Effect of adrenalectomy, pup removal, hypophysectomy and hydrocortisone acetate on mammary gland nucleic acid content in the near term pregnant rat

by

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INTRODUCTION

During pregnancy, mammary gland development may be divided into two phases (mammogenesis and lactogenesis). The proliferation of the lobuloalveolar system which occurs during most of pregnancy is defined as mammogenesis. The increase in cell number near term and the differentiation of these cells into competent secretory daughter cells is defined as lactogenesis.

During early and mid-pregnancy, there is a steady increase in the number of parenchymal cells which occurs at the expense of the fat pad portion of the gland (Harkness and Harkness, 1956; Paape and Sinha, 1971). This increase in cell number has been correlated with the development of alveolar structure (Dilley, 1971). Since the DNA content of mammary parenchymal cell nuclei remains constant (Tucker and Reece, 1962), cell number can be properly estimated by the DNA content of the gland (Griffith and Turner, 1957; Munford, 1963; Nagasawa et al., 1967).

Near term, mammary gland DNA continues to increase and represents an increase in the number of parenchymal cells per alveolus (Munford, 1963). Dramatic ultrastructural and biochemical changes accompany this increase in cell number (Wellings, 1969). These changes represent a transition from the non-secretory to the secretory state, and are associated with the initiation of milk secretion. An increase in RNA is characteristic of this phase of mammary gland development. This is associated with an hypertrophy of the rough endoplasmic reticulum (Banerjee and Banerjee, 1971) and can be used to evaluate the development

of secretory capability.

Studies on the hormonal regulation of mammary gland development in vivo have been reviewed by several authors in recent years (Cowie, 1969; Turner, 1970; Denamur, 1971; Kuhn, 1971; Ceriani, 1974; Meites, 1974). Conclusions based on in vitro studies have also been reviewed (Turkington, 1972; Turkington et al., 1973; Topper, 1970, Topper and Green, 1971; Porter, 1974).

The role of estrogen and progesterone in stimulating extensive lobuloalveolar growth during early and mid-pregnancy has been clearly defined in experimental mammogenesis (Moon et al., 1959). However, the hormonal events which signal the time at which lactogenesis should occur are less clearly understood. Kuhn (1971) has defined two basic concepts which permeate studies on lactogenesis. One is that lactogenesis is follows the withdrawal of the ovarian or placental steroids which promote growth, but restrain the onset of lactation. The other is that lactogenesis is the response to a positive stimulus in the form of increased secretion of pituitary hormones. The work of Liggins et al. (1973) on the mechanism of the initiation of parturition in the ewe has added a third perspective. There is sufficient evidence in the sheep (Drost and Holm, 1968; Basset and Thorburn, 1969; Liggins, 1973) and goat (Currie et al., 1973) to implicate fetal adrenal activity near term as the event which precipitates the multiple hormonal changes leading to parturition. Increased fetal adrenal activity near term has also been described in humans (Smith and Shearman, 1972), dogs (Jackson et al., 1973), pigs (Dvorak, 1972), guinea pigs (Jones, 1974) and rats

(Cohen, 1973; Milkovic et al., 1973). The signal which directs and coordinates the multifactorial changes leading to parturition may also lead to lactogenesis. Glucocorticoids have been implicated in the initiation of milk secretion both *in vivo* (Talwalker et al., 1961; Davis and Liu, 1969; Ferreri and Griffith, 1969; Kumaresan et al., 1967) and *in vitro* (Banerjee et al., 1971). One of the aims of this investigation is to examine the possibility that glucocorticoids of fetal origin may be involved in the initiation of milk secretion. The role of maternal adrenal activity near term is also considered, because a reciprocal relationship between the maternal and fetal hypophyseal-adrenal axes has been described in the rat (Jones et al., 1953; Christianson and Chester Jones, 1957).

In order to evaluate fetal and maternal adrenal involvement in mammary gland development near term, the effect of hypophysectomy, adrenalectomy, pup removal and treatment with hydrocortisone acetate on mammary gland DNA and RNA content was determined. The effect of pup removal on maternal adrenal secretion rate and plasma corticosterone levels was also evaluated.

LITERATURE REVIEW

Stages in Mammary Gland Development

The first anlage of the mammary gland (the mammary crest) appears as a thickening of ectoderm on the ventral surface of the embryo. Proliferation and constriction of this ectoderm results in the formation of mammary buds. These buds "sink" in to the mesenchyme, but remain connected to the ectoderm by a cylindrical shaft of ectodermal cells. The buds elongate to form a primary sprout which will eventually become the milk duct. Secondary sprouts form and are the anlagen of the duct system and glandular tissue (Raynaud, 1971).

After birth and until day 22, the rat mammary gland increases in area at the same rate as body surface area (Cowie, 1949). From the time of weaning until the onset of puberty, DNA increases and mammary gland area increases at a rate 3.5 times greater than body surface area (Sinha and Tucker, 1966). Pubertal growth of the mammary gland represents an increase in the rudimentary duct system (Nagasawa et al., 1967) and an increase in adipose and connective tissue portions of the gland (Paape and Sinha, 1971).

During pregnancy, there is a steady increase in the volume of glandular tissue (Munford 1963) and the DNA content of the gland (Griffith and Turner, 1961, Tucker and Reece, 1963). Growth of the mammary gland during pregnancy, unlike pubertal growth, is reflected only in the parenchymal portion of the gland and occurs at the expense of the fat pad portion (Harkness and Harkness, 1956; Paape and Sinha, 1971).

It is upon the fully developed lobuloalveolar gland of mid- and late pregnancy that the essential hormones of lactogenesis are competent to act. Near term, the transition from non-lactating to lactating tissue occurs. There is a striking fall in the number of alveoli per unit area, a corresponding rise in alveolar diameter, and a two-fold increase in the number of nuclei in the average alveolar cross section (Munford, 1963). Accompanying the increase in cellularity, there are ultrastructural and biochemical changes in the mammary parenchymal cells.

The ultrastructural changes include (Wellings, 1969; Holmann and Verley, 1972):

1. An increase in mitochondrial volume;

2. a rapid increase in the extensiveness of the endoplasmic reticulum;

3. an hypertrophy and supranuclear localization of the Golgi apparatus;

4. an increase in the number of cytoplasmic droplets and granules, and a migration of these inclusions to the apical surface of the cell;

5. an increase in the number of microvilli.

The biochemical changes include (Denamur, 1969; Baldwin and Milligan, 1966; Kuhn, 1972):

1. An increase in the RNA content of the cell;

2. an increase in the activity of citrate cleavage enzyme, fatty acid synthetase, glycerol kinase, glycerol-P-dehydrogenase, aspartic and glutamic acid amino transferase, and UDPG pyrophosphorylase;

3. an increase in the synthesis of α- and β- casein, β-lactoglobulin,

fatty acids and lactose;

4. an increase in the transport of milk serum albumin, immunoglobulin, salts, glucose, amino acids and fatty acids from the plasma to the mammary parenchymal cell.

The Hormonal Control of Pregnancy and Parturition

Control of luteal function

Selye et al. (1935) described the essentiality of the ovary in the maintenance of pregnancy in the rat, and postulated that the placenta determined the length of gestation either by an effect on the corpus luteum or by its own progestin secretion. Subsequent investigations have elaborated upon the endocrine interrelationships which govern pregnancy, but the basic understanding has remained unchanged since the time of Selye.

In the estrous cycle of the rat (Schwartz, 1969), a new set of follicles begins growing on the day of estrus under the influence of FSH. Follicular growth continues during metestrus under stimulation of FSH and low levels of LH. Pituitary secretion of LH during diestrus stimulates the secretion of large amounts of estrogen. On the day of proestrus, the ovulatory surge of LH occurs. LH begins to act on the ovary to bring about pre-ovulatory swelling of the follicle, a decrease in estrogen secretion and an increase in progesterone secretion. Mating behavior is elicited and ovulation takes place after midnight of proestrus. The estrous cycle must be interrupted if pregnancy is

to continue. Spies et al. (1971) suggested that stimulation of the cervix during coitus causes the reflex release of prolactin which is luteotrophic in the rat. This suggestion is based on the observation that severence of the pelvic nerve to the cervix results in failure of the blastocyst to implant unless the animal is treated with prolactin. The presence of the conceptus in the uterus may inhibit the luteolytic action of the uterus, as in the sheep (Anderson et al., 1969) and/or may induce uterine luteotrophic activity as does decidualization of the rat uterus (Gibori et al., 1974).

The role of prolactin during pregnancy has been described by many authors. Plasma prolactin levels are relatively higher during the first few days of pregnancy than they are during most of the estrous cycle (Bast and Melampy, 1972), and often show an erratic pattern of change during this period (Morishige et al., 1973). Prolactin has been repeatedly described as the luteotrophic hormone of early pregnancy and pseudopregnancy in the rat (Takayama and Greenwald, 1973). Administration of ergocornine free base (Morishige and Rothchild, 1974) or ergocryptine mesylate (Ford and Yoshinaga, 1975a) which suppress prolactin release from day 1 to day 8 of pregnancy results in a decline in circulating progesterone levels and uniformly induces abortion. After day 8, prolactin is no longer essential for luteal maintenance and treatment with ergocornine or ergocryptine does not result in abortion. Prior to complete control of luteal function by the placenta on day 11, hypophysectomy results in abortion of the fetuses (Pepe and Rothchild,

1972). It has been suggested that luteal maintenance from day 8 to day 11 is a function of LH secretion. Serum LH concentrations during the first eleven days of pregnancy is distinctly higher than concentrations between days 13 and 19 (Morishige et al., 1973). Administration of LH antiserum from day 8 to day 11 results in abortion, but it does not induce abortion prior to day 8 or after day 11 (Ford and Yoshinaga, 1975b; Morishige and Rothchild, 1974).

Placental control of luteal function after day 12 may be attributed to the prolactin-like properties of rat chorionic mammotrophin (Ray et al., 1955), and is evidenced by failure of hypophysectomy after day 12 to induce abortion (Selye et al., 1935). The role of rat chorionic mammotrophin (RCM) in pregnancy and mammary gland development has recently been reviewed by Matthies (1974). In situ production of a progestin by the rat placenta has also been described (Chan and Leathem, 1975). This may account for failure of ovariectomy after day 18 to induce abortion, but does not offer sufficient progestational support prior to day 18.

Changes in ovarian secretion near term

The progestational potency of ovarian secretions during pregnancy is reflected in the ratio of progesterone to its inactive metabolite, 20α hydroxy pregn-4en-3one (Fajer and Barraclough, 1967) and in changes in estrogen secretion (Yoshinaga et al., 1969). Progesterone levels rise to a peak during early pregnancy while 20α hydroxy pregn-4en-3one (20αOHP) levels decrease. At mid-gestation, there is a second rise in

plasma progesterone accompanied by an increase in 20αOHP. Between days 19 and 21, progesterone levels drop and there is an increase in peripheral levels of 20αOHP (Wiest et al., 1968).

An increase in LH levels near term has been correlated with an increase in the activity of 20α hydroxy steroid dehydrogenase (Bast and Melampy, 1972), and changes in serum progesterone levels are inversely related to those in serum LH from day 11 to term (Morishige et al., 1973). Hypophysectomy after day 12 delays the increase in the activity of 20α hydroxy steriod dehydrogenase (20αOHSDH) and treatment with LH restores enzyme activity to normal (Wiest et al., 1968). Hypophysectomy also prevents the rapid fall in progesterone which normally precedes parturition (Pepe and Rothchild, 1972). Hence, an increase in LH near term may direct progesterone catabolism to 20αOHP in the ovary as a mechanism for the prompt withdrawal of progesterone.

Except for the pre-implantation surge in 17β estradiol, there is no significant change in estradiol levels until day 18 of pregnancy. From day 18 through term, there is a steady increase in estradiol levels (Yoshinaga et al., 1969). It has been suggested (Waynforth and Robertson, 1972) that the increase in estradiol is also controlled by pituitary gonadotrophins and represents the advent of a post-partum ovulation. Hypophysectomy abolishes the increase in the estradiol content of ovarian venous blood and the extraluteal component of the ovary between days 16 and 21. The increase in estradiol coincides with an increase in $PGF_{2\alpha}$ (Labhsetwar and Watson, 1974). $PGF_{2\alpha}$ has been

found to cause degeneration of the corpora lutea of pregnancy and induces the formation of a fresh set of corpora lutea (Labhsetwar, 1972).

Fetal adrenal activity

The role of fetal adrenal activity in the precipitation of the endocrine events which precede parturition has been most comprehensively studied in the sheep, and has been recently reviewed by Liggins et al. (1973). The observation that gestation could be prolonged in ewes after fetal adrenalectomy (Drost and Holm, 1968) has directed attention to fetal physiology and its possible control of parturition.

The pituitary-adrenal system of the fetal lamb becomes active in late gestation. The increase in ACTH, which is noted after 140 days gestational age (Alexander et al., 1971), has been attributed to hypothalamic maturation (Liggins et al., 1973) or fetal asphyxia (Dawes, 1973). Before 130 days gestational age, fetal plasma levels of corticosteroids are low (Basset and Thorburn, 1969) and then increase markedly near term (Drost et al., 1973). The increase in secretory activity of the adrenal cortex coincides with morphological changes in the adrenal gland near term (Alexander et al., 1968). It has been suggested that ACTH is responsible for an increase in the activity of an 11β hydroxylase enzyme, which, in turn, enhances cortisol synthesis (Anderson et al., 1973).

The rise in fetal adrenal corticosteroids precedes all the other hormonal changes seen in the maternal plasma just prior to parturition.

It may be responsible for the decrease in progesterone and the increase in estrogen which is accompanied by an increase in $PG_{F2\alpha}$ (Currie et al., 1973). Infusion of ACTH to fetal lambs at gestational ages of 88 to 129 days (Liggins, 1968) or dexamethasone (Liggins, 1969) results in premature parturition. Liggins (1973) has proposed the following model to describe the initiation of parturition in the sheep. Fetal cortisol, by some unknown means, stimulates release of unconjugated estrogens from the placenta and causes a fall in the secretion of progesterone. The effect of estrogen in the presence of progesterone is to stimulate the potential for synthesis of $PGF_{2\alpha}$ in the maternal placenta. The local action of progesterone is to block the release of $PGF_{2\alpha}$. The net effect of changes in the estrogen to progesterone ratio preceding parturition is the release of large amounts of $PGF_{2\alpha}$ which reach the myometrium by an unknown, but direct route. $PGF_{2\alpha}$ may have a direct oxytocic effect and may increase myometrial sensitivity to oxytocin.

