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ABSTRACT
by
ESPERANZA SANCHO

PLASMA AND URINARY CORTICOSTEROIDS
AND ANDROGENS IN MURINE MAMMARY CANCER

Plasma corticosterone (PC) levels and urinary corticosteroids (UC) and androgen (UA) amounts were determined in male and female high mammary cancer C3H/HeJ (H) and low mammary cancer C3HeB/FeJ (F) mice. Before the tumor appeared, the PC levels were significantly depressed, became significantly elevated when it first manifested and again decreased significantly during tumor development. The UC amounts also increased when the tumor first appeared but the values were not subnormal prior to its appearance nor did they decline during its development. The UA amounts followed the same pattern except that they decline as the tumor developed. Females had significantly higher UC and significantly lower UA amounts, but the PC level showed no reliable sex difference. F mice had significantly higher PC levels, significantly lower UC amounts and lower UA amounts of borderline significance.

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ESPERANZA SANCHO

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Department of Investigative Medicine,
McGill University, Montreal.

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To my Parents,
for their kindest thoughts
and patience.

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LIST OF ABBREVIATIONS

ABG .....Androgen Binding Globulin
ACTH .....Adrenocorticotropic Hormone
ADREX .....Adrenalectomy
B .....Corticosterone
BW .....Body Weight
CBG .....Corticosterone Binding Globulin
CH .....Cholesterol
CHO .....Carbohydrates
DOC .....11- desoxycorticosterone
F .....C3HeB/FeJ Mice
G .....Grams
H .....C3H/HeJ Mice
MCG% .....Micrograms per 100 milliliters
ML .....Milliliter
NG .....Nanogram
HRS .....Hours
PC .....Plasma Corticosterone
RC .....Renal Clearance
SBG .....Steriod Binding Globulin
T .....Testosterone
UA .....Urinary Androgen
UCS .....Urinary Corticosteroids
µg .....Microgram
UV .....Urine volume

PART I

HISTORICAL AND LITERATURE REVIEW

PART I

CHAPTER I

THE CORTICOSTEROIDS

1. HISTORICAL BACKGROUND

The adrenal gland consist of two tissues which are distinct in their embryological origin, histologic structure and function. They are triangular bodies situated at the upper pole of the kidneys. On section, one distinguishes the capsule, the adrenal cortex of yellow color and the thin adrenal medulla of reddish-brown color (Paschkis, Rakoff, Cantarow, Rupp, 1967).

In 1563 (Ibanez, 1952), Bartholomaeus Eustachius first described the adrenal gland and called it "Glandulae renibus incumbentes." From then on, several names were coined such as "Glandulae renales," "capsulae renales" and "supra-renal capsules."

In the 17th century the idea arose that the adrenals functioned only in fetal life. In 1697, Sampson associated the adrenals with the production of tumors. Tilesius, 1803, reported the first recorded case with autopsy finding of a tumor of the left adrenal in a four-year old obese girl (Soffer, Dorfman and Gabrilove, 1961). In 1811, Cooke similarly described a tumor of the left adrenal in another four-year old obese girl, who died at the age of 7.

In 1855, Claude introduced the idea of internal secretion- the secretion into the circulation by a ductless

organ (Jones, 1957).

The adrenal cortex is indispensable for life and adrenalectomy is fatal. In 1855, Thomas Addison first described a disease which is the result of destruction of the adrenal cortex. Brown-Sequard (1856) showed that cats, dogs, and rabbits die soon after bilateral adrenalectomy. In 1913, Biedl proved that removal of the cortex in dogs and rabbits resulted in death.

2. STEROIDS SECRETED BY ADRENAL CORTEX

In 1927, Ragoff and Stewart extracted fresh dog adrenal gland with 0.9% salt solution and glycerol and found that the clear extract could be injected intravenously with no evident ill-effects. They also stated : "It is impossible to draw any other conclusion than that the extracts in some way prolonged the life of the animals in the absence of the adrenals." This importance of the cortical portion led the quest for the active principle named "cortin" and investigations along these lines were begun by Hartman, Brownell, Hartman, Dean and MacArthur in 1928. Later, Swingle and Pfiffner (1930) demonstrated that adrexed animals can be maintained by injections of adrenocortical extracts. With other workers, they simultaneously traced the activity to the lipid fractions.

These experiments proved that adrenal cortical extracts contain one or more hormones and these adrenal cortical hormones are steroids. More than 40 compounds have been extracted

from the adrenal cortex. Some are biologically active and the rest can be artifacts. The biologically active compounds isolated are the following: 1) Adrenal cortical hormone proper (corticoids), 21-carbon-atom compound; 2) Androgenic 17-ketosteroids, 19-carbon-atom compound; 3) Estrogens, 18-carbon-atom compound; 4) Progesterone, 21-carbon-atom compound.

2.1. THE CORTICOSTEROIDS

The seven biologically active members of this group are the following: corticosterone (Compound B); 11-dehydrocorticosterone (Compound A); 11-dehydro-17-hydroxycorticosterone (Compound E, cortisone); 17-hydroxycorticosterone (Compound F, hydrocortisone, cortisol); 11-deoxycorticosterone (Compound S); 17-hydroxy-11- deoxycorticosterone (11 deoxycortisol) and aldosterone.

Compounds B, A, E and F possess all oxygen atom at the C_{11} position. They are active in carbohydrate and protein metabolism and have relatively less effect on electrolyte and water metabolism. The 11-deoxycorticosteroids exert a profound effect on electrolyte and water metabolism and no activity in carbohydrate or protein metabolism. Aldosterone is the most active known mineralocorticoid but also has some glucocorticoid effects.

Although these active compounds have been isolated from cortical tissue, all are not actually secreted into the blood stream

By analysis of adrenal-vein blood of various species, three compounds have been consistently identified: cortisol, corticosterone and aldosterone.

In 1951, Nelson identified cortisol as the main component of human systemic blood. Two years later, Farrell and Lamus (1953) were able to identify cortisol and corticosterone from the 14 fractions which were isolated in dog adrenal vein blood. That same year Bush and Sandberg (1953) produced definite chemical proof that the main adrenal steroid circulated in the venous blood of human subjects stimulated by ACTH was indeed cortisol. They were the first to give clear estimates of the cortisol plasma level on stimulated subjects with values ranging from 22 to 66 micrograms %. In normal subjects, an average of 10 micrograms per 100 ml was obtained. Later in 1953, Baylis and Steinbach reported a mean of 9.5 micrograms per 100 ml.

Compound F is the major corticosteroid present in the sheep (Bush and Ferguson, 1953); (McDonald and Reich, 1959); hamster (Schindler and Knigge, 1959); monkey (Bush, 1953; Holzbauer, 1957; Lanman and Silverman, 1957); dog (Reich, Nelson, Zaffaroni, 1950; Bush, 1953; Holzbauer, 1957); hog (Dobriner, Katzenellebogen and Schneider, 1954; Heard et al., 1956); and guinea pig (Bush, 1951, Heard et al, 1956).

Corticosterone (Compound B) is present in the cirlation of the humans at about 1/10 the level of cortisol (Peterson,

1960). The ox and ferret (Hechter, Zaffaroni, Jacobsen, Levy, Jeanloz, Shenker and Pincus, 1951; Schindler and Knigge, 1959) also secrete both B and F. In the rat, mouse and rabbit, the major hormone secreted is corticosterone (Bush, 1953; Hofmann and Davison, 1954; Elliot and Schally, 1955; Southcott, Bendy, Newsom and Darrach, 1956; Cohen, Bloch and Celozzi, 1957; Bloch, Cohen and Furth, 1960; Kass, Hechter, Macchi and Moon, 1954; Vogt, 1955; Reif and Longwell, 1958).

Aldosterone was first isolated in 1952 by Grundy and Simpson. It is 500 times as potent as cortisol in maintaining adrenalectomized dogs in salt and water balance and it can exert its effects in very small quantities. Simpson and Tait (1954) showed in the pooled peripheral blood of 20 normal men, the aldosterone level was 0.08 micrograms per 100 ml while the cortisol level was 2.5 micrograms. With a normal sodium intake, it is secreted at the rate of 100 micrograms per day in adults.

2.2. BIOSYNTHESIS OF CORTICOIDS

Evidence show that cholesterol is utilized for the biosynthesis of corticoids (Hechter, 1953). Zaffaroni, Hechter and Pincus (1951) obtained cortisol and corticosterone from cholesterol - $4\text{-}C^{14}$ by perfusion in the isolated cow adrenal. Incubation of the substrate with cow adrenal cortex tissue was done by Hayano and group (1956) and also with hog adrenal homogenates (Heard, Bligh, Cann, Jellinck, O'Donnell, Rao and Webb, 1956). Werbin and Le Roy in vivo demonstrated

the utilization of cholesterol in man for the synthesis of cortisol by administration of labelled cholesterol and isolation of labelled ring - A tetrahydro derivatives (1956). Cholesterol is an intermediate factor in the biosynthesis but whether it is an obligatory intermediate is questionable. Progesterone is also considered as an intermediate in the conversion (Long, 1947).

The sequence of reactions involved in the biosynthesis of corticoids are still not quite known. Present evidence indicates that they are normally synthesized from acetyl Coenzyme A (Co A) via cholesterol, thus, Acetate → Acetyl Co A and Acetoacetyl Co A → Mevalonic Acid → Squalene → Lanosterol → Zymosterol → Cholesterol → Pregnenolone → Progesterone → Corticosteroids.

2.3 METABOLISM OF CORTICOIDS

The Gallagher group (1958) studied the metabolism of labelled cortisol and have shown that the substance is metabolized rapidly. The biological half-life is about 3 hours. These metabolic changes occur predominantly in the liver. The factor which protects the corticoids from rapid metabolic inactivation and conjugation in the liver is the corticosteroid binding globulin (CBG) also known as "transcortin." The glucocorticoids are present in two physico-chemical states: a) bound to protein and b) unbound. This protein-binding capacity helps the transport of these steroids and they serve to limit the activity of these hormones at the cellular level by restricting

their diffusion across the capillary wall (Paschkis, Rakoff, Cantarow, Rupp, 1967).

In man, the excretory products of the corticosteroids are present mainly in the urine. They are in the following forms: 1) biologically active compounds in their original form such as cortisol and corticosterone which are largely conjugates of glucuronic acid with small amounts of conjucates of sulphuric acid; 2) biologically inactive reduction products, free and conjugated such as tetra-hydrohydrocortisone and tetra-hydrocortisone; 3) 17-ketosteroids; 4) pregnanediol and pregnanetriol in small amounts.

Reduction at C-20 maybe the major step in the metabolic transformation of B and its 11-keto analogue (Engel, Carter, and Fielding, 1955). Cleavage of the side chain has not been reported for B. Hydroxylation of B at C-18 is known to occur in adrenal tissues but could not be demonstrated in peripheral tissues. Dehydroxylation of C-21 of B has been demonstrated by the isolation of 11-keto-5β - pregnane-3a, 20a-diol (Baulieu and Jayle, 1957), a reaction analogous to that observed with aldosterone (Engel et al, 1955).

Aldosterone is excreted chiefly as conjugates of glucuronic acid. Most urinary conjugates are tetrahydroaldosterone. Primarily, the liver is the site of conjugation but some acid-labile conjugates are also formed in the kidney (Siegenthaler, Dowdy and Leutscher, 1962; Leutscher et al., 1965).

3. THE ROLE OF CORTICOSTEROIDS IN METABOLISM

The corticosteroids play an important role in electrolyte carbohydrate, protein and lipid metabolism. It has an influence on water turnover, on blood and tissues cells, and on the central nervous system. Its effects are manifested on membrane permeability and on inflammatory tissue reactions.

3.1. ELECTROLYTE METABOLISM

Several of the corticosteroids act as mineralocorticoids, an action which is at least partially explicable on the basis of its chemical structure. Thus, compounds oxygenated at C_{11} and C_{17} (cortisol and cortisone) have a weak action on electrolyte metabolism. Compounds oxygenated only in one position like corticosterone, 11-dehydrocorticosterone, 11-deoxy-17 hydrocorticosterone have a borderline reaction but 11-deoxycorticosterone (Compound S) has a powerful effect. On the other hand, aldosterone with a structure that is entirely different has the most powerful salt-hormone action discovered to-date.

Compounds with salt-hormone activity promote the urinary excretion of potassium and retention of sodium and chloride and consequently retention of water. The kidney tubule is the site of action. Marine and Baumann (1927) showed that the sodium content of cat's blood decreased following adrenalectomy. Britton (1930) stated that saline injections relieved the symptoms of adrenal insufficiency

in dogs. Baumann and Kurland (1927), Hastings and Compere (1931) had reported that removal of the adrenal glands was followed by a rise in the serum K level and at death, very high concentrations were reached. The therapeutic value of a diet low in K in the treatments of Addison's disease or for the maintenance of adrexed animals has been demonstrated repeatedly (Allers et al., 1936;Allers and Kendall, 1937; Nilson, 1937; Wilder, Kendall, Snell, Kepler, Rynearson and Adams, 1937). Despite the considerable amount of work conducted up to 1950, some crucial facts relationg to the regulation of electrolyte metabolism by the adrenal cortex were still lacking.

3.2. CARBOHYDRATE METABOLISM

As early as 1910, Porges pointed out the frequency with which hypoglycemic episodes occurred in adrenalectomized dogs. Simpson (1932) observed that patients with Addison's disease failed to show a rise in the blood sugar level comparable to that of normal individuals following the injection of a standard dose of epinerphrine. Long, Katzin and Fry (1940) demonstrated in normal and adrexed fasting rats and mice an increase of liver glycogen and blood glucose following the administration of adrenal cortical extracts.

During the early periods of investigation of the functions of the adrenal cortex, the relationship of the cortex to CHO metabolism was a source of great conflict between those groups who insisted that the CHO disturbances observed in adrexed

animals were fundamentally related to the absence of the adrenal cortex, and their opponents who postulated that these disturbance were non-specific in character and rather related to malnutrition so commonly present in the adrexed animals. Today, there is no question concerning the fundamental role which the adrenal plays in CHO metabolism, inasmuch as the administration of glucocorticoids can reverse the effects of adrex on CHO metabolism.

The chief effects of the 11-oxygenated corticosteroids are the following : 1) they increase the blood glucose concentration by increasing the output of glucose by the liver; 2) they diminish glucose tolerance by diminishing the peripheral uptake and utilization of glucose; 3) they increase liver glycogen due to increased gluconeogenesis. Cortisol has the highest activity in carbohydrate metabolism followed by cortisone while corticosterone and compound A is about 1/3 as active. Aldosterone's activity is about 1/3 that of cortisone and 2/3 that of corticosterone (Paschkis, Rakoff, Cantarow, Rupp, 1967).

3.3. LIPID METABOLISM

The effects of glucocorticoids to lipid metabolism is just secondary to their effects on carbohydrates metablism. The glucocorticoids cause an increase in release of unesterified fatty acids from adipose tissues. This is presumably due to their action in depressing utilization of glucose (Brown, Englert, 1961).

3.4. PROTEIN METABOLISM

In 1940, Long et al found that administration of adrenocortical extracts to fasting intact animals resulted in an increase urinary nitrogen excretion. In the liver, glucocorticoids cause increased incorporation of amino-acids into protein. Intensive stimulation with ACTH of the adrenal cortex in man is followed by a considerable increase in the urinary excretion of various amino acids (Roberts, Ronzoni, and Frankel, 1951).

