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AUTORADIOGRAPHIC ANALYSIS OF DNA AND RNA SYNTHESIS IN THE NEONATAL RAT MAMMARY GLAND AFTER TRANSPLACENTAL EXPOSURE TO DIETHYLSTILBESTROL

City University of New York PH.D. 1984

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AUTORADIOGRAPHIC ANALYSIS OF DNA AND RNA SYNTHESIS IN THE NEONATAL RAT MAMMARY GLAND AFTER TRANSPLACENTAL EXPOSURE TO DIETHYLSTILBESTROL.

by

BARBARA STERN BERGMAN

A dissertation submitted to the Graduate Faculty in Biology in partial fulfillment of the requirements for the degree of Doctor of Philosophy, The City University of New York.

1983

This manuscript has been read and accepted for the Graduate Faculty in Biology in satisfaction of the dissertation requirement for the degree of Doctor of Philosophy.

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ABSTRACT

The administration of DES during pregnancy has been associated with benign and malignant changes in target tissues of the lower genital tract in humans and rodents. This study examined the effects of exposure to DES in utero on another estrogen target tissue, the mammary gland. Pregnant rats were injected with 1.2 µg DES during the second (days 10 & 13) or third (days 15 & 18) week of pregnancy. Offspring were injected with either 3H-thymidine or 3H-uridine at 5 days of age. The second and fifth pairs of nipples were fixed, serially sectioned, and coated with NTB-3 liquid emulsion (Kodak). In the nipple hood, the upper and lower mammary duct, the upper and lower mesenchyme, and in the epithelium lateral to the nipple opening, number of grains was counted for uridine analysis and number of labeled cells was used for thymidine. The Mann-Whitney U Test was applied to these data. In 3H-thymidine injected pups exposed to DES in the second week, label

uptake was reduced significantly ($p<0.05$) in the upper and lower duct regions, while in the third week, no difference in labeling was found in any tissue examined. 3H-uridine labeled pups had reduced labeling in the upper and lower duct, the nipple hood, and the nipple epithelium after DES exposure during weeks 2 and 3. Mesenchyme did not incorporate much label in either experimental or control animals. This evidence of early inhibition of DNA and RNA biosynthesis is consistent with recent data showing a reduction in ductal branching after neonatal exposure to DES or estradiol in the mouse (Tomooka & Bern 1982).

DEDICATION

To my children, Brandy and Thomas, and my parents, whose support, encouragement, and love have made the completion of this thesis possible.

ACKNOWLEDGEMENTS

I would like to express my sincere thanks and deep appreciation:

to Dr. Elizabeth S. Boylan for her constructive advice and kind encouragement in guiding this project from start to finish.

to Jacqueline Doody for her patient technical instruction and prodigious assistance.

to Dr. Michael Seidel for providing thoughtful and constructive suggestions.

to Dr. Jeanne Szalay for the use of her darkroom.

and to the rest of the faculty and laboratory workers at Queens College whose warm helpfullness has made my time there so pleasant.

PREFACE

Diethylstilbestrol (DES) administration during pregnancy has been associated with benign and malignant changes of the lower genital tract in rodents and humans. The teratogenic effects of prenatal DES may be visible early in life while the induction of cancer by DES follows a long latent period (Herbst et al. 1971).

Like the lower genital tract, the mammary gland is also an estrogen target tissue. Considering the large population of DES-exposed women, it is of interest at this time to examine the transplacental influence of DES on developing breast tissue. This is particularly timely as the greatest prescription of this drug occurred from the 1940's through the 1950's and the population of DES-exposed women is just reaching the age (over 40) when the risk of breast cancer greatly increases.

In addition, establishing the period during differentiation when a tissue is particularly sensitive to a carcinogen might shed some light

on the entire process of carcinogenesis. For these purposes this thesis examines the early effects of transplacental hormone exposure. Using autoradiography, and the rat as a model system, effects of prenatal DES exposure on rates of DNA and RNA synthesis in the neonatal mammary gland were studied.

TABLE OF CONTENTS

LIST OF TABLES

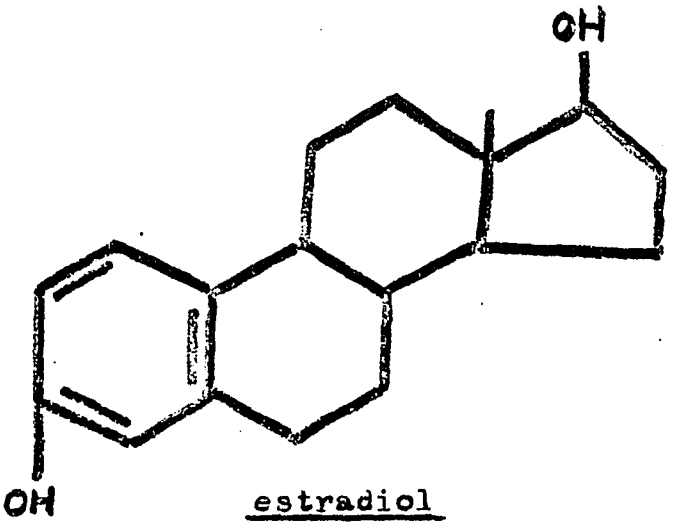
LIST OF FIGURES

INTRODUCTION

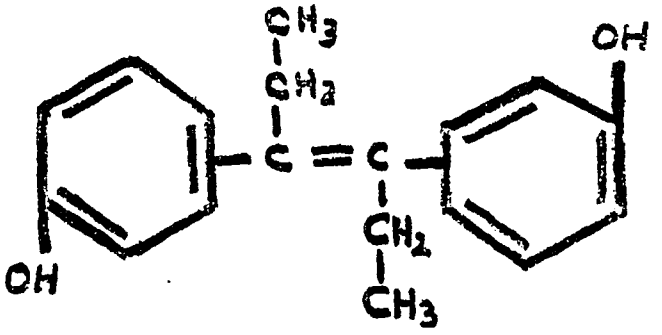
DES Structure: Diethylstilbestrol (4,4'dihydroxy-α-β diethylstilbene), or DES, is a synthetic alcohol with high estrogenic activity. With a molecular weight of 268.4, it is a small molecule which is capable of crossing the placenta (Nissen & Goldstein 1973; Fischer et al. 1976; Shah & McLachlan 1976; Metzler & McLachlan 1978).

Synthesized in London by Sir E. Charles Dodd in 1938, DES rapidly gained wide use as it is inexpensive, orally active, and physiologically as effective as the most potent of the natural estrogens. DES has the greatest steroidal effect of any of the molecules of the stilbene family which also includes hexestrol and dienestrol, each of which is lacking the double bond found in DES. Its high estrogenicity depends on the presence of its two free hydroxyl groups and on the trans position of the ethyl moieties (Heftman 1970) (Figure 1).

FIGURE 1. STRUCTURES



estradiol



diethylstilbestrol

DES Metabolism: After ingestion, DES is rapidly absorbed from the small bowel and distributed throughout the organism. In the pregnant female it crosses the placenta as evidenced by the transfer of radioactively labeled DES from the maternal to the fetal compartment in both the mouse (Shah & McLachlan 1976) and the rat (Fischer et al. 1976). In fetal and adult rodents and humans, DES is extensively and rapidly metabolized, primarily by the liver (Metzler & McLachlan 1978; McLachlan & Dixon 1977). The liver conjugates the DES with glucuronic acid producing diethylstilbestrol monoglucuronide (DESG) as a major metabolite. Along with this, a small sulfate-conjugated fraction is produced. These conjugates are excreted in the urine (Metzler 1976, McLachlan & Dixon 1977). Fecal excretion of DES consists of unconjugated material (Metzler 1976) which is probably formed as a result of the hydrolysis of bile conjugates by glucuronidase produced by intestinal flora (Fischer et al. 1976). In addition to conjugation, the production of oxidative metabolites has been demonstrated in

various species (Metzler & McLachlan 1978).

It is the DES compound itself, rather than one of its oxidative or conjugative metabolites, that has the greatest estrogenicity. Nonetheless, the metabolites are of interest in that, though most are inactive, some retain estrogenicity, some behave as antiestrogens, and some even demonstrate genotoxic activity (Glatt et al. 1979, Rudiger et al. 1979).

DES Action: DES acts as an estrogen analogue. Target tissues such as the breast, vagina, oviduct, and uterus have protein receptor molecules in the cytoplasm which bind DES and estrogen preferentially (Jensen & DeSombre 1972; Jensen et al. 1974), and with approximately equal binding affinity (Lipsett 1977; McLachlan & Dixon 1977). It has been shown that steroids act by diffusing into target cells and combining with the receptor molecules (Gorski et al. 1968; Jensen & DeSombre 1972; O'Malley & Schrader 1976). After binding, which probably causes a conformational change, the hormone-receptor

complex is translocated to the nucleus (Jensen et al. 1974; Gorski et al. 1968) where it binds at acidic non-histone protein acceptor sites on the interphase chromosome. Transcription follows, leading to the production of new RNA, its association with polysomes, and subsequent protein synthesis (Gorski et al. 1968; Jensen & DeSombre 1972; Mueller et al. 1972; Milgrom et al. 1973; Buller & O'Malley 1976; O'Malley & Schrader 1976).

It is reasonable to consider that the DES-receptor complex may act to initiate protein synthesis via the same route as that utilized by natural steroid. Though it should be mentioned that, in the case of corticosterone, the synthetic analogue dexamethesone was found to bind with a physiochemically distinct cellular receptor from those to which the natural steroid binds (Agarwal 1976). Nonetheless, the route of subsequent translocation to the nucleus and DNA interaction may be identical to that used by the natural steroid and its receptor.

DES, like natural estrogen, has an

overall anabolic effect on target tissues leading to increased activity of most metabolic pathways. After administration, levels of alkaline phosphatase, NADH oxidase, glucose 6-phosphate, lactic dehydrogenase, and RNA polymerase in the rodent vagina and uterus are elevated (Jensen & DeSombre 1972; Anderson et al. 1973; Heftman 1970; Mori 1976). Increased mitosis, RNA, and protein are observed in the uterus and vagina of the rat and mouse (McLachlan & Dixon 1977; Buller & O'Malley 1976; O'Malley & Schrader 1976; Heftman 1970). Increased growth of mammary duct and stroma occurs, as does proliferation of uterine, oviduct, and vaginal epithelium. Also, increased oviduct motility is seen (Morrell & Hart 1941; Heftman 1970; Notides & Gorski 1966; Baird 1972). Estrogenic compounds induce estrus. They yield an increase in the amount and viscosity of cervical mucus and increased fat deposition occurs.

In primates, their administration results in an increase in glycogen and mucopolysaccharride in the vagina, along with

enhanced nipple pigmentation (Heftman 1970).

Continued estrogen causes an increase in progesterone receptors rendering the exposed tissue more responsive to subsequent hormone exposure (Jensen & DeSombre 1972; Milgrom et al. 1973; Chan & O'Malley 1976). Estrogen has been shown to increase serum prolactin levels as well (Meites 1974).

DES History: After its development in 1938 the use of DES in pregnancy became fairly widespread. It was easily and inexpensively manufactured. Unlike natural estrogen, it was not labile to stomach acid, and could be taken orally, rendering it much easier to administer to patients.

Based on the theory that much fetal wastage was due to inadequate estrogen production, Doctors George and Olive Smith in 1946 recommended the prophylactic administration of DES in pregnancy. They proposed that it would prevent fetal loss and hemorrhage and, therefore, should be given to "at risk" pregnant females. A woman who was

staining during pregnancy, cramping, or with a history of previous miscarriage, was considered to be at risk (Smith et al. 1946). A 1948 report by Dr. Olive Smith, pooling the observations of 117 obstetricians who had given DES to 632 pregnant women, stated that a regimen of DES in increasing doses from weeks 7 to 35 of pregnancy would protect against toxemia and eclampsia, and increase the chance of a successful pregnancy in women who have previously miscarried, or who have high blood pressure or diabetes (Smith 1948). Between 1940 and 1960 hundreds of thousands, and possibly millions, of women were treated. Some women were given a small dose, while many took DES throughout their pregnancies in continually increasing doses until term (Herbst et al. 1975b; Ulfelder 1973). It appears that the Smith endorsement was the impetus leading to mass prescription of the drug for use in pregnancy. This usage occurred despite studies appearing as early as 1938 which contraindicated this practice.