There are striking similarities between the changes in the hormonal status of the dam which precedes parturition in the rat and those which precede parturition in the sheep. However, in the rat, the control of estrogen and progestin secretion must be exerted on the ovary and not the placenta. Uterine vein $PGF_{2\alpha}$ does increase near term (Labhsetwar and Watson, 1974). This increase coincides with a decrease in progesterone and an increase in 17β estradiol, and has been shown to be luteolytic in the rat (Labhsetwar, 1972). The advent of fetal adrenal activity near term has been described in the rat, but its role is not as clearly defined as it is in the sheep.

The adrenal primordium of the rat is recognizable on the 13th day of intrauterine life, and is a discrete organ by the 15th day (Bloch, 1968). It increases in weight from day 16 to day 20 and reaches a peak of activity between days 18 and 20 (Jost, 1966). Fetal plasma corticosterone levels are highest between days 19 and 20 (Cohen, 1973). Fetal adrenals are 6 times more sensitive to ACTH stimulation than are maternal adrenals (Milkovic and Milkovic, 1962). Stimulation of maternal adrenals by cold stress or ACTH results in a decrease in fetal adrenal activity indicating that corticosteroids may pass the placental barrier, but that ACTH may not (Jones et al., 1953). The reactiveness of the fetal adrenal cortex has been shown to be dependent upon fetal adrenocorticotrophic activity. Since high maternal plasma levels of corticosterone, stimulated by ACTH tumor implants, are paralleled by markedly lighter fetal adrenal weights after the 18th day but not on the 17th day, fetal adrenocorticotrophic activity is said to begin between the 17th and 18th day of intrauterine life (Milkovic et al., 1973). The responsiveness of the fetal pituitary adrenal axis to changes in glucocorticoid levels in the dam has been demonstrated by a number of authors. Adrenalectomy of the dam results in an increase in fetal adrenal weight (Knobil and Briggs, 1955) and the survival of pregnant adrenalectomized rats is longer than survival of non-pregnant adrenalectomized rats (Davis and Plotz, 1954). Treatment of adrenalectomized dams with cortisone acetate (Davis and Plotz, 1954) prevents fetal adrenal hypertrophy. The enlargement of

fetal adrenals after maternal adrenalectomy is due to increased activity of the fetal hypophysis since it does not occur when the fetuses are decapitated (Christianson and Chester Jones, 1957). It is not dependent upon the maternal hypophysis since hypophysectomy of adrenalectomized dams does not prevent fetal adrenal hypertrophy (Christianson and Chester Jones, 1957).

Hormonal Control of Lactogenesis

In vivo studies

There is substantial evidence to implicate the hormonal changes which occur prior to parturition as being essential for lactogenesis.

Ovariectomy after mid-pregnancy, which represents the experimental withdrawal of progesterone, results in a marked increase in protein phosphorus in the mammary gland (Kuhn, 1972), an increase in total RNA, an increase in the RNA to DNA ratio, and an increase in the heavy polysome fraction (Davis et al., 1972). The increase in each of these indicators of lactogenesis is prevented by treatment of the ovariectomized animal with progesterone. In the intact pregnant rat, treatment with estradiol benzoate and progesterone near term also inhibits the normal increase in RNA (Stefaniw, 1971). Although a combination of these steroids inhibits the lactogenic response, extensive lobuloalveolar growth continues (Meites and Sgouris, 1953), and the DNA content of the gland is not affected (Stefaniw, 1971).

Serum prolactin levels rise near term (Bast and Melampy, 1972;

Nagasawa and Yanai, 1972). The increase in prolactin is correlated with the pre-partum increase in estrogen and decrease in progesterone (Denamur, 1971). Furthermore, intrapituitary implants of estrogen stimulate prolactin release and the initiation of milk secretion (Bruce and Ramirez, 1970).

An increase in prolactin is not sufficient for lobuloalveolar differentiation unless it is accompanied by adrenal cortical activity (Ben-David et al., 1971). Similarly, experimental withdrawal of progesterone, by ovariectomy after mid-pregnancy does not result in lactogenesis in the adrenalectomized rat (Davis and Liu, 1969). The effect of glucocorticoids, in turn, is a function of the endocrine status of the dam (Ahren and Jacobson, 1957). During late pregnancy, treatment with hydrocortisone acetate results in an increase in RNA, indicative of lactogenesis (Ferreri and Griffith, 1969), and is capable of inducing mammary secretions (Talwalker et al., 1961). During early and mid-pregnancy, treatment with hydrocortisone acetate does not result in this increase.

The hormonal interrelationships which may affect lactogenesis are represented schematically in Figure 1 and Figure 2 and can be summarized as follows (Meites, 1954; Meites, 1974; Kuhn, 1971):

1. The high ratio of progesterone to estrogen which prevails during early and mid-pregnancy stimulates intense lobuloalveolar growth and thereby renders the mammary gland refractory to lactogenic stimulation.

2. Prolactin and/or adrenal steroids are produced in inadequate amounts during most of pregnancy.

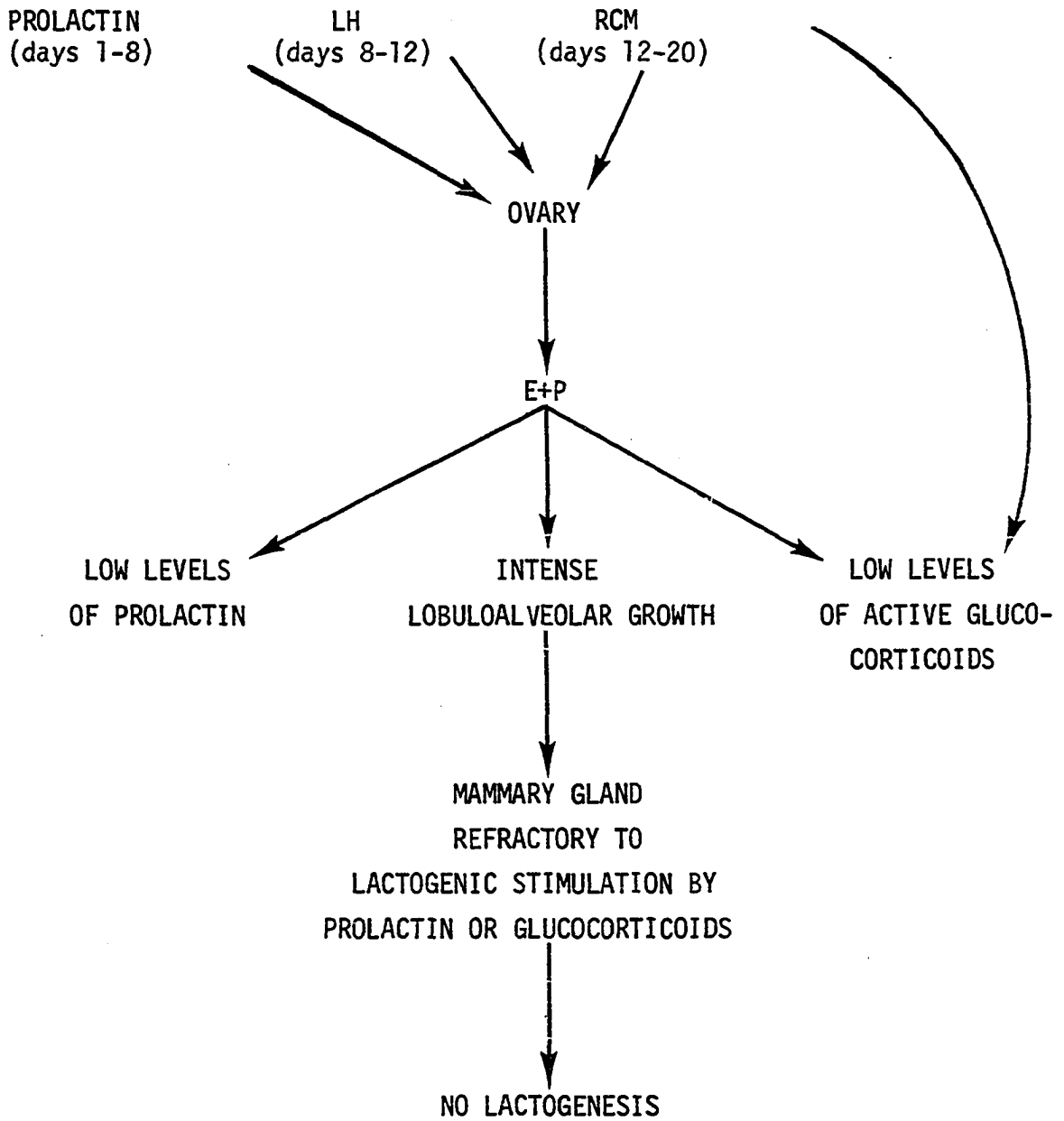

Fig. 1. Hormonal factors inhibiting lactogenesis during early and mid-pregnancy.

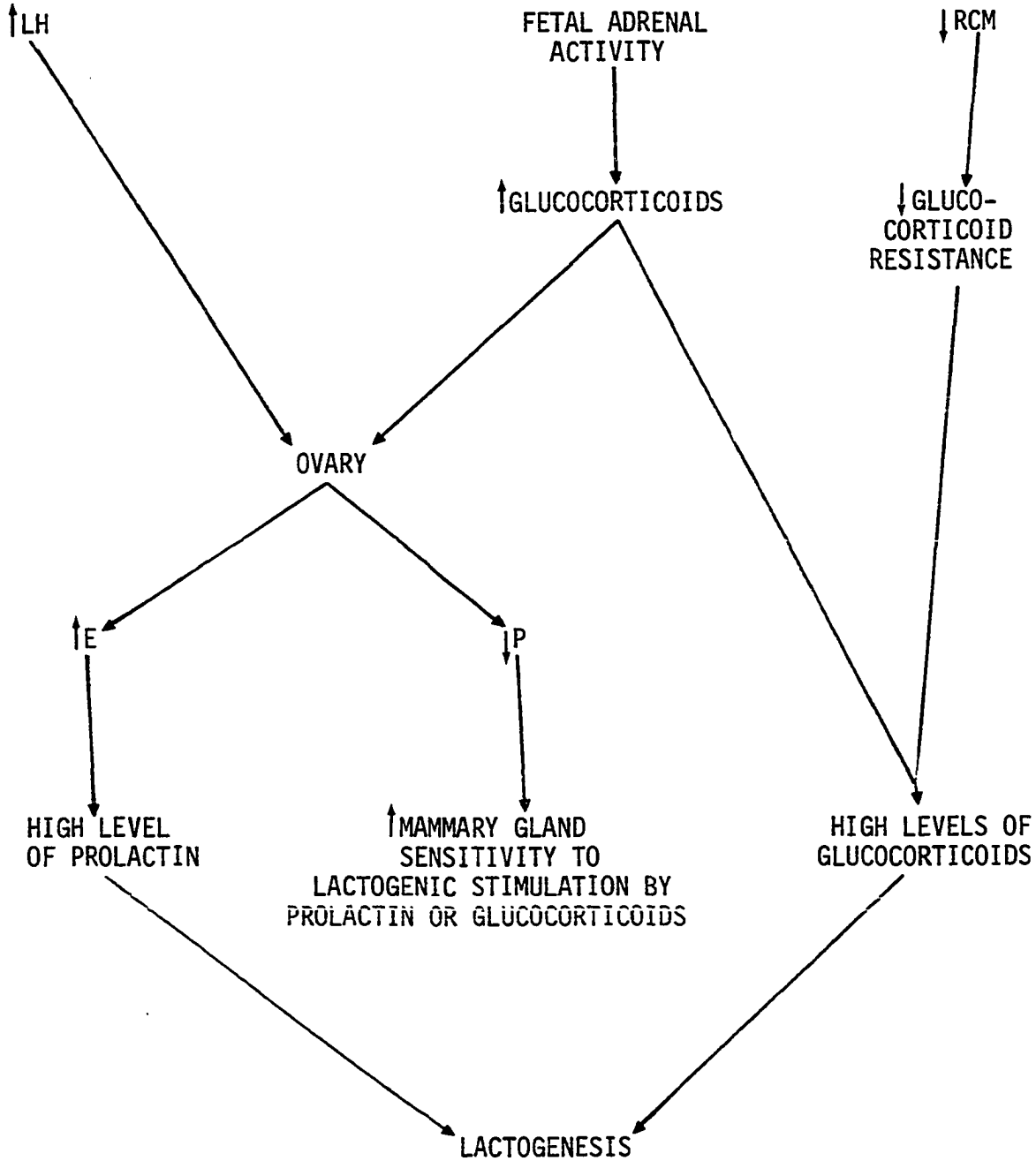

Fig. 2. Hormonal factors precipitating lactogenesis near term

3. The fall in progesterone near term and the increase in estrogen renders the gland more sensitive to lactogenic stimulation.

4. The secretion of prolactin increases near term due to direct stimulation by estrogen and the withdrawal of progesterone inhibition.

5. There is an increase in the level of biologically active glucocorticoids.

6. Once the mammary gland has developed sufficient alveoli, it responds to stimulation by prolactin and an adrenal cortical steroid.

7. Lactogenesis results from increased secretion of prolactin and adrenal steroids, which are themselves liberated and allowed to act by the withdrawal of inhibitory steroids.

In vitro studies

A proposed mechanism of hormone action in lactogenesis has been described on the basis of biochemical and ultrastructural changes which occur in pregnant mouse mammary gland explants cultured in hormonally defined media. Many of the investigations in this area of research have been reviewed by Turkington et al. (1973) and Topper (1970).

