SUBTLE EFFECTS OF PERINATALLY ADMINISTERED SMALL DOSES OF CORTISONE ACETATE ON SURVIVAL AND GROWTH OF YOUNG AND MAMMARY FUNCTIONS IN MICE

A Thesis

Presented to the Faculty of Graduate Studies,

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of the Requirements for the Degree of

Master of Sciences

by

Richard William Currie

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I ABSTRACT

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The possible teratogenic and long term effects of cortisone acetate at low doses comparable to the human clinical doses were investigated in A/j, C3H, CBA, C57B1 and BALB/c strains of mice.

The prenatal treatment of 0.75 mg had a significant detrimental effect on the survival of the C3H mice, but not in the CBA or A/j mice. In contrast, the neonatal treatment of 0.075 mg had a significant detrimental effect on the A/j mice, but not on the C3H mice.

In CBA mice the prenatally administered cortisone acetate caused some significant growth retardation in offspring which could be attributable to either maternal or fetal effects, or both.

The long term effects of prenatally administered cortisone acetate upon the female offspring were subtle and statistically not significant.

The transplantability of neonatal mammary gland, with or without pretreatment of cortisone acetate, was comparable to that of adult donors.

Mammary grafts at recovery always exhibited features of functional development similar to the host's own mammary gland. The earliest recovery of neonatal grafts were made 19 and 30 days after transplantation, from a 12 day pregnant host and a 1 day postpartum host mouse, respectively. Each graft showed characteristics similar to the hosts mammary gland.

The present study suggests the necessity of further detailed exploration of transplacental teratogenesis and detrimental effects of cortisone acetate upon neonates.

II INTRODUCTION

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Cortisone acetate has been used extensively in humans, even during pregnancy. Although cortisone acetate has not been proven to be a teratogen in humans, it is known to be a strong teratogen in mice, particularly causing cleft palate (Wilson, 1973). In these previous experiments, doses per body weight used were much greater than the clinical doses used in humans.

Glucocorticoids have been reported to be essential hormones for the differentiation of mammary epithelium and biosynthesis of Casein both *in vitro* (Ceriani, 1969) and *in vivo* (Nandi, 1958). Cortisone acetate has been found to cause a wasting syndrome when administered to neonates, similar to the runt disease (Schlesinger and Mark, 1964).

Abnormalities in mammary glands following prenatal exposure to testosterone, 17 β estradiol benzoate and progesterone, have called attention to the hormonal teratogenesis of mammary gland (Hoshino, 1965; 1966; and 1967).

Considering these facts, it was of interest to investigate the effects of a small dose of cortisone acetate, comparable to a human dose, upon the fate of pregnancy, offspring, and neonatal mammary gland in mice.

Transplacental carcinogenesis of vaginal and uterine cervical tissue with diethylstilbestrol in young women have caused much concern about hormonal treatments during pregnancy (Herbst *et al.*, 1971). Thus it was felt necessary to include some long term investigations of the prenatally treated offspring. Two preliminary reports from this line of work have been presented (Hoshino and Currie, 1975; Currie *et al.*, 1977).

III LITERATURE REVIEW

III LITERATURE REVIEW

1. Embryogenesis and Postnatal Development of Mammary Gland

According to Grausmann (1950) there is on either side of the trunk of an 11 day old mouse embryo, a zone of raised epidermis, which corresponds to the mammary band. However, Grausmann goes on to say that the mammary band is a derivative of ectodermal differentiation, and not specifically a mammary tissue.

In the 11.5 to 12 day old mouse embryo a slightly raised mammary crest differentiates, forms a line, and has a transient existence (Turner and Gomez, 1933).

Balinsky in 1949 and 1950 reported that the mammary crest was either difficult to recognize or absent and that the individual mammary anlagen develop independently from one another.

Raynaud in 1961 wrote that the mammary anlagen of a 12 day embryo are connected in the thoracic region to form a band of thickened epidermis. However, Raynaud found no connection between the thoracic and inguinal mammary anlagen. So the thoracic anlagen at least do not develop in an independent manner.

Raynaud, (1961) wrote that even in the 12 day embryo there are 3 thoracic and 2 inguinal mammary anlagen, which are easily recognizable.

Balinsky (1950) stated that there are 3 essential phases distinguishable in the development of the mammary anlagen.

1. Formation of the individual anlagen, through which their growth rate rapidly decreases.

2. A phase of retarded growth, with slight differentiation of the anlagen. In the mouse this phase is from day 11 to 15.
3. A phase of rapidly increasing growth corresponding to the lengthening of the mammary bud, and the formation of the primary mammary cord or sprout.

In mice, from the 12th to the 14th day of fetal life the mammary anlagen develop similarly in both sexes. However, on the 15th day sexual differences appear, as described by Raynaud (1961). In the female the mammary bud begins to sink into the mesenchyme, and remains connected to the epidermis. In the male the mammary bud sinks into the mesenchyme and becomes disconnected from the epidermis. The disconnection is complete by about the 19th day of fetal life (Hardy, 1950).

The primary sprout begins to be canalized at 18 or 19 days, and secondary sprouts begin to develop from the primary sprout on day 20 of fetal life. Development of the duct system is most rapid in the first week after birth. In the first day after birth the secondary sprouts become canalized, and in the next 6 days tertiary and quaternary sprouts appear. From the second week until about six weeks, the ducts slowly increase only in size and number. No alveoli are formed until after puberty. The nipple does not invert until 6 weeks after birth (Hardy, 1950).

After puberty the growth of the mammary gland is controlled by the hormones of the reproductive fertile female.

In 1967, Sekhrai et al., described and illustrated prenatal, neonatal, pubertal, prelactating, lactating and involuting stages of mammary gland in mice.

2. Histogenesis of Mouse Mammary Gland

Histogenesis of the mouse mammary gland has been studied in *in vivo* and *in vitro* systems:

A. by transplantation of segments of mammary gland ducts into other isogeneic animals; and

B. by *in vitro* studies of growth and differentiation of the mammary gland tissue.

A. *In Vivo* Studies

The earliest studies on mammary gland transplantation were only concerned with carcinogenesis since Fischer in 1937 first reported the transplantation of mammary gland. Shimkin *et al.*, (1946) transplanted mammary gland segments into the subcutaneous tissue of mice and rats.

DeOme and his associate in 1959 first reported the transplantation of mammary gland segments into the 4th mammary gland-free fat pad. They described the method for removing the parenchyme from the 4th mammary glands of a 3 week old mouse to prepare a site for future transplantation.

Mammary gland segments have been transplanted into:

1. Subcutaneous tissue (Faulkin and DeOme, 1958; DeOme *et al.*, 1959; Hoshino, 1962; Hoshino, 1964 a)
2. Fourth mammary gland-free fat pad (DeOme *et al.*, 1959; Hoshino, 1962). (This site has been extensively used by DeOme's and Hoshino's groups.)
3. Retroauriclar fat pad (Hoshino, 1962).
4. Pararenal fat pad (Hoshino, 1962, 1964a and Hoshino *et al.*, 1965).
5. Under the kidney capsule (Sakakura *et al.*, 1976).
6. Anterior chamber of the eye (Hoshino *et al.*, 1976).

7. Interscapular brown fat (Hoshino, 1967a).

8. Mesometrial fat layer (Hoshino, 1964a).

Faulkin and DeOme (1958) transplanted small pieces (each 1 x 1 x 0.5 mm) of mammary gland, taken from the edge of the gland of a multiparous female, into the subcutaneous connective tissue of C3H mice. They reported a recovery rate of viable grafts to be 154/179 or 86%. DeOme (1959) used adult C3H mice and he reported a recovery rate of the mammary gland grafts into the 4th mammary gland-free fat pad at 11/19 or 58%.

In 1964, Hoshino reported the rate of recovery of mammary grafts from normal CBA female donors of 3 to 11 weeks of age was 37/60 or 62%, and that of a 734 day old virgin female was 12/21 or 57%. These grafts that Hoshino used were mammary ducts, segments of known size, from 0.2 mm to 3.0 mm long, transplanted into the 4th mammary gland-free fat pad, by the Hoshino quantitative transplantation method (1963a).

In 1970, Hoshino reported that mammary gland, which had been serially transplanted through 6 hosts, over 1414 days (nearly 4 years) have a recovery rate of over 70%. The host mice were hybrid females (♀ BC_B x ♂ CBA) F-1 and the donor was a 135 day old virgin CBA female.

Hoshino's rate of recovery after nearly four years (much longer than the normal life span of a mouse) indicates that the age of the mammary gland has little effect on its transplantability. One of the aims of this study was to determine the transplantability of neonatal mammary gland and compare it to the recovery rates of adult mammary gland.

The regeneration and functional capabilities of the transplanted grafts in a pregnant and postpartum female were indistinguishable from

the host's own mammary gland (Hoshino, 1962 & 1964).

According to the experiments of Mori; Mills and Bern (unpublished, and cited by Bern *et al.*, 1976), when mammary glands of neonatally estrogen exposed BALB/cfC3H female mice are transplanted into the 4th mammary gland-free fat pad of normal mice on the day after completion of the neonatal treatment for 5 days, three months later the neonatal grafts were indistinguishable from the host's mammary glands.

Single mammary glands at each site of transplantation can be reproduced by implantation of a monolayer-cultured mammary cell (Daniel and DeOme, 1965) and small mammary gland duct segments (Chew and Hoshino, 1970; Hoshino *et al.*, 1976).

Small mammary duct-segments were reported to dissociate, scatter into the adjacent stromal tissue within 24 hours of transplantation into the 4th mammary gland-free fat pad, and aggregate into epitheloid masses from which a new mammary gland was reconstructed (Chew and Hoshino, 1970; Hoshino, *et al.*, 1976). However, to the best of my knowledge no data has been reported on the *in vivo* study of mammary isografts derived from prenatal mammary gland of mice.

B. *In Vitro* Studies

From *in vitro* studies, mammary glands have been shown to be able to develop from a day 10 fetal explant, survive up to 25 days and reach a final stage similar to a 7 day old mouse mammary gland (Hardy, 1950).

Lasfargues *et al.*, (1959) also reported the organ culture of 10 - 15 day old embryonic mouse mammary glands and concluded that estradiol and

progesterone inhibit growth of the mammary epithelium, and growth hormone and mammotropin promote active growth of mammary epithelium, and cortisol conditions the gland for secretion.

In 1967, Voytovich and Topper reported the *in vitro* synthesis of casein in mammary explants from 3 week old mice. This occurred in the absence of lobuloalveolar development.

Kratochwil (1969 and 1971) observed that 12 to 14 day old mouse embryo rudiments developed typically in organ culture, forming a nipple, nipple sheath, and a ramifying duct system in adipose tissue.

Ceriani (1969 and 1970) reported that the cultured mammary anlagen differentiated and synthesized a casein-like substance under hormonal stimulation *in vitro*.

From the literature, it can be seen that embryonic mouse mammary glands can be stimulated to grow and differentiate *in vitro*.

Voytovich and Topper (1967) showed *in vitro* data for 3 week old mouse mammary gland, and much work has been done by Topper's group on the *in vitro* growth and differentiation of adult mammary gland of pregnant mice.

3. Effects of Perinatally Administered Hormones

A. Effects of Perinatally Administered Glucocorticoids on Pregnancy and Offsprings

Cortisone was first shown to be teratogenic by Baxter and Fraser in 1950, and other early reports of the production of congenital defects, cleft palate in particular, produced by cortisone were made by Fraser and Fainstat (1951), Fraser (1951), Kalter and Fraser (1952), Fraser *et al.*, (1954).

Other reports (Robson and Sharaf, 1951, 1952; Glaubach *et al.*, 1951; Glaubach, 1952) indicated that cortisone treatment during pregnancy had a lethal effect on the fetuses, and if born alive, they died soon after.

The doses of cortisone used to cause death of all young was reported to be 2 mg/day for 5 days beginning on Day 11 of pregnancy (Robson *et al.*, 1951) and 2 injections of 2.5 mg of cortisone 7 - 8 days before parturition in R III mice (Glaubach *et al.*, 1951).

Fraser *et al.*, (1954) used 2.5 mg of cortisone given daily for 4 days to Strain A and C57BL mice, which produced cleft palate in 100% of A mice and 17% of C57BL mice. What soon becomes apparent from these and other more recent reports (Francis, 1973; Dostral and Jelinek, 1973; Sakizlioglu *et al.*, 1974; Greene and Kochhar, 1973; Chaudhry *et al.*, 1967; Spain *et al.*, 1975) is that the expression of the congenital abnormalities, particularly cleft palate, depends upon several things including:

1. Strain differences in sensitivities to glucocorticoids;
2. Fetal ages at the time of treatment; and
3. The routes of the administration of the hormone.

The teratogenicity of other glucocorticoids has also been reported in mice. Lahti *et al.*, (1972) reported the production of cleft palate with hydrocortisone in A mice but the resistance of CBA mice. Zimmerman & Bowen (1972) used triamcinolone acetonide to study the mechanism of cleft palate in A/J, C3H and CBA mice. Walker in 1965 reported that triamcinolone acetonide at low dose levels of 0.0125 and 0.001 mg/day for 4 days produced 100% and 18% cleft palate in A/J mice, respectively.