In 1938 Lacassagne reported mammary adenocarcinomas in male mice given DES.

Similar findings soon followed (Shimkin & Grady 1940). Still the greatest use of DES in pregnancy occurred from the late 1940's to the early 1950's.

Serious question as to the drug's effectiveness arose in 1952 with the first controlled study of the use of DES in pregnant women. This study showed DES ineffective as the experimental group of 200 women had a slightly higher incidence of premature birth and miscarriage (Ferguson 1953). These findings were further supported by a large double blind study conducted at the Chicago Lying-in Hospital which indicated that DES was ineffective in preventing fetal loss, prematurity, toxemia, or eclampsia. Again the DES-exposed patients faired slightly less well than those exposed to placebo (Dieckman et al. 1953).

Following the Dieckman study, which was repudiated by Doctors Smith and Smith (Smith & Smith 1954), prescription of DES for use in pregnancy declined greatly. Still, it continued until this use of the compound was finally banned by the FDA in 1971 (FDA

Bulletin 1971). It should be noted that DES is still used today in males with prostate cancer, in postmenopausal women, as a morning-after pill, and to prevent lactation after parturition.

Though the effectiveness of DES was doubted as early as 1952 (Ferguson 1952), it appears that its safety was unquestioned until much later. Even the tests of its effectiveness done by Ferguson and Dieckman exposed hundreds of pregnant women to DES, an unconscionable act if its safety was at issue (Ferguson 1952; Dieckman et al. 1953).

The unusual clustering in young women of an extremely rare form of cancer, clear cell adenocarcinoma of the vagina, was the fortuitous event which aroused suspicion (Herbst et al. 1971). Previously, this form of cancer was almost exclusively observed in the uterus of older women. Prior to 1970 only a few cases of clear cell adenocarcinoma of the vagina and cervix in females under 25 years old had ever been recorded in the medical literature. Surprisingly, in 1970, seven such cases were observed at one hospital

center in women whose ages ranged from 15 to 22 (Herbst & Scully 1970).

Adenocarcinoma is a tumor of glandular epithelium. The normal vaginal lining is stratified squamous epithelium and lacks the glandular elements one would expect to find in the cervix or uterus. This makes the occurrence of adenocarcinoma in the vagina unexpected. A retrospective study was conducted to determine commonality amongst the women with this disease. Transplacental exposure to DES was implicated (Herbst et al. 1971; Greenwald et al. 1973). More evidence supporting the relationship between fetal DES exposure and vaginal carcinoma soon came to light (Lanier et al. 1973; Herbst et al. 1975a; Fowler & Edelman 1978). Registries were set up to attempt to reach as many of the exposed women as possible, and to determine the overall extent of the consequences of this transplacental DES exposure (Herbst et al. 1979; Robboy et al. 1979; Tilley et al. 1978). It is estimated that anywhere from 4 to 6 million American women received this drug during their prenatal life (Nordqvist et al.

1979).

Results of Intrauterine Exposure to DES in Daughters: Prenatal exposure to DES and other similar synthetic estrogens resulted in a constellation of abnormalities in the female and male reproductive tracts of humans, rodents, and other mammals. In human female offspring of mothers who ingested DES, the incidence of clear cell adenocarcinoma of the vagina, after an average latency period of 19 years, has been estimated to be anywhere from 0.14 to 1.4 per thousand through the age of 24 (Herbst et al. 1977, 1979; Nordqvist et al. 1979). Carcinoma is rare before age 14, though the youngest reported case was in a 7 year old. There is an incidence peak between 17 and 21 years of age (Herbst et al. 1975b; Robboy et al. 1977).

Vaginal adenosis, the presence of glandular columnar epithelium or its mucinous products, particularly in the anterior upper wall of the vagina, is a consequence reported in about 80% of the exposed daughters (Antonioli & Burke 1975; Mattingly & Stafl

1976; Tilley et al. 1978). In the general population the incidence of adenosis is less than 4% (Bibbo et al. 1977). It appears that the occurrence of adenosis increases as the age of fetal exposure decreases, and it is relatively independent of DES dose level (Bibbo et al. 1977; Herbst et al. 1977). Exposure before week 18 is a better prognosticator of subsequent harm than either dose level or total duration of exposure (Poskanzer & Herbst 1977; Herbst et al. 1977; O'Brien et al. 1979; Robboy et al. 1977). The columnar epithelium of adenosis is composed of mucin rich cells like the endocervix. With this condition a chronic inflammatory infiltrate may be present resulting in heavy mucus discharge.

Cervical ectropion or erosion, the presence of glandular tissue or its mucinous products on the outside of the cervix (cervical adenosis) appears either alone, or along with the vaginal adenosis, with which it will most likely be contiguous (Herbst et al. 1975b; Bibbo et al. 1977; Ng et al. 1977; O'Brien et al. 1979).

With time, adenosis is known to regress as the columnar epithelium undergoes metaplastic squamous replacement. Glycogen poor squamous epithelium, the result of this metaplasia, is commonly observed. There is a question of increased risk of malignant transformation in this atypical glandular tissue (Fetherston 1975; Herbst et al. 1975a; Stafl & Mattingly 1974). Dysplastic and neoplastic changes have been observed by some (Nordqvist et al. 1979; Bibbo et al. 1977; Fowler & Edelman 1978; Mattingly & Stafl 1976; Stafl & Mattingly 1974). They suggest that there may be an increased risk of squamous carcinoma in these offspring. However, there have been reports based on follow-up of offspring (O'Brien et al. 1979; Antonioli & Burke 1975; Greenwald et al. 1973) and on DNA studies (Fu et al. 1978) that this enlarged transformation zone did not result in increased squamous neoplasm or carcinoma in situ.

Structural malformations of the upper and lower genital tract have been observed in DES daughters. Hypoplastic T-shaped uteri with

anomalous constricting bands and synechiae have been reported in offspring after transplacental DES exposure (Kaufman et al. 1977; Haney et al. 1979; Bibbo 1979). Abnormal transverse vaginal ridges across the normally smooth upper wall of the vagina have also been reported in excess in DES exposed daughters (Goldstein 1978). About one fifth of those daughters with early exposure to DES will present with either a collar-like formation of tissue called a vaginal hood, or a hypertrophic deformity consisting of a series of raised ridges known as a cock's comb cervix (Nordqvist et al. 1979; Bibbo 1979). In addition, protrusions of the central portion of the ectocervix surrounded by circular grooves, called cervical pseudopolyps, are often observed (Nordqvist et al. 1979).

Benign abnormalities of the vaginal and cervical lining such as leukoplakia (white epithelium), mosaicism and punctation (abnormal capillary patterns in the lining tissue), and Nabothian cysts (cysts formed as the surface of the small glands become plugged

by squamous epithelium overgrowth) have been reported in excess in the DES-exposed females compared to their incidence in the general population. Menstrual problems, including dysmenorrhea and oligomenorrhea (Barnes 1979; Haney et al. 1979; Bibbo et al. 1977); and reduced fertility, including both difficulty in conceiving (Berger & Goldstein 1980; Bibbo et al. 1977), as well as increased fetal wastage (Goldstein 1978), have been reported in DES-exposed female offspring. Fetal loss may be related to hypoplasia of the cervix and its incompetence during gestation (Barnes 1980, Berger & Goldstein 1980; Goldstein 1978).

Results of DES Exposure in Sons: The male offspring arising from transplacental DES exposure have not gone unscathed. They present with an increased incidence of epididymal cysts (Bibbo et al. 1977; Cosgrove et al. 1977; Gill et al. 1976), hypospadias (Bibbo et al. 1977; Gill et al. 1976), urethral stenosis (Henderson et al. 1976;

Cosgrove et al. 1977), cryptorchidism and testicular hypoplasia (Gill et al. 1976), capsular incuration (Bibbo et al. 1977; Gill et al. 1976), and pathological semen (Gill et al. 1976; Bibbo et al. 1977). Though no DES-associated carcinogenesis has been demonstrated in the male, there is an association between undescended testes and testicular cancer.

Perinatally Estrogenized Female Rodents: Even though the reproductive tract and mammary gland are not identical from one species to another, lesions produced in animal models are indicative of the consequences to humans of intrauterine DES and estrogen exposure. Extrapolation from experimental animal systems to humans must be done cautiously as the reproductive system of the neonatal rodent is the developmental equivalent of the human reproductive system at about 3 to 4 months of gestation. In addition, placental metabolism and deliverence of various substances show interspecific differences. Also there is

variation among species in the presence of serum binding proteins for estrogenic hormones. Nevertheless, sequelae in rodent models often mimic well the results of prenatal exposure to estrogenic compounds in humans.

The teratogenicity of perinatal exposure to estrogenic compounds is well established. Early experiments showed that high transplacental estrogen doses in rats prevented parturition, inhibited the development of the ovarian capsule and the urogenital sinus, caused hypospadias, and preserved vestiges of Wolffian ducts (Greene et al. 1940). If given early enough, resorption of pregnancy occurred (Greene et al. 1940). Similarly, high doses of DES during gestation caused virilization of female offspring. These progeny had large distended uteri, bare gonads, Wolffian duct remnants, and inhibited development of the lower vagina (Greene et al 1939, 1940). More moderate prenatal doses of DES (0.5 to 120µg per animal) proved teratogenic in both the mouse and the rat. In mice, exposure resulted in

persistence of the urogenital sinus (rather than its differentiation into a separate vagina and urethra), hypertrophy of the portio vaginalis (Nomura & Kanzaki 1977), hypospadias (Lamb et al 1979; McLachlan et al. 1980), and retention of Wolffian duct remnants (McLachlan et al. 1980). In an attempt to pinpoint a critical time of susceptibility to DES during the development of a structure (the palate), pregnant mice were treated with single doses of DES on any one of days 8 through 15. DES proved to be a teratogen leading to cleft palates as it prevented the fusion of the palate buds after just a single small dose given on the critical day (Gabriel-Robez et al. 1970). DES in 3 injections (days 9 to 11) in mice produced aortic and ventricular communication anomalies in exposed fetuses (Clavert & Gabriel-Robez 1968).

In the rat, transplacental DES produced females with enlarged cystic ovaries, with distended uteri and vaginae, and with developmental abnormalities of the urogenital sinus epithelium including urethrovaginal cloaca formation due to incomplete fusion of

the tissue along the ventral surface of the urethra (hypospadias) (Boylan 1978; Vorherr et al. 1979).

Acyclic anovulatory vaginal cornification often called "persistant estrus" has been observed in the golden hamster (Rustia & Shubik 1976), the rat (Napalkov & Anisimov 1979; Vorherr et al. 1979), and in the mouse (Nomura & Kanzaki 1977; McLachlan et al. 1980) after prenatal exposure to DES. In mice treated neonatally with low doses of estrogen or DES, the persistant vaginal cornification was shown to be ovary-dependent (Bern et al. 1975; Forsberg 1976, 1977). When cornification was the result of treatment with high dosage, ovariectomy had no palliative effect on the persistant cornification syndrome. The latter suggests a permanent disturbance of hypothalamic gonadotrophin secretion (Kimura 1975; Bern et al. 1975; Herbst & Bern 1981).

Glandular elements in the vagina (adenosis), particulary in the fornices, have been reported as a consequence of prenatal (Walker 1980; Mclachlan & Dixon 1977;

Plapinger & Bern 1979; Lamb et al. 1979) or neonatal (Forsberg 1976, 1977) DES exposure in the mouse, and after transplacental and/or transmammary exposure in the rat (Vorherr et al. 1979). In addition, DES-exposed female mice progeny in one study had large transverse vaginal ridges (Walker 1980). Precocious vaginal opening was observed in the mouse after prenatal DES exposure (Nomura & Kanzaki 1977).