3.5. OTHER METABOLIC EFFECTS

3.5.1. ON WATER TURNOVER

Swingle, Remington, Hays, and Collins (1941) and Eversole, Jaunt and Kendall (1942) demonstrated that DOC has a capacity to prevent water intoxication in adrexed animals but whole glands extracts or the corticoids are more effective. The adrenocortical hormones influence water excretion by these mechanisms-the retention of sodium causes increased water retention through tubular reabsorption and the direct action on water excretion of the C_{11}- oxygenated steroids especially cortisol causes water loss.

3.5.2. ON BLOOD AND TISSUE CELLS

Injections of C_{11}- oxygenated cortical

steroids or excessive secretion of these compounds due to ACTH stimulation is followed by decrease in size of the lymphatic tissue and a rapid drop in the number of circulating lymphocytes (Dougherty, 1952). Thus in adrenalectomized animals, the lymphatic tissue is large and the number of circulating lymphocytes is increased. Jaffe (1924) found that the thymus of rats hypertrophied after adrex. Marine, Manley and Baumann (1924) demonstrated similar results in rabbits and noted that even if the adrex had been performed when the thymus was already undergoing involution, regeneration occurred. Other workers (Zwemer and Lyons, 1928; Corey and Britton, 1932) showed that adrex not only increases the size of the thymus, but it results in an increase in circulating lymphocyte and a decrease in circulating polymorphonuclear neutrophies.

Following the injection of Compounds E and F, the number of circulating eosinophils and basophils drops rapidly but there is a rise of neutrophils. There is also an increase in erythrocytes (White and Dougherty, 1945). However, in adrenalectomized animals, the number of circulating eosinophils is increased (Forsham, Thorn, Prunty and Hills, 1948).

3.5.3. ON MEMBRANE PERMEABILITY

The 11-oxygenated corticosteroids exert an inhibiting effect on membrane permeability. The spread of dyes is

inhibited by a pretreatment of cortisone or 11-dehydrocorticosterone (Compound A). It is believed that the adrenal steroids act upon the ground substance of membranes, hyaluronate, making it unresponsive to the enzymes, hyaluronidase. This is especially important in the pathogenesis of rheumatoid arthritis and of collagen diseases in which cortisone has a therapeutic value (Paschkis, Rakoff, Cantarow and Rupp, 1967).

3.5.4. ON INFLAMMATORY TISSUE REACTION

Cortisol and cortisone by inhibiting the basic processes of the inflammatory reaction suppress the local and systemic reactions to inciting inflammatory agents. Thus perivascular exudation and edema are diminished and also the formation of pus. Blood histamine levels have been found to be raised after adrenalectomy (Wilson, 1941). Following treatment with cortical extract the ability to inactivate histamine is restored to normal (Rose, 1939).

3.5.5. ON CENTRAL NERVOUS SYSTEM

The influence of adrenocortical steroids on brain excitability was studied extensively in the rat by means of the electroshock threshold (EST). It was found that deoxycorticosterone acetate raised the EST while hydrocortisone, cortisone and corticosterone lowered it.

Cortisone or ACTH administration in man caused euphoria and sometimes precipitated psychosis (Sourkes, 1962).

Williams (1962) stated that about 40% of patients with Cushing's syndrome suffer from major psychologic abnormalities. Soffer, Dorfman and Gabrilove (1961) added that minor mental symptoms and major psychoses were observed in 2/3 of patients with Cushing's syndrome and also a prominent feature in patients with Addison's disease. Cleghorn (1951) reviewing cases of Addison's disease found apathy and negativism in more that 75% of the patients, seclusiveness, depression and irritability as symptoms in 50%, with suspiciousness, agitation and paranoid delusions present to a very limited extent. Such personality changes can be relieved by combinations of cortisone and DOC or with corticosterone.

3.5.6. ON TUMOR TISSUE

The corticosteroids have an effect on tumor tissue but this will be discussed in a later chapter.

CHAPTER 2

THE ANDROGENS

1. HISTORICAL BACKGROUND

Compounds have been isolated from the urine of male, females and eunuchs and from testicular extracts, which by injection into castrated or immature males cause restoration or development of the male genital organs and secondary sexual characteristics. These are the so-called male hormones or androgens (Greek, Andro, male) of which testosterone, androsterone and dehydroepiandrosterone are the most important (Shoppee, 1964).

Investigators in the early centuries were able to relate the physiological influence of castration and the loss of a biologically active substance, the androgen. In 1775, Bordeu, specifically ascribed the characteristics of castrated men and women and of capons to the absence of the gonads and declared that the testis emanated certain active principles into the chyle. In 1849, Berthold, a physiologist, first demonstrated the truth of such a theory by transplanting testicular tissue into capons. Later, in 1889, Brown-Sequard demonstrated the concept of internal secretion by the injection of testicular extracts conducted on himself. In 1911, Pezard was able to show that extracts of testis could actually stimulates the capon's comb. The first convincing demonstration of androgens in testis was performed in the laboratory of Koch, where McGee in 1926 succeeded in extracting a powerful androgen from the lipoidal extracts of bull testis. In 1931, Butenandt succeeded

in isolating an androgen from male urine and named the hormone androsterone. In 1934, Ruzicka and other workers produced androsterone by chemical means. In 1935, David of Laquer's group isolated a more potent androgen from the steer testis tissue and named it testosterone. This was verified by synthetic preparation reported by Butenandt (1935) and Ruzicka (1935). Following the discovery of testosterone, several research groups like Butenandt and Ruzicka, Schering in Berlin, Ciba in Basel prepared and submitted to assay numerous analogues of the hormone; yet no substance of higher physiological activity was encountered.

2. SOURCES OF ANDROGEN

Androgens are produced in at least 3 tissues; testis, ovary and adrenal.

2.1. TESTIS

As early as 1926 McGee demonstrated an androgenic material extracted from the lipids of bull testis. The site of hormone production is in the interstitial cells of Leydig (Bouin, 1903). Several experiments have shown the activity of the male sex hormones in conditions where the germinal epithelium is damaged as in spontaneous cryptorchidism (Moore and McGee, 1928), in testis exposed to X-rays (Talbot and Butler, 1940) in the Klinefelter's syndrome (Wilkins, 1965) without impairment of the interstitial tissue. Finally, Venning, Hoffman and Browne (1942) have demonstrated

an increased urinary excretion of androgens in cases of interstitial-cell tumors.

2.2. OVARY

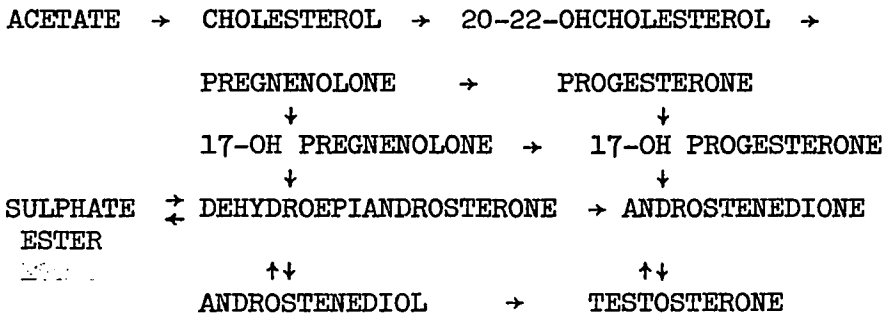
Androgenically active materials were isolated from the fat-soluble extracts of sow ovarian tissue (Parker, 1937). Transplanted ovaries of mice can be induced to produce androgen with the proper environmental temperature (Hill, 1937). Deanesley (1938) confirmed these findings in experiments with rats. Some virilizing tumors of the ovary can produce considerable amounts of androgens (Paschkis, Rakoff, Cantarow and Rupp, 1967).

2.3. ADRENAL

C_{19} steroids have been isolated from extracts of adrenal glands. Block, Dorfman and Pincus (1957) isolated androstenedione and its 11-β-OH derivative from beef adrenal glands perfused with ^{14}C - acetate and ^{14}C - cholesterol. ACTH stimulation of the adrenal cortex in both adult men (Migeon, 1955; Wieland, Courcy, Levy, Zala and Hirschmann, 1965) and women (Wieland et al, 1965; Short, 1969) leads to an increased secretion rate of free and esterified dehydroepiandrosterone and androstenedrione. Female CE mice spayed at 1 to 3 days of age, develop tumors which appear to secrete androgenic material as shown by the growth and development of the accessory sex organs (Wooley, and Little, 1945).

3. BIOSYNTHESIS AND METABOLISM

The precursor of androgens in all 3 glands is probably cholesterol derived from acetate (Menon, Dorfman and Forchielli, 1965), but Hall (1964), Shimizu and Gut (1965) studied a direct pathway via zymosterol or desmosterol to pregnenolone. At the present time, it is believed that the pathway of synthesis is as follows:



The site of androgen inactivation is the liver, skin and skeletal musculature. A group of workers have shown that free dehydroepiandrosterone can be converted into androstenedione and testosterone (Mahesh and Greenblatt, 1962; Camacho and Migeon, 1964) mainly in the liver (Klempien, Voigt and Tamm, 1961; Lipsett and Korenman, 1964). Some enzymes in the liver act on certain C_{21} steroids producing circulating androstenedione (Forchielli, Rosenkrantz and Dorfman, 1955). Also, Gallagher, Fukushima, Berry, and Dobriner (1951), Wotiz, Lemon and Voulgaropoulous (1954) and Breuer, Nocke and Pechthold (1959) have shown that androstenedione and testosterone are readily inconvertible in the body.

The conjugation of androgen metabolites with glucuronic or sulphuric acid occurs mainly in the liver. Baulieu, Corpechot, Dray, Emiliozzi, Lebeau, Mauvais-Jarvis and Reobel (1965) have shown that certain steroids are secreted in esterified forms and principally dehydroepiandrosterone. In the circulation about 5% of plasma free testosterone is converted to testosterone glucuronoside (Robel, Emiliozzi and Baulieu, 1964) and androstenedione can be converted to the same product in the liver (Korenman, and Lipsett, 1965). Thus, this conjugate is not a unique metabolite of plasma testosterone.

Androgen glucuronosides are excreted rapidly and are probably filtered through the glomerulus and the sulphates secreted by the renal tubules (Bongiovanni and Eberlien, 1957; Kellie and Smith, 1957; Cox and Kellie, 1965). The sulphate esters are excreted more slowly, aetiocholanolone sulphate being cleared first then dehydroepiandrosterone sulphate. About 15% of an infused esterified androgen metabolite like androsterone sulphate is excreted into the bile and about 80% are reabsorbed to undergo enterohepatic circulation (Baulieu et al, 1965).

4. THE ROLE OF ANDROGENS

The male sex hormones exert manifold influences in the body mainly on the reproductive, skeletal, cardiovascular and nervous systems.

4.1. THE REPRODUCTIVE SYSTEM

Testosterone, the most potent of the androgens, is responsible for the changes in the male genitalia from puberty to maturation. This includes development of the penis, seminal vesicles and prostate, growth and pigmentation of the scrotum and the appearance of sexual hair. Somatic sex differentiation is believed to be hormone dependent (Bouin and Ancel, 1903). The work of Jost in embryo revealed that androgen derived from the foetal testis is responsible for the development of the Wolffian ducts in males and regression of the Mullerian ducts in females (Jost, 1954). Simpson and Evans have presented evidence that this hormone governs spermatogenesis (Simpson and Evans, 1946).

Androgens are inhibitory to mammary development and function in man (Kurzrok and O'Connell, 1938). Ulrich (1939), Lisser (1946), Farrow and Woodard (1942), Fels (1944), Adair and Herrmann (1946) have described pronounced improvement with testosterone therapy in women with mammary cancer.

Gonadotrophic effects of androgens on the ovary have been observed in intact guinea pigs and rats as shown by the maturation of follicles and formation of corpora lutea (Salmon, 1941).

4.2. THE SKELETAL SYSTEM

Testosterone increases the mass and strength of the skeletal muscle due to its protein-anabolizing effect (Thompson and Heckel, 1939) and hence has been utilized as treatment in dwarfism (Werner and West , 1943). Androgens have been administered in cases of acromegaly (Goldberg and Lisser, 1953). Prager (1940) observed clinical improvements in otosclerosis with testosterone and estrogen therapy. Testosterone accelerates epiphyseal closure (Gardner, 1969).

4.3. THE CARDIOVASCULAR SYSTEM

Testosterone affects the cardiac muscle by improving its functional activity (Thompson, 1955). Korenchevsky, Hall, Burland and Cohen (1941) observed a decrease in the size of the heart and the potential energy of the heart muscle after castration in the rat (Prager, 1940). Hamm (1942), Lesser (1942), Levine and Likoff (1943) and Waldman (1945) have reported the use of testosterone in angina pectoris.

4.4. THE NERVOUS SYSTEM

Kearns (1941) have claimed that the male sex hormone specifically increases the resistance of the nervous system against fatigue. Testosterone therapy has been used in the

male climacteric (Turner, 1939) and in cases of involuted melancholia (Danziger, Schroeder and Unger, 1944). Torda and Wolff (1944) have studied the influence of androgen in the synthesis of acetylcholine by minced frog brain. Kral and Wigdor (1959) studied the effects of androgen on senescent memory function. They have shown that this substance produced a feeling of well-being and had some sedative effects in an experimental group. Also, significant improvement was found in recall of logical material but there was no rise in memory scores.

4.5. METABOLIC EFFECTS

Androgen is a protein-anabolizer. Following its administration, there is nitrogen retention and weight increase due to retention of sodium, chloride and water. Kochakian (1946) has demonstrated these effects in castrated dogs, rats and man. Studies of Litwack and Kritchevsky (1964) have shown that testosterone produces an increased rate of amino acid incorporation into proteins of various tissues.

An early study on the effects of male hormones on carbohydrate metabolism have led to confusing results-the glucose tolerance of animals was decreased and liver glycogen reduced after methyl testosterone administration but not after testosterone, which increased liver glycogen (Lewis and McCullagh, 1942).

4.6. MISCELLANEOUS EFFECTS

Androgens may stimulated oxygen consumption during hypo and hyperthyroid states and this depends on the dose of thyroxine given simultaneously. Thus, Eidelsberg and Ornstein (1940) showed that thyroxine adminstration was more effective during testosterone propionate therapy than when given alone. Kinsell, Hertz and Riefenstein (1944) found that in three hyperthyroid patients daily doses of 25,50 and 100 mgm. of testosterone propionate had no effect on the high metabolism where as methyl testosterone caused a further rise in the already elevated heat production of such patients.

The profound stimulating effect of the androgens on the secondary sex organ (prostate, seminal vesicles) is well known. Knowledge of this fact proved useful in the therapy of neoplasms of the prostrate where orchiectomy and estrogen administration has proved to be an effective treatment (Huggins, 1967). The influence on stimulating hair growth is also well known. Hamilton (1951) carried out a series of experiments relating hair growth with titres of urinary androgens and ketosteroids. However, the androgens have widespread morphological effects apart from their effect on the secondary sex organ or characteristics.

Thus, testosterone has been shown to increase the size of the serous tubules of the submaxillary gland of mice (Lacassagne, 1940a, 1940b, 1940c,) and of the rat (Grad and Leblond, 1949).

Testosterone also increases the size of the kidney, this effect being especially on the brush border of the proximal convoluted tubules: here the effects of testosterone and thyroxine are additive (Grad, 1949). A similar additive effect of testosterone and thyroxine is apparent in the decrease in the weight of the hypophysis which either hormone produces alone (Grad, 1949). Testosterone causes the disappearance of so-called castration cells in the anterior pituitary, enlarged, slightly vacuolated cells with a basophilic cytoplasm.

Testosterone also stimulates the growth of the cornified layers of skin and in this respect, its effect is opposite to that of thyroxine (Eartly, Grad and Leblond , 1951). Thus, the effect of androgens are widespread and varied.

