THE EFFECT OF HIGH AMBIENT TEMPERATURE ON ADRENAL CORTICAL AND TESTICULAR ENDOCRINE FUNCTION IN HEREFORD BULLS

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CHAPTER I

INTRODUCTION

Cattle are the most economically important domestic animals in the world. Their distribution is almost world-wide, but they are found in greatest numbers in temperate, subtropical, tropical, and semi-desert areas. In these climates environmental heat frequently impairs productivity. High ambient temperatures can decrease body growth, milk production, and general reproductive performance.

The reproductive performance of the bull is more vulnerable to environmental heat stress than that of the cow. The testes must be maintained at a temperature slightly lower than the rest of the body. Failure to maintain the testes at the proper temperature decreases production of fertile spermatozoa; prolonged exposure to adverse heating may damage the germinal epithelium permanently.

Extensive research has been conducted in an effort to define the physiological mechanisms involved in impariment of spermatogenesis by heat. However, no conclusive studies have been accomplished to elucidate the effects of hot environment on testicular androgen production.

This neglect has not been due to a lack of interest on the part of researchers but is the consequence of a lack of suitable hormone assays. Reliable quantitative methods were recently developed for measurement of minute amounts of testosterone. The author has adapted these techniques for use with bovine plasma.

One principle objective of this study was to measure plasma testosterone concentrations and production rates during long term exposure of bulls to an ambient temperature sufficiently high to damage spermatogenesis. Another important objective was to measure plasma concentrations of the glucocorticoid hormones, cortisol and corticosterone, determining if their absolute and/or relative levels are changed during heat exposure.

Interest in this particular research was engendered by Selye's work on the "stress syndrome". His research initiated a tremendous amount of investigation on the role of adrenal corticoids in acclimation of animals to various environmental stressors. The role played by these hormones in adjustment to cold has been extensively investigated. In contrast, their function in acclimation to hot environments has received little attention. Since cortisol and corticosterone are known to exert important influences on protein and carbohydrate metabolism and electrolyte balance, it is logical to assume that the response of the bovine to heat might be mediated through these hormones.

The purpose of this investigation was to study the effects of a hot environment on adrenal cortical and testicular endocrine function. This was accomplished by exposing eight Hereford bulls continuously for seven weeks to a temperature of 96° F, 50% relative humidity, after they had previously been maintained at 70° F, 50% relative humidity.

The response to heat by individual bulls was evaluated by measuring several physiological parameters. Rectal temperature and respiration were measured and recorded daily throughout both temperature periods. The physiological characteristics of semen samples, collected before and after heat treatment, were compared in order to evaluate the effects

of heat on spermatogenesis. Cortisol, corticosterone, and testosterone peripheral plasma concentrations were assayed at weekly intervals throughout both temperature periods, and daily during the first seven days of exposure to 96° F temperature. Twenty-four hour testosterone production rate studies were conducted at the beginning and end of each temperature exposure period.

CHAPTER II

REVIEW OF LITERATURE

Introduction

The purpose of this review of literature is to discuss some of the homeostatic adjustments which must be made by cattle in response to environmental heat. Special emphasis is placed on those physiological responses to heat that appear to influence, or to be influenced by, adrenal cortical and testicular endocrine function.

Whenever possible, studies in the bovine have been cited and compared with results found in other species. The amount of definitive measurement of endocrine adjustments to heat is small, and there is a virtual absence of information on sex hormones. Many indirect, rather than direct hormonal measurements will be noted in this review.

There are several comprehensive surveys available on the physiological responses by mammals to heat compiled by Dill, *et al.* (1964), Euler (1961), Hardy (1961), and Bianca (1965). Collins and Weiner (1968) and MacFarlane (1963) have reviewed endocrine involvement in high temperature exposure. Reproduction in livestock under climatic stress has been reviewed or discussed by Ulberg (1958), Hafez (1959), and Hart (1955).

Physiological Adjustments to a Hot Environment

Mammals living in naturally hot climates, or those subjected to artificially hot environments, must undergo functional adjustments to maintain thermal balance. Both rapid and prolonged changes occur in neural, circulatory, and metabolic processes.

The nervous system makes rapid reflex responses to heat which are necessary for the survival of the individual. Acute homeostatic adjustments to heat eventually give way to, or are modified by, more prolonged metabolic changes associated with growth and reproduction. Endocrine glands are involved, either directly or indirectly, in these latter adaptive processes. Hormones provide a background upon which nervous or metabolic adjustments can take place. Therefore, a thorough knowledge of the overt physical signs (rectal temperature, respiration rate, heart rate, etc.) of heat stress aids in evaluating the meaning of hormonal changes which take place simultaneously.

Physical Components of an Applied Heat Stress

There is a range of environmental temperature within which body temperature is maintained constant with minimal effort from thermoregulatory mechanisms. The sensation of heat or cold is absent, and respiration rate and body core temperatures are normal.

In man this range is usually designated as the "comfort zone". Demarcation of this thermo-neutral zone is more difficult in animals than in man. When moving outside the zone of thermal comfort towards "heat stress", the animal activates its defense mechanisms in a sequential manner.

The thermal load imposed upon homeotherms is the result of the

combined actions of several meteorological elements, including high temperature, humidity, solar radiation, and air movement. When experiments are conducted within an environmental chamber, temperature and humidity are usually the most important elements. However, Williams, et al. (1960), in studying physiological responses of heifers of various breeds, demonstrated that solar radiation and air movement become powerful heat stress factors under outdoor conditions.

It has been demonstrated that high air humidity at high environmental temperature acts as an extremely effective heat stress factor. Various attempts have been made to evaluate the importance of air humidity and air temperature in causing heat stress in cattle. Barrada (1957) expressed the combined effect of temperature and humidity by constructing "lines of equal effect" on psychrometric charts. Bianca (1962) determined weighting factors for dry and wet bulb temperature. His findings indicated that in cattle the effect of wet bulb temperature is approximately twice as large as that of dry bulb temperature (65% wet bulb and 35% dry bulb). The fact that in man the wet bulb temperature effect exceeds the dry bulb temperature effect by a factor of about 6 (Provins, 1963) is probably caused by the difference in the capacity for evaporation of moisture in the two species.

Vasomotor, Respiratory, and Rectal Temperature Response During Heat Exposure

When environmental temperature rises above the thermal comfort zone, physical defense mechanisms, such as general vasodilation, sweating, and panting, come into operation. With increasing ambient temperature, sweating and panting are intensified, and there may be a compensatory decrease in metabolic heat production. In response to further

heat rises, sweating and panting are both increased, but cooling is insufficient to maintain homeothermy. The body temperature begins to rise, leading to uncontrolled hyperthermy and death. Most mammals die when deep body temperature reaches 42 to 45° C (Carlson and Hsieh, 1970). Limits of thermo-regulation are reached more readily in hot than in cold environments.

Vasodilation occurs at relatively low environmental temperatures, resulting in a large increase in blood flow to the periphery. Beakley and Findlay (1955) found that ear skin temperature of calves increases suddenly by 18° C when environmental temperature reaches 15 to 20° C. Peripheral vasodilation is associated with a compensatory splanchnic vasoconstriction. Compensation is usually not complete; so the volume of the total vascular bed increases. As a result, blood pressure declines, and cardiac output diminishes due to the reduced blood return to the heart. However, normal conditions of circulation become reestablished through a compensatory increase in blood volume.

Riek and Lee (1948) noted that in cows exposed to various conditions of heat stress, respiratory rate began to rise before rectal temperature. Barrada (1957) found that the air temperature at which an increase occurred was significantly lower for respiratory rate than for rectal temperature. Bligh (1957) recorded a marked rise in respiratory rate of calves before there was an increase in the temperature of the blood in the bicarotid trunk.

Thresholds of environmental temperature for the onset of hyperthermia are modified by a variety of external factors which have been discussed by Bianca (1965). The environmental temperature at which deep body temperature begins to rise in response to heat in most species

of domestic animals, lies in the range of 28 to 32° C (Bianca, 1968). In lactating Holstein, Jersey, Brown Swiss, and Brahman cows, it is given by Worstell and Brody (1953) as 21° C, 24° C, 27° C, and 35° C, respectively.

There is evidence from studies by Nielson (1938) and Bianca (1966) that the temperature which the body seeks to maintain, the "set point", is not rigidly fixed but can change within limits. With a moderate heat load, a new equilibrium body temperature is established and regulated at the new level. Acclimation may be brought about by raising body temperature (Carlson and Hsieh, 1970) in combination with other physiological adjustments. Acclimation to heat is well established in cattle. The diminution of physiological strain (heat stress), resulting from prolonged exposure to heat, manifests itself in body temperature, respiration rate, and heart rate, declining to lower levels as exposure is continued.

Bianca (1959) found that exposing calves to a 45° C dry bulb temperature at 30% RH for a period of five hours on each of 21 successive days, caused progressively smaller increases in rectal temperature, heart rate, and respiration rate. Moreover, breathing changed from a labored to a less-labored type with acclimation. Most acclimation in rectal temperature occurred during the first ten days, but subsequently declined during nine weeks of continuous exposure to 29° C. Similar adjustments to chronic heat stress in cattle were noted by McDowell, *et al.* (1953), Thompson, *et al.* (1963), and Johnson and Kibler (1963).

Information derived from the foregoing studies indicates that rectal temperature and respiration rate are the simplest, most reliable measurements of heat stress. Thus, these criteria offer a means of

evaluating the significance of the more subtle and elusive endocrine ajustments occurring in response to heat stress.

Thyroid and Adrenal Cortical Adjustments to a Hot Environment

There is some direct and much indirect evidence of changes in mammalian endocrine activity in hot environments. Few adequate measurements of hormone production rates in response to heat have been made. Attention has focused on target organ response since many blood hormone assays were unavailable or impractical until recent years. However, target organ responses are not adequate indices of specific endocrine changes. For instance, change in the size of the testes or adjustments in the basal metabolic rate (BMR) may be the result of complex endocrine interraction, possibly involving serveral hormones. Thus, observations concerning end organ changes may offer only indirect evidence of specific hormonal adjustments.

Basal Metabolic Rate

The BMR of mammals represents the sum of the body's cellular turnover of energy modified by some component factor due to endocrine secretion. Thyroxine (T_4) and triiodothyronine (T_3), cortisol, and corticosterone, and to some degree, estrogens and androgens influence the resting metabolic rate by promoting calorigenesis. Therefore, BMR observations should give a clue to the overall endocrine state of the animal when exposed to heat. Lowered BMR may indicate that one or more of these hormones is present in smaller quantities or that they are unable to exert maximal activity.

The direct effect of heat in lowering the human BMR was first

reported by Masters (1930). Burton, *et al.* (1940), Eichna (1950), and Robinson, *et al.* (1943) all reported that hot room exposure served to reduce the BMR of man in a uniform manner. Benedict (1929) showed that the BMR of albino rats declined linearly with rise of temperature between 10 and 32° C. The BMR of Holstein, Jersey, and Sindhi-Holstein lactating cows was shown by Johnston, *et al.* (1958) to be significantly lower at 35° C than at 21° C.

The Thyroid Gland

The thyroid seems to provide an important part of the background regulation of metabolic rate. Experiments have repeatedly shown that thyroid function is depressed by moderate thermal exposure. Kamal, *et al.* (1959) demonstrated that dairy calves raised at 27° C had only half the quantity of protein-bound iodine (PBI) observed in calves raised at 10° C. Thompson, *et al.* (1963) found that PBI, T_4 secretion, and turnover rate decreased significantly when Holstein heifers were exposed to 75 to 95° F, after previous exposure to a 38 to 65° F environment. Approaching the problem from a different tack, Maqsood (1957) found that thyroidectomy lowered the rectal temperature of young rams and rabbits.

Since the evidence favors a reduced output of T_3 and T_4 that may persist for a long time in a hot environment, it follows that negative feed-back stimulation of the thyroid by the pituitary, in response to low plasma T_4 levels, must be inhibited. Andersson, *et al.* (1962) showed that warming the preoptic hypothalamic area during cold stress completely inhibited the normal increase in thyroid hormone in goats. The thyroid stimulating hormone (TSH) regulatory area is also located in this same region. Therefore, a tenable hypothesis is that direct

heating of the preoptic area by warmed blood could be responsible for inhibiting TSH, consequently reducing the release of thyroid hormone.

Adrenal Glucocorticoid Activity

Very little is known of the effects of heat stress on the adrenal cortical response in man and animals. This is due largely to the inadequacy of analytical methods commonly employed in early investigations. During the past ten years, reliable quantitative and qualitative chemical assays have been developed for the major glucocorticoids.

Cortisol and corticosterone plasma concentrations can be measured directly. Adrenal secretion rates can be calculated by utilizing injectable radioisotopes to evaluate the biological half-life of individual glucocorticoids (Yates and Urquhart, 1962).

