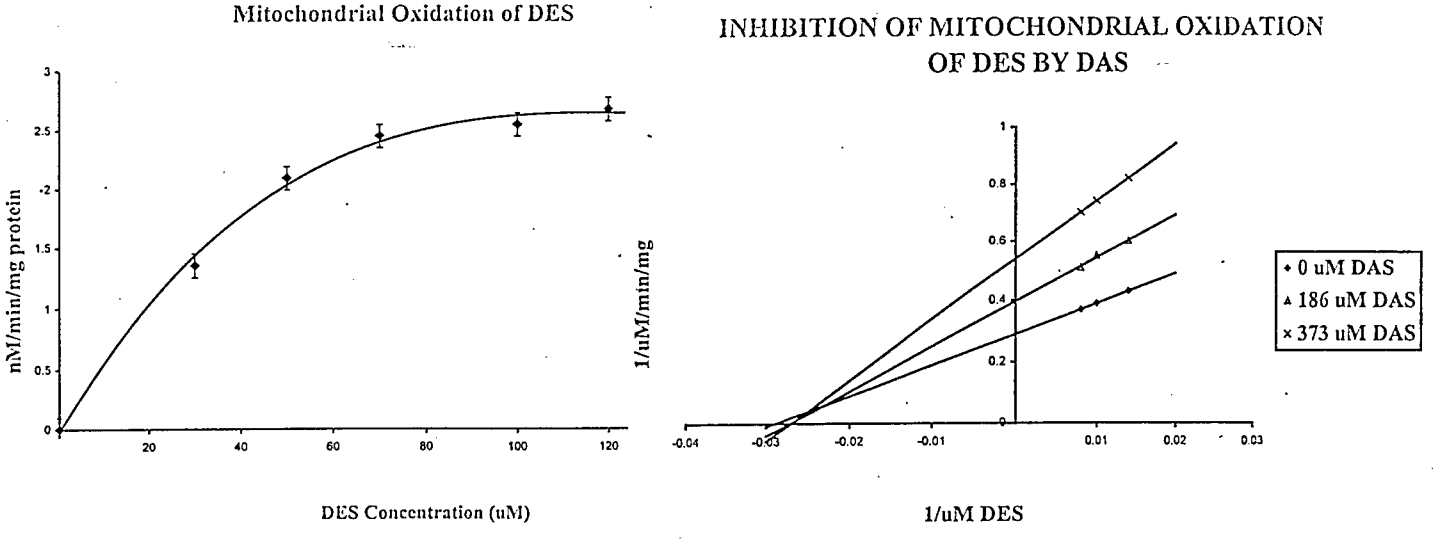

FIG 2. Influence of various concentrations of on the rate of oxidation of DES to DES Quinone by mitoplasts. The reaction mixture consisted of mitoplast (0.42 mg equivalent protein), 120 μM cumen hydroperoxide, and various concentrations of DES (0-100 μM) in a final volume of 1 ml of 10mM phosphate buffer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

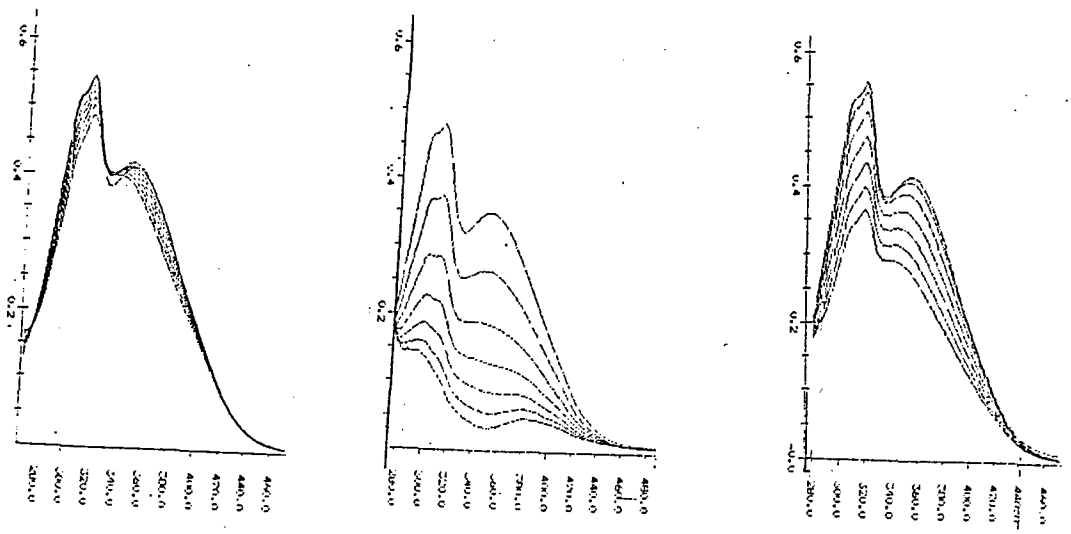

FIG 3. Reduction of DES Quinone to DES catalyzed by mitoplasts: Panel A represents the control reaction *i.e.* contains no NADH. Panel B represents the complete reaction. Panel C represents the complete reaction with 373 μM DAS. The reduction was monitored by U.V. spectroscopy. The highest absorbencies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