At maturity, prenatally DES-exposed rodents had reduced reproductive capabilities (McLachlan et al. 1975b; Boylan 1978).

Endometrial squamous metaplasia, sometimes with cystic hyperplasia, has been reported in the mouse (McLachlan & Dixon 1977; Lamb et al. 1979) and in the rat (Vorherr et al. 1979) after transplacental DES exposure. It has been argued that squamous metaplasia is significant in its association with later squamous cell carcinoma and that the presence of abnormal glandular columnar elements is related to the eventual development of adenocarcinoma (Forsberg 1976, 1977, 1981; Herbst & Bern 1981). Golden hamsters given

gastric intubations with DES during the last third of gestation produced female offspring with both hyperplastic and neoplastic reproductive tract lesions such as hypertrophy of the cervix along with an inflammatory exudate, uterine polyps and squamous cell metaplasia, adenomyosis (invasion of the uterine musculature by the endometrium), and one adenocarcinoma of the uterus (Rustia & Shubik 1979).

Early evidence that DES is tumorigenic in rodents came from the production of kidney clear cell carcinomas in the golden hamster after continuous DES treatment of adults (Mathews et al. 1974). Forsberg (1977) found squamous carcinomas of the uterus in mice neonatally treated with DES and estrogen. In another study prenatal DES in mice resulted in large vaginal tumors (Nomura & Kanzaki 1977). Uterine adenocarcinoma was produced in CD-1 and NMRI mice stock after prenatal DES (Forsberg & Kalland 1981; McLachlan & Dixon 1977, McLachlan et al. 1980).

In the rat prenatal DES resulted in squamous cell carcinoma of the vagina, one

adenocarcinoma of the uterus and one of the ovary (Vorherr et al. 1979). Subcutaneous DES treatment of pregnant rats increased the tumor response of their female progeny to subsequent DES exposure to 3 times the rate of tumorigenesis found in controls and only the experimental group had tumors of the ovary and the corpus uteri (Napalkov & Anisimov 1979).

Prenatal estrogenic exposure appears to predispose the animal to a neoplastic response to subsequent carcinogen treatment during postnatal life (Rustia & Shubik 1979; Boylan & Calhoun 1979). DMBA (7,12-dimethyl benz(a)anthracene) treatment of hamsters prenatally exposed to DES and those not exposed to DES resulted in a greater "multiplicity of tumors per tumor bearing animal" and in a higher incidence of malignancy in the DES-exposed group. These tumors included malignant carcinomas and benign adenomas of the mammary gland, benign polyps and malignant carcinomas and sarcomas of the uterus, benign squamous cell papillomas of the vagina and cervix, dermal melanomas, and squamous cell carcinomas and papillomas of

the forestomach (Rustia & Shubik 1979).

The rodent mammary gland is also an estrogen target tissue and there is evidence that estrogenic compounds are also teratogenic to the mammary gland, with the severity of the anomaly produced correlating with the time of exposure and with the dose administered. The growth and development of mammary anlagen in rat progeny were inhibited following prenatal administration of large amounts of steroid or DES (Greene et al 1939, 1940). In mice, estradiol benzoate (0.5µg) given days 8 and 9 of gestation inhibited development of mammary ducts and nipples in both sexes (Hoshimo & Connally 1967). Inhibited mammary gland development and nipple malformations including amastia, micromastia, coelomastia (cutaneous pockets), and premature lifting were observed in female mice after transplacental estrogen (Jean & Delost 1964; Jean 1971, Raynaud 1971). Mice treated neonatally with 17β-estradiol had distended mammary ducts (Mori 1968a). In female rat neonates treated with 17β-estradiol or prolactin, the mammary glands regressed and consisted only of rudimentary duct systems on

examination of whole mounts (Nagasawa et al. 1974). The precocious appearance of prominant nipples after prenatal DES in rats (Greene et al 1939; Boylan 1978), and after estrogen dipropionate in mice (Raynaud 1971) was reported.

Changes in nipple morphology in prenatally DES-exposed rodent offspring were dose and time dependent (Nomura & Kanzaki 1977; Boylan 1978). Inhibition of the nipple hood (epithelial projections on either side of the duct which penetrate the underlying tissue) was observed at high doses (120μg); low DES doses (1.2μg) in the third week of gestation resulted in reduced epithelial hood projections, but yielded normal hoods when administered in the second week (Boylan 1978). The hood is the rudiment of the nipple sheath and its inhibition may be related to the observation that, on maturing, female offspring had difficulties nursing their young (Boylan 1978). Reduced branching of mammary gland ducts was an early consequence of neonatal DES or 17β-estradiol in mice (Tomocka & Bern 1982).

Experiments done as early as the 1930's and 1940's demonstrated estrogen induction of mammary gland cancers in adult rodents after prolonged and/or high doses, by either injection or pellet implantation (Lacassagna 1932; Shimkin & Grady 1940; Eisen 1942). In female mice bearing mammary tumor virus, perinatal estrogen yielded an earlier onset and a higher incidence of mammary gland tumors (Bern et al. 1975; Mori 1968a, 1968b). Contradictory results were obtained in neonatally estrogenized female mice of virus free strains (Dunn 1963). Mice receiving estradiol as neonates and carcinogen as adults had more carcinogen-induced dysplasias than controls (Warner & Warner 1975).

Varying results have also been reported in the rat. In one experiment the administration of 10 to 40 µg estradiol to neonatal female rats inhibited carcinogen-induced adenocarcinomas as of 180 days (Nagasawa et al. 1974). Others reported enhanced tumorigenic response of rat mammary gland tissue to subsequent carcinogen exposure after early estrogenization. Progesterone

treatment of 5 day old female Sprague-Dawley rats increased the incidence of DMBA-induced adenocarcinoma (Shellabarger & Soo 1973). More palpable mammary tumors after DMBA treatment were reported in rats prenatally exposed to DES (Boylan & Calhoun 1979). In Sprague-Dawley rat neonates, estradiol benzoate and testosterone propionate decreased the mammary adenocarcinoma response and increased the mammary fibroadenoma response to DMBA (Shellabarger & Soo 1973). Prenatal DES in the golden hamster increased DMBA induction of mammary gland cancers (Rustia & Shubik 1979).

Other ramifications of perinatal estrogenic exposure are diverse and far-reaching. They include altered brain differentiation and behavior (Gorski et al. 1977), enzyme inductions (Lamartiniere & Lucier 1977), and changes in hormone receptor levels. Estrogen treatment was reported to yield an increase in both estrogen and progesterone receptor levels in the guinea pig uterus (Milgrom et al. 1973; Freifeld et al. 1974; Jensen & DeSombre 1972). An increase in

prolactin cells in the pituitary of mice neonatally treated with estrogen and/or progesterone has been demonstrated (Kawashima et al. 1978). Neonatal DES also resulted in higher blood plasma prolactin levels in mice (Nagasawa et al. 1978) as well as in rats (Nagasawa et al. 1973).

It has been demonstrated in mice that prenatal exposure to DES compromised the immune system. Decreased thymic weight and changes in B and T cell responses were reported after interuterine exposure (Kalland et al. 1979; Ways et al. 1980; Luster et al. 1979).

Perinatally Estrogenized Male Rodents: Male rodents show lesions of the reproductive tract and mammary gland after perinatal estrogenization. Large doses of DES given to pregnant rats feminized their male offspring. These progeny had external genitalia of the female type, they lacked prostates; and development of the seminal vesicles, epididymis, and vasa deferentia was inhibited.

In addition, parts of the uterine horns (Mullerian duct derivatives) were retained (Greene et al 1939, 1940). Prenatal DES given during the last third of gestation in the golden hamster produced progeny with spermatic granulomas of the epididymis (70%) and testes (40%) (Rustia & Shubik 1976). Prenatal DES in mice resulted in undescended hypoplastic testes (Nomura & Kanzaki 1977), epididymal cysts (McLachlan et al. 1975a), reduced reproductive capacity (McLachlan and Shah 1975), and the retention of Mullerian duct vestiges (evident in the appendix testes and prostatic utricle) (McLachlan et al. 1975a).

In the rat transplacental DES led to hypospadias (cleft phallus and urethra), undescended testes and their hypogenesis, and the persistance of Mullerian structures (Morrell & Hart 1941; Vorherr et al. 1979). Rudimentary uterine horns were found. Wolffian duct structures (seminal vesicles, vas deferentia, epididymis) were partly absent or underdeveloped (Vorher et al. 1979).

The male mammary gland is also an estrogen target tissue. Male rats prenatally

exposed to DES were born with prominent visible nipples (Boylan 1978). Estrone benzoate and DES proved mammary gland carcinogens in male mice (Lacassagna 1932, 1938).

In summary, rodent experiments provide convincing evidence that perinatal estrogenic compounds are genital tract and mammary gland teratogens and carcinogens. They affect females and males, are active physiologically on target tissues of the reproductive tract, mammary gland and hypothalamo-hypophyseal axis, and they affect biochemical systems and immune function.

TABLE 1: AREAS EFFECTED BY PERINATAL ESTROGENIZATION

NEUROENDOCRINE SYSTEM	
	HYPOTHALAMUS
	HYPOPHYSIS-GONADOTROPHINS
	PROLACTIN LEVELS
	HORMONE RECEPTOR LEVELS
MAMMARY GLAND	
	MORPHOLOGY
	HORMONE SENSITIVITY
	TUMORS
REPRODUCTIVE TRACT	
	MORPHOLOGY
	HORMONE SENSITIVITY
	TUMORS
IMMUNE SYSTEM	
	THYMUS
	CELL MEDIATED IMMUNITY
	HUMORAL IMMUNITY
BIOCHEMICAL SYSTEMS	
	ENZYME REGULATION

Embryology of the Genital Tract and Mammary Gland: One must consider the development of the normal genital tract and mammary gland in order to begin to explain the changes induced by early hormone exposure. The embryology of the genitalia and mammaries of both humans and rodents is similar and will be discussed together except where marked developmental differences occur.

The gonads: The genital ridges form as thickened strips of mesodermal epithelium from the upper edge of the visceral layer of lateral plate mesoderm. These genital rudiments are invaded by migratory primordial germ cells of extra-embryonic (endodermal) origin which give rise to the gametes. The genital ridges thicken and protrude into the coelomic cavity to form the cortex (surface) of the gonads. Cells of the mesonephrogenic cord migrate to the gonad rudiments to produce the primitive sex cords of the medulla (interior) of the gonad. The indifferent gonad with two sets of ducts, the mesonephros (Wolffian ducts) and paramesonephros (Mullerian ducts), persists in humans until

about the seventh week of gestation (Torrey 1945; Blandau et al. 1963; van Tienhoven 1968; Balinsky 1975; Fujimoto et al. 1977). The testes differentiate earlier than the ovaries do and evidences of testicular organization are apparent in 7 week embryos. Absence of testes at this time presages the development of an ovary during the eighth week.

Rodent development is similar and histologic signs of testicular differentiation of the gonad are evident in hamster, mouse, rat and guinea pig embryos on days 11, 12, 15, and 22 respectively. This precedes ovary development and coincides with the onset of androgen secretion in male embryos (Price & Ortez 1965; Forsberg 1981).

In both humans and rodents the seminiferous tubules arise from the primitive sex cords. These are invaded by the primordial germ cells which give rise to the spermatozoa. The sex cords produce the Sertoli cells. Thus, the medulla dominates in males and becomes the functional part of the testes while the cortex regresses and becomes a thin epithelial surface covering. In the

human the testes remain in the groin and do not descend through the inguinal canal until the seventh month reaching their scrotal sac position in the eighth month (Balinsky 1975; Forsberg 1981).

The human ovary begins to differentiate in the eighth week. The situation is the reverse of that in the male in that the primary sex cords degenerate and the gonadal cortex proliferates and predominates. The medulla becomes reduced and fills with loose mesenchyme. The primordial germ cells remain in the cortex to become oocytes (Balinsky 1975).

