DIETHYLSTILBESTROL INHIBITION OF SUCCINOXIDASE OF TUMOR AND TISSUE MITOCHONDRIAL ELEMENTS AND PROGESTERONE AND TESTOSTERONE REVERSAL OF THIS INHIBITION

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McSHAN AND MEYER[1], working with liver homogenates, and REILLY[2], using slices and homogenates of cat heart, showed that diethylstilbestrol inhibits succinoxidase and that this action was effected through the cytochrome–cytochrome oxidase system since brilliant cresyl blue (0.5 %), acting as a hydrogen acceptor in place of cytochrome *c*, eliminated the inhibition. CASE AND DICKENS[3], however, have found that steroids act upon succinoxidase also. Other investigators have shown that steroids act upon enzyme systems such as malicoxidase which requires DPN, fumarate, and oxalacetate (REILLY[2], ERWAY *et al.*[4]), the aerobic oxidation of α-glycerophosphate (GORDAN *et al.*[5]), and the aerobic oxidation of sodium lactate in a yeast-brain system (HOCHSTER AND QUASTEL[6]). GORDAN *et al.*[5] found that methylene blue reversed diethylstilbestrol inhibition of succinoxidase in cat brain, but not inhibition of glucose oxidation. Thus there is uncertainty as to the mode of action of diethylstilbestrol on the succinoxidase system of various mammalian tissues.

McSHAN *et al.*[7] studied the mechanism of hormone action by examining the biochemical properties of subcellular fractions of anterior pituitary gland from normal and castrate adult female rats. No greatly significant differences were found in the case of succinoxidase.

Data are here presented concerning the mode of action of diethylstilbestrol inhibition of the succinoxidase system at cellular and subcellular levels in various mammalian tissues and reversal by progesterone and testosterone.

METHODS

Conventional Warburg techniques were employed. The respiration is reported as Q_{O_2}. The gas phase was air and the center well of each Warburg vessel contained filter paper and 0.20 ml 25% sodium hydroxide. The vessels were filled to contain 0.50 ml mitochondrial suspension, 0.10 ml 0.2 *M* sodium succinate (Merck reagent) or paraphenylenediamine (Eastman Kodak), 0.10 ml 10^{-4} *M* cytochrome *c* (Viobin), 0.20 ml 0.50 *M* phosphate buffer (Baker's) or diethylstilbestrol, progesterone, or testosterone and 0.10 ml saline (0.85% sodium chloride) or sucrose. The hormones (Schering) were dissolved in 0.50 ml water plus 0.04 ml 2 *M* sodium hydroxide which was neutralized with 2 *M* hydrochloric acid (Baker's).

The tissues, liver, brain, and heart of mice (Webster strain N.I.H.), the Harding-Passey and S-91 melanomas (from c and dba strains of mice respectively) and the C_3HBA adenocarcinoma (from C_3H mice) were employed. The mice were sacrificed by dislocating the neck; the tissues, removed immediately, weighed, and placed in various fluids at 0° C.

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The homogenates were prepared by grinding the tissues with a mortar and pestle for about fifteen minutes and filtering through glass wool. The ratios of tissue to suspension medium were as follows: liver, heart, and brain, 1:3 in 30% sucrose; C_3HBA adenocarcinoma, 1:3 in 8.5% sucrose; S-91, 1:3 in saline; and the Harding-Passey melanoma, 1:4, in saline.

The mitochondria were obtained from the filtrates by centrifugal fractionation first separating the nuclei. The centrifugations were performed with the Servall superspeed angle centrifuge type SS-1 and all preparations were examined microscopically to determine their homogeneity. The following centrifugal speeds were used for the various tissues: C_3HBA, 400 r.p.m. for ten minutes followed by two successive runs at 9,000 *g* for some seconds to remove the nuclei, 12,000 *g* for 20 minutes to obtain mitochondria. For the Harding-Passey melanoma, the nuclei were separated by centrifugation at 400 r.p.m. for ten minutes followed by 9,000 *g* for some seconds and the mitochondria, by centrifugation of the supernatant for 30 minutes at 9,000 *g*. In the case of the S-91 melanoma, the nuclei were removed from the homogenate by centrifugation at 400 r.p.m. for ten minutes and the mitochondria for one half hour at 19,000 *g*. The nuclei of liver were removed by centrifuging the homogenate at 12,000 r.p.m. for ten minutes followed by 9,000 *g* twice each for one minute. For brain, the homogenate was centrifuged twice at 9,000 *g* for two minutes and once for one minute and for heart, 9,000 *g* for one minute. The mitochondria of liver and brain were obtained by centrifuging the supernatant for twenty minutes at 19,000 *g* and of heart for one half hour at 19,000 *g*. All mitochondrial preparations were washed once and the volumes were about one-third those of the homogenates.