Other investigators used corticosterone (Hachman and Brown, 1972), 17-hydroxycorticosterone acetate (Kalter and Fraser, 1952), and triamcinalone acetonide (Andrew *et al.*, 1973; Zimmerman *et al.*, 1970) to induce cleft palate in mice.

Other animals studied for the induction of cleft palate were rabbits (Fainstat, 1954) and rats (Nanda, 1969) with cortisone acetate, and hamsters with hydrocortisone (Chaudhry and Shah 1972, 1973; Shah and Chaudhry 1973).

For humans, the recommended dose of cortisone acetate range from 25 to 50 mg per day for chronic, non-fatal diseases, to 300 mg or more per day for acute, life threatening diseases (Cortone; Merck, Sharp and Dohme).

Most studies concerning teratogenesis were concerned with morphological defects present at birth or just shortly before birth. There are no reports of long term, subtle effects of any single small dose of glucocorticords prenatally administered to mice.

Glaubach in 1952 reported that three daily doses of 2.5 mg of cortisone given to puerperal mice starting 1 to 3 days after delivery had a lethal effect on the litter.

In animals, the effect of neonatally administered cortisone acetate was reported to induce the runting syndrome similar to that seen following neonatal thymectomy (Schlesinger and Mark, 1964).

The effects of neonatally administered cortisone acetate were studied in rats, and growth retardation and/or growth failure, immunological impairment and decreased weights of spleen, thymus and adrenals were observed (Schapiro, 1965; Fachet *et al.*, 1967; Winick and Coscia, 1967; Schapiro and Huppert, 1967; and Ioachim, 1971). Other steroids, in particular, estradiol, have also been reported by Reilly *et al.*, (1967) to induce a runting syndrome when neonatally administered to mice.

In his literature review Wilson (1973) placed sex steroids in a category of drugs, "positively implicated as teratogenic". It is a well accepted fact that treatment with androgens prior to the 12th week of gestation in humans tends to cause masculinization of the female fetuses. Diethylstilbsterol has been implicated in the development of adenocarcinoma of the vagina in young women prenatally exposed to this drug (Herbst *et al.*, 1971). As recently as April 1977, Adams *et al.*, have reviewed transplacental carcinogenesis of human vagina and cervix with diethystilbsterol.

The administration of corticosteroids to pregnant mice often produces cleft palate in the young. This has caused concern about the use of corticosteroids during human pregnancy. Reilly in 1958 reports a slight rise in abortion, stillbirth and immaturity above the background level, of more than 300 women treated with corticosteroids during pregnancy, Bongiovanni and McPadden (1960) reported only 5 cases of cleft palate. Considering the extensive use of corticosteroids and the failure of any epidemiologic surveys to show statistical correlation, Wilson (1973) claimed that steroids must be regarded as having little if any teratogenic potential in man. Yet there are no studies concerning the long term effects of perinatally administrated glucocorticoid in the human or other animals.

B. Effects of Perinatally Administered Hormones on Mammary Glands

(a) Sex Hormones

The long term effects of neonatal exposure of mice to sex steroids and the effects on reproductive structures and mammary glands were reported by Bern *et al.*, (1975; 1976).

The mammary tumor incidences in intact mammary tumor virus carrying mice neonatally exposed to androgen and estrogen were significantly higher

than that of the control. When the animals were ovariectomized no tumors developed (Bern *et al.*, 1975; 1976). They stated that whether this is a direct effect by changing the sensitivity of the mammary gland, or by changing the level of ovarian function, or by changing the output of pituitary hormones has yet to be determined.

Mori *et al.*, (1976) concluded that neonatal steroid exposure results in an increased mammary tumor risk in mice, but only in the presence of both murine mammary tumor virus and ovaries.

Bern *et al.*, (1976) proposed that the steroid treated neonatal mouse represents a valuable model in many regards for the human situation of diethylstrilbestrol induced vaginal adenocarcinoma.

After steroid exposure on the first 5 days of life, the permanent changes may not be induced in the mammary gland itself as indicated by a transplantation experiment. When the mammary glands of neonatally estrogen exposed BALB/cfC3H female mice were transplanted into the gland-free mammary fat pads of normal mice on the day after completion of the neonatal treatment, 3 months later the transplanted glands were indistinguishable from the host glands (Mori, Mills, and Bern, unpublished, but cited by Bern *et al.*, 1976).

In 1976, Warner reported the effects of neonatally administered 17B estradiol given to new born mice for the first 5 days of life, at the dose of 25 µg, 35 µg or 70 µg per day, on the development of the mammary gland at 3 weeks and 5 weeks of age. The high doses of estrogen showed no effect on the 3 week old mice, but the 5 week old mice exhibited increased areas of mammary gland and increased branching of mammary gland.

Prenatal treatment with corticosteroids can interrupt pregnancy and cause congenital abnormalities. Prenatal exposure to androgens induces masculinization of female fetuses (Wilson, 1973).

As the prenatal treatment, 5 mg testosterone propionate or 1 ug of estradiol benzoate was given to Day 12 pregnant mice followed by a post-natal treatment to the offspring immature mice of 0.3 ug of estradiol benzoate at 14-20 days of age, at 21 days of age the testosterone treated female young had mammary glands which were often masculinized, but those of male young were stimulated to grow. The estradiol benzoate treated male mice had fewer mammary glands and there was no influence on growth of the persisting mammary glands (Hoshino, 1963b).

The effects of prenatally administered progesterone was also reported (Hoshino, 1966). He reported an increase in the absence of the nipples and ducts in males, but a greater growth of the remaining mammary glands of treated males versus the controls. Growth of mammary glands of treated females was not different from the controls.

The effects of 5 mg of testosterone proprionate given to pregnant mice either on Day 12, 15, 16 or 17 of pregnancy upon prenatal and post-natal mammary development were observed (Hoshino, 1965). Day 12 was the most sensitive. In female fetuses the development of the nipples and mammary anlagen was most effectively inhibited. Vaginal atresia and retention of serous fluids in the uterine horns were also observed in females treated on Day 12 of fetal life.

From the experiments on the effects of a prenatal dose of 0.5 ug of estradiol benzoate given for 2 days on days 8 - 9, and 1.0 ug on days 11 - 12, or 13 - 14 of gestation, Hoshino (1967b) concluded that estradiol

benzoate was detrimental to development of nipple and ducts in both male and female young, particularly when given on Days 8 and 9 of gestation. Of the persisting mammary ducts in male, however, the same treatment enhanced their growth.

(b) Glucocorticoids

The effects of cortisone on the growth and development of mammary glands *in vivo* have been reported as follows.

Flux (1954) observed that cortisone acetate inhibited the growth of the mammary glands of intact and estrogen-treated ovariectomized mice, whereas cortisone acetate did not affect the mammary glands of ovariectomized females without estrogen treatment.

Selye (1954) and Johnson and Meites (1955) found glucocorticords to stimulate the growth of the mammary gland in intact rats. In 1957, Ahren and Jacobson reported that the effect of cortisone in hypophysectomized rats was to promote enlargement and proliferation of the epithelial cells lining the inner walls of mammary ducts, but normal growth and differentiation did not occur. When cortisone was given to intact animals, it stimulated secretion but not growth of the mammary gland. The *in vitro* effects of cortisone and other hormones on fetal mammary glands (Lasfargues *et al.*, 1959; and Ceriani, 1969 and 1970), 3 week old virgin mouse mammary gland (Voytovich and Topper, 1967), and 12 day pregnant mouse mammary gland (Turkington *et al.*, 1965; Turkington *et al.*, 1967 and Lockwood *et al.*, 1967), were reported.

In the fetal explanted mammary gland of the mouse, cortisol conditions the gland for secretion, inducing the distention of ducts to form alveoli (Lasfargues *et al.*, 1959). Fetal rat mammary glands, under appropriate conditions in organ culture can be stimulated to synthesize a casein-like material (Ceriani 1969 and 1970).

Explants from the mammary glands of 3 week old mice can be induced to synthesize casein *in vitro* in the absence of lobuloalveolar development. Differentiation required the presence of insulin, hydrocortisone, and prolactin in the culture medium (Voytovich and Topper, 1967).

Twelve day pregnant mouse mammary gland when explanted, can be induced to differentiate and synthesize casein *in vitro*, under the stimuli of insulin, hydrocortisone and prolactin. Insulin is required for the growth of the explants, cortisone is required for the development of the rough endoplasmic reticulum to prepare for the synthesis of the milk proteins, and prolactin is required for the synthesis and secretion of the milk proteins (Turkington *et al.*, 1965; Turkington *et al.*, 1967; and Lockwood *et al.*, 1967).

The experimental system using the sequential hormonal exposures to mammary explants has been well established by Topper and his associates (1975). In addition, progesterone and estrogen were reported to be important in the normal growth and development of the alveoli and ducts of the mammary gland (Nandi, 1958).

4. Circulation and Metabolism of Glucocorticoids

In humans, cortisol is the principle glucocorticoid circulating in the blood stream. According to Netter (1974) cortisol circulates in the blood either free or bound to transcortin or albumin. The bound cortisol is relatively stable and inactive. Free cortisol is biologically very active, but is metabolized quickly, with a half life of about 8 hours. Only the free cortisol is easily metabolized. In the liver, cortisol is inactivated by the reduction of the double bonds within the

molecule and by its conjugation with glucuronic acid. The product, tetracortisol and dihydrocortisol glucuronide is released into the blood stream, and excreted by the kidney.

In rodents, the principle glucocorticoid is corticosterone which is also metabolized in the liver by reduction of the double bonds and conjugation to glucuronic or sulfuric acid. The conjugated products are excreted by way of the bile in the feces (Samuels and Eik-Nes, 1968).

The conjugated metabolites are relatively inactive and do not enter cells easily. On the other hand, steroids free or unconjugated form enter cells freely and are active in the metabolism of proteins, lipids and carbohydrates. Unless they are bond to larger protein molecules, unconjugated steroids cross the placental membranes and enter into the fetal circulation (Moore, 1973). Their exact role in the human fetus is not known, but in the rodent glucocorticoids are involved in glycogen synthesis in the fetal liver (Favard and Jost, 1966; Dupouy, 1970).

5. Determination of Pregnancy in Mice

The presence of the vaginal plug, body weight gains and intravaginal bleeding have been used to determine the clinical onset and process of pregnancy in rodents.

(a) Vaginal Plug

Copulation in the mouse is accompanied by the formation of a vaginal plug, which usually persists for 18 to 24 hours (Snell, 1941).

(b) Body Weight Gain

Average body weights increase during normal pregnancy. In the first 4 days after a vaginal plug, the pregnant mice weigh less than on

the day of mating. From Day 6, body weights increased gradually for the next 3 or 4 days, and then increase more rapidly (Hoshino, 1964b).

(c) Intravaginal Bleeding

In normal pregnancies of mice, more than 95% show intravaginal bleeding during mid-pregnancy. This frequency of detectable intravaginal bleeding indicates that in the mouse it may be a physiological process or manifestation (Hoshino, 1964b). Long and Evans (1920) first reported that the appearance of blood in the vagina could be used to detect pregnancy in rats.

6. Litter Weight and Weight Gains

Multiparious mice usually have larger litters, and heavier litters than do primiparous mothers. However the smaller the number of young in a litter, the heavier the weight of the individual young (Hoshino, 1964b; Reading, 1966).

In 1967, Kumaresan studied the effect of different litter sizes in rats on the milk production of the lactating female on days 14, 16, 18, and 20 of lactation. The larger the number of young in a litter, the more milk was produced by the mother, but the milk available to each young decreased. Therefore, larger litters would have a slower weight gain per young.

7. Foster Nursing in Mice

Foster nursing has been used extensively since Bittner's work in 1936, for the investigation of the transmission of the milk agent, which is presently recognized as mammary tumor virus in mice.

In experiments concerned with lengthening the lactational interval to maintain the stimulus of sucking, growing litters are replaced from time to time with very young litters (Snell, 1941).

There have been apparently no reports concerned with the fostering of entire litters at birth among nursing animals in different experimental groups in order to isolate maternal and fetal factors.

IV METHODS AND MATERIALS

IV METHODS AND MATERIALS

1. Animals

In this study five strains of mice were used, namely, CBA, C3H, C57BL, BALB/c and A/J. The CBA, C57BL, BALB/c and C3H strains of mice were pedigreed, inbred and raised in Hoshino's breeder colony. The A/J strain of mice were obtained from the Jackscn Laboratory. All animals were kept in metal cages under controlled environmental conditions (temperature 22 - 24 C; relative humidity approximately 50%; 12 hours of fluorescent illumination, 12 hours of dark) and provided with the Wayne Lab-Blox F6 and water, *ad libitum*.

The female mice were placed with isogeneic males and thereafter checked daily, at about 9:30 a.m., for the presence of a vaginal plug. The day when a vaginal plug was found was considered to be Day 0 of pregnancy. Daily vaginal examinations and measurements of body weight were carried out during pregnancy in order to find vaginal bleeding and a weight gain of several grams. Body weight was measured with a dialogram scale, manufactured by OHAUS Scale Corporation.

2. Hormonal Treatments

Pregnant mice were divided into four groups as follows:

1. Control group without treatment.
2. Prenatally treated group receiving a single injection of cortisone acetate of 0.75 mg on Day 12 pregnancy.
3. Neonatally treated group receiving a single injection of cortisone acetate of 0.075 mg within 16 hours after birth.