Mills and Topper (1970) have reported the following ultrastructural observations. Explants cultured in insulin and hydrocortisone (IF medium) for 96 hours contain more epithelial cells and less adipose tissue than zero time samples taken from freshly killed fifteen day pregnant mice. There is an increase in the number of alveoli within the explant as well as a two-fold increase in the average number of cells per alveolar cross section. The lumina of the alveoli are wider

and there is a slight increase in the amount of rough endoplasmic reticulum (RER) and the Golgi apparatus (G). There is no change in the polarity of cellular organelles. Explants placed in a second incubation medium containing insulin plus hydrocortisone plus prolactin (IFP medium) for up to 96 hours contain less adipose than explants after the first incubation. The alveoli are still larger and contain even more cells per alveolar cross sectional area. The alveoli lumina are greatly distended and filled with lipid and protein. The cells are larger and show a polarity of cellular organelles. The bulk of the RER is located in the basal portion of the cell along with the nucleus. The Golgi is supra-nuclear and filled with secretory proteins. From these observations, Topper and Green (1971) have suggested the following sequence of events:

1. Insulin promotes the formation of a pair of daughter cells from each alveolar cell.

2. Hydrocortisone, in the presence of insulin, promotes the formation of an extensive RER system; but it has no effect on the number of daughter cells.

3. Insulin and prolactin stimulate synthesis of certain secretory proteins by the newly differentiated cells.

Turkington's group has monitored changes in several biochemical parameters upon incubation with insulin, hydrocortisone and prolactin. These studies provide evidence for the essentiality of cell division prior to functional differentiation, and describe a possible model for the mechanism of hormone action in effecting gene expression of a unique mammary gland function.

After 96 hours of incubation in IF medium, mouse mammary gland

cells do not produce casein unless prolactin is added. If mitosis during the IF incubation period is prevented by the addition of colchicine, prolactin does not stimulate casein synthesis (Turkington, 1968). Similarly, the prolactin induced increase in polyribosomes does not occur in a colchicine pre-treated incubation or in the absence of previous exposure to IF medium (Turkington and Riddle, 1970). An increase in prolactin stimulated phosphorylation of nuclear proteins occurs only when the tissue is pre-incubated with I+F (Turkington and Riddle, 1969).

Insulin stimulation of mitotic activity during the first incubation period is accompanied by increased phosphorylation of existing nuclear proteins, increased RNA polymerase activity, increased RNA synthesis, increased nuclear protein synthesis (Turkington and Riddle, 1969), and increased DNA synthesis (Turkington et al., 1971). Hence, in these studies, insulin-directed DNA synthesis and mitosis is a prerequisite for differentiated, prolactin stimulated, mammary gland activity.

The selective effect of prolactin on gene expression is evidenced by the fact that induction of casein and lactalbumin synthesis by prolactin is abolished by the incorporation of thymidine analogues during the pre-incubation period, whereas synthesis of proteins with a more general distribution in cells is not decreased. It has been suggested that the genomes for specific milk protein synthesis are thymidine rich and more seriously affected by the incorporation of thymidine analogues during replication (Turkington et al., 1971).

The mechanism by which specific genes are expressed may involve the selective phosphorylation of histones under prolactin stimulation, which is coupled with RNA and protein synthesis. Prolactin stimulates the phosphorylation of specific histone fractions, in contrast to insulin stimulation of histone phosphorylation in all fractions (Turkington and Riddle, 1969). With respect to phosphorylation of histones and activation of subsequent protein synthesis, neither cAMP nor adenyl cyclase activity has been found to be rate limiting. Instead, protein kinases and cAMP binding proteins seem to be rate limiting. Prolactin induces a rapid increase in protein kinase and cAMP binding activity (Majumder and Turkington, 1971) which precedes nuclear protein phosphorylation and casein synthesis. Figure 3 summarizes the results obtained from the *in vitro* studies cited herein, and describes a possible mechanism of hormone action in lactogenesis.

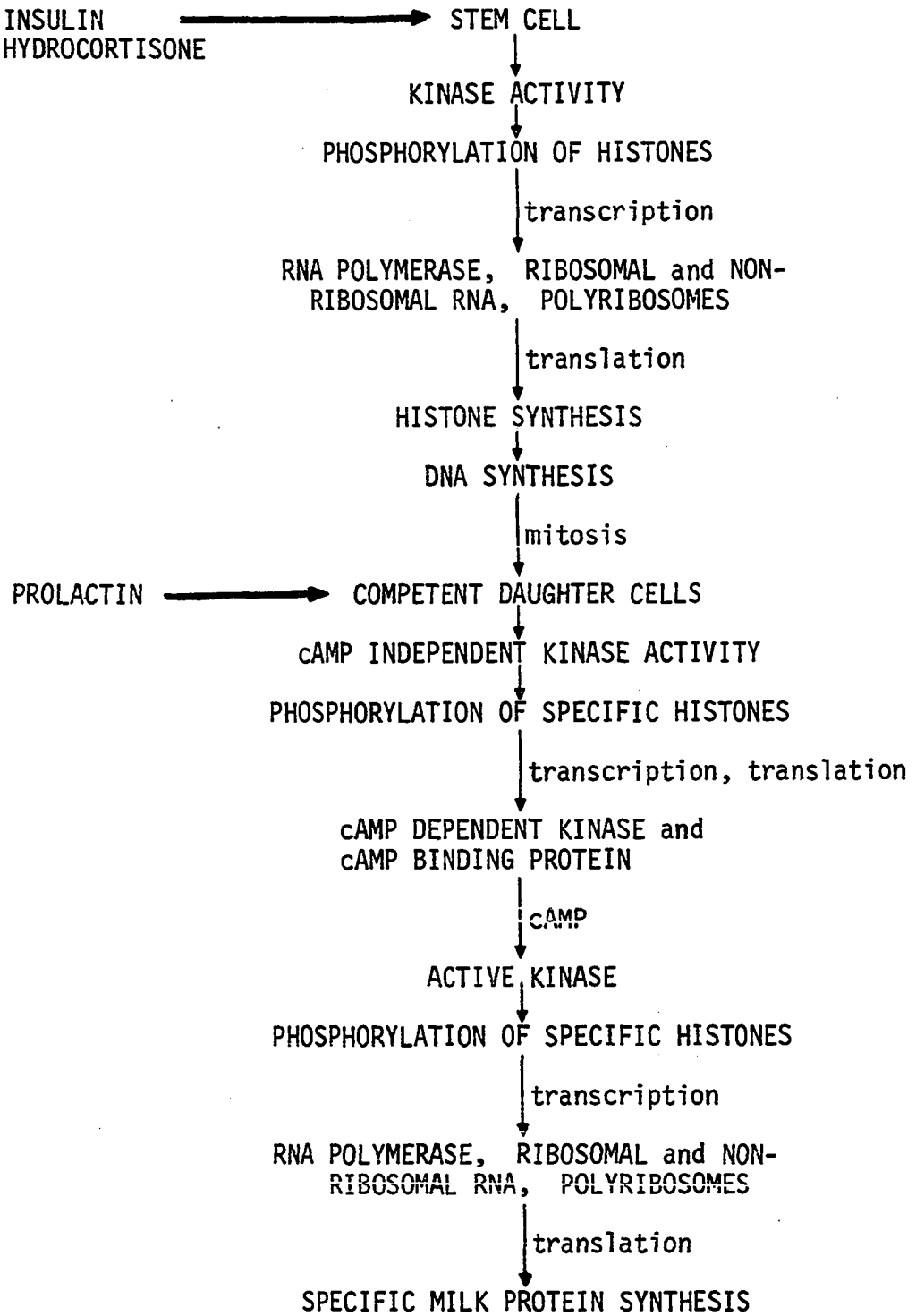

Fig. 3. A possible mechanism of hormone action in lactogenesis.

METHODS

Treatment of Animals

Six adult female rats and one mature male rat of the Sprague-Dawley strain were placed in large cages for breeding. The presence of sperm in a vaginal smear was considered day 1 of pregnancy. Pregnant animals were housed individually for the length of gestation, and all were given food and tap water ad libitum until day 18 of pregnancy. From day 18 to day 22, tap water was replaced by water containing 5% glucose and 1% NaCl for the hypophysectomized and adrenalectomized groups, respectively. Animals continued to be fed ad libitum.

On the 18th day, animals were either sacrificed or subjected to one of four types of surgical manipulation. Half of the animals within each of the surgical treatment groups were injected with 250 µg hydrocortisone acetate (F), subcutaneously, in a 0.1 ml saline suspension; the other half received no hormone therapy. Animals were sacrificed by decapitation on day 22, with the exception of the 18 day control group. Treatment groups and symbols used to identify them in the rest of the text are listed in Table 1.

Surgical Procedures

Adrenalectomy was performed under ether anaesthesia according to the method described by Zarrow et al. (1964). Two incisions were made on the dorso-lateral aspect of the animal, below the last rib. The supra-renal fat pad was grasped with a curved forceps and the adrenal gland

Table 1. Treatment groups

Group Symbol	Surgical Procedure	Hormone Treatment	Time of Sacrifice
A	Adrenalectomy on day 18	None	Day 22
AF	Adrenalectomy on day 18	250 µg F daily from days 18-21	Day 22
H	Hypophysectomy on day 18	None	Day 22
HF	Hypophysectomy on day 18	250 µg F daily from days 18-21	Day 22
P	Pup removal on day 18	None	Day 22
PF	Pup removal on day 18	250 µg F daily from days 18-21	Day 22
C	Sham operation on day 18	None	Day 22
CF	Sham operation on day 18	250 µg F daily from days 18-21	Day 22
E	None	None	Day 18

teased free. Upon sacrifice, the aorta and supra-renal fat were visually examined for residual adrenal tissue.

Hypophysectomy was performed by the intra-auricular method, using the Hoffman-Reiter Hypophysectomizer stereotaxic device. The ear canal was swabbed with 70% ethanol. The pinna was snipped above and below the ear canal in order to facilitate positioning of the ear bars. An 18 gauge, thin walled, wide beveled needle attached to a 10 cc syringe was inserted through the right ear canal and guided to a position above the sella turcica. The pituitary was removed by aspiration. Upon sacrifice, the sella turcica was examined for the presence of residual tissue. Reduced adrenal weights were also used as an indication of the effectiveness of hypophysectomy.

Pups were removed according to the method described by Pritchard and Huggett (1947). The uterus was exposed by laparotomy on day 18. A small incision was made on the mesometrial part of the uterus. The fluid-filled membranes were perforated and the fetuses were allowed to escape through the uterine incision. It was not necessary to suture the uterus.

Mammary Gland Nucleic Acid Analysis

Preparation of dried fat free tissue (DFFT)

The six abdominal-inguinal glands were excised; defatted in chloroform and methanol (2:1, v/v) for two twelve-hour periods, and in ether for two subsequent twelve-hour periods. The jars contained approximately

50 ml solvent and were kept under constant agitation. After the final solvent was decanted, the tissue was allowed to air dry.

Nucleic acid extraction

The method used for nucleic acid extraction was that described by Schneider (1945) and modified by Ferreri (1971).

1. Twenty-five mg of dry fat-free tissue suspended in 5 ml of 5% trichloro-acetic acid (TCA) in thick walled 15 ml volumetric centrifuge tubes was allowed to stand at room temperature until the DFFT became saturated and "fell" to the bottom of the tube with gentle tapping.

2. Samples were covered with glass marbles and placed in a hot water bath (90-95^{o}C) for 15 minutes and then transferred to a cold water bath.

3. Samples were centrifuged for 30 minutes, and the supernatant was poured into 12 ml volumetric centrifuge tubes.

4. A second extraction in hot TCA was made on the centrifugate, and the supernatant was added to that recovered from the first extraction.

5. The total volume was brought to 10 ml by the addition of cold 5% TCA, transferred to glass storage tubes, and stored at 4^{o}C.

Colorimetric determination of DNA content

The method used was that described by Burton (1956) and modified by Ferreri (1971).

1. Diphenylamine reagent was prepared immediately before use. It consisted of 1.5 g of reagent grade diphenylamine in 100 ml of A.R.

acetic acid and 1.5 ml of concentrated sulfuric acid. Five-tenths ml of acetaldehyde (16 mg/ml) was added to the reagent mixture prior to its addition to the samples.

2. One ml of nucleic acid extract or DNA standard was combined with 4 ml of diphenylamine reagent and 1 ml of 1N perchloric acid (9 ml of 70% perchloric acid in 91 ml of distilled water).

3. A blank was prepared with 1 ml of 5% TCA, 4 ml of diphenylamine reagent, and 1 ml of 1N perchloric acid.

4. Color was developed after 16 to 20 hours incubation at room temperature, and absorbance was measured against the blank at 600 mμ.

5. The concentration of DNA in the sample was calculated from a least squares estimation of the slope and Y intercept of the standard curve (absorbance vs concentration).

Colorimetric determination of RNA content

The method used was that described by Mejbaum (1939) and outlined in Schneider (1957).

1. Immediately before use, 1 g of orcinol was added to 100 ml of $FeCl_3$ in HCl (0.5 g of $FeCl_3$ per 100 ml of concentrated HCl).

2. Five-tenths ml of nucleic acid extract or 0.5 ml of DNA or RNA standard were added to 2.0 ml of 5% TCA.

3. Orcinol reagent (2.5 ml) was then added to the samples which were then placed in a boiling water bath for 20 minutes to develop color.

4. A blank was prepared with 2.5 ml TCA (5%) and 2.5 ml of orcinol reagent. Absorbance was measured against the blank at 660 mμ.

5. Standard curves and least squares estimations of the slopes and Y intercepts for both the RNA and DNA standards were made. Corrections for absorbance due to DNA were made using those concentrations for the unknown samples as previously determined by the Burton method.

In Vitro Incubation Procedures

Preparation of incubation medium (Cohen, 1957)

The following stock solutions were prepared and stored under refrigeration:

1. NaCl (4.5 g/500 ml distilled water)
2. KCl (1.73 g/150 ml distilled water)
3. $CaCl_2 \cdot 2HOH$ (2.43 g/150 ml distilled water)
4. KH_2PO_4 (3.17 g/150 ml distilled water)
5. $MgSO_4 \cdot 7HOH$ (5.73 g/150 ml distilled water)
6. $NaHCO_3$ (1.95 g/150 ml distilled water)

Prior to use, stock solutions were mixed in the following proportions:

100 parts NaCl
4 parts KCl
3 parts $CaCl_2 \cdot 2HOH$
1 part HK_2PO_4
1 part $MgSO_4 \cdot 7HOH$
21 parts $NaHCO_3$

Glucose was added to give a final concentration of 200 mg/100 ml incubation medium. ACTH (25 units/mg) was added to half of the incubation medium to give a final concentration of 250 milliunits/ml incubation medium.