4.7. EFFECTS ON TUMOR

The androgens have an effect on tumor tissue but this will be discussed in a later chapter.

CHAPTER 3

THE MAMMARY TISSUE

1. NORMAL TISSUE

1.1. MORPHOLOGY IN THE HUMAN

1.1.1. ANATOMY

The breast in the adult women refers to the eminence on the anterior chest wall which has a conical, discoidal or hemispherical shape (Cutler, 1962). A condensation of fibrous stroma forms the suspensory ligaments which anchor the breast to the deep fascia of the thoracic wall. This extends from approximately the 2nd to the 6th or the 7th rib and from the lateral border of the sternum to the axilla.

The breast contains the mammary glandular tissue and filling in between and around this is a considerable amount of adipose and connective tissue. There are 15 to 20 separate branching glands which drain through a separate main excretory or lactiferous ducts into the nipple, a conical or cylindrical structure proecting from the surface of the breast at about the level of the 4th intercostal space just below the centre of the breast. The skin of the nipple appears to be pigmanted and wrinkled and extends radially from 1 to 2 cm. to form the areola. During rest, bundles of unstriated muscle fibers described as myo-epithelial cells by Kuzma (1943) through which the ducts pass to reach the surface, form the main mass of the nipple. In lactation the nipple become enlarged due to the presence of a crowding acini. Surrounding the outside

of the acini and all the ducts is the pericanalicular and peri-acinous connective tissue.

1.1.2. HISTOLOGY

The areola and nipple are lined by stratified squamous epithelium which extends superficially into the mouth of the main lactiferous ducts. It changes into a pseudo-stratified columnar and double-layered cuboidal epithelium in the major breast ducts. Then as the ducts branch and become smaller, the epithelium tends to become a single layer of cells. In the smaller ducts and in the gland buds, a basement membrance, a layer of myoepithelial cells, low and flattened and on the internal surface a row of low columnar glandular cells can be seen, The interlobular connective tissue is the dense type while the intralobular and periductal connective tissue has a loose mucoid and myxomatous appearance, containing fewer collagenous fibers and almost no fat.

1.1.3. BLOOD AND NERVOUS SUPPLY

The internal mammary artery, the lateral thoracic artery and the intercostal arteries from the aorta give rise to the arteries which supply the blood to the mammary gland. They pass mainly along the larger ducts and on the external surface of the basement membranes of the secretory portions, they break up into very dense capillary networks. Salmon, Gitis, Livshits and Marcus (1936) studies the arterial blood supply of the human breast. The

work of these investigators was later reviewed and summarized by Maliniac (1943).

The mammary gland has secretory nerve endings connected with the glandular elements; these are the nerves which supply the smooth muscles of the blood vessels and of the papilla. Also, there are numerous sensory nerve endings of the nipple.

1.2. MORPHOLOGY IN THE MOUSE

Female mice have five pairs of nipples and mammary glands, three in the thoracic and two in the abdominal region(Hummel, 1966). The mammary glands consist of an extensive duct and alveoli system which open into a nipple. Variation in number and arrangement of nipples is frequent in some strains. Little and MacDonald (1945) found that there are less than 5 pairs in strain A females and more than five pairs in BALB/c females. Male mice have no nipples but only four pairs of rudimentary glands consisting of branching ducts with no alveoli or openings to the exterior.

The mammary glands of prepuberal mice consist of branching ducts lined by low columnar or cuboidal cells with dark staining oval nuclei and a small amount of cytoplasm. Pearson and Richardson (1954) demonstrated the presence of myoepithelial cells with an intense alkaline phosphatase reaction, between the epithelium and basement membrane. Postpuberal development reaches the maximum in virgin females of 4 to 7 months with further duct proli-

feration and the formation of a few isolated alveoli(Nandi,1958). Alveolar development in the glands of virgin female differs in strain (Richardson and Hummel, 1959). The glands of strain R111 virgins have few alveoli while the glands of C3H mice and of hybrids between C3H and R111 contain lateral buds and clusters of alveoli (Richardson and Hall, 1960). Complete lobuloalveolar development occurs during pregnancy and lactation periods. Growth reaches a peak by the end of the second week when numerous lobules made up of alveoli lined with single layers of low columnar and cuboidal cells are present (Wellings, DeOme and Pitelka, 1960). In the completely regressed and resting glands, capillaries are inconspicuous, adipose tissue fills the spaces and the duct lumina are narrow. In the aged, the glands undergo gradual involution, distal duct branches become atrophic leaving only the main ducts and a few secondary branches (Hummel, 1966).

1.3. PHYSIOLOGY

1.3.1. DEVELOPMENT

At birth, the mammary gland of the newborn is about 4 or 5 mm. in diameter. The flat nipple enlarges in the first days of extra-uterine life. The breast reaches a maximum size of 2 to 4 cm. in diameter in the 2nd week.

The first changes noted with hypertrophy of the mammary gland at puberty are the swelling of the areola and a flatten-

ing of the nipple due to tension of the skin. There is hyperplasia of the pericanalicular and periacinous connective tissue and of the epithelium lining the ducts and acini. These are followed by an increase in the amount of adipose tissue.

The development of the mammary gland is dependent upon hormonal control. The ovarian hormones, estrogen and progesterone are responsible for these changes. Folley and Malpress (1941) have shown that certain phases of mammary tissue growth is directly due to estrogen stimulation. Turner (1950) has confirmed that estrogen has a direct local vasodilatory effect on breast tissue. The effects of estrogen are as follows: 1) inducing the proliferation of the duct system hence stimulating the growth of the breast; 2) increased pigmentation of the areolae; 3) growth and development of the nipple; 4) development of the alveoli. Progesterone in combination with estrogen causes a greater increase in the size of the breast and in the development of the alveoli.

Other hormones such as the pituitary hormones prolactin, growth hormone and corticotropin also influence the development of the mammary glands.

Turner (1950) proposed the mammogen theory which states that the pituitary gland upon stimulation by estrogen and progesterone secretes mammogenic hormones which acts in the mammary glands. Lyons (1952) studied the effects of these hormones in rats and showed that they lead to a considerable growth of the lobular-

alveolar system. Deschin (1946) discovered that prolactin stimulates the development of acini in spayed female rats.

1.3.2. INITIATION OF SECRETION

The glandular system of the breast is the site of secretion. It is composed of 2 structure namely, the acini (alveoli) and the ducts. The secretion, milk, passes through a channel of 15 to 20 main ducts into the nipple. This secretion comes from multiple lobules, the smallest division of which consists of a cluster of acini, the secretory organ. The glandular elements exhibit different degrees of secretory activity.

1.3.2.1. The Nervous Influences

The suckling stimulus initiates milk secretion in the prepared breast and the cessation of suckling leads to cessation of milk secretion. The initiation of milk secretion is not under direct nervous control though it is susceptible to psychological influences (Keele and Neil, 1965).

1.3.2.2. Hormonal Influences

Estrogen in synergism with progesterone prepares the breast for the action of lactogenic hormone, also referred to as prolactin, luteotropin and mammogenic hormone. Meites and Turner (1948) have shown that estrogen relieved of inhibition by progesterone stimulates the anterior pituitary to secrete an increased amount of prolactin thus inducing lactation. Folley and Malpress (1948) pro-

pounded a double threshold action of estrogen on lactation, a high threshold inhibiting lactation and a low one stimulating it. At parturition, the estrogen level falls between the 2 thresholds, thus relieving direct inhibition on the mammary glands and stimulating secretion of prolactin and other hormones by the anterior pituitary gland. Cowie (1955) studied the effect of anterior pituitary hormones on hypophysectomized rats during lactation.

1.3.3. CESSATION OF SECRETION

The glandular elements return to a resting stage when the mammary gland regresses. The production of milk ceases with the remaining secretion being absorbed rapidly by the glands. The ducts shrink and the size of the breast diminishes remarkably. Yet, a return to the normal virginal breast cannot occur. There remains a permanent residue of some glandular parenchyma. It is presumed that this cessation mechanism is due to the absence of prolactin secreted by the anterior pituitary and other factors induced by the suckling stimulus.

1.3.4. INVOLUTION DURING AGING

In old age, the mammary gland undergoes involution gradually. The gland tends to return to the prepubertal condition of which the epithelium of the secretory portions and part of the excretory ducts atrophies. Thus, there are just a few scattered ducts and a few acini. Changes also occur in the interstitial connective tissue.

They become less cellular. There is a decrease in the number of collagenous fibers and the whole mass becomes homogeneous. In the very aged, the gland buds may sometimes partially disappear creating a pattern similar to the male breast. Yet, this is back fired by some persistent estrogenic stimulation of adrenal origin possibly in most women, maintaining the vestigial remnants of the gland buds, thus differentiating even the very aged female breast from the male.

2. NEOPLASMS OF THE BREAST

Neoplasms constitute the most important lesions of the female breast due to their potential significance to the patient (Robbins, 1964). These tumors can be classified into fibroadenoma, papilloma and carcinoma.

2.1. MORPHOLOGY OF TUMORS IN HUMAN

2.1.1. FIBROADENOMA

Fibroadenoma is a new growth of both fibrous and glandular tissue. It is the most common benign tumor of the female breast. The increased sensitivity of a focal area of the breast to estrogen results in the development of this tumor. It grows as a centrifugal, small nodule that is sharply circumscribed and freely movable from the surrounding breast substance. The histologic pic - ture is of a delicate, cellular, fibroblastic stroma enclosing glandular and cystic spaces lined by epithelium. Intact, round to oval gland spaces are present, lined by a single or multiple layers

of cells. The glandular lumens are collapsed and compressed to slit-like clefts. The connective tissue element is scanty. Thus, the entire tumor is composed of fairly dense packed glandular or acinar spaces lined by a single or double layer of cells (Robbins, 1964). Cheattle and Cutler (1931) have presented details of individual cases.

2.1.2. PAPILLOMA

This is a tumor of a central stalk of connective tissue covered by epithelial cells. It may develop within ducts and cysts, hence intraductal or intracystic papillomas. This papilloma is very isolated and solitary. It is a very minute lesion, rarely more than 1 cm. in diameter. Anatomically and clinically, it is extremely difficult to locate. The central connective tissue framework is covered by one to two layers of small, regular cuboidal epithelial cells. A non-developed stalk and a stromal framework is absent in a difuse papillomatosis.

Estes and Phillip (1949) reported a series of cases of intraductal papilloma of the breast with simple mastectomy for treatment.

Haagensen, Stout and Phillips (1951) reported 110 cases of gross papillomas and advised local excision and radical amputation if carcinoma is present.

2.1.3. CARCINOMA

This is the most common form of malignancy in women. Carcinomas arise mostly in the upper outer quadrant (Haimov, Kark and Lesnick, 1968). The central subareolar are in the next favored location but they can extend progressively in all direction. The tumors are bilateral, although in many instances a solitary tumor arises from multicentric foci that later coalesce (Qualheim and Gall, 1957).

Breast carcinoma is divided into two groups - those of ductal or of lobular origin. (Foote and Stewart, 1946). Since carcinomas of lobular origin are very rare with a morphological pattern resembling those arising in ducts, a more adequate classification can be considered by dividing the carcinomas of the mammary ducts into the non-infiltrating and infiltrating varieties. Then non-infiltrating type includes the papillary carcinomas (discussed previously under papilloma) and duct carcinoma. The infiltrating variety may also be subdivided into scirrhous, medullary, colloid carcinoma and duct or comedocarcinoma.

2.1.3.1. Scirrhous Carcinoma

This is the most common variety and accounts for over a half of all mammary carcinomas. The growth consist of nodules of stony hard consistency with a diameter averaging 2cm. but rarely exceed 4 to 5 cm. The form of malignancy is ill-defined. The mass is retracted below the cut surface, has a hard cartilagenous consistency and produces a grating sound when scraped. There are small pin-point foci

or streaks of chalky white, necrotic tumor. A histological picture of the tumor shows a dense, collagenous, hyaline fibrous stroma with epithelial cells, scattered or isolated in small nest or long, filamentous, irregularly disposed strands. The individual cells are round, polygonal and compressed with small, deeply chromatic nuclei, uniform in size and shape and often do not show a mitotic picture. At the border of the main tumor mass, the neoplastic cells infiltrate into the surrounding fibrofatty tissue, the perivascular and perineural lymphatics and also the blood vessels.

2.1.3.2. Medullary Carcinoma

This is a very uncommon variant characterized by large, bulky, soft, fleshy tumor masses with a diameter of 5 to 10 cm. They are more yielding on external palpitation because they do not have the characteristic formation of fibrous tissue found in scirrhous carcinoma. The tumor do not shrink when cut but bulges above the level of the tissue. The dominant features histologically are a scant stroma and the large irregular masses, sheets and cord of cells that grow in no particular arrangement and form an ill-defined gland or papillary structure. There is a striking lymphocytic infiltration in the scant connective tissue stroma within these tumors.

2.1.3.3. Colloid or Mucinous Carcinoma

This is another unusual variant which tends to grow slowly over the course of many years. It produces large, bulky, soft,

gelatinous masses which appear to be circumscribed on palpitation but on cut sections, they are blended into the surrounding tissue zones. The tissue is extremely soft with a consistency and appearance of a pale gray-blue gelatin. The tumor undergoes central cystic softening and hemorrhage. Histologically the tumor carries one of 3 patterns of growth. There are large lakes and masses of basophilic, amorphous mucin that dissects and extends into continguous tissue spaces and planes of cleavage. Floating within this mucin are small islands and isolated neoplastic cells which sometimes form glands. Vacuolation of the cells is characteristic. In other colloid tumors, there is a well-defined gland whose lumens contain most of the mucinous secretions. Some mucin can be found in the interglandular spaces and in the fibrous stroma. The cells lining the glands are also vacuolated. The 3rd picture shows a tumor with an undifferentiated mass of cells, most of which are distended with large vacuoles of mucin producing a characteristic signet ring pattern.

2.1.3.4. Duct Carcinoma

This tumor can be non-infiltrating or infiltrating. It is believed that these tumors begin as non-infiltrating anaplastic proliferations of ductal epithelium that eventually fill and plug the ducts with neoplastic cells. Thus, in the non-infiltrating stage, the tumor exists as a poorly defined focus of slightly increased consistency caused by the marked dilatation and solidification of the ducts. On cut section, the cord-like ducts are filled with a cheesy and necrotic tissue which can be easily extruded with slight pressure, hence, the name come-

docarcinoma. Histologically, the ducts are dilated, filled with neoplastic epithelial cells which plugs the lumens completely. The cells are compressed and anaplastic solid cords are visible. The central cores of these cell masses are necrotic. As the lesion advances, the neoplasia extends into the basement membrane thus becomes infiltrative. This infiltrative duct carcinoma has characteristic of the scirrhous type.

There are other morphologic features common to all these breast carcinomas regardless of histology. The breast carcinoma is fixed in position due to its adherence to the deep fascia of the chest wall. The extension to the skin causes not only fixation but also retraction and dimpling of the skin- a very important characteristic of malignant growth. Simultaneously, the lymphatics become involved by blocking the area of skin drainage. This causes lymphedema and thickening of the skin characteristically referred to as the "orange peeling."

2.2. MORPHOLOGY OF MAMMARY TUMORS IN C3H MICE

The characteristic feature of a precancerous lesion is a hyperplastic nodule consisting of a localized area of acinar and alveolar proliferation. Jones (1950) demonstrated that hyperplastic nodules are numerous in very old female mice. A cut section shows a grayish white tumor tissue, soft with many blood-filled cysts and central necrosis. The tissue shows a well differentiated and orderly arrangement of small and uniform-sized cells. In the benign stage, there is paucity

of mitotic figures. However, the tumor metastasize readily. Vacuoles appear in the cell cytoplasm and the luminal spaces are filled with fluid.

