SOME ASPECTS OF STEROID METABOLISM AND CONJUGATION IN MAMMALS

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CHAPTER I

INTRODUCTION

Interest in problems concerning impaired fertility in the bull has stemmed from the development of artificial insemination programs where a single ejaculate may be used to inseminate many cows. Frequently a good, proven bull may experience temporary infertility due to age, season of year, or endocrine imbalance. One possible approach to the treatment of infertility is through the use of hormonal therapy. One such attempt to improve fertility in humans and certain laboratory animals is by means of the "rebound phenomenon". This phenomenon is brought about through injections of testosterone which temporarily blocks the spermatogenic processes. Some time after injections have been stopped, the animal goes through a period of increased spermatozoa numbers with a corresponding increase in fertility. The mechanism for the depression of spermatogenesis is now thought to be through the control of pituitary gonadotrophins which in turn control spermatogenic processes. Pituitary gonadotrophins influence Leydig cells to produce testosterone, the male sex hormone. Testosterone in turn is responsible for development of the male sex characteristics, stimulation of germinal epithelium favoring spermatogenesis, and suppression of gonadotrophin production by the pituitary. Therefore, this experiment was designed to test this phenomenon in normal bulls to see if it could be applied later to abnormal bulls.

The experiment was conducted in two parts. The first part was designed to evaluate spermatogenic and testicular function. These data

have been reported by Meinecke (75) and Meinecke and McDonald (76). The second part consisted of following the overall metabolism of steroids during the experiment and the correlation of these data with the reproductive data from the first part of the experiment. These data were collected to see if the clinician and researcher could follow reproductive processes in the bull during the rebound phenomenon using chemical tests instead of purely histological and gross anatomical observations. Since very little is known concerning the metabolism of testosterone in the bull and how it affects other steroids and their metabolism, one of the prime objectives was to gain information as to how the ruminant metabolizes and excretes steroidal material. The results of these experiments form the first part of the thesis.

Several questions arose during the above studies in connection with the mode of conjugation and metabolism of steroids and carotenoid compounds from the diet, henceforth referred to as ionone derivatives. Therefore, research was undertaken to evaluate several probable sites of conjugation in addition to evaluating some factors influencing conjugation of steroids in these animals. In the process of working out methods for evaluating the foregoing problems, additional studies were undertaken in the dog and rat. The data collected from these animals have made possible certain pertinent comparisons with similar data collected from sheep. The results of these experiments form the second part of the thesis.

CHAPTER II

REVIEW OF LITERATURE

Effects of Steroids on Spermatogenesis

Prolonged administration of exogenous testosterone has been shown to inhibit spermatogenesis in man and rat (12, 47, 48, 71, 112, 132); however, degeneration of the testes was more pronounced following smaller doses than from larger doses of this steroid (49, 71, 146). Studies of a similar nature had not been reported in the bull until Meinecke and McDonald reported their findings from this joint investigation. However, Holtz (57) has reported briefly on the urinary excretion of steroids following intramuscular injections of testosterone propionate in oil to a bull, steer, and cow. Slow absorption of testosterone from the injection site into the blood was assumed since the urine, collected three months later, contained marked amounts of etiocholanolone, which is considered to be a testosterone metabolite.

Intramuscular injections of 500 mg. of cortisone tri-weekly for 12 weeks has been reported to increase the sperm count and the fructose levels in the semen from a normal bull (19). Injections of cortisone and hydrocortisone have been reported to increase the number and decrease the percentage of abnormal forms of spermatozoa of two bulls with poor semen quality (18). Hydrocortisone was effective at a lower level than cortisone. Deoxycorticosterone acetate had no measurable effect on semen quality of one bull. Of six bulls treated with testosterone, only one

responded with an increased libido and a rise in seminal fructose. Motility of the spermatozoa was not altered by any of the hormones studied.

Measurements of Urinary Steroids

Considerable work in the area of steroid metabolism has been accomplished in humans but relatively little work has been done in dairy cattle (84), especially in bulls.

The isolation of pregnanediol-3(a)-20(a), allo-pregnanediol-3(a)-20(a), allo-pregnanediol-3(b)-20(a), alpha and beta equistanol, androsterone, and dehydroandrosterone from urine of pregnant cow's urine was accomplished by Marker (73, 74).

The so-called neutral steroid fraction of cattle and horse urine was investigated photometrically by Meschanks (77) using the Zimmerman and Pincus reactions. Although variations in steroid content were noticed with respect to specific gravity, diurnal variation, and certain metabolic states of the animal, the absorption curves of the colors produced by these reactions did not correspond with androsterone or other typical 17-ketosteroids.

The use of zinc and HCl for the hydrolysis of cow urine prior to extraction of 17-ketosteroids was reported by Arrhenius (2); however, no data were reported to establish the steroid nature of the extracts.

Gassner et al. (36) isolated a steroid fraction from venous blood of cows following intravenous injections of ACTH. This fraction was characterized by a positive androgenic bioassay and a positive Zimmerman reaction for 17-ketosteroids.

Preliminary work on the extraction, separation, and spectrophotometric analysis of the neutral steroid fractions of cow urine was reported by

Mixner (80) and Mixner and Saunders (82, 83). Although definite absorption spectra were obtained with the Zimmerman, Pincus, and Allen color reactions, these spectra did not correspond to those obtained with the 17-ketosteriods in man.

Holtz (56) noticed the presence of several ionone derivatives in extracts of cow urine which were identified as (2,3,6-trimethyl-benzol)-acetone, 5-hydroxy-cis-tetrahydro-ionone, and 5-oxo-cis-tetrahydro-ionol; other ionone derivatives were not definitely established. When introduced into the Zimmerman reaction, some of these compounds gave absorption curves similar to those reported by Meschanks (77) and Mixner (80) for their crude extracts which undoubtably contained these ionone derivatives. A blue-violet color with an absorption maximum at 550 mμ was obtained with 5-hydroxy-cis-tetrahydro-ionone and the Pincus reagent as previously reported (77, 80) for urine extracts. Dehydroisoandrosterone, etiocholanolone and 11-hydroxyandrosterone were also demonstrated as 17-ketosteroids. The ionone derivatives were attributed to the carotenoid derivatives from animal feed rich in carotene.

Others have reported on the fractionation of steroids and their determination (53, 55, 113) without establishing the steroid nature of the various fractions.

Mixner et al. (84), using column chromatography and infra red spectrum analyses, found that the ionone derivatives were present in the conjugated form in urine and that they may be hydrolyzed by either acids or β-glucuronidase. Following hydrolysis, the ionone derivatives and steriods were extractable with lipid solvents. Specific absorption spectra were obtained with these chromogens using Zimmerman, Pincus, and Allen color reactions. The spectra differed from that of the true 17-ketosteroids.

At least six chromogens were found which reacted similarly to 17-keto-steroids on alumina chromotographic columns. Distinct diurnal rhythms were found in the urinary excretion pattern of steroids from these animals.

Holtz (57) analyzed urine of cattle following acid hydrolysis and isolation of the neutral steriod fractions. Purification and separation were accomplished using Girard's reagent T and column and paper chromotography. The main compounds isolated from the neutral lipid fraction of cattle urine were recognized as the ionone derivatives which were first obtained from pregnant mares' urine by Prelog et al. (see reference 57). Those identified by use of paper chromatography were: (2,3,6-trimethyl-benzol)-acetone (ketone J), 5-hydroxy-cis-tetrahydro-ionone (hydroxyketone G), and 5-oxo-cis-tetrahydro-ionol (hydroxyketone E). Two other spots were due probably to 5-oxo-cis-tetrahydro-ionone (diketone D), 2,2,8-trimethyl-bicyclo-/$\bar{0}$,0,4$\bar{/}$-dekadien-5,7-one-4 (ketone K), the bicyclic hydroxyketone C of unknown structure, or a mixture of these compounds. All of these substances react with the Zimmerman reagents. Ketone J gave a spectrum almost identical with that of typical 17-ketosteroids. It was estimated that 90 per cent of the total ketosteroid content of urine was due to these non-steroidal substances. The maximal concentrations of true 17-ketosteroids were estimated to be about 0.3 mg. per liter of urine. Three 17-ketosteroids were identified with some degree of accuracy: dehydroepiandrosterone, etiocholanolone, and 11-ketoetiocholanolone. No differences of steroid levels were found between bulls, cows, steers, and cows with sexual disturbances.

Holcombe (54) has reported on an assay of neutral and reducing corticosteroids; however no data were given as to the specificity of the

method he used. The values he obtained suggest that far more corticosteroids are excreted than any other steroids in these urines.

Ungar et al. (130) analyzed calf urine by use of hot acid hydrolysis, snail juice sulfatase, β-glucuronidase hydrolysis and column and paper chromatography. It was found that the crude ketonic fractions gave an average 17-ketosteroid value of 21.5 mg. per 24 hours. Following chromatography on an adsorbent column, an average value of 13.3 mg. per 24 hours was obtained. The average value obtained after eluting zones from paper chromatograms was 2.6 mg. per 24 hours. Following the same purification procedures as indicated above, the 17-hydroxycorticosteroids decreased proportionately to the 17-ketosteroids. The crude extract contained 0.9 mg. corticosteroids per 24 hours, 0.3 mg. per 24 hours after column chromatography, and 0.11 mg. per 24 hours after paper chromatography.

From the literature cited above, it can be seen that due to the presence of ionone derivatives from the diet, any interpretation of urinary steroid excretion using existing methods is particularly hazardous (130).

General Aspects of Steroid Metabolism in Bulls

Holcombe (52) reported low urinary levels of corticoids in parturient, paretic cows and interpreted the results as being indicative of a low adrenal cortical output of steroids. Holm and Firch (55) found a decreased ketosteroid excretion following parturition. In other studies, Holcombe (53) found an increased excretion of reducing compounds in urine during pregnancy. During the last month of pregnancy there was a 2-3 fold increase in excretionary rate. After parturition, the excretionary rate fell rapidly to the non-pregnant rate within 3-4 days. In cows with

retention of the placenta there was little or no fall in the excretionary rate. Cows with milk fever showed a much lower excretionary rate than healthy cows at the same stage of reproduction.

Pregnant cows were shown by Putriano (94) to have an increase while acetonemic cows showed a decrease of adrenal activity as compared to normal, lactating cows.

Robertson et al. (99) reported that ketotic cows had greater levels of plasma 17-hydroxycorticosteroids than did the controls. It was postulated that low urinary levels of corticoids as reported by Putriano (94) could result if the liver function of his cattle were impaired.

Robertson and Mixner (100) reported the plasma levels of 17-hydroxycorticosteroids in cattle and found a difference between dry, pregnant cows, and milking pregnant cows. The increase in steroid content associated with pregnancy was considered to be similar to that reported for humans. Bush (7) also has reported an increase in the plasma 17-hydroxycorticosteroids during the last month of pregnancy. However, Shaw et al. (114), while studying the plasma steroid levels of healthy cattle during various physiological states, concluded that, with the exception of estrual values, there were no significant differences in plasma 17-hydroxycorticosteroid levels. In addition, Robinson and Macgregor (101) found no significant difference in the urinary excretion of steroids in four ewes from the time of conception until a few weeks after parturition as compared with seasonal fluctuations. This study was complicated further by the fact that two of the ewes received from 20-40 IU of ACTH during the final three weeks of pregnancy. The steroid levels were high in winter and low in summer with an abrupt fall occurring towards the end of pregnancy regardless of treatment. The amount of steroids excreted in

urine remained very low immediately after parturition, rose during lactation, and dropped again upon artificial termination of lactation.

Bovine blood and urine are noticeably lower in sex hormones in comparison to human or equine urine. According to Meschanks (77), the blood of steers and cows contains less than one unit of chick comb growth promoting substance per liter in contrast to almost 20 units per liter of human blood. A positive comb growth test from bull blood requires from 300 to 600 ml. of blood per day.

Fluctuations in total ketosteroid excretion due to season or quality of grass were found by Holtz (57). Differences in the metabolism of testosterone were found in cattle in contrast to man since the gonadal metabolites, androsterone and etiocholanolone, do not occur in cattle urine in large quantities as they do in human urine (56). Using paper chromatography, spots were found corresponding to the 17-ketosteroids dehydroepiandrosterone, etiocholanolone, and 11-ketoetiocholanolone. These spots were, however, not considered as conclusive evidence for the presence of these compounds. Administration of testosterone or corticotrophin increased the excretion of these compounds. Every group of animals tested contained these three steroids. The possibility of adrenal origin for these compounds was suggested by the presence of etiocholanolone, a typical testosterone metabolite, in urine from steers. Testosterone, when administered in a large dose, was found unaltered in the urine. However, unaltered testosterone was not found in the urine of animals receiving lower doses of testosterone. No significant difference in total ketosteroids could be observed following the injection of ACTH into two bulls. Column and paper chromatograms from injected animals did, however, differ markedly from those of normal animals, especially the peak

corresponding to 11-ketoetiocholanolone, dehydroepiandrosterone, etiocholandolone, and a fourth 17-ketosteroid though to be androsterone (57).

For a discussion of testosterone metabolism in man, the reader is referred to Fieser and Fieser (32).

It has also been shown (53) that cortisone administration to adrenalectomized sheep restored the excretory rate of steroids in urine to preoperative levels. ACTH and experimentally produced stress resulted in increased excretion of reducing corticoids in healthy sheep and cattle.

Bush (8), likewise, found an increase in the 17-hydroxycorticosteroids in the blood of ruminants following the injection of 200 IU of ACTH. The levels rose from 0-5 to 20-25 ugm. per 100 ml. of blood. From this study it was concluded that cortisol is the main Porter-Silber chromogen present in the plasma of the cow. However, a 1:1 ratio of cortisol and corticosterone in the blood has been reported (7) from studies using perfused bovine adrenals. Although no other corticosteroids have been reported in large amounts, Glick (39) has reported on the isolation and identification of tetrahydrocortisone and tetrahydrocortisol from cattle bile. These steroids were considered to be metabolites of adrenal cortical steroids and were obtained from pooled bile after treatment with bacterial β-glucuronidase. An increase in the 17-ketosteroid content in the urine from diethylstilbesterol-treated lambs was reported by Ruliffson et al. (105). Similarly, Seekles et al. (113) concluded that the 17-ketosteroids of cow and horse urine were predominantly of adrenal origin and that gonadal steroids constitute only a small part of this fraction. In addition, Holm and Firch (55) has shown that surgical stress may increase the 17-hydroxycorticosteroid as well as the 17-ketosteroid excretion in animals. The urinary concentration of 17-hydroxycorticosteroids was,

however, very low. Robertson et al. (99) also have reported low levels of Porter-Silber chromogens in the plasma of cows.

Miller and Turner (79), using the chick assay procedure, followed blood steroid levels after progesterone injections in the ruminant. Sexually mature and pregnant cows were found to excrete appreciable amounts of androgen, whereas males and immature females did not. Injections of progesterone into males and immature females increased androgenic activity in the feces.

For a more complete discussion of progesterone metabolism see references 56, 73, and 74.

In man, the 17-ketosteroids arise primarily from two organs with approximately one-third coming from the testes and the remaining two-thirds coming from the adrenal cortex (1). Thus the 17-ketosteroids are of interest clinically since they are derived from the metabolism of both the sex and adrenal steroids.

Fecal Excretion of Steroid

The importance of fecal excretion of steroid metabolites has been increasingly recognized in the past few years. The chick assay test has been used almost exclusive for fecal steroid assays (13, 29, 41, 97). Isotopically labeled steroids have been used during the last few years to verify the presence of steroids and steroid metabolites in the feces (142). Among the steroids isolated from the bile or feces are metabolites of estrogens (29), progesterone (79), adrenal steroids (39, 54, 55, 58, 130), and testosterone (57).

Hyde and William (58) have shown differences in the amounts of steroids excreted in the urine and feces of the rat which depended upon the route

of administration. Hydrocortisone-4-C^{14} in 50 ugm. doses was given intravenously, intramuscularly, sublingually, or intragastrically with a stomach tube. More than one-half of the C^{14} given was found in the feces of normal animals; the remainder was found in the urine with no activity being found in the expired air. The rate of excretion was greatest after intravenous injection and decreased in order with sublingual, intramuscular, and intragastric routes of administration. About 91 per cent of the C^{14} given intravenously was excreted in the bile of animals with biliary fistulas, whereas only 80 per cent was eliminated via the liver after administration by the other routes.

In the rodent, biliary excretion was predominant while in man only a small percentage of administered radioactivity was excreted or detected in the feces (39). Biliary excretion in ruminants may be more important than the urinary route (53, 55, 79, 100, 130). Based on a nine minute half-life of cortisol in the blood (81, 100), adrenal perfusion studies (129), and measurements of radioactive steroids in the feces and urine, a low urinary excretion of steroids was confirmed in the calf.

Wright (142) has reviewed the literature concerning the possibility of microbial transformations of steroids in the digestive tract of the ruminant giving rise to androgens. However, very little work has been done to verify this possibility.

Although an enterohepatic pathway for resorption of some steroids may exist, the presence of these steroids in the feces indicates that resorption is incomplete (64). Since some steroids appear in the bile and not in the urine, the fecal route must be important in steroid excretion.

Diurnal Variations

Considerable variation in day-to-day excretion of 17-hydroxycorticosteroids both in single samples and in 24-hour samples is characteristic of manmalian organisms (55). A variety of factors influence the excretionary rate, e.g., environment, somatic conditions, variations due to differences in activity of endocrine systems, and genetic differences (53).

The concentration of steroids in the blood of an animal at any one time is considered to be the product of the secretory rate, distribution into the tissues, and the losses due to metabolism and excretion (62, 90, 106, 131).

A urinary diurnal variation was reported by Meschanks (77) for the bovine. In this study, a low excretion rate was observed in the morning and higher rates in late afternoon and evening. However, Holm and Firch (55) encountered a different situation where the greatest concentration of steroids occurred in the urine at 8 a.m. rather than at 4 p.m. The latter observation corresponds closely to that found in humans where the plasma content was higher in the morning than during the day, evening, or night (89, 106). The highest content of 17-hydroxycorticosteroids was found between 4-9 a.m., usually at 8 a.m., with a rapid decline before 10 a.m. The decrease continued slowly until late evening or early night and then began to rise again. Although the exact cause of the diurnal rhythm is unknown at present, it is believed to be due to fluctuations in the production of 17-hydroxycorticosteroids by the adrenal glands.

That the foodstuff being used for energy could induce changes in steroid excretion has been shown by Kurth (66). This does not, however, explain the diurnal variations in ionone chromogens in urine (84) or the

close correlation of dirunal values in night workers and normal subjects as reported by Samuels et al. (106).

Role of Kidneys on Plasma Steroid Levels

In humans the "free" steroids in the urine were considered to be resorbed up to 80-90 per cent in the kidney tubule under normal conditions (110), whereas the glucuronide conjugated corticosteroids were not. These results were based on the rates of excretion of these steroid fractions before and after a water load.

In uremia, both free and conjugated steroids accumulated in blood concomitant with a decreased urinary excretion. However, the conjugating powers for the 17-hydroxycorticosteroids appeared to be normal (62, 106).

Mode of Steroid Transport in Blood

It has been shown in man that 96 per cent of the 17-ketosteroids was associated with the plasma and no more than four per cent was associated with the red cells (136). It was considered highly probable that all of the conjugated steroids are in the plasma since this four per cent could be due to trapped plasma in the red cell fraction. More recently, the in vitro distribution of some 17-hydroxycorticosteroids in blood has been studied. Extracts of red cells and plasma showed significant amounts of steroids associated with red cells. Of the total steroid content of the blood, 25.1 per cent was reported in the red cell fractions, whereas all of the 17-hydroxycorticosteroid glucuronides were found in the plasma.

It has been shown, however, in human adrenal perfusion studies that the blood, after passing through the adrenal gland, contained the same amount of conjugated steroids as the arterial blood. The main difference

was in the free steroid content which was found to be as high as 280 µgm./100 ml. of plasma. As much as 30 per cent of the total steroids of this blood was found in the red cell fraction. In the peripheral circulation where the levels dropped to 2-23 µgm./100 ml. of plasma, the amount of steroids associated with the red cells was similar to occluded blood (106). Much lower levels of steroids were found in the body fluids of subjects; this difference was attributed to binding of steroids to the plasma proteins.

Protein Binding of Steroids

Albumin has an extraordinary ability to bind a number of chemical substances. Human albumin does bind all the steroid hormones, but the strength of binding varies greatly. The binding of estrogenic hormones is much greater than the comparatively weak binding of corticosteroids. The strengths of the bonds for progesterone and testosterone lie between these two extremes. The number of available binding sites on the albumin molecule is so great that saturation of the binding sites with steroid hormones is never achieved under physiologic conditions. It was found that the human albumin molecule appeared to have two primary binding sites for corticosterone and testosterone and only one binding site for progesterone and estrone. On this basis albumin would be capable of binding in excess of 40 mg. of corticosterone per 100 ml. of human plasma (20).

It would appear that albumin is the only serum protein that binds testosterone (20, 69, 70). Albumin, either bovine or human, has been shown to bind the urinary steroid conjugates, especially the conjugates of estradiol, estrone, progesterone, and testosterone (117).

In human plasma, the 17-ketosteroids have been shown to be associated primarily with fractions IV-1, IV-4, and V; the predominant fraction was found to be fraction V (35, 118).

Little is known concerning the specificity of the binding sites of human albumin for individual steroids, but the "saturation" of albumin with corticosterone did not alter the binding of estrone, progesterone, and testosterone (20). In this respect, an ion-dipole interaction has been suggested (70) and the ϵ-amino group of the bovine serum albumin molecule ruled out as the part of the molecule reacting with the steroids. Van der Waal's forces have also been implicated in the steroid-protein binding (137). Szego (125) has shown that some steroid-protein complexes are apparently synthesized in the liver by an enzyme-catalyzed reaction closely associated with the glucuronosyl transferase system.

Corticosterone and cortisol, which are loosely bound to albumin, have been shown to be tightly bound to the globulin fraction of plasma referred to as the corticosteroid-binding glubulin (20) or transcortin (107, 118). Even though the concentration of transcortin is lower than that of albumin, the former binds cortisol approximately 6,000 times stronger than does the latter (107). The conjugates of these and other steroids were not significantly bound by transcortin. Transcortin has also been demonstrated in other animals (rat, guinea pig, rabbit, and alligator) and with competition studies two types of sites were demonstrated for the binding of cortisol and corticosterone. At one site, cortisol is definitely bound more strongly than corticosterone, while at the second, they are approximately equal in strength of binding. The binding appears to be quite specific for these two steroids (118). The configuration of the oxygen group at carbon-11 is critical in the binding of steroids to transcortin

(20). If the oxygen is present as a ketone group or an 11-alpha hydroxyl group in place of the normal 11-beta hydroxyl group, the affinity for transcortin is decreased. The introduction of a 9-alpha fluro or aldehyde group at carbon-19 (aldosterone) greatly decreased the binding of steroids to this protein.

Distribution of Steroids in the Tissues

Since forces holding the steroids to the plasma proteins are, for the most part, comparatively weak (electrostatic and Van der Waal's forces), the complex formed would be in equilibrium with the free steroid. As the steroid level rises in the blood the proportion taken up by the extravascular tissues increases. However, the difference in distribution of the hormone between vascular and extravascular spaces may not be due solely to the plasma proteins. The proportion leaving the blood stream would be greater with increasing concentration of steroid if absorption occurred on extravascular surfaces of if cells such as fibroblasts engulfed diffusible steroid. Thus a very effective system exists where the increase in hormone content at the tissue level is greater than the increase in plasma concentration providing the extent of protein binding in the blood remains unaltered (106).

Factors Affecting Protein Binding of Steroids in the Blood

Important alterations in corticosteroid metabolism occur during pregnancy or during treatment with large doses of estrogens (11, 30, 51, 98, 118, 133). These include an increase in plasma cortisol concentration, and exaggerated response of plasma levels of cortisol to ACTH, and a decrease in removal of cortisol from plasma (20). The administration of

estrogens caused greatly increased protein binding of steroids with greatly increased levels of the 17-hydroxycorticosteroids. A decreased total daily production of hydrocortisone by the adrenal has been reported following estrogen administration (11). Protein binding of the steroids probably prevents hypercorticism during estrogen therapy by rendering the steroids biologically inactive through restricting their distribution, thereby rendering them unavailable for metabolism (98, 107). Concomitant with this increase of steroids in the blood was a decreased excretion of the conjugates in the urine (11, 51, 98, 133).

Currently there are two main concepts as to the nature of increased protein-bound steroids in the plasma. One group (20) believes that there are three plasma proteins involved in protein-steroid binding (albumin, transcortin, and a corticosteroid-binding globulin similar to transcortin). This third protein cannot be separated using electrophoretic techniques, but was differentiated on the basis of physical properties. The second group (107, 118) believes that there are only two plasma proteins responsible for steroid binding and that the increase in steroid-protein binding is due primarily to increased levels of transcortin in the blood. The main difference of interpretation between the two groups appears to be due to differences in methodology.

A somewhat similar phenomenon occurs with respect to the thyroxine binding protein (30, 42, 95). Pregnancy, as well as estrogen injections, increase the amount of circulating thyroxine-binding protein in the blood (95). Administration of estrogen increases the thyroxine-binding capacity of serum, increases the concentration of PBI, increases pituitary secretion of thyrotropin, and increases thyroid activity (30).

Thus it can be seen that the plasma steroid level does not aid in elucidating the finer differences in adrenal function since the rate of steroid synthesis, degradation, and the distribution volume of the steroid are all factors that modify the plasma concentration. However, measurements of turnover rates make it possible to ascertain if abnormally high concentrations of plasma steroid are the result of increased rates of synthesis or decreased metabolism of circulating steroids (62, 90, 131, 106).

Role of the Liver in Steroid Metabolism

The rate of removal of steroids from the blood is markedly influenced by liver function (31, 106, 136). Changes in blood flow through the liver alters the rate of removal and conjugation of steroids (106). Shunting blood from the portal vein to the vena cava decreased cortisol removal while shunting aortal blood into the vena cava increased its removal. This increased removal from the blood was attributed to accelerated reduction and conjugation of the steroids. When liver function was reduced, there was a significant change in the slope of the removal curve. Similarly, a slower rise and longer duration in the appearance curve was observed for the conjugated 17-hydroxycorticosteroids (106). Acute, hepatic venous congestion severely impaired the enzymatic reduction of ring A of various steroids in both the male and female (144). Impairment of conjugation due to liver damage appears to be due to impaired reduction of steroids and not to the conjugation system. This is evident by the fact that the tetrahydro derivatives of various steroids were conjugated strikingly faster than cortisol when infused into the animals (31). A close correlation has been noted between the size of the adrenal glands

and the capacity of the liver to inactivate adrenal cortical steroid hormones by ring A reduction *in vitro*. Neither ACTH nor adrenal steroids increased the capacity of the liver to inactivate corticosteroids. However, diminution of hepatic capacity for steroid inactivation by surgically removing two-thirds of the liver resulted in secondary adrenal atrophy (131).