Methods Used to Evaluate Glucocorticoid Activity. Early workers were dependent upon observing changes in the size, shape, and morphology of the adrenals to assess their secretory activity (Cramer, 1916; Sundstroem, 1927). Later, investigators utilized the depression of eosinophils and lymphocytes in the circulating blood for a rough estimate of glucocorticoid secretion (Long, 1947). Porter and Silber (1950) and Nelson and Samuels (1952) developed the first useful chemical assays which could be used to estimate glucocorticoid activity from blood and urine. These determinations are based on the fact that the 17, 21-dihydroxy-20-ketone grouping, found on the common glucocorticoids, will form colored compounds when reacted with phenylhydrazine in an acid solution. However, results from such assays must be interpreted carefully since many non-corticoid steroidal molecules possess these same groups and may be present in blood or urine in varying amounts.

Sweat (1954) was one of the first workers to report on the fluorescent properties of compounds formed by reacting cortisol or corticosterone with sulfuric acid. The reaction is apparently specific for the 11-OH group found on these steroids and forms the basis for a variety of specific fluorimetric assays for glucocorticoids (Silber, *et al.*, 1958; Moor, *et al.*, 1960; Ganjam, *et al.*, 1970). Murphy (1963) developed a particularly sensitive competitive protein-binding radioassay which allowed glucocorticoids to be measured from extremely small blood plasma samples. The development of these quantitative assays has furnished the investigator valuable tools with which to study the effects of heat on glucocorticoid production.

Effects of Acute Exposure to High Heat. Increased levels of plasma glucocorticoids have been found in experiments where animals have been acutely exposed to severe heat stress. Hubler (1952) used heat lamps to warm rats to the point of near heat exhaustion, demonstrating eosinopenia and lymphopenia in response to hyperthermia. This was taken as circumstantial evidence that glucocorticoid secretion was enhanced since adrenocorticotropic hormone (ACTH) mediated similar changes. Plasma 17-OHS increased seven-fold when anesthetized dogs were immersed in hot water until rectal temperature reached 42° C for an hour or more. Marked increases in plasma cortisol and corticosterone were seen in unacclimated men who were exposed to a wet bulb temperature of 35° C for two hours (Collins, 1963). Collins found that the enhanced secretion of glucocorticoids was accompanied by an increased rate of hepatic removal from the plasma under these same conditions. Plasma glucocorticoids of Holstein steers increased from 30 to 46 μg/l after four hours of exposure to 42° C, 60% RH (Christian, 1970).

It was previously stated that a number of physical factors such as air temperature, humidity, and solar radiation combine to modify heat loss from the body, thus constituting heat stress. In reviewing this subject one often neglected factor is psychological strain. Perhaps this is the most important factor in the distinction between the effects of acute exposure to severe heat and the processes involved in long term acclimation to moderate heat loads. Harris (1955) and Mason (1959) presented an impressive array of data from animal experiments, emphasizing the marked adrenocortical stimulation that follows even simple procedures, such as handling or transferring animals to a new environment. Thus, it seems possible that increased levels of plasma glucocorticoids, in response to actue heating, may result from non-specific stressors due to psychological strain.

Effects of Prolonged Heat Exposure. Attention was first drawn to adrenal cortical response to high environmental temperatures by morphological changes, observed in the adrenal glands of animals maintained under hot conditions (Cramer, 1928). The effect of high temperature on the pituitary-adrenal system has received less attention than that of low temperatures. This is probably due to the fact that glucocorticoids, like thyroxine, fulfill a specific function in cold adaptation when the BMR and the need for carbohydrates are increased. In contrast, heat exposure leads to suppression of thermogenesis with depression of thyroid activity, BMR, and the need for carbohydrate fuels for heat production.

Studies in men, rats, sheep, and cattle imply that glucocorticoid activity is reduced during heat acclimation and with seasonal increases in environmental temperature. Stein, et al. (1949) observed that

acclimation to 41.5° C resulted in reduced 17 ketosteroid (17-KS) urinary excretion in human males. Bass (1955) reported a 15% fall in 17-KS excretion in men during artificial heating. Reduced 17-KS excretion was detected by Streeten (1960) when young men were exposed to 38° C for three weeks. Students, exposed to 40° C, showed a 15% reduction in 17-KS excretion the first day of treatment (Robinson, et al., 1955).

Heroux (1959), comparing seasonal and thermal acclimation in rats, found that adrenal weights were lower in animals reared at 30° C for 12 weeks than in controls reared at a lower room temperature. Itoh and Nishimura (1963) observed that rats, exposed to 32° C for three weeks, had slightly lower concentrations of plasma corticosterone than rats kept at 20° C.

Seasonal variation in adrenal activity was studied in Romney Marsh sheep by Robinson (1960). Urinary 17-OHS was measured in 24 hour samples, collected every two weeks for a year. Seventeen-hydroxysteroid levels were low during the summer and high in the winter. Artificial heating in a psychrometric room for seven hours per day during the winter reduced 17-OHS to the low summer values. Moreover, reversal of the seasonal lighting trend and length of day did not affect the seasonal urinary 17-OHS levels.

Two major efforts were made to determine the nature of adrenal glucocorticoid responses in cattle to prolonged heat exposure (Thompson, et al., 1963; and Bergman and Johnson, 1963). Both studies employed environmental chambers and prolonged exposure of cattle to varying degrees of moderately increased heat load.

Thompson (1963) exposed ten yearling Holstein heifers of uniform age and weight to a controlled environment. During an interval of 48

days, the temperature was maintained at 38° F for 12 hours, followed by 12 hours at 65° F. Vapor pressure was constant at 10 mm of mercury (Hg). Subsequently, the heifers were exposed to hot conditions for 72 days. Throughout this period, ambient temperature was maintained at 75° F for 12 hours, followed by 12 hours at 95° F. Vapor pressure was held constant at 20 mm Hg. Plasma 17-OHS concentration was 1.7 μg/100 ml and 4.5 μg/100 ml during the cool and hot periods, respectively. This represented a three-fold increase in glucocorticoids found in the peripheral plasma of the cattle, placed under a moderate thermal load.

Bergman (1963) evaluated the effects of prolonged, moderately elevated temperature on adrenal glucocorticoid production in six lactating Holstein cows. The cows were exposed to a control environment of 65° F, 50% RH for six weeks, a heat treatment of 84° F, 50% RH for nine weeks, and subsequently, to a second three weeks of control conditions. Blood plasma cortisol concentration was monitored and 24 hour cortisol production rate measured during each treatment period. Mean plasma cortisol concentration was 2.07 and 1.66 μg/100 ml during the control and heat treatment periods, respectively. Thus plasma cortisol decreased significantly ($P < .05$) during heat exposure. The cortisol 24 hour production rate was significantly ($P < .01$) lower during the 84° F treatment period, but the half-life of injected radioactive cortisol remained unchanged. These results indicate that plasma cortisol was lowered during prolonged heat exposure. Moreover, it appeared that this decrease was due to reduced secretory activity of the adrenal cortex.

The results of these two experiments seem to be contradictory. However, differences in methodology used make comparison of the

experiments difficult. These observations typify the ambiguities which generally pervade the literature concerning heat stress and adrenal function in hot environments.

It appears that there is some evidence for increased glucocorticoid secretion during certain types of acute heat stress. There is also evidence of a seasonal decrease in corticoid secretion from winter to summer in animals reared under natural conditions. Laboratory experiments on animals, particularly cattle continuously exposed to heat, are few in number and somewhat ambiguous; but these experiments support the hypothesis that heat stress results in lowered plasma glucocorticoid levels.

Testicular Response to Hot Environment

Control of Intratesticular Temperature

During the course of vertebrate evolution, body temperature has progressively increased (Hafez, 1968). However, concurrent tolerance for elevated temperature failed to occur in the processes engendering male gametogenesis in most mammals. This intolerance to heat by mammalian testes has been circumvented by the acquisition of an effective thermo-regulatory mechanism, whereby intratesticular temperature may be maintained at a level below that of the body core. This has been accomplished by locating the testes outside the abdominal cavity in a supportive and protective pouch, the scrotum.

Scrotal Function

The scrotum behaves much like a thermostat, holding the testes closer to the body when the air temperature is below the zone of thermal

neutrality, and allowing the testes to drop a greater distance from the body when the air temperature is above the zone of thermal neutrality. This thermo-regulatory action does not commence until an animal approaches puberty (Phillips and Andres, 1936) and is influenced by testicular androgens (Almquist and Andrews, 1944).

An important factor contributing to the cooling and warming of the arterial blood pumped into the testes is the counter-current heat exchange mechanism, achieved by the position of the spermatic artery and the surrounding venous pampiniform plexus (Harrison and Weiner, 1949). Arterial blood entering the testes is pre-cooled by heat transfer to the cooler venous blood returning through the pampiniform plexus. The result is a cooling, or warming, of the blood supply to a point midway between the abdominal-testicular temperature difference. The temperature achieved is dependent on the scrotal temperature, and thus indirectly upon environmental temperature.

Relationship Between Rectal, Scrotal, and Intratesticular Temperature

Riemerschmid, *et al*. (1941) observed that scrotal temperature was lower than intratesticular temperature in bulls. The lowest temperature was observed at the bottom of the scrotum. Quinlan (1941) observed that intratesticular temperature was approximately midway between body temperature and the temperature at the bottom of the scrotum. Low scrotal temperatures result from surface evaporation of moisture, convection, circulation of lower temperature air, and heat loss by radiation.

Although it is remarkably efficient, there are limits to the temperature control mechanisms of the scrotum; thus, the scrotal skin temperature increases as the environmental temperature goes up. In fact,

the rise in scrotal temperature is more rapid than that of rectal temperature, the difference between them becoming smaller as ambient temperature increases. Beakley and Findlay (1954) observed that rectal-scrotal temperature difference of Ayrshire bulls was 6.5° C at 15° C ambient temperature, but only 2.5° C when the ambient temperature was raised to 35° C. Therefore, rectal, scrotal, and intratesticular temperatures are highly correlated and dependent on environmental temperature.

Effects of High Temperature on Spermatogenesis

When the environmental temperature becomes elevated so that the abdominal-testicular temperature gradient required for normal spermatogenesis cannot be maintained, degeneration of the germinal epithelium results. Crew (1922) first suggested the necessity of a testes temperature lower than abdominal temperature for active spermatogenesis. These observations were confirmed by Moore (1923, 1924), and the subject was reviewed by Moore (1951) and Nelson (1951).

Cells were lost from the germinal epithelium, and spermatozoa disappeared from the ejaculate altogether when severe heat was applied to laboratory animals, such as the guinea pig (Young, 1927), rat (Cunningham and Osborn, 1929), and the dog (Guieysse-Pellessier, 1937). Germinal degeneration following exposure to high ambient temperature, is recognized as a major source of infertility in sheep (Moule and Waites, 1963), cattle (Skinner and Louw, 1966), and swine (Thibault, *et al.*, 1966).

The general conclusion from examination of heat-damaged testes is that spermatogonia survive high temperatures longer than any other germ

cell (Clegg, 1963; Young, 1927; Ortavant, 1958; and Waites and Ortavant, 1967). There is also general agreement that stages of the long meiotic prophase of primary spermatocytes are sensitive to high temperature (Young, 1927; Clermont and Leblond, 1955; Waites and Ortavant, 1967). Early stages of spermiogenesis are likewise adversely affected by temperature elevation (Young, 1927; Chowdhury and Steinberger, 1963; Waites and Ortavant, 1967; Skinner and Louw, 1966).

Semen Production in Bulls Exposed to High Environmental Temperature

Erb, *et al*. (1942) noted that semen volume, initial motility, sperm concentration, and survival time were lowest during July, August, and September in Jersey, Holstein, Guernsey, and Ayrshire bulls in Indiana. Weeth, *et al*. (1949) found that summer temperatures produced deleterious changes in spermatozoa motility and morphology in Missouri. Johnson, *et al*. (1953) concluded that sperm concentration and motility from dairy bulls began to drop in July, reaching a low in September in Louisiana. Kelly and Hurst (1963) concluded that spermatogenesis in dairy bulls was impaired when ambient temperatures exceeded 30° C in North Carolina.

Casady, *et al*. (1953) reported that following 17 days exposure to 100° F in a climate control chamber, one Guernsey bull was sterile for 100 days, and another had reduced sperm concentration and motility for a similar period. Continuous exposure to a mean temperature of 86° F also produced lowered sperm concentration and motility in two bulls.