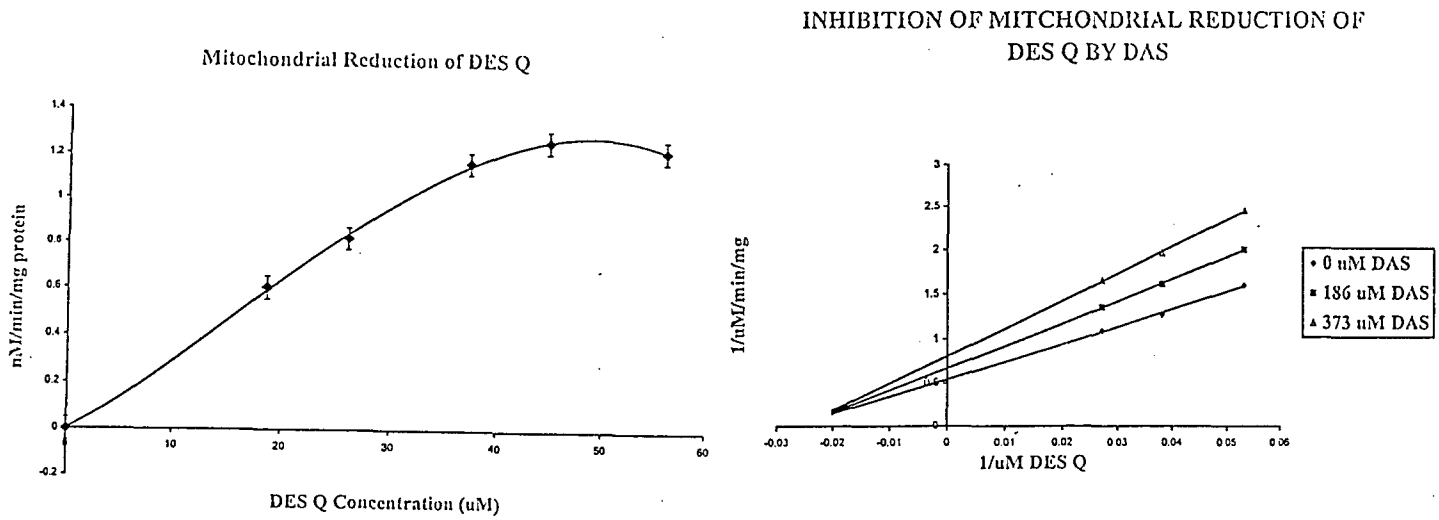

FIG 4. Influence of various concentrations of on the rate of reduction of DES Quinone to DES by mitoplasts. The reaction mixture consisted of mitoplast (0.42 mg equivalent protein), 50μM NADH, and various concentrations of DES quinone (0-44 μM) in a final volume of 1 ml of 10mM phosphate buffer pH 7.5.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

Table1 : KINETIC CONSTANTS OF OXIDATION ANDREDUCTION REACTIONS

	Oxidation		Reduction	
	Microsomes	Mitochondria	Microsomes	Mitochondria
Km	80 μM	35.7 μM	100 μM	50 μM
$Vmax_0$ 0 μM DAS	5.56 p.mol	3.45 p.mol	12 p.mol	2.0 p.mol
$Vmax_I$ 186 μM DAS	4.16 p.mol	2.44 p.mol	14 p.mol	1.5 p.mol
$Vmax_{II}$ 373 μM DAS	3.33 p.mol	1.82 p.mol	22 p.mol	1.25 p.mol

Table 2: HPLC ANALYSIS OF OXIDATION REACTIONS

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

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

Table 3: HPLC ANALYSIS OF REDUCTION REACTIONS

REDUCTION PRODUCTS
(DES) pmoles/min/mg protein

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

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MEMORANDUM FOR Administrator, Defense Technical Information Center (DTIC-OCA), 8725 John J. Kingman Road, Fort Belvoir, VA 22060-6218

SUBJECT: Request Change in Distribution Statement

1. The U.S. Army Medical Research and Materiel Command has reexamined the need for the limitation assigned to technical reports written for this Command. Request the limited distribution statement for the enclosed accession numbers be changed to "Approved for public release; distribution unlimited." These reports should be released to the National Technical Information Service.

2. Point of contact for this request is Ms. Kristin Morrow at DSN 343-7327 or by e-mail at Kristin.Morrow@det.amedd.army.mil.

FOR THE COMMANDER:

Encl

PHYLLIS M. RINEHART
Deputy Chief of Staff for
Information Management

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