2.3. HORMONAL INFLUENCES

2.3.1. EARLY HISTORY

In 1896, Beatson performed ovariectomy in women with advanced breast cancer at the time when there was no knowledge of hormones. Lett (1905) studied 99 patients with breast carcinoma treated by ovariectomy. In 1919, Loeb established that the occurrence of mammary tumors in mice was limited to females alone and that ovariectomy reduced their incidence. Murray (1928) demonstrated the production of mammary tumors in castrated male mice grafted subcutaneously with an ovary. With the advent of the carcinogenic polycyclic hydrocarbons whose structure resembles the steroid hormones, Lacassagne in 1932 reported the induction of mammary tumor in male mice injected with folliculin benzoate in oil. Between 1939 and 1942, several investigations showed the beneficial effects of ovariectomy and of androgens in advanced carcinoma of the breast in women and of orchidectomy in mammary carcinoma (Loeser, 1941; Farrow and Adair, 1942). In 1944, Haddow, Watkinson and Paterson surprisingly reported that synthetic estrogen produced significant retardation in mammary cancer which was contrary to the belief that estrogens were presumably involved in the cause of cancer of the breast. In 1951, Huggins and Bergenstal introduced adrenalectomy for advanced neoplasms of the breast and the prostate.

Luft and Olivercrona (1955) introduced hypophysectomy as experimental study of the hormone-neoplasm relationship. In 1962, Bulbrook and Hayward investigated a possible association between abnormal urinary hormone excretion and the subsequent development of breast cancer. Later in 1965, they described a discriminant function based on the urinary excretion of 17 hydroxy-corticosteroids and etiocholanolone with a promising prediction of response to ablative treatment (Hayward and Bulbrook, 1965).

2.3.2. PITUITARY HORMONES

The pituitary synthesizes and releases into the blood stream protein hormones which influence growth, differentiation and function. Some of these pituitary hormones have a direct or indirect effect on tumors of the mammary gland (Segaloff, 1967). Marked regression of human mammary cancer has been brought about by hypophysectomy (Atkins, Bulbrook, Falconer, Hayward, McLean, Schurr, 1964).

2.3.2.1. Mammotropin

The studies of Furth (1956) and collaborators with mammotropin (LTH) secreting tumors have drawn attention to the possible role of the pituitary in mammary tumor production. Stimulation of mammary tissue with mammotropin induced tumors in Fischer strain of rats. Mammary tumors have also been induced with graftings of mammotropin secreting pituitary tumors with subthreshold doses of methylcholantrene or with use of X-rays. The induction of mammary tumors

and their progressive growth are influenced by mammotropin levels (Yokoro and Furth, 1961). Kim and Yannopoulous (1963) have shown that stimulation of the adult mammary gland and of mammary tumors in rats by small doses of estrogen is indirect and results from a direct stimulation of pituitary mammotropes. The mammotropins are undoubtedly powerful promoters of neoplasia in mammary tissue.

2.3.2.2. Growth Hormones

The induction of mammary fibroadenomas in rats with prolonged administration of growth hormones had been studied by Evans and Simpson (1931). In 1950, Moon, Simpson, Li and Evans reported mammary fibroadenomas in female rats after administration of purified somatotropin (STH). With similar experiments using hypophysectomized animals, no tumors were observed (Moon, Simpson, Li and Evans, 1951). Mirand and Hoffman (1957) injected purified STH in mice bearing 4 different mammary tumors - all showed increased in growth due to the hormone treatment.

2.3.2.3. Other Pituitary Hormones

The thyroid-stimulating hormone plays an unknown role in mammary carcinogenesis. Some if not all the effect of ACTH on mammary carcinogenesis is mediated by its stimulation of the adrenal cortex to secrete corticosteroids whose effects on mammary tumorgenesis will be discussed later. Further studies are obviously required on this

and other pituitary hormones.

2.3.3. THE SEX HORMONES

2.3.3.1. Estrogen

There were several attempts by various investigators to clarify the relationship between estrogens and breast cancer. As early as 1896, Beatson stated that after a bilateral oophorectomy, there was a striking remission of metastatic carcinoma of the breast in 2 women. These remissions are generally considered to be due to removal of estrogens stimulating the growth of tumors. This concept was supported by Huggins and Bergenstal (1952) who found similar results after bilateral adrenalectomy and by Luft and Olivercrona (1953) after hypophysectomy. Pearson, Li, McLean and West (1955) demonstrated that certain malignant tumors of the breast seem to be "estrogen dependent" in the sense that extirpation of the organs secreting estrogens can cause a regression in a primary tumor. Further studies on the relationship of estrogen to breast cancer were carried out by Kalomiris (1967) by measuring the plasma level of estrone, estradiol and estriol in 32 patients with breast cancer and in 8 normal subjects. He showed that there is no significant difference between the normal plasma levels of estrone and estradiol in the 2 groups. The plasma estriol however was considerably higher in breast cancer patients. Okey and Gass (1968) reported that mammary carcinoma incidence in male or castrated male C3H mice was lower when diethylstilbestrol was given in the diet in

intermittent cycles than when given on a continuous basis. A paradox seemed involved in the finding of Haddow, Watkinson and Paterson who demonstrated that phenolic estrogens have an ameliorative effect in human mammary cancer. The resolution of this apparent paradox is that the favorable therapeutic effect of both deficiency or excess of the estrogens is not due to any effect of these hormones directly on the tumor but on differential response of normal and cancer cells to a modification of the hormonal milieu interieur of the body. This is a basic proposition at the present time in the field of hormone restraint of malignant disease.

2.3.3.2. Progesterone

Large doses of progesterone have a suppressive effect on tumor growth in patients with metastatic breast cancer. Studies by Heiman (1945) and Lerner and co-workers (1965) also indicated such inhibition of tumor growth in rats and mice. Huseby (1965) using a high dose of 6-alphamethly, 17-hydroxyprogesterone (Provera) in post-menopausal cases of metastatic mammary carcinoma obtained objective remissions lasting to an average period of eight months. On the other hand, Kaufman (1964) reported exacerbation of tumor growth in patients treated with Provera.

2.3.3.3. Androgens

Farrow and Adair (1942) established that testis

function can sustain mammary cancer in the human male inasmuch as considerable therapeutic benefit followed orchiectomy in such condition. Huggins had reviewed the known androgenic effects upon mammary tumors, listing the inhibitions of neoplasms in rats and mice, the regression of mammary cancer in women and its acceleration in one case in a man (1944).

Rawson and Rall (1955) have observed that testosterone induced a remission of breast cancer in the ovariectomized, adrenalectomized patient.

In 1961, Segaloff reported some tumor suppressing effects with the use of delta - 1 - testololactone, an analogue of testosterone.

A technique of introducing androgens in oil into the surgical wound-bed at the time of primary operation has given improved 5 year survival in certain stages of mammary cancer (Krauss, 1962).

Joseph H. Farrow (1962) suggested androgen therapy in premenopausal and early post menopausal patients with widely disseminated breast cancer. Objective improvements were noted in 19% of 133 patients. Likewise, Pearson, 1967 stated that large doses of androgens produced objective remission in about 20% of post menopausal women with metastatic breast cancer lasting approximately

6 months on the average.

Benard et al (1962) found no differences between the plasma 17- oxosteroid levels in normal pre-menopausal women and patients with breast cancer, although there was a tendency for the latter to have slightly higher levels. Yet, in post-menopausal women with breast cancer, the plasma 17-oxosteroids were significantly raised above those of the controls. However, Desphande's findings (1965) were not in agreement with Benard's : no significant divergence from normality was found but there was a tendency for the plasma 17- oxosteroids of the cancer patients to be at the lower end of the normal range.

Forchielli, Thomas, Freymann, Parsons and Dorfman (1967) have found that in the advanced stage of mammary breast cancer, only one of every five patients treated responded satisfactorily to testosterone treatment; in these the tumor continued to be testosterone responsive if the necessary testosterone concentration in the cell was maintained.

2.3.4. CORTICOSTEROIDS

A half century after the classic intervention of Beatson it was found out that adrenal function can maintain and promote growth of human mammary cancer. The adrenal factor supporting growth of cancer was identified (Huggins and Bergenstal, 1952)

when it was shown that bilateral adrenalectomy with glucocorticoids as substitution therapy can result in profound and prolonged regression of mammary carcinoma in men and women who do not possess gonadal function. The idea of adrenalectomy for treatment of advanced cancer in men had been influenced by the discovery of Wooley et al (1939) that adrenals can evoke cancer of the breast in the mouse.

The adrenal steroids have been used in the treatment of breast cancer because of a possible direct action on the tumor or an indirect one through inhibition of pituitary ACTH and estrogen formation in the adrenal cortex (Block, 1958; Smith, 1954).

Increased plasma 17- OHCS and high cortisol binding have been reported in patients with breast cancer (Sandberg, 1960).

In 1961, Burton and Begg studied the influence of cortisone upon the growth and metabolism of spontaneous and transplanted mammary tumor in C3H mice. The inhibition of tumors by cortisone was more striking in transplanted than in spontaneous tumors.

Schubert, Bacigalupo and Frankenberg, 1961, found high resting levels of plasma corticosteroids in advanced cases of breast cancer and this was associated with a marked response

to ACTH. Benard, Bourdin and Saracino in 1962 noted a great variability between the plasma corticosteroid levels but nevertheless found that the mean level was significantly raised above that in their controls.

In 1965, Deshpande, Hayward and Bulbrook have reported high corticosteroid levels in about half of their advanced breast cancer patients. Yet, Beck, Blair, Griffiths, Rosenfeld and McGarry (1965) found normal plasma corticoid levels in their patients. Both Desphande et al, 1965 and Beck et al, 1965, have commented on the lack of correlation between plasma levels and the amounts of 17-OHCS in the urine.

2.4. HORMONAL IMBALANCE

It has been shown repeatedly in laboratory experiments that tumors can be induced in almost all organs that respond to hormones, by alteration of the hormonal environment; the alterations maybe brought about by hormone excess or by deprivation (Bulbrook, 1965). Thus, favorable effects were obtained both with oophorectomy and injections of large doses of estrogen as mentioned earlier.

Moreover, an interaction between the androgens and the corticosteroids in providing favorable or unfavorable

conditions for breast tumor remissions is suggested by the findings that the levels of etiocholanolone relative to that of the urinary corticosteroids maybe useful in predicting the outcome of various therapeutic procedure utilized at the present time.

The adrenals have been shown to play a role in the development of mammary tumor, and the reasons for this may be two-fold. First, it has long been known that the normal adrenal gland may functionally simulate the ovary by secreting sex hormones, most strikingly when hormone production of the normal ovary declines (Thung, 1962). This could explain why mice ovariectomized at 2 or 3 months of age developed tumors 5 months later than non-operated controls. The reason why tumor development was not totally inhibited but developed later was probably because the adrenals were beginning to secrete sex hormones (Pilgrim, 1957). For these reasons, adrenalectomy has been found to be a useful therapeutic procedure in selected patients with breast cancer (Atkins and Bulbrook, 1964). Secondly, some corticoids such as cortisol have an inhibitory effect on the growth of mammary tumor (Sparks, Deane and Hayashida, 1955; Scholler, Philips, Stenberg and Bittner, 1956; Fauklin and DeOme, 1958) and others such as cortisone produced increased well-being despite advanced disease. Moreover, cortisone has a very favorable effect on the symptomatic hypercalcemia (Segaloff and Gordon, 1956).

Recently, a group of British workers presented evidence that the level of etiocholanolone relative to that of the 17-OHCS in the urine may be useful in predicting the outcome of adrenalectomy or hypophysectomy (Bulbrook, Greenwood and Hayward, 1960; Atkins, Bulbrook, Falconer and Hayward, 1964 or radical mastectomy (Bulbrook, Hayward and Thomas, 1964). More recently still, members of the same group showed in a prospective study that in a substantial number of cancer patients, the excretion of androgen and corticosteroid metabolites was abnormal before any disease was apparent (Bulbrook and Hayward, 1967). Our own studies discussed in the experimental section attempted to investigate the relationship in mice of the tumor-bearing strain.

Finally, clinicians have utilized the principle of altering the hormonal balance in the therapy of breast cancer patients (Segaloff, 1967).

2.5. THERAPY

Cutler and Myers (1967) in a review of the "Clinical Classifications of Extent of Disease in Cancer of the Breast" have stated that precise clinical determination of the extent of the disease and the anatomical site of the original tumor as well as its histological characteristics are the most important pre-requisites for the planning of treatment. Several different treatments are currently in vogue but most patients with mammary

cancer undergo mastectomy. Irradiation either before but especially also utilized hormonal intervention represents a more recent approach. These methods will be summarized now.

Radical mastectomy as a treatment for mammary cancer dates back to 1876 when Moore maintained that mammary cancer required careful extirpation of the entire organ. Thereafter, the operation technique underwent numerous modifications until the present day procedure. Radical mastectomy is usually in conjunction with radiotherapy or hormonal modifications (Ochsner, 1966). Some physicians advocate simple mastectomy in certain conditions (Ravdin, 1966).

Irradiation as an adjunct to mastectomy is in widespread use at the present time as a therapy of breast cancer. While postoperative irradiation for carcinoma of the breast has been widespread in the past, some data indicate that this improved the five year salvage rate for patients and proposals for preoperative irradiation have been made (Nickson, 1966).

Manipulation of the hormonal milieu interieur of the body has been found useful. The best results in this field were obtained by hypophysectomy which produced remissions in 40% of patients lasting an average of $1\frac{1}{2}$ years. Adrenalectomy and oophorectomy produced the same incidence of remission but only of 1 year duration. The administration of estrogens, progesterone, androgens and corticoids were all found to be useful but less so than the

ablation techniques (Segaloff, 1966).

Myer in 1967 stated that the goal of primary therapy is to give each patient the simplest effective treatment. The selection of patients for a particular kind of therapy has proved to be a problem. However, Bulbrook had offered some solution for selection. He developed a discriminant function as follows: 80-80 x 70-OHCS (mg/24 hours) plus etiocholanolone (µg/24 hours). He divided the patients into 2 groups, one with a positive discriminant and the other group of negative discriminants. If patients with positive discriminants are treated by radical mastectomy, the disease will not recur in most. If it does, the recurrent tumors would respond well to adrenalectomy or hypophysectomy. If patients with negative discriminants have radical mastectomy, recurrence will develop in most of them and will not respond favorably to hormonal therapy (Bulbrook, 1965). Thus, proper integration of hormone therapy with surgery, radiation and chemotherapy is but one method which maybe useful in the management of breast cancer.

2.6. USE OF MURINE MAMMARY TUMOR AS AN EXPERIMENTAL MODEL

1903, Jensen performed the first successful serial transplantation of the mammary tumor in the mouse, thus, marking a significant event in experimental cancer research.

Since then the mammary tumor of the mouse has been the most completely studied of all tumors (Dunn, 1959). Its accessibility to palpation, predictable frequency in a number of inbred strains, and ready transplantability have made the MT an invaluable tool for investigations in genetic, viral, hormonal, chemotherapeutic, nutritional and other facets of cancer research. The discovery of the milk agent (Staff, Jackson Memorial Laboratory, 1933) greatly stimulated research on mammary tumors. The agent has the characteristic of a virus and is transmitted by the milk of high mammary tumor strain females to the young. It appears to modify the responsiveness of the mammary gland tissue so that with a favorable genetic constitution and the proper hormonal stimulation, tumors develop at a comparatively early age.