Johnston, *et al*. (1963) exposed three year old bulls to controlled cyclic climatic conditions for seven days. Maximum room temperature was 104° F at 54% RH. This was maintained for eight hours and then

lowered to 82° F, 74% RH for the balance of the 24 hours. They found that scrotal temperature reached a maximum of 100° F, while rectal temperature rose to an average of 103.9^{o} F during the hot period. Semen concentration and motility began to decline after four days of heat exposure and remained depressed for a month after cessation of the heat treatment.

Skinner and Louw (1966) determined the duration of exposure to 40° C, 35% RH, required to reduce spermatogenesis in Friesland and Afrikaner bulls. They concluded that as little as 12 hours of exposure to 40° C, 35% RH was sufficient to cause damage to the seminiferous tubules. Spermatids seemed to be the most severely affected cell types, but there was also significant damage to spermatozoa and spermatocytes.

Austin, et al. (1961) increased intratesticular temperature of 12 bulls by insulating the scrotum to prevent heat loss. The animals were maintained at an ambient temperature within the thermal neutral zone and showed no rise in rectal temperature. The insulation period lasted from 12 to 72 hours. Four days later, semen concentration, motility, and percent normal spermatozoa rapidly declined from preinsulation levels. These characteristics remained depressed for eight to twelve weeks but eventually returned to normal.

Okomoto, et al. (1962) exposed dairy bulls to 32° C, 55% RH for five weeks. These investigators found that continuous cooling of the scrotum with water, so that scrotal skin temperature did not rise above values found at ambient temperatures of 26° C, did not completely prevent depression of spermatozoa concentration and total spermatozoa per ejaculate, due to the temperature treatment. Sperm motility and percent abnormal sperm did not change as a result of the treatment. They

concluded that not all of the deleterious effects of high thermal environment on semen were due to local hyperthermia. The possibility was suggested that some centrally mediated neural endocrine mechanism might also be involved.

These experiments proved that local hyperthermia of the testes causes deleterious changes in spermatogenesis. Furthermore, they indicate that hyperthermia, resulting from exposure of the animal to high ambient temperature causes damage to specific cells in the seminiferous tubules. This damage is reflected by degenerative changes observed in the physical characteristics of seminal spermatozoa.

Testicular Androgen Production

Researchers have been unable to determine whether androgen production by mammalian testes is affected by heat stress and acclimation to hot environments. Historically, observations and conclusions have been based on obtuse evidence gathered from indirect indices of hormone activity.

Techniques for Evaluating Testicular Endocrine Function.

Changes in testicular androgen production were first investigated by quantitating changes in weight or histology of accessory sex glands (e.g., seminal vesicles, prostate, coagulating glands) or the testis itself (Jeffries, 1931; Nelson, 1937; Moore, 1944).

Biochemical methods, including estimation of urinary 17-ketosteroids (Kimeldorf, 1948) and analysis of certain components of semen and accessory gland secretions (Antliff and Young, 1957), were used later.

Specific and sensitive methods have been developed for measuring

testosterone and other androgens in biological samples in recent years. These techniques include: 1) gas-liquid chromatography with electron capture detection (Brownie, *et al.*, 1964); 2) double isotope derivative methods (Rivarola, 1966); 3) radioisotope competitive protein-binding using testosterone-binding globulin (Nugent and Mayes, 1970); and 4) radioimmunoassay (Furuyama, 1970).

The biological half-life of testosterone is determined by injecting known amounts of radioactive testosterone into an animal and monitoring its disappearance from the peripheral circulation. Testosterone 24 hour production rates are calculated, utilizing plasma testosterone concentration and biological half-life (Southren, *et al.*, 1965).

It should be possible to elucidate the interrelationships between heat stress, acclimation to heat, and testicular endocrine function, utilizing these quantitative techniques and psychrometric chambers.

Experiments in Cryptorchid Animals. Cryptorchid animals make excellent subjects in which to study the direct effects of heat on testis function. Cryptorchidism can be created experimentally in most laboratory and domestic animals. The advantage of studying the effect of experimental cryptorchidism on testis function is that it allows one to maintain animals at normal room temperature while one or both of the testes is being subjected to abdominal heat. Many researchers have used cryptorchid testes to study the effects of heat on testicular androgen production.

Lluarado and Dominguez (1963) demonstrated that an abdominal testis is less able to convert progesterone to testosterone than its scrotal control; its enzyme system for this conversion is less responsive to gonadotropin. Kormano (1964) showed that Δ^5-3β-hydroxysteroid

dehydrogenase activity was not histochemically detectable in the abdominal testis of experimental unilateral cryptorchid rats. Inano and Tamaoki (1968) also examined enzymes of the cryptorchid testis and concluded that *in vivo* androgen production was suppressed. Morehead (1967) found that the incorporation of tritiated thymidine into the epithelium of accessory sex glands was much slower in cryptorchid rats than in scrotal controls. In contrast to the above experiments, Antliff and Young (1957) and Eik-Nes (1966) published data, supporting the contention that cryptorchid testes are capable of secreting normal, or nearly normal, levels of androgens in species that are not cryptorchid during a non-breeding season (e.g., dog, bull, stallion).

Albert (1961) reviewed the results obtained from research involved with experimental and spontaneous cryptorchidism. He concluded, "The cryptorchid testis is capable of producing androgens, but depending on species, probably in amounts less than normal." Since that time, most research results have supported Albert's conclusions. However, additional research is needed to elucidate the effects of heating on testicular androgen production.

Androgen Production in Hot Environments. Experiments with cryptorchid animals cannot reveal the effect of hot environments on testicular endocrine function. This is because heat stress and acclimation to hot environments may affect testicular endocrine function via two possible routes. One route, exemplified by cryptorchidism or scrotal insulation, is through direct local heating of the scrotum or testes. The other, exemplified by a sojourn in a hot room, includes elements of the first, but superimposes upon it other physiological changes, such as effects mediated through neuroendocrine secretions of the hypothalamo-

hypophyseal-gonadal axis.

The former situation has been investigated more thoroughly than the latter. This reviewer has been unable to find a single published experiment which evaluates androgen secretion by testes during acclimation to heat in mammals. However, data from various heat stress experiments offer some oblique insight into the situation.

Urinary 17-KS excretion rates reflect the metabolism and production of several steroids, including androgens. Stein, et al. (1949), Bass (1955), Robinson, et al. (1955), and Robinson and MacFarlane (1958) found that 17-KS excretion declined 15 to 50% within a few days after men were exposed to hot environments. Robinson (1960) observed that 17-KS excretion was depressed in rams during the hot summer months in Australia. The percentage depression of 17-KS that may be attributed to decline in androgen production is speculation.

Pituitary gonadotropins, especially luteinizing hormone, have important functions in the regulation of testicular androgen production. Experiments by MacFarlane (1957), suggest that acclimation to heat may be associated with reduced gonadotropin secretions by the pituitary in female rats.

MacFarlane bred and reared several generations of female rats at 35° C or 22° C. When cycling female rats were first exposed to 35° C, the number of ovarian corpora lutea were significantly reduced. The number of corpora lutea gradually increased as the rats acclimated to the 35° C temperature, but still remained below the 22° C controls. MacFarlane stated, "This suggests that gonadotropins may be produced in smaller quantities during acclimation to heat." He further indicated that the second and third generations of animals exhibited signs of

general pituitary depression.

It is not unlikely that the pituitary of both males and females might be affected similarly by exposure to heat. It follows that suppression of androgen secretion might result if pituitary depression takes place in males exposed to high environmental temperatures.

Summary and Conclusions

The pattern of endocrine involvement in the physiological adjustments of homeotherms exposed to high temperature conditions has been examined. Particular attention has been directed toward adrenal cortical and testicular endocrine function, especially in the male bovine.

Some direct and much indirect evidence suggests that hormonal output from certain endocrine glands is reduced by ambient temperatures above 27° C. This may include the main functions of the anterior pituitary, thyroid, fascicular and reticular layers of the adrenal cortex, and the testes. However, the evidence is far from conclusive in most instances.

Experimentation is required to clarify changes which occur in endocrine gland function during heat stress and acclimation to heat. New techniques for direct quantitation of hormone levels in blood and tissues, along with half-life and secretion rate studies, promise to facilitate answering the following questions: 1) What changes take place in the plasma concentration of specific glucocorticoids during heat stress and acclimation? 2) Are the adrenal cortical hormones necessary for acclimation to heat? 3) What effect does heat have on peripheral concentration of androgens? 4) Does heat exposure alter androgen secretion rate? 5) If so, does it alter peripheral utilization or hepatic

removal, and do changes in testicular androgen production result in the depression of spermatogenesis by heat?

CHAPTER III

MATERIALS AND METHODS

Materials

The sensitivity of the assays employed in this study necessitated the use of only the purest reagents and other materials. In some cases, even the highest quality commercial products available contained excessive contamination, requiring further purification before use in the laboratory.

Reagents

Most of the solvents were "nanograde" in quality (Mallinckrodt Chemical Works) and included: dichloromethane, ether, benzene, hexane, toluene, and methanol. Iso-octane and chloroform were "spectrograde" in quality (Mallinckrodt Chemical Works). Ether and dichloromethane (nanograde) were further purified by distillation over glass. Water used in the procedures was distilled two times over glass, ether-washed, and finally redistilled. Ethyl alcohol was 100% analytical grade (U. S. Industrial Chemical Co.). Concentrated hydrochloric and sulfuric acids were also analytical grade (Baker Chemical Co.). Scintillation fluid contained 3.79 l toluene, 15.14 g of 2,5-diphenyloxazole (PPO), and 0.1514 g of 1,4-his-1-(5 phenoxazolyl)-benzene (POPOP). The mixture was allowed to equilibrate 24 hours before use.

Silica Gel

Silica gel (Silicar TLC-7GF, Mallinckrodt) was washed three times with dilute acetic acid over a scintered glass filter, followed by three rinses in boiling triple distilled water, and finally washed two times with boiling methanol. It was dried 24 hours at 110° C. Thin layer chromatography (TLC) plates were poured (0.25 mm thickness) with slurry, containing 33 g silica gel to 78 ml water.

Steroids

Cortisol (11β,17α,21-trihydroxypregn-4-ene-3,20-dione), corticosterone (11β,21-dihydroxypregn-4-ene-3,20-dione), testosterone (17β-hydroxyandrost-4-ene-3-one), and 20β-OH progesterone (20β-hydroxypregn-4-ene-3,20-dione), obtained from Steraloids, were recrystallized to their constant melting points and dissolved in ethanol at a concentration of 1 mg steroid/ml solvent.

Cortisol-1,2-^{3}H, corticosterone-1,2-^{3}H, and testosterone-1,2-^{3}H were obtained from New England Nuclear Corporation, Boston, Massachusetts, and were purified via thin layer chromatography. Purity was checked by a radiochromatogram scanner (Packard, Model 7201) and identity by the use of authentic standards in adjacent lanes on the thin layer plate. They were stored in ethanol at 5° C and their purity checked at two to four month intervals.

Glassware

Glassware was rinsed immediately after use, soaked in detergent, brushed and placed in chromic acid overnight. Following seven to eight tap water rinses, the glassware was soaked in detergent, rinsed again

with tap water, and placed in dilute hydrochloric acid. After being rinsed ten times with tap water, ten times with distilled water, and two times with nanograde methanol, the glassware was air dried. The microcuvettes, used for the fluorimetric analyses of glucocorticoids, were soaked in concentrated nitric acid, instead of chromic acid, rinsed with tap and distilled water, and air dried, following an ethanolic wash.

Methods

General Procedures

The eight Hereford bulls used in this study were selected at random from bulls included in the Oklahoma State University Department of Animal Science herd, located at Fort Reno Research Station near El Reno, Oklahoma. They were raised under range conditions, and varied in age from 20 to 26 months at the time they were utilized for experimentation. Since the environmental chamber in which the bulls were to be housed during the experiment would hold only four animals at a time, they were divided into two groups. Each group of animals was treated in similar fashion.

On the morning of the initial day of the experiment, a group of four bulls was driven from their pasture to the central pens of the research station. That afternoon the bulls were weighed individually, injected with tritiated testosterone, and blood was collected for testosterone 24 hour production rate studies. The next day the bulls were transported via truck from Fort Reno to Oklahoma State University. They were housed indoors where light and temperature where controlled. The bulls were exposed to 12 hours of light and 12 hours of darkness at a

constant temperature of 70° F, 50% RH. These conditions, representing an optimum environment, were maintained for a period of seven weeks. Following this control period, the bulls were placed in an adjacent Space Corporation Environmental Chamber, where they were exposed to a 96° F, 50% RH environment for seven weeks of heat treatment. Temperature and humidity were constantly monitored by dry bulb and dew point recording thermometers.