RESULTS AND DISCUSSION

At concentrations of 0.02 *M* succinate and 10^{-5} *M* cytochrome *c*, diethylstilbestrol (0.38 millimole–1.0 millimole/ml) significantly decreased the Q_{O_2} of tumor mitochondria 50–100% for three tumors: Harding-Passey, S-91, and C_3HBA with pH changes of not greater than 0.20 pH unit. The succinoxidase system of liver, brain, and heart mitochondria also was inhibited by diethylstilbestrol from 30 to 90% at concentrations of 10^{-4} *M*—$3 \cdot 10^{-5}$ *M*. See Table I.

TABLE I

EFFECT OF DIETHYLSTILBESTROL ON SUCCINATE AND PARAPHENYLENEDIAMINE OXIDATION BY TUMOR AND NORMAL TISSUE MITOCHONDRIA OF MICE

Tissue	*Substrates: succinate (0.02 M); cytochrome c (10^{-5} M)*								
	Hormone concentrations, millimoles								
	1.0	*0.50*	*0.38*	*0.25*	*0.125*	*0.10*	*0.06*	*0.03*	*None*
Harding-Passey	1.5	1.5	2.1	5.6	—	—	5.4	6.1	5.7
S-91	0.1	0.1	5.5	—	8.0	—	8.0	11.1	11.5
C_3HBA	0.0	0.7	1.4	1.4	—	—	2.0	—	2.8
Liver	0.8	3.8	—	6.5	9.0	—	—	—	8.9
Brain	—	—	—	—	—	0.2	—	0.9	2.2
Heart	—	—	—	—	—	0.2	—	1.2	4.3
	Substrates: PPDA (0.02 M); cytochrome c (10^{-5} M)								
Brain	—	—	—	—	—	1.6	—	2.3	4.4
Heart	—	—	—	—	—	0.7	—	3.5	3.8

With paraphenylenediamine PPDA (0.02 *M*) and cytochrome *c* (10^{-5} *M*), oxygen consumption was inhibited 50 to 80% for mouse brain and heart mitochondria respectively (Table I). Action of stilbestrol on cytochrome oxidase is suggested.

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TABLE II

INFLUENCE OF TIME OF ADDITION OF SUBSTRATE AND DIETHYLSTILBESTROL UPON OXYGEN CONSUMPTION OF MOUSE LIVER MITOCHONDRIA

Substrates: succinate (0.02 M); cytochrome (10^{-5} M					
Time	*20′*	*35′*	*1 h*	*1 h 20′*	
Conditions	*µl oxygen consumed/mg tissue*				*Diethylstilbestrol concentrations*
Diethylstilbestrol added initially with substrates	1.5 1.8	2.9 2.8	4.9 3.4	6.3 3.8	$1.5 \cdot 10^{-4}$ *M* 10^{-3} *M*
Diethylstilbestrol added initially; substrates added at 35′	1.4 0.08	0.2 0.2	1.2 0.4	2.6 0.8	$1.5 \cdot 10^{-4}$ *M* 10^{-3} *M*
Diethylstilbestrol added at 35′; substrates added initially	2.7 1.0	5.9 2.2	7.9 4.1	8.3 5.7	$1.5 \cdot 10^{-4}$ *M* 10^{-3} *M*
Substrates only	2.8	5.6	8.0	9.7	

The effect of time of addition of the substrates, succinate and cytochrome *c*, and diethylstilbestrol on the Q_{O_2} of liver mitochondria is reported in Table II. For a given time interval the hormone was more inhibitory to succinoxidase when added before the substrate than when added afterwards. REILLY[2] has obtained similar results. HOCHSTER AND QUASTEL[6] have presented evidence to show that diethylstilbestrol may compete with cytochrome *c* as a carrier and that this competition was dependent upon the particular dehydrogenase under consideration.