Although the adrenal steroids do not affect ring A reduction in the liver, thyroxine and the sex hormones do alter this ability (131, 143, 145). Female rats have larger adrenals than males and the *in vitro* ring A reduction capacity is also greater in the female. There is, however, a reversal of the hepatic-adrenal-sex difference in the hamster with the male having larger adrenals and a greater capacity to inactivate steroids than does the female.

Castration increases while testosterone decreases the Δ^4-steroid hydrogenase activities in male rats. Neither castration nor estrogen administration had any appreciable effect in females. The sex difference persists, although diminished, in animals castrated when young and allowed to grow to maturity (143). The sex difference in reduction of the 17, 21-dihydroxy-20-ketone side chain of cortisone has been shown to be greater in male rats than in females (127). It was also shown that the male hormore promoted the ability of the liver to reduce the sidechain of cortisone while the female hormone had the opposite effect.

Thyroid hormones increase total hepatic activity for *in vitro* ring A reduction of cortisol in both male and female rats. Alterations in thyroid state affected the total amount of Δ^4-steroid hydrogenase, liver size, the amount of enzyme per gram of liver, and the availability of

TPNH[1] (145). In addition, it has been postulated that triiodothyronine increases bilirubin excretion by accelerating maturation of the glucuronosyl transferase system in the newborn human (138).

Other tissues in addition to the liver can metabolize steroids. Kidney and spleen were found to be active in metabolizing 11-dehydro-17-hydrocortisone while blood serum, brain, and muscle were inactive (67). Rat brain, diaphragm, heart, gastrointestinal tract, thymus, leg muscle, and kidney were assayed for their ability to metabolize cortisol and cortisone. Only the kidney was found to be active to any extent (72). However, cortisol has been found to be metabolized by loose connective tissue (3). In addition, extensive metabolism of intravenously administered progesterone and cortisol has been shown to take place in eviscerated rats where the liver, spleen, and gastrointestinal tracts have been removed (4, 5). Of the metabolites isolated, neither the water soluble conjugates nor the tetrahydro derivatives were found.

Mechanism of Glucuronide Formation

One of the pathways for the excretion of hormones and other alcoholic or phenolic compounds in the urine or the bile is through conjugation with glucuronic acid (84). In this form, they possess little or no physiological activity and an enhanced water solubility (59). The physiologically

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[1]The abbreviations used throughout the text are: TPNH, reduced triphosphopyridine nucleotide; UDPGA, uridine diphosphoglucuronic acid; UTP, uridine triphosphate; UMP, uridine monophosphate; UDPglucose, uridine diphosphoglucose; ATP, adenosine triphosphate; ADP, adenosine diphosphate; iPP, inorganic pyrophosphate; DPN, oxidized diphosphopyridine nucleotide; DPNH, reduced diphosphopyridine nucleotide; PAPS, adenosine-3'-phosphate-5-phosphosulfate; H_4E, tetrahydrocortisone; H_4F, tetrahydrocortisol; and PBI, plasma bound iodine.

active hormone may in some instances serve as the substrate for conjugation; in other cases, metabolism and inactivation take place prior to conjugation. The glucuronosyl transferase system resides in the particulate fraction of cells referred to as the microsomes (60).

The formation of a glucuronide conjugate requires "activated" glucuronic acid; the glucuronide containing compound is referred to as UDPGA. Glucuronic acid per se is poorly utilized in conjugation studies (59). Glucose, however, is a good precursor of UDPGA (27, 28). The sequence of events leading to the formation of this compound is as follows (26, 124, 126):

$$\text{Glucose} + \text{ATP} \longrightarrow \text{Glucose-6-phosphate} + \text{ADP}$$

$$\text{Glucose-6-phosphate} \longrightarrow \text{Glucose-1-phosphate}$$

$$\text{Glucose-1-phosphate} + \text{UTP} \longrightarrow \text{UDPGlucose} + \text{iPP}$$

$$\text{UDPGlucose} + \text{DPN} \longrightarrow \text{UDPGA} + \text{DPNH} + H^{+}$$

The oxidation step for the removal of hydrogen and the addition of oxygen at C-6 of UDPGlucose is a two step system (124).

The transfer of glucuronic acid from UDPGA requires a transferase enzyme. At present it is not known whether there is only one enzyme for all substrates or a specific enzyme for each substrate (124). The following reaction characterizes the transfer of glucuronic acid from UDPGA to its acceptor (26, 59, 123, 124).

$$\text{UDPGA} + \text{Aglycone} \longrightarrow \text{Aglycone glucuronide} + \text{UDP}$$

Through this system ether, ester, and normal glucuronides of foreign or endogenous compounds can be formed.

Tissues other than the liver have been implicated in glucuronide conjugation (33). The kidney of man, mouse, and guinea pig (25), the duodenal mucosa in rat (43, 45), skin in mouse and guinea pig (122), and

the avian adrenal gland (87) have been reported as being able to conjugate various substrates with glucuronic acid. However, in vivo studies have failed to show significant amounts of the conjugates being formed when the liver, kidney, and intestinal tract were removed (4, 5). Studies using liver, kidney, and intestine of mice have shown that the kidney and liver of this animal conjugate steroids while the intestine does not. It also has been shown that the parenchymal cells of the liver are the cells responsible for controlling the conjugation of steroids, and that reticuloendothelial cells have a large capacity to oxidize and reduce the substituted groups of the steroid nucleus. The liver, which contains approximately one-third reticuloendothelial cells, actively reduces ring A to form tetrahydro steroids which are subsequently cojugated by the hepatic cells. The kidney, reducing ring A to a much lesser extent than the liver, does not conjugate Δ^4-3-ketosteroids to any appreciable extent. It can, however, conjugate the tetrahydro compounds as readily as the liver (121).

A partial listing of the many compounds that are known to be conjugated with glucuronic acid can be found in the article by Sie and Fishman (115).

Mechanism of Sulfate Conjugation of Steroids

There is now abundant evidence to show that many compounds are conjugated with sulfate as well as with glucuronic acid (6, 21, 88, 103, 104, 108). In the presence of ATP, Mg^{++}, and SO_4^{--} ions, yeast and presumably mammalian cells synthesize PAPS by a two-step reaction as follows (120):

$$ATP + SO_4^{--} + Mg^{++} \longleftrightarrow \text{Adenosine-5'-phosphosulfate} + iPP$$

$$\text{Adenosine-5'-phosphosulfate} + ATP \longrightarrow PAPS + ADP$$

The first step in this sequence is reversible while the second step is non-reversible.

Kidney, spleen, heart, lung, and testes have been shown to produce PAPS and transfer the sulfate from this compound to endogenous acceptors (120). Evidence also was obtained suggesting that some of the endogenous acceptors were phenols and possibly steroids. In addition, evidence has been put forth indicating that there are many sulfokinases in mammalian liver cells with specific enzymes for specific substrates (88). These same workers demonstrated the presence of phenol sulfokinase in kidney and intestine, but the steroid sulfokinase was demonstrated only in the liver.

The transfer of SO_4^{--} from PAPS requires a specific sulfokinase in a reaction as follows:

$$PAPS + ROH \longrightarrow ROSO_3H + ADP$$

CHAPTER III

MATERIALS AND METHODS

PART I:

The following experimental procedures were designed to determine the levels of steroids in the urine, feces, and plasma of bulls before, during, and after intramuscular injections of testosterone propionate.

Experimental Bulls

Seven, Hereford, range bulls, 21-26 months old at the beginning of the experiment, were selected on the basis of physical and genetic uniformity. One bull was discarded because of temperament. The experimental animals were kept in a one-half-acre exercise lot containing a 16 x 16 ft. shelter equipped with an evaporator cooler to give year-round relief from the elements. Daytime temperatures in the shed did not exceed 85° F.

The bulls were fed a ration of prairie hay and a protein-vitamin-mineral supplement to permit normal growth.

Experimental Treatment

The experiment consisted of a 17-week control period, an 18-week injection period, and a 34-week recovery period. The preliminary period started January 10, 1958. Collections of blood, urine, and feces were taken every other week throughout the course of the experiment which ended April 27, 1959. The first 17 days of the control period served to acquaint

the bulls with the exercise pens, personnel, stalls, and other equipment used in collecting the samples.

During the injection period, the bulls received individual doses of 250 mg. of testosterone propionate in cottonseed oil, intramuscularly, three times weekly (68). The dosage was calculated, from that used in man, with consideration given to weight differences (12, 47, 112).

The collection of data was carried over through the recovery period in order to follow steroid metabolism after injections had ceased.

Collection of Samples

Approximately 100 ml. of blood were collected using 100 ml., heparinated centrifuge tubes. The animals then were placed in stalls provided with feed and water which were given ad libitum. Two bulls were placed in the stalls daily. The animals were assigned to the stalls at random but were placed in the stalls the same day of the week in order to keep the time interval between samples constant. Twenty-four-hour urine samples were collected using rubber urinals suspended under the animal with heavy straps.[2] The urinal was connected to a garden hose which passed through a hole in the floor of the stall to a five-gallon galvanized tank placed under each stall. Being next to the concrete floor and insulated by the wooden floor of the stall, the urine was kept several degrees below room temperature. The urine was measured volumetrically, subsampled, and taken to the central laboratory where the samples were stored in the freezer until analyzed.

Metal trays were placed at the rear of the stalls to collect the feces during the 24-hour period. The feces were weighed, mixed thoroughly,

[2]Davol Veterinary Rubber Products, Providence, Rhode Island.

subsampled, and taken to the central laboratory where they were stored in the freezer until analyzed.

Semen samples collected with an artificial vagina, were taken weekly. Testicular biopsies were taken May 5, 1958, September 5, 1958, and April 27, 1959. Periodic measurements of testicular size were reported by Meinecke and McDonald (76).

Blood Analysis

After centrifuging the blood for 45 min. at 3,000 RPM in an International centrifuge, type SB, and plasma was aspirated off and analyzed for steroid content as described below. Ten ml. of plasma were extracted with 10 ml. followed by two five ml. aliquots of chloroform. The chloroform layers were pooled and washed with five ml. aliquots of 0.1 N NaOH and 0.1 N HCl according to the method of Nelson and Samuels (86). This fraction was designated as the "free steroids".

The "glucuronide-bound" steroids were liberated by adjusting the samples to pH 5.0 with acetate buffer, adding several drops of chloroform, and incubating the mixture with 0.5 ml. Ketodase (2,500 units beef liver β-glucuronidase - Warner Chilcott) at 45° C for 15 hours (38). Chloroform was added to the mixture since it has been shown that greater yields of glucuronide-bound materials are obtained when incubated in the presence of small amounts of chloroform (116). The steroids were extracted and purified as described above.

The "sulfate-bound" steroids were liberated by adding four ml. of concentrated HCl and the mixture incubated at 80° C for 20 min. The steroids were extracted and purified as indicated above.

The three steroid fractions were blown to dryness and analyzed using the Isonicotinic Acid Hydrazide (INH) reaction of Weichselbaum and Margraf (134) using a battery operated Spectronic "20" photometer and one-ml. microcuvettes (Pyrocell).

The "total" steroid fraction was obtained by summation of the "free", "glucuronide", and "sulfate-bound" fractions.

An additional 40 ml. aliquot of plasma was adjusted to pH 5.0 using acetate buffer. Several drops of chloroform were added and the mixture incubated with two ml. of Ketodase (10,000 units) for 15 hours at 47° C. After being extracted three times with 15 ml. chloroform per extraction, the extracts were pooled and washed with acid and base as indicated earlier. The 17-ketosteroids were separated from the 17-hydroxycorticosteroids using column chromatography (86).

The 17-ketosteroids were analyzed with the Zimmerman reaction. The method of Callow et al. (9) was modified by using methanol instead of ethanol as the solvent in preparing the 2.5 N KOH (63) and the mixture stabilized with ascorbic acid (44). The two per cent m-dinitrobenzene was made up in methyl cellosolve to increase the stability of the color (140). After stoppering the tubes, the mixture was incubated for three hours in an ice bath at 0° C. The ice bath containing the samples was placed in the refrigerator to avoid light during the incubation period. Following incubation, one ml. of 80 per cent ethanol was added to each tube and the samples read in the Spectronic "20" photometer using microcuvettes as indicated earlier.

Fecal Analysis

Approximately five grams of feces were weighed out from each sample, ten ml. of water were added, and the samples adjusted to pH 5.0 using acetate buffer. After incubating with one ml. of Ketodase (5,000 units) for 15 hours, the mixture was extracted, washed with acid and base, and the chloroform extract run through a Florisil column of which contained two cm. of activated Norit (charcoal) on top of seven cm. of adsorbent to remove interferring pigments. Using this procedure, the neutral steroids were separated into 17-ketosteroids and 17-hydroxycorticosteroids containing both the free and glucuronide-bound steroids. The steroids were eluted from the column using positive pressure. Norit was activated according to the method of Roe and Kuether (102); the Florisil was activated by heating overnight at 375° C and washing with chloroform before using (34). After separation, the 17-ketosteroid samples were split and analyzed using both the INH reaction and the modified Zimmerman reaction for comparative purposes. The 17-hydroxycorticosteroids were analyzed with the INH reaction. All samples were read in the Spectronic "20".

An additional five-gram aliquot of feces was analyzed according to the procedure for plasma free, glucuronide, and sulfate-bound steroid fractions. The pooled extracts for each fraction were filtered through two cm. of activated Norit in chromatographic columns to remove interfering pigments before assaying for steroid content.

Urine Analysis

A five ml. sample of urine was adjusted to pH 5.0 with acetate buffer and incubated with one ml. of Ketodase (5,000 units). Following incubation,

the urine samples were extracted and run through Florisil columns in a manner similar to that described above. The column consisted of five cm. of adsorbent and two cm. of 1.6:10 (W/W) ratio of Norit:Florisil. Both compounds were activated before use as described earlier. Positive pressure was applied to elute the steroids.

The 17-ketosteroid samples were split and assayed by both the modified Zimmerman reaction and the INH reaction as previously described. The 17-hydroxycorticoids were assayed using the INH reaction.

Additional five ml. samples of urine were assayed for free, glucuronide-, and sulfate-bound steroids using the same procedure as described for the plasma samples. One ml. of Ketodase (5,000 units) was used for the glucuronide hydrolysis while four ml. of concentrated HCl were used for the sulfate hydrolysis.

PART II:

The following procedures were followed in order to evaluate the glucuronic acid transferase mechanism in liver and adrenal tissues from rats, dogs, and sheep. Female experimental animals were used in this part of the experiment since it has been shown (145) that female rats have more active enzyme systems in respect to steroid inactivation and metabolism. Cattle were replaced by sheep for economic reasons while rats and dogs were used for comparative purposes.

Experimental Rats

Mature, non-pregnant, female rats (Holtzman strain) were caged in groups and fed Laboratory Chow ad libitum. The rats were sacrificed by decapitation, the chest massaged to facilitate the removal of blood from the body, and the body cavity opened by making a "V" shaped incision

extending from the pelvic region to the ribs. The liver or adrenals were quickly removed, chilled, weighed, minced, and homogenized.

Experimental Dogs

Mature, non-pregnant, female, mongrel dogs were purchased locally and held in kennels until sacrificed. The animals were fed Purina Dog Chow. The dogs were killed by electrocution, the body cavity quickly opened, and the adrenals or liver quickly removed, chilled, minced, weighed, and homogenized. In order to get enough tissue for conjugation studies, the adrenals from two animals were pooled for each assay.

Experimental Sheep

Six, mature, western type, non-pregnant, female sheep were obtained locally and fed a ration of prairie hay and a vitamin-mineral-protein supplement to permit normal growth. The sheep were killed by electrocution, the body cavity opened, and the adrenals or liver removed, chilled, minced, weighed, and homogenized.

Tissue Preparation

Tissues were homogenized in ice cold KCl solutions in a Potter-Elvejhem homogenizer. A 0.1 M KCl solution was used for microsomal preparations (61) while isotonic KCl (1.12 per cent) was used for homogenate preparations (93). A 30 per cent homogenate was prepared for all tissues except the rat adrenals. Microsomes were prepared by centrifuging the homogenate in a Servall centrifuge at 10,800 x g for 10 min. in a cold room (1-2° C) to remove nuclei, mitochondria, and cell debris. The supernatant was centrifuged for one hour in a Spinco Model L

ultracentrifuge at 105,000 x g. The supernatant was discarded and the microsomes resuspended in 0.1 M KCl using a Potter-Elvejhem homogenizer. Each ml. of resuspended microsomes represented two and one-half grams of original tissue. Due to the low yield of microsomes from rat adrenal tissue, conjugation studies using microsomes from this tissue were omitted.

Incubation of Homogenates

One ml. of homogenate was added to a Warburg flask containing 25 mg. glucose, 0.5 ml. buffer, 0.5 ml. UDPGA (0.086 μMoles - Sigma), and 0.5 ml. aqueous solution containing 0.140 μMoles of steroid. In a like manner, one ml. of homogenate was added to a 30 ml. test tube containing 0.5 ml. buffer, 0.5 ml. distilled water in place of the UDPGA, and 0.5 ml. steroid solution (0.140 μMoles). This tube, containing no UDPGA, served as a control to ascertain the amount of steroid that disappeared during incubation. The buffer of Potter et al. (93) was used in preference to Krebs-Ringer-Phosphate buffer (128) because the Potter buffer has a higher concentration of potassium ions and lower sodium content. This buffer was used since it has been shown that larger QO_2 resulted using this buffer with bovine liver homogenates (109). Greater transferase activity was also achieved with the isotonic KCl instead of 0.25 M sucrose as the homogenizing medium.

Incubation of Microsomes

Incubations were carried out in 15 x 125 mm. pyrex test tubes under constant shaking at 37° C for one hour. The substrates, dissolved in methanol, were added to the tubes and blown to dryness before adding the other ingredients. Two tubes were used for each sample. One tube

containing 0.1 ml. UDPGA (0.4 μMoles), 0.55 ml. Tris buffer (61), and 0.1 ml. microsomes was incubated to measure transferase activity. Similarly, a second tube containing 0.1 ml. distilled water, 0.55 ml. Tris buffer, and 0.1 ml. microsomes was not incubated but served as a control in order to calculate the disappearance of the substrate.

Inhibition Studies

Sheep liver microsomes were prepared as described above. The incubation mixture and procedure were the same as indicated above except for the addition of 0.4 μMoles of competitive substrate (H_4E) and the addition of the substrates in 0.0, 0.1, 0.2, 0.3, and 0.4 μMole increments. The substrates were phenolphthalein and p-nitrophenol. Competitive conjugation incubations were carried out in duplicate. Separate assays using the same enzyme preparation were used to establish normal rates for the enzyme preparation for comparative purposes.

Substrate Determinations

Steroid determinations for both the homogenate and microsomal studies were analyzed by boiling the test tubes containing the incubation mixture minus the UDPGA in boiling water for two min. to inactivate the enzymes and precipitate the proteins. The contents of the Warberg vessels after incubation were transferred to test tubes and boiled as indicated above. Both series of tubes were extracted three times with two and one-half volumes of chloroform and the extracts pooled, washed with acid and base, evaporated to dryness, and the steroids separated from interfering materials using column chromatography. The columns were prepared according to the method of Nelson and Samuels (86). The columns were washed

with chloroform (34), the sample in five ml. of chloroform was added to the column, and the column developed using 25 ml. of chloroform followed by 10 ml. of three per cent (v/v) methanol in chloroform. This was followed by 60 ml. of three per cent (v/v) methanol in chloroform and finally by 20 ml. of 40 per cent (v/v) methanol in chloroform. Only the last two fractions contained the steroids. These two fractions were combined, blown to dryness, split, and analyzed for steroid content using the Robertson-Mixner modification of the Porter-Silber method (100). The samples were read in a Beckman model DU spectrophotometer using micro cells (Pyrocell).

Phenolphthalein and p-nitrophenol determinations were accomplished after precipitation of the protein with three ml. of 95 per cent ethanol. The samples were centrifuged to remove the precipitate and one ml. aliquots were taken from each tube for substrate assays. Phenolphthalein samples were assayed in duplicate according to the method for β-glucuronidase assay (116). p-Nitropnenol was assayed in duplicate using the method outlined by Isselbacher and McCarthy (61). The samples were read in a Coleman Jr. spectrophotometer.

Microsomal protein determinations were accomplished using the Biuret test as outlined by Gornal et al. (40).

CHAPTER IV

RESULTS AND DISCUSSION

PART I:

Data were collected from six Hereford bulls over a period of 64 weeks; samples of blood, feces, and urine were collected once every two weeks during the course of the experiment except for the last two months when they were collected once every four weeks. A 12-week control period was followed successively by an 18-week injection period and a 34-week recovery period. The samples collected during these three periods were analyzed according to the procedure outlined in Table I; the results are summarized in Table II. Since very little is known concerning the metabolism of testosterone in the male ruminant, steroid levels were followed in the plasma, urine, and feces. In addition, many of the conventional tests used to follow steroid metabolism in humans are of little value in ruminants; therefore, various fractionation procedures as well as colorimetric tests were used to follow variations in the steroid fractions of the plasma, urine, and feces. In this connection, a search of the literature has failed to show any attempt to correlate blood, urine, and fecal levels of any steroid fraction in the bull over a prolonged period of time.

Steroid levels, determined by the INH method, for plasma, urine, and feces are plotted in Figures 1, 2, and 3, respectively. Inspection of these figures shows large variations in all fractions, but especially in the conjugated and total fractions. Since it has been shown previously

TABLE I. ANALYTICAL PROCEDURES FOR ISOLATION AND ASSAY OF STEROID FRACTIONS

Samples	Procedure for Isolation of Steroids	(Neutral) Steroid Fractions Obtained	Testsutilized
Plasma	(10 ml.)		
	1. Extracted with $CHCl_3$	Free	INH
	2. Incubated with β-glucuronidase & re-extracted with chloroform	Glucuronide	INH
	3. Boiled in acid & re-extracted	Sulfate	INH
	4. Summation of the above	Total	INH
	(40 ml.)		
	1. Incubated with β-glucuronidase, extracted with $CHCl_3$, & separation using column chromatography. (Florisil)	17-Ketosteroids (Free and glucuronide)	Zimmerman
Feces	(5 gm.)		
	1. Extracted with $CHCl_3$	Free	INH
	2. Incubated with β-glucuronidase & re-extracted with $CHCl_3$	Glucuronide	INH
	3. Boiled in acid & re-extracted	Sulfate	INH
	4. Summation of the above	Total	INH
	(5 gm.)		
	5. Incubated with β-glucuronidase, extracted with $CHCl_3$, & separation using column chromatography (charcoal and Florisil).	17-Ketosteroids (Free and glucuronide)	INH & Zimmerman
		17-OH Corticosteroids (Free and glucuronide)	INH
Urine	(5 ml.)		
	1. Extracted with $CHCl_3$	Free	INH
	2. Incubated with β-glucuronidase & re-extracted with $CHCl_3$	Glucuronide	INH
	3. Boiled in acid & re-extracted	Sulfate	INH
	4. Summation of the above	Total	INH
	(5 ml.)		
	5. Incubated with β-glucuronidase, extracted with $CHCl_3$, & separation using column chromatography (charcoal and Florisil).	17-Ketosteroids (Free and glucuronide)	INH & Zimmerman
		17-OH-Corticosteroids (Free and glucuronide)	INH & Zimmerman

TABLE II. AVERAGE STEROID CONTENT OF PLASMA, URINE, AND FECES OF VARIOUS STEROID FRACTIONS DURING THREE EXPERIMENTAL PERIODS

	Control Period							Infection Period								
								1958								
	1-27	2-10	2-24	3-17	3-31	4-14	4-28	5-12	5-26	6-9	6-23	7-7	7-21	8-4	8-18	9-1
Plasma (INH) Free (ugm./ml.)							0.13	0.00	0.11	0.67	0.90	3.98	6.29	2.79	4.34	2.00
Gluc (ugm./ml.)							18.71	17.60	29.34	5.26	11.51	24.82	26.39	27.07	36.97	38.06
SO_4 (ugm./ml.)							19.39	33.01	41.93	14.61	32.08	12.29	11.36	3.16	11.38	3.31
Total (ugm./ml.)							38.23	50.61	71.38	20.54	44.49	41.09	44.04	33.02	52.69	43.37
Urine (INH) Free (ugm./ml.)	2.48			2.51			2.68			3.40			6.20			8.09
Gluc (ugm./ml.)	8.97			7.85			9.77			8.11			11.68			5.64
SO_4 (ugm./ml.)	14.09			15.49			11.83			12.11			15.29			16.70
Total (ugm./ml.)	25.54			25.85			24.28			23.62			33.17			30.43
Feces (INH) Free (ugm./gm.)	116.10			96.99			89.29			80.67			79.13*			66.01
Gluc (ugm./gm.)	103.21			63.95			77.15			63.87			47.30*			28.43
SO_4 (ugm./gm.)	71.99			71.23			52.16			41.96			33.80*			37.52
Total (ugm./gm.)	291.31			232.17			218.59			186.50			160.23*			131.96
Plasma (Zimmerman) 17-Ketosteroids (ugm./ml.)			0.26	0.34	0.18	0.44	1.30	0.99	1.64	1.72	3.79	1.30	1.37	0.56	0.48	0.71
Urine (Zimmerman) 17-Ketosteroids (ugm./ml.)	25.61	23.68	25.80	31.92	23.66	21.37*	19.34	21.03*	22.09	22.68	29.38	39.56	20.37	38.70	52.00	48.37
Urine (INH) 17-Ketosteroids (ugm./ml.)	8.72	7.08		8.43	5.59	10.57*	8.09	10.73*	5.39	7.78	9.45	13.84	6.32	7.18	13.07	11.83
Urine (INH) 17-OHCS (ugm./ml.)	0.88	0.20	2.00	0.48	0.66	0.70*	0.65	1.01*	0.24	0.62	0.78	0.93	0.38	0.34	0.58	0.72
Feces (Zimmerman) 17-Ketosteroids (ugm./gm.)			102.21	40.26	40.92	159.71	209.27	156.38	128.40	81.84	116.35	22.20	192.72	140.83	80.09	224.60
Feces (INH) 17-Ketosteroids (ugm./gm.)				39.59	37.86	139.43	177.04	116.67	104.09	55.60	69.83	116.23	128.26	72.73	76.44	98.18
Feces (INH) 17-OH (ugm./gm.)			7.17	10.07	16.51	31.95	38.56	21.65	23.89	25.99	30.04	19.02	24.92	8.69	10.08	14.24

*Indicates average on five instead of six animals.