During the 14 week experimental interval, the bulls were allowed to run loose in an area which measured 20 feet wide, 30 feet long, and nine feet high. They were supplied water *ad libitum* and fed twice daily. A complete built-in roughage ration was fed at the rate of two pounds of feed/100 pounds of body weight/day. Table I (Appendix A) shows the detailed composition of the ration.

Measuring Rectal Temperature and Respiration Rate. The bulls were fastened in their individual stanchions each afternoon at 5:00 P.M. to be fed. This was a convenient time to record their rectal temperatures and respiration rates. In order to simplify tabulation, these daily observations were averaged for each seven day interval and recorded as "mean weekly temperature" and "mean weekly respiration rate".

Collection of Blood Samples for Endocrine Assay

Blood samples were collected in the afternoon when the bulls were fastened in their individual stanchions to be fed. The head of each bull was pulled to one side and immobilized, while a 100 ml sample of jugular vein blood was drawn into a large glass syringe through a 15 gauge needle. The blood was transferred from the syringe to 80 ml centrifuge tubes, kept in an ice bath. The blood was then centrifuged so

that the cellular elements separated from the plasma within minutes. The plasma was drawn off with a syringe and needle, placed in plastic vials, frozen, and stored at -20° C until such time as it was convenient to assay the hormone content.

No blood samples were collected during the first 20 days of the 70° F control period because psychological stress of adjustment to confinement in strange surroundings might have resulted in abnormal peripheral levels of glucocorticoid hormones. However, by the end of week three, all bulls were quite gentle to handle and were not disturbed by the bleeding procedure. On the last day of weeks three through seven of the 70° F control period, a single 100 ml blood sample was collected from each bull. The same size blood sample was also collected from each bull at the end of each of the seven weeks of the 96° F heat treatment period. In addition similar blood samples were collected each day from day one through day seven of 96° F heat treatment. On day 49 of both the control and heat treatment periods, testosterone 24 hour production rate studies were conducted on each bull.

Semen Collection and Evaluation

Individual semen samples were collected from the bulls during week seven of the control period and one the day following the termination of the heat treatment period. The bulls were ejaculated by rectal massage, using the technique described by Goodwin (1970).

The semen was collected in graduated centrifuge tubes which were kept in a 90° F water bath while being transported to the laboratory for study. The criteria used for evaluating the physical characteristics of the semen were those recommended by the American Veterinary

Society for the Study of Breeding Soundness (Hill, 1956). This system assigns weighted scores to various subjective components of semen quality, according to the following standards: spermatozoa motility, 40 points possible; spermatozoa morphology, 30 points possible; spermatozoa concentration, 20 points possible; percent spermatozoa alive, 10 points possible; total, 100 points possible. Thus, if a semen sample were perfect in every respect, it would receive a score of 100. This scoring system made it possible to evaluate numerically the effects of heat treatment on spermatogenesis.

Collection and Preparation of Tissues for Histological Examination

The 96° F heat stress period of this study was terminated by removing the bulls from the psychrometric chamber and transporting them to a nearby abatoire for slaughter. At the time of slaughter, representative portions of the testis, epididymis, prostate, seminal vesicle, and adrenal were removed and placed directly into Bouin's solution. The tissues were allowed to "fix" in Bouin's solution for 18 hours, after which they were removed, washed under running tap water for 24 hours, and stored in several volumes of 70% ethanol. A technician in the Oklahoma State University Department of Veterinary Pathology Tissue Laboratory subsequently embedded, sectioned, and stained the tissues with hematoxylin and eosin.

Detailed Fluorimetric Method Used to Measure Cortisol and Corticosterone

The fluorimetric method used in this study to measure corticosteroids was based on the method described by Ganjam, *et al.* (1970) for use with small volumes of rabbit plasma. A number of modifications

were necessary in order to adapt the assay for use with relatively large volumes of bovine plasma.

Fifty µl of each of two tritium labeled glucocorticoid standards (1,500 dpm of both cortisol-1,2-^{3}H and corticosterone-1,2-^{3}H) were pipetted into 80 ml extraction tubes and evaporated to dryness under a stream of nitrogen. Similar aliquots (in duplicate) of the same radioactive standards were placed in scintillation vials to be used to monitor losses through the method and permit calculations of recovery.

Preparation of Plasma for Solvent Extraction. Plasma samples were thawed and 15 ml of plasma was added to extraction tubes containing the labeled standards. The tubes were gently vortexed and the contents allowed to equilibrate for 30 minutes at 5° C. Subsequently, each sample was washed with 30 ml of iso-octane (2,2,4-trimethyl pentane). The iso-octane layer was aspirated with a Pasteur pipette, using mild vacuum suction, and then discarded. Prewashing the plasma with iso-octane removed lipids less polar than C-21 glucocorticoids.

Extraction and Alkali Wash. Prewashed plasma was extracted three times with 45 ml of ice-cold dichloromethane and discarded. The organic extract was then evaporated under a nitrogen stream, and the residue dissolved in 30 ml ethyl ether.

Alkali washing of the ether extract by the addition of 4 ml of 0.1 N sodium hydroxide solution followed. The alkali was allowed to contact the organic extract for not over 30 to 40 seconds, after which it was aspirated and discarded. Immediately after the alkali wash, the ether extract was washed three times with ten ml of H_2O and evaporated to dryness under nitrogen.

Chromatographic Isolation of Glucocorticoids. Thin layer chromatography

(TLC) plates were divided into nine lanes, each two cm wide. Six lanes were used for unknown samples, one for a blank, and the two end lanes for standards. Samples were spotted on the TLC plates with capillary tubes, utilizing dichloromethane methanol (9:1, V/V) as solvent. The TLC plate was developed in chloroform:methanol:water (90:10:1, V/V). After development, the TLC plate was briefly exposed to ultraviolet light so that areas corresponding chromatographically to standard cortisol and corticosterone could be identified. Cortisol had an approximate R_f of 0.4 and corticosterone 0.65 in this solvent system.

Elution of Cortisol and Corticosterone From Thin Layer Chromatography Plates. Cortisol and corticosterone were eluted separately, using a scintered disc eluter (Dependable Scientific Co.), with ten ml of dichloromethane:methanol (9:1, V/V). The eluate was then evaporated to dryness under nitrogen.

Fluorimetric Measures. One ml of methanol was added to the eluate residue and 0.2 ml was removed for scintillation counting. The remaining 0.8 ml was evaporated to dryness, and one ml of H_2SO_4:C_2H_5OH (65:35, V/V) was added to the residue. Standard tubes were prepared simultaneously which contained 0.04, 0.08, and 0.12 μg of cortisol. Additional tubes contained 0.01, 0.02, and 0.04 μg of corticosterone. Background fluorescence was determined with blank tubes containing only fluorescing solution. Fluorescence was measured with an Aminco Bowman Spectrophotofluorometer. The following instrument settings were used: 1) activating wave length 472 mμ through a three mm slit; 2) emission wave length 526 mμ through a three mm slit; 3) turret setting two; 4) meter multiplier 0.003; and 5) sensitivity 25-35.

Monitoring for Losses Through the Method. Corticosteroid loss prior to

fluorimetry was determined by measuring the loss of tritiated standards added to the plasma sample before extraction. All sample 0.2 ml aliquots were evaporated to dryness in the counting vials and 15 ml of scintillation fluid were added. Radioactivity was determined as described in the section on single label scintillation counting.

The following formula was used to calculate the percent recovery for the corticosteroids through the method.

$$\% \text{ recovery} = \frac{\text{net sample CPM}}{\text{net standard CPM}} \times \frac{100}{0.2}$$

where:

0.2 = sample aliquot taken for scintillation counting.

Calculation of Corticosteroid Concentration.

$$\text{Steroid (nanogram/ml plasma)} = \frac{C}{St} \times \frac{S}{A} \times \frac{1}{V} \times \frac{100}{R}$$

where:

C = concentration of standard (nanograms/ml);

St = reading of standard (fluorescence units);

S = reading of sample (fluorescence units);

A = sample aliquot (ml);

V = volume of plasma sample extracted (ml);

R = percent recovery (%).

Detailed Method Used to Measure Testosterone

The method used to quantitate testosterone in this study was adapted from methods described by Brownie, *et al.* (1964) and Kirschner, *et al.* (1968) for use with human plasma. Several modifications were necessary in order to adapt the assay for use with large volumes of

bovine plasma

Preparation of Plasma for Solvent Extraction. Fifty µl tritium labeled testosterone standard (1,500 dpm of testosterone-1,2-^{3}H) was pipetted into 80 ml extraction tubes and evaporated to dryness under a stream of nitrogen. Plasma samples were thawed and 15 ml added to the extraction tube containing labeled standard. The tube was then gently vortexed and allowed to equilibrate for 30 minutes at 5° C.

Extraction and Alkali Washing. The plasma was extracted three times with 45 ml of ice-cold dichloromethane. The extract was evaporated under nitrogen and the residue was taken up in 30 ml ethyl ether.

Alkali wash was accomplished by adding 20 ml of 1.0 N sodium hydroxide solution to the ether extract and gently inverting the tube several times. After removing the alkali phase, the organic phase was washed twice with 20 ml of water and evaporated to dryness under nitrogen.

Chromatographic Isolation of Testosterone. TLC plates were marked into nine lanes, eight for samples, and one for standard testosterone. The samples were spotted in hexane and developed in benzene:ethyl acetate (55:50, V/V). Since samples did not contain sufficient testosterone to visibly absorb ultra-violet light, areas corresponding to the standard testosterone were eluted. In this solvent system testosterone had an R_f of 0.3.

Appropriate areas of silica gel containing testosterone were scraped from the TLC plate and placed in 35 ml conical centrifuge tubes. This silica gel was extracted three times with a benzene:water partition system, using one ml of benzene with five drops of water. The benzene fraction was pooled in 12 ml conical centrifuge tubes and evaporated to

dryness under nitrogen.

Formation of the Heptafluorobutyrate Derivative. One-tenth ml of a 2% solution of heptafluorobutyric anhydride in hexane and tetrahydrofuran was added to the sample tube. The tube was sealed and incubated in a 60° C water bath for 30 minutes. After incubation, the sample was evaporated to dryness.

Chromatographic Isolation of Testosterone Heptafluorobutyrate (THFB). Following concentration by hexane, the residue was spotted on a TLC plate, using hexane, then developed in benzene:ethyl acetate (87.5:12.5, V/V). In this solvent system THFB had an approximate R_f of 0.5. The silica gel in the area corresponding chromatographically to the THFB standard was placed in a 35 ml tube and extracted three times with a benzene:water partition system. These extractions were carried out, using one ml benzene with five drops water. The benzene was pooled and dried under nitrogen. Following the addition of one ml benzene, a 0.2 ml aliquot was taken for scintillation counting, a 0.5 ml aliquot for gas liquid chromatography, and the balance of the sample volume was pooled for structural confirmation studies.

Gas Liquid Chromatography. A Selecta Series 5000 Barber-Coleman gas chromatograph equipped with a Model 5120 electron capture detector was used to measure the heptafluorobutyrate derivative of testosterone. Samples were applied in five to ten µl of benzene. A U-shaped glass column (three feet long, four mm internal diameter), silanized with a 5% solution of dimethyldichlorosilane in toluene and packed with Gas-Chrom Q (80 to 100 mesh) coated with 3% OV-225 (Applied Science Laboratories Inc.), was used to effect separation of steroids. Column bath temperature was 200° C. Pure, extra dry nitrogen was used as a carrier

gas with an outlet velocity of approximately 100 ml/minute. The detector bath temperature was 240° C.

The electron capture detector was operated in the DC mode and voltage was adjusted to give a standing current 60% of that obtained at the plateau of the voltage input curve. The detector bath temperature was 240° C. Sensitivity and linearity of detector response to standard HFB derivatives of testosterone and 20β-OH progesterone were ascertained each day of data collection on GLC. Samples were not run unless the response was linear over the range of expected THFB concentration. The size of peaks was calculated by multiplying one-half the height (measured by a perpendicular line from the vertex to the base) by the base.

Monitoring Testosterone Loss Through the Method. Testosterone loss prior to GLC was determined by measuring the loss of testosterone-1,2-^{3}H added to the plasma sample before extraction. Adjustments for losses incurred during quantitation by GLC were made by the addition of an internal standard (25 nanograms 20β-OH progesterone HFB per sample) as outlined by Horning, *et al.* (1963).