4. Neonatally treated group receiving a single injection of cortisone acetate of 0.15 mg within 16 hours after birth. Treatment was given dorsal subcutaneously with a Hamilton syringe. The cortisone acetate was Cortone (50 mg/cc) (Merch, Sharp and Dohme).

3. Statistical Analysis

Statistical analysis of the data was made using the unpaired *t*-test and Duncan's New Multiple-Range Test, computed by the CDC 700 computer utilizing the programs prepared by Mr. R. Rollwagen (The Department of Medical Computer Sciences, Faculty of Medicine, University of Manitoba).

4. Experimental Designs

This thesis consists of four different series of experiments as listed below.

A. Preliminary Investigations: The Effects of 0.75 mg of Cortisone Acetate Given on Day 12 of Pregnancy upon Pregnancy and Young

B. Determination of the Effects of Treatment on the Offspring Young Mice

C. Determination of the Effects of Treatment on the Offspring Mice after their Maturation and Parturition

D. Determination of the Effects of Treatment on Survival and Growth of Mammary GlandsTransplanted from Neonate into Adult Mice.

A. Preliminary Investigations: The Effects of 0.75 mg of Cortisone Acetate Given on Day 12 of Pregnancy upon Pregnancy and Young

To determine the effect upon the conceptus of a small dose of cortisone acetate prenatally administered, four strains of mice C3H, C57BL, CBA and BALB/c were treated.

All pregnant females were treated on Day 12 of pregnancy with 0.75 mg of cortisone acetate. The number of young per litter was determined and recorded daily at birth (Day 0) and Days 1, 2, 3, 4 and 5 postpartum. All dead young were fixed in Bouin's fixative, and examined for cleft palate.

B. Determination of Effects of Treatment on the Offspring Young Mice

To separate the effects of the prenatal treatment on the young mice from the effects of the treatment on the mother mouse, the following groups of fostering young mice were set up using CBA and A/J mice.

Group 1. Control young nursed by Control own mother.

Group 2. Control young nursed by Control foster mother.

Group 3. Control young nursed by Treated foster mother.

Group 4. Treated young nursed by Control foster mother.

Group 5. Treated young nursed by Treated foster mother.

Group 6. Treated young nursed by Treated own mother.

In addition, normal Young neonatally treated were nursed by their own non-treated mother in C3H and A/J mice (Group 7).

The number of young per litter and weight per litter were determined and recorded daily at birth (Day 0) and Days 1, 2, 3, 4, 5, 7, 14 and 21 postpartum.

Analysis of Data -

(a) Average Litter Size was determined by the following criteria and steps:

- Only the number of live young were included in Young per Litter.

- If all young in a litter died then the number of litters in the group were decreased by one.
- The Young per Litter for all the litters in a group were computed to give Average Litter Size $\pm$ S.E.M.

$$\text{Average Litter Size} = \frac{\Sigma \text{ No. of Young per Litter at Day X}}{\text{No. of Litters at Day X}}$$

(b) Group Average Percentage Survival Rates of Young per Litter were determined by the following criteria and steps.

- The number of live young at birth per litter was taken as 100% of neonates in that litter, to use as a reference point for future comparisons.
- The percentage of surviving young per litter was determined for each day.
- If all young in a litter died, the percentage of surviving young per litter was zero, and 0% was included in the calculation.
- The percentages of surviving young per litter of all litters involved on the day in question in each group were computed to give Group Average Percentage Survival Rates of Young per Litter $\pm$ S.E.M.

$$\text{Group Average Percentage Survival Rates of Young per Litter} = \frac{\Sigma \text{ \% of Surviving Young in Each Litter}}{\text{No. of Litters at Day 0 Birth}}$$

(c) Group Average Body Weight per Young at Birth was determined by the following criteria and steps.

- Only live young were weighed at birth to give a Total Body Weight for each Litter.
- The Total Body Weight for each Litter was divided by the number of live young to give -

$$\text{An Average Body Weight Per Young at Birth} = \frac{\text{Total Body Weight for Each Litter at Birth}}{\text{No. of Live Young in Litter at Birth}}$$

for each litter.

- The Average Body Weight per Young at Birth for each litter was averaged in each group to obtain Group Average Body Weight per Young at Birth $\pm$ S.E.M.

$$\text{Group Average Body Weight per Young} = \frac{\Sigma \text{ Average Body Weight per Young of all Litters}}{\text{No. of Litters in a Group}}$$

(d) Group Average Body Weight Gain per Young was determined by the following criteria and steps.

- Average Body Weight per Young at Birth for each litter was used as a reference point to obtain Average Body Weight Gain per Young at different ages.

$$\text{Average Body Weight Gain per Young} = \frac{\Sigma \text{ Total Body Weight of Litter at Day X}}{\text{No. of Live Young in Litter at Day X}} - \text{Average Body Weight per Young at Birth}$$

- The Average Body Weight Gain per Young for each litter in the group were averaged to give Group Average Body Weight Gain per Young $\pm$ S.E.M.

$$\text{Group Average Body Weight per Young} = \frac{\Sigma \text{ Average Body Weight Gain per Young}}{\text{No. of Litters in Group}}$$

(e) Group Average Percentage Body Weight Gain of Young was determined by the following criteria and steps.

- The Average Body Weight per Young at birth for each litter was set at 100% as a reference point.
- The Average Body Weight per Young at Days 2 or 5 were divided by Average Body Weight per Young at Birth and multiplied by 100 to obtain Percentage Body Weight Gain per Young for the day in question for each litter.

$$\text{Percentage Body Weight Gain per Young} = \frac{\text{Average Body Weight per Young (at Day 2 or 5)}}{\text{Average Body Weight per Young (at Birth)}} \times 100$$

- The Percentage Body Weight Gains per Young for each litter in a group were averaged to obtain Group Average Percentage Body Weight Gain of Young $\pm$ S.E.M.

$$\text{Group Average Percentage Body Weight Gain per Young} = \frac{\Sigma \text{ Percentage Body Weight Gain per Young}}{\text{No. of Litters in Group}}$$

The data was analyzed for significant differences as follows:

1. Differences in the data among the prenatally treated young and the Control young in which some young had foster mothers were analyzed by Duncan's multiple-range test.
2. Differences in the data between the neonatal treatment group (group 7) and the control group (group 1) were analyzed by *t*-test.

C. Determination of the effects of Treatment on the Offspring Mice After Their Maturation and Parturition

To determine if the prenatal treatment had any effect on the reproductive ability of the prenatally treated female versus the control female, a control and treated group of females were allowed to mate with isogeneic males and have young.

The number of Young per Litter and Total Body Weight for each litter were determined and recorded daily at birth (Day 0) and Days 1, 2 and 3 postpartum.

Analysis of Data -

Average Litter Size, Group Body Weight per Young at Birth and Group Average Body Weight Gain per Young were determined by the same criteria and steps as described in the previous Section (B).

The data were analysed for significant differences using a *t*-test and testing for differences between the treated group versus the control group.

The data was analysed for differences in Group Average Body Weight Gain per Young, Average Litter Size and Group Average Body Weight per Young in each litter.

D. Determination of the Effects of Treatment on Survival and Growth Of Mammary Gland Transplanted from Neonate into Adult Mice

The adult C3H and A/J mice which were to receive the mammary gland transplants had been prepared by a simple operation for the removal of the parenchyma from the fourth and fifth mammary gland (Appendix I) and to leave a fourth mammary gland free fat pad (the remaining fat pad). This operation was performed when the mice were 21 days of age or younger under Nembutal anesthesia (Appendix II).

The neonatal mammary glands were obtained for transplantation from the three groups of newborn mice, namely:

1. Control without treatment;
2. Prenatally treated with 0.75 mg of cortisone acetate on Day 12 of fetal life; and
3. Neonatally treated with 0.075 mg of cortisone acetate within 16 hours after birth.

The neonatal mammary glands were obtained from the 1 day old mice and transplanted immediately into the 4th mammary gland free fat pad of the 3 month old female host mice (Appendix III), under Nembutal anesthesia.

Three days after transplantation the C3H host mice were mated with their siblings and checked daily to detect vaginal plugs. The day when a vaginal plug was found was considered as Day 0 of pregnancy. The host mice were killed on Day 12 of pregnancy, on Day 0 postpartum, and the remaining mice which did not become pregnant were killed as non-pregnant within 6 months of mammary isografting. All the A/J host mice were killed as non-pregnant 6 months after isografting.

When the animals were killed, they were skinned and their hides were fixed in Bouin's solution. Viable mammary grafts and some of the host's own mammary gland were removed and examined histologically, (Appendix IV) or by whole mount preparation (Appendix V).

Nine C3H female mice, which had been operated on for the preparation of the 4th mammary gland free fat pad received no grafts. These animals were killed when they were about 1 year old, and their 4th mammary gland free fat pad was examined for the absence of mammary gland parenchyma.

V RESULTS

V RESULTS

1. Preliminary Investigations: The Effects of 0.75 mg of Cortisone Acetate Given on Day 12 of Pregnancy upon Pregnancy and Young

The results of the preliminary investigation are shown in Table 1. No abortions were observed in C3H and BALB/c mice, and only 8% and 11% of pregnancies aborted in CBA and C57BL mice respectively. All other pregnancies and parturitions were normal.

From birth to 5 days of age, varying rates of infantile deaths including neonatal deaths, were observed in these strains of mice. The rate of infantile death was highest in C3H mice, and lowest in BALB/c mice. The order of infantile death rates was BALB/c < CBA < C57BL < C3H.

In all four strains of mice used no cleft palates were found in the young which died during this period.

2. Effects of the Treatement on the Offspring Young Mice

In the A/j mice, the incidence of cleft palate in the prenatally treated young was not different from the incidence of naturally occurring cleft palate in the Control young.

A. Litter Size and Number of Surviving Litters

Tables 2, 3 and 4 show the data concerning Average Litter Size of surviving young and the number of surviving litters.

Table 2 shows the effects of cortisone treatments and fostering of young in CBA mice. The numbers of surviving young and of surviving litters at three weeks was apparently less than those at birth. There are no significant differences in Table 2.

Table 3, concerning A/j mice, shows similar data as in Table 2, with the addition of a neonatally treated (0.075 mg) group. There are significant differences between the neonatally treated young and both the prenatally treated and control young. The number of surviving litters at 21 days of age as compared with that at birth was significantly less in the neonatally Treated litters (Group 7), than Control litters (Group 1). Table 4, concerning C3H mice, shows similar data as Table 3, with the addition of a second neonatally treated (0.15 mg) group but without the fostering of young.

In Tables 2, 3 and 4, Average Litter Sizes were in some instances increased because they were calculated using a fewer number of Surviving Litters due to the entire death of small litters. For example, in Table 4 Average Litter Size at 5 days of age in Group 2 increased from 6.0 to 6.9 while the number of surviving litters dropped from 10 to 8. There are significant reductions in numbers of surviving litters in two treated groups.

B. Percentage Survival Rates of Young per Litter

Tables 5, 6 and 7 show a percentage survival rate of young in the CBA, A/j and C3H mice, respectively. In Tables 5 and 6, the Control young nursed by Treated foster mother (Group 3) have the lowest survival rate of the young at 3 weeks of age. The highest survival rate was in the Control young nursed by own Control mother (Group 1). Although there are no significant differences between the prenatally Treated groups and the Control groups, more surviving young appeared to be in Groups 1, 2 and 3, than in Groups 4, 5 and 6.

Table 6 also shows the survival rates of young neonatally treated with 0.75 mg of cortisone (Group 7) which is significantly lower than that of the control young (Group 1).

Table 7 shows the survival rates of C3H mice for the Control and the three Treated groups. The survival rates of the prenatally treated (Group 2) was significantly lower than the Control young (Group 1). The young neonatally treated with the low dose (0.075 mg) (Group 3) was not different from the Control Group (1). However, the survival rates of the young neonatally treated with the high dose (0.15 mg) (Group 4) was significantly lower than that of the Control (Group 1).

Data selected from above are compared in Table 8 and Figure 1.

Percentage survival rates of young per litter at 21 days of age were not significantly different in the prenatally treated groups from the Control Groups in CBA and A/j mice. The difference however, in C3H mice was significant.

C. Body Weight Gain of Young

Table 9 and 10 show the weight at birth and weight gain of control mice and the young mice prenatally and neonatally treated with cortisone acetate.

The significant differences in weight per young at birth are shown on Tables 9 and 10. Weight gains between birth and 2 days of age are all insignificant among groups in both CBA and A/j mice.

Weight gains between birth and 5 days of age in control and prenatally treated young show significant differences only among the groups

in CBA mice (Table 9) but not in the A/j mice (Table 10). In the neonatally treated A/j mice (Group 7) weight gains between birth and 5 days of age was significantly different from those of the Control young (Group 1) (Table 10).

The Percentage Body Weight Gains from birth to 2 and 5 days of age are shown in Figure 2 and 3, for CBA and A/j mice respectively. Those in the prenatally treated young (Groups 4, 5 and 6) showed no differences from the Controls (Groups 1, 2 and 3) in both CBA and A/j mice. However, in A/j mice, the neonatal treatment (Group 7) caused a significant reduction in percentage body weight gain at 5 days when compared to the Control young nursed by Control own mother (Group 1).