The genital ducts: The pronephros appears in about the third week of human embryogenesis. The development of the mesonephros (Wolffian ducts) follows. The Wolffian ducts, which degenerate in females, persist in the male to form the epididymis, vas deferans, and seminal vessicles. In the fifth week the paramesonephros (Mullerian ducts) arises from coelomic epithelium. The Mullerian ducts grow caudally, parallel and lateral to the Wolffian ducts which they cross

so that the caudal ends of the Mullerian ducts lie central to the two Wolffian ducts in the lower pelvis (Gruenwald 1959; Forsberg 1981).

At this indifferent stage (6 weeks) embryos of both sexes possess a double set of genital ducts (mesonephros and paramesonephros). Differentiation in response to sex hormones secreted by somatic cells of the gonads causes regression of the genital ducts of the opposite sex and retention and proliferation of the appropriate ducts (Balinsky 1975; Ratzan & Waldon 1979; Resko 1977). In the female, estrogen secretion causes the Wolffian duct regression. Testicular antimullerian hormone causes the Mullerian ducts to regress in the male except for cranial remnants which form the appendix testes and the caudal ends which persist as the prostatic utricle (Jost et al. 1973; Josso et al. 1977). In the female, the Mullerian ducts survive and are involved in the formation of the Fallopian tubes, the uterus, and the vaginal primordia (Jost et al. 1973, Resko 1977).

The vagina and uterus: In 7 or 8 week

female embryos the Mullerian ducts fuse beginning at the caudal ends and progressing cranially. As they unite the common wall disappears so that an epithelial tube (the uterovaginal canal) with a single lumen is formed. The caudal tip of the fused Mullerian ducts forms the hymen. Cranial to the hymen, the lower portion of the uterovaginal canal forms the rudimentary vagina lined by columnar Mullerian epithelium. This simple Mullerian epithelium of the vaginal rudiment undergoes transformation into a stratified Mullerian epithelium of polygonal cells. Subsequently, starting caudally, the upgrowing vaginal plate replaces the transformed stratified Mullerian epithelium so that the vagina is finally lined with stratified squamous epithelium leaving the squamocolumnar junction at the level of the external os of the cervix (Forsberg 1972; Ulfelder & Robboy 1976; Prins et al. 1976; Herbst 1975a; Forsberg 1981).

Though there is some disagreement as to whether the vaginal plate arises from the Wolffian ducts or from the urogenital sinus, the consensus supports a sinus origin. This

epithelial vaginal plate proliferates and expands as it proceeds cranially to resorb and replace the Mullerian epithelium and the vaginal lumen is filled in the process. Cavitation occurs in the center of the plate recreating the vaginal lumen and forming a vagina lined with stratified squamous epithelium of urogenital sinus origin (Prins et al. 1976; Ulfelder & Robboy 1976; Raynaud 1977; Forsberg 1977, 1981).

Organogenesis of the rodent vagina is slightly different. The hollow upper two-fifths (rat) to three-fifths (mouse) of the vagina forms from the fused Mullerian ducts while the remaining solid region below this is derived from urogenital sinus epithelium. The urogenital sinus portion divides into a dorsal sinus vagina and a ventral urethral portion. Decreased intercellular adhesion, rather than cell degeneration, leads to lumen formation in the sinus vagina (Cunha 1975; Forsberg 1977, 1981). Thus, the immature rodent vagina consists of cranial Mullerian columnar epithelium and caudal ectodermal urogenital

sinus epithelium. The Mullerian columnar epithelium is transformed into stratified squamous epithelium. This transformation proceeds cranially up into the cervical canal and continues in the neonate after birth (Forsberg 1973; Cunha 1975; Raynaud 1977).

Human and rodent development is similar in that both combine Mullerian duct and urogenital sinus elements, and both involve a transformation of Mullerian epithelium into stratified squamous epithelium. The uterus arises from the upper part of the uterovaginal canal formed from the fused Mullerian ducts. The vaginal fornices form as solid outgrowths of the vaginal plate which later hollow out.

It is of interest that the human vagina shows estrogen sensitivity before the uterus does and this sensivity is related to increased estrogen binding by the vagina compared to the uterus between 4 to 7 months (Boving et al. 1978).

The mammary gland: In humans the first evidence of the mammary glands appears as a mammary band in 4 mm embryos. This region of raised ectoderm broadens and a medial ridge of

tall cells, the milk line (mammary crest), is present resting on condensed mesenchyme at the 7 mm stage. In the 10-12 mm embryos the milk line is visible cranially but fades caudally. In the thoracic region hillocks form in the milk line. These hillocks are biconvex collections of ectodermal cells surrounded by condensed mesenchyme. The line continues to thicken cranially and primitive epithelial buds form, increase in volume, and become globular in shape by the 20-30 mm stage. These primitive mammary buds are quite well differentiated by the end of the third month.

By the fourth month nipple primordia appear as slight hollows surrounded by raised epidermis in the mammary buds. During the fifth month the surfaces of the buds spread out, depressions form, and duct formation begins. The bud epithelium proliferates to produce secondary buds. These secondary buds give rise to cellular cords which bore into the mesemchyme to produce ducts, the future galactophores. In 5-6 month embryos the cords further bifurcate and internal degeneration results in lumen formation. The mesenchyme

forms septa dividing the mammary ducts into lobules. In the 7-8th month, the apex of the nipple desquamates forming a hollow and the nipple increases in width and flattens. In the ninth month the underlying dermis pushes out the depressed nipple surface so that the galactophores open. At term the galactophores have further lengthened and branched so that the gland is a complex of 15-25 separate openings surrounded by a connective tissue capsule (Raynaud 1961).

Mammary gland development in the female rat begins during the second week of gestation and shows rapid growth during the third week (days 15-22). The first evidence of the mammary primordia appear in the 11 day embryo as two subcutaneous thickenings of the ectoderm which extend from the shoulder to the inguinal region. These thickenings, the mammary streaks, grow downward into the underlying mesenchyme and, by day 14, rudiments of 6 pairs of mammary glands are visible. By day 15 the rudiments have enlarged and appear as club-shaped hillocks (Young & Hallowes 1973; Myers 1916, 1917a).

At day 17 a depression (the mammary pit) forms above the developing hillock. Epidermal downgrowths surrounding the primary duct form on days 18-19. These projections called the nipple hood are the rudimentary nipple sheath. The mammary pit of cornified epithelium deepens at this time toward the primary duct (Myers 1917a; Green et al. 1939). By day 20 ductal branching has progressed to the level of tertiary ducts and lumina have formed. Underlying mesenchymal cells have condensed about the branched ducts. Though nipples are not obvious at birth, they become visible in the female after 4-5 days (Myers 1917b).

Rat mammary glands show more sexual dimorphism than humans mammaries do. Androgen secretion in the male inhibits mammary gland development so that at birth, in the male rat, there is less branching of ducts, the primary duct does not break the surface, and the nipple and nipple hood are missing (Myers 1917b, 1920; Elger et al. 1979).

The results of transplacental exposure to estrogenic compounds in humans and rodents have been discussed along with a brief

examination of the normal embryology of the lower genital tract and mammary gland. This was presented to provide a framework for the present study which examines the effects of transplacental DES exposure on the developing rat mammary gland.

MATERIALS AND METHODS

Animal Care and Background: Sprague-Dawley derived (CD) rats obtained from the Charles River Breeding Laboratories (Wilmington, MA) or rats bred from CD stock were used in this experiment. They were maintained in temperature controlled quarters and exposed to alternating 12 hour periods of light and dark. Rats were provided with Purina Rat Chow (St. Louis, MO) and tap water ad libitum.

Mating Procedures: In the late afternoon 3 to 4 month old virgin females were weighed and placed in the cages of males of proven fertility, 4 to 5 females per male. Presence of sperm in vaginal smears taken the next morning established day zero of pregnancy. At day 10 these rats were weighed again. Weight gain of 40 grams or more was taken to confirm their gravid state. Those which were pregnant were randomly assigned to one of 4 groups for experimental treatment.

Prenatal Exposure to Hormone: Diethylstilbestrol (DES) was purchased from Sigma Chemical Co (St. Louis, MO). To achieve a total dose of 1.2 µg DES per rat, each rat received two subcutaneous injections (0.3 ml each) of a 2.0 µg/ml solution of DES in sesame oil. These two injections were given during the second week of pregnancy (days 10 and 13) or third week (days 15 and 18) in order to coordinate with either the first evidence of the development of the paired mammary primordia, or with their differentiation respectively (Myers 1917a). One third (n=14) of the gravid rats were injected with the DES solution during week two (DES2) and one third (n=14) received the DES during week three (DES3); and, to serve as controls, approximately one sixth (n=8) of the remaining rats were injected with vehicle alone during week two, and one sixth (n=9) during week three (V2 and V3). Rats were left to deliver at term.

Healthy offspring were segregated based

on sexual dimorphism in anogenital distance. All males except two per treatment group were sacrificed. The spared males were retained for examination for Mullerian duct remnants at 5 days of age (Appendix A). Females were returned to their mothers.

Radioactive Labeling: Radioactive nucleoside precursors of DNA and RNA were used to label newly synthesized DNA and RNA in the neonates. Sterile aqueous solutions of thymidine (methyl-3H) with a specific activity of 20.0 Ci/mM and of uridine (5-3H) with a specific activity of 30.0 Ci/mM, each at a concentration of 1 mCi/ml, were obtained from New England Nuclear (Boston, MA). These were diluted with sterile saline to provide a suitable volume for intraperitoneal injection. Pups were given a dose of 1μCi/gm body weight (Gude 1968).

At day five, when nipples became visually apparent, female pups were removed from their mothers and kept warm using incandescent light. They were tagged and weighed so that

weight specific injection volumes could be calculated. Very fine gauge (#27) hypodermic needles were used to inject these pups so as to minimize tearing of the extremely thin neonatal ventral skin and to reduce "oozing" of radioactive fluid from the injection site. Each of the 4 previously established rat groups (DES2, DES3, V2, V3) was divided into 3 subgroups depending on whether pups were injected with 3H-thymidine or 3H-uridine or left untreated to provide background control values. Thus, 12 different treatment classes were established (Table 2).

TABLE 2: TREATMENT CLASSES

TITLE	WEEK OF GESTATION	PRENATAL TREATMENT	LABEL
DES2T	2	DES	THYMIDINE
DES2U	2	DES	URIDINE
DES2	2	DES	NONE
V2T	2	VEHICLE	THYMIDINE
V2U	2	VEHICLE	URIDINE
V2	2	VEHICLE	NONE
DES3T	3	DES	THYMIDINE
DES3U	3	DES	URIDINE
DES3	3	DES	NONE
V3T	3	VEHICLE	THYMIDINE
V3U	3	VEHICLE	URIDINE
V3	3	VEHICLE	NONE

Treatment and Sacrifice of Pups: ^{3}H-uridine injected pups were sacrificed by submersion in ice water followed by severence of the cervical spine with a sharp scalpel. Using a dissecting microscope, the second, third, and fifth nipple pairs were removed.

The second and fifth were taken for examination of hormone effects. The third pairs provided test tissue on which exposure time and label specificity was determined (Appendices B, D & E). Each nipple was placed skin side up on aluminum foil and allowed to dry for one minute so as to adhere and remain flat for later sectioning. These nipple and foil combinations were then fixed in vials of 10% neutral buffered formalin to await further processing for autoradiography.

3H-thymidine labeled pups were similarly sacrificed and their nipples removed and processed as above. A portion of each of the thymidine and uridine labeling solutions was subjected to paper chromatography to test for purity; radioactivity in chromatograph samples was measured using a Nuclear-Chicago Mark II scintillation counter (Appendix C).

Histotechnique: All nipples remained in the 10% buffered formalin fixative for a minimun of 48 hours. Nipples were passed through an 80 minute dehydration series in

tetrahydrofuran (THF) consisting of 4 successive 20 minute baths (one 50% THF/50% water, 3 pure THF).