TABLE II. (Continued)

	Recovery Period														
	1958								1959						
	9-15	9-29	10-13	19-27	11-10	11-24	12-8	12-22	1-5	1-19	2-2	2-16	3-2	3-20	4-27
Plasma (INH) Free (ugm./ml.)	2.97	1.18	3.31	0.33	0.63	1.69	2.30	0.00	0.41	0.00	0.00	1.12	1.36	4.37	3.66
Gluc (ugm./ml.)	38.39	47.42	56.82	46.51	57.03	39.95	19.02	118.40	147.02	63.09	91.88	102.91	74.51	43.87	38.37
SO_4 (ugm./ml.)	17.90	12.42	16.89	17.01	19*.36	17.76	23.06	39.79	97.09	45.59	82.74	24.95	40.58	3.38	12.36
Total (ugm./ml.)	59.26	61.02	77.02	63.85	70*.62	59.40	44.38	158.19	244.52	108.68	174.98	128.98	116.45	51.62	54.39
Urine (INH) Free (ugm./ml.)			4.40			3.44			3.92			4.41			8*.14
Gluc (ugm./ml.)			10.20			8.14			7.68			8.47			6*.33
SO_4 (ugm./ml.)			15.10			14.82			18.67			15.72			17*.28
Total (ugm./ml.)			29.70			26.40			30.27			28.60			31*.75
Feces (INH) Free (ugm./gm.)			51.45			67.93			52.73			62.72			25.17
Gluc (ugm./gm.)			30.16			63.10			32.76			45.39			31.78
SO_4 (ugm./gm.)			40.12			53.02			29.81			46.73			45.21
Total (ugm./gm.)			121.73			184.05			115.30			154.84			102.16
Plasma (Zimmerman) 17-Ketosteroids (ugm./ml.)	0.31	0.44	0.40	0.49	0.33	0.72	0.16	0.66	1.27	0.24	0.20	1.68	0.67	2.21	2.52
Urine (Zimmerman) 17-Ketosteroids (ugm./ml.)	37.07	42.96	41.18	32.91	40.88	38.32	37.08	22.51	34.97	26.66	36.48	36.69	50.76	41.28	48*.72
Urine (INH) 17-Ketosteroids (ugm./ml.)	9.16	15.87	9.25	10.83	8.07	9.59	12.29	5.92	10.62	6.21	12.43	8.30	10.32	10.31	12*.29
Urine (INH) 17-OHCS (ugm./ml.)	0.76	1.28	0.65	0.72	1.45	1.01	1.20	0.13	0.83			1.11	0.74	0.80	1*.05
Feces (Zimmerman) 17-Ketosteroids (ugm./gm.)	227.02	228.97	217.53	164.02	174.46	49.71	42.90	236.76	202.05	311.79	103.83	158.54	143.95	183.31	145.28
Feces (INH) 17-Ketosteroids (ugm./gm.)	97.08	95.62	109.76	106.66	131.97	75.24	65.01	96.09	98.18	93.80	104.66	75.08	76.75	61.26	68.83
Feces (INH) 17-OH (ugm./gm.)	23.89	17.70	19.64	14.24	24.31	9.31	10.49	13.30	15.84	10.55	12.17	7.02	7.84	7.98	9.37

*Indicates average on five instead of six animals.

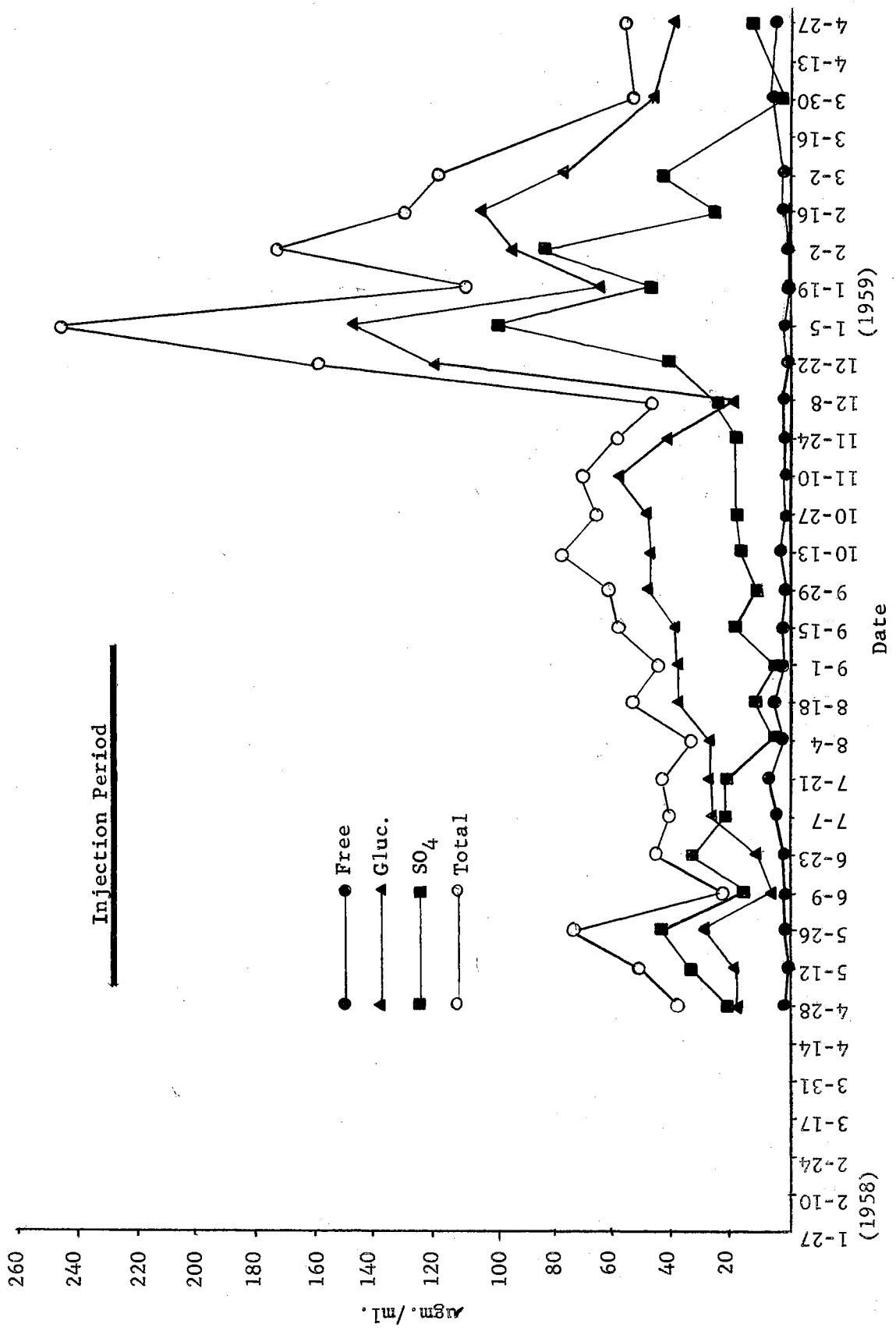


Figure 1. Free, Glucuronide, Sulfate, and Total (INH) Steroid Content of Plasma During Three Experimental Periods

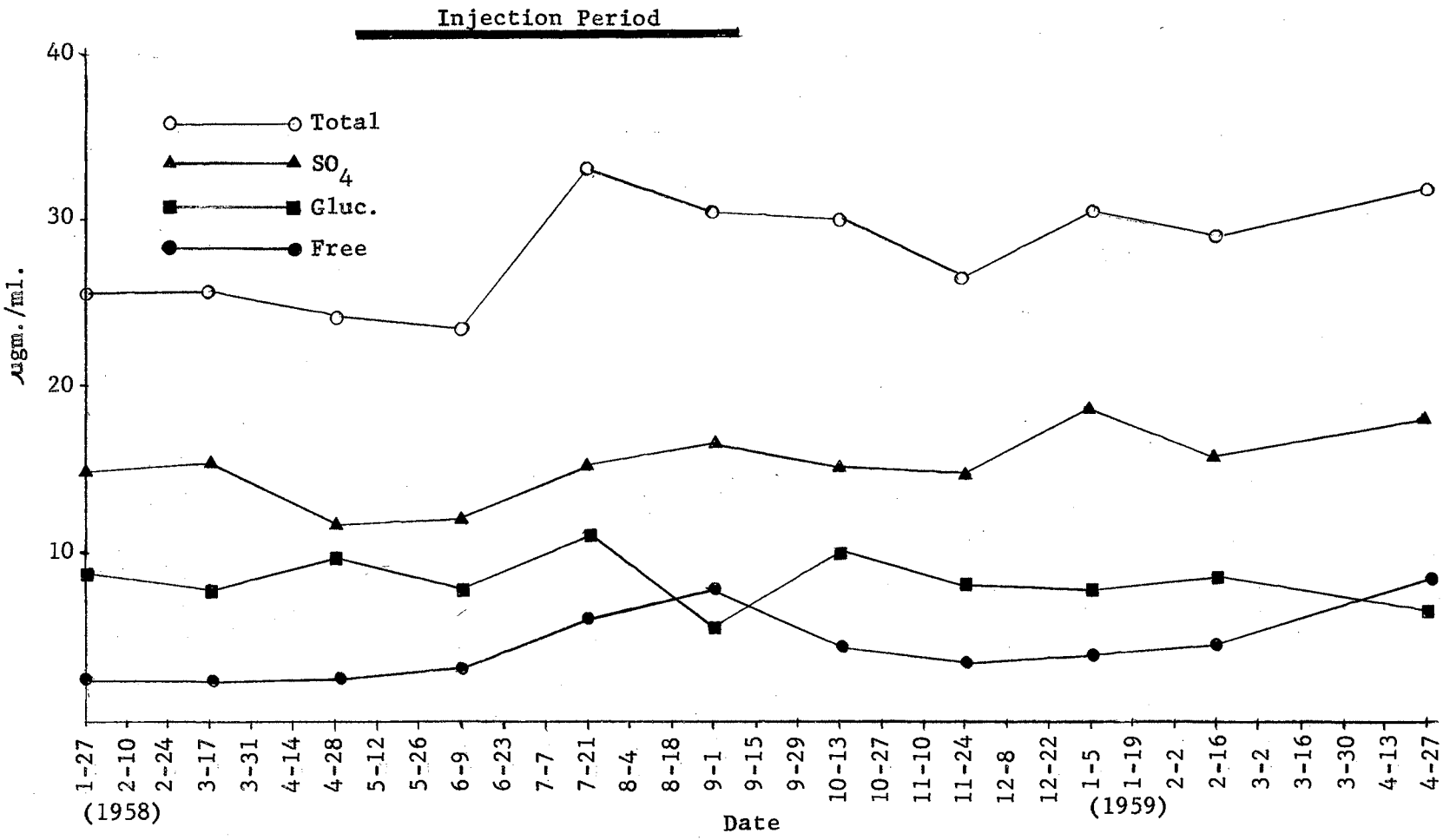


Figure 2. Free, Glucuronide, Sulfate, and Total (INH) Steroid Content of Urine During Three Experimental Periods

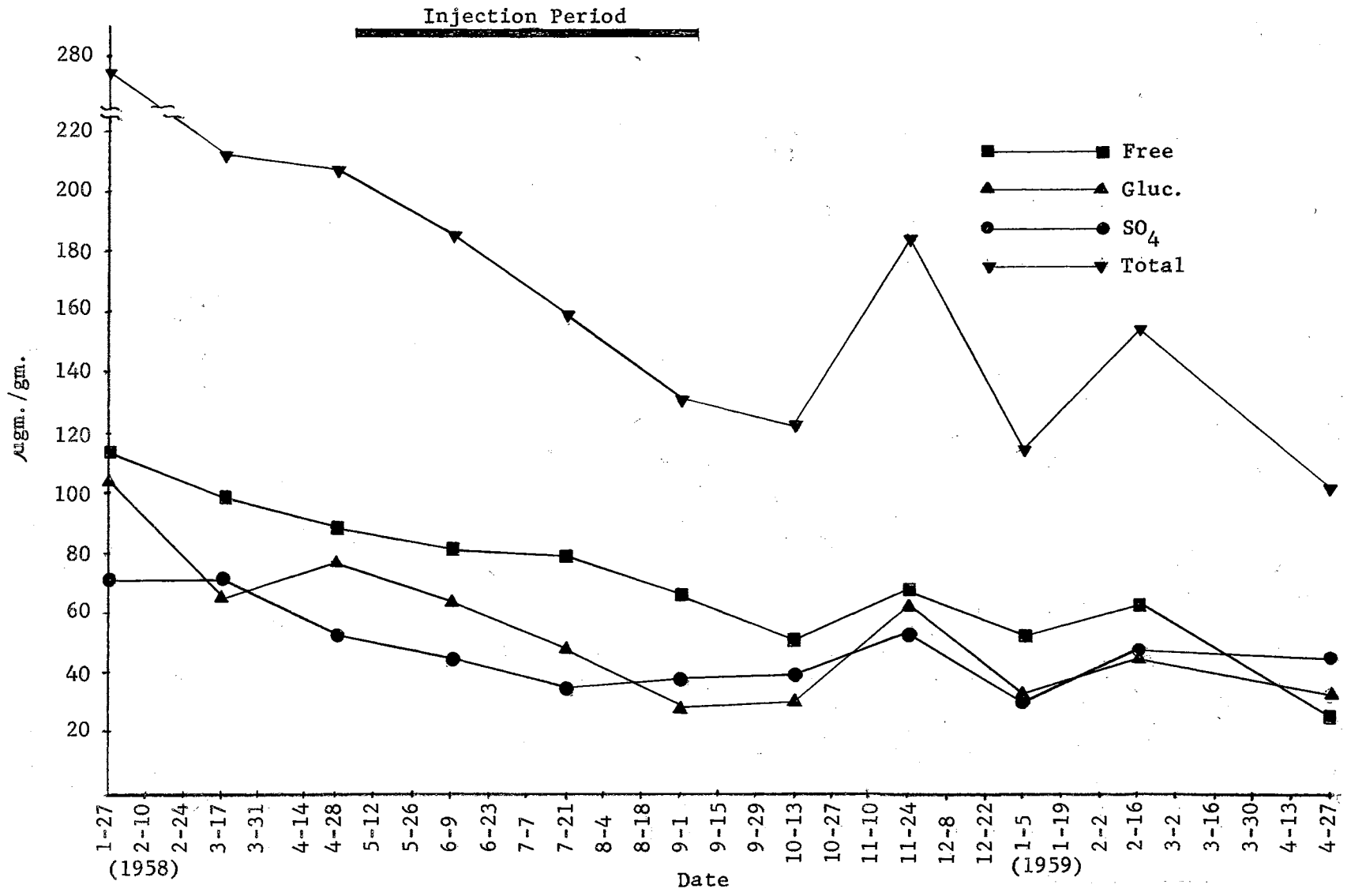


Figure 3. Free, Glucuronide, Sulfate, and Total (INH) Steroid Content of Feces During Three Experimental Periods

that all fractions are comprised mainly of ionone derivatives and metabolically inactive steroids (56, 57, 13), the free steroids were reported in Figure 4 using an expanded scale to give more detail as to the nature of the fluctuations in this more active fraction. Many factors affect the levels of steroids in the blood (62, 90, 106, 131) tending to give erroneous impressions as to their physiological significance; therefore, it also was deemed necessary to plot the glucuronide, sulfate, and total (INH) steroid content of the plasma, urine, and feces in separate graphs (Figures 5, 6, and 7, respectively). This was done since steroids can build up in the plasma due to protein binding and still not be metabolically active (106). In addition, this method of plotting the data also helps in visualizing which route of excretion predominated for each steroid fraction.

The data from the plasma (Zimmerman) and urine (INH and Zimmerman) were plotted in Figure 8 for comparative purposes. The data for the fecal 17-ketosteroid (Zimmerman) and 17-ketosteroid (INH) were plotted in Figure 9. Likewise, the urinary 17-hydroxycorticosteroids (INH and Zimmerman) were plotted in Figure 10. The reproductive data obtained from the same animals used in this experiment are summarized in Figure 11 for the semen values and Figure 12 for testicular volume. Similarly, the fecal dry-matter excreted and the volume of urine voided were plotted in Figure 13.

The values obtained from the plasma, urine, and feces have been expressed in terms of μgm. per ml. for plasma and urine while values for the feces were expressed as μgm. per gm. of feces on a dry-matter basis. This method was used since there was considerable variation in daily

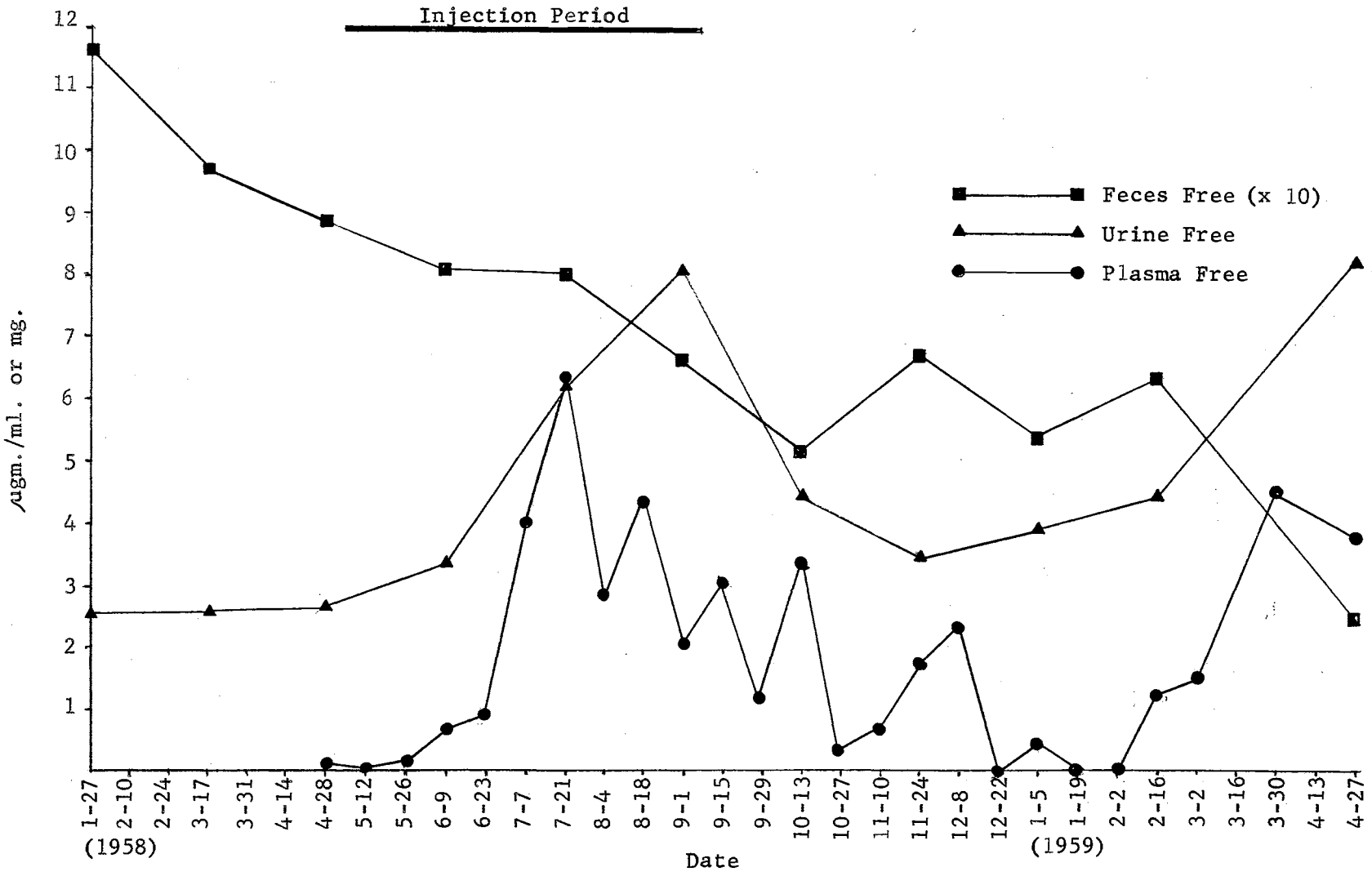


Figure 4. Free (INH) Steroid Content of Plasma, Urine, and Feces During Three Experimental Periods

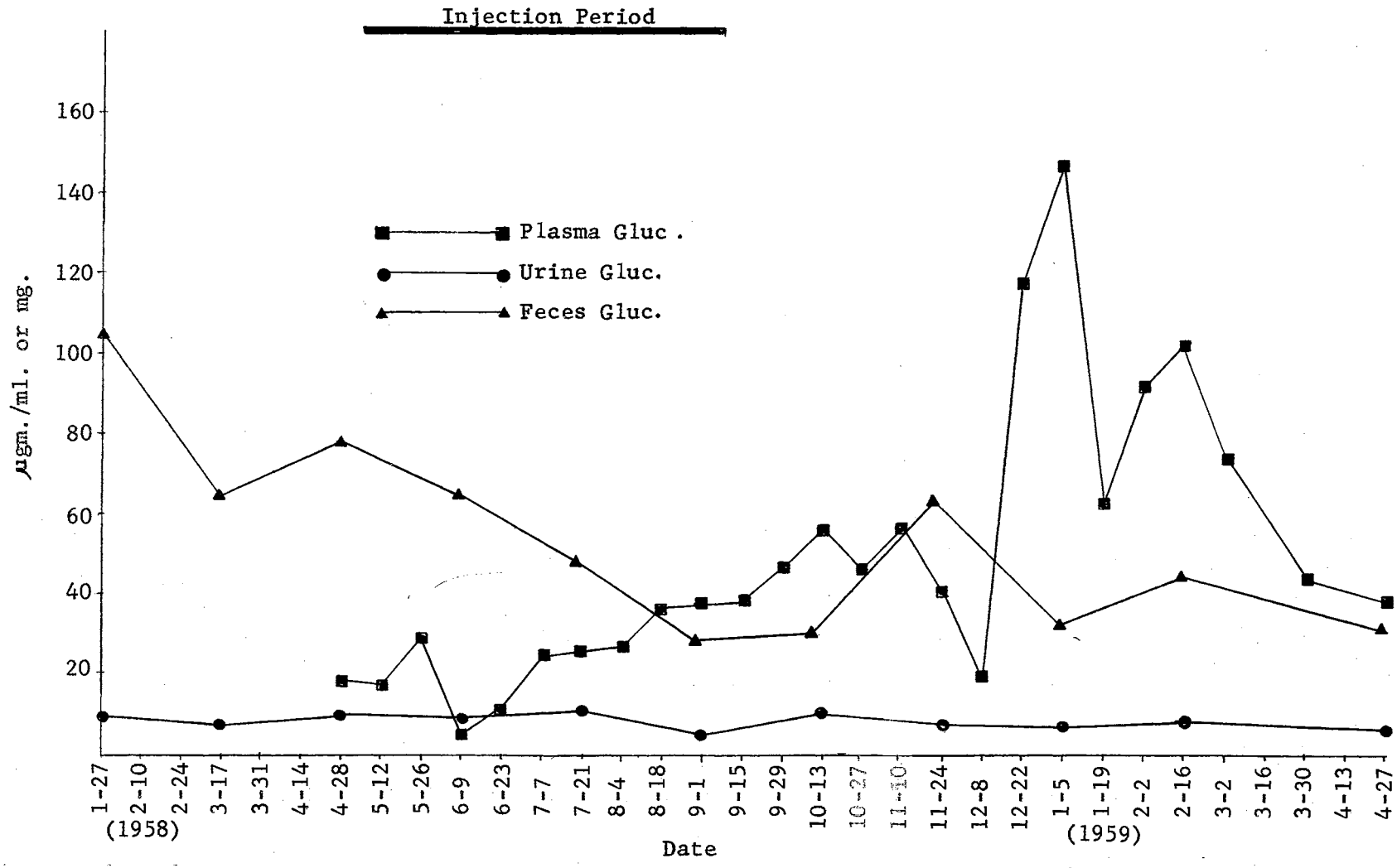


Figure 5. Steroid Content of Glucuronide (INH) Fractions of Plasma, Urine, and Feces During Three Experimental Periods

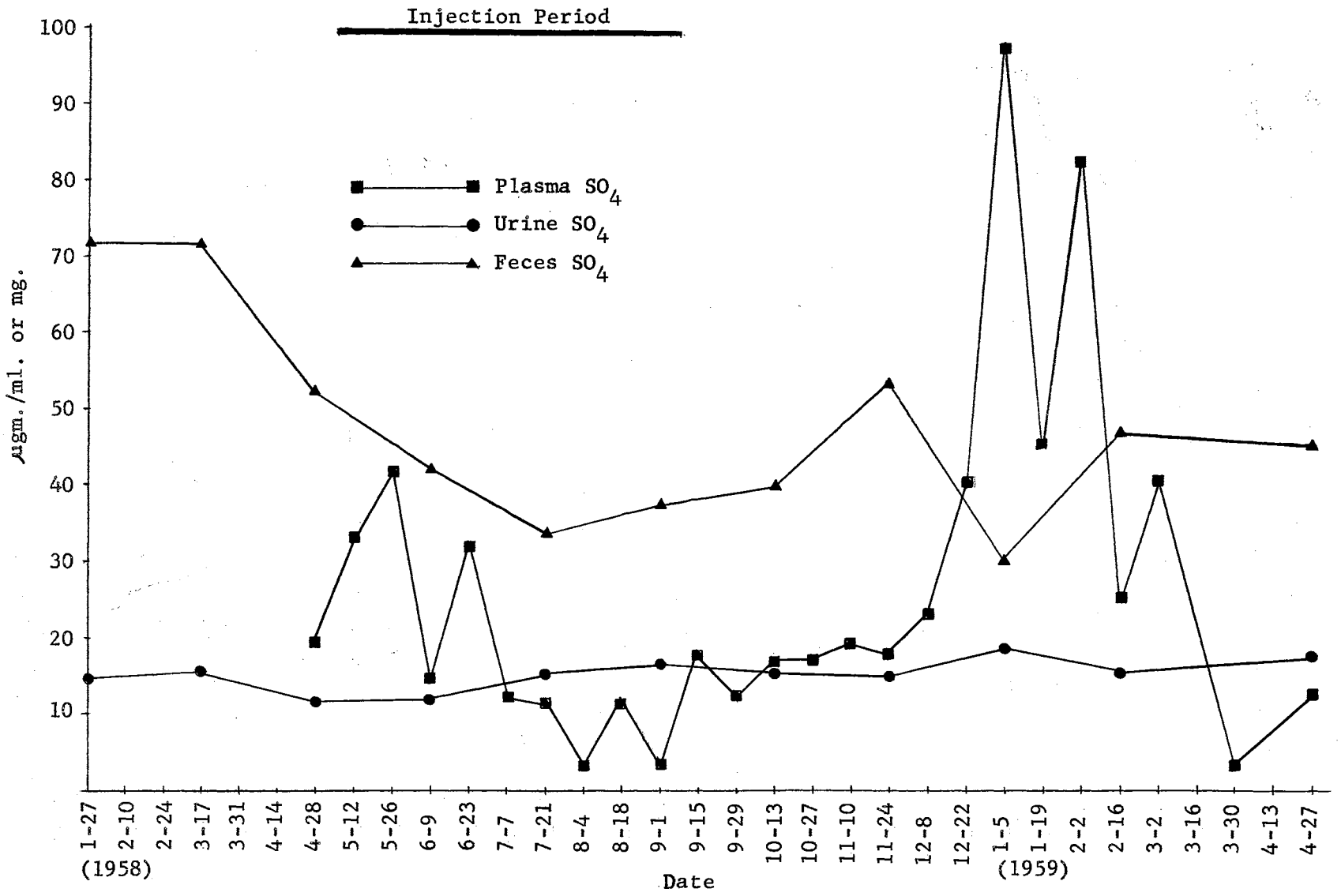


Figure 6. Steroid Content of Sulfate (INH) Fractions of Plasma, Urine, and Feces During Three Experimental Periods