3. Effects of Treatment on the Offspring Mice After Their Maturation and Parturition

Tables 11 and 12 show the Average Litter Size, Average Body Weight per Young, and Average Body Weight Gain per Young for Control young and young of mothers who were treated with cortisone on Day 12 of their fetal life for CBA and C3H mice respectively. Although no significant differences were found in the data, there was a consistant tendency that the control animals were at an advantage over the treated animals.

The Average Litter Size of the CBA mice was higher at birth in the Treated group than in the Control group (Table 11). However, at 2 and 3 days there were smaller sizes of litters surviving in the Treated Group. There were higher mortality rates in the Treated Group.

In Table 12, the litter size of the C3H mice at birth appeared to be slightly higher in the Control. At 3 days of age the difference became greater. The mortality rate of young from the treated mother was higher than in the Controls.

Concerning the weight per young, there was a subtle tendancy that the Control Groups were slightly heavier than the Treated Groups in both CBA and C3H mice (Tables 11 and 12), however statistically insignificant.

The weight gain per young observed in surviving young in Treated groups were not different from those in the Control groups in both CBA and C3H mice (Table 11 and 12).

There were higher mortality rates (litter size) and larger litter size in C3H mice than in CBA mice (Tables 11 and 12).

4. Effects of Treatment on Survival and Growth of Transplanted Mammary Gland from Newborn-Mouse into Adult Mice

The nine C3H female mice with fourth mammary gland-free fat pads did not receive neonatal grafts. When these animals were killed and examined, no mammary gland parenchyma was found in the fourth mammary gland-free fat pad.

The transplantability rates of the newborn mouse mammary gland are shown in Table 13 for C3H and A/j mice.

There were no significant differences in the recovery rates of the surviving mammary gland from the host at different physiological conditions, and also between the pretreated groups and the Controls in C3H

mice (Table 13). In A/j strain, mammary transplantability from neonates which had been pretreated with 0.75 mg of cortisone acetate on Day 12 of fetal life was lowest (27.3%). The overall recovery rates of the grafts were higher in C3H mice than in A/j mice.

Development of the neonatal grafts recovered from non-pregnant hosts (Figure 4a, b, c) were comparable to the host's own gland (Figure 4d). Similarities were seen in the branching of mammary ducts with bud-like structures at the ends of the ducts. In grafts recovered from 12 day pregnant host mice (Figure 5a, b), the beginnings of lobuloalveolar growth were observed, however, patent lumen were not readily apparent. These features were also noted in the host's own gland (Figure 5c). Neonatal grafts (Figures 6a, b, c) recovered from post-partum host mice revealled features similar tc the host's own gland (Figure 6d), namely, well developed, distended alveoli, with dark rims and light centers. Neonatal mammary grafts (Figures 7a, b, c) recovered post-partum all showed well developed alveoli, which was comparable to the host's own gland (Figure 7d).

The functional development of the prenatally treated mammary grafts (Figures 4b, 5b, 6b, and 7b) and the neonatally treated mammary grafts (Figures 4c, 6c and 7c) were similar to that of the non-treated control mammary grafts (Figures 4a, 5a, 6a, and 7a).

The duration of the transplantation depended upon varying time intervals between the time of transplantation and the time when the host became pregnant. The shortest period from transplantation to recovery was 19 days in a Day 12 pregnant host C3H mouse, and 30 days in a Day 1 postpartum host C3H mouse, Figures 5b and 7b respectively.

TABLE 1

NUMBER OF LITTERS AFFECTED BY 0.75 mg OF CORTISONE ACETATE

GIVEN ON DAY 12 of PREGNANCY

Fate of Conceptus	Strains of Mice Investigated							
	C3H		C57BL		CBA		BALB/c	
ABORTION	0		2/18	(11%)	1/13	(8%)	0	
NORMAL BIRTH	54/54	(100%)	16/18	(89%)	12/13	(92%)	16/16	(100%)
INFANTILE DEATH								
Death of all young in a litter	39/54	(72%)	9/18	(50%)	5/13	(39%)	4/16	(25%)
Total*	40/54	(74%)	13/18	(72%)	7/13	(54%)	4/16	(25%)
INFANTILE DEATH								
at 0 and 1 day old	25		9		3		3	
at 2 day old	11		2		3			
at 3 day old	2							
at 4 day old								
at 5 day old	2		2		1		1	

* Includes death of all young in a litter and death of some young in a litter.

TABLE 2

LITTER SIZE FROM BIRTH TO 3 WEEKS OF AGE IN CBA MICE

MEAN ± S.E.M. (*)

Experimental Groups	Birth 0 Days	2 Days of Age	5 Days of Age	7 Days of Age	14 Days of Age	21 Days of Age
1. Control young nursed by Control own Mother	5.4 ± .5 (9)	5.4 ± .5 (9)	5.4 ± .5 (9)	5.4 ± .5 (9)	5.3 ± .4 (9)	5.3 ± .6 (8)
2. Control young nursed by Control foster mother	6.1 ± .5 (8)	6.1 ± .5 (8)	6.1 ± .5 (8)	6.1 + .5 (8)	5.9 ± .7 (8)	5.3 ± .7 (7)
3. Control young nursed by Treated foster mother	6.1 ± .5 (9)	5.4 ± .6 (9)	5.9 ± .4 (8)	5.9 ± .4 (8)	5.6 ± .6 (7)	5.5 ± .7 (6)
4. Treated young nursed by Control foster mother	6.4 ± .6 (9)	6.0 ± .6 (8)	6.0 ± .6 (8)	6.0 ± .6 (8)	5.8 ± .5 (8)	5.7 ± .5 (7)
5. Treated young nursed by Treated foster mother	6.8 ± .4 (12)	6.8 ± .4 (12)	6.6 ± .5 (12)	6.6 ± .5 (12)	6.0 ± .5 (10)	5.7 ± .5 (10)
6. Treated young nursed by Treated own mother	6.5 ± .5 (11)	6.0 ± .8 (9)	6.6. ± .6 (8)	6.6 ± .6 (8)	6.5 ± .6 (8)	6.1 ± .7 (8)

Treated young were prenatally treated with 0.75 mg of Cortisone Acetate administered on Day 12 of pregnancy to the female mouse, which is the Treated mother.

No significant difference

(*) Number of surviving litters

TABLE 3

AVERAGE LITTER SIZE

FROM BIRTH TO 3 WEEKS OF AGE IN A/j MICE

MEAN ± S.E.M. (*)

	Experimental Groups	Birth 0 day	2 days of age	5 days of age	7 days of age	14 days of age	21 days of age
1.	Control young nursed by Control own Mother	5.9 ± .4 (20^{X})	5.2 ± .4 (20)	5.1 ± .4 (20)	4.9 ± .4 (20)	4.9 ± 4^{a} (19)	4.9 ± .4 (18^{x})
2.	Control young nursed by Control foster Mother	6.4 ± .5 (12)	5.8 ± .5 (12)	5.6 ± .4 (12)	5.3 ± (12)	5.4 ± $.4^{b}$(11)	5.4 ± $.5^{A}$ (10)
3.	Control young nursed by Treated foster Mother	5.3 ± .6 (9)	4.1 ± .8 (8)	4.6 ± .8 (7)	4.6 ± .8 (7)	4.4 ± .7 (7)	4.2 ± .9 (6)
4.	Treated young nursed by Control foster Mother	4.7 ± .6 (9)	4.3 ± .4 (7)	4.1 ± (7)	4.1 ± (7)	4.1 ± .4 (7)	4.1 ± .4 (7)
5.	Treated young nursed by Treated foster Mother	5.6 ± .6 (9)	4.7 ± .6 (9)	4.1 ± .4 (8)	4.1 ± .4 (8)	3.9 ± .4 (7)	3.7 ± .4 (7)
6.	Treated young nursed by Treated own Mother	5.5 ± .3 (25)	5.2 ± .3 (23)	5.2 ± .3 (22)	5.1 ± .3 (22)	4.8 ± $.3^{c}$ (21)	4.7 ± $.3^{B}$ (19)
7.	Treated at birth (neonatally)	6.0 ± .6 (11^{Y})	4.8 ± .6 (10)	4.4 ± .6 (8)	4.1 ± .4 (7)	3.0 ± $.6^{d}$ (6)	3.2 ± $.7^{C}$ (5^{y})

(*) Number of Surviving Litters

Differences $\frac{y}{Y} < \frac{x}{X}$ ($p < .005$)

d < a
d < b
($p < .01$)
d < c
($p < .05$)

C < A
C < B
($p < .05$)

Treated young were prenatally treated with 0.75 mg of cortisone acetate administered on Day 12 of pregnancy to the female mouse, which is the Treated Mother.

Treated at birth (Group 7) were neonatally treated with 0.075 mg of cortisone acetate administered within 16 hours of birth.

TABLE 4

AVERAGE LITTER SIZE

FROM BIRTH TO 3 WEEKS OF AGE IN C3H MICE

MEAN $\pm$ S.E.M. (*)

Experimental Groups	Birth 0 days	2 days of age	5 days of age	7 days of age	14 days of age	21 days of age
1. Control young nursed by Control own Mother	6.4 ± 0.4 (14^{W})	6.2 ± 0.5 (14)	6.1 ± 0.5 (14)	6.1 ± 0.5 (14)	6.1 ± 0.5 (14)	5.6 ± 0.6 (14^{W})
2. Treated young nursed by Treated own Mother (0.75 mg)	6.3 ± 0.6 (12^{X})	6.0 ± 0.8 (10)	6.9 ± 0.7 (8)	6.9 ± 0.7 (8)	6.8 ± 0.7 (8)	6.6 ± 0.8 (8^{X})
3. Treated at Birth (0.075) mg)	6.6 ± 0.5 (18^{Y})	6.6 ± 0.5 (18)	6.4 ± 0.5 (18)	6.2 ± 0.6 (17)	6.1 ± 0.6 (17)	6.1 ± 0.6 (17^{y})
4. Treated at Birth (0.15 mg)	6.5 ± 0.4 (17^{Z})	5.9 ± 0.6 (14)	5.5 ± 0.6 (14)	5.6 ± 0.6 (11)	5.1 ± 0.8 (9)	4.4 ± 0.8 (9^{z})

(*) Number of Surviving Litters Differences $\frac{x}{X} < \frac{w}{W}$ ($p < .05$); $\frac{x}{X} < \frac{y}{Y}$ ($p < .01$); $\frac{z}{Z} < \frac{w}{W}$ and $\frac{z}{Z} < \frac{y}{Y}$ ($p < .005$)

Treatement is with cortisone acetate.

TABLE 5

GROUP AVERAGE PERCENTAGE SURVIVAL RATES OF YOUNG PER LITTER

FROM BIRTH TO 3 WEEKS OF AGE IN CBA MICE

MEAN ± S.E.M.

	Experimental Groups	Birth 0 day	2 days of age	5 days of age	7 days of age	14 days of age	21 days of age
1.	Control young nursed by Control own Mother	100% (9)*	100%	100%	100%	98.4 ± 1.6%	81.8 ± 11.6%
2.	Control young nursed by Control foster Mother	100% (8)	100%	100%	100%	93.8 ± 6.3%	75.1 ± 12.2%
3.	Control young nursed by Treated foster Mother	100% (9)	89.2 ± 7.4%	84.7 ± 11.4%	84.7 ± 11.4%	70.9 ± 14.1%	57.9 ± 15.1%
4.	Treated young nursed by Control foster Mother	100% (9)	87.3 ± 11.0%	87.3 ± 11.0%	87.3 ± 11.0%	84.6 ± 11.0%	69.9 ± 13.5%
5.	Treated young nursed by Treated foster Mother	100% (12)	99.1 ± 0.9%	96.3 ± 2.8%	96.3 ± 2.8%	75.5 ± 11.1%	72.0 ± 10.9%
6.	Treated young nursed by Treated own Mother	100% (11)	72.7 ± 13.1%	71.2 ± 13.9%	71.2 ± 13.9%	69.9 ± 13.7%	64.5 ± 12.7%

Treatment is with Cortisone Acetate

No significant differences

* ( ) Number of litters at birth

TABLE 6

GROUP AVERAGE PERCENTAGE SURVIVAL RATES OF YOUNG PER LITTER

FROM BIRTH TO 3 WEEKS OF AGE IN A/j MICE

MEAN ± S.E.M.

	Experimental Groups	birth 0 day	2 days of age	5 days of age	7 days of age	14 days of age	21 days of age
1.	Control young nursed by Control own mother	100% (20)*	88.1 ± 3.9%	87.0 ± 3.8%	84.0 ± 5.9%	78.9 ± 5.8%[a]	75.1 ± 7.0%[A]
2.	Control young nursed by Control foster Mother	100% (12)	90.0 ± 4.2%	88.2 ± 4.2%	83.2 ± 7.1%	76.8 ± 8.5%	70.4 ± 10.6%
3.	Control young nursed by Treated foster Mother	100% (9)	67.4 ± 11.5%	63.8 ± 13.3%	63.8 ± 13.3%	62.1 ± 13.1%	51.4 ± 13.9%
4.	Treated young nursed by Control foster Mother	100% (9)	69.0 ± 13.4%	66.8 + 13.4%	66.8 ± 13.4%	66.8 ± 13.4%	66.8 ± 13.4%
5.	Treated young nursed by Treated foster Mother	100% (9)	80.6 + 10.9%	74.0 + 10.4%	70.8 + 10.4%	59.7 + 12.3%	57.8 + 12.3%
6.	Treated young nursed by Treated own Mother	100% (25)	84.8 ± 5.7%	81.4 ± 6.5%	80.4 ± 6.4%	73.2 ± 7.4%	65.8 ± 8.1%
7.	Treated at birth (0.075 mg)	100% (11)	75.5 ± 9.3%	56.6 ± 12.5%	55.5 ± 12.4%	28.4 ± 9.9%[b]	26.1 ± 10.2%[B]

Treatment is with cortisone acetate.