Also important is the fact that mouse strains with a high incidence of mammary tumors can be studied before and during the period of the appearance of the tumor, thus, providing a valuable tool for the study of the aetiology and pathogenesis of the disease closely related to the disease in humans.

Control mice of the same strain not developing the tumor due to a lack of the initiator of the disease (such as the milk factor in C3H mice) can also be obtained.

Further advantages in the use of mice in experimental research stem from their small size, thus, allowing many

animals to be kept in a small space and reducing the costs of feeding and cleaning.

Moreover, short life span of these animals relative to that of other mammals permits a saving of time in conducting the experiments.

PART II

EXPERIMENTS

PART II

1. AIMS OF THE THESIS

The breast is the leading cancer site among women. In Ontario, 3853 death were attributed to breast cancer among females; this is 20.5% of all female cancer death and 3.6 % of all female deaths during the five-year period 1957 to 1961 (Sellers, 1965). During this period, the female death rate for cancer of the breast was 26.0 per 100,000 population. The lifetime probability of death from breast cancer for a female born in Ontario is 3.3%; that is, one in every 30 women will die of breast cancer (Sellers, 1965). Canada has one of the highest incidence of breast cancer in the world.

In the United States, 64,000 new cases of breast cancer are being reported per year. It appears that there is a death-rate of 22 per 100,000 population or 28,000 cases per year (Hess, 1969). Except for a period between the ages of 60 and 64, a woman is more likely to develop breast cancer as she grows older. Five females out of 1,000 of 45 years of age, although asymptomatic, already may have a breast cancer in the early stages (Mersheimer, 1969).

Early diagnosis seems to be the key to survival, yet there remains 25% of patients who, with early diagnosis, do not survive five years. Several methods have been established

to aid in diagnosis. These are the following: cytological examination of nipple discharge, transillumination, mammography, thermography, ultrasonography and the use of liquid crystals in detecting temperature changes. Yet, some of these are still experimental. Undoubtedly, physical examination is still the most efficient method of detecting breast cancer. However, the depressing mortality associated even with early diagnosis suggests that early is not soon enough.

Many neoplasms of the breast are hormonally dependent and their growth can be modified by the removal of a hormonal stimulus especially in their early stages (Meakin, 1969). The relationship of hormones to carcinogenesis has been very actively investigated in mammary tumors of mice (Dunn, 1945) and both the ovarian and testicular hormones have been shown to play a role in mammary carcinogenesis (Dmochouwski, 1953; Bonser, 1961).

Thus, ovarian hormones tend to favor MT development while the androgens have the reverse effect. Noble's comprehensive review on this subject contains a series of relevant references (Noble,1964).

That there maybe an interaction between the androgens and the corticosteroids is suggested by the findings that the level of etiocholanolone relative to that of the urinary corticosteroids (UC) maybe useful in predicting the outcome of adrenalectomy of hypophysectomy (Bulbrook, Greenwood and Hayward, 1964;

Atkins, Bulbrook, Falconer, Hayward, McLean and Schurr, 1964). or radical mastectomy (Bulbrook, Hayward, and Thomas, 1964) in patients with breast cancer. More, recently, it was shown in a prospective study that in a substantial number of MT patients the excretion of androgen and corticosteroid metabolites was abnormal before any neoplastic disease was apparent (Bulbrook and Hayward, 1967).

In view of these studies, the investigations of UC and urinary androgen (UA) was begun in C3H mice, in which a high percentage of females develop MT spontaneously. Plasma corticosterone (PC) levels were also determined in these animals.

2. MATERIALS

2.1. ANIMALS

Eight month old C3H male and female mice belonging to two substrains and of known age were purchased from the Jackson Memorial Laboratory in Bar Harbour, Maine for use in this study. A high proportion of the females of the C3H/HeJ substrain (hereafter referred to as H mice) develop MT spontaneously while the females of the C3HeB/FeJ substrain (hereafter referred to as F mice) do not. The F mice as well as healthy male and female H mice served as controls for the tumor bearing female H mice. Pregnant mice were excluded from study because their PC levels are elevated.

All animals were housed in a room at 26 to 27 degrees centigrade with a humidity of 45 to 55% and illuminated from 7:15 a.m. to 8:30 p.m. EST. The animals were fed Purina Fox Chow and water ad libitium. The collections of urine were made on each of two consecutive days with the animals isolated in metabolic cages containing food and water. Between 10:30 a.m. and 11:00 a.m. of the third day, blood for the PC determination was collected by orbital bleeding. Each day the volume of urine collected was measured following which the cage bottom was washed down with distilled water and collected in the same bottle containing the urine. The volume of urine and distilled water was also measured. Many of the animals were studied at repeated intervals from 9 to 15 months.

3. METHODS

3.1. THE MICRO ASSAY

3.1.1. THE PRINCIPLE

The corticosterone and androgen levels in urine and plasma were determined in the mice by Murphy's ultramicro assay based on competitive protein-binding. The principle of the method involves the competitive binding of labelled and unlabelled steroid by a globulin normally found in the serum or plasma which specifically binds these steroids. A solution containing the steroid binding globulin (SBG) from a standard plasma

or serum and labelled steroid (corticosterone or testosterone) was added to the standards and unknowns and equilibration immediately occurred between the unbound steroid and that bound to the SBG. The amount of labelled steroid now bound to the SBG was inversely proportional to the amount of unlabelled steroid originally present in the unknown. Florisil was then added which removed the free steroids, both labelled and unlabelled, and the amount of labelled steroid bound to the SBG was estimated by an appropriate radioactive-detecting device. The amount of steroid originally present in the unknown was read off from a standard curve which had a range of from 0-8 ng in the determinations of PC and urinary corticosteroids (UCS) and 0-6 ng for urinary androgens (UA).

3.1.2. PREPARATION OF SBG

3.1.2.1. Preparation of CBG-Isotope Solution (Solution A)

Into a 100 ml volumetric flask containing 15-20 ml of distilled water, add 2.5 ml dog's plasma or serum. Then add 0.4 ml of a solution of corticosterone-H^3 (10 µc/ml) in ethanol (specific activity 158 µc/µg). Make up to a final volume of 100 ml with distilled water.

3.1.2.2. Preparation of Androgen Binding Globulin (ABG)

Add 0.5 ml of a solution of testosterone-1-2-H^3 (10μc/ml) in ethanol (specific activity 46.5 Ci/mmol) into a 100 ml volumetric flask partially filled with a phosphate buffer, pH6. Then add 0.5 ml late pregnancy plasma and make up with phosphate buffer to the 100 ml mark.

3.1.3. PREPARATION OF STANDARDS

3.1.3.1. PC and UCS Standards

From a corticosterone (B) solution of 100 mμg/ml in ethanol, pipette in triplicate 0, 0.01, 0.02, 0.03, 0.04, 0.08 ml to give a 0,1,2,3,4,8 mμg standards. Evaporate under a gentle stream of filtered air.

3.1.3.2. UA Standards

From a testosterone (T) solution of 10 mμg/ml in ethanol, pipette in triplicate 0, 0.05, 0.1, 0.2, 0.3, 0.4, 0.6ml to give 0, 0.5, 1,2,3,4,6mμg standards. Evaporate to dryness.

3.1.4. PREPARATION OF UNKNOWNS

3.1.4.1. For PC Determinations

Pipette 0.01 ml plasma into centrifuge tubes containing 1.0 ml ethanol in duplicate, mix and centrifuge for 4 minutes. Decant supernatant carefully into test-tubes. Add 1.0 ml ethanol to the precipitate and repeat process. Evaporate to dryness.

3.1.4.2. For UCS Determinations

Into a centrifuge tube, pipette 0.15 ml urine in duplicate. Add 1.0 ml methylene chloride, mix and centrifuge for 5 minutes. Transfer the extract into a test tube. Repeat extraction with another 1.0 ml of methylene chloride. Evaporate to dryness.

3.1.4.3. For UA Determinations

Pipette 0.3 ml urine in triplicate into separatory tubes. Add 1.0 ml di-ethyl ether. Shake thoroughly. Transfer the ether layer into a Kimax tube. Repeat extraction. With 1.0 ml di-ethyl ether rinse the sides of the separatory tubes and collect final extract. Evaporate to dryness.

3.1.5. PREPARATION OF COUNTING SOLUTION

The counting solution used was Bray's solution, prepared as follows: to 800 ml of dioxane in a litre measuring flask, add 100 ml of anhydrous methanol, 20 ml of ethylene glycol, 200 mg of POPOP (4-methyl-5-phenyloxazolyl benzene); shake well to dissolve POPOP. Add 4 gm PPO (2,5-diphenyloxazole), 60 gm of naphthalene and make up the total volume to 1 litre by adding more dioxane.

3.1.6. PREPARATION OF STANDARD CHECK

To check on the amount of radioactivity of the SBG-isotope solution, 1.0 ml is added to 10 ml of Bray's solution for PC and UCS while 0.5 ml is added for UA and counts are recorded. An Ansitron liquid-scintillation spectrometer with an efficiency of 10.5% for tritium was used for counting.

3.1.7. DETERMINATION OF STANDARDS AND UNKNOWNS

Into each test tube containing the dried solutions of standards and unknowns, add 1.0 ml of SBG isotope solution. Mix well. Warm to 45 degrees centigrade for 5 minutes. Cool to 10 degrees centigrade for 20 minutes. To each test tube, add 40 mg Florisil (measured with a specially-designed plastic

spoon). Shake for 2 minutes on automatic shaker; then return to the 10 degrees centigrade bath for 10 minutes. Florisil settles to the bottom. Pipette 0.5 ml supernatant into 10 ml Bray's solution and shake well. Count each sample to 5,000 twice in a liquid scintillation counter. Plot the time required to count the standards as ordinates versus the concentration (mμg) of the unlabelled steroids as abscissae. This yields a standard curve off which the unknowns are read.

3.1.8. CALCULATIONS

PC: The value read off from the curve x 10 (for 0.01 ml plasma) equals μg/100ml.

UCS: The value read off from the curve divided by 0.150 (for 0.15 ml urine) x the total volume of urine collected in 24 hours equals ng/24 hours

UA: The value read off from the curve divided by 0.3 (for the 0.3 ml urine) x the total volume of urine collected in 24 hours equals ng/24 hours

3.1.9. THE PRECISION, ACCURACY AND SPECIFICITY OF THE ASSAY

The precision of the assays was estimated by calculating the indices of precision of 5 standard curves selected at random from the numerous runs made in this study in

C3H mice. Five such curves were selected for study from the corticosterone series and another 5 from the androgen series of runs. Another measure of the precision of the assays was obtained by calculating the standard deviation of duplication determinations of test plasmas used for the corticosterone determinations in C3H mice as well as of test urines used for the determinations of corticosteroids and androgens in the same strain of animals.

The accuracy of the method was determined by measuring the recovery of added corticosterone or testosterone. One nanogram of B was added to the plasma and urine of 5 healthy male and 5 healthy female, F and H mice and of 5 H females bearing the mammary tumor. Similarly, 1 ng of testosterone was added to the urine of other animals taken from the same groups. Each of the groups also contained 5 animals to which B or T were not added and these served as controls. The % recovery was then calculated.

The specificity was determined previously for the corticoids by Murphy (1967) and by Grad and Khalid (1968) and for androgens by Murphy (1968).

3.2. STATISTICAL METHODS

The data was analyzed as follows: means and standards errors were calculated for various groups of data. Two

types of analysis of variance were conducted - a one-way analysis on the data of the H mice, and a two-way analysis on the data of healthy H and F mice, the latter involved groups with unequal or disproportionate subclass numbers, and the technique used was described by Ferguson (1959). When the F ratio was significant in variables involving more than 2 groups, t test were conducted and correlation coefficients were also calculated. A probability of $P<0.05$ was considered significant.

4. RESULTS

4.1. THE PRECISION OF THE ASSAYS FOR THE DETERMINATION OF CORTICOSTERONE AND ANDROGENS

4.1.1. FOR THE STANDARD CURVES

The index of precision (λ) of 5 standard curves utilized in the determination of corticosterone varied from 0.097 to 0.128 with a mean and standard error of 0.110 ± 0.007. The corresponding values for the standard curves for the determination of the androgens varied from 0.063 to 0.247 with a mean standard error of 0.150 ± 0.040.

4.1.2. FOR THE UNKNOWNS (TABLE 1)

The precision of the determinations of the unknowns was obtained by calculating the standard deviations (SD) of duplicate determinations over a reasonable range of values. Thus, the precision of the determination of plasma corticosterone in the test

TABLE 1

STANDARD DEVIATION (S.D.) OF DUPLICATE DETERMINATIONS OF PLASMA AND URINARY CORTICOIDS AND URINARY ANDROGENS

Variable						
Plasma Corticosterone (mcg %)	0-10	10-20	20-30	> 30		
S.D.	6	7	7	8		
No. of Pairs	9	10	9	9		
Urinary Corticoids (ng/24 hours)	0-100	100-200	200-300	300-400		
S.D.	26	20	20	23		
No. of Pairs	16	17	15	15		
Urinary Androgens (ng/24 hours)	0-0.75	0.75-1.00	1.00-1.25	1.26-2.00	> 2.00	
S.D.	0.10	0.14	0.15	0.16	0.15	
No. of Pairs	12	14	14	13	14	

samples gave an SD of 6 in samples ranging in value from 0-10ng% to an SD of 8 in samples with values greater than 30 ng%.

For the determination of UC, an SD of 26 was obtained in samples ranging from 0 to 100 ng per 24 hours to an SD of 23 in samples with a range of 300-400 ng per 24 hours.

The SD of the urinary androgens was 0.10 in samples ranging from 0 to 0.75 ng per 24 hours and was 0.15 in samples greater than 2 ng per 24 hours.

4.2. THE ACCURACY OF THE METHODS (TABLE 2)

The % recovery of one ng of compound B added to the plasma of male and female F and H healthy mice and H mice with MT varied from 87% in male F mice to 97% in female H healthy mice, a non-significant difference according to analysis of variance ($0.30 > P > 0.20$). The corresponding percent recoveries of one ng of compound B from the urine of same groups of animals varied from 85% in healthy female H mice to 90% in healthy female F mice, also a non-significant difference according to an analysis of variance ($0.50 > P > 0.30$).

The recovery of one ng of T added to the urine of male and female F and H healthy and sick mice varied from 87% in male F mice to 98% in H mice bearing a MT, a significant difference

TABLE 2

THE PERCENT RECOVERIES OF 1 NANOGRAM OF CORTICOSTERONE OR TESTOSTERONE FROM PLASMA AND URINE OF C3HeB/FeJ AND C3H/HeJ MICE

Variable	C3HeB/FeJ		C3H/HeJ		
	Male N=5	Female N=5	Male N=5	Female N=5	Mammary Tumor N=5
Plasma Corticosterone (mcg %)	87[1] ±3[2]	96 ± 3	89 ± 2	97 ± 4	90 ± 5
Urinary Corticoids (ng/24 hours)	88 ±2	90 ± 1	96 ± 3	85 ± 2	89 ± 3
Urinary Androgens (ng/24 hours)	87 ±3	93 ± 1	90 ± 2	89 ± 3	98 ± 2

[1] Mean; [2] Standard Error

by analysis of variance ($0.025 > P > 0.01$). The only significant difference occurred between mice bearing a MT (98%) and male F mice (87%, $0.05 > P > 0.02$).

4.3. HORIZONTAL INVESTIGATIONS ON PLASMA AND URINARY CORTICOSTEROIDS AND ANDROGENS AND OTHER PARAMETERS IN C3HeB/FeJ AND C3H/HeJ MICE.

Table 3 contains the data of 94 C3H male and female mice of which 33 had a MT. Determinations were made in 34 of these animals at least twice during a maximum period of 6 months and in such instances the mean per animal was calculated and utilized in further calculations of the group means presented in Table 3. The individual values of each animal are presented in Tables 4-9 inclusive.