Calculation of Testosterone Concentration. The amount of testosterone in one ml of plasma was calculated according to a formula used by Stabenfelt (1968). The formula used is as follows:

$$\text{Testosterone (ng/ml)} = R \times C \times U \times A \times 0.596 \times Df \times X \times P$$

where:

$$R = \frac{\text{cpm testosterone-1,2-}^3\text{H added to plasma}}{5 \times \text{cpm in 20\% aliquot obtained prior to GLC}};$$

$$C = \frac{\text{peak area (Cm}^2\text{) 10 ng 20}\beta\text{-OH progesterone HFB standard}}{\text{peak area (Cm}^2\text{) 10 mg THFB standard}};$$

$$U = \frac{\text{peak area (Cm}^2\text{) THFB in sample}}{\text{peak area (Cm}^2\text{) 20}\beta\text{-OH progesterone HFB in sample}};$$

A = ng 20β-OH progesterone HFB added as internal standard;

$$0.596 = \frac{\text{molecular weight testosterone}}{\text{molecular weight THFB}};$$

$$Df = \frac{\text{(100-hematocrit)(blood volume)}}{\text{[(100-hematocrit)(blood volume)] - (milliliters anticoagulant)}}$$

$$X = \frac{1}{\text{GLC aliquot}};$$

$$P = \frac{1}{\text{milliliters plasma extracted}}.$$

Scintillation Counting

After radioactive samples were placed in scintillation vials, they were evaporated to dryness under nitrogen, and 15 ml of scintillation fluid was added to each vial. The scintillation mixture was 3.77 l of toluene, containing 15.14 g of 2,5-diphenyloxalate (PPO) and 0.1514 g of 1,4-bis-2-(5 phenyloxasolyl)-benzene (POPOP). All samples were counted in a three channel Packard Tri-Carb Liquid Scintillation Spectrometer, Model 3003, equipped with a ^{137}Cs Automatic External Standard device.

Single Label Counting. In this study all single labeled samples contained one of the following tritium labeled compounds: testosterone-1,2-^{3}H, cortisol-1,2-^{3}H or corticosterone-1,2-^{3}H. The liquid scintillation spectrometer operated at an efficiency of 41% for tritium with a background of 17 cpm. Ratio of background cpm to sample cpm varied with the activity of the samples, but usually fell within the range of 0.005 to 0.02. Quenching was minimal and uniform between samples; therefore, it was disregarded as being a significant factor in calculations. Each sample was counted for three periods of ten minutes each. Appropriate background and standard vials were included each time

unknown samples were counted.

Double Label Counting. Double labeled samples in this study contained both testosterone-1,2-^{3}H and testosterone-4-^{14}C dissolved in 15 ml of scintillation fluid. The Packard Tri-Carb Spectrometer operated at approximately 26% efficiency for ^{3}H and 60% for ^{14}C with double labeled setting. Background in both channels was similar (10 to 15 cpm). The ratio of background to sample cpm was never greater than 0.01. Each sample was counted for three periods of 20 minutes each. Appropriate background and standard vials were included each time samples were run. The level of quenching was low and varied from sample to sample. Therefore, quench correction was made by the automatic external standardization method utilizing ^{137}Cs as a radiation source.

Measurement of Testosterone Metabolic Clearance Rate (MCR) and 24 Hour Production Rate (PR)

Preparation of Tritiated Testosterone for Injection. Testosterone-1,2-^{3}H (50 Ci/mM) and testosterone-4-^{14}C (58.8 mCi/mM) were purchased from New England Nuclear Corporation, Boston, Massachusetts, and purified by TLC in benzene:ethyl acetate (55:50, V/V). Purity was checked by a radiochromatographic scanner; identity by the use of standard preparations in adjacent lanes on the TLC plate. Tritiated testosterone was diluted with 10% ethanol in normal saline, prior to use, so that each individual 20 ml dose contained approximately 60 μCi or 300 ng. Duplicate 0.2 ml aliquots of the diluted testosterone-1,2-^{3}H were placed in scintillation vials and counted in order to be able to determine the exact amount of radioactivity (dpm/ml) in the solution.

Installation of the Jugular Catheter. Injection of the tritiated testosterone and subsequent withdrawal of blood samples was made through a

polyethylene cathetrer which was inserted into the jugular vein. Clay Adams PE 205 Intramedic Polyethylene tubing (I.D. 0.062", O.D. 0.082") was used.

The bull was fastened in a stanchion and his head pulled to one side and tied securely. Hair over the jugular vein area was removed with a No. 40 Oster Animal Clipper; the skin was disinfected with 70% alcohol. A two inch 12 gauge hypodermic needle was then inserted into the vein and directed toward the heart. Approximately 12 inches of a 42 inch long polyethylene catheter was passed through the needle into the vein. The needle was withdrawn, leaving about 30 inches of tubing protruding from the skin.

The catheter was then passed up the outside of the animal's neck and taped to his horn so that 12 to 18 inches extended beyond the animal's head. A blunt, thin-walled 15 gauge needle was inserted in the end of the catheter to act as a syringe adapter. Once the catheter was fixed in place, the head tie of the bull was loosened so that the animal had free movement in the stanchion.

Injection of Tritiated Testosterone and Withdrawal of Blood Samples. An initial 100 ml blood sample was withdrawn through the catheter and placed in oxylated 80 ml extraction tubes. This sample was used to determine the plasma concentration of endogenous testosterone prior to the injection of radioactive material.

At zero time a 60 μCi (300 ng) dose of testosterone-1,2-^{3}H was rapidly injected, and the catheter was flushed with 20 ml of 10% sodium citrate solution to remove any trace of radioactivity clinging to the inside wall.

Seventy ml blood samples were drawn at 5, 10, 15, 30, 40, and 50

minutes after the radioactive testosterone injection. The jugular catheter was flushed with 5 ml of 10% sodium citrate solution, following the withdrawal of each blood sample, and plugged to prevent coagulation of blood in the tubing. The individual blood samples were placed in oxylated 80 ml extraction tubes, cooled in an ice-bath, and centrifuged. The plasma was removed with a syringe and needle, placed in storage vials, and frozen at -20° C until extraction. Zero-time plasma samples were assayed for testosterone concentration, using the assay method which has been previously described.

Extraction of Testosterone-1,2-^{3}H From the Plasma Samples. Fifty µl of testosterone-4-^{14}C (1,500 dpm) and 50 µl of standard testosterone-^{12}C solution (1 µg/µl) were pipetted into 80 ml extraction tubes and dried under nitrogen. Similar duplicate aliquots of testosterone-4-^{14}C and testosterone-^{12}C were placed in scintillation vials. These vials were used to monitor losses through the method and permit calculation of recovery rate. Plasma samples were thawed, and 20 ml aliquots were measured into each extraction tube. The tube was vortexed, and the contents were allowed to equilibrate for 30 minutes at 5° C.

Each sample was extracted twice with 50 ml of ice-cold dichloromethane. The extract was dried under nitrogen, residue dissolved in 30 ml ethyl ether, and washed with 20 ml of 1.0 N sodium hydroxide. The alkali phase was discarded; the organic phase was washed twice with 20 ml water, and dried under nitrogen.

The residue was spotted on TLC plates which were developed in benzene:ethyl acetate (55:50, V/V). Areas of silica gel containing testosterone were visualized under ultra-violet light and eluted with benzene:H_2O partition. The eluate was placed in scintillation vials,

evaporated to dryness under nitrogen, and 15 ml of scintillation fluid added to each vial. The vials were then counted, using the method previously described in the section entitled "Double Label Counting".

Correcting for Testosterone-1,2-^{3}H Losses Through the Method. Tritiated testosterone loss through the method was assumed to be identical to the loss of testosterone-4-^{14}C from the internal standard which was added to each sample, prior to purification. Recovery of ^{14}C internal standard radioactivity varied between 70 and 85%. The following formula was used to calculate the recovery rate of ^{14}C-testosterone:

$$\text{\% testosterone-}^{14}\text{C recovered} = \frac{\text{Net sample dpm }^{14}\text{C}}{\text{Net dpm }^{14}\text{C standard}} \times 100 .$$

Assume:

$$\text{\% testosterone-}^{14}\text{C recovered} = \text{\% testosterone-}^{3}\text{H recovered.}$$

After the serial blood samples from each bull containing testosterone-^{3}H were counted, and the percent recovery rate for testosterone -^{3}H calculated, it was possible to calculate how many dpm of testosterone-^{3}H were present in each sample prior to purification. The following formulas were used:

$$\begin{array}{l}\text{dpm as testosterone-}^{3}\text{H in 20 ml} \\ \text{plasma samples after purification}\end{array} = \frac{Rt - \left[\frac{Gt \times Cr}{Cg}\right]}{Hr}$$

where:

Rt = total corrected cpm recorded in red channel of scintillation spectrometer;

Gt = total corrected cpm recorded in green channel of scintillation

spectrometer;

Cg = counting efficiency of ^{14}C in green channel (from quench curve);

Cr = counting efficiency of ^{14}C in red channel (from quench curve);

Hr = counting efficiency of ^{3}H in red channel (from quench curve).

$$\text{dpm as testosterone-}^{3}\text{C in 20 ml plasma (corrected for loss)} = \frac{\text{dpm } ^{3}\text{H in 20 ml after purification}}{\%\text{ recovery } ^{3}\text{H}} \times 100$$

In order to arrange these data into the standard form used to study steroid dynamics, it was necessary to express the total corrected ^{3}H radioactivity of each sample as a percent of the original dose of testosterone-1,2-^{3}H, recovered per liter of plasma. This was calculated in the following manner:

$$\text{dpm testosterone-}^{3}\text{C per liter plasma} = \text{dpm as testosterone-}^{3}\text{C in 20 ml plasma corrected for loss through method} \times \frac{1000}{20}$$

$$\%\text{ testosterone-}^{3}\text{C dose recovered per liter plasma} = \frac{\text{dpm testosterone-}^{3}\text{H/l plasma}}{\text{dpm testosterone-}^{3}\text{H in administered dose}} \times 100$$

Plotting the Disappearance Curves of Testosterone-1,2-^{3}H From Bull Plasma and Calculating Metabolic Clearance Rate, Volumes of Distribution, and Rate Constants. The mathematical treatment and discussion of steroid dynamics that is relative to this investigation has been fully reviewed by Tait (1963), Tait and Burstein (1964), and Baird (1969). The presentation of data in this study was modeled after the reports by Horton, *et al.* (1965) and Southren, *et al.* (1965).

The values for the radioactivity, specifically as testosterone,

and fully corrected for recovery in plasma taken at 5, 10, 15, 30, 40, and 50 minutes after injection of 60 μCi (300 ng) of testosterone-1,2-^{3}H, were plotted as the logarithm of percent dose injected per liter of plasma, against time after injection. The disappearance curve (Figure 1) consisted of two distinct slopes, requiring analysis in terms of a two compartment model as described by Tait, et al. (1961). Figure 1 shows the composite curve for the disappearance of testosterone-1,2-^{3}H in the plasma of eight Hereford bulls. A + B is the intercept of the initial curve, plotted from the 5, 10, and 15 minute plasma concentrations. B is the intercept of the final curve, calculated from the 30, 40, and 50 minute concentrations. The slope of this line is β. Since the initial curve (intercept A + B) is influenced by the final curve (intercept B), a third curve (intercept A, slope α) is constructed by subtracting the calculated radioactivity, contributed by the final curve, from that which was actually observed.

A reasonable model for the transport and metabolism of testosterone consists of an inner pool, which probably includes the plasma, extracellular fluid, and liver volume; and an outer (tissue) pool. Transport between the pools can be described by the rate constant, K_1. The total volume of the outer pool is V_2. Metabolism in the outer pool is assumed to be negligible because analyses in the outer pool are, at present, impossible. If all the measured radioactivity is due to testosterone, then K_1 equals the fraction of injected dose per ml of plasma transported to the outer pool in unit time; K_2 equals the fraction of injected dose per ml of plasma metabolized in the inner pool per unit time.