* ( ) Number of Litters at Birth

b < a ($p < .01$)

B < A ($p < .01$)

TABLE 7

GROUP AVERAGE PERCENTAGE SURVIVAL RATES OF YOUNG PER LITTER FROM BIRTH TO 3 WEEKS OF AGE IN C3H MICE

MEAN ± S.E.M.

Experimental Groups	birth 0 day	2 days of age	5 days of age	7 days of age	14 days of age	21 days of age
1. Control young nursed by Control own Mother	100% (14)*	95.9 ± 3.0%[a]	94.9 ± 3.1%[d]	94.9 ± 3.1%[g]	93.5 ± 3.2%[k]	85.3 ± 5.1%[p]
2. Treated young nursed by Treated own Mother	100% (12)	72.0 ± 12.0%[b]	62.0 ± 13.7%[e]	62.0 ± 13.7%[h]	60.7 ± 13.4%[l]	58.6 ± 13.0%[g]
3. Treated at Birth (0.075 mg)	100% (17)	100%[c]	96.8 ± 1.7%[f]	94.3 ± 3.3%[i]	91.7 ± 4.2%[m]	85.9 ± 6.4%[r]
4. Treated at Birth (0.15 mg)	100% (15)	88.1 ± 7.2%	81.7 ± 8.1%	70.0 ± 10.7%[j]	49.3 ± 11.9%[m]	42.1 ± 10.3%[s]

*( ) Number of Litters at Birth
Treatment is with Cortisone Acetate

b < c ($p < .01$)	e < f	h < i	m < m	s < r
b < a ($p < .05$)	e < d ($p < .01$)	h < g	m < k ($p < .01$)	s < p ($p < .01$)
		j < i	l < k ($p < .05$)	g < p
		j < g ($p < .05$)		g < r ($p < .05$)

TABLE 8

LITTER SIZE AND SURVIVAL RATES OF YOUNG IN MICE

MEAN ± S.E.M.

	Litter Size at Birth	Litter Size at 21 Days	% Survival of Young at 21 Days
CBA			
Control	5.4 ± 0.5 (9)	5.3 ± 0.6 (8)	81.8 ± 11.6%
Prenatal treatment (0.75 mg*)	6.5 ± 0.5 (11)	6.1 ± 0.7 (8)	64.5 ± 12.7%
A/j			
Control	5.9 ± 0.4 (20)	4.9 ± 0.4 (18)	75.1 ± 7.0%[c]
Prenatal treatment (0.75 mg*)	5.5 ± 0.3 (25)	4.7 ± 0.3 (19)	65.8 ± 8.1%[b]
Neonatal treatment (0.075 mg*)	6.0 ± 0.6 (11)	3.2 ± 0.7 (5)	26.1 ± 10.2%[a]
		a < c ($p < .01$); a < b ($p < 0.5$)	
C3H			
Control	6.4 ± 0.4 (14)	5.6 ± 0.6 (14)	85.3 ± 5.1%[d]
Prenatal treatment (0.75 mg*)	6.3 ± 0.6 (12)	6.6 ± 0.8 (8)	58.6 ± 13.0%[c]
Neonatal treatment (0.075 mg*)	6.6 ± 0.5 (18)	6.1 ± 0.6 (17)	85.9 ± 6.4%[b]
Neonatal treatment (0.15 mg*)	6.5 ± 0.4 (17)	4.4 ± 0.8 (9)	42.1 ± 10.3%[a]
	a < b, a < d ($p < .01$); c < d, c < b ($p < .05$)		

* Cortisone Acetate

( ) Number of Litters with Surviving Young

TABLE 9

GROUP AVERAGE BODY WEIGHT GAIN PER YOUNG

BETWEEN BIRTH AND 5 DAYS OF AGE IN CBA MICE

MEAN ± S.E.M. (*)

Experimental Group	Body weight(g) at birth (0 day old)	Body weight gain(g) between 0 and 2 days of age	Body weight gain(g) between 0 and 5 days of age
1. Control young nursed by Control own Mother	1.4 ± .04[a] (9)	.7 ± .09 (9)	2.1 ± .08[A] (9)
2. Control young nursed by Control foster Mother	1.3 ± .04[b] (8)	.6 ± .06 (8)	2.0 ± .09[B] (8)
3. Control young nursed by Treated foster Mother	1.2 ± .02[c] (9)	.5 ± .06 (9)	1.7 ± .13[C] (8)
4. Treated young nursed by Control foster Mother	1.3 ± .04[d] (9)	.5 ± .07 (8)	1.6 ± .16[D] (8)
5. Treated young nursed by Treated foster Mother	1.1 ± .03[e] (12)	.5 ± .05 (12)	1.6 ± .11[E] (11)
6. Treated young nursed by Treated own Mother	1.2 ± .04[f] (11)	.4 ± .09 (9)	1.7 ± .08[F] (8)

Treated young were prenatally treated with 0.75 mg of cortisone acetate administered on Day 12 of pregnancy to the female mouse.

(*) Number of Surviving Litters

e < a
f < a
(p< .01)
e < c
e < b
e < d
(p< .05)

E < A
D < A
(p< .01)
E < B
C < A
F < A
(p< .05)

TABLE 10

GROUP AVERAGE BODY WEIGHT GAIN PER YOUNG

BETWEEN BIRTH AND 5 DAYS OF AGE IN A/j MICE

MEAN ± S.E.M. (*)

Experimental Groups	Body weight(g) at birth (0 day old)	Body weight gain(g) between 0 and 2 days of age	Body weight gain(g) between 0 and 5 days of age
1. Control young nursed by Control own Mother	1.2 ± .03[a] (20)	.17 ± .03 (20)	1.0 ± .08[A] (20)
2. Control young nursed by Control foster Mother	1.2 ± .02[b] (12)	.20 ± .03 (12)	1.1 ± .10 (12)
3. Control young nursed by Treated foster Mother	1.3 ± .04[c] (9)	.14 ± .05 (8)	.9 ± .13 (7)
4. Treated young nursed by Control foster Mother	1.3 ± .05[d] (9)	.07 ± .11 (7)	.9 ± .17 (7)
5. Treated young nursed by Treated foster Mother	1.2 ± .03[e] (9)	.21 ± .06 (8)	.9 ± .10 (8)
6. Treated young nursed by Treated own Mother	1.1 ± .02[f] (25)	.23 ± .05 (23)	1.1 ± .10 (21)
7. Treated at Birth (neonatally)	1.2 ± .03 (11)	.06 ± .05 (10)	.6 ± .09[B] (8)
(*) Number of Surviving Litters	b < c f < c f < d e < c a < c (p< .01)		B < A (p< .01)

TABLE 11

CBA MICE

AVERAGE LITTER SIZE, BODY WEIGHT, AND WEIGHT GAIN

OF YOUNG FROM FEMALE MICE* WHO HAD BEEN

PRENATALLY TREATED WITH CORTISONE ACETATE

LITTER SIZE

	DAY 0	1	2	3
Control (7)	5.0 ± .58	4.9 ± .63	4.9 ± .63	4.9 ± .63
Treated (13)	5.5 ± .46	5.0 ± .42	4.8 ± .47	4.6 ± .46

WEIGHT/YOUNG

	DAY 0	1	2	3
Control (7)	1.3 ± .04	1.4 ± .06	1.7 ± .08	2.0 ± .09
Treated (13)	1.2 ± .04	1.3 ± .05	1.6 ± .07	1.9 ± .09

WEIGHT GAIN/YOUNG

	DAYS 0-1	DAYS 0-2	DAYS 0-3
Control (7)	0.1 ± .05	0.4 ± .06	0.7 ± .07
Treated (13)	0.1 ± .05	0.3 ± .06	0.7 ± .07

( ) Litters / Group

* These female mice were treated prenatally on Day 12 of their fetal life with 0.75 mg of cortisone acetate.

TABLE 12

C3H MICE

AVERAGE LITTER SIZE, BODY WEIGHT, AND WEIGHT GAIN

OF YOUNG FROM FEMALE MICE* WHO HAD BEEN

PRENATALLY TREATED WITH CORTISONE ACETATE

LITTER SIZE

	DAY 0	1	2	3
Control (9)	6.4 ± .56	5.9 ± .56	5.9 ± .56	5.7 ± .53
Treated (7)	6.3 ± .47	5.9 ± .26	5.3 ± .20	5.1 ± .14

WEIGHT/YOUNG

	DAY 0	1	2	3
Control (9)	1.3 ± .04	1.4 ± .04	1.6 ± .06	1.9 ± .09
Treated (7)	1.3 ± .03	1.3 ± .04	1.6 ± .05	1.8 ± .07

WEIGHT GAIN/YOUNG

	DAYS 0-1	DAYS 0-2	DAYS 0-3
Control (9)	0.1 ± .03	0.3 ± .03	0.6 ± .07
Treated (7)	0.1 ± .03	0.2 ± .05	0.5 ± .08

( ) Litters / Group

* These female mice were treated prenatally on Day 12 of their fetal life with 0.75 mg of cortisone acetate

TABLE 13

TRANSPLANTABILITY OF MAMMARY GLANDS OF NEONATES

WITH OR WITHOUT PRETREATMENTS OF CORTISONE ACETATE

Pretreatment of Neonatal Donors	State of Host at Recovery	Number of Successful Grafts			
		C3H		A/j	
Non Pretreated Control	Not Pregnant	6/8	75%	12/28	42.9%
	12 Day Pregnancy	2/4	50%		
	Postpartum	8/12	67%		
	Sum	16/24	67%	12/28	42.9%
Prenatal Treatment (0.75 mg) on Day 12 of Fetal Life	Not Pregnant	6/10	60%	6/22	27.3%
	Postpartum	6/10	60%		
	Sum	12/20	60%	6/22	27.3%
Neonatal Treatment (0.75 mg) at Birth	Not Pregnant	5/6	83.3%	5/10	50%
	Postpartum	6/10	60%		
	Sum	11/16	68.8%	5/10	50%