4.3.1. DIFFERENCES DUE TO SEX AND SUBSTRAIN IN HEALTHY F AND H MICE (TABLE 3)

The body and adrenal weight data showed no significant difference due to sex or substrain. Males excreted significantly higher urine volumes per day than females ($0.01 > P > 0.005$) and H mice had similarly higher values than F mice ($0.025 > P > 0.01$).

The PC levels showed no significant difference due to sex but they were significantly higher in F than in H mice ($P = 0.05$). The UC data were significantly higher in female than in male mice ($0.001 > P > 0.0005$) and significantly higher in H than in F mice ($0.05 > P > 0.025$). Renal clearances of corticoids were

significantly higher in females than in males ($0.005 > P > 0.001$) and significantly higher in H than in F mice ($0.01 > P > 0.005$).

UA values were significantly higher in males than in females ($P < 0.005$) and tended to be higher in H than in F animals ($0.10 > P > 0.05$). The UA/UC values were definitely higher in male than in female mice ($0.005 > P > 0.001$). However, there was no significant difference between substrains in this regard.

Finally, males survived longer than females in F mice, and the reverse was true in H mice but in neither case was the sex difference significant. However, the interaction was significant ($P < 0.0005$), the only interaction of all the above parameters which achieved statistical significance.

4.3.2. DIFFERENCES DUE TO THE PRESENCE OF THE MAMMARY TUMOR (TABLE 3)

An analysis of variance of the data between the various groups of H mice, male and female, healthy as well as those bearing a MT, revealed that mice bearing a MT weighed significantly more than healthy females ($0.05 > P > 0.02$). The adrenals of males weighed significantly more than those of the 2 female groups ($P< 0.05$) and similarly the daily urine volume excreted by the male mice was significantly higher than those secreted by the females, sick or healthy ($P < 0.005$).

TABLE 3

VARIOUS PARAMETERS OF MALE AND FEMALE C3HeB/HeJ MICE WITH AND WITHOUT MAMMARY TUMOR DETERMINED SEVERAL TIMES BETWEEN 9 AND 15 MONTHS OF AGE

	C3HeB/FeJ		C3H/HeJ		
	MALE N=10	FEMALE N=16	MALE N=18	FEMALE N=17	MAMMARY TUMOR N=33
BODY WEIGHT (g)	30.3[1]±1.3[2]	29.3±0.6	29.1±0.7	28.2±0.5	30.7±0.8
ADRENAL WEIGHT (mg)	6.5±0.3	6.2±0.5	7.2±0.5	6.6±0.3	6.5±0.2
URINE VOLUME (ml/24 hours)	3.3±1.0	2.2±0.3	5.4±0.9	2.8±0.3	2.3±0.3
PLASMA CORTICOSTERONE (mcg %)	20.1±3.6	22.0±2.7	17.5±2.5	13.8±2.1	25.7±1.4
URINARY CORTICOIDS (UC) (ng/24 hours)	59±10	153±17	122±23	173±23	210±18
RENAL CLEARANCE OF CORTICOIDS (ml/24 hours)	0.5±0.1	1.1±0.3	1.0±0.2	2.1±0.3	0.9±0.1
URINARY ANDROGENS (UA) (ng/ 24 hours)	81±17	38±5	117±13	43±23	41±3
UA/UC	2.8±1.4	0.3±0.1	1.8±0.4	0.3±0.1	0.3±0.01
LIFE SPAN (Days)	512±4	4o8±12	413±13	461±11	429±11

[1] mean; [2] standard

Tumor-bearing mice had significantly higher PC values than did healthy female ($P < 0.001$) or male H mice ($0.01 > P > 0.001$), and significantly higher UC values than males ($0.01 > P > 0.001$). Renal clearances of corticoids were significantly higher in healthy than in sick females or healthy males ($P < 0.001$).

Male mice had the highest UA values ($P < 0.001$), there being no significant difference in this respect between healthy and sick female mice. The same was true of the UA/UC data ($P < 0.001$).

Healthy females survived significantly longer than males ($0.05 > P > 0.02$), but other differences in this regard were not statistically significant. The weight of the tumor itself at death was 6.8 ± 0.3 grams.

4.4. LONGITUDINAL INVESTIGATIONS ON THE PLASMA AND URINARY CORTICOSTEROIDS AND ANDROGENS AND DERIVED PARAMETERS IN C3H/HeJ AND C3HeB/FeJ MICE.

4.4.1. IN FEMALE C3H/HeJ MICE BEFORE AND DURING THE APPEARANCE OF THE MAMMARY TUMOR (TABLE 4)

Table 4 presents the daily UA and UC amounts and the PC levels determined in each of four female H mice before and during the period of the appearance of the MT.

The UA values were higher after the appearance of the tumor in 3 out of 4 mice than prior to this time. Moreover, in

TABLE 4

URINARY ANDROGENS, URINARY AND PLASMA CORTICOIDS
AND DERIVED PARAMETERS DETERMINED IN THE SAME FEMALE C3H/HeJ MICE
BEFORE AND DURING THE APPEARANCE OF A MAMMARY TUMOR

MOUSE	REMARKS	AGE (Days)	URINE ANDROGENS (UA) (ng/24hrs)	URINE CORTICOIDS (UC) (ng/24hrs)	URINE UA/UC	PLASMA CORTICOSTERONE (mcg %)	RENAL CLEARANCE OF CORTICOIDS (ml/24hrs)
Loff	Tumor Absent	284	18	180	0.10	8.7	2.07
		311	21	131	0.16	4.5	2.91
		334	32	209	0.20	8.3	2.52
		375	32	263	0.12	22.1	1.19
		402	28	114	0.25	29.7	0.38
	Mean	341	26	147	0.17	14.7	1.81
	Tumor First Observed	452	40	232	0.17	24.0	0.97
		468	38	392	0.10	36.1	1.09
	Mean	460	39	312	0.15	30.1	1.03
	Died	470					
Loff R1	Tumor Absent	291	73	174	0.42	7.3	2.38
	Tumor First Observed	472	85	328	0.27	41.0	0.80
	Died	520					
4L2	Tumor Absent	326	142	145	0.98	6.3	2.30
	Tumor First Observed	417	60	376	0.16	17.0	2.21
		458	50	234	0.21	23.8	0.98
		465	45	390	0.12	30.6	1.27
	Mean	447	52	333	0.16	27.2	1.49
	Died	518					
6L1	Tumor Absent	459	32	142	0.22	4.3	3.30
	Tumor First Observed	465	40	243	0.16	20.3	1.19
	Died	465					

two of the 4 mice with several determinations during the tumor period the UA values declined the longer the mice bore the tumor.

In the UC of animal 1 LOFF, the 2 values obtained after the tumor was observed were higher than 4 out of the 5 values obtained prior to its appearance. In the remaining 3 animals, the values obtained after the appearance of the tumor were in every case higher than the values found prior to its appearance. The same was found to be true of the PC level.

The UA/UC values were lower during the tumor-bearing period in every value obtained in 3 out of 4 mice when comparisons were made with the tumor free period. In the 4th animal, the mean value of the "tumor-bearing" period was smaller than that obtained during the "tumor-free" period but here the differences were not as striking as in the other 3 animals.

The renal clearances of corticoids were lower in 3 out of 4 mice during the tumor-bearing period. In the 4th animal, both values of the postperiod were lower than 4 out of 5 pre-tumor values.

4.4.2. IN FEMALE C3H/HeJ MICE BEARING MAMMARY TUMORS (TABLE 5)

Table 5 contains the UA and UC amounts and the PC levels in each of 8 female H mice bearing a MT.

In all 8 mice, the UA values tended to decline the

TABLE 5

URINARY ANDROGENS, URINARY AND PLASMA CORTICOIDS AND DERIVED PARAMETERS DETERMINED IN FEMALE C3H/HeJ MICE BEARING A MAMMARY TUMOR

MOUSE	REMARKS	AGE (Days)	URINE: ANDROGENS (UA) (ng/24 hrs)	URINE: Corticoids (UC) (ng/24 hrs)	UA/UC	PLASMA CORTICOSTERONE (mcg%)	RENAL CLEARANCE OF CORTICOIDS (ml/24 hrs)
R2 Loff	Tumor First Observed	367	35	75	0.77	34.3	0.22
		417	25	296	0.09	28.0	1.06
		430	20	94	0.21	20.0	0.47
	Mean	405	27	155	0.36	27.4	0.58
	Died	440					
Roff	Tumor First Observed	375	70	200	0.35	43.9	0.46
		402	50	180	0.28	22.0	0.82
		438	35	78	0.45	15.0	0.52
	Mean	405	52	153	0.36	27.0	0.60
	Died	454					
R2	Tumor First Observed	438	35	87	0.40	14.5	0.60
		445	32	298	0.11	35.0	0.85
	Mean	442	34	193	0.26	24.8	0.73
	Died	501					
Roff	Tumor First Observed	465	50	102	0.49	---	---
		472	40	106	0.38	14.3	0.74
		478	38	241	0.16	15.3	1.58
	Mean	472	43	174	0.34	14.8	1.16
	Died	507					

TABLE 5

URINARY ANDROGENS, URINARY AND PLASMA CORTICOIDS AND DERIVED PARAMETERS DETERMINED IN FEMALE C3H/HeJ MICE BEARING A MAMMARY TUMOR

MOUSE	REMARKS	AGE (Days)	URINE: ANDROGENS (UA) (ng/24 hrs)	URINE: CORTICOIDS (UC) (ng/24 hrs)	URINE: UA/UC	PLASMA CORTICOSTERONE (mcg%)	RENAL CLEARANCE OF CORTICOIDS (ml/24hrs)
2L1R1	Tumor First Observed	291	80	231	0.34	20.7	1.11
		312	13	144	0.10	24.6	0.59
		334	26	359	0.10	12.1	2.97
	Mean	312	40	245	0.18	19.1	1.56
	Died	363					
2L2	Tumor First Observed	305	59	278	0.21	30.5	0.91
		319	29	113	0.26	23.3	0.48
		334	27	322	0.10	36.7	0.88
		340	16	322	0.10	23.2	1.39
		375	22	322	0.10	25.9	1.24
	Mean	335	31	271	0.15	27.9	0.98
	Died	375					
3L1R2	Tumor First Observed	326	65	176	0.37	25.5	0.69
		347	55	102	0.54	23.5	0.43
	Mean	337	60	139	0.46	24.5	0.56
	Died	421					
3L2R1	Tumor First Observed	347	50	92	0.55	26.6	0.35
		367	45	45	1.00	33.4	0.13
		417	32	203	0.16	30.0	0.68
		438	30	163	0.18	21.3	0.77
		445	30	117	0.26	18.6	0.63
	Mean	403	37	124	0.43	26.0	0.51
	Died	501					

longer the animals bore the tumor. On the other hand, the UC value showed no definite trend either up or down. The UA/UC values showed a definite tendency down in 6 out of the 8 animals while in 2 the tendency was up.

The initial PC level tended to be high and showed a downward drift during the period of tumor bearing in 6 out of the 8 animals. One showed no change during this period and the other increased. In the 2 mice which did not show a decline in PC level during the period of observation, the time interval between the 1st and the last determination were only 6 or 7 days whereas in the others that showed a decline, the time interval between the 1st and the last varied from 21 to 98 days.

The renal clearances of corticoids declined in 2, rose in 2, showed no initial decline followed by a rise in 3 mice and the opposite pattern on the remaining mouse.

4.4.3. IN NON-TUMOR-BEARING FEMALE C3H/HeJ MICE (TABLE 6)

The UA data tended to decline during the repeated taking of the determination in 2 of the 4 animals and to increase in the other two. The UC declined in 3 of the 4 animals and increased in one. The ratio of UA/UC decreased in one and increased in the remaining 3 animals. The PC increased in 1, decreased in another and showed relatively no change in the remaining two. The renal clearances of corticoids declined in 2 and increased in two.

TABLE 6

URINARY ANDROGENS, URINARY AND PLASMA CORTICOIDS AND DERIVED PARAMETERS IN NON-TUMOR BEARING FEMALE C3H/HeJ MICE

MOUSE	REMARKS	AGE (Days)	URINE ANDROGENS (UA) (ng/24 hrs)	URINE CORTICOIDS (UC) (ng/24 hrs)	UA/UC	PLASMA CORTICOSTERONE (mcg%)	RENAL CLEARANCE OF CORTICOIDS (ml/24 hrs)
5Loff		305	68	219	0.31	5.0	4.38
		319	20	110	0.18	7.4	1.48
		472	35	146	0.24	19.3	0.76
	Mean	365	41	158	0.24	10.6	2.21
	Died	511					
6L2		347	43	226	0.19	26.4	0.86
		472	45	109	0.41	25.2	0.43
	Mean	410	44	168	0.30	25.8	0.65
	Died	495					
3L2R2		417	48	470	0.12	25.0	1.88
		472	35	208	0.17	5.0	4.16
	Mean	445	42	339	0.15	15.0	3.02
	Died	475					
2 Roff		438	20	111	0.18	15.0	0.74
		445	44	154	0.28	15.5	0.99
	Mean	442	32	133	0.23	15.3	0.87
	Died	455					

4.4.4. IN NON-TUMOR-BEARING MALE C3H/HeJ MICE (TABLE 7)

Of the UA data of the 7 male H mice in this table, 3 showed a decline in values during repeated determinations, 3 showed no essential change and 1 showed an increase with subsequent decline. The UC values increased in 5, decreased in one and increased and then decreased in the remaining animal. The UA/UC values decreased in 4, increased in one and showed a more complex pattern in the remaining two. The PC levels showed an increase in 3, and an initial increase and a decrease in one and no essential change in the third. Data was missing in the remaining 2 animals so that comparisons with time cannot be made in them. The renal clearances of corticoids declined in 3, increased in one and declined first and later increased in the remaining animal.

4.4.5. IN NON-TUMOR-BEARING FEMALES C3HeB/FeJ MICE (TABLE 8)

The UA increased in 5 mice and decreased in 3, although the changes in either direction were rather small compared to those in the previous groups of animals. The UC essentially decreased in 4 compared to the first value and increased in the remaining 4 and the same was true of the UA/UC. Five of the 7 animals showed an increase in PC level, 2 showed a slight decrease and one showed no change. The renal clearances of corticoids declined in 6, increased in one and remained unchanged in one.