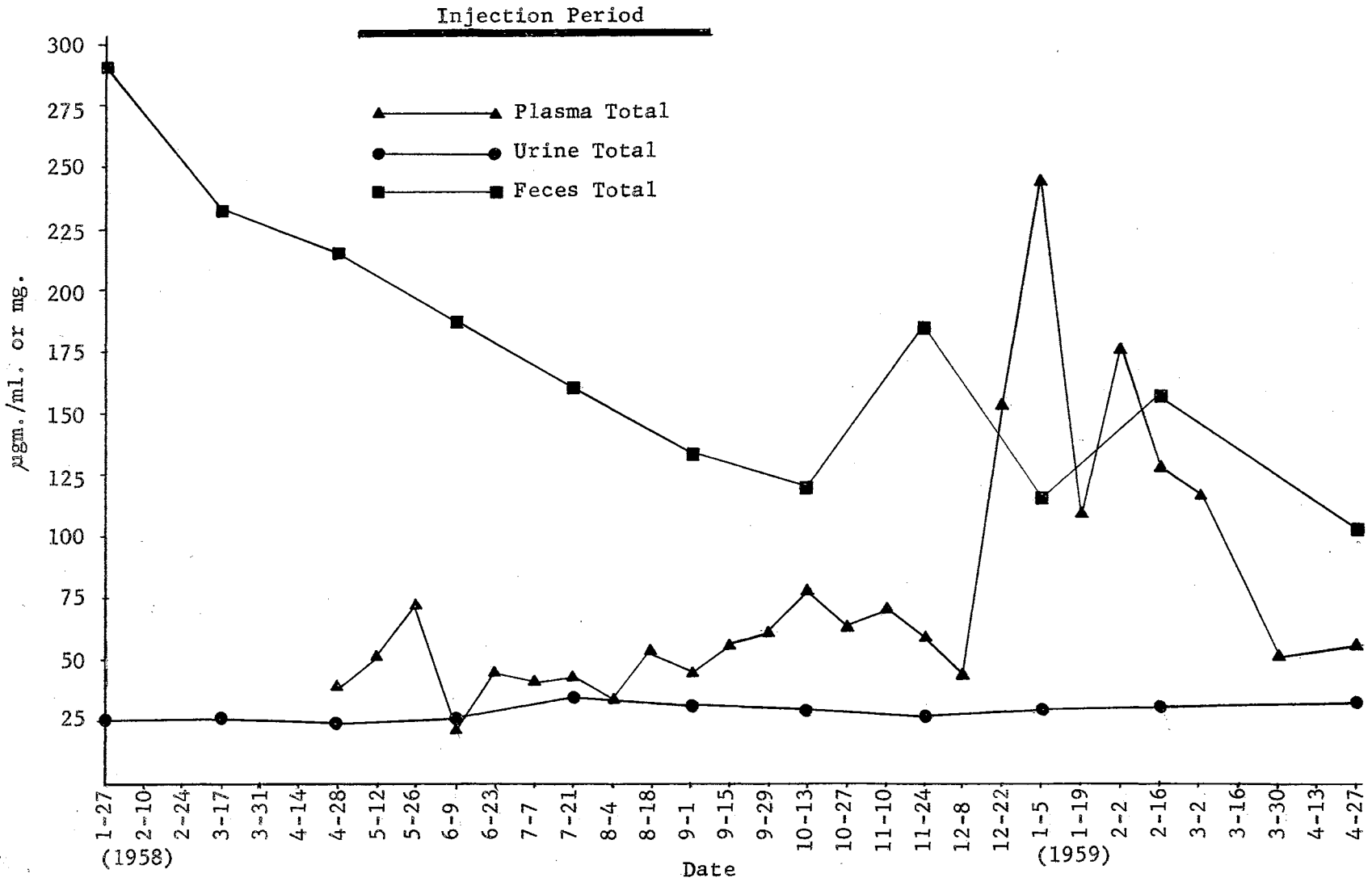


Figure 7. Total (INH) Steroid Content of Plasma, Urine, and Feces During Three Experimental Periods

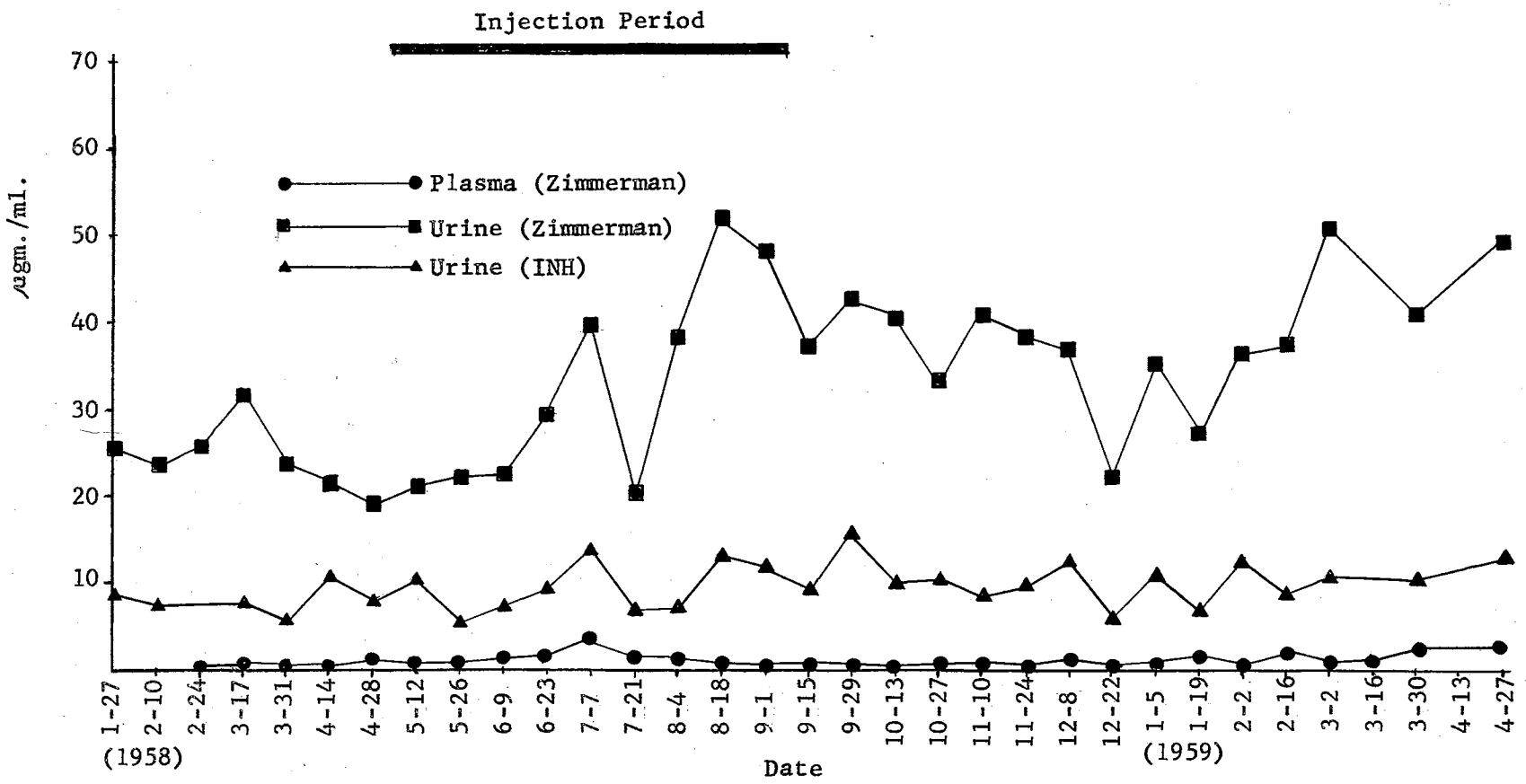


Figure 8. Plasma and Urinary 17-Ketosteroid (Zimmerman and Urinary 17-Ketosteroid (INH) Content During Three Experimental Periods

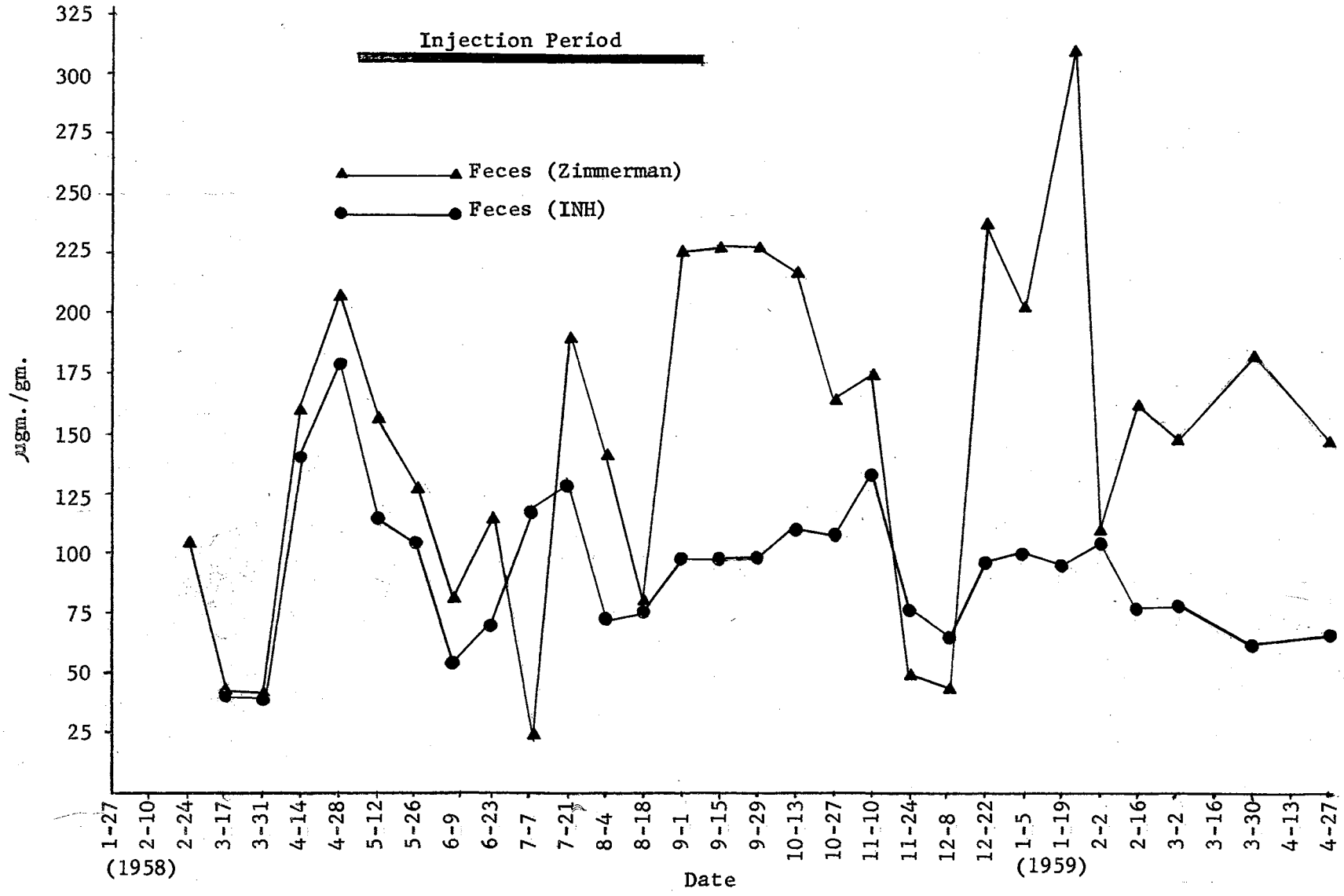


Figure 9. Fecal 17-Ketosteroid (Zimmerman and INH) Content During Three Experimental Periods

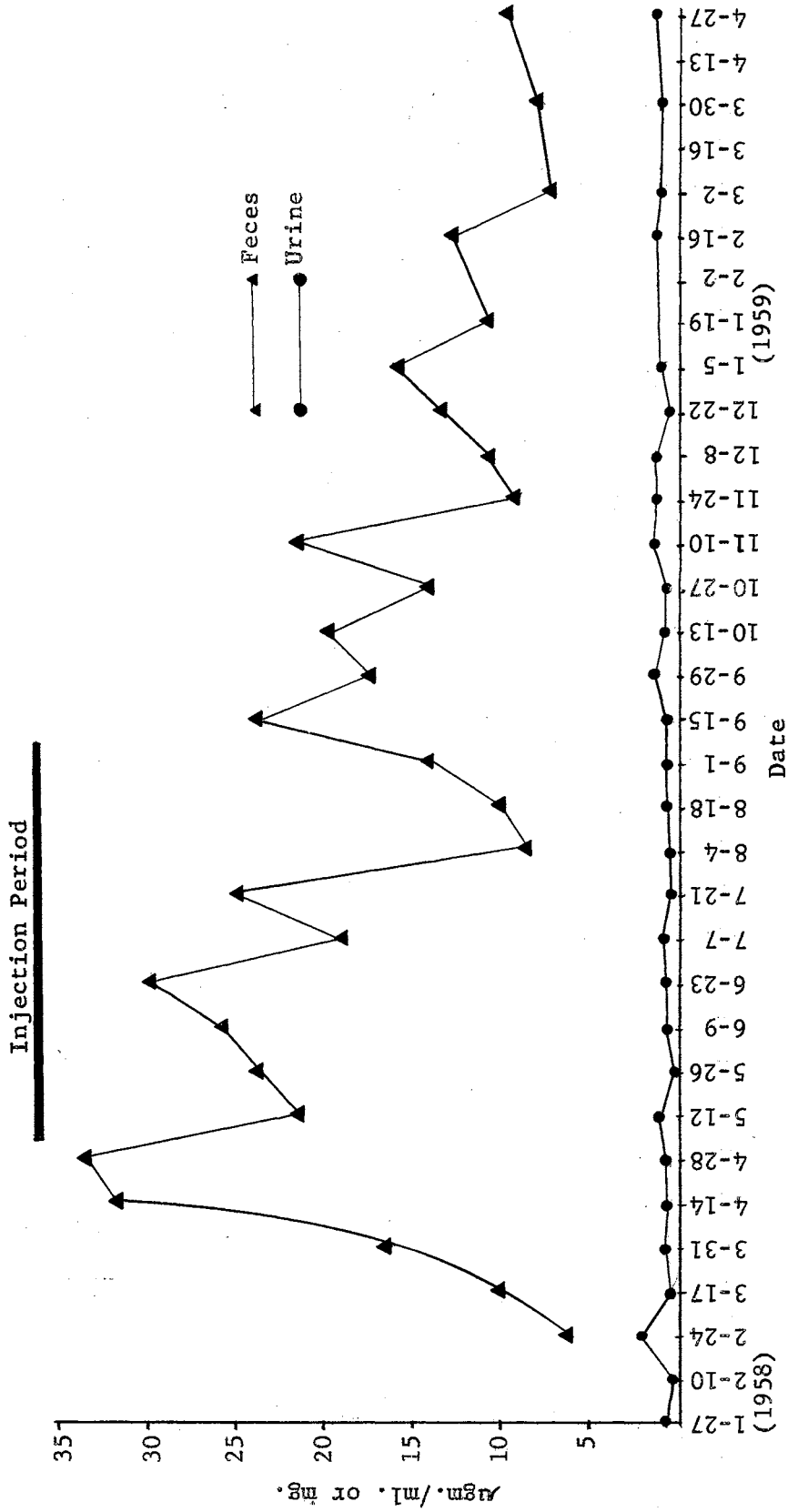


Figure 10. Urinary and Fecal 17-Hydroxycorticosteroid (INH) Content During Three Experimental Periods

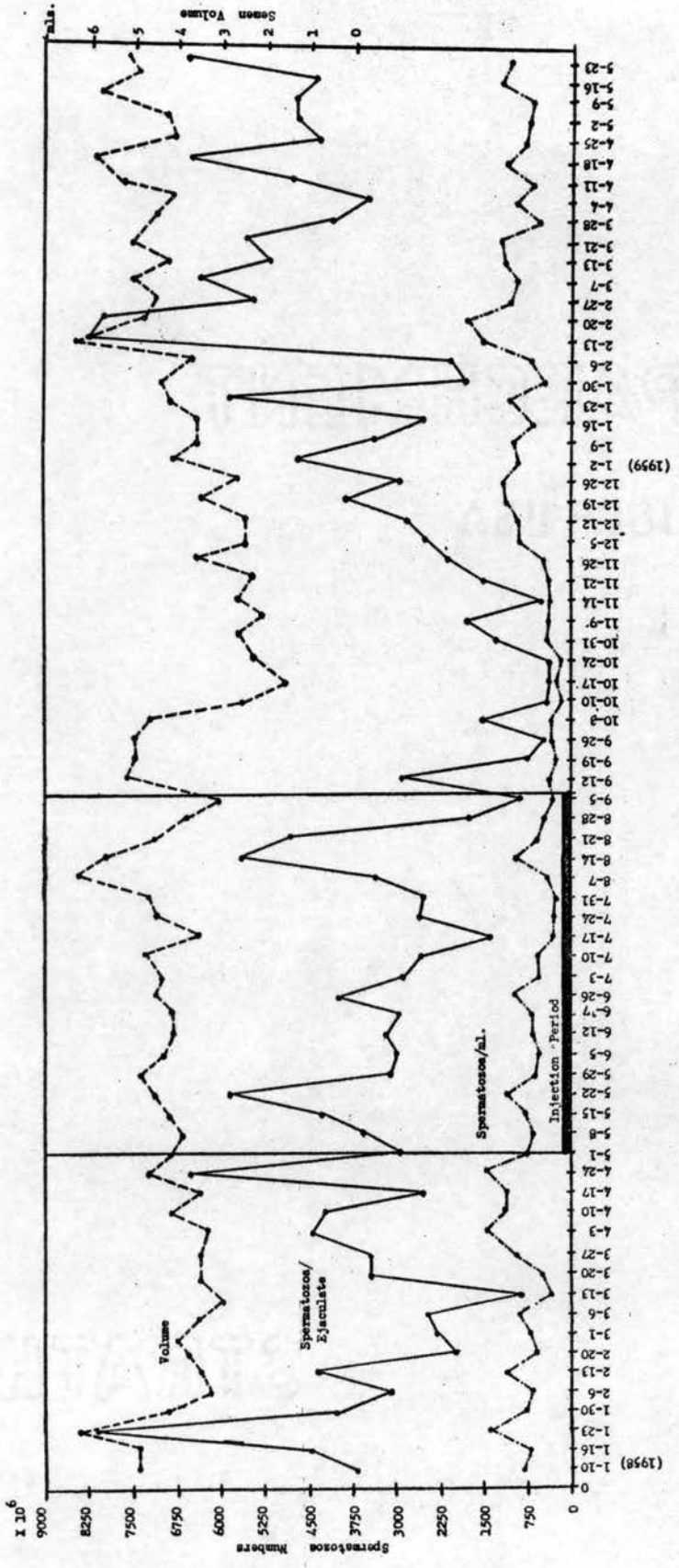


Figure 11. Weekly Average Values of Semen Collected from Four Hereford Bulls (Calculated for the number obtained if four samples not collected)

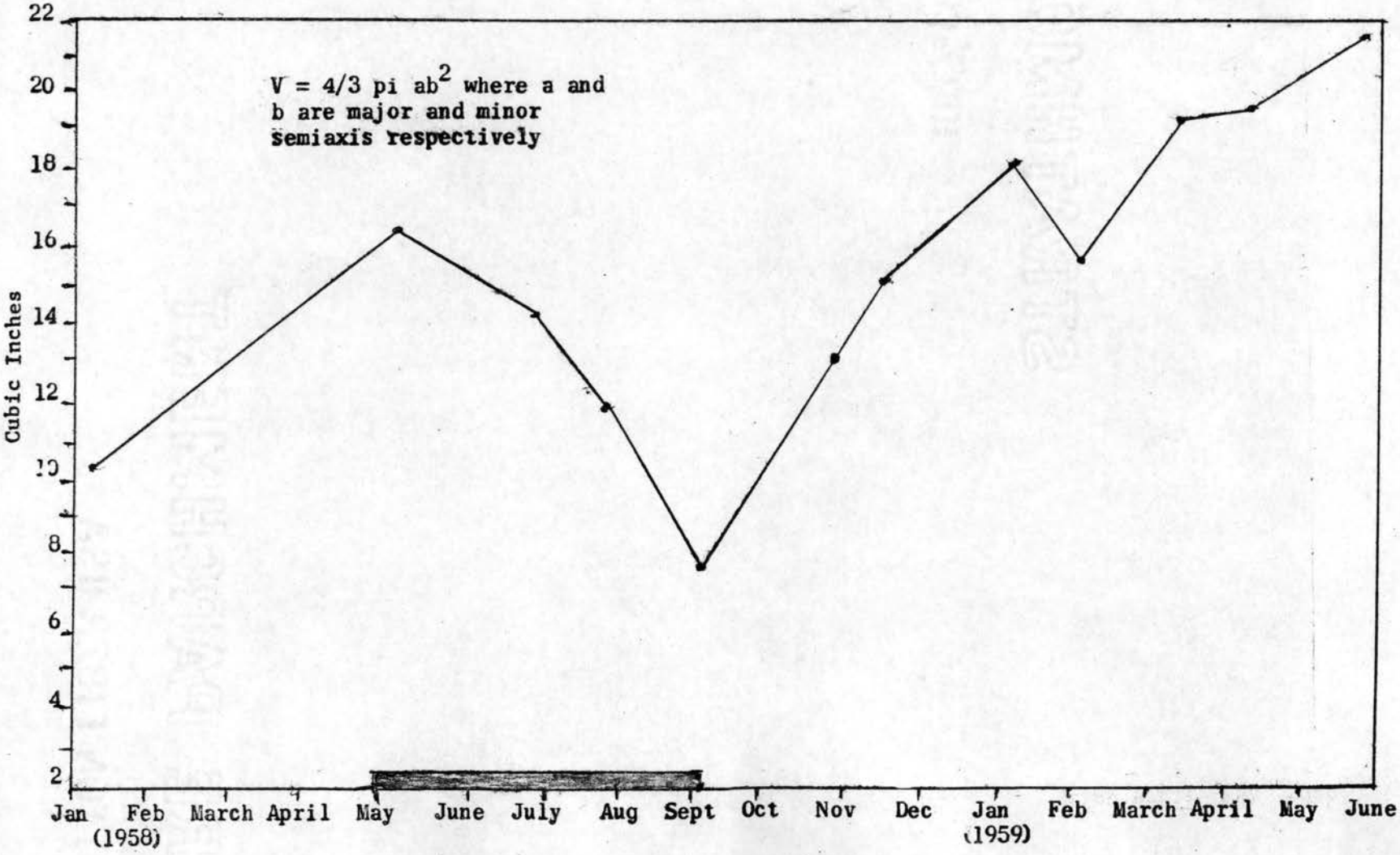


Figure 12. Average Testicular Volume for Four Bulls

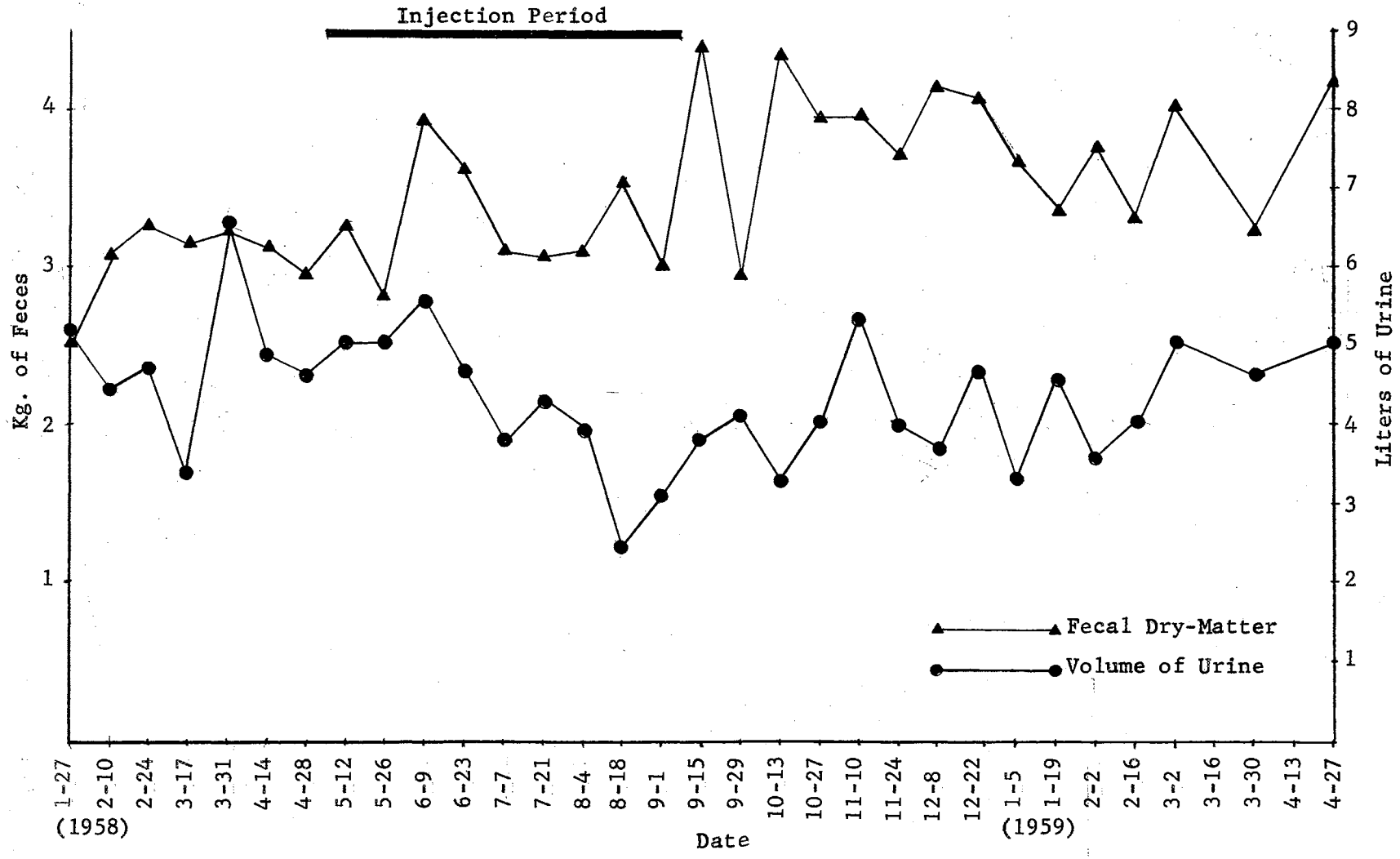


Figure 13. Volume of Urine and Fecal Dry-Matter Excreted During Three Experimental Periods

excretion of both urine and feces (Figure 13). It was felt that the content of steroids per ml. or gm. of material would contain less variation and be better for comparative purposes than total steroids excreted per day since the amount of steroid excreted per day increased, irrespective of treatment, as the experiment progressed. There was also an increase in total chromogens excreted per day due to an increased feed intake associated with an increased maintenance requirement of these animals.