Figure 1

SURVIVAL RATES OF YOUNG FOLLOWING VARIOUS TREATMENTS WITH CORTISONE ACETATE

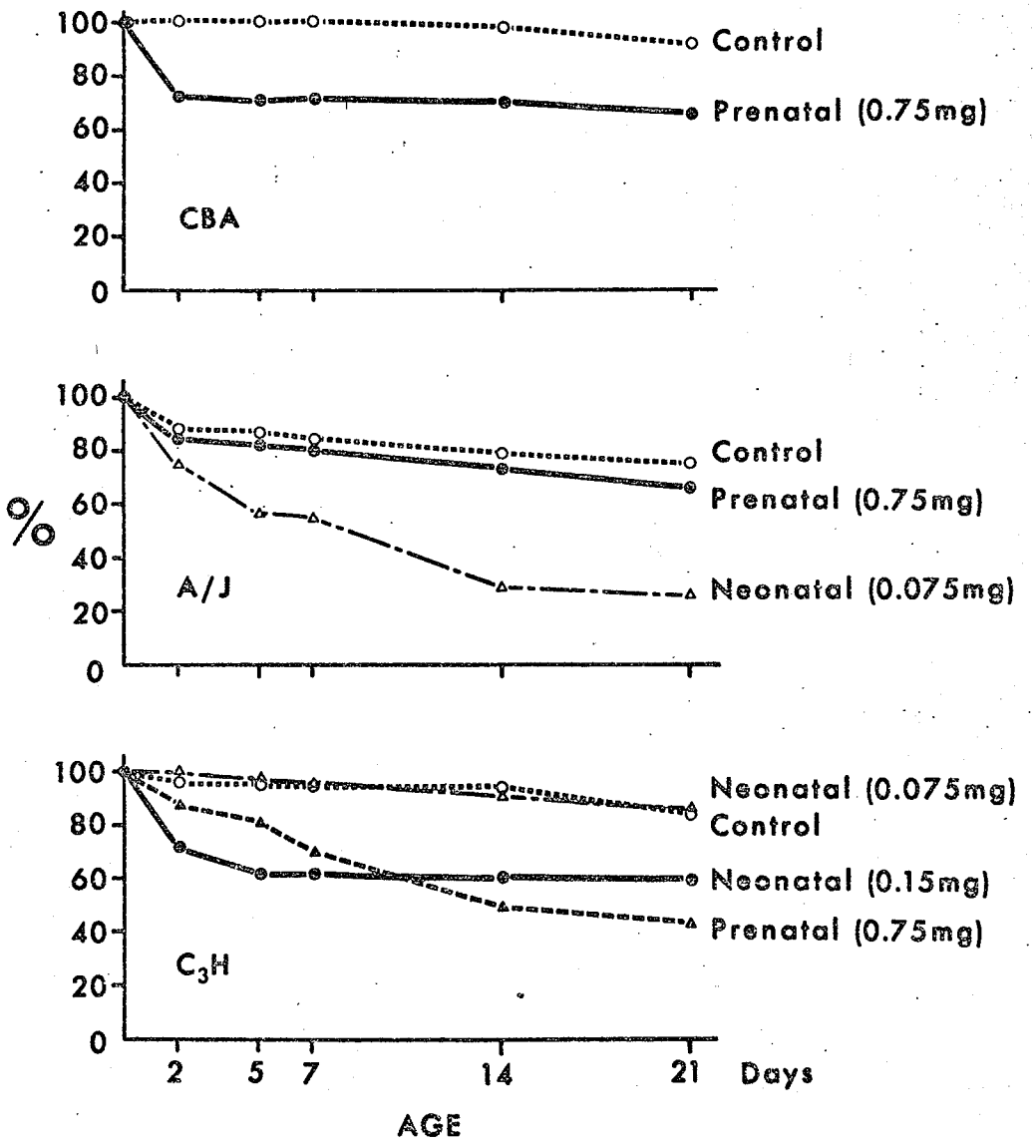

Figure 2

PERCENTILE BODY WEIGHT GAIN OF YOUNG BETWEEN BIRTH AND 5 DAYS OF AGE IN CBA MICE

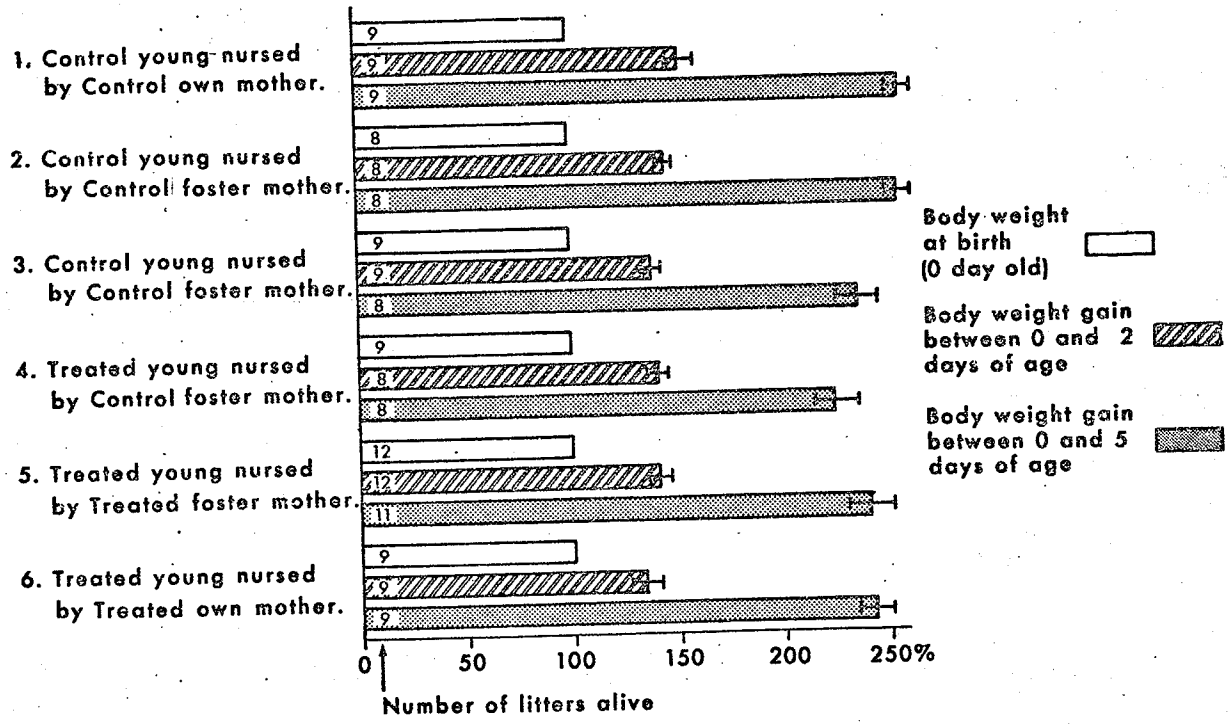

Figure 3

PERCENTILE BODY WEIGHT GAIN OF YOUNG BETWEEN BIRTH AND 5 DAYS OF AGE IN A/J MICE

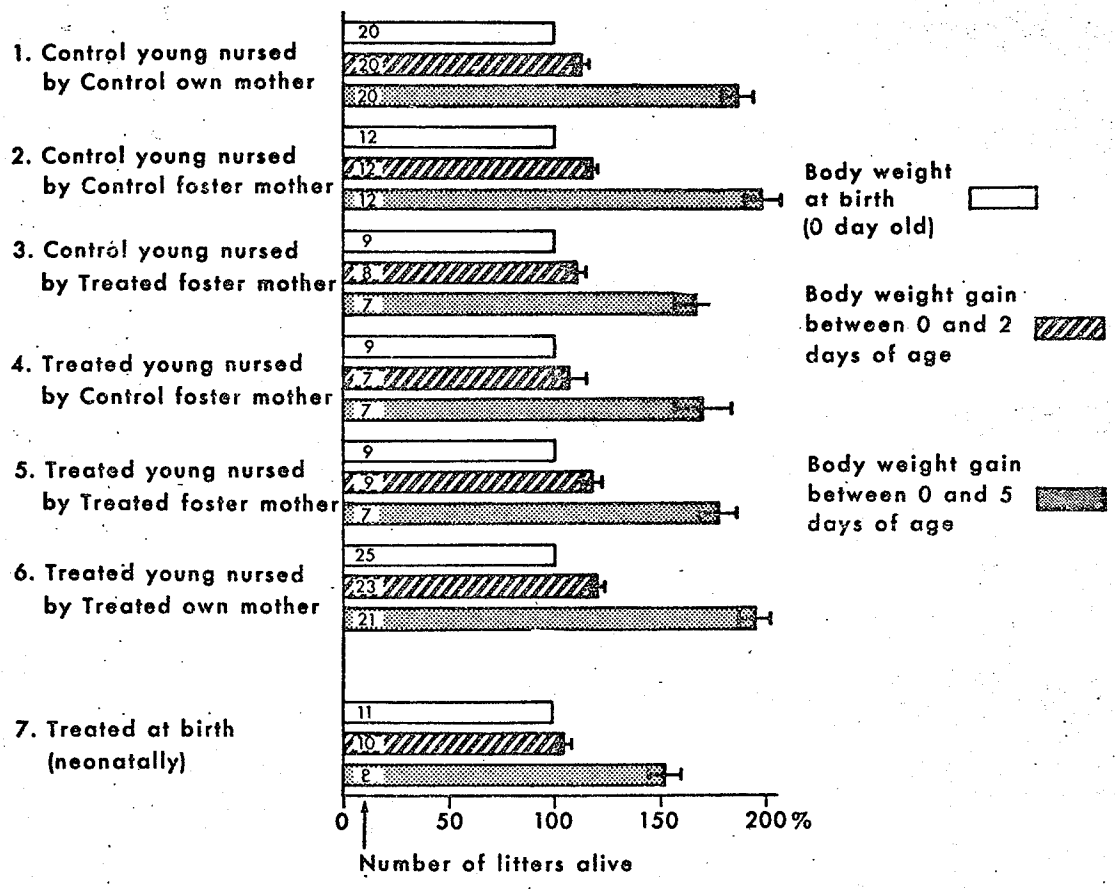

FIGURE 4

Whole mount preparations of mammary glands from non-pregnant C3H host mice. Alum Carmine staining X 12.

A. Mammary Graft from a Control Neonate

B. Mammary Graft from a Prenatally Treated Neonate

C. Mammary Graft from a Neonatally Treated Neonate

D. Mammary Gland from a Host's Own Mammary Gland

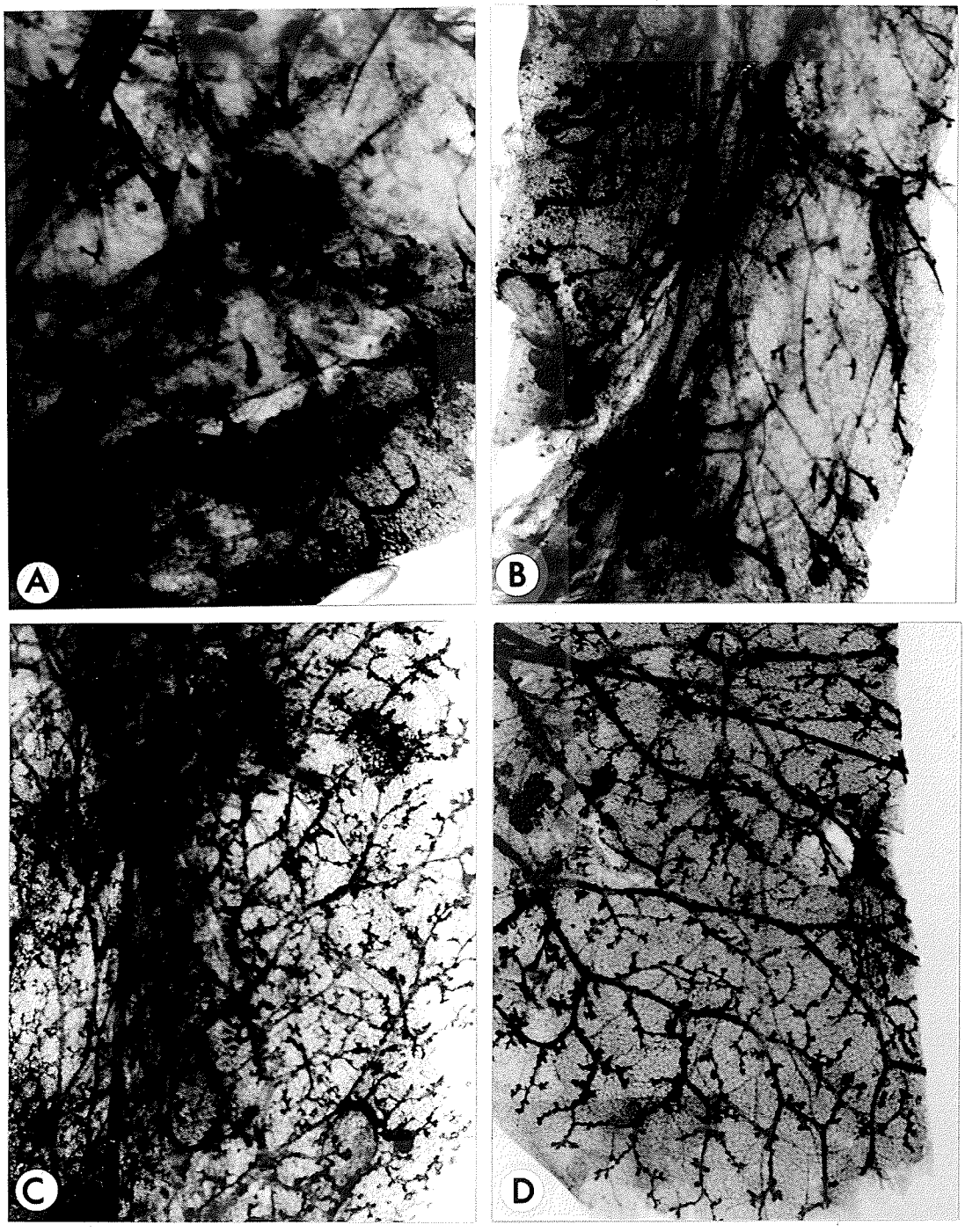

FIGURE 4

FIGURE 5

Photomicrographs of mammary glands from 12 day pregnant C3H Host mice. Hematoxylin and eosin staining after plastic embedding X525.