TABLE 7

URINARY ANDROGENS, URINARY AND PLASMA CORTICOIDS
AND DERIVED PARAMETERS DETERMINED IN NON-TUMOR BEARING MALE C3H/HeJ MICE

MOUSE	REMARKS	AGE (Days)	URINE ANDROGENS (UA) (ng/24 hrs)	URINE CORTICOIDS (UC) (ng/24 hrs)	UA/UC	PLASMA CORTICOSTERONE (mcg%)	RENAL CLEARANCE OF CORTICOIDS (ml/24 hours)
9 Loff		284	31	33	0.92	14.6	0.22
		291	23	167	0.14	11.4	1.46
	Mean	288	27	100	0.53	13.0	0.84
	Died	330					
9 Roff		291	85	62	1.37	11.9	0.52
		375	142	42	3.38	32.2	0.13
		402	125	25	5.00	43.0	0.06
		472	115	166	0.69	19.3	0.86
	Mean	385	117	74	2.61	26.6	0.39
	Died	480					
10L2		305	169	70	2.41	6.5	1.08
		319	29	121	0.24	18.6	0.65
		417	25	307	0.08	40.0	0.77
	Mean	347	74	166	0.91	21.7	0.83
	Died	420					
8L2 Roff		326	190	139	1.37	12.3	1.13
		364	150	64	2.35	19.3	0.33
	Mean	345	170	102	1.86	15.8	0.73
	Died	364					

TABLE 7

URINARY ANDROGENS, URINARY AND PLASMA CORTICOIDS
AND DERIVED PARAMETERS DETERMINED IN NON-TUMOR BEARING MALE C3H/HeJ MICE

MOUSE	REMARKS	AGE (Days)	URINE: ANDROGENS (UA) (ng/24 hrs)	URINE: CORTICOIDS (UC) (ng/24 hrs)	UA/UC	PLASMA CORTICOSTERONE (mcg%)	RENAL CLEARANCE OF CORTICOIDS (ml/24 hours)
8L2		334	148	165	0.90	--	--
		340	149	322	0.46	8.0	4.03
	Mean	337	149	244	0.68	8.0	4.03
	Died	340					
7L2Ro		347	153	67	2.28	10.0	0.67
		430	153	279	0.55	--	--
		452	152	102	1.49	20.5	0.50
	Mean	410	153	149	1.44	15.3	0.58
	Died	465					
10R1		445	140	25	5.60	12.3	0.20
		452	135	45	3.00	--	--
	Mean	449	138	35	4.30	12.3	0.20
	Died	456					

TABLE 8

URINARY ANDROGENS, URINARY AND PLASMA CORTICOIDS
AND DERIVED PARAMETERS DETERMINED IN NON-TUMOR BEARING FEMALE C3HeB/FeJ MICE

MOUSE	REMARKS	AGE (Days)	URINE			PLASMA CORTICOSTERONE	RENAL CLEARANCE OF CORTICOIDS
			ANDROGENS (UA) (ng/24 hrs)	CORTICOIDS (UC) (ng/24 hrs)	UA/UC	(mcg%)	(ml/24 hours)
23L1R1		334	19	149	0.13	8.2	1.82
		375	50	220	0.23	29.9	0.74
	Mean	355	35	185	0.18	19.1	1.28
	Died	380					
18R2		347	47	131	0.58	29.2	0.45
		367	38	101	0.38	31.1	0.32
	Mean	357	43	116	0.48	30.2	0.39
	Died	453					
17P1		402	38	235	0.16	30.0	0.78
		430	40	151	0.26	30.0	0.50
	Mean	416	39	193	0.21	30.0	0.64
	Died	442					
18P1		438	35	63	0.55	25.0	0.25
		445	30	118	0.25	24.0	0.49
	Mean	442	32	91	0.40	24.5	0.37
	Died	467					

TABLE 8

URINARY ANDROGENS, URINARY AND PLASMA CORTICOIDS
AND DERIVED PARAMETERS DETERMINED IN NON-TUMOR BEARING FEMALE C3HeB/FeJ

MOUSE	REMARKS	AGE (Days)	URINE ANDROGENS (UA) (ng/24 hrs)	URINE CORTICOIDS (UC) (ng/24 hrs)	UA/UC	PLASMA CORTICOSTERONE (mcg%)	RENAL CLEARANCE OF CORTICOIDS (ml/24 hours)
16P1		284	21	169	0.12	7.5	2.25
		312	34	83	0.41	4.3	1.93
	Mean	298	28	126	0.27	5.9	2.09
	Died						
17L1		291	96	183	0.52	11.0	1.66
		340	70	306	0.23	18.6	1.65
	Mean	316	83	245	0.38	14.8	1.66
	Died	342					
1812R1		305	22	215	0.10	4.6	4.67
		319	25	138	0.14	18.1	0.76
	Mean	312	24	177	0.12	11.4	2.72
	Died	416					
19Loff		325	17	63	0.27	12.3	0.51
		472	28	116	0.24	29.0	0.40
	Mean	399	23	90	0.26	20.7	0.46
	Died	475					

4.4.6. IN NON-TUMOR-BEARING MALE C3HeB/FeJ MICE (TABLE 9)

The UA increased in all 3 mice. The UC decreased in one and increased initially and then decreased in the other 2, while the UA/UC showed a pattern of initial decrease and then an increase prior to death in 2 out of 3 mice. The third animal showed an increase. The PC levels showed no consistent pattern in the 3 animals with repeated determination over a considerable period of time. The renal clearances of corticoids declined in 2 mice and increased initially in one followed by a decrease.

4.5. CORRELATION COEFFICIENTS BETWEEN THE VARIOUS PARAMETERS

4.5.1. BETWEEN BODY WEIGHTS AND OTHER PARAMETERS

A significant correlation coefficient (r) between the body weights (BW) and the UA occurred in all the healthy male and female mice of the H substrain ($r = + 0.3541$, $0.05 > P > 0.02$) and between BW and UC in tumor-bearing mice ($r = + 0.3780$, $0.05 > P > 0.02$), in all females substrains ($r = + 0.2737$, $0.02 > P > 0.01$), in healthy H mice ($r = - 0.3209$, $0.05 > P > 0.02$), in all healthy H and F animals taken as one group ($r = - 0.3282$, $0.01 > P > 0.001$), and in all the males of both substrains ($r = - 0.5127$, $P < 0.001$). In all the females, significant r's between body weights, UA/UC were observed in all healthy H animals ($r = + 0.4019$, $0.02 > P > 0.01$), in all females, sick and healthy

TABLE 9

URINARY ANDROGENS, URINARY AND PLASMA CORTICOIDS
AND DERIVED PARAMETERS DETERMINED IN NON-TUMOR BEARING MALE C3HeB/FeJ

MOUSE	REMARKS	AGE (Days)	URINE ANDROGENS (UA) (ng/24 hrs)	URINE CORTICOIDS (UC) (ng/24 hrs)	UA/UC	PLASMA CORTICOSTERONE (mcg%)	RENAL CLEARANCE OF CORTICOIDS (ml/24 hours)
21R2		291	24	59	0.40	15.3	0.39
		311	29	97	0.30	6.5	1.49
		312	32	105	0.31	12.9	0.81
		367	30	25	1.20	42.5	0.06
	Mean	320	29	72	0.55	19.3	0.69
	Died	430					
24 Loff		326	91	57	1.60	12.3	0.46
		347	120	83	1.36	35.7	0.25
		374	158	55	2.87	26.5	0.21
		402	160	11	14.50	16.1	0.07
	Mean	362	132	53	5.08	22.7	0.25
	Died	455					
18P1		500	80	71	1.13	18.0	0.39
		521	82	18	4.54	15.0	0.12
	Mean	511	81	45	2.83	16.5	0.20
	Died	521					

($r = + 0.2517$, $0.05 > P > 0.02$), and in all healthy mice ($r = + 0.2648$, $0.05 > P > 0.02$). Significant r's occurred between BW and renal clearance of the corticoids in tumor-bearing mice ($r = + 0.4311$, $0.01 > P > 0.02$) and in all males ($r = - 0.3990$, $0.02 > P > 0.01$). Significant r's between BW and adrenal weights were observed in female H mice with MT ($r = + 0.5143$, $P < 0.001$), in all H mice ($r = + 0.2583$, $0.05 > P > 0.02$) and in all females ($r = + 0.3964$, $P < 0.001$).

4.5.2. BETWEEN URINARY ANDROGENS AND OTHER PARAMETERS

A significant r between UA and UC was observed only in all H mice ($r = - 0.3062$, $0.02 > P > 0.01$). There were also several significant r's between the UA and urine volume (UV) excreted in 24 hours in MT animals ($r = + 0.3698$, $0.05 > P > 0.02$), in all the healthy mice of the H substrain ($r = + 0.5582$, $P < 0.001$) and in both sick and healthy H mice ($r = + 0.6066$, $P < 0.001$). There was also a significant r between the UA excreted in 24 hours and the tumor weight at death ($r = + 0.4010$, $0.02 > P > 0.01$). The significant r between the UA and BW have been mentioned in section 4.5.1..

4.5.3. BETWEEN URINARY CORTICOIDS AND OTHER PARAMETERS

The only significant r between UC and PC in C3H mice occurred in all the H mice ($r = + 0.2522$, $0.05 > P > 0.02$). There was a significant r between UC and UV of all the males of both substrains ($r = + 0.3332$, $0.05 > P > 0.02$). Other significant r's involving UC were

mentioned in previous section.

4.5.4. BETWEEN PLASMA CORTICOSTERONE AND OTHER PARAMETERS

Besides the significant r's between the PC and other parameters already mentioned, a significant r between the PC level and age in all H mice, sick and healthy ($r = -0.2243$, $0.05 > P > 0.02$) occurred.

4.5.5. BETWEEN RENAL CLEARANCE OF THE CORTICOSTEROIDS AND OTHER PARAMETERS

There was a significant r between renal clearance of the corticosteroids (RC) and life span in all healthy mice of this study ($r = +0.9953$, $P < 0.001$) and in all females ($r = +0.9953$, $P < 0.001$). There were 5 significant r's between the RC and the age of the mice. These occurred in all the healthy H mice ($r = +0.6990$, $P < 0.001$), in all H mice, sick and healthy ($r = +0.7025$, $P < 0.001$), in all F mice ($r = +0.9965$, $P < 0.001$), in all healthy mice of both the H and F substrains ($r = +0.9916$, $P < 0.001$) and in all females ($r = +0.9916$, $P < 0.001$). Other significant r's between the RC and BW were mentioned in section 4.5.1..

4.5.6. BETWEEN URINE VOLUME AND OTHER PARAMETERS

There were significant r's between UV and life span in the F substrain, both males and females taken together ($r = +0.9969$,

$P < 0.001$), in all the healthy mice of both F and H substrains ($r = + 0.9936$, $P < 0.001$), in all the males of either substrain ($r = - 0.3603$, $0.05 > P > 0.01$) and in all females ($r = + 0.9937$, $P < 0.01$). There was a significant r between the UV and the age of the mice bearing MT ($r = + 0.3838$, $0.02 > P > 0.01$), in healthy male and female H mice ($r = + 0.6954$, $P < 0.001$) in all H mice, sick and healthy ($r = + 0.6994$, $P < 0.001$), in all F mice, ($r = + 0.9970$, $P < 0.001$), in all healthy mice ($r = + 0.9929$, $P < 0.001$) and in all females ($r = + 0.9930$, $P < 0.001$). Other significant r's between the UV and other parameters were mentioned in earlier sections.

4.5.7. BETWEEN LIFE SPAN AND OTHER PARAMETERS

There were significant r's between life span and adrenal weights in all healthy H mice ($r = - 0.4293$, $0.01 > P > 0.001$), and all H mice, healthy and sick ($r = - 0.2970$, $0.01 > P > 0.001$) and in all males of the F and H substrains ($r = - 0.5243$, $P < 0.001$). Other significant r's between life span and other parameters were mentioned in section 4.5.5. and 4.5.6..

4.5.8. BETWEEN ADRENAL WEIGHTS AND OTHER PARAMETERS

There was a significant r between adrenal weight and age of the male mice in both substrains ($r = - 0.3214$, $0.05 > P > 0.02$). Other significant r's between adrenal weights and other parameters were mentioned in earlier sections.

5. DISCUSSION

5.1. THE PRECISION OF THE ASSAYS FOR THE DETERMINATION OF CORTICOSTERONE AND ANDROGENS.

5.1.1. FOR THE STANDARD CURVES

The mean and standard error of the λ of the 5 standard curves for the determination of corticosterone was 0.110 ±0.007 which was of the same order as the 0.195 ± 0.22 reported earlier for 20 such standard curves (Grad, Khalid, 1968). Similarly, the corresponding values for 5 standard curves for the determination of androgens was 0.150 ± 0.040, again a satisfactory result especially in view of the ultramicro range of the assays, and in view of the fact that λ's of less that 0.300 were acceptable with assays much less sensitive than the ones utilized in the present study.

5.1.2. FOR THE UNKNOWNS (TABLE 1).

The precision of the determination of the unknowns was assessed by calculating the SD of duplicate determinations was somewhat higher at the lower range of values in the determination of corticosterone in the plasma of C3H mice (6 mcg. % in the 0-10 mcg. % range) than it was in the plasma or serum of C57Bl/6J or AKR mice (0.6 mcg. % in the 0-2.5 mcg. % range) reported earlier (Grad and Khalid, 1968).

At the higher ranges, the precision was of the same order in both the present and earlier studies. However, fewer pairs formed the basis of the calculations in the present study and this could perhaps partially account for the apparent lesser precision at the lower ranges.

The precision of the determination of UC in the test samples from mice in this ultramicro assay was as good as that reported earlier for a semimicro assay of the urinary corticosterone in human (Grad, Kral, Payne and Berenson, 1967).

The precision of UA in the test samples was as good as that of determining the standard error of the determination of plasma androgens in human plasma which over the ranges 0 to 0.20, 0.20 to 0.40 and 0.40 to 0.90 µg/ml were 0.02, 0.04 and 0.06 µg/100 ml respectively (Murphy, 1969a).

5.2. THE ACCURACY OF THE ASSAYS FOR THE DETERMINATION OF THE PLASMA AND URINARY CORTICOSTERONE AND ANDROGENS (TABLE 2).

The recovery of 1 ng of corticosterone from the plasma or urine of C3H mice was very similar to that recovered from the serum or plasma of C57Bl/6J or AKR mice utilizing the same ultramicro assay (Grad and Khalid, 1968) and was at least as good as the recovery of 100 to 300 ng added to pooled urine utilizing

a semimicro assay (Grad, et al., 1967).

The % recovery of 1 ng testosterone from the mouse urine was very similar to that of the recovery of 1 ng corticosterone from the same liquid. Murphy (1969a) recovered 95 ± 1% of 0.005 μc tritiated testosterone added to human plasma. Thus, the increased sensitivity achieved by the ultramicro methods was not at the expense of precision or accuracy.

Tests for specificity for the corticosteroid assays were reported earlier (Murphy, 1967; Grad and Khalid, 1967). Murphy (1969b) who devised the assays utilized in the present study, claimed that the major material tested in the assay was testosterone but a small amount of other material believed to be androgen was also being determined.

5.3. PLASMA AND URINARY CORTICOSTEROIDS AND ANDROGENS AND OTHER PARAMETERS IN C3HeB/FeJ AND C3H/HeJ MICE.

5.3.1. DIFFERENCES DUE TO SEX.

The lack of a significant difference in PC level in the F and H mice is in contrast with the significantly higher values observed in females of the C57Bl/6J (Grad, Khalid, 1968), AKR (Grad and Khalid, 1969a) and in WLO mice (Solem, 1966). A similar difference has been reported to occur in rats but not in the human (Grad et al., 1967). There was also no significant sex

difference in C3H adrenal weight which in other strains of mice are said to be heavier in the female (Deansley, 1938) and were found to be so in the C57Bl/6J and AKR strains (Grad and Khalid, 1969a). However, neither in the present study nor in earlier ones issuing from our laboratory was a significant correlation between adrenal weight and the PC level observed (Grad and Khalid, 1968, 1969a). On the other hand, the significantly higher UC amounts in female C3H mice is in accord with that observed in C57 and AKR animals (Grad and Khalid, 1969b).

The higher UA values in male mice than in females is comparable to that reported in the human urine (Murphy, 1969b) but no other report on the UA in mice has been reported.

The higher renal clearance of corticosteroids in the healthy female of both the F and H substrain and the higher UA/UC in the male is the consequence of the sex differences in the PC, UC, and UA values already discussed.

Male mice weighed more than females in the C57Bl/6J and AKR strains (Grad and Khalid, 1969b): however, there was no significant difference in the body weight between the sexes of the C3H mice of this study. Therefore, the higher urine volumes in males could not be explained as being due to differences in BW. No explanation can be offered for the sex X strain interaction in the life span of the F and H mice.