For infiltration, nipples were placed in successive 20 minute baths of 50% THF/50% paraffin and pure paraffin at 56°C. They were embedded in individual paraffin containing boats to prepare for sectioning and mounting on slides.

Each nipple containing paraffin block was chilled (refrigerator, then freezer), trimmed to a square, and than serially sectioned at 5 microns on a Spencer 820 microtome.

To prepare for section mounting, glass slides were cleaned with 95% alcohol, carefully dried to avoid streaking, and placed on a warming tray set at 45° C. The ribbons were affixed using a flood of dilute albumin solution as an adhesive agent. The sections were stretched and the rows arranged so that they occupied the right-hand two-thirds of the slides at least 4 mm from each edge (Rogers 1979; Gahan 1972; Gude 1968). For drying, the slides were moved to a warming tray set at 36° C where they remained overnight.

Completely dry slides were stored in wooden slide boxes.

Autoradiographic Technique: Slides were deparaffinized by passage through several changes of xylene and hydrated using a series of graded ethanols to distilled water. To show that the label was specifically incorporated into nucleic acid, four slides from each batch were subjected to enzymatic digestion, two by bovine pancreatic deoxyribonuclease (DNASE) and two by bovine pancreatic ribonuclease (RNASE) from Sigma Chemical Company (St. Louis, MO). Slides were incubated at 37°C in the DNASE (0.05 mg/ml) in 0.1M Tris maleate buffer at pH 6.5 for 24 hours (Humason 1967; Daoust 1964). Incubation with RNASE (0.3 mg/ml) in 0.01M phosphate buffer at pH 7.6 was done for 3 hours at 37° C (Carmago & Plaut 1967; McDonald & Kaufman 1954) (Appendix D).

The remaining deparaffinized slides, along with those subjected to enzymatic hydrolysis, were allowed to dry prior to

application of liquid photographic emulsion in a light-tight darkroom equipped with a 15 watt bulb with a #2 Wratten filter (Kodak, Rochester, NY). The NTB3 liquid emulsion (Kodak, Rochester, NY) was removed from the refrigerator one hour before use and melted in a thermostatically controlled water bath at 44° C for 30 minutes in total darkness (Gahan 1972) The emulsion was poured into a narrow glass vial designed to hold one slide at a time. Warmed slides (42° C) were carefully dipped for one second and slowly removed so that excess emulsion dripped back into the vial. The backs of the slides were wiped with tissue and the edges and bottoms blotted. The slides were hung from clothespins on a wire rack.

Slides were stored horizontally, emulsion side up, in light-tight wooden boxes which were wrapped in aluminum foil. Drierite wrapped in gauze was added to each box to prevent fading (Gude 1968; Kopriwa & Leblond 1961). Boxes were refrigerated at 4° C for an exposure period of two weeks for the 3H-thymidine labeled tissues and four weeks

for the 3H-uridine labeled tissues (Appendix E).

Slides were developed with agitation in Dektol (Kodak, Rochester, NY) for two minutes at 18°C and dipped for 10 seconds in a 1% acetic acid stop bath followed by agitation in Kodak fixer for 5 minutes. A rinse in cold tap water for at least 15 minutes followed (Gude 1968; Kopriwa & Leblond 1961; Rogers 1979).

Dried slides were stained with Ehrlich's hematoxylin for 12 minutes, rinsed with distilled water, dipped into Scott's solution for two minutes, dehydrated through graded ethanols up to 70%, stained with eosin for 10 minutes, and completely dehydrated through graded alcohols followed by two final two minute xylene baths. Coverslips were applied immediately with Permount (Fisher Scientific Company). When thoroughly dry, excess emulsion was cleaned from the edges and the backs with a razor and the slides were stored in wooden boxes.

Counts: Visual grain counting was

undertaken on all slides. Counts were taken within a 6 box area (90 square microns) of a grid using an oil immersion lens (970X). Nipples were subdivided into 6 sections: upper mesenchyme (UM), lower mesenchyme (LM), upper duct (UD), lower duct (LD), hood (H), and epithelium (E) (Figures 2 & 3). A careful attempt was made to evaluate identical areas in each nipple using the most longitudinal section available. In 3H-thymidine labeled tissues the number of labeled cells per total number of cells present in the test area was counted. A cell whose nucleus contained greater than 5 grains was considered labeled (Mori et al. 1983; Rogers 1979; England et al. 1973). This number clearly differentiated between labeled and unlabeled cells as the former generally had many grains and the latter none, or one or two at most (Figure 4). In 3H-uridine exposed nipples, where labeling was much more diffuse, the total number of grains present per number of cells in the area evaluated was tallied (Figure 5). Similar counts were made on unlabeled and enzyme treated control slides.

FIGURE 2. AREAS EVALUATED
Counts were made in a 6 box area (90 sq μ) in all regions. The centermost section was used (where the nipple duct breaks the surface).
UD=upper duct. Counts were taken in a 1 x 6 box area beginning 4 boxes below the surface of the nipple.
LD=lower duct. Counts were taken in a 1 x 6 box area beginning at the left margin of the duct, or (if contiguous with the upper duct) beginning at the point where the lower duct turns and becomes approximately perpendicular to the upper duct.
UM=upper mesenchyme. Counts were taken in a 2 x 3 box area centered between the nipple hood and the upper duct at a distance one box below the epithelium.
LM=lower mesenchyme. Counts were taken in a 2 x 3 box area, 2 boxes above the lower duct, centered over the region talleyed for the lower duct.
H=hood. Counts were taken in a 1 x 6 box area beginning 4 boxes below the surface of the nipple.
E=epithelium. Counts were taken in a 1 x 6 box area beginning 2 boxes to the right of the nipple pore.
*This same format was adhered to even when the lower duct and lower mesenchyme were found on a different section from the section on which the rest of the counts were taken.

FIGURE 2. AREAS EVALUATED

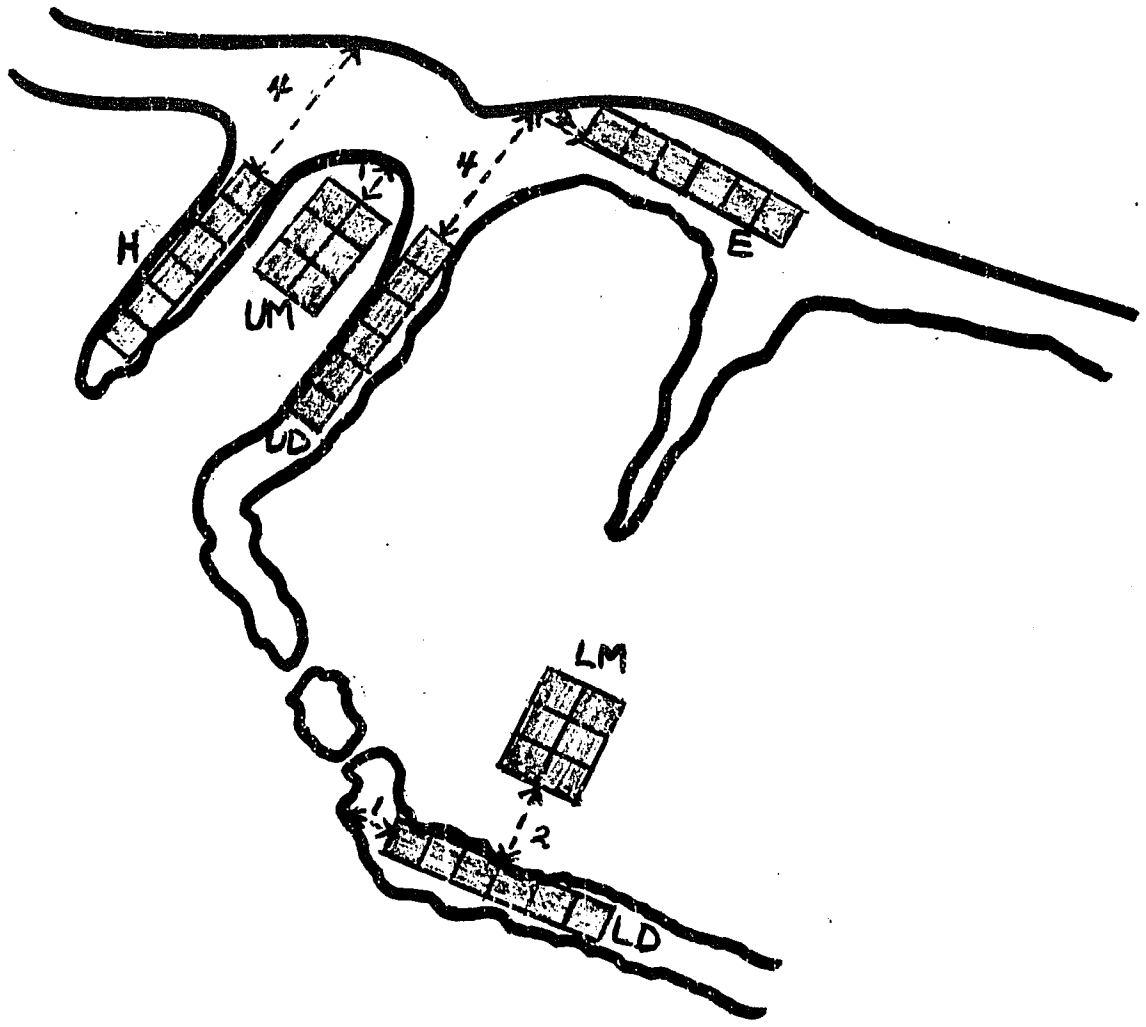


FIGURE 3. Photomicrograph of typical nipple section.

FIGURE 4. 3H-Thymidine labeling.

FIGURE 5. 3H-Uridine labeling.

Organization of Data and Statistical Analysis: Data for each nipple taken was entered into the computer listing the total number of cells per 6 box area for the upper mesenchyme, lower mesenchyme, upper duct, lower duct, hood, and epithelium. For 3H-thymidine exposed nipples and their controls, the number of labeled cells present in each of these areas was entered; and for 3H-uridine exposed tissues and their controls, the total number of grains present in each area was listed. Data for all 6 test areas was entered for 115 nipples. Of the total of 690 entries, 6 values were missing where nipples taken lacked the required region of lower mesenchyme or lower duct. In these cases the mean value was inserted in place of the missing value. For comparison purposes ratios were computed of the number of labeled cells for 3H-thymidine, or number of grains present for 3H-uridine, each divided by the total number of cells present in the area in question. Background levels were subtracted from each entry (Appendix F). To eliminate zero and negative values 0.5 was added to all

values. Even following various reasonable transformations, the data failed to be normally distributed. Therefore, using the Statistical Package for the Social Sciences (SPSS, 1979), the nonparametric Mann-Whitney U Test was applied. This is a semi-graphical test of equality of location of two samples. Critical values of U, the test statistic, are tabulated for two samples sizes n1 and n2. The two groups are combined and cases are ranked in order of increasing size. The statistic U is computed as the number of times a score for group one precedes a score for group two. If the samples are from the same population, the distribution of the scores for the two groups should be random. Extreme values of U indicate a non-random pattern (Sokal & Rohlf 1969; Hull & Nie 1979).

TABLE 3: DATA MANIPULATIONS*

1. Raw data consisted of the number of cells present in the measured area of UD, LD, UM, LM, H or E (for each of 115 nipples) along with the number of labeled cells present in each of these areas (thymidine) or the total number of grains present in each area (uridine).

2. Data for the LM or LD was missing on six nipples. The appropriate means were inserted in these cases.
   LM mean number cells = 15
   LD mean number cells = 48
   Mean number labeled cells or grains present was computed for each treatment class and entered.*

3. Subfiles were created.

4. Ratios were computed of the number of labeled cells per number of cells present for thymidine, or number of grains per number of cells for uridine.