Correlation of Reproduction with Steroid Metabolism

Since the reproductive data gathered by Meinecke and McDonald (76) are to be compared with the data on steroid metabolism reported in this study, the results of the previous study are summarized below. Semen samples were collected and analyzed for volume, spermotozoa per ml., total spermatozoa per ejaculate, motility, and per cent abnormal forms. Testicular size was measured periodically throughout the course of the experiment. Testicular biopsies were taken prior to testosterone injections, following injections and at the termination of the experiment. No great change in average semen volume was observed during the experiment. A depression in spermatozoa per ml. during the preliminary period was observed which continued through approximately 10 weeks of the recovery period which in turn was followed by an increase to slightly above pre-injection levels at the end of the experiment. An increase in testicular size up to the time of testosterone injection was observed; this increase was followed successively by a decrease in size until the end of the injection period and an increase in size up to the time the experiment was terminated. Other histological changes such as an increase in connective

tissue content, an increase in number and size of Sertoli cells, and loss of germinal epithelial cells of the seminiferous tubules immediately following the injection period were reported. Permanent damage to the testes did not result from this treatment as indicated by histological sections taken at the end of the experiment.

It has been previously shown by Holtz (57) that the urinary content of total ketosteroids increased only slightly after a large (1 gram) injection of testosterone propionate. He also postulated that 90 per cent of the total ketosteroid values obtained from urine was due to non-steroidal substances. Calculations from the data reported by Ungar et al. (130) show that only about 12 per cent of both the 17-ketosteroid fraction and 17-hydroxycorticosteroid fraction remained after column and paper chromatography.

Since it also has been shown that many factors such as protein binding, liver function, kidney function, distribtuion into the tissues, and dietary components affect the level of steroids in the blood and urine (20, 53, 57, 84), it was deemed necessary to use a more specific assay than the Zimmerman or Porter-Silber assay to follow metabolism of testosterone.

Shortly before this experiment was started, Weichselbaum and Margraf (134) published a report on the method of determination of Δ^4-3-ketosteroids using the INH reaction. This method was reported by the authors to be quite specific for Δ^4-3-ketosteroids (135). Since the tetrahydro derivatives, as well as the 17-ketosteroids, are generally considered to be inactive and do not contain the Δ^4-3-keto group, this test, when applied to appropriately prepared samples, should give a measure of biologically active steroids. However, the possibility of ionone derivatives reacting

with the INH reagent has not been ruled out. Since a double bond conjugated with a ketone group has been postulated as being required for a positive test with this reagent (134), there are probably only two of the ionone derivatives (ketone K and ketone J) listed by Holtz (57) that would give a positive test with this assay. It is highly probable that other compounds such as progesterone and adrenal steroids might react with this reagent. It was assumed in this experiment, however, that during the injection period testosterone would be the principle steroid being metabolized since it has been shown that the 17-hydroxycorticosteroid content of plasma is very low (81, 99). In addition, very little progesterone should be present in the male. Any estrogens, if present, would have been removed when the chloroform extracts were washed with acid and base. Even if the estrogens were present, they should not give a positive test since they do not contain the Δ^4-3-keto group.

The 17-hydroxycorticosteroids were followed in plasma, urine, and feces to see if they contributed to the free, glucuronide, sulfate, and total INH steroid factions during the course of the experiment. The 17-hydroxycorticosteroid content of the plasma was determined using the Robertson-Mixner procedure. The values which were obtained were so low that they are not reported. However, at no time during the experiment did the 17-hydroxycorticosteroid content of the plasma show any appreciable change in content. Essentially the same low results were found with the urine (Figure 10). The urinary level of 17-hydroxycorticosteroids was very low and never showed any appreciable change during the course of the experiment. The fecal content of 17-hydroxycorticosteroids, however, was somewhat higher and showed much greater fluctuations. The significance of the fecal 17-hydroxycorticosteroids is not know at present

since very little is known concerning fecal excretion of steroids in the ruminant. However, the ionone derivatives and other materials in the feces could have contributed significantly to the amount of material present in the fecal extracts. That materials other than steroids are found in the 17-hydroxycorticosteroid fraction after column chromatography and do give a positive test with the Porter-Silber reaction was shown by Ungar and Dorfman (132). Approximately 12 per cent of the Porter-Silber positive material was present after the original crude extract of calf urine was purified using column and paper chromatography.

Holtz (57) has shown that free testosterone appeared in the urine of cattle after large doses of this compound. It has also been shown that testosterone can be conjugated per se without previously being metabolized. In this respect, Wotiz et al. (141) have presented evidence for the conjugation of testosterone at the carbon-17 position. If testosterone is conjugated in the 17 position without the 3-keto group being reduced, the compound after hydrolysis would be free to react with the INH reagent. Therefore, the INH test could be useful in following testosterone as such in the blood, urine, and feces.

From the data in Figures 1, 2, 3, 4, 5, 6, 7, 8, 9, and 10, it can be seen that the only values that correlate in any way with the data recorded by Meinecke and McDonald (76) are the urinary and plasma levels of free steroids measured by the INH method of Weichselbaum and Margraf (134). This, undoubtedly, is due to the fact that ionone derivatives in the other steroid fractions overshaddow any change affected by the injected steroid. When comparing the data in Figure 4 with the reproductive data (Figures 11 and 12), the fluctuations of the free INH steroids of the plasma and urine correspond more closely to the spermatozoa per

ejaculate than do the other steroid fractions. The urinary free steroids rose shortly after testosterone injections were initiated while the plasma free steroids failed to rise until after about four weeks. The plasma steroid level rose moderately at first then rose very abruptly after June 23 and reached a maximum on July 21. The urinary steroids, however, failed to peak until August 1, some six weeks later. The fact that the urinary steroid content reached a peak some six weeks after that of the plasma would tend to indicate that the amount of hormone being absorbed into the blood increased throughout the injection period while the ability of the kidney to excrete this compound in the free state was enhanced. Actually, the free steroids in the urine are thought to be resorbed up to 90 per cent under normal conditions (110). Therefore, a possible explanation for this lag would be due to a steroid pump mechanism for reabsorption of testosterone from the kidney tubule, which was depressed during the prolonged exposure to excessive amounts of this compound. This would account for the increase in free steroid content in the urine during the time when the content in the blood was decreasing. This decrease in free steroid content in the plasma took place in spite of the fact that the amount of steroid in the tissue was building up due to repeated injections of testosterone. Unless there was a local reaction at the sites of injection preventing the absorption of the hormone, there should have been an increased absorption of the steroid during this period of time. In this respect, Holtz (57) has found no free testosterone in the urine after a small dose, however, significant amounts were present in the urine after a large dose of testosterone.

As will be shown later, the INH-positive, conjugated fractions are thought to contain ionone derivatives as well as steroids. Whether the

presence of ionone derivatives extends to the free INH levels of the plasma and urine is not known. However, since the urinary, free (INH) levels were so high during the preliminary period, ionone derivatives must have been present in these two fractions. The plasma, free (INH) fraction was very low at the start of the injection period. Therefore, the question arises as to whether the large increase in free, INH-positive materials in this tissue was due to testosterone or the ionone derivatives. Since the urine and feces did not show marked depressions in the various fractions during this period of time, it is unlikely that all of material present in the plasma was due to ionone derivatives. It is highly probable that a good portion of the free (INH) fraction in the urine after injections of testosterone were started was testosterone. If, however, the free (INH) fractions in the plasma and urine are not comprised primarily of testosterone, then one would suspect that the ionone derivatives and the steroids would behave similarly in the way they were excreted and reabsorbed from the tubules. The results of this experiment are interpreted on the premise that the rise in free (INH) materials of the plasma and urine were due primarily to testosterone. Thus, a homeostatic mechanism would appear to be present in the kidney to decrease the rate of absorption of steroids from the tubules during chronic exposure to free steroids. This would affect an increased excretionary rate of the unaltered steroid to lower the level of circulating hormone in the blood.

It is of interest to note the same observation during the period from March 30, 1959 to April 27, 1959. The plasma (INH) content rose to a peak and dropped off while the urinary content of INH-positive materials continued to rise. Unfortunately, samples were not collected after this date to see if the urinary level would decrease at it had done previously.

If the free (INH) content of the plasma after February 2, 1959 were due to exogenous testosterone, this would tend to indicate that the recovery from the injected testosterone was just reaching a peak at the time the experiment was terminated. Inspection of the reproductive data (Figures 11 and 12) shows that testicular volume continued to increase after testosterone injections, up to the time the experiment was terminated. The semen picture looked somewhat different. The peak in the spermatozoa per ejaculate was reached about February 13, 1959. This corresponds with the time when the free (INH) values had started to rise again. The period of highest spermatozoa numbers per ejaculate lags by about 48 days, the period of cold weather where thyroxine secretion would have been greatest. In this respect, it requires at least 48 days for a germinal cell to form a spermatozoa (76). Thus thyroxine could have had a potentiating action on the action of FSH from the pituitary. Thyroxine is known to have a pronounced action on reproduction in the male (85).

The reason for the fact that the steroid levels in the plasma failed to remain at a high level during the entire injection period and shortly after, has been attributed to the ability of the animal to metabolize and rapidly excrete the hormone. This failure to maintain high levels of steroid in the blood would also tend to indicate that after a certain level of steroid is reached in the tissue, a maximum level will result in the blood. Injecting testosterone beyond this point would result in decreased levels of steroid in the blood through an increased rate of excretion of the free steroid and possibly other routes of metabolism and excretion. Although it has been shown in man that pretreatment with testosterone before surgery failed to demonstrate alterations in plasma, free or conjugated corticosteroids (119), prolonged administration could have a demonstratable affect as far as testosterone metabolism and excretion are concerned.

The data from this experiment would tend to substantiate the claims that small doses of testosterone are more effective in producing the rebound phenomenon than larger doses (49, 71, 146) by altering the rate of elimination of this hormone. Thus it may be easier to maintain a certain level of steroid in the blood using smaller doses rather than massive doses of the steroid. From this standpoint, it may be advantageous to give one or two massive doses to bring the steroid content in the tissues up quickly, followed by periodic injections of small doses of the hormone to maintain the level in the tissue where absorption takes place.

The fact that steroids were being excreted in the urine concomitant with a rise in steroids in the plasma indicates that the rise in free steroids in the plasma was not due to protein binding. If the steroids in the plasma were protein bound, the free steroids in the urine would have decreased (20, 106).

The fact that the steroid levels in the plasma and urine did not return immediately to the preinjection level after injections were stopped indicates a slow absorption of the steroid from the tissues. This is also evident in the slow rise in free steroids in plasma and urine after injections were started. Holtz (57) has reported on the slow rate of absorption of testosterone after a large single injection. He reported the presence of testosterone metabolites in the urine three months following the injection. If this period of time were added to the time when injections were stopped in this experiment, the date would be about December 7. This date corresponds quite closely with the time when the plasma and urinary steroid content reached a minimum. Therefore, it would appear that most of the injected steroid had been absorbed from the tissues

during this three-month period. Judging from the urinary excretion, the steroid probably was almost entirely absorbed by November 24.

There is a possibility that the hot weather during the summer months could have lowered the thyroxine secretion rate thereby decreasing the rate of conjugation and metabolism of testosterone (85). This would explain the marked increase in steroid content in the plasma after June 23, 1958. This possibility should be considered since it has been shown that thyroxine increases total hepatic activity for in vitro ring A reduction of cortisol (145). It also accelerates the maturation of the glucuronosyl transferase system in young animals (138). The data from this experiment correlate well with the findings of Robertson and McGregor (101) who observed a decreased excretion of steroids in the urine during summer months. This mechanism might explain why there is a tendency for some farm animals to experience a temporary infertility during hot summer months. Through this mechanism a decreased thyroxine secretion would increase the testosterone level in the blood which in turn would decrease FSH and LH production by the pituitary. Due to the decreased secretion of these two gonadotrophic hormones, spermatogenesis could be interrupted during hot weather. There is, however, another possibility to consider. This would be through a cellular action whereby thyroxine augments or potentiates the action of these two gonadotrophic hormones. Thus, an increased response would be noted in the winter and a decreased response during the hot summer months (85).

The reason for the fact that the plasma level of free (INH) steroids almost reached the preinjection level on October 27 then rose to a new peak on December 8 is not known. However, it probably reflected endogenous secretion and metabolism. There is a possibility that the thyroid may

have been implicated in the response noted here since this time of year was characterized by cooler weather. This cooler weather could have induced an increased thyroxine secretion which in turn would have increased the rate of steroid metabolism, thereby tending to flush the steroids from the bloodstream. This would be consistent with the data reported by Robinson and McGregor (101) who reported an increased urinary secretion of steroids in sheep during the cold, winter months. Although the free (INH) steroids in the plasma decreased during this period of time, thyroxine could have potentiated the action of the surviving testosterone or the pituitary gonadotrophins. This potentiating action of thyroxine would help explain the observed response in respect to testicular volume which increased during this period of time. The same reasoning would apply also to the spermatozoa per ejaculate which increased during this period of time. This reasoning does not, however, explain the tremendous increase in conjugated, INH steroids in the plasma (Figure 1). This period of time would also be characterized by an increased intake of feed; however, this increased feed consumption would not be able to account for the three-fold increase in total (INH) steroid content of the plasma. Since the feed was given ad libitum, the total dry-matter excreted was calculated from the data to see if the fecal dry-matter would reflect any gross changes in feed consumption (Figure 13). Although a considerable variation was experienced throughout the experiment, no definite trend could be established in connection with seasons of the year. There was, however, a slight overall increase in the dry-matter content of the feces during the experiment. This overall increase in dry-matter excretion reflects an increased size of the bulls with a corresponding increase in the maintenance requirements for these animals.

Unfortunately the INH test was not employed on all of the samples throughout the trial. It would have been much better had the INH test been used on the plasma samples throughout all of the control period; however, it was used to see if it could be of use in following exogenous steroid metabolism in the ruminant. This question was answered in the affirmative. Nevertheless, the INH test should be evaluated in terms of its specificity in order to evaluate its usefulness as a tool in evaluating steroid metabolism in the ruminant. More information could be gained when using the INH test if the crude extract were purified using column and paper chromatography.

Some General Aspects of Ionone Metabolism in Bulls

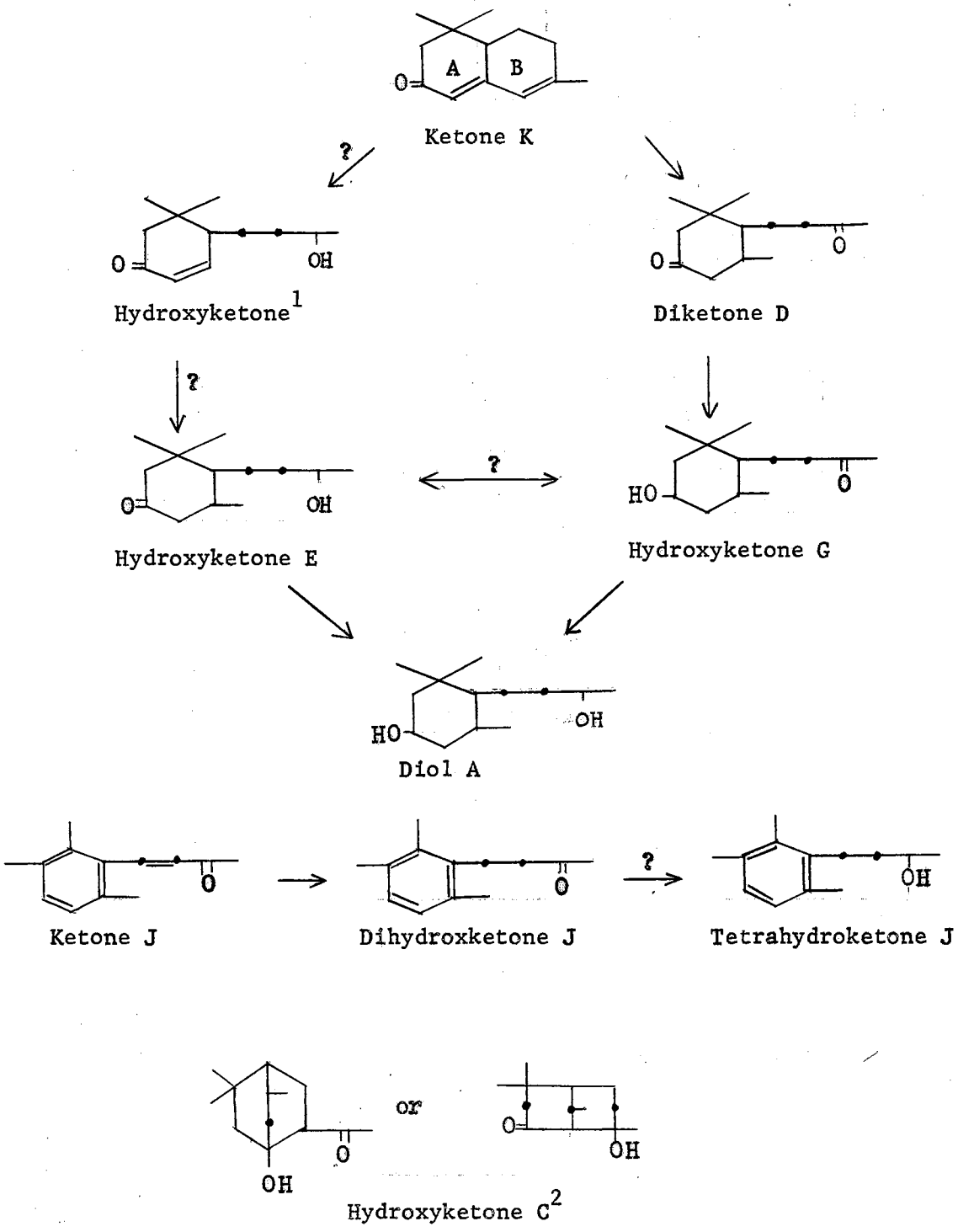
Inspection of Figures 1 through 10 shows that the fecal steroids were high at the beginning of the experiment with a continual drop throughout the experiment. The only exception to this observation was during the period from October 13, 1958 to February 16, 1959. This decrease in steroid content was evident in spite of a general increase in fecal dry-matter content (Figure 13). Since the INH test measures predominantly Δ^4-3-ketosteroids or compounds with a double bond conjugated with a carbonyl group, it was suspected that perhaps the liver or bacteria in the digestive tract were involved in metabolizing compounds of this nature occurring in the digestive tract. That the Δ^4-3-ketosteroids or similar materials were being reduced so as not to give a positive test with the INH procedure is quite evident in Figure 8. Here the urinary extract was separated into 17-ketosteroid and 17-hydroxycorticosteroid fractions using column chromatography. The resulting 17-ketosteroid fractions were split and analyzed by both Zimmerman and

INH procedures. During the control period, the two values were almost identical. However, the differences obtained by use of the two methods progressively increased throughout the experiment. This indicated that compounds with the double bond conjugated with a carbonyl group were disappearing and reappearing as a reduced compound that did not give a positive INH test. To support further this observation, a similar decline in 17-hydroxycorticosteroids was observed in the feces (Figure 10). Since this fraction was assayed using the INH procedure, it would appear that some of this INH-positive material was disappearing due probably to metabolism. Similarly, the urinary extract was separated into 17-ketosteroid and 17-hydroxycorticosteroid fractions using column chromatography. The resulting 17-ketosteroid fraction was split and assayed using both the INH and Zimmerman reactions. Here again (Figure 8) a general increase in the 17-ketosteroid Zimmerman positive materials can be seen with very little change in the 17-ketosteroid INH-positive materials.

It is of interest to note that all the free, glucuronide, sulfate, and total, fecal, INH-positive materials declined throughout the experiment. This would indicate that the compounds were capable of being conjugated with either glucuronic acid or sulfate. It has been shown that all of the known steroids except testosterone and estrogen are conjugated at the three position after the Δ^4-3-keto group has been reduced to the corresponding tetrahydro derivative (10, 111, 141). It also has been implied that urinary and biliary losses of metabolically active steroids are, under normal conditions, negligible (131). This would indicate that the material being metabolized was of dietary origin and probably not a steroid. Of the ionone derivatives isolated from cattle

urine by Holtz (57), only two compounds (ketone K and ketone J) would give a positive test under the requirements listed by Weichselbaum and Margraf (134). However, it is possible that there are other plant steroids which could be metabolized, as ketone K and ketone J could be metabolized, to the tetrahydro derivatives giving the observed results. Other plant steroids could have been present in the crude extract since Ungar and Dorfman (130) have shown that the Porter-Silber values from calf urine decreased by about 88 per cent after column and paper chromatography. Thus, it would appear that there were in this experiment materials of plant origin capable of being metabolized and conjugated with glucuronic acid or sulfate. Furthermore, it would appear that there was an age factor as far as the ability to metabolize these compounds were concerned. It would appear that the ability of the animal to reduce these compounds increased with age.

Since the free, INH-positive chromogens in the feces declined at a rate similar to that for glucuronide and sulfate-conjugated fractions (Figure 3), it would appear that the precursors for all three fractions were being metabolized at a similar rate. A proposed metabolic scheme for the metabolism of ionone derivatives can be found in Figure 14. The structures of the compounds used in this scheme are based on the structures of compounds previously isolated from cattle urine (57). It should be kept in mind that these compounds, in all probability, do not give a positive test with the Porter-Silber reaction. Therefore, this proposed scheme was not intended to be used to explain the results observed by Ungar and Dorfman (130) in regard to the decrease in Porter-Silber positive materials following column and paper chromatography.



[1]Hypothetical structure similar to hydroxyketone C except for a double bond.

[2]The structure of this compound not fully established at present (32).

Figure 14. Proposed Schemes for the Metabolism of Ionone Derivatives in the Ruminant

If a double bond conjugated with a carbonyl group is required for a positive INH test, only ketone K and ketone J would be expected to give a color with the INH reagent (Figure 14). However, the Zimmerman test requires a $-CH_2CO-$ grouping (carbonyl group) for a positive test (9). Of the compounds listed in Figure 14, only the following would give a positive test with this method: ketone K, hydroxyketone, diketone D, hydroxyketone E, hydroxyketone G, ketone J, dihydroketone J, and hydroxyketone C. In this proposed scheme, only hydroxyketone, dihydroketone J, and tetrahydroketone J are theoretical compounds used to explain the observed results. It should be kept in mind that the other compounds in these schemes have been isolated and fully characterized. Other compounds did exist in the urine extracts of Holtz (57) which were not fully characterized or identified.

The decrease in INH-positive chromogens in the feces in the free form could well be attributed to ketone K or ketone J. These two compounds may have been present in the plasma and urine as well. The 2.5 µgm. per ml. of free, INH-positive materials in the urine at the start of the experiment (Table I) could be due to these compounds. If these two compounds were not readily resorbed from the kidney tubules, this would explain in part why the free (INH) chromogens in the plasma on April 28, 1959 were so low (0.13 ugm. per ml. of plasma). If ring B of ketone K were opened, this could, through a series of reductive steps, give rise to diketone D and hydroxyketone. Similarly, ketone J could be reduced to dihydroketone J. These three compounds (diketone D, hydroxyketone, and dihydroketone J) would be capable of giving a positive INH test. This would explain the increase in 17-ketosteroids (Zimmerman) concomitant with a decrease in 17-ketosteroid (INH) in the feces. Since a large portion of the INH-

positive chromogens in the feces were conjugated with either glucuronic acid or sulfate, it would require a compound with a structure similar to hydroxyketone to explain the positive INH test and the ability to be conjugated. This compound must have a -OH group for conjugation and a double bond conjugated with a carbonyl group for a positive INH test. Hydroxyketone would fulfill these requirements.

Both hydroxyketone E and hydroxyketone G have been isolated from cattle urine; therefore, it would be probable that hydroxyketone E could be formed through reduction of hydroxyketone. Similarly, hydroxyketone G could be formed through reduction of diketone D. In addition, it would be possible for hydroxyketone E to be formed from hydroxyketone G through a reversible enzyme catalyzed reaction. Nevertheless, both of these two compounds would give a positive Zimmerman test, but not a positive INH test. The formation of these two compounds help to explain the increase in 17-ketosteroid (Zimmerman-positive) chromogens in the feces during the time when the 17-ketosteroid (INH-positive) chromogens were decreasing in the feces. It should be remembered that most of these compounds, including diol A and tetrahydroketone J, could be conjugated even though the latter two would not be detected using either the Zimmerman or INH test. As stated earlier, the only three not capable of being conjugated are ketone K, diketone D, and ketone J.