TABLE III

REVERSAL OF DIETHYLSTILBESTROL INHIBITION OF SUCCINOXIDASE BY PROGESTERONE AND TESTOSTERONE FOR TUMOR MITOCHONDRIA

Substrates: succinate (0.02 M); cytochrome (0.00001 M)										
Tumor: Harding-Passey										
Hormone	*Concentrations, millimoles*									
	1.0	*0.5*	*0.38*	*0.25*	*0.20*	*0.125*	*0.10*	*0.06*	*0.03*	*None*
	Q_{O_2} *(based on 1st 60′)*									
Diethylstilbestrol	1.5	1.5	2.1	5.6	—	—	—	5.5	6.1	5.7
Progesterone	—	—	—	—	7.5	—	5.4	—	5.2	
Diethylstilbestrol + progesterone (0.10 millimole)	1.3	2.3	6.9	—	—	6.2	—	6.1	—	
Tumor: S-91										
Diethylstilbestrol*										
Expt. 1	0.1	0.1	5.5	11.1	—	10.9	—	10.9	—	11.5
Expt. 2	2.0	3.0	5.0	5.0	—	6.0		9.0	8.0	11.0
Diethylstilbestrol* + testosterone (0.20 millimole)										
Expt. 1	0.9	5.3	5.1	7.5	—	11.5	—	10.9	—	
Expt. 2	7.0	10.0	11.0	—	—	12.0	—	10.0	9.0	

* Q_{O_2} values with 0.1 millimoles of testosterone alone were 12.0 and 10.0 in expts. 1 and 2 respectively.

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The effect of testosterone and progesterone on the inhibition by stilbestrol of the succinoxidase system of mitochondria of the Harding-Passey and S-91 melanomas was studied. Reversal of the stilbestrol inhibition by at least 30% is reported in Table III at $2 \cdot 10^{-4} M$ testosterone, $10^{-4} M$ progesterone, $5 \cdot 10^{-4} M$ stilbestrol, 0.02 M succinate, and $10^{-5} M$ cytochrome c. The quantities, $2 \cdot 10^{4} M$ and $10^{-4} M$ of testosterone and progesterone respectively were used because larger amounts sometimes inhibited succinoxidase while smaller quantities were not usually sufficient to produce reversal.

These experimental results were correlated with the growth studies of HERTZ[8] who found that progesterone inhibited the growth of a chick oviduct which was stimulated by stilbestrol. Because progesterone has been shown to reverse stilbestrol inhibition of succinoxidase of tissue mitochondria and inhibit chick oviduct growth stimulation by stilbestrol, the common mechanism of action may be the action of these hormones on the terminal oxidase system of the subcellular mitochondria of the tissue cells involved.

SUMMARY

1. In the presence of 0.02 M succinate and $10^{-5} M$ cytochrome c, diethylstilbestrol ($10^{-3} M$ — $3 \cdot 10^{-5} M$) inhibited by 30–95% the oxygen consumption of the mitochondria of C_3HBA adenocarcinoma, S-91 and Harding-Passey melanomas, liver, brain, and heart of mice; and with paraphenylenediamine instead of succinate, the mitochondria of mouse heart and brain.

2. The inhibition of the succinoxidase of liver mitochondria was more effective if the diethylstilbestrol was added to the tissue before the substrates.

3. The inhibition by diethylstilbestrol ($5 \cdot 10^{-4} M$) of succinoxidase of the S-91 and Harding-Passey melanomas was reversed by testosterone ($2 \cdot 10^{-4} M$) and progesterone ($10^{-4} M$). This can be correlated with the progesterone inhibition of stilbestrol stimulation of growth of chick oviducts as reported by HERTZ[8].

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