A. Mammary Graft from a Control Neonate

B. Mammary Graft from a Prenatally Treated Neonate

C. Mammary Gland from a Host's Own Mammary Gland

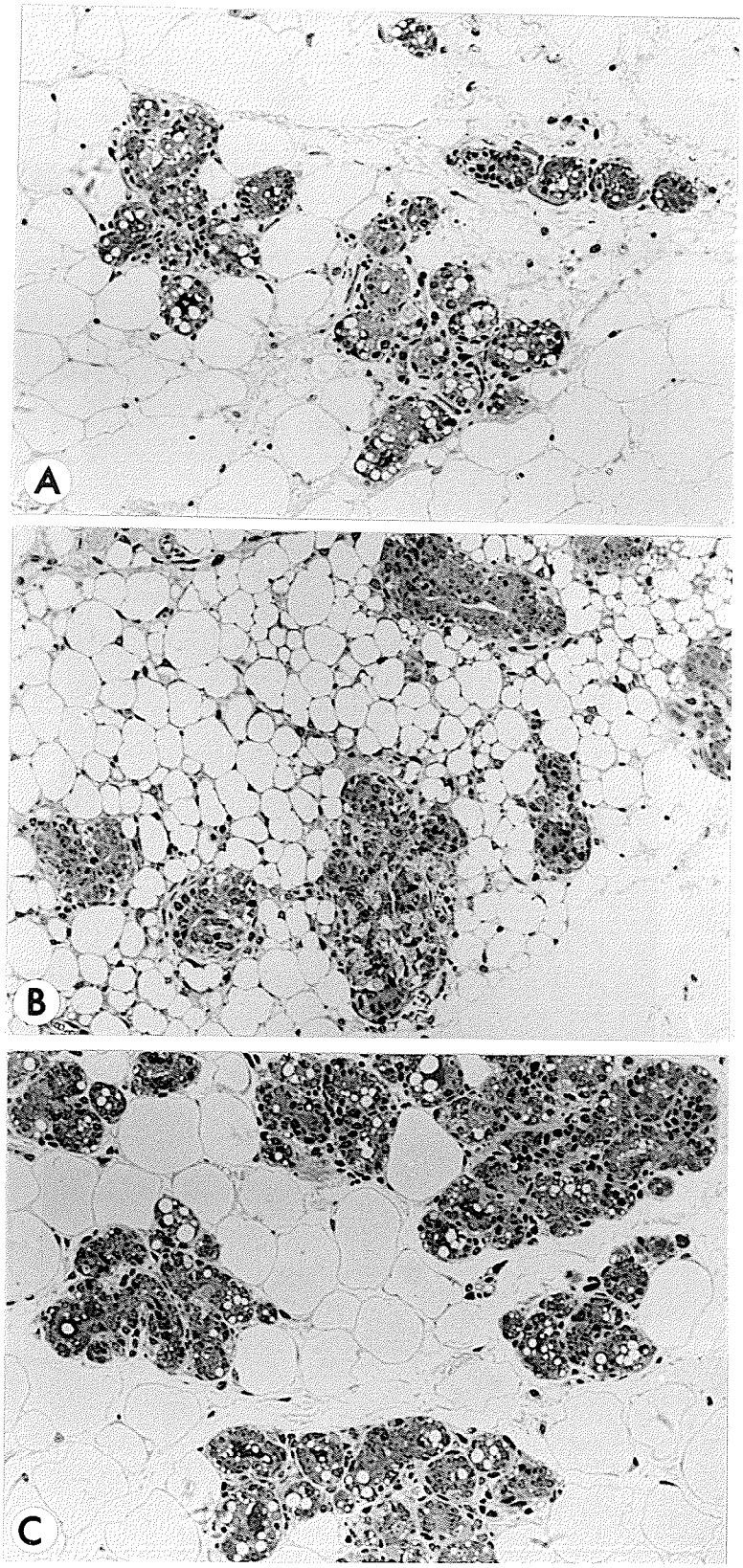

FIGURE 5

FIGURE 6

Photomicrographs of mammary glands from 1 day postpartum C3H Host mice. Hematoxylin and eosin staining after paraffin embedding X525.

A. Mammary Graft from a Control Neonate

B. Mammary Graft from a Prenatally Treated Neonate

C. Mammary Graft from Neonatally Treated Neonate

D. Mammary Gland from a Host's Own Mammary Gland

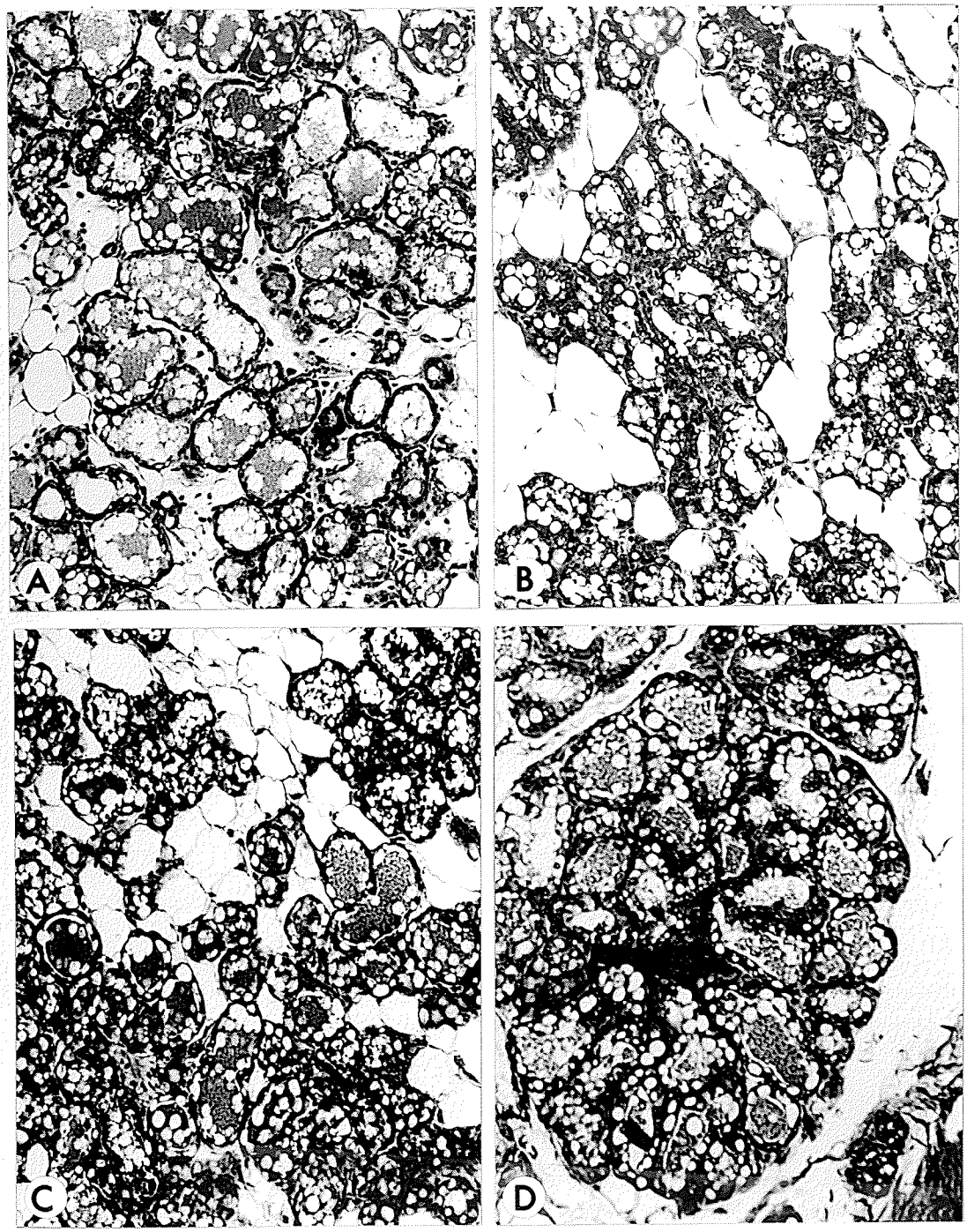

FIGURE 6

FIGURE 7

Whole mount preparations of mammary glands from 1 day postpartum C3H Host mice. Alum Carmine staining. X12.

A. Mammary Graft from a Control Neonate

B. Mammary Graft from a Prenatally Treated Neonate

C. Mammary Graft from a Neonatally Treated Neonate

D. Mammary Gland from a Host's Own Mammary Gland

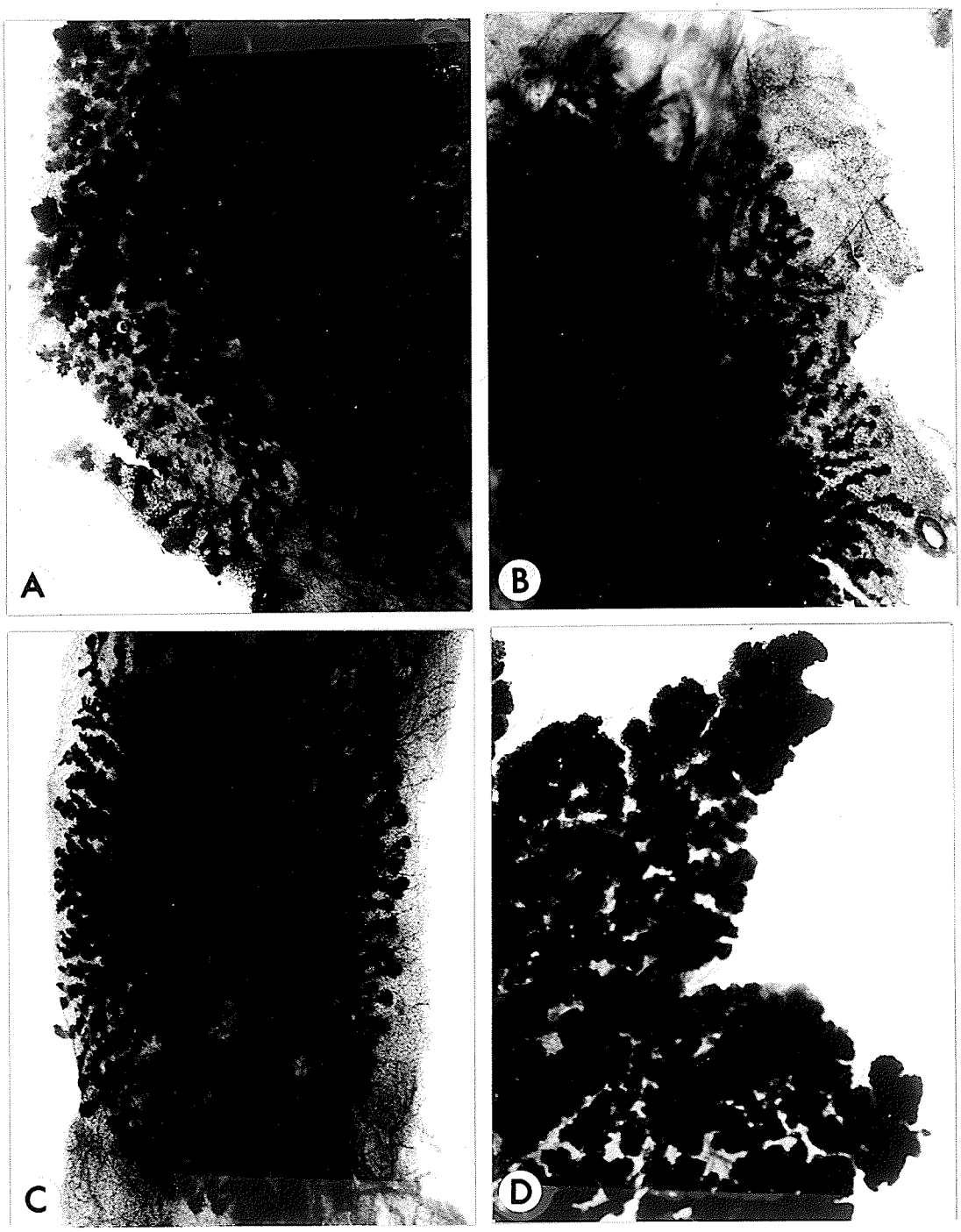

FIGURE 7

VI DISCUSSION

VI DISCUSSION

1. Animals

The prenatal treatment was given on Day 12 of fetal life, thus it was necessary to identify the day of pregnancy. The presence of vaginal plugs has been widely accepted as the criterion for the commencement of pregnancy since Parkes described it in 1926. In order to confirm the normal progress of pregnancy, the presence of intravaginal bleeding and a steady weight gain (Hoshino, 1964b) were used as additional criteria.

The strain difference in mice has been recognized by a number of different studies, e.g. teratogenicity (Wilson, 1973) and reproductive function (Hoshino, 1964b).

The strains of mice used in the preliminary experiment were CBA, C3H, C57Bl and BALB/c, whose characteristics have been thoroughly investigated in our laboratory.

After the preliminary experiments, CBA mice which showed a moderate infantile death rate after 0.75 mg of cortisone acetate on Day 12 of fetal life, were chosen for the next experiment, to determine the effects of the treatment on the offspring young mice. In addition, the A/j strain of mice were obtained from Jackson Laboratory because of their extremely high sensitivity to cortisone acetate (Francis, 1973).

Finally, the C3H mice were compared with the other strains because, in the preliminary study, the C3H mice were found to be the most sensitive to the cortisone among the strains of mice available in our laboratory.

2. Hormonal Treatments

Human doses of cortisone acetate can be 300 mg or higher per patient per day for several days. If the patient weighs 60 Kg., and equivalent dose in a 30 gm mouse becomes a daily dose of 0.15 mg per mouse. In the present study only single doses of 0.75 mg were given to pregnant mice on Day 12 of gestation as a prenatal treatment of the fetuses. This single dose of 0.75 mg was less than one-half the daily dose used by other investigators for 4 or 5 consecutive daily injections in order to induce cleft palate in mice.

The purpose of our use of a small dose of cortisone acetate was to avoid inducing cleft palate in the offspring so we could study the subtle long term effects of cortisone acetate on the offspring mice. From the results, no cleft palates were seen in the CBA, C3H, C57Bl and BALB/c mice, and in the A/j mice the incidence of cleft palate in the treated young was not different from the natural incidence of cleft palate in the control offspring. Therefore, our expectation of observing no cortisone induced cleft palate was confirmed. The incidence of naturally occuring cleft palate and cleft lip in A/j mice is 7.3% of young (Green, 1968).