Considering these chemical configurations and proposed metabolic schemes, it is highly probable that the decrease in INH-positive materials in the feces during the experiment was due to the metabolic changes of these compounds. Since the ionone derivatives account for roughly 90 per cent of the urinary 17-ketosteroid (Zimmerman) chromogens (57, 130), the question arises as what affect these compounds might have on steroid

metabolism. Since these compounds have a structure similar to the isoprene nucleus of steroids, it is highly probable that the ionone derivatives could be accounted for by similar mechanism for steroids. If these compounds are reduced with TPNH as are the steroids (131), how much affect can these ionone derivatives have on TPNH availability in the liver cell? It has been postulated that under normal conditions, the reduction of the Δ^4-3-keto grouping is the rate-limiting step in the metabolism and excretion of many steroids (121, 131, 145). If this is true and if the ionone derivatives are metabolized by the same enzyme mechanism, a very significant alteration in the rate of metabolism of these steroids could result from sudden appearance of these compounds in the blood from the diet.

Since there are other compounds such as testosterone, thyroxine, estrogens, etc. that are not reduced before conjugation, it is highly probable that a substrate competition for UDPGA and PAPS could exist. In this respect, it has been shown that following injections of estrogen, the disappearance rates of various steroids including tetrahydrocortisone were altered within a matter of minutes (11, 30, 42, 51, 98, 133). This would tend to rule out protein binding as the only causative agent in decreasing the removal time of these compounds. It has also been shown that n-acetyl-p-aninophenol increased the plasma 17-hydroxycorticosteroid levels when given in connection with cortisol and prednisolone (17). This effect was presumed to be due to inhibited steroid-glucuronide formation in the liver. It also was shown that this compound increased conjugated glucuronic acid in the urine (16). This increase in glucuronic acid was due primarily to the glucuronide of n-acetyl-p-aminophenol.

Plasma Steroid Levels

Unfortunately, very few studies have been conducted on the steroid levels in the blood of ruminants. Meschanks (77) has reported low levels of sex steroids in blood using bioassays while Robertson and Mixner (100), Robertson et al. (99), and Shaw et al. (114) have reported low 17-hydroxycorticosteroid levels in the blood of cattle. These workers have reported 17-hydroxycorticosteroid values of about 5 µgm. per 100 ml. of plasma. The values found during this investigation were considerably lower than these values which were around 1-2 µgm. per 100 ml. of plasma. Although very little information is available in the literature concerning 17-ketosteroid values, the values obtained during this investigation were found to be low in contrast to humans but higher than the 17-hydroxycorticosteroid (Porter-Silber) obtained during this experiment. The extreme high for the entire experimental period was a mean value of 379 µgm. per 100 ml. on June 23, 1958.

The question arises as to why the values are so much lower for ruminants than they are for non-ruminants. Do the ionone derivatives have any influence on the steroid levels in the blood? This is only one of the many questions that need to be answered as far as steroid metabolism in the ruminant is concerned.

It is of interest to note that the glucuronide and sulfate-conjugated steroids in the plasma (INH) are about equal at the beginning of the experiment (Figure 1). These values seem to fluctuate back and forth as far as which one predominated as the main fraction during the experiment. When the experiment was terminated, there were three times as much glucuronide-conjugated as there were sulfate-conjugated materials.

Urinary Steroid Excretion

Holm and Firch (55) have reported an average of 18.4 mg. per liter of glucuronide-conjugated 17-ketosteroids (Zimmerman) and an average of 21.0 mg. per liter of free 17-ketosteroids (Zimmerman) for six cows. This would correspond to an average of 39.4 mg. of total 17-ketosteroids (Zimmerman) per liter. Meschanks (77) reported a mean of 22.0 mg. of total 17-ketosteroids (Zimmerman) per liter for bulls and 16.0 mg. per liter for cows.

The 17-ketosteroid (Zimmerman) excretion on April 28, 1958 of this experiment was found to be 19.3 mg. per liter. However, this fraction contained only the free and glucuronide-conjugated steroids since β-glucuronidase instead of hot HCL was used to hydrolyze the bound steroids. Other values for the urine during the preliminary period were within the range of values reported by these two groups.

It is of interest to note that the urinary steroid levels did not, for the most part, reflect gross changes in steroid metabolism which would have been expected during the period of injection of testosterone. Since the 17-ketosteroids (Zimmerman) increased throughout the experiment, it would appear useless to compare given periods of time with amounts of steroids excreted in the urine since the age affect and the change in metabolism of ionone derivatives might overshaddow any real change in steroid metabolism. There is also the possibility that a misinterpretation of the data might result from the age effect.

Fecal Steroid Excretion

Total steroid excretion per day (Table III) was calculated from the data for the last day of collection for each period; namely, April 28, 1958,

TABLE III. AVERAGE DAILY EXCRETION OF VARIOUS STEROID FRACTIONS TAKEN FROM THE LAST COLLECTION PERIOD OF THREE EXPERIMENTAL PERIODS*

Steroid Fractions		Dates 4-28-58	9-1-58	4-27-59
		(mg.)	(mg.)	(mg.)
Urine (INH)	Free	12.3	24.9	41.1
	Gluc.	45.0	17.4	32.0
	SO_4	54.5	51.5	87.4
	Total	111.8	93.8	160.5
Feces (INH)	Free	262.5	197.4	108.5
	Gluc.	226.8	85.0	137.0
	SO_4	153.4	112.2	194.9
	Total	642.7	394.5	440.4
Urine (Zimm.)	17-Keto.	89.0	149.0	246.3
Urine (INH)	17-Keto.	37.2	36.4	62.1
Urine (INH)	17-OHCS	3.0	2.2	5.3
Feces (Zimm.)	17-Keto.	615.2	671.6	626.2
Feces (INH)	17-Keto.	520.5	293.6	296.7
Feces (INH)	17-OHCS	113.4	42.6	40.4

*Values calculated for the number obtained if all six samples were not collected.

TABLE IV. FECAL:URINARY RATIOS OF EXCRETED STEROIDS USING AVERAGE DAILY EXCRETION VALUES OF VARIOUS ASSAYS TAKEN ON THE LAST COLLECTION PERIOD OF THREE EXPERIMENTAL PERIODS

Steroid Fractions	Dates 4-28-58	9-1-58	4-27-59
	(mg.)	(mg.)	(mg.)
Total (INH)	5.75	4.20	2.74
17-Keto. (Zimm.)	6.91	4.51	2.54
17-Keto. (INH)	13.99	8.06	4.78
17-OHCS (INH)	37.80	19.36	7.62

August 1, 1958, and April 27, 1959. This was done for comparative purposes. In rodents, biliary excretion predominates while in many only small amounts of steroids are excreted in the feces (39). Biliary excretion in ruminants has been said to be more important than the urinary route (53, 55, 79, 81, 100, 130). The data obtained from this experiment substantiate these claims (Tables II, III, and IV). In Table IV, the fecal urine ratios were calculated for the three representative days April 28, 1958, September 1, 1958, and April 27, 1959. In all cases the fecal elimination exceeded the urinary elimination. There were differences in sepcific steroid fractions as to the preferred route of excretion. There also appears to be a specific age effect as to the specific route of elimination. The 17-hydroxycorticosteroids (INH) were more predominantly excreted in the feces followed by 17-ketosteroids (INH), 17-ketosteroids (Zimmerman), and total (INH) steroids in decreasing order. It is of interest to note that the total (INH) and 17-ketosteroids (Zimmerman) parallel each other very closely during the three observation periods listed.

On comparing the excretion of fecal steroids in Figures 9 and 10, an overall decrease in steroid excretion was observed. This overall decrease in excretion was undoubtedly due to the metabolism of ionone derivatives as mentioned earlier. Some of these derivatives may be metabolized to the point where they no longer give a positve test with either the Zimmerman or INH method. Such a compound would be Diol A (Figure 14) which is known to be conjugated. No noticeable change was observed in the urinary excretion during the period from December 8, 1959 to April 30, 1959 (Figures 5, 6, and 7). The plasma levels of INH positive conjugated materials rose to tremendous heights during this period of time. There was also a significant change in the fecal excretion during this period.

It has been shown in humans that up to 90 per cent of the free steroids in the urine was resorbed, whereas the glucuronide conjugated steroids were not resorbed. If this were true in cattle, then a rise in the conjugated steroids should have occurred in the urine during this period of time. This was not the case. The only apparent explanation that could explain this observation would be an increase in protein binding of the conjugated materials. The conjugates of steroids are known to be protein bound in plasma (117). However, if this situation were present, then the levels in the feces should also be correspondingly low. This was not the case in this experiment. If, on the other hand, a pump mechanism is present in the kidney tubules for resorption of the conjugated steroids, this could account for the failure to observe a rise of these compounds in the urine. Since the conjugated materials, as well as the free materials, were found to be predominantly excreted by the feces, a pump mechanism in the liver biliary duct cells must be present. If this pump mechanism is more specific for some compounds than others, then the piling up in the plasma could be attributed to a competition of substrates for the pump mechanims. It should be kept in mind that this period was also characterized by cold weather which would have increased the circulating level of thyroxine. This increase of thyroxine must be handled by the liver also. Other compounds which do not give a positive test with either the INH or Zimmerman methods could also be present giving rise to further competition. Not being excreted, these compounds would build up in the plasma until such time as they could be excreted.

When comparing the data in Figures 5, 6, and 7, the conjugated materials in the feces were found to be lowest (January 5, 1959) when the conjugated material reached a peak in the plasma. As the level of

conjugated material in the plasma dropped, the fecal content increased. It cannot be argued that a depressed liver function in all of the animals was present during this period of time and thereby accounting for the observed results since there were low plasma levels of free INH materials at this time (Figure 4).

It is interesting to note that in the studies reported by Ungar and Dorfman (130) about 90 per cent of the 17-hydroxycorticosteroids (Porter-Silber) and 17-ketosteroids (Zimmerman) were ionone derivatives. Even though most of the ionone derivatives were removed by column and paper chromatography, the purified steroid fractions still reflected many of the gross variations present in the crude extract. Thus, it may be concluded that even though the ionone derivatives may not be metabolically active, they may have a pronounced effect on hormone metabolism.

Inspection of the various steroid fractions from individual animals indicates that each animal has its own characteristic excretion pattern. However, it was hoped that by pooling the data from six animals, the fluctuations of one animal would be offset by those of other animals so that the mean values would be meaningful in evaluating steroid metabolism in the ruminant. This was accomplished with a moderate degree of success.

PART II:

Conjugation of Steroids by the Liver

In Part I of these experiments a tremendous amount of steroids and ionone derivatives were found in the conjugated state. Most of these conjugates were found in the feces and urine as excretory products. Thus it seemed advisable to investigate the nature of the glucuronosyl

transferase system in the liver of the ruminant. Sheep were used in these experiments for economic reasons and ease of handling.

Initial studies were undertaken in the sheep and the dog; however, these studies were temporarily abandoned due to technical difficulties which were later worked out in the rat. After working out these difficulties, studies were conducted on the three species. Since the dog and rat are not ruminants and were not fed diets high in carotenoids, this presented the possibility of comparing the glucuronosyl transferase activity of these three species.

There are, however, two parameters to consider when evaluating transferase activity of a given tissue; the rate of synthesis of UDPGA and the amount of transferase activity in the tissue in question. In order to evaluate the first parameter, homogenates of the tissue were prepared and assayed as described earlier. In the tissue homogenates, the enzymes for synthesis of UDPGA were present as well as the transferase enzyme. In these studies, UDPGA was added in catalytic amounts (50 μgm.) so that UDPGA would be the rate-limiting factor in the overall synthesis of steroid-glucuronides.

The second parameter was evaluated using microsomal preparations and larger quantities of UDPGA (0.4 μMoles) so that the rate of synthesis of glucuronides would be related to transferase activity instead of UDPGA synthesis.

The data from these assays are recorded in Table V for homogenates and Table VI for microsomal preparations. From the data in Table V it can be seen that the amount of steroid disappearing upon incubation and reappearing after β-glucuronidase hydrolysis is greatest for the sheep liver. This is consistent with the findings in Part I where it was found

that these ruminants had tremendous amounts of materials in the urine and feces which were apparently conjugated by the animal. Therefore, the liver of the ruminant rather than that of the dog or rat would be expected to have the greatest ability to synthesize UDPGA for the conjugation of steroids and ionone derivatives.

In preliminary studies designed to expand upon this previous observation, glucose and galactose were assayed with sheep liver homogenate to see which would be most effective in the production of UDPGA since it has been shown that galactose is a better precursor for glucuronic acid synthesis than glucose (126). The results from this study indicated that glucose was a much better precursor than galactose. This is as would be expected since the ruminant probably does not metabolize as much galactose as it does glucose. When glucose was used as the substrate, 0.040 μMoles of phenolphthalein disappeared in contrast to 0.020 μMoles of indicator which disappeared when galactose was used as the subsrate for UDPGA synthesis. When p-nitrophenol was being conjugated, 0.041 μMoles of the dye disappeared on incubation with glucose in contrast to 0.034 μMoles of dye which disappeared when galactose was used as the precursor for UDPGA synthesis.

A comparison of the data in Table VI shows that sheep liver had the greatest transferase activity based on the amount of steroid disappearing on incubation and reappearing on hydrolysis with β-glucuronidase. It can also be seen that the ovine liver conjugates both H_4E and H_4F to approximately the same extent. In this respect, it is interesting to note that Glick (39) has isolated both H_4E and H_4F from bile of cattle after β-glucuronidase hydrolysis. From the data in Tables V and VI it also can be seen that the steroid recovered after β-glucuronidase hydrolysis was

TABLE V. GLUCURONIDE CONJUGATION OF VARIOUS SUBSTRATES USING HOMOGENATES OF LIVER AND ADRENAL TISSUES FROM SHEEP, DOG, AND RAT

Animal	Tissue	Substrate	Number of Observations	Substrate Disappearing On Incubation (μMoles)[1]	Substrate Recovered as Glucuronide (μMoles)
Sheep	Liver	H_4E	10	0.101 ± .025*	0.047 ± .017*
Sheep	Adrenal	H_4E	10	0.032 ± .006*	0.008 ± .008*
Dog	Liver	H_4E	5	0.038 ± .012*	(-)0.005 ± .003*
Dog	Adrenal	H_4E	5	0.065 ± .003*	(-)0.003 ± .001*
Rat	Liver	H_4E	5	0.073 ± .022*	0.009 ± .005*
Rat	Liver	H_4F	5	0.012 ± .007*	0.005 ± .004**
Rat	Adrenal	H_4E	4	0.040 ± .019*	0.004 ± .001*

[1]Denotes the amount of substrate in μMoles followed by the S.D. and statistical Probability.

*Denotes a statistical Probability < 0.05 as compared with non-incubated controls.

**Denotes a statistical Probability < 0.10 as compared with non-incubated controls.

TABLE VI. GLUCURONIDE CONJUGATION OF VARIOUS SUBSTRATES USING MICROSOMAL PREPARATIONS OF LIVER AND ADRENAL TISSUES FROM VARIOUS ANIMALS

Animal	Tissue	Substrate	Number of Observations	Substrate Disappearing On Incubation	Substrate Recovered as Glucuronide
				(μMoles)[1]	(μMoles)
Sheep	Liver	H_4E	5	0.178 ± .006*	0.092 ± .003*
Sheep	Liver	H_4F	5	0.126 ± .023*	0.104 ± .058*
Sheep	Liver	Phenolphtha.[2]	5	0.184 ± .049*	
Sheep	Liver	p-NP[3]	5	0.140 ± .028*	
Sheep	Adrenal	H_4E	5	0.006 ± .003**	0.002 ± .002*
Dog	Liver	H_4E	5	0.040 ± .007*	0.0002 ± .0001**
Dog	Liver	Phenolphtha.	5	0.072 ± .015*	
Dog	Liver	p-NP	5	0.063 ± .017*	
Dog	Adrenal	H_4E	5	0.051 ± .022*	(-)0.0004 ± .0002**
Rat	Liver	H_4E	5	0.025 ± .015*	0.006 ± .003*
Rat	Liver	Phenolphtha.	5	0.028 ± .015*	
Rat	Liver	p-NP	5	0.020 ± .007*	

[1] Denotes the amount of substrate in μMoles followed by the S.D. and statistical Probability.

[2] Phenolphthalein.

[3] p-Nitrophenol.

* Denotes a statistical Probability < 0.05 as compared with non-incubated controls.

** Denotes a statistical Probability < 0.10 as compared with non-incubated controls.

was always lower than the amount of steroid disappearing upon incubation. This difference could be due to the failure to hydrolyze all of the steroid-glucuronides formed during incubation or it could be due to metabolism of the steroid to a derivative that no longer gives a positive test with the Porter-Silber reaction. Metabolism of the steroid could be accomplished through reduction of the side chain or through cleavage of the side chain to give the corresponding 17-ketosteroid. That metabolism of the steroid molecules took place during these incubations is highly probable since the enzymes for the metabolism of the steroids are found in microsomal fractions of disrupted cells. (96).

The problems of quantitatively assaying for glucuronosyl transferase activity are many. One of the biggest problems lies in the fact that many competing enzyme reactions occur simultaneously during the incubation period. These reactions have been summarized in Figure 15. It has been shown by Pogell and Krisman (91) that a pyrophosphatase exists in skin extracts which rapidly destroys UDPGA to form UMP and glucuronic acid-1-phosphate. The UMP and glucuronic-1-phosphate are broken down further to glucuronic acid, uridine, and phosphate by a phosphatase. In addition, DeDuve et al. (22) have demonstrated the presence of β-glucuronidase in microsomal fractions of disrupted cells. This enzyme was attributed to the lysosome, a particulate fraction of the cell which contains many hydrolytic enzymes. Other enzymes isolated from this structural entity of the cell are phosphatase and sulfatase. Therefore, any glucuronide formed during incubation could be hydrolyzed and the UDP destroyed so that no net synthesis of steroid would result. These same observations have been extended to the rat liver microsomal preparations with all of the competing reactions taking place (92).

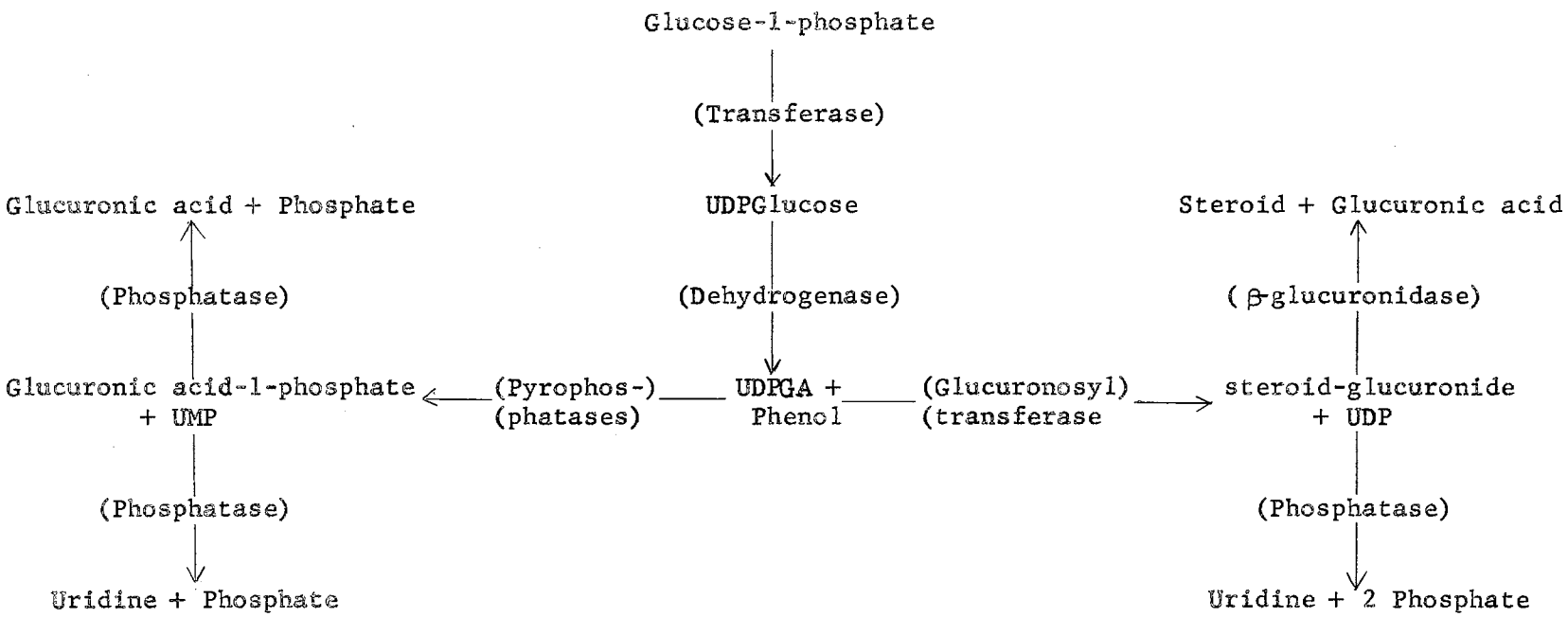


Figure 15. Competing Reactions in Rat Liver for Synthesis, Transfer, and Degradation of UDPGA*

*Based on the findings of Pogell and Leloir (92).

It has been shown in preliminary studies in connection with this present series of experiments that UDP assays failed to correspond to either the amount of steroid disappearing on incubation or the amount of steroid liberated after hydrolysis with β-glucuronidase. Therefore, UDP assays were not run on the experiments reported in Tables V and VI. Furthermore, glucuronic acid production was observed with rat liver homogenates and electrophoretic separation of the compounds formed after incubation. Electrophoretic separations were accomplished according to the method of Cohn and Bondy (15). Using these electrophoretograms, the presence of a sulfatase also was demonstrated. These electrophoretograms also were used to confirm the fact that the steroids reappearing after hydrolysis with β-glucuronidase were, in fact, true conjugates and not artifacts.

Of particular interest is the apparent lack of conjugation of steroids in dog liver and adrenal preparations. Many attempts were made to demonstrate transferase in liver and adrenal tissues from this animal. Various buffer combinations as well as incubation procedures were used without success; however, a positive assay was obtained for phenolphthalein and p-nitrophenol. That steroid conjugation does take place in dog liver is evident from indirect methods reported in the literature (14, 65). Willoughby et al. (139) have shown that the rate of removal of C^{14}-labeled corticosterone from plasma was clearly reduced in hepatectomized dogs. Therefore, the negative results obtained with dog liver and adrenal tissues are undoubtably due to the presence of competing reactions which either destroy the UDPGA so fast that conjugates cannot be formed or that the product is hydrolyzed after being formed. The latter explanation is highly probable since a decrease of endogenously conjugated steroids

was observed in the glucuronide fraction after incubation. Thus there is good reason to believe that there was present in these dog liver and adrenal homogenates and microsomal preparations a β-glucuronidase which hydrolyzed the glucuronides as fast as they were formed (Tables V and VI). The positive assay for conjugation of phenolphthalein and p-nitrophenol probably was due to a substrate specificity for the β-glucuronidase resulting in less destruction of the newly formed conjugates of these two dyes than for the steroid. The possibility does exist that the enzyme activity in the dog liver is greater for these dyes than for steroids since it has been shown that considerable in vivo conjugation of steroids takes place in the kidney of this animal.

Conjugation of Steroids by the Adrenal

In Part I it was shown that there were many compounds present in the urine and feces in the conjugated state. Since these compounds are true excretory products having been detoxified by the animal, the presence of these compounds in the blood would pose a tremendous burden for the liver to conjugate them all before excretion. Therefore, it seemed probable that other organs may participate in steroid conjugation besides the liver. The adrenal gland was suspected of being able to conjugate steroids since Newcomer and Heninger (87) have put forth evidence for glucuronosyl transferase activity in the avian adrenal.

Homogenate and microsomal preparations were assayed for UDPGA synthesis and glucuronosyl transferase activity as previously outlined. Rat adrenal microsomal preparations were not used in this study because of the failure to obtain enough adrenal microsomes to complete an assay.

Several attempts were made to prepare microsomes from this tissue without success.

Inspection of the data in Tables V and VI shows that only the sheep adrenal and rat adrenal homogenates were capable of demonstrating a net synthesis of steroid-conjugates. In the case of the dog homogenate, the presence of β-glucuronidase could have prevented a positive assay. It also is quite likely that this reaction as well as the other competing reactions mentioned earlier (Figure 15) prevented the demonstration of glucuronosyl transferase activity using microsomal preparations from the sheep and dog. These data also show that the sheep liver had the greatest ability to synthesize UDPGA as well as the greatest transferase activity.

The role of tissues other than the liver in the overall conjugation of steroids is not known at present. However, it is not likely that the conjugation of steroids could be a mechanism in the process of steroid secretion since most of the steroids in the gland were in the free form with very small amounts of endogenous steroid-glucuronides being present. In this connection, it has been shown in human adrenal perfusion studies that the venous blood collected from the perfused adrenals contained the same amount of conjugated steroids as arterial blood. Furthermore, it has been shown in the rat that only small amounts of steroid were conjugated after removal of the liver, digestive tract, and spleen (5).