5. Background values were subtracted for each autoradiographic batch (4 dipping times). For thymidine slides there was no correction needed as none of the test slides had cells which were labeled. The following values were subtracted from ratios for uridine slides for dip 3 and dip 4:

	Dip 3	Dip 4
UM	0.5444773	0.4901960
LM	0.6563231	0.3075000
UD	0.1993122	0.1119976
LD	0.1854520	0.1203703
H	0.3548951	0.4153846
E	0.2870773	0.2614130

6. To eliminate any zero or negative values, 0.5 was added to all entries.

7. Merged files were created in order to make the desired comparisons as follows:
   DES and vehicle trimester 2 (thymidine).
   DES and vehicle trimester 3 (thymidine).
   DES and vehicle trimester 2 (uridine).
   DES and vehicle trimester 3 (uridine).

8. The Mann-Whitney U test was applied to these merged files. The two groups compared for each trimester were represented as N1 (DES) and N2 (Vehicle).

*Detailed discussion of statistical analysis in Appendix (F).

RESULTS

Outcome of pregnancies: forty-five pregnant rats (28 DES treated and 17 controls) produced 284 pups (191 DES-exposed and 93 vehicle-exposed) (Table 4). Most delivered normally on the expected day of parturition (day 22). Those few which were more than one day postmature belonged to the DES group. The latter were sacrificed and their uteri examined for fetuses and implantation sites. It had previously been established that animals who do not deliver on time will not do so even if left several days beyond day twenty-two. Five of the DES treated mothers appeared to have resorbed their fetuses, and two had retained their litters *in utero*, common among DES treated animals but a very rare condition in untreated rats (Boylan, 1978). Twenty-one pups of DES treated mothers were stillborn whereas none of the controls had stillbirths.

TABLE 4. Outcome of Pregnancies after Injection of 1.2μg DES or Vehicle During Week 2 and Week 3 of Gestation.

TREATMENT	NUMBER PREGNANT*	PUPS BORN	STILLBORN	PUPS/ LITTER	SURVIVORS/ LITTER**
DES	28	191	21	6.8	6
VEHICLE	17	93	none	5.5	5.5
TOTALS	45	284	21	6.3	5.8

*Judged by sperm present in vaginal smears taken the morning after mating and by weight gain 10 days later.
**Pups surviving at 5 days after birth.

Labeling Patterns: Prior to drawing contrasts between the labeling of DES-exposed and vehicle-exposed tissues, the pattern of labeling within the nipple tissues was analyzed.

When a comparison was made between the amount of labeling in the second week and the third week, no significant difference was found in any of the thymidine treated tissues. After uridine treatment, there was more labeling of the hood and epithelium during week 2 than during week 3 (Tables 5 & 6).

To determine whether data taken from tissues of the thoracic nipples (second pairs) could be lumped together with data taken from tissues of the inguinal nipples (fifth pairs); these data were analyzed and compared. No difference in either thymidine or uridine uptake was found between the second and fifth nipples in any of the tissues examined (Tables 7 & 8) and, as a result, they were considered together in all other analyses.

Various individual tissues were compared to elucidate differences in activity between them. Thymidine labeling was significantly greater in the hood than in the epithelium. Uridine labeling in these 2 tissues was approximately equal (Tables 9 & 10). There was no difference between the upper and lower duct in their ability to take up either nucleoside (Tables 9 & 10). When uptake of label by the duct was compared to mesenchymal uptake, there was no difference in thymidine labeling, while uridine labeling was much greater in the duct tissue (Tables 9 & 10).

TABLE 5. Means of 3H-Nucleoside Labeling During Week 2 Compared to Week 3 in 5 Day Old DES and Vehicle Exposed Pups.

3H-Nucleoside Treatment					
3H-THYMIDINE			3H-URIDINE		
MEAN # LABELED CELLS PER 100 cells			MEAN # GRAINS PER CELL		
Tissue	Week 2 (n=20)	Week 3 (n=18)	Tissue	Week 2 (n=20)	Week 3 (n=20)
UM	59	61	UM	.95	.91
LM	64	61	LM	.86	1.07
UD	60	64	UD	1.74	1.66
LD	61	61	LD	1.71	1.75
H	66	68	H	1.39	1.49*
E	61	62	E	1.78	1.46*

*There was a significant difference ($p<0.05$) in uridine label uptake between week 2 and week 3 in the hood and the epithelium (Table 6).

TABLE 6. Mann-Whitney U Test: 3H-Nucleoside Labeling Week 2 Compared to Week 3 in DES-Exposed and Vehicle-Exposed 5 Day Old Pups.

	3H-THYMIDINE					3H-URIDINE				
Tissue	Mean Rank Week2 n=20	Mean Rank Week3 n=18	U	Prob	Tissue	Mean Rank Week2 n=20	Mean Rank Week3 n=20	U	Prob
UM	18.8	20.3	166	.68	UM	21.9	19.2	173	.47
LM	21.7	17.1	137	.21	LM	17.7	23.4	143	.12
UD	16.7	22.6	125	.11	UD	21.5	19.5	180	.58
LD	19.4	19.6	178	.95	LD	20.0	21.1	189	.77
H	17.7	21.5	145	.30	H	24.4	16.6	123	.04
E	19.3	19.7	177	.92	E	24.8	16.3	115	.02

Lower bounds of critical values are tabulated; the sample statistic U has to be less than the given critical value (Siegel 1956; Hull & Nie 1979).

TABLE 7. 3H-Nucleoside Label Uptake by Tissues of the Second Nipple Pairs Compared with Tissues of the Fifth Nipple Pairs in DES and Vehicle Exposed 5 Day Old Pups.

	3H-THYMIDINE		3H-URIDINE	
	MEAN # LABELED CELLS PER 100 CELLS		MEAN # GRAINS PER CELL	
Tissue	Nipple 2 (n=19)	Nipple 5 (n=19)	Nipple 2 (n=20)	Nipple 5 (n=20)
UM	60	59	.93	.92
LM	64	62	.97	.97
UD	63	61	1.69	1.71
LD	62	61	1.74	1.73
H	67	66	1.75	1.63
E	59	64	1.70	1.54

As there were no differences between the two (Table 8), data for the second nipple pairs was lumped together with data for the fifth nipple pairs for subsequent analyses.

TABLE 8. Mann-Whitney U Test: 3H-Nucleoside Label Uptake of Nipple 2 Tissues Compared to Nipple 5 Tissues of DES and Vehicle Exposed 5 Day Old Pups.

	3H-THYMIDINE					3H-URIDINE				
Tissue	Mean Rank Nip2 n=19	Mean Rank Nip5 n=19	U	Prob		Tissue	Mean Rank Nip2 n=20	Mean Rank Nip5 n=20	U	Prob
UM	20.4	18.6	163	.61		UM	20.5	20.5	200	1.00
LM	20.4	18.6	163	.61		LM	20.0	21.0	190	.79
UD	21.4	17.6	144	.29		UD	20.1	21.0	191	.81
LD	20.0	19.1	172	.80		LD	19.5	21.6	179	.57
H	20.5	18.5	162	.59		H	21.5	19.5	180	.58
E	16.0	23.0	115	.06		E	22.3	18.7	164	.32

As there was no difference between the two, data for the second and fifth nipple pairs was lumped together for subsequent analyses.

TABLE 9. Tissue Specific Variations in 3H-Nucleoside Label uptake by DES and Vehicle Exposed 5 Day Old Pups.

3H-THYMIDINE			3H-URIDINE		
MEAN # LABELED CELLS PER 100 CELLS			MEAN # GRAINS PER CELL		
	mean	n		mean	n
Hood	67*	38	Hood	1.69	40
Epithelium	61*	38	Epithelium	1.62	40
Upper Duct	62	38	Upper Duct	1.70	40
Lower Duct	61	38	Lower Duct	1.73	40
Mesenchyme	63	76	Mesenchyme	.97	80*
Both Ducts	61	76	Both Ducts	1.73	80*

*Thymidine labeling was greater ($p<0.05$) in the hood than in the epithelium; uridine labeling was greater ($p<0.05$) in the ducts than in the mesenchyme (Table 10).

TABLE 10. Mann-Whitney Comparisons of 3H-Nucleoside Uptake by Various Tissues Taken from DES and Vehicle Exposed 5 Day Old Pups.

	3H-THYMIDINE				3H-URIDINE			
	H vs E		Z*	Prob	H vs E		Z	Prob
Mean Rank	45.0	32.0	-2.56	.01	40.9	40.1	-.144	.89
Sample Size	38	38			40	40		
	UD vs LD		Z	Prob	UD vs LD		Z	Prob
Mean Rank	38.7	38.3	-.078	.94	40.1	40.9	-.154	.88
Sample Size	38	38			40	40		
	MES vs DUCT		Z	Prob	MES vs DUCT		Z	Prob
Mean Rank	74.5	78.5	-.551	.58	49.2	111.8	-8.54	.00
Sample Size	76	76			80	80		

*For larger samples (N>30) U is transformed into the normally distributed statistic Z (Hull & Nie 1979; Siegel 1956).

Experimentals vs Controls: Lumping data for the equivalent second and fifth nipple pairs (no differences in label uptake were found), experimental and control tissues were compared for nucleoside uptake. In 3H-thymidine injected pups exposed to DES in the second week, label uptake was reduced significantly ($p<0.05$), compared to vehicle exposed controls, in the upper and lower duct regions; while in the third week, no difference in labeling was found in any tissue examined. The 3H-uridine labeled pups had reduced labeling in the upper and lower duct, the nipple hood, and the nipple epithelium after DES exposure during both week two and week three (Tables 11, 12 & 13).

TABLE 11. Mean Labeling of Nipple Tissues after Injection of 3H-Thymidine or 3H-Uridine in Rat Pups Exposed to DES or Vehicle During Week 2 of Gestation

3H-THYMIDINE**			3H-URIDINE**		
TISSUE	PRENATAL EXPOSURE		TISSUE	PRENATAL EXPOSURE	
	DES n=12	VEHICLE n=8		DES n=12	VEHICLE n=8
UM	7	12	UM	.34	.60
LM	12	18	LM	.25	.53
UD	7	16*	UD	.93	1.70*
LD	7	17*	LD	.89	1.69*
H	16	15	H	.99	1.99*
E	10	13	E	1.05	1.62*

* Label uptake was significantly reduced ($p<0.01$) in DES-exposed group (Table 13).
** Mean number of labeled cells per 100 cells for thymidine; mean number of grains per cell for uridine.

TABLE 12. Mean Labeling of Nipple Tissues After Injection of 3H-Thymidine or 3H-Uridine in Rat Pups Exposed to DES or Vehicle During Week 3 of Gestation

3H-THYMIDINE**			3H-URIDINE**		
TISSUE	PRENATAL EXPOSURE		TISSUE	PRENATAL EXPOSURE	
	DES n=10	VEHICLE n=8		DES n=12	VEHICLE n=8
UM	10	10	UM	.39	.42
LM	11	11	LM	.43	.78
UD	12	17	UD	.81	1.68*
LD	11	11	LD	.89	1.80*
H	19	16	H	.74	1.37*
E	10	14	E	.78	1.23*

* Label uptake was significantly reduced ($p<0.05$) in these tissues after DES-exposure (Table 13).

** Thymidine values are expressed as the number of labeled cells per 100 cells; uridine values are expressed as the number of grains per cell. Values were corrected for the appropriate background counts (Appendix F).

TABLE 13. Mann-Whitney U Test: Experimentals versus Controls. Label uptake of nipple tissues after injection of 3H-thymidine or 3H-uridine in rat pups exposed to DES or vehicle during week 2 or week 3 of gestation. Lower bounds of critical values are tabulated. The sample statistic U must be less than a given critical value (Siegel 1956).

(a) DES THY-WEEK2; URI-WEEK2 & 3 n=12; THY-WEEK3 n=10.
(b) VEH n=8.
(c) Exact probabilities in Appendix F.