Incubation procedure (Saffran et al., 1952; Pritchett, 1973)

Adrenal glands were rapidly excised from the animal, following decapitation and exsanguination, and placed on filter paper moistened with incubation medium (minus ACTH). The petri dish containing the adrenal glands was kept on ice while the adipose tissue was carefully trimmed away from the glands. Each gland was weighed, quartered and placed into 25 ml Ehrlenmeyer flasks containing either 2 ml of incubation medium minus ACTH or 2 ml of incubation medium plus ACTH. Right and left adrenal glands were alternated with respect to ACTH stimulation. The flasks were gassed with 95% O_2 plus 5% CO_2 for 60 seconds, placed in a holder designed for the Warburg by Mary McMahon at Iowa State University, Ames, Iowa (private communication, 1974), and incubated with shaking for 90 minutes at 37°C. At the end of the incubation period, the medium was transferred to screw-capped vials and frozen.

Fluorometric Determination of Corticosterone

The method used for the determination of corticosterone in plasma and in the incubation medium was based on the development of fluorescence by 11-hydroxycorticosteroids when exposed to ultraviolet light in concentrated sulfuric acid (Mattingly, 1962). The Mattingly method has been outlined by Sherrick et al. (1971). Modifications and the reagent purification steps used in this investigation have been described by Craig (1972).

1. Dichloromethane purification
    a. Dichloromethane (CH_2Cl_2) was allowed to stand over one-tenth

its volume of concentrated H_2SO_4 for three days with occasional shaking.

b. Dichloromethane was washed three times with one-tenth its volume on 2N NaOH and then three times with one-tenth its volume of distilled water.

c. Dichloromethane was dried for 24 hours with anhydrous sodium sulfate.

d. Dichloromethane was distilled through a Dufton column. The fraction which came over between 40 and 41°C was collected.

2. Ethanol purification

Ethanol was double distilled in a Dufton column. The fraction which came over at 75°C was collected.

3. Preparation of fluorescence reagent

Three parts concentrated H_2SO_4 were slowly added to one part purified ethanol kept on ice.

4. Preparation of corticosterone standards

Ten mg of corticosterone was dissolved in 5 ml of purified ethanol and brought to 100 ml with distilled water. This solution was stored under refrigeration and served as the stock solution. Aliquots of the stock solution were diluted with distilled water to give a series of standards containing 0.25, 0.5, 1.0, 2.0, and 4.0 µg/ml.

5. Corticosterone determination

   a. Frozen plasma samples or incubation medium were allowed to come to room temperature.

   b. Duplicate 0.5 ml aliquots of samples or standards were transferred to glass stoppered centrifuge tubes.

   c. Steroids were extracted in 5 ml purified dichloromethane. Samples were shaken for sixty seconds, layers allowed to separate, and shaken again for thirty seconds.

   d. Samples were centrifuged at 2000 RPM for two minutes. The aqueous layer was removed by aspiration.

   e. The remaining extract was washed with 200 µl of 0.1N NaOH and centrifuged for sixty seconds. NaOH was rapidly removed by aspiration to avoid breakdown of the steroid.

   f. Anhydrous sodium sulfate (enough to cover the bottom of the tube) was added for ten minutes to dry the extract.

   g. Two ml of the Na_2SO_4 dried extract was mixed with 5.0 ml of fluorescence reagent by shaking for thirty seconds.

   h. The mixed samples were centrifuged at 1000 RPM for two minutes. The organic (top) layer was removed by aspiration.

   i. One hour after mixing with the fluorescence reagent (step g), fluorescence was read in a Turner model 110 fluorometer using a Primary 110-813 (47B) filter and a Secondary 110-818 (2A-12) filter.

   j. Micrograms corticosterone/milliliter sample was calculated from a standard curve.

RESULTS

For the purpose of clarity in the reporting of results, the data have been grouped into the following sections:

1. Body weights and mammary gland weights
2. Mammary gland DNA
3. Mammary gland RNA
4. Adrenal and ovarian weights
5. Pup number, pup weights and placental weights
6. Adrenal secretion rates and plasma corticosterone

Each section will include a text describing the effect of treatment on particular parameters, a table listing the average values and standard errors for these parameters, tables of comparisons indicating tests of significance, and bar graphs illustrating the comparisons.

Body Weights and Mammary Gland Weights

Final body weights (Tables 2 and 3, Fig. 4)

Final body weights of adrenalectomized (265 g) and pup removal (276 g) animals were similar to those of controls (270 g). Body weights of hypophysectomized animals (225 g) were lower ($P<0.001$) than those of controls (270 g). Treatment of adrenalectomized, hypophysectomized, pup removal or sham operated controls with hydrocortisone acetate (F) had no effect on final body weight when compared to the respective surgical treatment groups that were not given hormone replacement.

Table 2. Effect of adrenalectomy, pup removal, hypophysectomy and hydrocortisone acetate on final body weight and mammary gland dried fat free tissue weight

Group[a](#)[b]	B.W.[c]	s.e.[d]	DFFT[e]	s.e.
A (9)	265	12.0	568.23	36.04
AF (10)	276	5.7	675.52	37.26
P (9)	257	6.3	618.56	24.40
PF (9)	243	6.3	668.75	43.61
H (10)	225	5.9	426.92	16.98
HF (9)	216	6.6	472.40	13.71
C (10)	270	9.2	688.37	29.08
CF (10)	260	8.4	730.04	46.42

[a]Group = symbol used corresponds to treatment described in Table 1.

[b](#) = number of animals included in the group.

[c]B.W. = final body weight (g).

[d]s.e. = standard error.

[e]DFFT = mammary gland dried fat free tissue weight (mg).

Table 3. Statistical comparisons of body weights using t-test

Groups compared	Difference between means	Significance
A and AF	11	not significant
P and PF	14	not significant
H and HF	9	not significant
C and CF	10	not significant
C and A	5	not significant
C and AF	6	not significant
C and P	13	not significant
C and PF	27	* significant ($P<0.05$)
C and H	45	* significant ($P<0.001$)
C and HF	54	* significant ($P<0.001$)

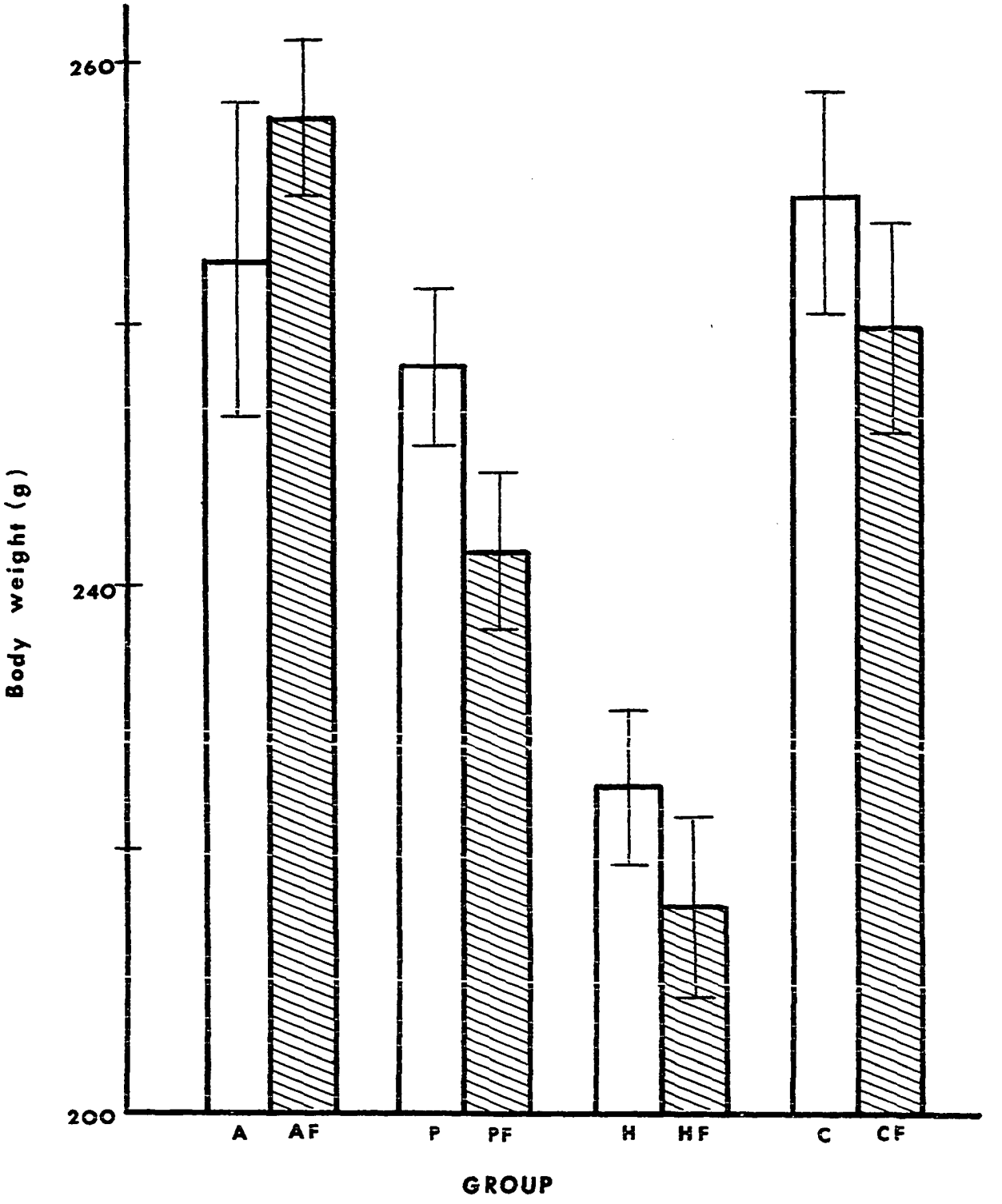

Fig. 4. Body weights

Mammary gland dried fat-free tissue weights (Tables 2 and 4, Figure 5)

Mammary gland dried fat free tissue weights (MG-DFFT) of adrenalectomized (568.23 mg) and hypophysectomized (426.92 mg) animals were significantly lower ($P<0.02$ and $P<0.001$, respectively) than those of controls (688.37 mg). MG-DFFT of pup removal animals (618.56 mg) was similar to MG-DFFT of the controls (688.37 mg). MG-DFFT's of F treated adrenalectomized (675.52 mg) and F treated hypophysectomized (472.40 mg) animals were greater ($P<0.005$) than the MG-DFFT's of the corresponding animals that did not receive F treatment. In the case of the F treated adrenalectomized animals, MG-DFFT was restored to the control level. In the case of the F treated hypophysectomized animal, MG-DFFT remained significantly lower ($P<0.001$) than the control value. MG-DFFT's of F treated pup removal (668.75 mg) and F treated sham operated (730.04 mg) animals were similar to the MG-DFFT's of the corresponding non-treated surgical groups.

Mammary Gland DNA

DNA on a per mg MG-DFFT basis (Tables 5 and 6, Fig. 6)

The average ug/DNA/mgMG-DFFT values of adrenalectomized (37.03), hypophysectomized (40.81) and pup removal (33.76) animals were significantly higher ($P<0.001$, $P<0.001$, $P<0.01$, respectively) than those of controls (27.93). Average ug/DNA/mgMG-DFFT values of F treated adrenalectomized (31.42), F treated hypophysectomized (36.50) and F treated pup removal (30.87) animals were significantly lower ($P<0.02$, $P<0.01$, $P<0.05$, respectively) than those of similarly operated

Table 4. Statistical comparisons of mammary gland dried fat free tissue weights using t-test

Groups compared	Difference between means	Significance
A and AF	107.19	* approaching significance ($P<0.05$)
P and PF	50.19	not significant
H and HF	45.48	* approaching significance ($P<0.05$)
C and CF	41.67	not significant
C and A	120.14	* significant ($P<0.02$)
C and AF	12.95	not significant
C and P	69.81	not significant
C and PF	19.62	not significant
C and H	261.45	* significant ($P<0.001$)
C and HF	215.97	* significant ($P<0.001$)