Although in vitro conjugation of steroids has been observed in skin (122), digestive tract (43, 45, 122), kidney (14, 67), spleen (67), liver (25), and adrenal (87), very little is known concerning the intracellular compartmentalization and metabolic control of the conjugation systems in these tissues and their relationship with other metabolic pathways. This

TABLE VII. DATA FROM INHIBITION STUDIES USING SHEEP LIVER MICROSOMAL PREPARATION, PHENOLPHTHALEIN AS THE SUBSTRATE, AND H_4E AS THE INHIBITOR

μMoles of Substrate Added	μMoles of Substrate Disappearing on Incubation (No Inhibitor)	μMoles of Substrate Disappearing on Incubation (Inhibitor Added)
0.0	0.000	0.000
0.1	0.076	0.070
0.2	0.146	0.142
0.3	0.231	0.195
0.4	0.294	0.232

TABLE VIII. DATA FROM INHIBITION STUDIES USING SHEEP LIVER MICROSOMAL PREPARATION, p-NITROPHENOL AS THE SUBSTRATE, AND H_4E AS THE INHIBITOR

μMoles of Substrate Added	μMoles of Substrate Disappearing on Incubation (No Inhibitor)	μMoles of Substrate Disappearing on Incubation (Inhibitor Added)
0.0	0.000	0.000
0.1	0.055	0.046
0.2	0.135	0.125
0.3	0.200	0.168
0.4	0.269	0.187

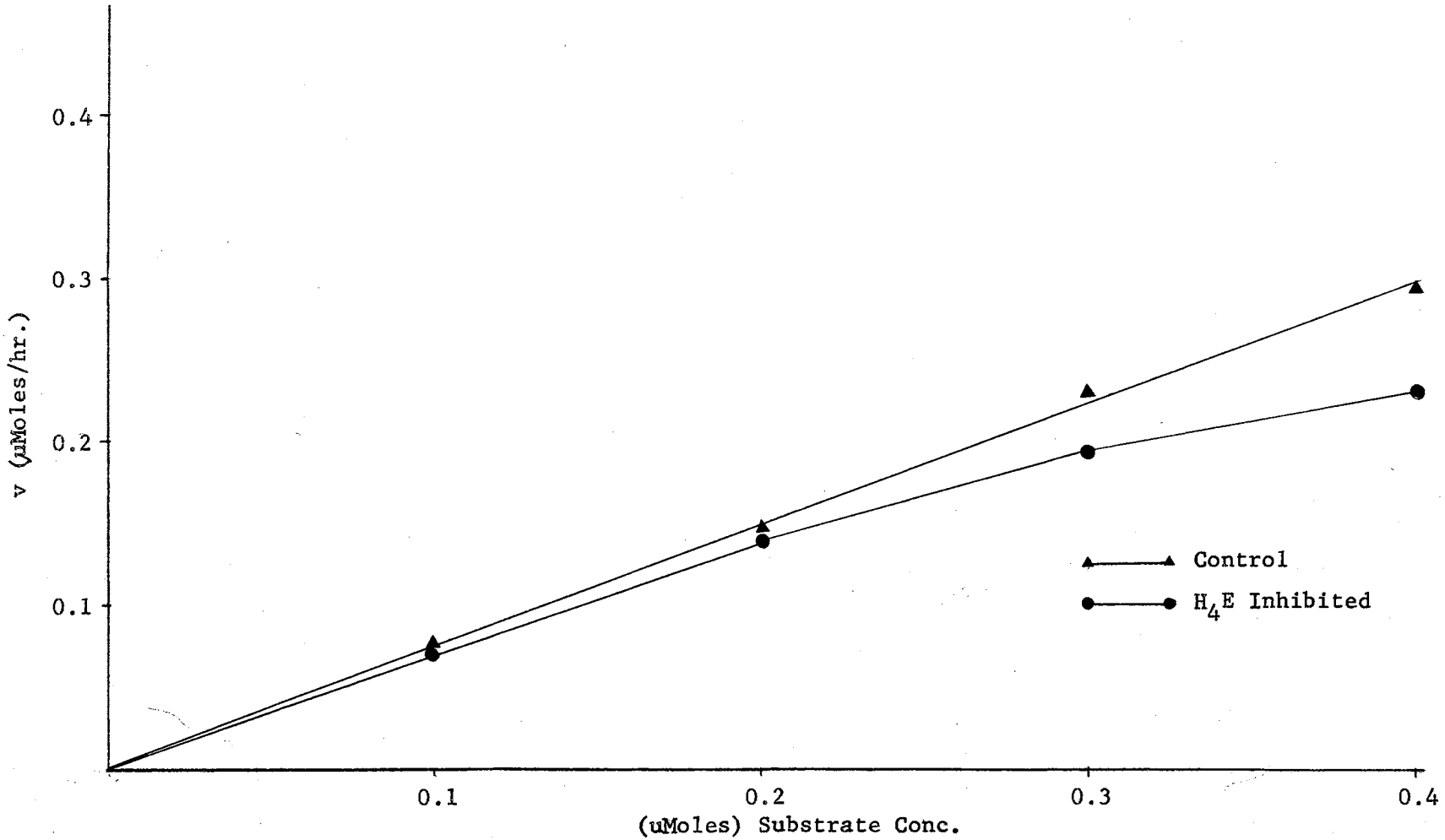


Figure 16. Effect of Increased Phenolphthalein Concentrations on Control and H_4E Inhibited Rates of Synthesis of Phenolphthalein-glucuronidate

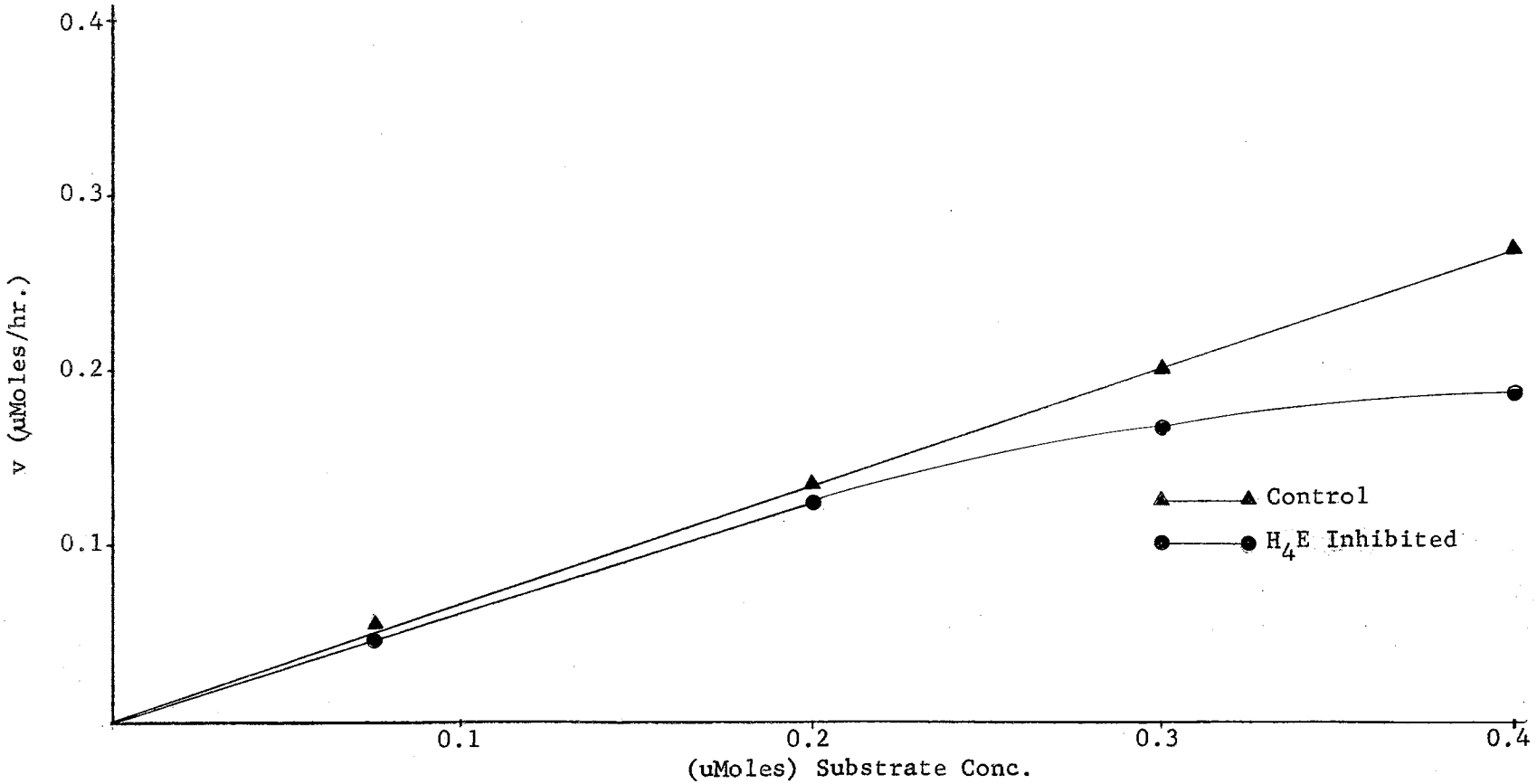


Figure 17. Effect of Increased p-Nitrophenol Concentrations on Control and H_4E Inhibited Rates of Synthesis of p-Nitrophenol-glucuronidate

is especially true of the ruminant where some 90 per cent of the steroid fractions is comprised of ionone derivatives from the feed.

Inhibition Studies

An attempt was made to evaluate the conjugation mechanism of the sheep liver to see if a substrate competition could be present. The substrates being measured were phenolphthalein and p-nitrophenol; the inhibitor was H_4E. The data from these experiments are recorded in Table VII for phenolphthalein and Table VIII for p-nitrophenol. An attempt was made to analyze the data using Lineweaver-Burk plots (23). The plots did not readily reveal the nature of the competition. The velocity vs substrate concentration plots in Figures 16 and 17 did, however, resemble what Dixon and Webb (23) refer to as mixed competition. Therefore, these experiments should be re-evaluated using inhibitors to block the competing reactions mentioned earlier (Figure 15).

CHAPTER V

SUMMARY AND CONCLUSIONS

In Part I of the thesis, steroid metabolism was followed in six, Hereford, range bulls over a 69-week period using the INH, Zimmerman, and Porter-Silber reactions. This 69-week experimental period was subdivided into a 17-week control period, an 18-week injection period, and a 34-week recovery period. Samples of plasma, urine, and feces, collected every other week throughout the experiment, were prepared for analysis using various fractionation procedures. These data were compared with reproductive data collected by co-workers during this joint investigation and published elsewhere.

The free (INH) steroid fractions of plasma and urine were the only steroid fractions that were found to be useful in correlating testosterone metabolism with spermatozoa per ejaculate and testicular size. The failure to be able to correlate the other steroid fractions with reproductive processes in the bull during this experiment was attributed to the presence of large amounts of ionone derivatives in these fractions. These ionone derivatives have been shown by other workers, after the initiation of this experiment, to originate from feed containing large amounts of carotenoid compounds. The plasma and urinary free (INH) steroid fractions were elevated during that time when spermatozoa numbers per ejaculate and testicular size were at a minimum. However, there was a secondary rise in these two fractions some 29 weeks after the cessation of testosterone

injections. This secondary increase in steroid content in the urine and feces was associated with an increase of spermatozoa numbers per ejaculate and testicular size. In addition, there also appeared to be a seasonal effect on steroid metabolism and testicular function. In this respect, a decreased rate of steroid metabolism and decreased testicular function were present during and immediately following the hot, summer months while an increased steroid metabolism and increased testicular function were present during the cold, winter months. Also associated with the winter months was a tremendous increase in plasma conjugated (INH) steroids. This increase in conjugated steroids in the plasma was thought to be due to a competition of conjugated materials (steroids, ionone derivatives, and thyroxine) for a pump mechanism in the process of biliary excretion of these compounds.

The plasma free (INH) steroids reached a peak during testosterone injections, whereas the urinary free (INH) steroids continued to rise until testosterone injections were stopped. This failure to observe a high level of free (INH) steroids in the plasma throughout the injection period was thought to be due in part to the rapid excretion of free steroids by the kidney. In this respect, a homeostatic mechanism was proposed whereby the steroid pump mechanism in the kidney tubule cells was depressed during prolonged exposure to excessive amounts of free steroids.

Metabolic schemes were presented to explain the apparent metabolism and conjugation of ionone derivatives from the feed. These schemes, based on compounds which have been isolated from urine of cattle by other workers, were proposed to explain the decrease in INH-positive chromogens in the feces throughout the experiment. Concomitant with this decrease

in INH-positive chromogens in the feces was an increase in the 17-keto-steroid (Zimmerman) material in the urine and feces. These schemes also were used to explain the lack of agreement between the 17-ketosteroid (Zimmerman) fraction and the 17-ketosteroid (INH) fraction in the feces. At the start of the experiment these two steroid fractions agreed quite closely but differed markedly at the end of the experiment. In this respect, there appeared to be an age effect on the metabolism of these ionone compounds; there was an apparent increase with age in respect to the ability of the animal to metabolize the ionone compounds. Concomitant with this increased ability to metabolize the ionone compounds was an increase in the 17-ketosteroid (Zimmerman) chromogens per ml. of urine and per gm. of feces. Compounded with this increase was an increase in total daily chromogen excretion in these two fractions associated with age. This increase was associated with an increased size of the animals and a corresponding increase in the maintenance requirements since the fecal dry-matter excreted per day increased throughout the experiment. The fecal route of excretion predominated during the experiment; however, the daily excretion of chromogens in the urine increased throughout the experiment so that the fecal:urinary ratios dropped considerably by the end of the experiment; this observation applied to all steroid fractions.

In Part II of the thesis, conjugation studies were undertaken to evaluate the liver and adrenal as possible sites of conjugation of steroids in the ruminant. Additional studies were undertaken to gain information concerning the nature of the glucuronosyl transferase enzyme system.

Sheep had a greater ability to synthesize UDPGA in both the liver and adrenal glands than did the dog or rat. Likewise, sheep had a greater ability to synthesize glucuronide conjugates of H_4E, phenolphthalein, and

p-nitrophenol. Glucose was shown to be a better precursor for UDPGA in sheep liver homogenates than galactose. This is opposite to what has been reported in rat liver preparations. Ovine liver microsomal preparations were found to conjugate H_4E and H_4F with almost equal ability. The adrenal glands of all three species had a much lower capacity to synthesize UDPGA as well as to conjugate H_4E.

Negative results were found in the dog with respect to the conjugation of steroids by either the liver or the adrenal glands. The failure to demonstrate conjugation of H_4E in this species was attributed to competing reactions. In this respect, it was shown that a sulfatase and a β-glucuronidase were present in dog liver and adrenal preparations. The dog liver microsomal preparations were, however, capable of synthesizing conjugates of phenolphthalein and p-nitrophenol.

Evidence was obtained which was suggestive of a complex type of competition when H_4E was used as an inhibitor and phenolphthalein and p-nitrophenol were used as substrates. An attempt was made to analyze the kinetics of these inhibition studies, but failed due to other competing reactions taking place during the incubation of the samples.

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A P P E N D I X

APPENDIX TABLE I. PLASMA (INH) FREE STEROID CONTENT BEFORE, DURING AND AFTER TESTOSTERONE INJECTIONS

Animal No.	1958							
	4-28	5-12	5-26	6-9	6-23	7-7	7-21	8-4
	(μgm./ml.)							
3	0	0	0	0.52	1.54	4.89	5.77	4.12
6	0	0	0	0.93	1.29	4.58	5.10	4.94
2	0	0	0	0.10	0	2.83	9.48	2.63
4	0	0	0	1.65	0.41	3.19	7.42	2.78
1	0	0	0.67	0.72	1.80	5.20	6.23	1.08
5	0.77	0	0	0.10	0.36	3.19	3.76	1.18
$\bar{x}$	0.13	0	0.11	0.67	0.90	3.98	6.29	2.79

Animal No.	1958							
	8-18	9-1	9-15	9-29	10-13	10-27	11-10	11-24
	(μgm./ml.)							
3	2.99	1.34	2.32	0.93	1.65	0	0	1.13
6	3.35	2.52	3.76	1.91	2.63	0	0.46	1.65
2	4.38	1.08	1.39	0.21	1.85	1.70	0	0.46
4	5.10	1.18	1.91	0.67	1.85	0	0.21	3.14
1	5.72	2.32	3.55	1.70	3.40	0.26	1.65	1.65
5	4.53	2.32	4.89	1.65	8.50	0	1.44	2.11
$\bar{x}$	4.34	2.00	2.97	1.18	3.31	0.33	0.63	1.69

APPENDIX TABLE I (Continued)

Animal No.	1958		1959						
	12-8	12-22	1-5	1-19	2-2	2-16	3-2	3-30	4-27
					(μgm./ml.)				
3	1.85	0	0	0	0	1.24	0.52	2.88	5.72
6	2.88	0	0	0	0	1.18	1.39	3.91	6.44
2	0.67	0	0	0	0	1.18	1.96	2.11	1.39
4	3.66	0	2.47	0	0	1.44	1.18	5.56	2.11
1	2.11	0	0	0	0	0.93	1.70	5.00	2.88
5	2.63	0	0	0	0	0.72	1.39	6.75	3.40
$\bar{x}$	2.30	0	0.41	0	0	1.12	1.36	4.37	3.66

APPENDIX TABLE II. PLASMA (INH) STEROID-GLUCURONIDE CONTENT BEFORE, DURING, AND AFTER TESTOSTERONE INJECTIONS

Animal No.	1958 4-28	5-12	5-26	6-9	6-23	7-7	7-21	8-4
	(μgm./ml.)							
3	16.79	18.59	15.04	5.97	0.77	18.85	28.89	40.32
6	18.13	3.09	0	3.91	6.18	43.11	47.48	45.78
2	0	70.97	21.32	1.96	3.86	17.61	5.34	16.38
4	38.83	3.04	60.31	14.06	2.47	4.17	8.50	43.00
1	0	8.60	0	3.60	52.02	8.24	8.39	16.94
5	38.52	1.34	79.26	2.06	3.76	56.91	59.64	0
$\bar{x}$	18.71	17.60	29.34	5.26	11.51	24.82	26.39	27.07

Animal No.	1958 8-18	9-1	9-15	9-29	10-13	10-27	11-10	11-24
	(μgm./ml.)							
3	65.35	33.99	37.85	11.38	69.37	40.63	60.82	49.65
6	56.86	56.97	57.32	51.50	45.16	15.76	43.26	54.85
2	2.78	12.46	3.35	30.54	28.17	44.24	42.59	22.35
4	72.46	47.02	48.92	63.34	60.82	9.06	59.79	45.58
1	4.12	44.05	52.84	56.08	50.09	77.10	47.48	40.79
5	20.24	33.89	30.08	71.69	79.31	92.29	88.22	26.47
$\bar{x}$	36.97	38.06	38.39	47.42	56.82	46.51	57.03	39.95

APPENDIX TABLE II (Continued)

Animal No.	1958		1959						
	12-8	12-22	1-5	1-19	2-2	2-16	3-2	3-30	4-27
					(μgm./ml.)				
3	15.19	93.16	133.49	28.31	96.78	125.10	87.07	53.61	1.54
6	12.26	77.69	120.49	37.36	95.47	44.77	77.86	63.60	8.60
2	7.31	3.13	103.86	2.96	9.71	23.87	20.58	34.35	42.95
4	15.76	63.74	192.75	65.18	136.12	144.35	51.85	49.54	45.58
1	12.98	174.64	160.20	99.09	77.87	110.12	55.47	52.22	47.95
5	50.62	195.22	171.35	119.01	135.30	169.21	154.23	9.89	83.58
$\bar{x}$	19.02	118.40	147.02	63.09	91.88	102.91	74.51	43.87	38.37

APPENDIX TABLE III. PLASMA (INH) STEROID-SULFATE CONTENT BEFORE, DURING, AND AFTER TESTOSTERONE INJECTIONS

Animal No.	1958							
	4-28	5-12	5-26	6-9	6-23	7-7	7-21	8-4
	(μgm./ml.)							
3	19.11	28.74	10.35	13.18	47.59	0.46	6.75	0.98
6	15.86	4.33	1.91	8.09	24.15	36.67	20.60	6.28
2	2.27	122.79	15.55	5.87	25.54	3.91	6.95	0.26
4	28.84	34.61	69.46	39.50	29.61	2.37	2.06	11.43
1	2.88	3.19	2.88	13.29	57.60	2.63	10.71	0
5	47.38	4.38	151.43	7.72	7.98	27.71	21.12	0
$\bar{x}$	19.39	33.01	41.93	14.61	32.08	12.29	11.36	3.16

Animal No.	1958							
	8-18	9-1	9-15	9-29	10-13	10-27	11-10	11-24
	(μgm./ml.)							
3	31.26	2.68	4.89	3.96	6.13	1.80	24.10	20.75
6	7.00	2.63	40.32	4.43	9.27	1.24	16.02	13.44
2	0	1.49	0.67	0.67	4.17	7.26	21.99	2.68
4	29.38	0.93	36.00	1.65	32.86	1.75	17.92	15.71
1	1.44	4.17	21.63	11.07	6.44	39.55	16.79	26.99
5	0.21	7.98	3.91	52.74	42.44	50.47		17.00
$\bar{x}$	11.38	3.31	17.90	12.42	16.89	17.01	19.36*	17.76

*Average of five instead of six animals.

APPENDIX TABLE III (Continued)

Animal No.	1958		1959						
	12-8	12-22	1-5	1-19	2-2	2-16	3-2	3-30	4-27
	(μgm./ml.)								
3	4.74	1.03	99.75	0	169.70	22.09	32.50	0	0
6	5.30	2.21	54.98	2.96	45.26	2.21	46.25	0.93	1.24
2	6.64	1.29	65.84	2.96	31.27	0.26	7.00	0.52	1.65
4	36.31	2.14	115.06	12.67	63.04	34.76	31.00	0	2.27
1	55.26	104.68	146.33	108.64	12.51	29.82	53.87	18.85	14.37
5	30.13	127.40	100.57	146.33	174.64	60.56	72.87	0	54.64
$\bar{x}$	23.06	39.79	97.09	45.59	82.74	24.95	40.58	3.38	12.36

APPENDIX TABLE IV. PLASMA (INH) TOTAL STEROID CONTENT BEFORE, DURING AND AFTER TESTOSTERONE INJECTIONS

Animal No.	1958							
	4-28	5-12	5-26	6-9	6-23	7-7	7-21	8-4
	(μgm./ml.)							
3	35.90	47.33	25.49	19.67	49.90	24.20	41.56	45.42
6	33.99	7.42	1.91	12.93	31.62	84.36	73.18	57.00
2	2.27	193.76	36.87	7.93	29.40	24.35	21.77	19.27
4	67.67	37.65	129.77	55.21	32.49	9.73	17.98	57.21
1	2.88	11.79	3.55	17.61	110.42	16.07	25.33	18.02
5	86.67	5.72	230.69	9.88	12.10	87.81	84.52	1.18
$\bar{x}$	38.23	50.61	71.38	20.54	44.49	41.09	44.04	33.02

Animal No.	1958							
	8-18	9-1	9-15	9-29	10-13	10-27	11-10	11-24
	(μgm./ml.)							
3	99.60	38.01	45.06	16.27	77.15	42.43	84.92	71.53
6	67.21	62.12	101.40	57.84	57.06	17.00	59.74	69.94
2	7.16	15.03	5.41	31.42	34.19	53.20	64.58	25.49
4	105.94	49.13	86.83	65.66	95.53	10.81	77.92	64.43
1	11.28	51.77	78.02	68.85	67.93	116.91	65.92	69.43
5	24.98	44.19	38.88	126.08	130.25	142.76		45.58
$\bar{x}$	52.69	43.37	59.26	61.02	77.02	63.85	70.02*	59.40

*Average of Five instead of six animals.

APPENDIX TABLE IV (Continued)

Animal No.	1958		1959						
	12-8	12-22	1-5	1-19	2-2	2-16	3-2	3-30	4-27
	(μgm./ml.)								
3	21.78	94.19	233.24	28.31	266.48	148.43	120.09	56.49	7.26
6	20.46	79.90	175.47	40.32	140.73	48.16	125.50	68.44	16.28
2	14.62	4.42	169.70	5.92	40.98	25.31	29.54	36.98	45.99
4	55.73	65.88	310.28	77.85	199.16	180.55	84.03	55.10	49.96
1	70.35	279.32	306.53	207.73	90.38	140.87	111.04	76.07	65.20
5	83.38	323.02	271.92	265.34	309.94	230.49	228.49	16.64	141.62
$\bar{x}$	44.38	158.19	244.52	108.68	174.62	128.98	116.45	51.62	54.39

APPENDIX TABLE V. URINE (INH) FREE STEROID CONTENT BEFORE, DURING, AND AFTER TESTOSTERONE INJECTIONS

Animal No.	1958								1959		
	1-27	3-17	4-28	6-9	7-21	9-1	10-13	11-24	1-5	2-16	4-27
	(μgm./ml.)										
3	2.21	0.75	2.07	3.94	3.54	4.64	4.78	4.34	4.80	3.27	5.21
6	3.14	2.38	2.58	2.52	5.57	5.42	6.24	3.33	3.89	3.23	6.17
2	1.35	2.86	3.06	2.71	6.46	7.64	4.15	2.80	3.57	4.44	9.26
4	1.79	3.89	2.97	3.61	8.49	5.53	3.47	2.73	3.81	3.39	10.23
1	3.88	2.19	2.26	2.74	7.36	11.31	4.24	4.60	4.43	6.78	9.84
5	2.50	2.98	3.17	4.91	5.75	13.98	3.51	2.83	3.01	5.34	
$\bar{x}$	2.48	2.51	2.68	3.40	6.20	8.09	4.40	3.44	3.92	4.41	8.14*

*Average of five instead of six animals.

APPENDIX TABLE VI. URINE (INH) STEROID-GLUCURONIDE CONTENT BEFORE, DURING, AND AFTER TESTOSTERONE INJECTIONS

Animal No.	1958								1959		
	1-27	3-17	4-28	6-9	7-21	9-1	10-13	11-24	1-5	2-16	4-27
	(μgm./ml.)										
3	10.11	9.73	8.75	10.33	22.78	6.73	7.75	6.86	8.47	9.36	9.06
6	7.55	7.47	7.28	10.65	10.57	6.46	8.78	7.45	2.70	15.94	7.85
2	8.81	8.30	12.09	3.48	9.60	5.95	9.74	10.35	11.32	6.63	3.73
4	9.28	6.76	10.84	7.32	9.09	5.72	14.91	10.24	9.67	6.18	6.76
1	9.50	5.76	11.60	6.84	10.54	5.82	10.42	8.40	8.11	6.62	4.26
5	8.55	9.08	8.04	10.06	7.52	3.13	9.63	5.55	5.82	6.09	
$\bar{x}$	8.97	7.85	9.77	8.11	11.68	5.64	10.20	8.14	7.68	8.47	6.33*

*Average of five instead of six animals.

APPENDIX TABLE VII. URINE (INH) STEROID-SULFATE CONTENT BEFORE, DURING, AND AFTER TESTOSTERONE INJECTIONS

Animal No.	1958								1959		
	1-27	3-17	4-28	6-9	7-21	9-1	10-13	11-24	1-5	2-16	4-27
	(μgm./ml.)										
3	14.36	14.05	15.91	17.24	14.45	26.99	21.53	14.24	18.59	15.87	19.69
6	13.63	18.98	10.13	11.79	14.01	14.87	7.22	11.79	18.47	15.35	15.76
2	14.67	14.62	12.60	8.74	4.67	11.58	18.13	9.44	17.73	17.25	17.73
4	13.44	14.98	12.84	11.92	20.70	27.24	18.47	18.96	19.86	18.03	16.49
1	15.64	12.01	10.95	6.59	23.90	9.37	14.68	17.81	17.15	12.56	16.71
5	12.82	18.28	8.55	16.38	14.80	10.14	10.56	16.71	20.24	15.23	
$\bar{x}$	14.09	15.49	11.83	12.11	15.29	16.70	15.10	14.82	18.67	15.72	17.28*

*Average of five instead of six animals.