After a rapid intravenous injection of testosterone-1,2-^{3}H into a

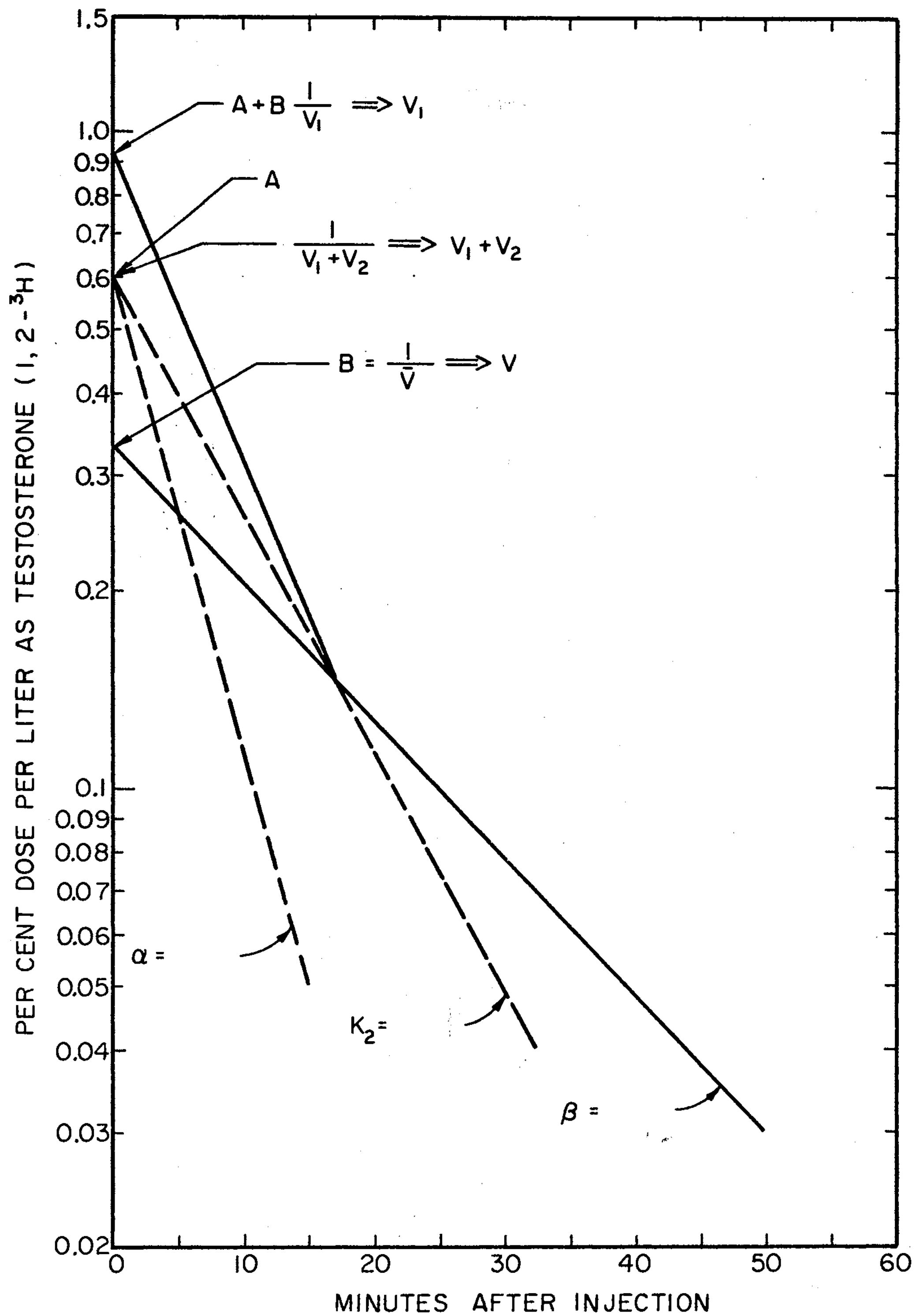


Figure 1. Average Disappearance Curve of Testosterone-1,2-^{3}H in Plasma of Eight Hereford Bulls

bull, the radioactive hormone is distributed extremely fast into the inner pool. Inner pool volume (V_1) is equal to body testosterone in rapid equilibrium, divided by plasma concentration ($\frac{1}{A + B}$). After this initial distribution, the radioactivity then appears to move more slowly into a larger total volume ($V_1 + V_2$). This volume ($V_1 + V_2$), multiplied by the blood concentration of steroid, will be equal to the total body content of non-isotopic steroid. $V_1 + V_2$ can be calculated from the expressions given in the latter part of this discussion. This volume is also the reciprocal of the intercept on the ordinate obtained by extrapolating, using the slope K_2, from the point where the initial and final disappearance curves intersect. The outer pool, (V_2), is equal to the testosterone in slow equilibrium, divided by the plasma concentration. In this study metabolism in the outer pool was assumed to be zero, and V_1 was subtracted from $V_1 + V_2$ to give V_2. The volume $\overline{V}$ represents a theoretical total volume of distribution and is calculated from the final disappearance curve. $\overline{V}$ is equal to $\frac{1}{B}$, and this volume, multiplied by the radioactive concentration in the plasma, will give the total radioactivity of the hormone at any particular time. However, this is not the true volume of distribution, since the radioactive concentration in the outer pool is greater than in the inner pool. Therefore, the volume $\overline{V}$ is greater than the more meaningful volume of distribution ($V_1 + V_2$).

Metabolic clearance rate (MCR) is defined as the volume of plasma, cleared completely of steroid per unit time; its value is obtained by integrating the area of the initial (intercept A, slope α) with final (intercept B, slope β) disappearance curves. MCR seems to be a reasonable measure of the entire metabolism of a steroid for purposes of

comparison.

The more pertinent equations used in this study are presented in Figure 1 and as follows:

1) The slope of the initial (α) and final curve (β) was expressed in units per day:

$$\text{slope} = \frac{2.3 \text{ x } (- \log X)}{t} \text{ x } 1400$$

where:

$$X = \frac{\text{\% dose per 1 at 10 min}}{\text{\% dose per 1 at zero min}};$$

t = 10 minutes;

1440 = minutes per day.

2) The half-life (t 1/2) of testosterone-1,2-^{3}H in the various compartments was expressed in minutes:

$$t(\tfrac{1}{2}) = \frac{0.6923\ (1440)}{\text{slope}}$$

where:

slope = α or β;

0.6923 = 2.3 (log 2).

3) Metabolic clearance was expressed in liters of plasma per day:

$$\text{MCR} = \frac{\alpha\beta}{A\beta + B\alpha}$$

4) Production rate (PR) was expressed in mg per day.

$$\text{PR mg/day} = \frac{\text{conc. x 1000 x MCR}}{10^6}$$

where:

conc. = concentration (ng/ml plasma of endogenous testosterone

found in plasma prior to injection of testosterone-1,2-^{3}H);

1000 = milliliter per liter;

10^6 = nanograms per milligram.

5) Pool volumes were expressed in liters:

$$V_1 = \frac{1}{A + B}$$

$$V_2 = \frac{\beta^2 A + B\alpha^2}{(A\beta + B\alpha)^2} - V_1$$

$$\overline{V} = \frac{1}{B}$$

6) Rate constants were expressed in units/day:

$$K_1 = V_2(A\beta + B\alpha)$$

$$K_2 = \frac{\alpha\beta\ (A + B)}{A\beta + B\alpha} = \frac{MCR}{V_1}$$

Statistical Analysis of Data

All experiments were arranged in completely randomized block design. Data were subjected to analysis of variance (AOV), (Snedecor and Cochran, 1967). When AOV revealed significant variation due to treatment, then differences in treatment means were detected by the Newman-Keuls Sequential Range Test (Snedecor and Cochran, 1967).

CHAPTER IV

RESULTS

The objectives of this study were: 1) to determine the adjustments of body temperature and respiration rate to continuously high ambient temperature and humidity within a psychrometric chamber; 2) to evaluate the effects of prolonged elevated ambient temperature on semen quality and testicular morphology; 3) to measure peripheral plasma levels of specific adrenal glucocorticoids and testicular androgens under optimum (70° F) and hot (96° F) temperatures; and 4) to determine and compare 24 hour testosterone production rates among bulls kept on pasture at 60° F and those maintained at 70° F and 96° F in a psychrometric chamber.

Physical Measurements During Exposure to Optimum and Hot Environments

Rectal temperature and respiration rate are outward expressions of internal regulatory adjustments made by homeotherms in response to their environment. Therefore, changes in rectal temperature and respiration rate can be used to indicate whether or not a particular heat treatment is effective.

Effects of Ambient Temperature on Rectal Temperature

Heat production, heat gain, and heat loss determine the temperature of a tissue; any change in these factors causes variation in the

temperature of a tissue. Rectal temperature is an excellent monitor of core temperature (Bianca, 1965) since small, transient changes are not registered due to thermal inertia of the tissues at the site.

Table II shows that rectal temperature of Hereford bulls during seven weeks exposure to 70° F was stable with a mean of 100.0° F. Rectal temperature rose rapidly in all bulls, reaching a maximum of 104.5° F on day three of the first week of exposure to the hot environment; it subsequently declined to 103.1° F by day seven (Table III). Table IV shows that exposing the bulls to 96° F, 50% RH for seven weeks caused their mean temperature to increase to 102.8° F. This was significantly higher ($P < .01$) than the control mean.

Weekly changes in rectal temperature as a result of heat treatment at 96° F are presented in Table IV. Rectal temperature was highest during the initial week of heat exposure and declined gradually to a low of 101.9° F by week four. However, during the last three weeks of heat treatment, rectal temperature rose, reaching 103.3° F by the end of week seven. Each rectal temperature recorded during the seven week (96° F) treatment period was significantly higher ($P < .01$) than the control value of 100.0° F.

Effects of Ambient Temperature on Respiration Rate

Panting accounts for approximately 35 to 50% of the total evaporative cooling in the bovine (Bianca, 1965). Respiratory evaporation depends on the volume of air moved over the moist surfaces of the respiratory passages per unit time. Ventillation is defined as the resultant of the frequency and the depth of breathing. When the body defends against heat, an increase in ventilation is effected. This is

TABLE II

RECTAL TEMPERATURE[1] OF EIGHT HEREFORD BULLS DURING EXPOSURE TO 70° F, 50% RH[2] ENVIRONMENT

Week of Exposure						
1	2	3	4	5	6	7
100.0[3]	99.9	100.2	99.9	99.5	100.1	100.0
± 0.3[4]	± 0.2	± 0.3	± 0.4	± 0.3	± 0.3	± 0.3
70° Treatment Mean ± SE = 100.0 ± 0.2						

[1]Degrees fahrenheit.

[2]Relative humidity.

[3]Each mean represents the average of seven daily observations on eight bulls.

[4]± standard error of mean (SE).

TABLE III

RECTAL TEMPERATURE[1] OF EIGHT HEREFORD BULLS DURING INITIAL SEVEN DAYS OF EXPOSURE TO 96° F, 50% RH[2] ENVIRONMENT

Day of Exposure							
0	1	2	3	4	5	6	7
100.0[3]	102.8	103.4	104.5	104.1	103.8	104.3	103.1
± 0.3[4]	± 0.5	± 0.3	± 0.5	± 0.5	± 0.6	± 0.5	± 0.3

[1]Degrees fahrenheit.

[2]Relative humidity.

[3]Zero value represents the mean of seven daily observations on eight bulls taken during final week of exposure to control (70° F, 50% RH) environment.

[4]± standard error of mean (SE).

TABLE IV

RECTAL TEMPERATURE[1] OF EIGHT HEREFORD BULLS DURING EXPOSURE TO 96° F, 50% RH[2] ENVIRONMENT

Week of Exposure							
0	1	2	3	4	5	6	7
100.0[3]	103.7[4]	102.9	102.4	101.9	102.3	102.8	103.3
± 0.3[5]	± 0.3	± 0.2	± 0.3	± 0.3	± 0.3	± 0.5	± 0.4
96° Treatment Mean ± SE = 102.8 ± 0.2							

[1]Degrees fahrenheit.

[2]Relative humidity.

[3]Zero value represents the average of seven daily observations on eight bulls taken during the final week of exposure to control (70° F, 50% RH) environment.

[4]Each mean represents the average of seven daily observations on eight bulls.

[5]± standard error of mean (SE).

accomplished by increased frequency and decreased depth of respiration. The rate of this fast, shallow breathing may be used as an indication of respiratory evaporation (Bianca, 1965).

Weekly mean respiration rates during seven weeks exposure to 70° F temperature are presented in Table V. The average value for the period was 33.0 ± 1.0 respirations per minute (R/m). This value agrees with the observations made by Williams and Bell (1963) who reported a "normal" of 34.0 R/m in cattle maintained at 70° F.

During heat treatment, the increased respiratory rates generally parallel the rise noted previously in rectal temperature. Respirations per minute rose rapidly during the first week of heat, reaching 81.0 R/m on day seven (Table VI). This rate was sustained throughout the second week, gradually decreased through the fourth week of exposure, and reached a low of 59.0 R/m (Table VII). Respiration rates of the bulls reached a plateau, varying between 61.0 to 67.0 R/m, during the last three weeks of heat treatment (Table VII). Mean respiration rate throughout the seven week heat treatment period was 69.3 R/m. This was approximately double the rate recorded for the control period. Each respiration rate recorded during the seven seek exposure to 96° F was significantly higher ($P < .01$) than the 70° F control rate of 33.0 R/m.

Effects of Ambient Temperature on Male Gametogenesis

Peripheral tissues of homeotherms sustain wide variation in temperature; whereas tissues nearer the body core enjoy a more stable thermal environment. Migration of the testis and epididymis in mammals means that embryologically abdominal structures must function at the temperature prevailing within the scrotum. Testicular temperatures

TABLE V

RESPIRATION RATE[1] OF EIGHT HEREFORD BULLS DURING EXPOSURE TO 70° F, 50% RH[2] ENVIRONMENT

Week of Exposure						
1	2	3	4	5	6	7
33[3]	33	32	32	34	32	33
± 2[4]	± 3	± 3	± 3	± 2	± 3	± 1
70° Treatment Mean ± SE = 33.0 ± 1.0						

[1]Respirations per minute.