The embryocidal effects of 5 mg cortisone acetate when administered to a 12 day pregnant mouse have been reported (Dostral and Jelinek, 1973).

In our present study, using a much smaller dose of cortisone acetate, little or no incidence of embryocidal death was observed. In the preliminary investigation, there were 2 and 1 abortions in C57Bl and CBA mice, respectively (Table 1), while in C3H and BALB/c mice, there were no embryocidal effects.

The neonatal treatment of 0.075 mg of cortisone acetate was given to the new born mice on the day they were born. This dose was one-tenth of the prenatal dose. Runting syndrome has been produced in new born C3H mice by a single injection of 0.25 mg of cortisone acetate (Schlesinger and Mark, 1964).

In the present experiment, we observed that the neonatal treatment of 0.075 mg had little or no effect on C3H mice (Table 4 and 7). An additional group of C3H mice were treated neonatally with 0.15 mg of cortisone which then caused the runting syndrome in C3H mice. Similar effects were elicited in A/j mice with the lower dose of 0.075 mg (Table 3 and 6).

Thus the A/j mice were more sensitive to the neonatally administered cortisone acetate than the C3H mice, in contrast to the prenatally administered cortisone acetate.

In the present experiment, strain differences in sensitivity to cortisone acetate were found to be less than the cleft palate inducing doses, dependant upon the time of its administration as follows:

Prenatally C3H < CBA = A/j mice

Neonatally (0.075 mg) A/j < C3H mice.

3. Foster Nursing of Young and Growth Retardation

The transplacental treatment of the 12 day old fetal mouse invariably involves the treatment of the pregnant mouse. In order to separate the maternal influence from the fetal effects of the treatment, foster nursing of the treated young and non-treated young were undertaken (Tables 2, 3, 5, 6, 8, 9, Figures 2 and 3).

In order to evaluate the effects of foster nursing, the data between Group 1 (Control young nursed by Control own Mother) and Group 2 (Control young nursed by Control foster Mother) and between Groups 5 (Treated young nursed by Treated foster Mother) and Group 6 (Treated young nursed by Treated own Mother) were compared. Concerning the Average Litter Size (Tables 2 and 3), Group Average Percentage Survival Rates of Young per Litter (Tables 5 and 6); Group Average Body Weight Gain per Young (Table 9 and 10) and Group Average Percentage Body Weight Gain per Young (Figure 2 and 3), fostering nursing of Young did not cause any significant effect on the young.

When the Group Average Body Weight Gain per Young between Birth and 5 Days of Age in CBA mice (Table 9) were compared, significant differences were observed. The significant difference between Groups 3 and 1 (Control young nursed by Treated foster Mother and Control young nursed by Control own Mother) appears to be due to the detrimental effect of the treatment on the maternal side. The significant difference observed between Groups 4 and 1 (Treated young nursed by Control foster mother and Control young nursed by Control own mother) seems to be due to the detrimental effect of the treatment on the fetuses. The significant differences between Groups 5 and 1 (Treated young nursed by Treated foster mother and Control young nursed by Control own mother); 5 and 2 (Treated young nursed by Treated foster mother and Control young nursed by Control foster mother); and 6 and 1 (Treated young nursed by Treated own mother and Control young nursed by Control own mother), could be due to the detrimental effects of the treatment on both the mother mouse

and the fetuses. This data seems to indicate that the subtle detrimental effect of the treatment may have an influence upon both the mother mouse and fetuses.

Cortisone acetate given to a 12 day pregnant mouse probably elevates the cortisone levels in the maternal circulation. Cortisone, not bound to larger proteins, crosses the placental membranes and enters the fetal circulation. Elevated levels of cortisone would inhibit the release of ACTH from the fetal pituitary, which in turn would decrease the release of corticosterone from the fetal adrenal gland.

The gluconeogeneic action of the glucocorticoids stimulate the breakdown of protein and fat in the peripheral regions of the body, to elevate blood glucose levels (Liddle, 1974).

The cortisone given to the pregnant mice might have a detrimental effect, postpartum, on the neonates.

When the cortisone treatment, 2.5 mg daily, was given to the nursing mother mouse on Day 4 - 6 postpartum, all young were reported to die within 3 - 5 days after the treatment (Glaubach *et al.*, 1951).

A single injection of 0.1 or 0.25 mg of cortisone acetate into 1 day old C3H mice resulted in a wasting syndrome strikingly similar to that developed in runt disease and in the post-thymectomy syndrome, the mortality rates of young and mean survival days after injections being 7/10, 12.4 $\pm$ 0.4 days and 19/19, 10.6 $\pm$ 0.7 days, respectively (Schlesinger and Mark, 1964).

4. Long Term Subtle Effects of Treatment on Offspring

In CBA and C3H mice (Tables 11 and 12) the treated mice received

the prenatal (0.75 mg) treatment during fetal life. When they matured these animals had young. Although there are no significant differences in the performance of the young (Average Litter Size, Average Body Weight and Average Body Weight Gain) between the Treated group and Control Group, the Control Group appears to be at an advantage over the Treated Group. It may be possible that these data do not reflect the true picture, since of the treated animals, only the least affected young of the Treated Group survived until maturation.

5. Transplantability of Neonatal Mammary Gland

The age of the mammary gland appears to have little effect on the transplantability of mammary gland. When mammary grafts from 3 to 4 weeks old CBA female donors were transplanted 62% survived, while mammary grafts from a 734 day old senile virgin CBA female donor, when transplanted, 57% survived (Hoshino, 1964). Adult C3H donor female mouse mammary grafts had a recovery rate of 58% (DeOme *et al.*, 1959). Even after serially transplanting mammary grafts through 6 generations, over 1414 days (Nearly 4 years) the recovery rate of the grafts was over 70% (Hoshino, 1970). In our study the recovery rates of mammary grafts from neonates, prenatally and neonatally treated with cortisone acetate, were for C3H and A/j mice, 62% and 27.3%; and 68.8% and 50% respectively, which were comparable to the transplantability of the mammary grafts from the control neonates, 67% and 43% of C3H and A/j mice respectively (Table 13).

The transplantability of the neonatal mammary glands is not different from that of adult mammary glands.

The functional state of the mammary grafts from the neonates (with or without pretreatment) were similar to that exhibited by mammary gland of the adult host mice. A mammary gland graft recovered from a 12 day pregnant host and that from a Day 1 postpartum host were in the functional state of mid-pregnancy and early lactation, respectively. The former was recovered only 19 days after transplantation (Figure 5b) and the latter was recovered 30 days after transplantation (Figure 7b).

These mammary grafts appear to be the youngest glands which exhibited the capability of differentiation into the prelactation and milk secretion stages in an *in vivo* system, responding to the endogenous gestational hormonal stimuli.

In *in vitro* studies fetal mammary explants (Ceriani, 1970) and three week old mammary explants (Voytovich and Topper, 1967) demonstrated the capability of casein synthesis following exogenous hormonal stimuli.

VII CONCLUSIONS

VII CONCLUSIONS

In the present study, the possible teratogenic and long term effects of cortisone acetate at low dose levels, comparable to the human clinical doses, were investigated in mice. The A/j, C3H, CBA, C57BL and BALB/c strains of mice were used for the study of strain differences in sensitivity to cortisone.

The preliminary experiment showed that the prenatal treatment was not embryocidal and did not induce cleft palate, however there was considerable infantile death.

The second part of the work, which examined the effects of the treatments on the survival and growth of the young, showed a significant difference in the survival of the prenatally treated C3H mice, but not in the CBA or A/j mice. In contrast, the neonatal treatment of 0.075 mg showed a detrimental effect in the A/j mice, but not in the C3H mice (Table 8, Figure 1). The prenatal treatment also revealled a significant decrease in weigh gain between Birth and 5 Days of Age in CBA mice (Table 9). Foster nursing of young had little or no effect on the survival and growth of the young mice.

The third part of the work, concerned with the long term effects of the prenatal treatment upon the reproductive ability of the mice, showed an apparent detrimental effect which was not statistically different from the control (Table 11 and 12).

From the fourth part of this work, the effect of cortisone treatment either prenatally or neonatally, on the transplantability of neonatal mammary gland was not significantly different from the control. However, in both C3H and A/j mice the prenatally treated grafts had the lowest recovery rates (Table 13). Cortisone acetate treatment apparently

had no effect on the functional development of the neonatal mammary glands.

The youngest mammary graft from the neonates which reached a mid-pregnant prelactational stage was recovered 19 days after transplantation; and that which demonstrated a functional stage comparable to Day 1 post-partum lactation, was recovered 30 days after isografting from the neonate (Figures 5b and 7b, respectively).

The present study revealled that such a low dose of cortisone acetate still exerted a subtle effect on the growth and survival of young mice. Further exploration of the teratogenesis of the prenatal treatment as well as the detrimental effect of the neonatal treatment of cortisone acetate is warranted in the light of the clinical implications.

VIII APPENDIX

VIII APPENDIX

I Surgical Procedure for the Preparation of a Fourth Mammary Gland-Free Fat Pad (DeOme and Faulkin, 1959; Hoshino, 1962)

Under Nembutal anesthesia, the hair was removed from the abdominal wall of the mouse.

The ventral skin of the mouse was incised along the mid-sagittal line and transversely at the suprapubic level in the shape of an inverted T. The skin flaps, including the mammary tissues, were reflected laterally. Using a cautery, the fourth mammary gland ventral to the ventral inguinal lymph node and all of the fifth mammary gland were removed. The blood supply to the fourth mammary gland-free fat pad remained intact.

The skin flaps were sutured back together with surgeon's silk thread.

This procedure was done only on mice of 21 days of age or younger.

II Procedure for Anesthetizing Mice

Veterinary Nembutal (60 mg/ml) was diluted 1 part to 12 with distilled water. The weight of the mouse was multiplied by a factor of 0.15 to give the number of cc of the diluted solution to be injected. The injection was given intraperitoneally using a 26 gauge hypodermic needle.

III Surgical procedure for the Transplantation of Neonatal Mammary Gland

Donors of the mammary glands were one day old female mice. Donors were killed by decapitation and pieces of skin and subcutaneous tissue surrounding the nipples of the first, second, third and fourth mammary gland were removed and placed in a petri dish moistened with tissue culture medium. Under a dissecting microscope the mammary gland anlagen was removed from the skin and transplanted into a host within 15 minutes.

Host mice were given Nembutal anesthesia and the hair over the lumbar region removed.

The dorsal skin of the mouse was incised along the mid-sagittal line in the lumbar region. By sliding the skin laterally the dorsal portions of the fourth mammary gland-free fat pad on each side can be alternatively exposed.

Using fine point forceps, a hole was made in the fourth mammary gland-free fat pad and then a mammary gland anlagen was inserted through the hole. Both sides received anlagen with the same treatment.

The skin was stapled back together with wound clips.

IV Procedure for Histological Preparation of Mammary Gland

(a) Paraffin embedding and sectioning at 4 μm

Samples of mammary gland were removed, found the skins previously fixed in Bouin's Fixative. Tissues were dehydrated and embedded

in paraffin by using 70%; 80%; 95% 100% ethanol (x2), 50/50 100% ethanol/xylol (x2); paraffin (x2), at 4 µm and Sorvall JB-4 microtome. Sections were dehydrated and coverslipped.

(b) Plastic embedding and sectioning at 2 µm, of 12 day pregnant mammary gland.

Mammary gland was removed from the mouse immediately after its death. The tissue was fixed in 10% neutral Buffered Formalin for a minimum of 24 hours. Dehydration was done using 70%; 95%; 100% ethanol (x2); 100% methanol, each for 10 minutes. Tissues were infiltrated with Solution A, for 2 - 3 hours in a vacuum. Embedding was in Solution A + B (25:1), which was allowed to polymerize at room temperature for several hours. Solutions A and B were from the JB-4 embedding kit (Polysciences, Inc.). Staining was done as follows:

Modified Harris Hematoxylin	10 min.
Tap Water	3 - 5 dips
Lithium Carbonate (Saturated)	3 - 5 dips
Tap Water	3 - 5 dips
70% Ethanol	10 dips
95%	10 dips
Alcoholic Eosin (95% Alcohol)	8 - 10 min.
100% Alcohol	1 min.
Xylol	2 min.
Xylol	2 min.

and finally coverslip.

V Procedure for Whole Mount Preparation of Mammary Gland

Skins of the mice were fixed in Bouin's solution for 24 hours, and then rinsed in water for 30 minutes and stored in 70% alcohol. The mammary glands were removed from the skins for processing, and also stored in 70% alcohol. Before processing, the tissues were placed in 50% alcohol for 30 minutes. The tissues were stained for 10 to 15 minutes in aluminum carmine preheated to 60 C. The tissues were rinsed in 1% acid alcohol. Under a dissecting microscope the connective tissue surrounding the mammary gland was removed. The tissue was dehydrated with alcohols and finally xylol, then placed on a glass slide and coverslipped.

Alum Carmine Staining

Mix 10 gm of carmine and 100 cc of 100% alcohol, and then add 50 cc of a 2% $CaCl_2$ solution (aq.). Add 90 cc of a saturated ammonium aluminum (aq.) solution, while shaking. Boil the mixture for one minute, filter, and allow to cool to room temperature. Add 50 cc of glacial acetic acid, and store at room temperature.

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