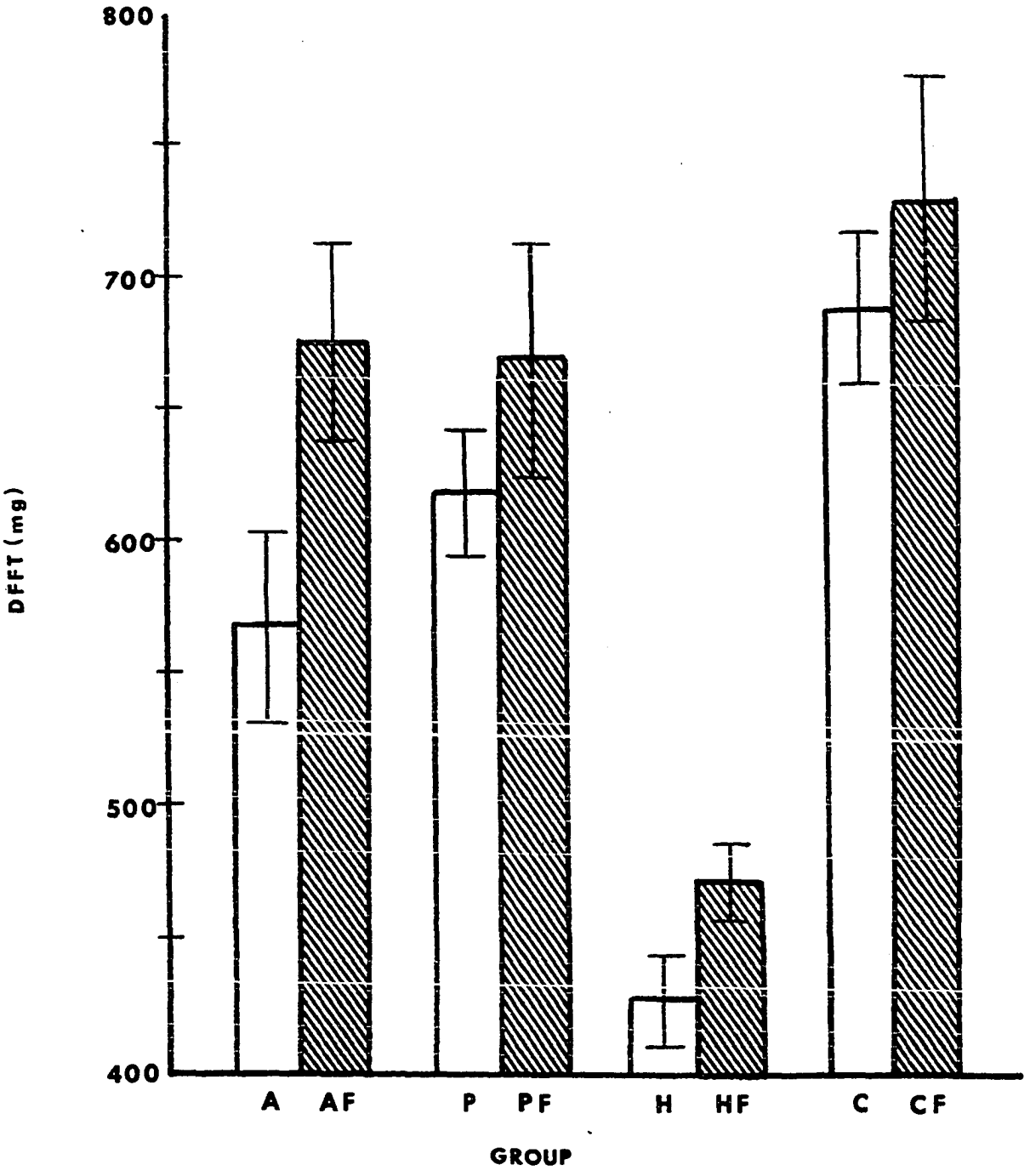

Fig. 5. Weights of dried fat free tissue (DFFT)

Table 5. Effect of adrenalectomy, pup removal, hypophysectomy and hydrocortisone acetate on mammary gland DNA content

Group (#)	$\frac{\text{ugDNA}}{\text{mgDFFT}}$	s.e.	Total mgDNA	s.e.
A (9)	37.03	1.69	20.61	0.60
AF (10)	31.42	1.05	20.96	0.71
P (9)	33.76	0.91	20.78	0.70
PF (9)	30.87	1.15	20.39	0.97
H (10)	40.81	0.75	17.42	0.77
HF (9)	36.50	1.07	17.23	0.69
C (10)	27.93	1.22	19.02	0.71
CF (10)	26.93	1.36	19.21	0.78

Table 6. Statistical comparisions of mammary gland DNA expressed as ugDNA/mgDFFT using t-test

Groups compared	Difference between means	Significance
A and AF	5.61	* significant (P<0.02)
P and PF	2.89	* approaches significance (P<0.05)
H and HF	4.31	* significant (P<0.01)
C and CF	1.00	not significant
C and A	9.10	* significant (P<0.001)
C and AF	3.49	* significant (P<0.05)
C and P	5.83	* significant (P<0.01)
C and PF	2.94	not significant
C and H	12.88	* significant (P<0.001)
C and HF	8.57	* significant (P<0.001)

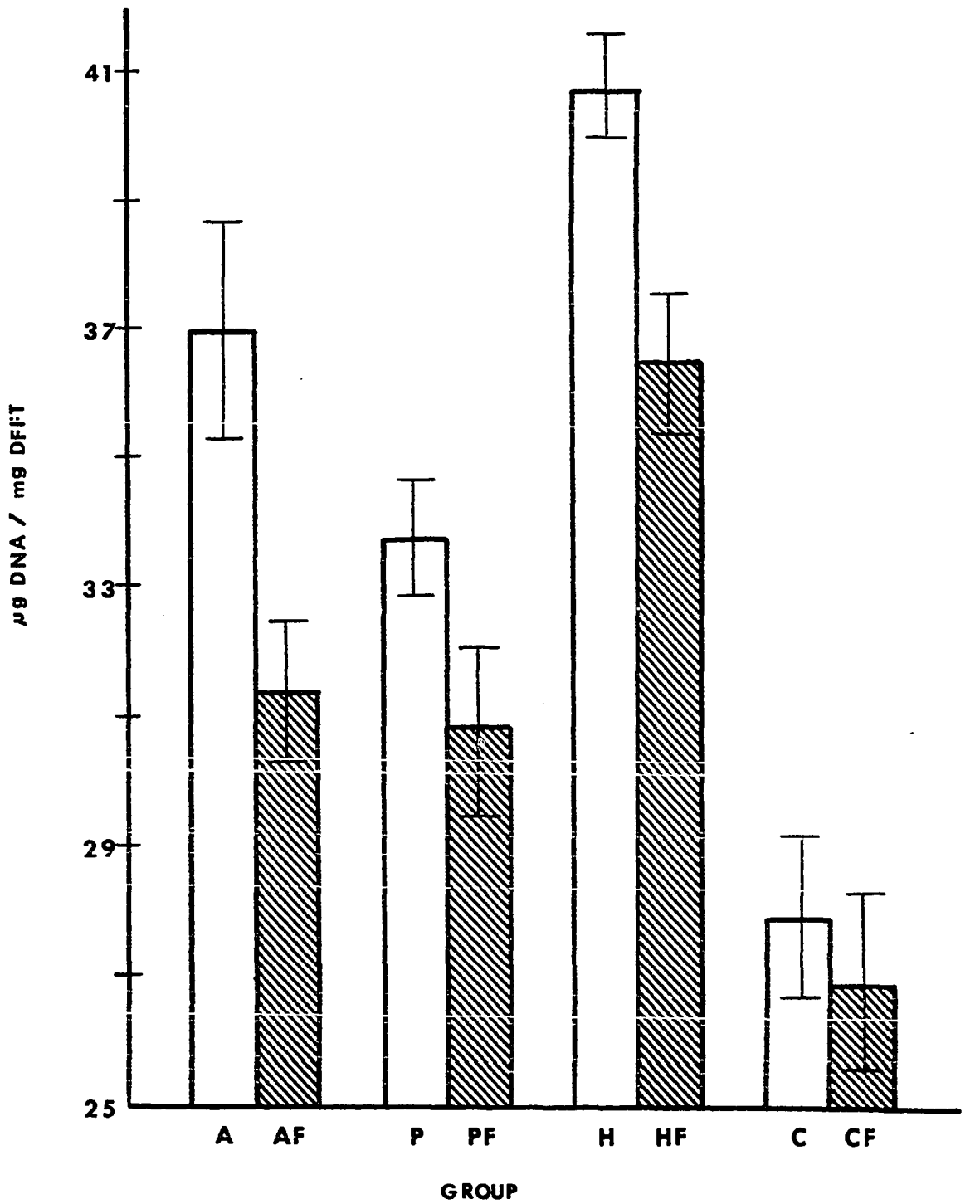

Fig. 6. DNA on a per mg DFFT basis

animals that did not receive F treatment. F treatment of the sham operated control resulted in a ugDNA/mgMG-DFFT (26.93) which was not significantly different from that of the control (27.93). F treatment of pup removal animals restored ugDNA/mgMG-DFFT to the control level. F treatment of hypophysectomized animals or F treatment of adrenalectomized animals did not completely restore ugDNA/mgMG-DFFT to the control level.

Total mammary gland DNA (Tables 5 and 7, Fig. 7)

No statistically significant differences were observed in any of the planned comparisons made (Table 7). However, the lower total mammary gland DNA of hypophysectomized (17.42 mg) and F treated hypophysectomized (17.23 mg) animals may have been biologically different from that control (19.02), and is statistically different from the total mammary gland DNA of adrenalectomized (20.61 mg), F treated adrenalectomized (20.96 mg), pup removal (20.78 mg) and F treated pup removal (20.39 mg) animals (Fig. 7).

Mammary Gland RNA

Total mammary gland RNA (Tables 8 and 9, Fig. 8)

Total mammary gland RNA (MG-RNA) of adrenalectomized (39.47 mg) and pup removal (44.37 mg) animals was not significantly different from that of sham operated controls (39.47 mg). MG-RNA of hypophysectomized animals (25.86 mg) was lower ($P<0.001$) than MG-RNA of controls. MG-RNA of F treated adrenalectomized animals (47.02 mg) and F treated

Table 7. Statistical comparisons of total DNA content expressed in mg using t-test

Groups compared	Differences between means	Significance
A and AF	0.35	not significant
P and PF	0.39	not significant
H and HF	0.19	not significant
C and CF	0.19	not significant
C and A	1.59	not significant
C and AF	1.94	not significant
C and P	1.76	not significant
C and PF	1.37	not significant
C and H	1.60	not significant
C and HF	1.79	not significant

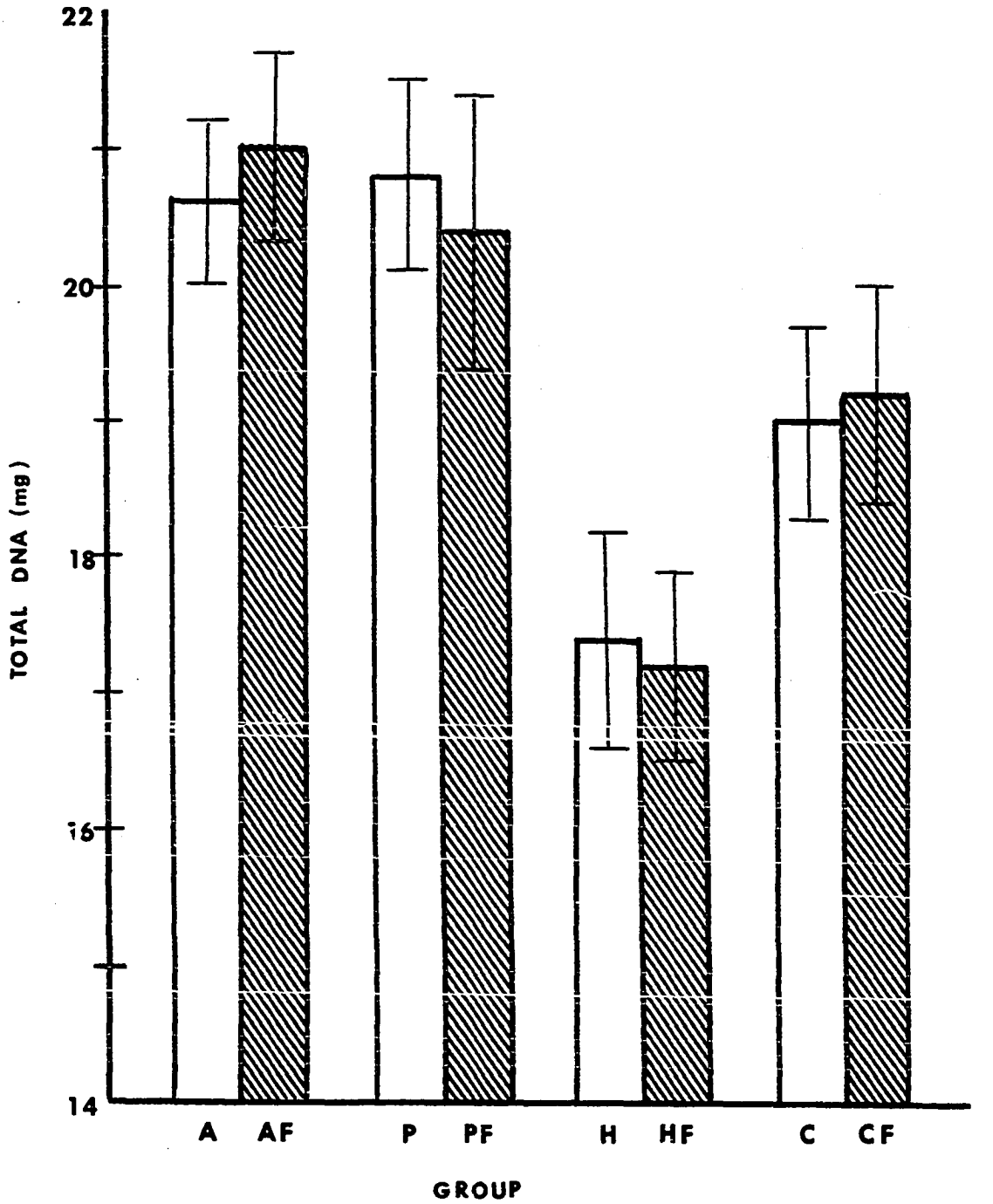

Fig. 7. Total DNA content of the six abdominal-inguinal mammary glands

Table 8. Effect of adrenalectomy, pup removal, hypophysectomy and hydrocortisone acetate on mammary gland RNA content

Group (#)	Total mgRNA	s.e.	RNA:DNA	s.e.
A (9)	39.47	2.41	1.91	0.13
AF (10)	47.02	2.70	2.25	0.11
P (9)	44.37	2.19	2.13	0.07
PF (9)	47.62	8.82	2.33	0.09
H (10)	25.86	0.83	1.56	0.05
HF (9)	30.42	1.40	1.78	0.08
C (10)	39.47	3.41	2.27	0.11
CF (10)	42.61	1.31	2.48	0.16

Table 9. Statistical comparisons of total mammary gland RNA content expressed in mg

Groups compared	Difference between means	Significance
A and AF	7.55	not significant
P and PF	3.25	not significant
H and HF	4.56	* significant (P<0.01)
C and CF	5.05	not significant
C and A	3.14	not significant
C and AF	4.41	not significant
C and P	1.75	not significant
C and PF	5.01	not significant
C and H	16.75	* significant (P<0.001)
C and HF	12.19	* significant (P<0.001)