APPENDIX TABLE VIII. URINE (INH) TOTAL STEROID CONTENT BEFORE, DURING AND AFTER TESTOSTERONE INJECTIONS

Animal No.	1958								1959		
	1-27	3-17	4-28	6-9	7-21	9-1	10-13	11-24	1-5	2-16	4-27
	(μgm./ml.)										
3	26.68	24.53	26.73	31.51	40.77	38.36	34.06	25.44	31.86	28.50	33.96
6	24.32	28.83	19.99	24.96	30.15	26.75	22.24	22.57	25.06	34.52	29.78
2	24.83	25.78	27.75	14.93	20.73	25.17	32.02	22.59	32.62	28.32	30.72
4	24.51	25.63	26.65	22.85	38.28	38.49	36.85	31.93	33.34	27.60	33.49
1	29.02	19.96	24.81	16.17	40.99	26.50	29.34	30.81	29.69	25.96	30.81
5	23.87	30.34	19.76	31.35	28.07	27.25	23.70	25.09	29.07	26.66	
$\bar{x}$	25.54	25.85	24.28	23.62	33.17	30.43	29.70	26.40	30.27	28.60	31.75*

*Average of five instead of six animals.

APPENDIX TABLE IX. FECES (INH) FREE STEROID CONTENT BEFORE, DURING, AND AFTER TESTOSTERONE INJECTIONS

Animal No.	1958								1959		
	1-27	3-17	4-28	6-9	7-21	9-1	10-13	11-24	1-5	2-16	4-27
	(μgm./gm.)										
3	91.26	87.12	73.30	81.30	49.32	83.18	29.50	53.58	47.16	23.48	15.88
6	148.08	98.06	88.10	136.54		87.52	40.94	92.94	43.12	67.88	23.78
2	97.28	108.12	69.94	63.94	79.02	41.94	63.54	45.18	73.70	50.32	5.62
4	84.56	101.22	82.78	67.68	78.04	52.58	34.62	61.36	52.38	79.44	22.50
1	134.66	102.50	119.38	64.12	178.88	46.66	86.72	69.36	55.34	88.40	42.02
5	140.78	84.94	102.20	70.44	100.40	84.16	53.38	85.14	44.70	66.80	41.24
$\bar{x}$	116.10	96.99	89.28	80.67	79.13*	66.01	51.45	67.93	52.73	62.72	25.17

*Average of five instead of six animals.

APPENDIX TABLE X. FECES (INH) STEROID-GLUCURONIDE CONTENT BEFORE, DURING, AND AFTER TESTOSTERONE INJECTIONS

Animal No.	1958								1959		
	1-27	3-17	4-28	6-9	7-21	9-1	10-13	11-24	1-5	2-16	4-27
	(μgm./gm.)										
3	73.30	49.82	68.38	49.32	14.00	46.56	6.32	45.38	14.90	58.02	14.90
6	150.66	83.76	99.64	117.60		43.60	17.36	58.12	42.92	19.54	42.22
2	58.70	61.66	36.20	54.26	75.58	19.04	38.28	59.00	38.88	41.94	32.56
4	99.26	65.32	54.96	51.70	46.66	23.18	22.88	71.52	26.94	49.82	30.28
1	123.90	68.38	95.70	58.12	60.78	21.80	54.36	75.48	51.80	52.38	28.32
5	114.06	54.76	108.04	52.20	39.46	16.38	41.74	69.08	21.12	50.62	42.42
$\bar{x}$	103.21	63.95	77.15	63.87	47.30*	28.43	30.16	63.10	32.76	45.39	31.78

*Average of five instead of six animals.

APPENDIX TABLE XI. FECES (INH) STEROID-SULFATE CONTENT BEFORE, DURING, AND AFTER TESTOSTERONE INJECTIONS

Animal No.	1958								1959		
	1-27	3-17	4-28	6-9	7-21	9-1	10-13	11-24	1-5	2-16	4-27
	(μgm./gm.)										
3	85.84	94.52	58.60	59.88	26.14	25.16	29.80	60.28	11.94	26.84	21.80
6	58.82	58.60	90.76	52.78		41.60	30.10	56.14	22.10	36.80	28.80
2	45.88	80.80	39.86	37.78	24.28	49.82	44.60	65.42	27.32	57.12	53.86
4	90.48	38.98	36.60	33.54	21.70	29.60	33.06	44.50	31.76	47.26	52.68
1	77.94	57.12	49.92	30.68	56.14	28.52	53.76	39.96	45.68	48.44	57.92
5	74.98	97.38	37.20	37.10	40.74	50.42	49.42	51.80	40.06	63.94	56.20
$\bar{x}$	71.99	71.23	52.16	41.96	33.80*	37.52	40.12	53.02	29.81	46.73	45.21

*Average of five instead of six animals.

APPENDIX TABLE XII. FECES (INH) TOTAL STEROID CONTENT BEFORE, DURING, AND AFTER TESTOSTERONE INJECTIONS

Animal No.	1958								1959		
	1-27	3-17	3-28	6-9	7-21	9-1	10-13	11-24	1-5	2-16	4-27
	(μgm./gm.)										
3	250.40	231.46	200.28	190.50	89.46	154.90	65.62	159.24	74.00	108.34	52.58
6	354.96	240.42	278.50	306.92		172.72	88.40	207.20	108.14	124.22	94.80
2	201.86	250.58	146.00	155.98	178.88	110.80	146.42	169.60	139.90	149.38	92.04
4	274.30	205.52	174.34	152.92	146.40	105.36	90.56	177.38	111.08	176.52	105.46
1	336.50	228.00	265.00	152.92	295.80	96.98	194.84	184.80	152.82	189.22	128.26
5	329.82	237.08	247.44	159.74	180.60	150.96	144.54	206.02	105.88	181.36	139.86
$\bar{x}$	291.30	232.17	218.59	186.50	160.23*	131.96	121.73	184.05	115.30	154.84	102.16

*Average of five instead of six animals.

APPENDIX TABLE XIII. PLASMA (ZIMMERMAN) 17-KETOSTEROID CONTENT BEFORE, DURING, AND AFTER TESTOSTERONE INJECTIONS

Animal No.	1958									
	2-24	3-17	3-31	4-14	4-28	5-12	5-26	6-9	6-23	7-7
	(μgm./ml.)									
3	0.57	0.08	0.06	0.27	4.20	0.89	4.51	0.52	5.30	0.34
6	0.49	0.31	0.09	0.23	0.86	1.96	0.59	0.55	4.66	1.85
2	0.18	0.54	0.40	0.25	0.47	0.51	1.60	0.18	0.03	0.29
4	0.14	0.09	0.16	0.01	0.48	1.02	0.83	1.50	3.43	0.46
1	0.16	0.88	0.05	0.30	0.43	0.77	1.25	0.99	4.96	2.50
5	0.03	0.13	0.30	1.56	1.38	0.79	1.06	6.60	4.37	2.37
$\bar{x}$	0.26	0.34	0.18	0.44	1.30	0.99	1.64	1.72	3.79	1.30

Animal No.	1958									
	7-21	8-8	8-18	9-1	9-15	9-29	10-13	10-27	11-10	11-24
	(μgm./ml.)									
3	0.61	0.74	0.37	0.50	0.40	0.58	0.15	0.30	0.41	0.48
6	1.38	0.15	1.13	0.81	0.61	0.77	0.32	0.15	0.30	0.74
2	0.32	0.47	0.23	1.81	0.07	0.18	0.23	0.20	0.10	0.52
4	0.70	0.61	0.32	0.61	0.26	0.46	0.26	0.30	0.36	0.48
1	2.01	1.13	0.37	0.18	0.34	0.23	0.20	0.36	0.41	0.98
5	3.21	0.26	0.48	0.34	0.18	0.43	1.21	1.61	0.41	1.13
$\bar{x}$	1.37	0.56	0.48	0.71	0.31	0.44	0.40	0.49	0.33	0.72

APPENDIX TABLE XIII (Continued)

Animal No.	1958		1959						
	12-8	12-22	1-5	1-19	2-2	2-16	3-2	3-30	4-27
					(μgm./ml.)				
3	0.15	0.29	2.06	0.07	0.29	1.25	0.34	0.26	2.12
6	0.02	0.32	0.58	0.05	0.15	1.70	1.75	3.60	3.82
2	0.29	0.20	0.34	0.15	0.23	0.20	0.32	0.34	0.55
4	0.07	1.60	1.42	0.34	0.07	0.81	0.07	0.52	0.77
1	0.20	1.02	0.67	0.40	0.32	1.13	0.23	2.37	0.95
5	0.23	0.55	2.57	0.40	0.12	4.99	1.29	6.18	6.89
$\bar{x}$	0.16	0.66	1.27	0.24	0.20	1.68	0.67	2.21	2.52

APPENDIX TABLE XIV. URINE (ZIMMERMAN) 17-KETOSTEROID CONTENT BEFORE, DURING, AND AFTER TESTOSTERONE INJECTIONS

Animal No.	1958									
	1-27	2-10	2-24	3-17	3-31	4-14	4-29	5-12	5-26	6-9
	(μgm./ml.)									
3	28.78	20.57	27.57	34.73	39.97	37.17	21.11	23.59	15.16	29.59
6	24.94	17.95	22.33	37.17	27.97	25.67	14.39	25.67	18.13	21.38
2	18.45	20.03	18.26	38.52	17.46	11.37	20.03	18.94	20.66	23.46
4	23.69	22.33	30.67	33.61	18.45		26.39	19.49	24.18	23.46
1	31.53	31.53	27.48	19.49	21.11	15.66	18.95	17.46	33.02	19.40
5	26.39	29.68	28.46	27.97	17.01	17.00	15.20		21.38	8.78
$\bar{x}$	25.61	23.68	25.80	31.92	23.66	*21.37	19.32	*21.03	22.09	22.68

Animal No.	1958									
	6-23	7-7	7-22	8-4	8-19	9-1	9-15	9-29	10-13	10-27
	(μgm./ml.)									
3	20.66	29.59	11.82	31.30	54.31	43.48	34.05	28.78	38.97	29.59
6	27.97	29.59	22.01	20.03	48.54	38.97	20.66	22.73	41.14	34.01
2	24.90	38.97	24.90	36.90	51.33	41.14	34.91	59.36	47.18	44.66
4	25.62	48.54	37.89	16.89	49.89	42.22	38.97	61.17	49.89	25.62
1	34.91	37.89	18.76	36.90	48.54	59.36	55.93	42.22	35.91	34.01
5	42.22	52.78	6.86	90.22	59.36	65.04	37.89	43.48	34.01	29.59
$\bar{x}$	29.38	39.56	20.37	38.70	52.00	48.37	37.07	42.96	41.18	32.91

*Average of five instead of six animals.

APPENDIX TABLE XIV (Continued)

Animal No.	1958				1959						
	11-10	11-24	12-8	12-22	1-5	1-19	2-2	2-16	3-2	3-30	4-27
	(μgm./ml.)										
3	37.89	33.02	35.90	12.90	38.43	28.78	36.90	40.06	47.18	34.91	35.91
6	43.48	37.89	45.92	14.52	30.40	25.62	40.06	29.59	45.54	44.66	33.02
2	37.89	37.89	38.97	33.02	27.15	21.38	38.97	33.02	59.36	40.06	49.89
4	43.48	38.74	44.66	29.59	37.89	30.40	35.91	30.40	55.93	43.48	57.56
1	48.54	52.78	32.12	19.40	31.30	29.59	34.91	41.14	51.33	41.14	67.21
5	34.01	29.59	24.90	25.62	44.66	24.18	32.12	45.92	42.22	43.48	
$\bar{x}$	40.88	38.32	37.08	22.51	32.97	26.66	36.48	36.69	50.76	41.28	48.72*

*Average of five instead of six animals.

APPENDIX TABLE XV. URINE (INH) 17-KETOSTEROID CONTENT BEFORE, DURING AND AFTER TESTOSTERONE INJECTIONS

Animal No.	1958 1-27	2-10	3-18	3-31	4-14	4-29	5-12	5-26	6-9	6-23
	(μgm./ml.)									
3	8.40	6.92	9.57	10.31	12.74	8.57	10.72	3.73	8.80	7.95
6	9.20	4.78	10.67	7.10	11.59	4.04	10.05	4.37	6.39	10.34
2	7.13	5.81	9.67	3.52	5.27	8.12	11.42	5.91	7.17	7.87
4	9.07	7.65	7.68	4.88		13.92	12.09	5.62	8.69	8.76
1	10.01	8.36	5.76	4.08	15.07	6.80	9.35	8.65	5.21	10.86
5	8.49	8.98	9.25	3.66	8.17	7.10		4.08	10.42	10.90
$\bar{x}$	8.72	7.08	8.43	5.59	10.57*	8.09	10.73*	5.39	7.78	9.45

Animal No.	1958 7-7	7-21	8-4	8-18	9-1	9-15	9-29	10-13	10-27	11-10
	(μgm./ml.)									
3	10.51	4.02	4.84	14.61	10.03	8.55	9.64	9.72	11.58	7.75
6	10.46	8.14	6.94	14.34	8.90	4.47	16.03	9.33	13.66	10.98
2	16.03	8.32	9.17	14.05	10.77	9.65	18.00	9.83	13.25	7.60
4	15.66	3.77	4.88	12.20	12.38	9.83	17.18	10.36	7.91	10.18
1	14.54	8.03	11.12	12.50	13.51	14.50	19.34	7.58	8.73	7.68
5	15.82	5.64	6.14	10.73	15.37	7.95	15.04	8.67	9.87	4.24
$\bar{x}$	13.84	6.32	7.18	13.07	11.83	9.16	15.87	9.25	10.83	8.07

*Average of five instead of six animals.

APPENDIX TABLE XV (Continued)

Animal No.	1958			1959						
	11-24	12-8	12-22	1-5	1-19	2-2	2-16	3-2	3-30	4-27
	(μgm./ml.)									
3	6.61	9.60	8.28	11.12	5.91	11.93	8.47	10.84	8.63	11.31
6	9.15	11.93	6.32	12.30	4.76	12.40	7.97	10.36	8.92	10.38
2	11.93	10.55	5.60	11.78	7.02	10.73	7.75	10.69	8.94	10.67
4	12.17	12.30	5.81	10.67	8.55	12.90	6.66	11.52	13.45	15.34
1	11.89	11.06	5.46	8.90	5.91	13.31	8.90	7.99	12.07	13.74
5	5.79	18.31	4.08	8.98	5.11	14.32	10.03	10.55	9.83	
$\bar{x}$	9.59	12.29	5.92	10.62	6.21	12.43	8.30	10.32	10.31	12.29*

*Average of five instead of six animals.

APPENDIX TABLE XVI. URINE (INH) 17-HYDROXYCORTICOSTEROID CONTENT BEFORE, DURING, AND AFTER TESTOSTERONE INJECTIONS

Animal No.	1958 1-27	2-10	2-24	3-17	3-31	4-14	4-28	5-12	5-26	6-9
	(μgm./ml.)									
3	1.04	0.19	1.62	0.20	0.98	0.89	0.90	0.99	0.13	0.81
6	0.94	0.07	1.83	0.65	0.83	0.76	0.42	1.05	0.05	0.46
2	0.54	0.16	2.44	0.46	0.31	0.50	0.72	1.04	0.16	0.23
4	0.97	0.45	2.46	0.35	0.38		0.76	0.90	0.35	0.69
1	0.95	0.24	1.69	0.73	0.46	0.64	0.25	1.09	0.39	0.43
5	0.81	0.09	1.94	0.47	0.36	0.73	0.83		0.34	1.13
$\bar{x}$	0.88	0.20	2.00	0.48	0.66	0.70*	0.65	1.01*	0.24	0.62

Animal No.	1958 6-25	7-7	7-21	8-4	8-18	9-1	9-15	9-29	10-13	10-27
	(μgm./ml.)									
3	0.55	0.75	0.42	0.18	0.79	0.80	0.84	0.56	0.75	0.39
6	0.68	1.05	0.32	0.32	0.88	0.78	0.58	0.72	0.57	0.74
2	0.66	0.90	0.23	0.42	0.28	0.68	0.75	1.24	0.63	1.23
4	1.12	0.84	0.88	0.29	0.62	0.65	1.05	1.55	0.65	0.60
1	0.82	0.88	0.36	0.35	0.31	0.86	0.74	2.56	0.55	0.97
5	0.86	1.17	0.09	0.51	0.57	0.52	0.58	1.02	0.74	0.39
$\bar{x}$	0.78	0.93	0.38	0.34	0.58	0.72	0.76	1.28	0.65	0.72

*Average of five instead of six animals.

APPENDIX TABLE XVI (Continued)

Animal No.	1958				1959				
	11-10	11-25	12-8	12-22	1-6	2-16	3-2	3-30	4-27
	(μgm./ml.)								
3	0.51	0.79	1.29	0.25	0.63	3.27	0.51	0.71	0.88
6	0.82	0.97	1.45		0.75	0.93	1.08	0.90	1.03
2	0.71	1.16	1.48		0.97	0.36	0.74	0.79	0.68
4	1.16	1.26	0.91	0.38	0.58	1.03	0.82	1.05	0.76
1	0.82	1.17	1.41		0.55	0.53	0.55	0.73	1.88
5	4.68	1.71	0.65	0.13	0.65	0.54	0.75	0.65	
$\bar{x}$	1.45	1.01	1.20	0.13	0.83	1.11	0.74	0.80	1.05*

*Average of five instead of six animals.

APPENDIX TABLE XVII. FECES (ZIMMERMAN) 17-KETOSTEROID CONTENT BEFORE, DURING, AND AFTER TESTOSTERONE INJECTIONS

Animal No.	1958									
	2-24	3-18	3-31	4-14	4-28	5-12	5-26	6-9	6-25	7-7
	(µgm./gm.)									
3	225.77	46.15	39.77	179.31	129.84	133.17	103.20	77.16	99.29	13.75
6	114.32	38.09	28.67	234.16	235.17	122.28	131.62	116.55	112.54	17.96
2	133.56	42.51	39.24	115.48	140.43	171.78	92.43	62.91	117.15	9.18
4	139.60	39.84	42.27	64.81	231.53	182.69	109.39	75.34	131.34	11.28
1	162.30	31.86	42.76	214.72	267.02	153.82	152.69	64.48	127.29	19.75
5	102.19	43.10	42.82	149.77	245.61	174.55	181.05	94.61	110.50	61.28
$\bar{x}$	102.21	40.26	40.92	159.71	209.27	156.38	128.40	81.84	116.35	22.20

Animal No.	1958									
	7-21	8-4	8-9	9-1	9-15	9-29	10-13	10-27	11-10	11-24
	(µgm./gm.)									
3	163.14	147.20	66.02	214.13	210.64	201.15	180.48	94.47	80.40	51.69
6	203.20	135.79	63.86	321.45	274.08	219.62	290.98	134.41	88.88	43.23
2	179.96	181.83	155.87	108.17	222.57	273.33	251.76	138.55	134.41	50.64
4	160.83	125.98	70.76	235.62	201.05	235.03	192.93	174.56	259.83	55.89
1	179.52	133.24	45.11	215.41	195.71	230.19	212.12	280.48	226.80	47.09
5	269.64	120.92	78.94	252.81	258.09	214.47	176.91	161.63	256.43	49.71
$\bar{x}$	192.72	140.83	80.09	224.60	227.02	228.97	217.53	164.02	174.46	49.71

APPENDIX TABLE XVII (Continued)

Animal No.	1958		1959						
	12-8	12-22	1-5	1-19	2-2	2-16	3-2	3-30	4-27
	(μgm./gm.)								
3	50.94	93.91	130.81	235.89	48.15	115.57	158.95	130.55	127.75
6	45.54	171.14	134.98	298.37	107.60	138.20	212.54	183.83	151.45
2	36.94	261.62	183.65	205.69	83.27	163.46	107.25	172.75	130.56
4	44.67	403.44	313.86	306.15	116.84	121.35	120.87	194.45	140.13
1	45.88	263.66	207.16	202.23	120.28	253.73	182.07	206.74	145.35
5	33.45	226.79	241.83	316.24	146.83	158.94	82.01	211.54	176.46
$\bar{x}$	49.90	236.76	202.05	311.79	103.83	158.54	143.95	183.31	145.28

APPENDIX TABLE XVIII. FECES (INH) 17-KETOSTEROID CONTENT BEFORE, DURING, AND AFTER TESTOSTERONE INJECTIONS

Animal No.	1958									
	3-17	3-31	4-14	4-28	5-12	5-26	6-9	6-23	7-7	7-21
	(μgm./gm.)									
3	35.41	33.90	157.42	102.68	97.04	92.38	71.34	69.68	98.45	108.92
6	39.86	30.63	213.23	180.81	150.44	105.04	40.90	71.10	118.25	130.14
2	38.07	44.70	95.34	172.20	114.98	113.72	70.30	71.18	153.05	112.00
4	40.68	46.67	110.74	150.17	112.60	99.59	46.46	83.70	86.87	119.15
1	33.56	30.71	100.28	239.82	127.23	110.48	38.42	58.50	109.10	143.53
5	50.28	40.57	159.57	216.56	97.73	103.34	66.20	64.84	131.67	155.82
$\bar{x}$	39.59	37.86	139.43	177.04	116.67	104.09	55.60	69.83	116.23	128.26

Animal No.	1958									
	8-4	8-18	9-1	9-15	9-29	10-13	10-27	11-10	11-24	12-8
	(μgm./gm.)									
3	65.15	50.32	92.97	90.91	80.96	99.90	84.78	102.02	77.25	43.07
6	77.38	135.59	104.06	120.70	88.12	129.74	95.40	121.68	72.75	60.57
2	76.12	81.08	84.42	86.34	110.51	121.35	88.68	109.17	82.79	68.57
4	53.79	63.07	93.73	94.06	94.60	106.26	97.44	168.31	71.87	81.80
1	85.66	46.94	112.87	100.89	100.45	104.20	159.39	131.06	62.16	59.59
5	78.28	81.61	101.06	89.60	99.05	97.13	114.30	159.57	84.51	76.45
$\bar{x}$	72.73	76.44	98.18	97.03	95.62	109.76	106.66	131.97	75.24	65.01

APPENDIX TABLE XVIII (Continued)

Animal No.	1958	1959						
	12-22	1-5	1-19	2-2	2-16	3-2	3-30	4-28
				(μgm./gm.)				
3	60.45	77.81	61.80	82.40	61.29	67.44	49.15	58.42
6	87.98	79.70	79.13	112.65	65.45	82.95	46.06	66.71
2	107.74	95.53	72.09	109.25	70.56	74.92	68.12	51.99
4	113.19	105.48	92.81	87.25	74.81	74.10	49.47	67.50
1	123.16	112.17	110.83	96.35	109.54	93.83	74.39	90.24
5	84.00	118.40	146.15	140.08	68.82	67.27	80.38	78.10
$\bar{x}$	96.09	98.18	93.80	104.66	75.08	76.75	61.26	68.83

APPENDIX TABLE XIX. FECES (INH) 17-HYDROXYCORTICOSTEROID CONTENT BEFORE, DURING, AND AFTER TESTOSTERONE INJECTIONS

Animal No.	1958									
	2-24	3-17	3-31	4-14	4-28	5-12	5-26	6-9	6-23	7-7
	(µgm./gm.)									
3	4.34	11.88	19.69	29.91	15.72	18.02	17.37	18.98	24.55	14.08
6	1.69	8.24	9.89	38.23	43.53	28.88	30.12	36.70	30.86	19.18
2	4.13	13.26	19.21	16.08	29.34	18.88	20.34	12.08	29.03	21.51
4	11.68	11.61	16.93	30.10	36.73	18.16	24.14	25.39	46.02	12.92
1	10.88	8.52	15.76	55.42	58.77	20.22	24.58	42.53	26.73	18.25
5	10.31	6.89	17.56	21.98	47.26	25.73	26.79	20.26	22.94	28.16
$\bar{x}$	7.17	10.07	16.51	31.95	38.56	21.65	23.89	25.99	30.04	19.02

Animal No.	1958									
	7-21	8-4	8-18	9-2	9-14	9-29	10-13	10-27	11-10	11-24
	(µgm./gm.)									
3	9.06	6.85	6.15	11.43	15.33	15.83	20.77	6.34	10.25	8.68
6	31.06	9.27	5.97	18.94	18.59	16.00	19.19	13.20	12.06	7.09
2	23.54	5.43	12.33	11.77	38.12	18.60	21.59	12.51	12.73	8.26
4	23.66	11.83	10.59	14.05	18.59	20.02	22.51	14.30	22.51	9.52
1	31.59	10.63	13.25	15.61	22.92	18.63	19.81	27.73	23.08	10.47
5	30.62	8.15	12.20	13.62	29.81	17.09	13.97	11.39	65.24	11.82
$\bar{x}$	24.92	8.69	10.08	14.24	23.89	17.70	19.64	14.24	24.31	9.31

APPENDIX TABLE XIX (Continued)

Animal No.	1958		1959						
	12-8	12-22	1-5	1-19	2-2	2-16	3-2	3-30	4-27
	(μgm./gm.)								
3	7.09	5.48	12.91	6.01	6.82	5.69	6.23	6.76	9.46
6	9.35	9.17	5.16	9.98	8.90	7.04	7.28	7.28	8.37
2	9.47	11.51	11.20	8.87	9.49	6.35	10.70	6.14	7.29
4	11.50	13.16	18.14	7.68	10.41	6.68	6.20	6.09	7.68
1	14.44	20.60	18.31	21.29	11.06	8.39	8.88	10.92	13.89
5	11.08	19.89	29.33	9.49	26.32	7.94	7.76	10.70	9.53
$\bar{x}$	10.49	13.30	15.84	10.55	12.17	7.02	7.84	7.98	9.37

VITA

LeGrande Clark Ellis

Candidate for the Degree of

Doctor of Philosophy

Thesis: SOME ASPECTS OF STEROID METABOLISM AND CONJUGATION IN MAMMALS

Major Field: Physiology

Biographical:

Personal Data: Born at Farmington, Davis County, Utah, June 20, 1932, the son of Owen W. and Mary L. Ellis.

Education: Attended elementary school at Farmington, Utah and junior high school at Bountiful, Utah; graduated from Davis High School at Kaysville, Utah, in 1950; received the Bachelor of Science degree from Utah State University in May, 1954 and the Master of Science degree from Utah State University in June, 1956, with a major in Physiology; completed the requirements for the Doctor of Philosophy degree in August, 1961.

Professional Experience: Employed as a Teaching Assistant in the departments of Physiology and Zoology at Utah State University from 1954-55, as a Graduate Research Assistant in the Institute of Nutrition at Utah State University from 1955-56, and as a Graduate Research Assistant in the Department of Physiology and Pharmacology at Oklahoma State University from 1956-57; appointed as an Instructor in Physiology in 1957 and as an Assistant Professor in 1960, both at Oklahoma State University.