	TISSUE	DES (a) MEAN RANK	VEH (b) MEAN RANK	2-TAIL P (c)	U
THY-WEEK2	UM	9.21	12.44	P>0.2	32.5
	LM	9.75	11.63	P>0.2	39.0
	UD	7.67	14.75	P<0.01	14.0
	LD	7.58	14.88	P<0.01	13.0
	H	10.96	9.81	P>0.2	42.5
	E	9.79	11.56	P>0.2	39.5
THY-WEEK3	UM	9.45	9.56	P>0.2	39.5
	LM	8.35	10.94	P>0.2	28.5
	UD	7.80	11.63	P>0.1	23.0
	LD	9.10	10.00	P>0.2	36.0
	H	10.40	8.38	P>0.2	31.0
	E	8.55	10.69	P>0.2	30.5
URI-WEEK2	UM	9.13	12.56	P>0.2	31.5
	LM	9.04	12.69	P>0.1	30.5
	UD	7.08	15.63	P<0.005	7.0
	LD	7.00	15.75	P<0.005	6.0
	H	7.08	15.63	P<0.005	7.0
	E	7.58	14.88	P<0.01	13.0
URI-WEEK3	UM	10.58	10.38	P>0.2	47.0
	LM	8.58	13.88	P>0.05	25.0
	UD	6.92	15.88	P<0.005	5.0
	LD	6.92	15.88	P<0.005	5.0
	H	8.17	14.00	P<0.05	20.0
	E	8.04	14.19	P<0.05	18.5

DISCUSSION

These results indicate that transplacental exposure to DES interferes with the normal development of the rat mammary gland. Though the mechanism of interference is unknown, the result has been an early inhibition of biosynthesis of DNA and RNA in epithelial tissues of the neonatal mammary gland. Reductions in DNA synthesis occurred in the upper and lower duct regions after exposure during week 2 of gestation. Reductions in RNA synthesis in the upper and lower duct, the nipple hood, and the nipple epithelium occurred after DES exposure during both weeks 2 and 3. DNA synthesis, as measured by labeling index, is a reflection of mitotic index; while the rate of RNA synthesis, measured by label uptake, is a monitor of cell metabolism (Schultze 1969; Miller & Emmens 1967). These results indicating early inhibition of mammary gland development are consistent with previous studies. Branching of mammary gland ducts was

seen to be inhibited in mice exposed neonatally to DES or estrogen (Tomooka & Bern 1982). Hoshino and Connolly (1967) reported inhibition of the development of both the nipples and the mammary ducts in young mice of both sexes exposed prenatally to 0.5µg estradiol benzoate on days 8 and 9 of gestation. Though the precise cause of the inhibitions observed is unknown, there are several possible explanations that may be offered.

Plasma contains specific carrier proteins for the transport of steroid hormones. These molecules may function as a buffer system protecting from excessive exposure to hormone by regulating the level actually reaching the target tissue. Small lipid soluble steroid molecules can cross the placenta, as does DES, while the larger carrier proteins and protein-hormone complexes do not do so. Thus, it is the unbound hormone which exerts biological activity. The interaction of hormone with the blood proteins causes the hormone to be retained in the vascular bed and significantly reduces hormone potency (Nunez

et al. 1979; Vannier & Raynaud 1975). In the rat, as the fetal carrier protein molecule concentration declines with advancing age, tissue uptake of estrogen has been observed to increase (Anderson et al. 1978). This glycoprotein carrier molecule, alpha-fetoprotein, is normally found in high concentrations in the plasma of human and rodent fetuses and neonates. Human plasma also contains a steroid hormone binding globulin as well. There is much evidence to indicate that DES has little affinity for these circulating carrier proteins. DES and its metabolites cannot compete with estradiol-17β for binding with either alpha-fetoprotein or steroid hormone binding globulin (Raynaud 1971; Sheehan & Young 1979; Savu et al. 1975).

This inability of DES to bind to carrier proteins implies that its inhibitory effects may arise from its enhanced bioavailability. It has been shown that binding with alpha-fetoprotein may be necessary to protect estrogen sensitive tissues of the fetus from maternal estrogens. Chandra produced birth

defects in the rat by using rabbit antiserum to make alpha-fetoprotein unavailable during development (Chandra 1979). The failure of DES to bind serum proteins suggests that DES could be active at lower doses than would be required of natural steroid.

DES-induced changes in the mammary gland may be mediated by alterations of the hypothalamo-hypophyseal-ovarian axis. There is some indication that early exposure to DES serves to make the hypothalamo-pituitary axis less sensitive later on to negative feedback by estrogens (Napalkov & Anisimov 1979). Compensatory ovarian hypertrophy occurs in hemiovariectomized rats due to an increase in the activity of the hypothalamic gonadotrophin system in response to the fall in estrogen level. This hypertrophy can be suppressed by hormone replacement. In rats prenatally exposed to DES, later DES treatment did not inhibit compensatory ovarian hypertrophy as it did in controls (Napalkov & Anisimov 1979).

Further evidence of neuroendocrine disturbance exists. A persistent estrus syndrome has been observed in rodents after

neonatal estrogen or DES treatment. Noncyclic gonadotrophin secretion leading to continuous estrogen production by the ovary produces persistent vaginal cornification (Forsberg & Kalland 1981; Dunn 1963; Herbst & Bern 1981). It appears as if a permanent hormonal derangement has resulted from exposure to estrogen during a critical developmental period. Rustia has suggested that this is the result of impairment of the normal sex differentiation of the hypothalamus (Rustia & Shubik 1976).

The toxicity of transplacental DES to the mammary gland may reside in its ability to induce alterations in prolactin levels. DES induced neuroendocrine disturbance may lead to excessive estrogen release. This increased continuous estrogen secretion is known to increase pituitary prolactin output (Frantz et al. 1972; Meites 1974). Cell counts in the anterior pituitary reveal prolactin cells to be more numerous after estrogen or progesterone treatment of mice neonates (Kawashima et al. 1978; Nagasawa et al. 1978). Lawson observed increased plasma

prolactin levels after estradiol-17β injection in ovariectomized rats (Lawson 1979). Antiestrogen exposure has be shown to reduce prolactin output in female rats (Meites 1974). Using a DNA hybridization probe, increased levels of preprolactin mRNA were found in the pituitary of estrogen treated rats indicating an estrogen induced rise in transcription (Ryan et al. 1979). However, our laboratory has recently reported that prolactin levels in 2 month old rats exposed to DES *in utero* were significantly reduced when compared to vehicle exposed controls (Boylan et al. 1983).

If elevated prolactin levels are involved, two different mechanisms for estrogen or DES influence on these levels may be envisioned. It may be that estrogenic stimulation increases the number of prolactin cells in the pituitary and/or it may be that it reduces hypothalamic release of prolactin inhibitory factor (dopamine) into the pituitary portal system (Meites 1974).

Whether the increased prolactin levels are the cause of enhanced mammary tumor incidence in DES-exposed rats remains to be

proven. However, it is established in rats that tranquilizer treatment, hypothalamic lesion, or pituitary grafting to elevate serum prolactin levels will all enhance tumor progression; whereas tumor inhibition can be demonstrated by those conditions which decrease the level of circulating prolactin (Nagasawa 1979). Chronic administration of 17-β estradiol in female mice has been shown to significantly increase the incidence of mammary tumors. Concurrent treatment with CB-154 (2-bromo-α-ergocryptine), an inhibitor of pituitary prolactin secretion, reversed this tumorigenic effect. This implicates prolactin as the vehicle for this estrogen induced tumorigenicity (Welsch et al. 1977).

When tumor bearing and non-tumor bearing mice were compared, more prolactin cells were observed in those with tumors, but no differences in gonadotropin levels were observed (Kawashima et al. 1978). Strain differences in prolactin and growth hormone levels correlate with the incidence of mammary tumors in mice (Sinha et al. 1975).

It is noteworthy that the changes

observed in the lower genital tract of the offspring of DES treated mothers are mostly epithelial lesions involving its altered morphogenesis or differentiation (adenosis, erosion, carcinomas). Similarly, epithelial tissues of the mammary gland are significantly altered by hormone exposure as well. Tomooka and Bern (1982) observed inhibition in the mammary duct tissue after neonatal treatment with estrogenic compounds. Necrosis of the mammary gland epithelium resulted after in vitro androgen exposure of mammary rudiments of mice (Kratochwil & Schwartz 1976). This is consistent with observations made in this study.

These epithelial changes may be indirect. That is, DES may exert its effects on epithelium by altering the normal stromal-epithelial interactions. There is ample evidence, from tissue separation and recombination experiments, of specific induction of the overlying epithelium by different regions of the fibromuscular stroma (mesenchyme) of the genital tract (Cunha 1976). The mesenchyme of the mammary gland

plays an important role in its differentiation as well (Balinsky 1949). It appears to be a steroid sensitive mesenchyme which specifies the morphogenesis of the epithelium. Uptake of transplacental DES into fetal mammary gland tissue has been demonstrated and localized. When pregnant Swiss albino mice were injected with tritiated DES, the mammary glands of their fetuses concentrated radioactivity in the mesenchyme surrounding the epithelial primordia rather than in the epithelium itself (Narbaitz et al. 1980). In tissue recombination experiments involving combinations of epithelium and mesenchyme from normal mice and androgen insensitive mutants, Kractochwil & Schwartz (1976) demonstrated that the mesenchyme of the mammary rudiment was the target tissue for testosterone. Testosterone exposure led to necrosis of the mammary gland epithelium but this epithelial change was indirect, mediated by the mesenchyme. Similarly, it may be that DES is inhibiting DNA and RNA synthesis as observed by first binding the mesenchyme and causing the mesenchyme to direct the biosynthetic

activity of the epithelium (even though the biosynthetic activity of the mesenchyme itself remained unaffected to the limits of detection by autoradiography).

The pivotal role of the stroma is further appreciated by considering that, even in adulthood, the overlying epithelium may require association with the appropriate stroma for self-maintenance or to be reestablished after trauma. Thus, stromal alteration early on may cause epithelial changes which prove teratogenic or carcinogenic later in life.

If one considers carcinogenesis as a two step process (tumor initiation and later tumor promotion possibly after a long latent period), then DES as a teratogen may be setting the stage for increased susceptibility to a later carcinogenic event and, as such, may serve as an early initiator. Herbst called DES an "incomplete carcinogen" (Herbst et al. 1971) in referring to its effect on the lower genital tract and it may, unfortunately, prove to have the same role in mammary gland malignancy. It is possible that the changing

hormonal milieu of puberty or menopause may prove to be a secondary event capable of promoting carcinogenesis in DES sensitized tissues.

This study was undertaken in an attempt to shed light on the possible role of transplacental DES as a mammary gland carcinogen and/or teratogen. It does not answer the question as to whether or not DES is a direct chemical carcinogen though sister chromatid exchanges and cell division delays have been reported recently after *in vitro* treatment of human lymphocytes with DES (Hill & Wolfe 1983). Vorherr has suggested possible mechanisms for carcinogenesis by DES. The unstable cis-isomer of DES, which exists in solution in a 30:70 equilibrium with the trans isomer, is strongly electrophilic. This attraction of electrons causes epoxide formation at the double bond. DES-epoxide, like other epoxide-forming chemical carcinogens, can bind DNA leading to transcription failures. It is also possible that epoxide-forming DES metabolites, such as hydroxydienestrol, may be the active agents

leading to misreading or non-function of a gene; ultimately, resulting in malignancy (Vorherr et al. 1979). It has been hypothesized that such a compound might activate a dormant type II herpes virus leading to carcinogenesis (Vorherr et al. 1979; Stafl & Mattingly 1974).

It is known that DES, as well as its oxidative and conjugative metabolites, reach the fetus (Metzler 1981; Fisher et al. 1976), but it would be necessary to further understand the metabolism of these compounds by the fetus in order to pinpoint the exact nature of their fetotoxicity.