[2]Relative humidity.

[3]Each mean represents the average of seven daily observations on eight bulls.

[4]± standard error of mean (SE).

TABLE VI

RESPIRATION RATE[1] OF EIGHT HEREFORD BULLS DURING INITIAL SEVEN DAYS EXPOSURE TO 96° F, 50% RH[2] ENVIRONMENT

Day of Exposure							
0	1	2	3	4	5	6	7
33[3]	73	76	83	73	78	84	81
± 1[4]	± 5	± 4	± 5	± 6	± 5	± 6	± 4

[1]Respirations per minute.

[2]Relative humidity.

[3]Zero value represents the average of seven daily observations on eight bulls taken during the final week of exposure to control (70° F, 50% RH) environment.

[4]± standard error of mean (SE).

TABLE VII

RESPIRATION RATE[1] OF EIGHT HEREFORD BULLS DURING EXPOSURE TO 96° F, 50% RH[2] ENVIRONMENT

Week of Exposure							
0	1	2	3	4	5	6	7
33[3]	81[4]	82	73	59	61	67	63
± 1[5]	± 4	± 2	± 4	± 2	± 2	± 3	± 3
96° Treatment Mean ± SE = 69 ± 2.0							

[1]Respirations per minute.

[2]Relative humidity.

[3]Zero value represents the average of seven daily observations on eight bulls taken during the last week of exposure to control (70° F, 50% RH) environment.

[4]Each mean represents the average of seven daily observations on eight bulls.

[5]± standard error of mean (SE).

measured in conscious bulls reveal a body-testicular difference, ranging from 2.5 to 6.5° C (Riemerschmid, 1941). High environmental temperatures can affect spermatogenesis adversely in the bovine, but precise information concerning the mechanism is lacking.

Semen Characteristics After Exposure to Heat

Table VIII shows that the mean total score for semen collected from Hereford bulls after seven weeks in the psychrometric chamber at 70° F was 80 $\pm$ 2.0. Immediately following a similar period of heat exposure at 96° F, the total semen score dropped to 28.0 $\pm$ 2.9. This was 35% of its former control value. The decrease in total semen score resulted from dramatic declines in all component factors evaluated by the scoring system used (Hill, 1956). Prolonged heat exposure resulted in a 69% reduction in morphology score, 80% decrease in motility, 70% fall in percent alive, and a 25% reduction in concentration score.

Testicular and Accessory Sex Gland Morphology After Exposure to Heat

Gross Pathology. Just prior to slaughter, the testes and epididymides of each bull were palpated through the scrotum; the prostate and seminal vesicles were palpated through the rectum. Immediately following slaughter, both testes and epididymides, the prostate, seminal vesicles, and adrenals were removed and examined for evidence of gross pathology. No abnormalities in testis volume or texture were detectable after the seven weeks of exposure to 96° F. Furthermore, the epididymides, accessory sex glands, and adrenals appeared normal in size, color, and texture. No gross pathology was found in any of the tissues examined by the author.

TABLE VIII

EFFECT OF ELEVATED AMBIENT TEMPERATURES ON SEMEN SCORES[1] OF EIGHT HEREFORD BULLS

Ambient Environment	Time Collected	Criteria Evaluated				
		Motility[2]	Concentration[3]	% Alive[4]	Morphology[5]	Total[6]
70° F, 50% RH[7]	After 7 Weeks Exposure	31.0[8] ± 1.3	16.0 ± 2.2	7.0 ± 1	26.0 ± 2.1	80.0 ± 2.0
96° F, 50% RH	After 7 Weeks Exposure	6.0 ± 1.6	12.0 ± 2.7	2.0 ± 0.4	8.0 ± 1.0	28.0 ± 2.9

[1]Scoring system recommended by the Veterinary Society for the Study of Breeding Soundness (Hill, 1956).

[2]Maximum possible - 40.

[3]Maximum possible - 20.

[4]Maximum possible - 10.

[5]Maximum possible - 30.

[6]Maximum possible - 100.

[7]Relative humidity.

[8]± standard error of mean (SE).

Histopathological Findings. No significant histopathological lesions were observed in hematoxylin-eosin stained sections of the adrenals, prostate, or seminal vesicles from bulls exposed to 96° F for seven weeks. The only abnormality noted in epididymal sections was that the lumina of the tubules were filled with degenerating cellular products. However, numerous histopathological changes were seen in the testes after heat treatment. Some degree of degeneration was seen in all tubules, but the degree of severity was variable.

Plate 1 is a photomicrograph of a section from the testis of a normal fertile Hereford bull. All stages of normal seminiferous epithelium are represented. Distinct spermatogonia, primary and secondary spermatocytes, spermatids, and spermatozoa are present. In contrast, Plates 2a and 2b are photomicrographs of representative areas seen in a section of a testis from one of the Hereford bulls exposed to 96° F for seven weeks. Both plates show the degenerative effects of heat on the germinal cells lining the seminiferous tubules.

Plate 2a is a photomicrograph of a moderately advanced stage of tubular degeneration in which there is an absence of mature spermatozoa. Note a degeneration of spermatids, that most spermatids are necrotic, and that others have produced characteristic spermatidic multinuclear phagocytic giant cells. Cytoplasmic vacuoles are present in many spermatocytes, and the lumina of the tubules are filled with degenerating cellular debris.

Plate 2b shows a stage of severe degeneration in which only a few spermatogonia and Sertoli cells remain lining the tubules. There is also a relative increase in interstitial tissue due to the decreased volume of the tubular tissue.

Plate 1. Photomicrograph of a Typical Area in a Hematoxylin-eosin Stained Section of a Testis From a Fertile, 24 Months old, Hereford Bull. (Magnification 100X).

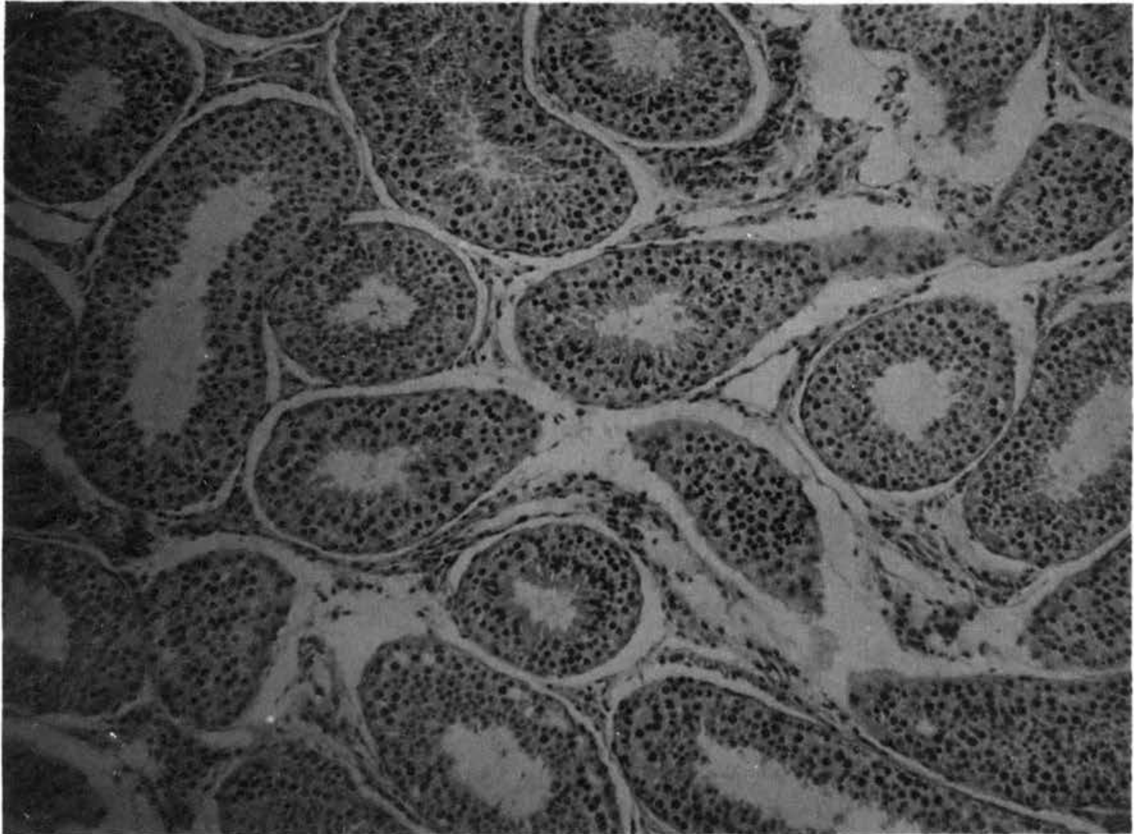
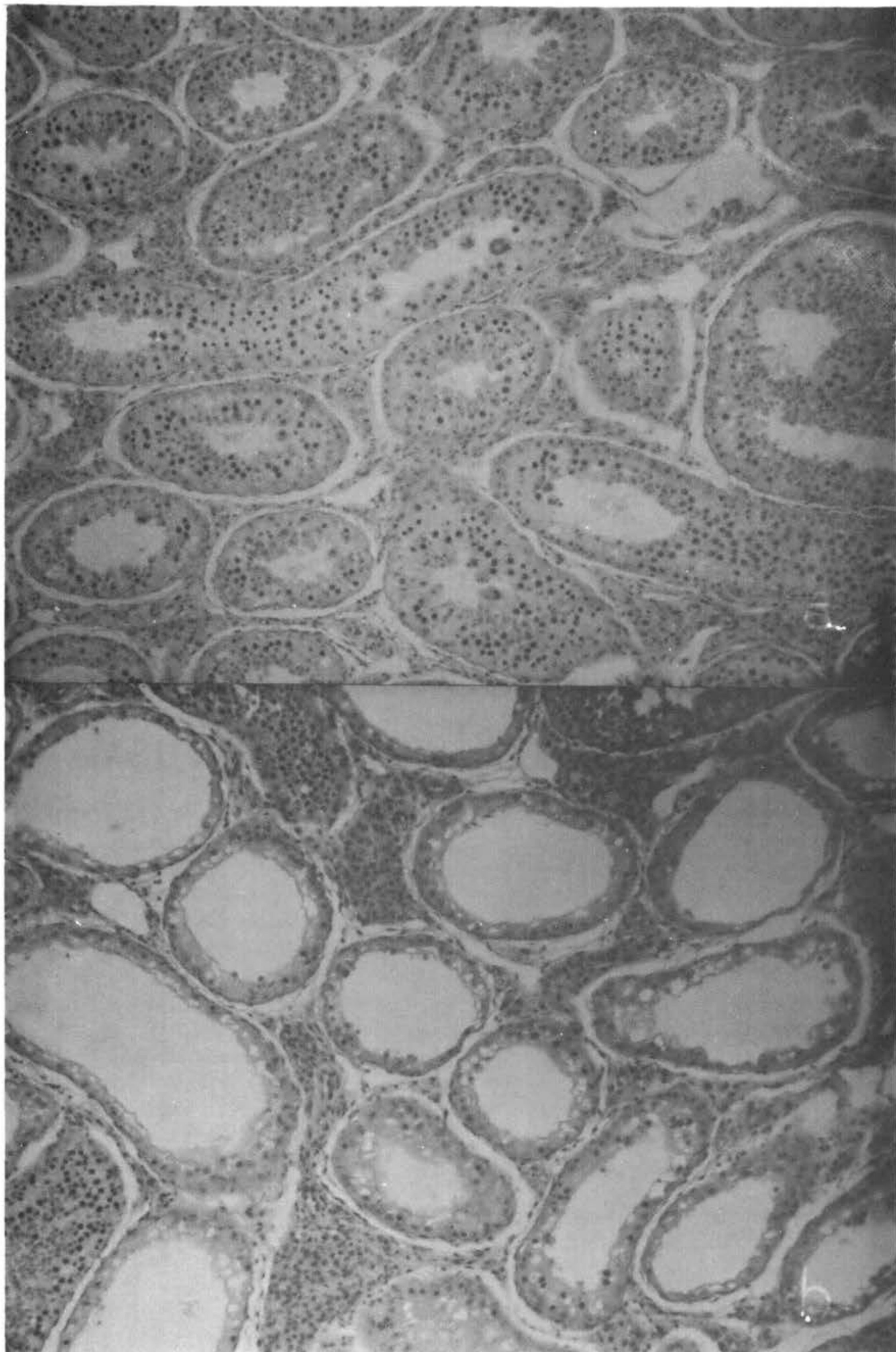


Plate 2. Photomicrographs of Representative Areas of Degenerating Germinal Epithelium in a Hematoxylin-eosin Stained Section of a Testis From a 24 Month old Hereford Bull Which had Been Exposed to 96° F, 50% RH for Seven Weeks. a) Shows moderately advanced stages of degeneration of germinal epithelium. Note an absence of mature spermatozoa and a degeneration of spermatids, with the formation of spermatidic multinuclear phagocytic giant cells; b) Shows advanced stage of degeneration of germinal epithelium. Note that only a few Sertoli cells and spermatogonia remain lining the tubules.


a
b

Identification of Isolated Steroids

Assays used for isolation and measuring of testosterone, cortisol, and corticosterone have been shown to be highly specific by the investigators who developed the techniques (Brownie, *et al.*, 1964; Kirschner, *et al.*, 1968; Ganjam, *et al.*, 1970). In this study, however, the steroids were isolated from bovine plasma, not from human or rabbit plasma, as had been done by previous investigators. It was deemed advisable to confirm the specificity of the modified assays. Whenever steroids from bull plasma were measured, aliquots of the individually isolated compounds were saved and placed in one of three pools: the testosterone pool, the cortisol pool, or the corticosterone pool. These pools were subsequently subjected to physico-chemical tests to demonstrate that the isolated compounds were testosterone, cortisol, and corticosterone.