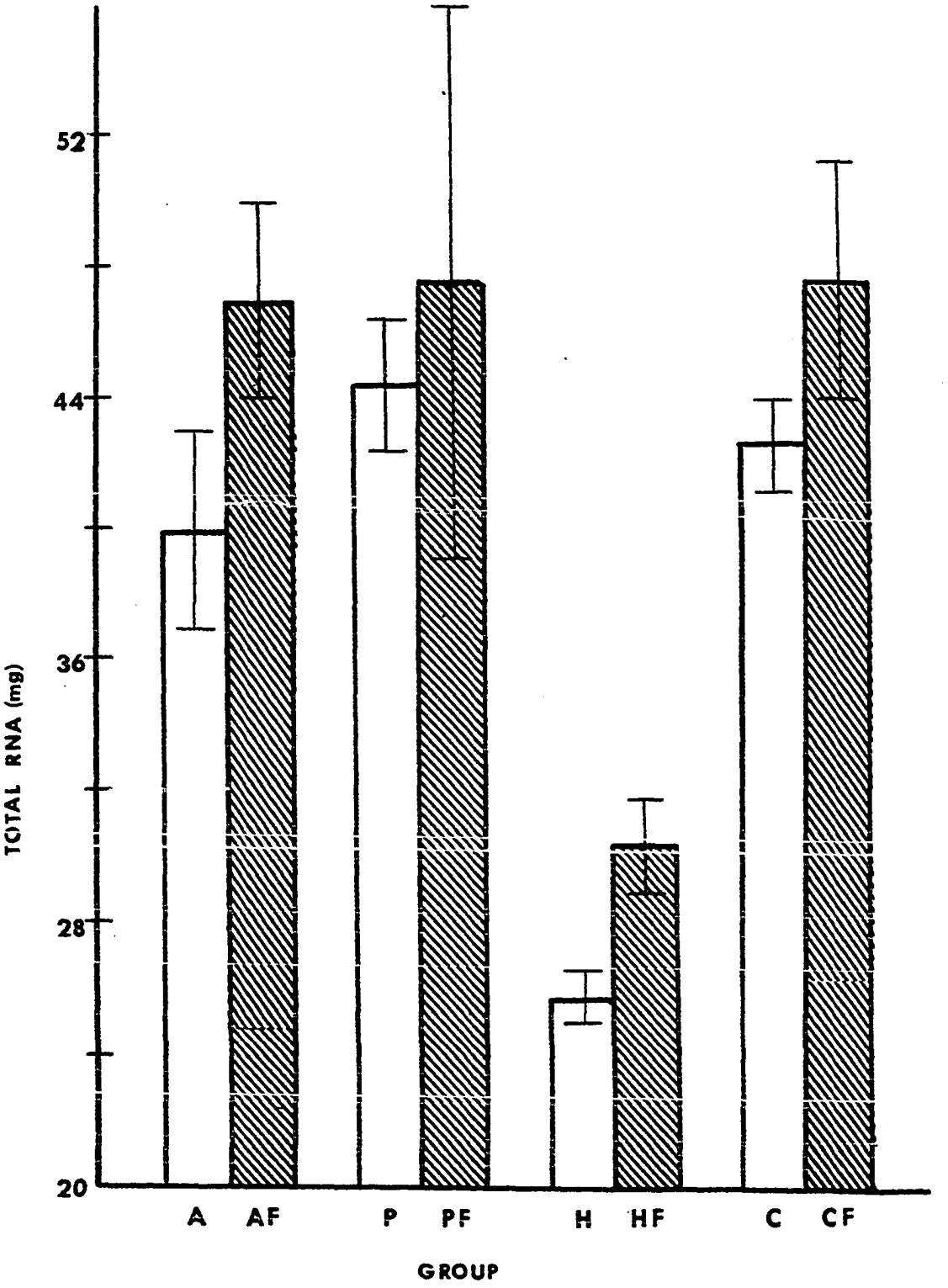

Fig. 8. Total RNA content of the six abdominal-inguinal mammary glands

pup removal animals (47.62 mg) was not significantly different from MG-RNA of adrenalectomized and pup removal animals that did not receive F treatment. MG-RNA of F treated hypophysectomized animals (42.61 mg) was significantly greater ($P<0.01$) than MG-RNA of hypophysectomized animals that did not receive F treatment, but was still lower ($P<0.001$) than MG-RNA of sham operated controls.

RNA to DNA ratio (Tables 8 and 10, Fig. 9)

The RNA to DNA ratios in the mammary gland (MG-RNA:DNA) of adrenalectomized animals (1.91) and hypophysectomized animals (1.56) were significantly lower ($P<0.05$ and $P<0.001$) than the MG-RNA:DNA of controls (2.27). The MG-RNA:DNA of pup removal animals (2.13) was not significantly different from that of the controls. MG-RNA:DNA's of F treated adrenalectomized animals (2.25), F treated pup removal animals (2.33) and F treated hypophysectomized animals (1.78) were significantly greater ($P<0.05$) than the MG-RNA:DNA's of similarly operated animals that did not receive F treatment. In the case of the F treated adrenalectomized animal, the MG-RNA:DNA was increased to the control level. In the F treated hypophysectomized animal the MG:RNA:DNA was lower ($P<0.001$) than that of the control.

Pup Number, Pup Weights and Placental Weights

The average litter sizes of all treatment groups were comparable to each other (Tables 11 and 12). Pup number ranged from 8.7 to 10.2, and no significant differences were obtained from any of the planned comparisons.

Table 10. Statistical comparisons of the RNA to DNA ratios using t-test

Groups compared	Difference between means	Significance
A and AF	0.34	* approaches significance ($P<0.05$)
P and PF	0.20	* approaches significance ($P<0.05$)
H and HF	0.22	* significant ($P<0.05$)
C and CF	0.21	not significant
C and A	0.36	* significant ($P<0.05$)
C and AF	0.02	not significant
C and P	0.14	not significant
C and PF	0.06	not significant
C and H	0.71	* significant ($P<0.001$)
C and HF	0.49	* significant ($P<0.01$)

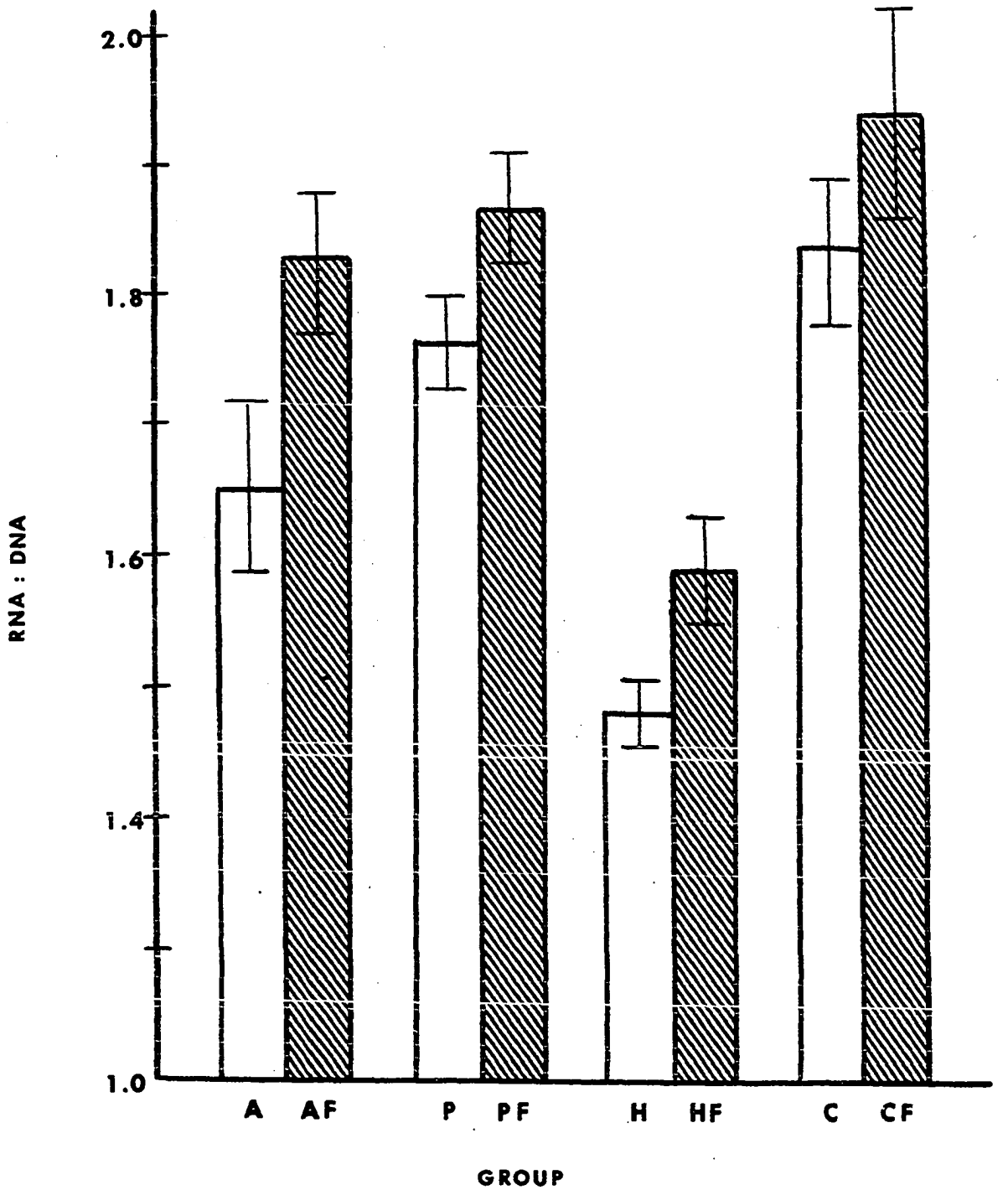

Fig. 9. RNA:DNA ratio

Table 11. Effect of adrenalectomy, pup removal, hypophysectomy and hydrocortisone acetate on pup number, unit pup weight and unit placental weight

Group (#)	# of pups	s.e.	Unit pup weight (g)	s.e.	Unit placental weight (g)	s.e.
A (9)	10.4	0.9	5.45[a]	0.20	0.70	0.02
AF (10)	10.4	0.6	5.77[a]	0.12	0.67	0.02
P (9)	8.8	0.8	0.88[b]	0.03	0.49	0.05
PF (9)	8.9	0.7	0.90[b]	0.03	0.51	0.02
H (10)	10.0	0.7	5.47[a]	0.25	0.69	0.03
HF (9)	10.2	0.8	5.05[a]	0.21	0.68	0.03
C (10)	9.9	0.7	5.66[a]	0.10	0.68	0.03
CF (10)	8.7	0.6	5.60[a]	0.11	0.72	0.03

[a]Pup weight on day 22.

[b]Pup weight on day 18.

Table 12. Statistical comparisons of litter size using t-test

Groups compared	Difference between means	Significance
A and AF	0.0	not significant
H and HF	0.2	not significant
C and CF	1.2	not significant
C and A	0.5	not significant
C and AF	0.5	not significant
C and P[a]	1.1	not significant
C and H	0.1	not significant
C and HF	0.3	not significant

[a]C and P a comparison between the number of pups on day 18 and day 22.

Individual body weights of pups (Tables 11 and 13) from adrenalectomized dams (5.45 g), F treated adrenalectomized dams (5.77 g), and hypophysectomized dams (5.47 g) were similar to body weights of pups from control animals (5.66 g). Body weights of pups from F treated hypophysectomized dams (5.05 g) were significantly lower ($P<0.02$) than body weights of pups from control animals. In the case of adrenalectomized and sham operated control animals, F treatment did not effect a decrease in pup body weight.

Individual placental weights (Tables 11 and 14) of adrenalectomized dams (0.70 g), F treated adrenalectomized dams (0.67 g), hypophysectomized dams (0.68 g), F treated hypophysectomized dams (0.68 g), and F treated sham operated dams (0.72) were all similar to individual placental weights of the sham operated control that did not receive F treatment (0.68 g). Individual placental weights of pup removal animals (0.49 g) and F treated pup removal animals (0.51) were significantly lower ($P<0.01$ and $P<0.001$, respectively) than individual placental weights of controls (0.68 g).

Adrenal and Ovarian Weights

Adrenal weights (Tables 15 and 16, Fig. 10)

Adrenal gland weights on day 18 of pregnancy (75.38 mg) were not significantly different from adrenal gland weights of control animals on day 22 of pregnancy (72.34). Adrenal gland weights of hypophysectomized animals (55.30 mg) were significantly lighter ($P<0.001$) than adrenal gland weights of controls on day 22 of pregnancy (72.34 mg).

Table 13. Statistical comparisons of individual pup weight using t-test

Groups compared	Difference between means	Significance
A and AF	0.32	not significant
H and HF	0.42	not significant
C and CF	0.06	not significant
C and A	0.21	not significant
C and AF	0.11	not significant
C and P[a]	4.78	* significant (P<0.001)
C and H	0.19	not significant
C and HF	0.61	* significant (P<0.02)

[a]C and P = a comparison between the pup weight on day 18 and day 22.

Table 14. Statistical comparisons of individual placental size using t-test

Groups compared	Difference between means	Significance
A and AF	0.03	not significant
P and PF	0.02	not significant
H and HF	0.01	not significant
C and CF	0.03	not significant
C and A	0.02	not significant
C and AF	0.01	not significant
C and P	0.19	* significant (P<0.01)
C and PF	0.17	* significant (P<0.001)
C and H	0.01	not significant
C and HF	0.00	not significant

Table 15. Effect of adrenalectomy, pup removal, hypophysectomy and hydrocortisone acetate on adrenal and ovarian weights

Group (#)	Adrenal gland weight (mg)	s.e.	Ovarian weight (mg)	s.e.
A (9)	75.38[a]	3.08	142.64	5.18
AF (10)	72.62[a]	3.46	143.72	5.91
P (9)	98.96[b]	4.16	159.21	6.27
PF (9)	86.69[b]	3.64	154.64	6.11
H (10)	56.34[b]	1.72	127.96	4.86
HF (9)	55.30[b]	3.46	136.68	7.72
C (10)	72.34[b]	2.35	156.52	8.68
CF (10)	66.30[b]	3.16	138.43	8.56

[a]Adrenal weight on day 18.

[b]Adrenal weight on day 22.