The work here does fulfill its immediate goal which was to determine if transplacental exposure to DES causes early changes in mammary gland development. These data indicate that it does, even at the low dose used here which is not associated with severe interference with reproductive tract structure or function (Boylan 1978, Boylan et al. 1983). It is evident that changes are occurring which affect both mitotic rate and cell metabolism. Though the precise nature of these changes

remains to be elucidated, DES has been shown to have a significant inhibitory effect on both DNA and RNA synthesis in the neonatal rat mammary gland. Considering the large human population of DES-exposed offspring, these results cast a dark shadow. As in the rat, it may be that similar developmental changes in the mammary gland have occurred in the human offspring transplacentally exposed. Whether or not these changes will act synergistically with normal mammary gland senescence to increase the incidence of malignancy in this population merits further study.

APPENDIX (A) Examination of Male Rat Pups for Mullerian Duct Remnants.

A total of 8 male rat pups, two from each treatment group (DES2, DES3, VEH2 and VEH3), were sacrificed at 5 days of age and dissected to examine for retention of remnants of the Mullerian ducts. No evidence of these ducts was present in either experimental or control male offspring.

APPENDIX (B) Treatment and Sacrifice of 5 Day Old Rat Pups.

Preliminary experiments were conducted to determine the length of time between label injection and pup sacrifice needed to maximize label incorporation. Pups were injected (1μCi/gm) with 3H-thymidine (n=8) and 3H-uridine (n=8). They were sacrificed either 2, 6, 10 or 24 hours after injection. Nipples were removed and processed for autoradiography. After a two week exposure period slides were examined. It was determined that optimal label incorporation occurred 10 hours after uridine injection, and 24 hours after thymidine injection and these times were used for this experiment.

APPENDIX (C)

TABLE 14. Results of Paper Chromatography of 3H-Nucleosides to Establish Purity of Labeling Solutions*

Radioactivity in Sections of Paper Chromatographs (CPM).**

THYMIDINE UNDILUTED		THYMIDINE HOT/COLD MIX		URIDINE UNDILUTED		URIDINE HOT/COLD MIX	
676.5	1	300.5	1	56,050.0	1	31,799.0	1
761.5	2	807.0	2	82,570.0	2	73,802.0	2
3,168.5	3	3,296.0	3	499,136.0	3	421,391.0	3
5,007.0	4	3,429.0	4	143,022.0	4	55,830.0	4
50,078.0	5	33,296.0	5	20.097.0	5	14,365.0	5
221,598.0	6	145,489.0	6	11,844.0	6	8,917.0	6
29,523.0	7	9,374.0	7	4,529.5	7	4,150.5	7
1,206.5	8	597.5	8	814.0	8	746.5	8
300.5	9	224.0	9	211.0	9	327.5	9
114.0	10	82.0	10	54.5	10	36.5	10

*Paper chromatography was conducted using a n-butanol: acetic acid: water solvent system (for thymidine 80: 12:30 and for uridine 40:10:10, V/V/V). Chromatographs were checked for absorbance at 260nm.

**Counts per minute above background are given for each sample ascending from the origin to the solvent front. Sections (1sq cm) of each paper strip were cut and placed in a 20 ml vial with 5 ml scintillation fluid. Each sample was read for two minutes using a Nuclear-Chicago Mark II Scintillation Counter.

APPENDIX (D) Enzymatic Digestion to Demonstrate Label Specificity.

In each autoradiographic batch deoxyribonuclease treatment of thymidine slides removed the label as did ribonuclease treatment of uridine slides. Incubation at 37° C with 0.05 mg/ml bovine pancreatic deoxyribonuclease (Sigma Chemical Co, St. Louis, MO) in 0.1M Tris maleate buffer at pH 6.5 was done for 24 hours (Humason 1967; Daoust 1964). Incubation at 37°C with 0.3 mg/ml bovine pancreatic ribonuclease (Sigma Chemical Co, St. Louis, MO) in 0.01M phosphate buffer at pH 7.6 was done for 3 hours (Carmago & Plaut 1967; McDonald & Kaufman 1954).

APPENDIX (E) Exposure Times for Autoradiography

Preliminary experiments were conducted to determine the optimal autoradiographic exposure time. After dipping them in photographic emulsion, thymidine slides (n=8) and uridine slides (n=8) were each left for an exposure period of either 1, 2, 3, or 4 weeks (2 thymidine slides and 2 uridine slides in each batch). Examination of these slides revealed a favorable balance between label incorporation and background fog after a two week exposure time for thymidine slides. A 4 week exposure period proved necessary for sufficient uridine incorporation.

APPENDIX (F) Data Manipulations.

Key: UM = upper mesenchyme
LM = lower mesenchyme
UD = upper duct
LD = lower duct
H = hood
E = epithelium

Each nipple was given an 8 digit code number as per the format below:

First digit (label): 1=thymidine, 2=uridine, 3= thymidine background, 4=uridine background.
Second digit (treatment); 1=DES week 2, 2=vehicle week2, 3=DES week 3, 4=vehicle week 3.
Third and forth digits (mother ID): numbered 1-52.
Fifth digit (pup ID): numbered 1-9.
Sixth digit (Nipple #): 2=second pair, 3=third pair, 5=fifth pair.
Seventh digit (enzyme treatment): 0=none, 1=DNase treated, 2=RNase treated.
Eighth digit (autoradiographic batch): 1=dipped 7/1/80, 2=dipped 7/2/80, 3=dipped 10/13/80, 4=dipped 12/13/80.

For each of 115 nipples coded, the number of cells followed by the number of labeled cells (for thymidine), or the number of grains present (for uridine), was entered for each of the 6 areas of the nipple examined. Two digits were reserved for each entry (see explanation of a sample entry below).

SAMPLE: 1226120160213014000336004130524O4

A one in the first column indicates thymidine labeling. Two in the second column indicates treatment with DES week 2. Twenty-six is the mother ID. One in column 5 is the pup ID. Two indicates the specimen was taken from the second pair of nipples. Zero in column 7 represents no enzyme treatment. One in column 8 is the first dipping date. The next two digits (16) are the number of cells counted in the UM test area. The next 2 columns contain the number 02 indicating that 2 of the 16 cells were labeled. Thirteen is the number of cells present in the LM test area. The next two digits are the number of those cells which were labeled. In the same manner the rest of the columns refer to counts taken for the UD, LD, H, and E respectively.

Six areas from each of 115 nipples produced 690 data entries. Data on the LM or LD was missing on 6 of these entries as the sections taken lacked this region of tissue. Values were missing on entries 5, 21, 26, 52, 69, and 81. Mean values were computed based

on the remaining entries and these means were inserted in place of the missing values. Mean number of cells in the LH test area was 15. Mean number of cells in the LD test area was 48. The file was divided into subfiles to compute the mean values for the number of labeled cells or number of grains present for each group.

Subfiles Created

1) Thymidine and DES week 2.
2) Thymidine and vehicle week 2.
3) Thymidine and DES week 3.
4) Thymidine and vehicle week 3.
5) Uridine and DES week 2.
6) Uridine and vehicle week 2.
7) Uridine and DES week 3.
8) Uridine and vehicle week 3.
9) Thymidine background
10) Uridine background
11) Thymidine DNase
12) Thymidine RNase
13) Uridine DNase
14) Uridine RNase

Based on these subfiles, means were computed for each group.

Mean values inserted:

Entry # 5: thymidine and vehicle week 2, mean number of labeled cells in the LD = 7.
Entry # 21: thymidine background. mean number of labeled cells in the LD region = 0.
Entry # 26: same as # 21.
Entry # 52: thymidine and RNase, mean number of labeled cells for LM = 2, for LD = 4.
Entry # 69: uridine and DES week 2, mean number of grains found in LD = 62.
Entry # 81: uridine and vehicle week 3, mean number of grains present in LM = 26.

Ratios were computed. For each area of nipple examined (UM, LM, UD, LD, H, E) the number of labeled cells (thymidine) or number of grains (uridine) was divided by the total number of cells counted in that test area. Thus, a sample entry would be: 333311503 .85714271 .12499901 .97674411 .94117641 .21951201 .26470571. In this sample the first 8 digits (33311503) represent the code for a uridine and DES week 3 sample from the fifth nipple pair of pup 1 of mother 31 with no enzyme treatment which was dipped on 10/13/80. The next number (.85714271) represents the ratio of the number of grains present divided by the number of cells present in the UM test area of this tissue sample. The next number

is the ratio of grains per cell for the LM and so forth for the UD, LD, H, and E.

Corrections were made for background. Mean background ratios were computed for each variable (UM through E). There was no correction necessary for thymidine exposed tissues for dips 1-4 as no cell met the labeling criterion of having 5 or more grains. There were no uridine slides in dipping batch 1 or 2. Corrections for background on uridine slides for dips 3 and 4 were made in accord with the chart below (background ratios were subtracted from ratios already computed for each test area).

URIDINE CORRECTIONS

Variable*		Dip3**		Dip4***
CRUM = RUM - Correction which is		.5444773	or	.4901960
CRLM = RLM - Correction which is		.6563231	or	.3075000
CRUD = RUD - Correction which is		.1993122	or	.1119976
CRLD = RLD - Correction which is		.1854520	or	.1203703
CRH = RH - Correction which is		.3548951	or	.4153846
CRE = RE - Correction which is		.2870773	or	.2614130

*CR = corrected ratio, R = ratio
**Values subtracted from ratios in the third autoradiographic batch.
***Values subtracted from ratios in the forth autoradiographic batch.

To eliminate zero and negative entries created by subtracting background values, 0.5 was added to all numbers. Histograms were produced and these clearly demonstrated that the data did not fit a normal distribution. Various appropriate transformations were tried in an attempt to normalize the data for parametrical statistical analysis. Log, Ln, and square root transformations all failed to produce normal distributions. The arcsin transformation often used on ratio data was inapplicable as the data did not fall between

zero and one. Therefore, distribution free statistics were utilized.

Appropriate subfiles were merged so that the desired comparisons could be made; (1) thymidine DES and vehicle week 2; (2) thymidine DES and vehicle week 3; (3) uridine DES and vehicle week 2; (4) uridine DES and vehicle week 3. Data was recoded so that the nonparametric Mann-Whitney U Test might be applied using SPSS (1979). This test uses the rank of each case to test whether two samples come from the same population. Two groups are combined for each variable and the cases are ranked according to their increasing size. If the two samples are from the same population the distribution of scores from the two groups being ranked should be random. The test statistic U is tabulated as the number of times a score from group 1 precedes a score from group 2. An extreme value of the test statistic indicates the samples are not from the same population (SPSS 1979).

TABLE 15. Mann-Whitney U Test Comparing 3H-Nucleoside Uptake in DES and Vehicle Exposed 5 Day Old Pups.

Exact probabilities are presented.

(a) DES THY-WEEK 2, URI-WEEK 2 & 3 N=12; THY-WEEK3 N=10
(b) VEH N=8

	TISSUE	DES (a) MEAN	RANK	VEH (b) MEAN	RANK	2-TAIL P	U
THY-WEEK2	UM	9.21		12.44		0.288	32.5
	LM	9.75		11.63		0.486	39.0
	UD	7.67		14.75		0.009	14.0
	LD	7.58		14.88		0.007	13.0
	H	10.96		9.81		0.671	42.5
	E	9.79		11.56		0.512	39.5
THY-WEEK3	UM	9.45		9.56		0.965	39.5
	LM	8.35		10.94		0.303	28.5
	UD	7.80		11.63		0.131	23.0
	LD	9.10		10.00		0.722	36.0
	H	10.40		8.38		0.424	31.0
	E	8.55		10.69		0.397	30.5
URI-WEEK2	UM	9.13		12.56		0.203	31.5
	LM	9.04		12.69		0.177	30.5
	UD	7.08		15.63		0.002	7.0
	LD	7.00		15.75		0.001	6.0
	H	7.08		15.63		0.002	7.0
	E	7.58		14.88		0.007	13.0
URI-WEEK3	UM	10.58		10.38		0.939	47.0
	LM	8.58		13.88		0.076	25.0
	UD	6.92		15.88		0.001	5.0
	LD	6.92		15.88		0.001	5.0
	H	8.17		14.00		0.031	20.0
	E	8.04		14.19		0.023	18.5

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