5.3.2. DIFFERENCES DUE TO STRAIN.

F mice had higher PC values than healthy H mice, and both F and H mice had higher PC values than C57Bl/6J (Grad and Khalid, 1968) and AKR mice not ill with lymphatic leukemia (Grad and Khalid, 1969a) all determined by the same method. Thus C57 or AKR mice of comparable age to the 9-15 month old C3H mice of this study did not have PC levels in excess of 7.2 ± 0.8 mcg. %, still significantly below the 13.8 ± 2.1 mcg % of female F mice ($0.01 > P > 0.001$) the group with the lowest PC values in the present study. More recently, Grad and Rafizadeh (1969) showed that CF_1 female mice, 12 to 15 months old had morning PC values of 6.8 ± 1.1 mcg%, significantly below the 13.8 ± 2.1 mcg % of female F mice ($0.01 > P > 0.001$). Levine and Treiman (1964) reported values between 8 and 9 mcg % and Solem (1966) reported PC values of 6.3 ± 0.6 and 12.1 ± 1.1 mcg % for male and female WLO mice. However, these were obtained from 2 to 3 month old mice by a fluorimetric techniques which are known to yield higher values due to the inclusion of a contaminating non-specific substance.

Healthy H mice had significantly higher UC amounts than F mice (Table 3) and higher also than that of apparently healthy AKR males (34 ± 17 ng/24 hrs.) and females (117 ± 21 ng/24hrs.) and C57Bl/6J males (48 ± 6ng/24 hrs.) and females (112 ± 32 ng/24 hrs.) (Grad and Khalid, 1969b). No other data on the UC of individual mice

or pooled mice could be found.

Based on the above PC and UC figures, the renal clearances of the corticosteroids would appear to be markedly lower in C3H than in AKR or C57 mice.

There are no reports of the UA of mice in the literature to which the data of the present study could be compared. Murphy (1969b) showed that the UA values in humans (0.65 ± 0.17 µg/100 ml in men and 0.18 ± 0.06 µg/100 ml in women) are higher than in mice no doubt due to their large size. There was no significant difference in BW between that F and H substrains, and the same was true when comparisons were made between the BW of the males of F and H mice (Table 3) and the BW of male C57 (29 ± 1 g) or male AKR mice (28 ± 1 g; Grad and Khalid, 1969b). On the other hand, the BW of female F and H mice was significantly greater than that of female C57 (24 ± 1 g) and female AKR mice (24 ± 1 g; Grad and Khalid, 1969b).

On the other hand, significant difference in adrenal weight between the C3H mice of the present study (Table 3) were apparent when comparisons were made with the males of the C57 (4.1 ± 0.1 mg) and AKR strains (4.6 ± 0.7 mg) but not with the females of these strains(5.9 ± 0.8 mg for C57 and 7.2 ± 0.5 mg for AKR mice (Grad and Khalid, 1969b).

Comparison of urine volumes with mice of other strains was not possible because of lack of such data in the literature.

The life span of the C3HeB/FeJ was found to be significantly less in our study than in those of Stoner (1966) whose males survived 652 ± 17 days and females 657 ± 15 days. The reason for the difference may be the housing conditions. Our mice were housed 4-5 per metal cages with wire meshed bottoms, his, in plastic cages providing more room per animal.

5.3.3. CHANGES BEFORE THE APPEARANCE OF THE TUMOR (TABLES 3 to 9)

Comparisons between the UA and the UC amounts and the UA/UC data of female H mice with the potentiality for breast cancer with those that did not develop it showed no significant differences either in the horizontal (Table 3) or the longitudinal studies (Table 4 to 9 inclusive). However, significant differences were observed during the pre-tumor-bearing period in the PC level and the renal clearance of the corticosteroids when compared with those of C3H mice that did not develop breast cancer.

Thus female H mice that were apparently healthy but had a high potentiality for developing breast cancer had significantly lower PC levels and significantly higher renal clearances than did female F mice that did not develop mammary cancer (Table 3).

This was also apparent when the pre-tumor PC levels of the 4 mice studied before and during the tumor period were compared with those of other groups that did not develop the tumor.

Thus, 6 out of 8 pre-tumor PC levels were less than 10 mcg % in the 4 mice that subsequently developed a mammary cancer (Table 4) but only 10 out of 51 had such low values in mice that did not develop the tumor (Table 6-9 inclusive) a significant difference ($0.01 > P > 0.001$).

Similarly, 6 out of 8 renal clearance determinations had values greater than 2 ml per 24 hours during the pre-tumor bearing period of the 4 mice that eventually developed mammary cancer (Table 4) while only 4 of 25 such determinations in C3H mice that did not develop the MT had such values (Table 6-8 inclusive) a significant difference ($0.01 > P > 0.001$). Moreover, when the same comparisons were made between the apparently healthy, but mammary tumor agent (MTA) bearing female H mice and non-MTA-bearing F females, the differences were highly significant (Table 3). Therefore, both the PC level and the renal clearance of the corticosteroids may have some prognostic value in the mammary cancer of mice at least.

5.3.4. COMPARISONS BETWEEN TUMOR-BEARING AND NON-TUMOR BEARING MICE (TABLES 3 TO 9).

Tumor bearing mice had significantly higher PC values than healthy female or male H mice (Table 3) and higher also

than that of their own PC values determined before they developed the tumor (Table 4). Bulbrook and Hayward (1967) also found elevated 17-OHCS in patients with advanced breast cancer. Higher PC values were also found in AKR sick mice with acute lymphatic leukemia than in healthy AKR and C57 controls (Grad and Khalid, 1969a).

Tumor-bearing mice had somewhat higher UC amounts than non-tumor bearing female H mice (Table 3) and the same results were reported by Bulbrook and Hayward (1967) in patients with advance breast cancer. However, definitely higher UC amounts were found in tumor-bearing mice when the results were compared with their own values prior to the appearance of the tumor (Table 4). Higher UC amount in AKR mice with acute lymphatic leukemia than in healthy AKR and C57 mice were also reported earlier (Grad and Khalid, 1969b). In this connection, Nadel and Burnstein (1956) showed that the urinary excretion of cortisol, 2-a-hydroxycortisol and 6-B hydroxycortisol in guinea pigs with a L_2C/NB leukemia or a transplanted liposarcoma was increased nearly three-fold, and that the adrenal production of the same three corticosteroids was significantly elevated in the leukemic guinea pigs (Burnstein and Nadel, 1967). The rise in PC level early in the MT period cannot be due to a decline in renal function because the UC did not decrease at the same time (Tables 4 and 5). However, whatever the cause, the rise in PC level may favor the sick animal by inhibiting the further MT development.

The renal clearance of the corticosteroids was significantly lower in MT-bearing mice than in healthy H females (Table 3) and lower in all 4 mice after the tumor appeared than it was before that time (Table 4). That is, although the PC and UC increased early in the tumor-bearing period, the former did more so than the latter.

The high PC level and UC amount in C3H mice bearing the MT may be a non-specific response to the stress of the disease. However, the corticosteroids are known to inhibit MT development (Burton and Begg, 1961). Therefore, the increased PC level may help the animal combat the disease and help prolong survival.

There was no significant UA difference between healthy and tumor-bearing female H mice (Table 3) and yet 3 out of 4 had higher UA values after the tumor appeared than before this time (Table 4). The reason for this apparent contradiction will be discussed in the next section. The same applies to the UA/UC which was also not significantly different in the MT-bearing mice from their non-tumor-bearing female controls and yet in all 4 mice in which the ratio was calculated, the values were lower after the tumor appeared than before.

Mice bearing an MT weighed significantly more than healthy females, and no doubt the tumor contributed much to this

Leaf 86 missing.

an inhibiting influence on the growth of the tumor.

The renal clearances of the corticosteroids were increased during the tumor-bearing period in 7 of 10 mice, the UA diminished in all 10 mice and the UA/UC diminished in 8 out of 10 mice.

To determine to what extent the above changes were characteristic of sick mice, comparisons of the patterns of change were made with non-tumor-bearing male and female F and H mice in whom determinations were made repeatedly (Tables 6 to 9). Thus, 6 of 7 MT-bearing mice had a downward trend in PC level over a longer than 3-week period, only 3 out of 15 mice showed a similar trend in the non-tumor-bearing mice, a significant difference by chi-square test ($0.02 > P > 0.01$). Again, while 7 out of 10 showed an increase in the renal clearance of corticosteroids, only 2 out of 22 showed the same pattern of change, again a significant difference ($0.01 > P > 0.001$). In the UA, all the 10 mice showed declines during the period when they had the tumor, while this occurred in only 8 of the 23 non-tumor-bearing controls ($0.01 > P > 0.001$). Finally, in the UA/UC, 8 out of 10 sick mice showed a downward trend, while 9 of 23 showed the same pattern, a difference of borderline significance ($0.10 > P > 0.05$). Therefore, these changes would appear to be characteristic of sick mice. Whether it is also true of MT-bearing mice specifically requires further comparison with mice sick from causes other than MT. In one tumor-bearing mouse in whom several

readings were taken before and after the appearance of the tumor (Table 4, Loff), the changes in the PC levels, the UA amounts, the UA/UC and the renal clearance before the tumor became evident were all in a direction opposite to those that occurred afterwards as described above. This is in evidence that the changes in the above 4 parameters are at least characteristic of sick mice, and could not be due to the effect on the animal of taking repeated samples.

Inasmuch as the origin of the UA in the female is the adrenal cortex, the decline observed in the UA during MT illness suggests that this aspect of adrenocortical function declines during this time. However, the UC amounts does not decline at this time as seen by the lack of a significant correlation between the UA and UC amounts ($r = \dagger 0.0357$, $P > 0.10$). Therefore, the adrenal is not secreting less corticosterone at this time. In this connection, when the data of both sick and healthy H mice are considered, there is a significant negative correlation between the UA and UC amounts ($0.02 > P > 0.01$) which indicates that the adrenal tends to secrete more of one when it is secreting less of the other.

There was no significant UA difference between healthy and tumor-bearing female H mice (Table 3) and yet 3 out of 4 tumor-bearing mice had higher UA values soon after the tumor appeared than before this time (Table 4). The reason for this apparent contradiction probably arises from the fact that the UA values declined

steadily the longer the mouse bore the tumor (Tables 4 and 5) and possibly, as suggested by the data of LOFF (Table 4) a steady increase before the tumor appears. The random sampling of 2 opposing trends would tend to yield means which did not differ reliably from each other. This argument also applies to the UA/UC.

6. GENERAL DISCUSSION

When comparisons were made between mice bearing an MT and other female mice of the same substrain, the PC levels were found to be significantly elevated, the UC amounts also elevated but not significantly so, the UA amounts and the UA/UC showed no change and the renal clearance of corticosteroids significantly depressed (Table 3).

When the same variables were compared in the same 4 mice before and soon after the appearance of the MT, the PC levels and the UC amounts were higher in all 4 cases, the UA amounts were higher in 3 out of 4 instances, while the UA/UC and the renal clearance of the corticosteroids were lower in all 4 instances after the tumor appeared.

Moreover, during the tumor-bearing period, the PC levels, the UA amounts and the UA/UC all declined, while the renal clearance of the corticosteroids increased. Non-tumor-bearing mice with repeated determinations had significantly different patterns. Thus, longitudinal studies appeared to provide the more precise information than the horizontal ones.

In connection with these studies, Bulbrook, Greenwood and Hayward (1960) found that the UC amounts were usually low and those of the 11- deoxy-17- oxosteroids (dehydroepiandrosterone, androsterone and etiocholanolone) were usually high in patients who subsequently responded to adrenalectomy or hypophysectomy, compared with the amounts of these steroids found in the urine of patients who subsequently failed to respond. Later, a greater efficiency was achieved by the development of a discriminant function which contained both UC and etiocholanolone amounts. A negative discriminant was obtained when the etiocholanolone was low relative to the UC, and a positive one in the reverse situation. Patients with negative discriminants were found to respond poorly to adrenalectomy or hypophysectomy (Bulbrook, et al., 1960; Atkins, Bulbrook, Falconer, Hayward, MacLean and Schurr, 1964). Later, the same was found to apply to mastectomy (Bulbrook, Hayward and Thomas, 1964). These studies support our findings in mice where it was found that the UA/UC steadily declined in 8 out of 10 mice bearing a MT but did so only in 9 out of 23 non-MT-bearing mice. However, our findings suggest that the PC level, the UA amounts and the renal clearance of the corticosteroids might also be useful as prognosticators of the progression of the disease.

A matter of primary importance in the attack on mammary cancer is the need for methods of detecting the disease before the tumor becomes manifested, for as indicated earlier, once the tumor appears, very little can be achieved towards producing a

permanent cure with present day therapies. In this connection, Bulbrook and Hayward (1967) reported that a substantial proportion of women who subsequently developed breast cancer excreted subnormal amounts of androgen metabolites. This was not supported by the UA data of the pre-tumor-bearing mice of the present study. Nor indeed, did the UC amounts or the UA/UC have any prognostic value in this condition.

However, both the PC level and the renal clearance of the corticosteroids appeared to have such prognostic value, the PC level being significantly depressed during the pre-tumor stage and the renal clearance significantly elevated prior to the appearance of the mammary tumor. Subnormal PC levels were also observed during incipient leukemia in the high leukemia AKR strain, although the values increased to significantly above normal levels when the mice became acutely ill with the disease (Grad and Khalid, 1969a). Inasmuch as the corticosteroids have an lymphocyto-karyorrhectic effect (Dougherty, 1952), the depressed PC levels in AKR mice may provide favorable conditions for the proliferation of the leukemia virus, the aetiological agent in AKR leukemia (Gross, 1960) and hence for the development of leukemia. The same conditions may also pertain to C3H mice where MT development is also viral in origin (Bittner, 1945) and where the corticosteroids have also been reported as inhibiting the growth of mammary tissue (Burton and Begg, 1961). Further studies in this direction are indicated.

7. SUMMARY AND CONCLUSIONS

PC levels and UC and UA amounts were determined in male and female C3H/HeJ and male and female C3HeB/FeJ mice, the former bearing the MTA, the latter not. Renal clearances of the corticosteroids and UA/UC values were also calculated.

The results showed that prior to the appearance of the tumor, the PC levels were significantly depressed and the renal clearances significantly elevated in the female H mice. However, there was no reliable change in the UA and UC amounts and in the UA/UC at this time.

When comparisons were made between the same animals before and soon after the tumor appeared, the PC, UC and UA values were all reliably higher in the tumor-bearing mice, while the UA/UC and the renal clearances of the corticosteroids were reliably depressed. Moreover, as the tumor developed, the PC, UA and UA/UC all declined, while the renal clearances increased. The patterns of change in non-tumor-bearing mice were significantly different.

When comparisons were made between different groups of tumor-and non-tumor-bearing animals, the tumor-bearing animals had significantly higher PC levels, higher but not significantly so, UC amounts, significantly depressed renal clearances and no significant differences in the UA amounts and in the UA/UC values.

Females had significantly higher UC amounts but

significantly lower UA amounts and therefore significantly lower UA/UC values. PC levels showed no reliable sex difference, while the renal clearance of the corticosteroids were reliably higher in females.

Mice of the F substrain had significantly higher PC levels and significantly lower UC amounts than H mice. Therefore, the renal clearances were also significantly lower in F than in H animals. The higher UA amounts in H than in F mice were of borderline significance, while the UA/UC values showed no significant substrain difference.

These studies indicate that the PC level and the renal clearance of the corticosteroids may have useful prognostic value in the mammary cancer of C3H mice, and suggest also that longitudinal studies are preferable to horizontal ones in such investigations.

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