Table 16. Statistical comparisons of adrenal gland weights expressed in mg using t-test

Groups compared	Difference between means	Significance
P and PF	12.27	* significant (P<0.05)
H and HF	1.04	not significant
C and CF	6.04	not significant
C and A[a]	3.04	not significant
C and P	26.62	* significant (P<0.001)
C and PF	14.35	* significant (P<0.01)
C and H	16.00	* significant (P<0.001)
C and HF	17.04	* significant (P<0.001)

[a]C and A = comparison between adrenal weights on day 18 and day 22.

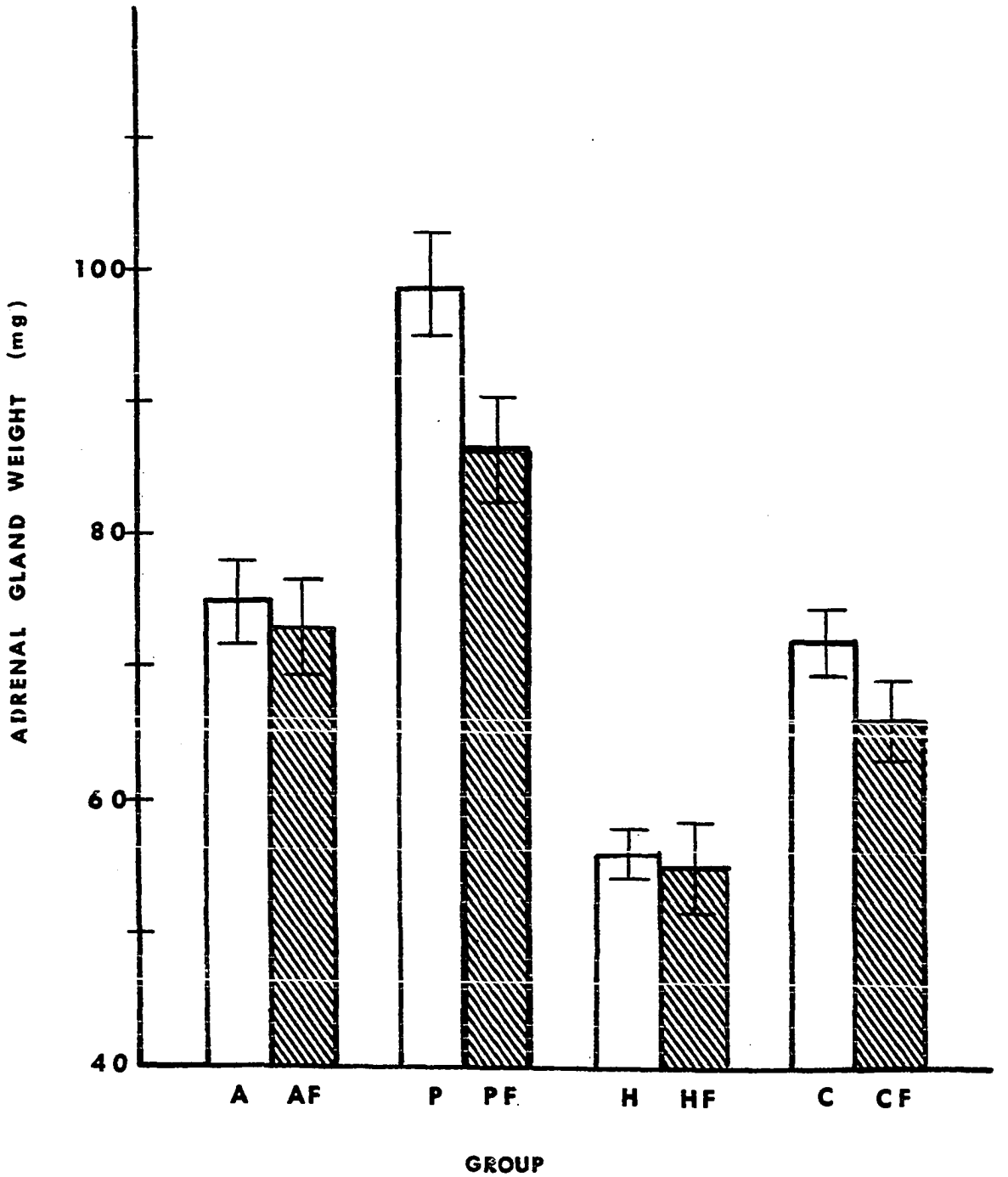

Fig. 10. Adrenal gland weight

Adrenal gland weights of pup removal animals (98.96 mg) and F treated pup removal animals (86.69 mg) were greater ($P<0.001$ and $P<0.01$, respectively) than those of the control animals on day 22 of pregnancy (72.34 mg). F treatment of pup removal animals resulted in adrenal gland weights (86.69 mg) which were significantly lower ($P<0.05$) than adrenal gland weights of pup removal animals that did not receive F treatment (98.96 mg). The difference between adrenal gland weights of F treated sham operated controls (66.30 mg) and sham operated controls that did not receive F treatment (72.34 mg) was not statistically significant at the 0.05 level, but may have been biologically significant.

Ovarian weights (Table 15 and 17, Fig. 11)

Ovarian weights of adrenalectomized animals (142.64 mg), F treated adrenalectomized animals (143.72 mg), pup removal animals (159.21 mg), F treated pup removal animals (154.64 mg) and F treated sham operated control animals (138.43 mg) were not significantly different from ovarian weights of sham operated control animals that did not receive F treatment (156.52 mg). Ovarian weights of hypophysectomized animals (127.96 mg) were significantly lower ($P<0.01$) than ovarian weights of controls (156.25 mg). The difference between ovarian weights of F treated hypophysectomized animals (136.68 mg) and controls (156.52 mg) was not statistically significant, but may have been biologically significant.

Table 17. Statistical comparisons of total ovarian weight expressed in mg using t-test

Groups compared	Difference between means	Significance
A and AF	1.08	not significant
P and PF	4.57	not significant
H and HF	8.72	not significant
C and CF	18.09	not significant
C and A	13.88	not significant
C and AF	12.80	not significant
C and P	2.69	not significant
C and PF	1.88	not significant
C and H	28.56	* significant ($P<0.01$)
C and HF	19.84	not significant

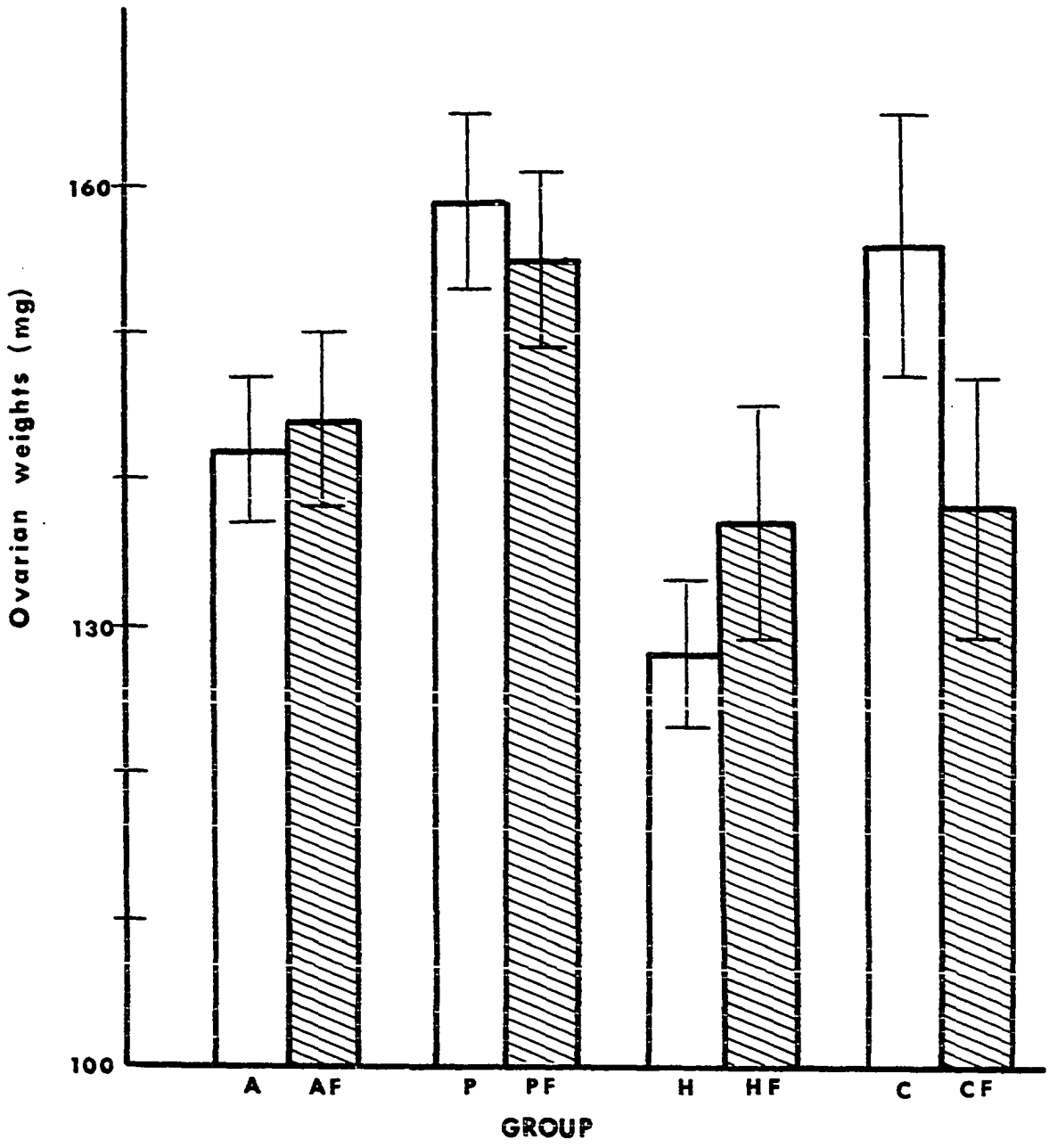

Fig. 11. Total ovarian weights

Adrenal secretion rates (Tables 18-22, Fig. 12)

In vitro adrenal corticosterone secretion rates (expressed as µg corticosterone/100 mg adrenal/hr) of 18 day controls (3.92) and pup removal animals (3.50) were not significantly different from those of 22 day controls (4.46). Similarly, ACTH stimulated adrenal corticosterone secretion rates of 18 day controls (9.42) and pup removal animals (7.44) were not significantly different from ACTH stimulated adrenal corticosterone secretion rates of 22 day controls (12.79). The differences between non-stimulated and ACTH stimulated adrenal corticosterone secretion rates within each group (18 day control, 22 day control and pup removal) were statistically significant at the 0.001, 0.005 and 0.005 levels, respectively.

Plasma corticosterone levels (Tables 23 and 24, Fig. 13)

Plasma corticosterone levels (expressed as µg corticosterone/100 ml plasma) of 18 day controls (41.16) and pup removal animals (30.72) were not significantly different from the levels of 22 day control animals (42.66). Plasma corticosterone levels of adrenalectomized animals (17.17) were significantly lower ($P<0.005$) than levels of 22 day control animals (42.66).

Table 18. *In vitro* adrenal corticosterone secretion rates of eighteen day controls, twenty-two day controls, and pup removal animals

Group (#)	µg corticosterone/100 mg adrenal/hr	s.e.
E (10)	3.92	0.56
C (8)	4.46	0.78
P (7)	3.50	0.50

Table 19. Effect of ACTH on *in vitro* adrenal corticosterone secretion rates of eighteen day controls, twenty-two day controls, and pup removal animals

Group (#)	µg corticosterone/100 mg adrenal/hr	s.e.
E (10)	9.24	1.22
C (8)	12.79	2.24
P (7)	7.44	0.78

Table 20. Statistical comparisons of *in vitro* adrenal corticosterone secretion rates

Groups Compared	Difference between means	Significance
E and C	0.54	not significant
P and C	0.96	not significant

Table 21. Statistical comparisons of ACTH stimulated *in vitro* adrenal corticosterone secretion rates

Groups Compared	Difference between means	Significance
E and C	3.37	not significant
P and C	5.35	not significant

Table 22. Statistical comparisons of ACTH stimulated and non-stimulated adrenal corticosterone secretion rates

Groups Compared	Difference between means	Significance
E(-ACTH) and E(+ACTH)	5.50	* significant ($P<0.001$)
C(-ACTH) and C(+ACTH)	8.32	* significant ($P<0.005$)
P(-ACTH) and P(+ACTH)	3.94	* significant ($P<0.005$)

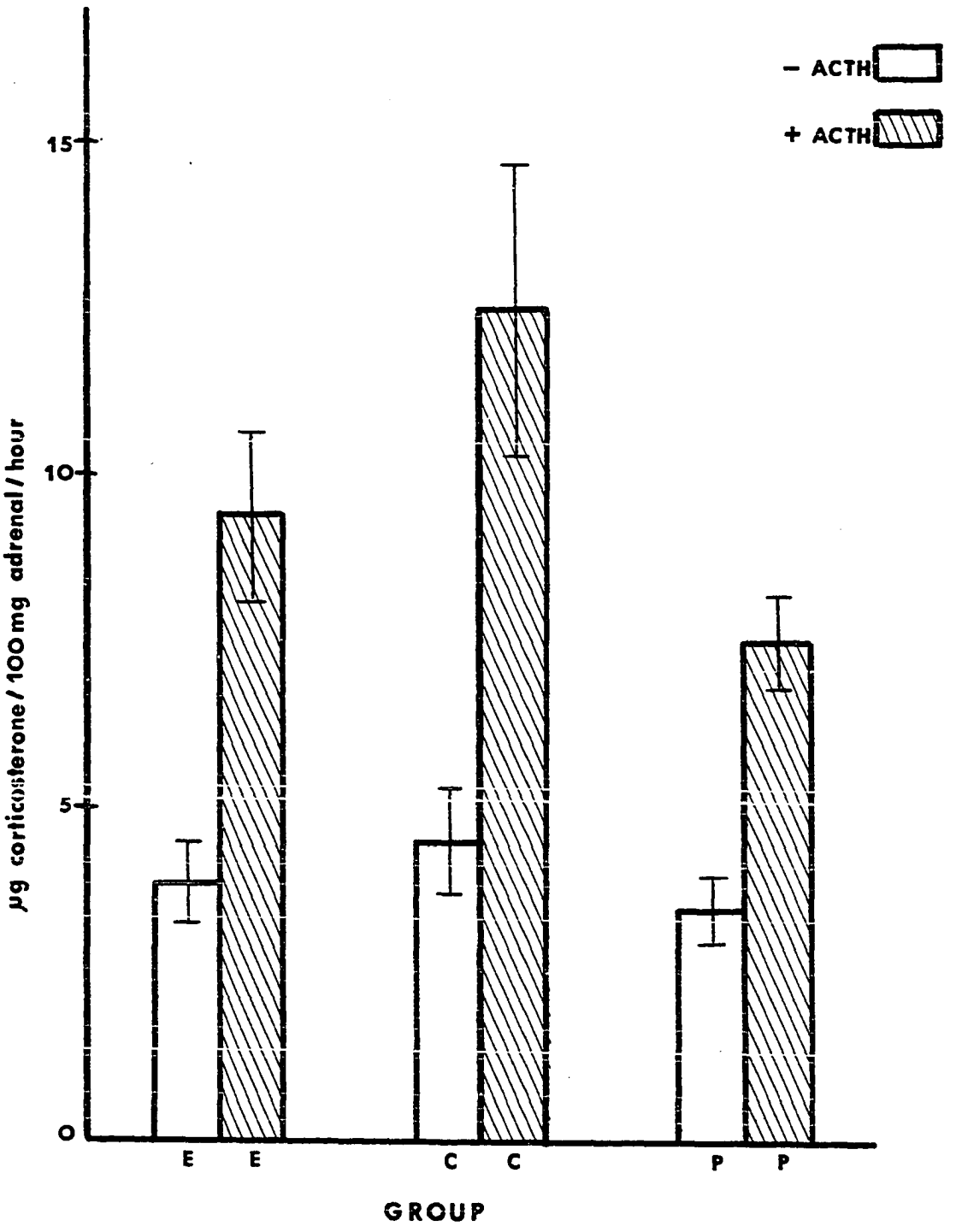

Fig. 12. In vitro adrenal corticosterone secretion rates

Table 23. Plasma corticosterone levels in eighteen day controls, twenty-two day controls, pup removal animals and adrenalectomized animals

Group (#)	µg corticosterone / 100 ml plasma	s.e.
E (11)	41.16	6.45
C (10)	42.66	5.58
P (7)	30.72	10.04
A (10)	17.17	3.56

Table 24. Statistical comparisons of plasma corticosterone levels

Groups Compared	Difference between means	Significance
E and C	1.50	not significant
P and C	11.94	not significant
A and C	25.49	* significant ($P<0.005$)

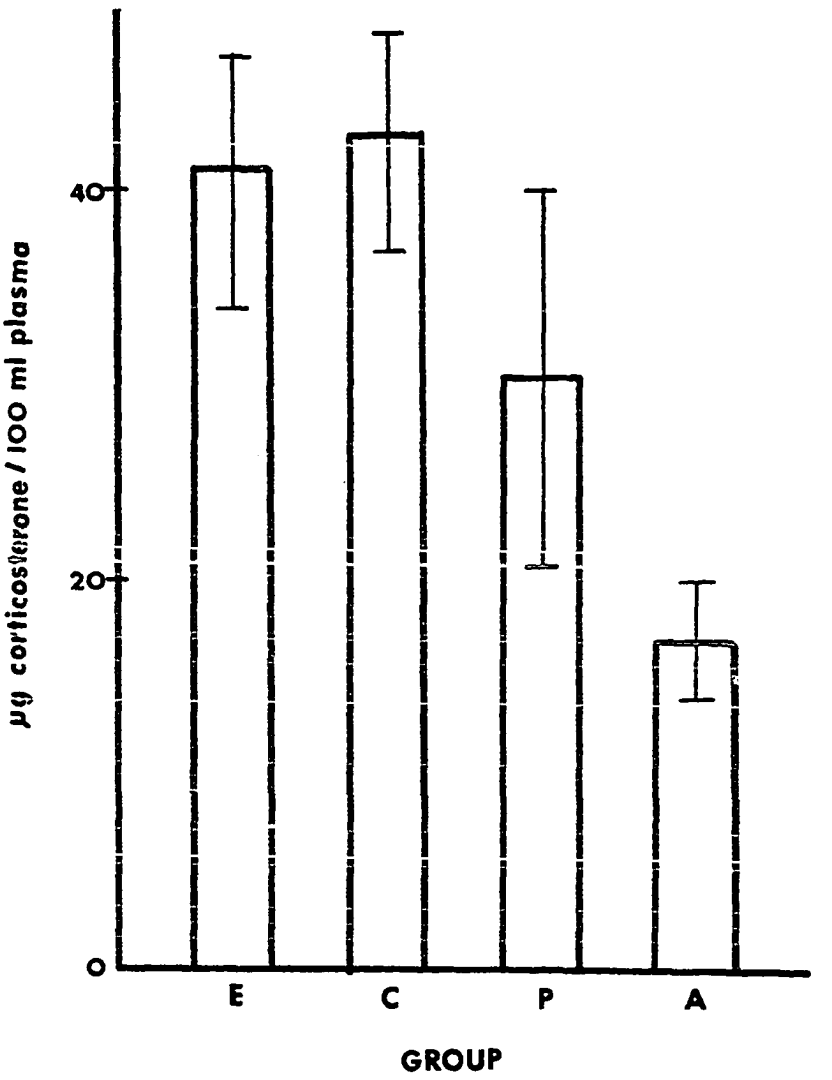

Fig. 13. Plasma corticosterone levels

DISCUSSION

It has been suggested (Banerjee et al., 1971) that RNA synthesis in the mammary gland is selectively more dependent upon adrenocortical hormones during its functional, rather than structural, development, and that DNA synthesis (indicative of cellular proliferation) is not affected by adrenocortical hormones during late pregnancy (Ferreri and Griffith, 1969). The results of this investigation agree with these earlier works. Adrenalectomy on day 18 of pregnancy resulted in a decrease in the RNA to DNA ratio in the gland, a decrease in individual cell size and a decrease in the overall size of the mammary gland. However, it had no effect on total mammary gland DNA, indicating that cell number was not affected. Treatment of adrenalectomized animals with hydrocortisone acetate restored the RNA to DNA ratio, individual cell size and overall gland size to normal, but did not effect an increase in total mammary gland DNA.

George et al. (1973) hypophysectomized rats on day 14 of pregnancy and reported a significant decrease in total mammary gland DNA and total mammary gland RNA, and found no difference between the RNA: DNA ratios of hypophysectomized animals and 20 day controls. In this study, mammary glands from animals hypophysectomized on day 18 and sacrificed on day 22 contained almost as much DNA as controls, but contained far less RNA. The RNA to DNA ratios of these hypophysectomized animals were significantly lower than those of controls. Treatment of hypophysectomized animals with hydrocortisone acetate resulted in an

increase in total RNA and the RNA to DNA ratio, but did not restore these to the control levels. As stated previously, mammary gland development during pregnancy can be divided into two phases: mammogenesis and lactogenesis. In the study by George et al. (1973) the cessation of cellular proliferation implicates anterior pituitary hormones in mammogenesis during mid-pregnancy. The lower day 22 mammary gland RNA to DNA ratios of animals hypophysectomized on day 18 implicates anterior pituitary hormones in the pre-partum phase of lactogenesis. Chadwick (1971) has reported that ACTH, given systemically, induces lactogenesis in pseudopregnant rabbits and that the ACTH effect is mediated by corticosteroid secretion. In this study, adrenal glands of rats hypophysectomized on day 18 were significantly lighter than adrenal glands of controls. Treatment of hypophysectomized animals with hydrocortisone acetate resulted in an increase in the RNA to DNA ratio, and supports Chadwick's conclusions concerning the role of ACTH. However, the RNA to DNA ratios of hydrocortisone treated hypophysectomized animals were still significantly lower than those of controls; suggesting that pituitary hormones, in addition to ACTH, are required for normal lactogenesis. Meites and Turner (1947) have reported that prolactin alone can induce lactation in pregnant rabbits and it has been suggested (Meites, 1954) that prolactin is an essential component of a lactogenic hormonal complex. Ben-David et al. (1971) and Chadwick (1971) have described a synergistic action of prolactin and adrenocortical hormones on mammary gland development in vivo. El-Darwish and Rivera (1970) have described the optimum requirements for RNA synthesis by pregnant

mouse mammary glands cultured *in vitro* as being insulin, prolactin and corticosterone. The inability of treatment of hypophysectomized animals with hydrocortisone acetate to restore total RNA and the RNA to DNA ratio to normal may reflect the absence of pituitary prolactin.

Desjardins et al. (1968) reported that removal of the fetus after mid-pregnancy (day 12) did not interfere with mammary gland DNA and RNA. In this investigation, pups were removed on day 18 and mammary gland DNA and RNA values were similar to the 22 day control values. Treatment of pup removal animals with hydrocortisone acetate had no effect on mammary gland DNA and RNA.

One of the aims of this investigation was to determine whether the onset of fetal adrenal activity could be considered a triggering mechanism for the initiation of milk secretion in the rat. The results obtained suggest that a fetal contribution to the level of circulating glucocorticoids is not essential for lactogenesis, but that sustained maternal adrenal activity is essential.

Plasma corticosterone levels of pup removal animals were similar to those of 22 day control animals. Mammary gland size, DNA content and the RNA to DNA ratio of pup removal animals were also similar to 22 day control values. In the initial stages of this investigation, an increase in adrenal gland weights of pup removal animals was noted, and suggested a compensatory hypertrophy of the maternal adrenal in the absence of the fetus. However, evidence to refute this observation was subsequently obtained. An increase in adrenal gland weights was not noted in a second group of pup removal animals, and *in vitro*

adrenal corticosterone secretion rates of pup removal animals were found to be similar to those of 22 day controls. ACTH stimulated adrenals of pup removal animals produced a significantly greater amount of corticosterone *in vitro* than non-stimulated adrenals of pup removal animals, again suggesting that the adrenal glands were not being maximally stimulated *in vivo*.

The essentiality of sustained maternal adrenal activity in lactogenesis, and the inability of fetal adrenal hypertrophy to compensate for the lack of adrenal steroids following adrenalectomy also was demonstrated in this study. Plasma corticosterone levels of adrenalectomized dams were significantly lower than levels of 22 day control animals. Mammary gland weights and the RNA to DNA ratios of adrenalectomized animals were smaller than those of the 22 day controls.

The possibility that increased maternal glucocorticoid secretion near term could serve as the triggering mechanism for the initiation of milk secretion was also considered. Plasma corticosterone and *in vitro* adrenal corticosterone secretion rates of 18 day controls were similar to those of 22 day controls, indicating that an increase in maternal glucocorticoid secretion is not necessary. Other workers have suggested that sensitivity to glucocorticoids, rather than secretion rates, may change near term. Gala and Westphal (1965) have reported that during most of pregnancy, the level of biologically active glucocorticoids may be low because of increased binding to corticoid binding globulin (CBG), but that during late pregnancy, the level of biologically active glucocorticoids increases as a result of decreased binding to CBG. Curry and Beaton (1958) have reported that pregnant

rats exhibit a marked resistance to the biological effects of the administration of cortisone, and that this resistance may be imparted by placental hormones. With particular reference to the mammary gland, Ferreri and Griffith (1969) have reported that treatment of pregnant rats with hydrocortisone acetate during early pregnancy has no effect on the mammary gland RNA to DNA ratio, whereas treatment during late pregnancy results in an increase in the RNA to DNA ratio. Therefore, an increase in mammary gland sensitivity to lactogenic stimulation by glucocorticoids and an increase in the level of biologically active glucocorticoids near term may be considered the mechanism which triggers the increase in the mammary gland RNA to DNA ratio at the time of lactogenesis, rather than an increase in maternal adrenal secretion rates.

SUMMARY

The present investigation was concerned with the effect of adrenalectomy, pup removal, hypophysectomy and treatment with hydrocortisone acetate on mammary gland nucleic acid content near term in the rat. It was designed to allow for a consideration of the onset of fetal adrenal activity or changes in maternal adrenal activity as a possible triggering mechanism for lactogenesis. Surgery was performed on day 18 of pregnancy; hydrocortisone treated animals were injected from day 18 to day 21; and animals (excluding the 18 day controls) were sacrificed on day 22. Mammary gland nucleic acid content was estimated colorimetrically and used to assess the extent of mammary gland development. Adrenal glands from 18 day controls, pup removal animals and 22 day controls were incubated for 90 minutes with or without ACTH stimulation. *In vitro* adrenal corticosterone secretion rates were estimated fluorometrically and used to assess adrenal gland function *in vivo*. Plasma corticosterone levels of 18 day controls, adrenalectomized animals, pup removal animals and 22 day controls were also estimated fluorometrically.

The results reported herein indicated the following:

1. Glucocorticoids are involved in the increase in the RNA to DNA ratio, individual cell size and overall gland size which occurs near term. Adrenalectomy abolishes these increases. Treatment of adrenalectomized animals with hydrocortisone acetate restores these values to normal.

2. Pituitary hormones, in addition to ACTH, are involved in the increase in the RNA to DNA ratio, individual cell size and overall gland size which occurs near term. Hypophysectomy abolishes these increases. Treatment of hypophysectomized animals with hydrocortisone acetate does not completely restore these values to normal.

3. A fetal contribution to the level of circulating glucocorticoids is not essential for lactogenesis. Pup removal has no effect on plasma corticosterone levels, in vitro adrenal corticosterone secretion rates, or mammary gland nucleic acid content.

4. Sustained maternal adrenal activity is essential for lactogenesis and the effects of adrenalectomy cannot by fully compensated for by fetal adrenal hypertrophy. Plasma corticosterone levels of adrenalectomized animals are significantly lower than plasma corticosterone levels of controls.

5. An increase in mammary gland sensitivity to lactogenic stimulation by the glucocorticoids and/or an increase in the level of biologically active glucocorticoids is probably involved in effecting mammary gland differentiation near term. An actual increase in maternal adrenal glucocorticoid secretion apparently does not occur. Plasma corticosterone levels and in vitro adrenal corticosterone secretion rates of 18 day control animals were not significantly different from those of 22 day controls.

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