Testosterone. Structure and purity of the isolated compound was established by comparing its mass spectrum with that of authentic testosterone heptafluorobutyrate (THFB). The mass spectrum of the unknown HFB derivative was determined by a mass spectrometer-gas chromatograph (LKB-90000, prototype). Details of its operation and construction have been reported by Waller (1967). Figure 2 presents the mass spectrum of authentic THFB and Figure 3 presents the mass spectrum of the derivative of the unknown compound isolated from bull blood plasma. The finding of a molecular weight of 484 with a fragmentation pattern similar to that of authentic THFB supports the identity of the compound isolated from bovine plasma as THFB.

Cortisol. The R_f value of the unknown compound was identical to that of standard cortisol in several TLC solvent systems. Likewise, the

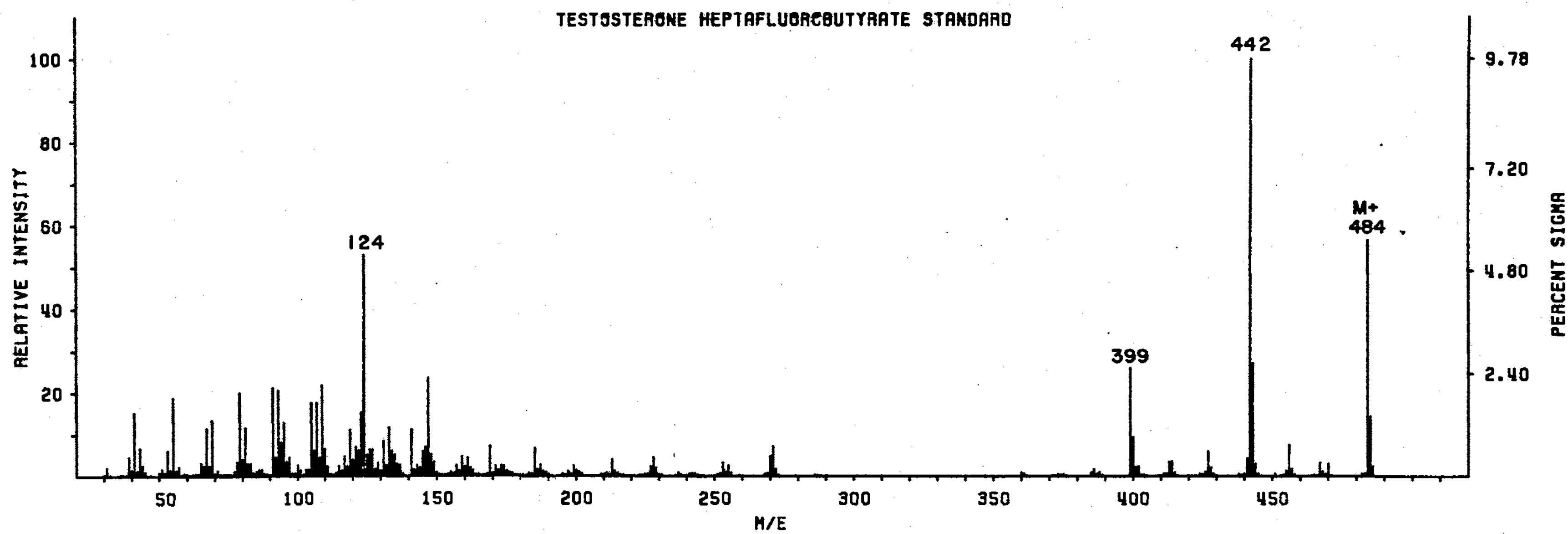


Figure 2. Mass Spectrum of Standard Testosterone Heptafluorobutyrate (MS 1970:5 - MS 1970:3).

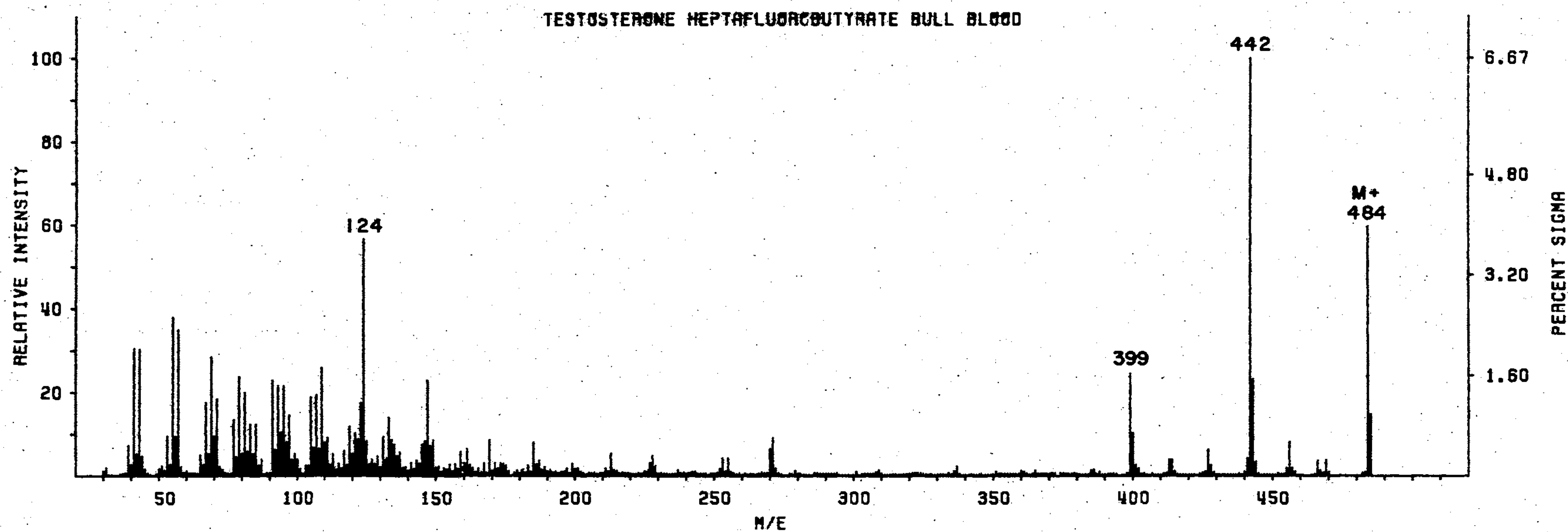


Figure 3. Mass Spectrum of Testosterone Heptafluorobutyrate Isolated as Testosterone From Peripheral Plasma of Hereford Bulls. (MS 1970:6 - MS 1970:3).

fluorescent spectrum of the unknown compound (excitation 472 mμ, emission, 528 mμ) coincided with that of authentic cortisol. More rigorous proof of the structure of the isolated compound was established by subjecting it to mass sepctral analysis. Figure 4 shows the mass spectrum of authentic cortisol and Figure 5 presents the mass spectrum of the unknown compound isolated from bull plasma. The molecular ion peak was 362 for both the standard and sample cortisol, and both compounds had similar fragmentation patterns. Thus it was concluded that the unknown compound, isolated from bull plasma, was cortisol.

Corticosterone. Data from this study and previous experiments (Venkataseshu, 1965) shows that the bovine adrenal gland primarily secretes cortisol. Plasma corticosterone concentrations remained relatively constant following heat treatment imposed on the bulls in this study. Therefore, the author accomplished only a tentative proof of structure on the isolated corticosterone. No mass spectral data is presented for unknown corticosterone. Nevertheless, the specificity of the method for corticosterone was evaluated by comparing the fluorescent spectrum of the isolated compound to that of corticosterone standards. Figures 6a and 6b present excitation (472 mμ) and emission (528 mμ) scans of authentic corticosterone. Figures 7a and 7b present the same type scans of the unknown compound. Standard and unknown corticosterone yielded almost identical scans. The similarity of fluorimetric spectra, and also the fact that the R_f of the unknown was identical to that of standards in several TLC systems offers strong evidence that the unknown was a relatively pure form of corticosterone.

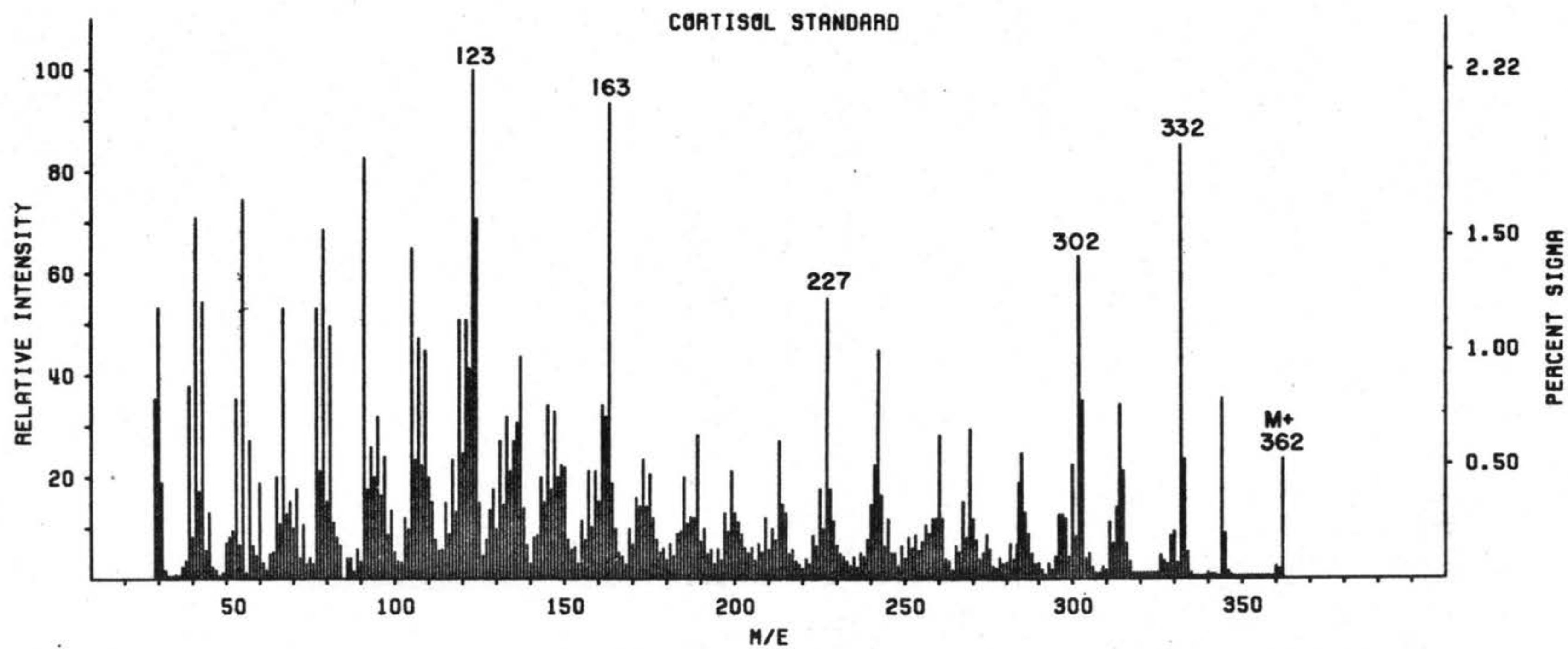


Figure 4. Mass Spectrum of Standard Cortisol. (MS 1970:9 - MS 1970:7).

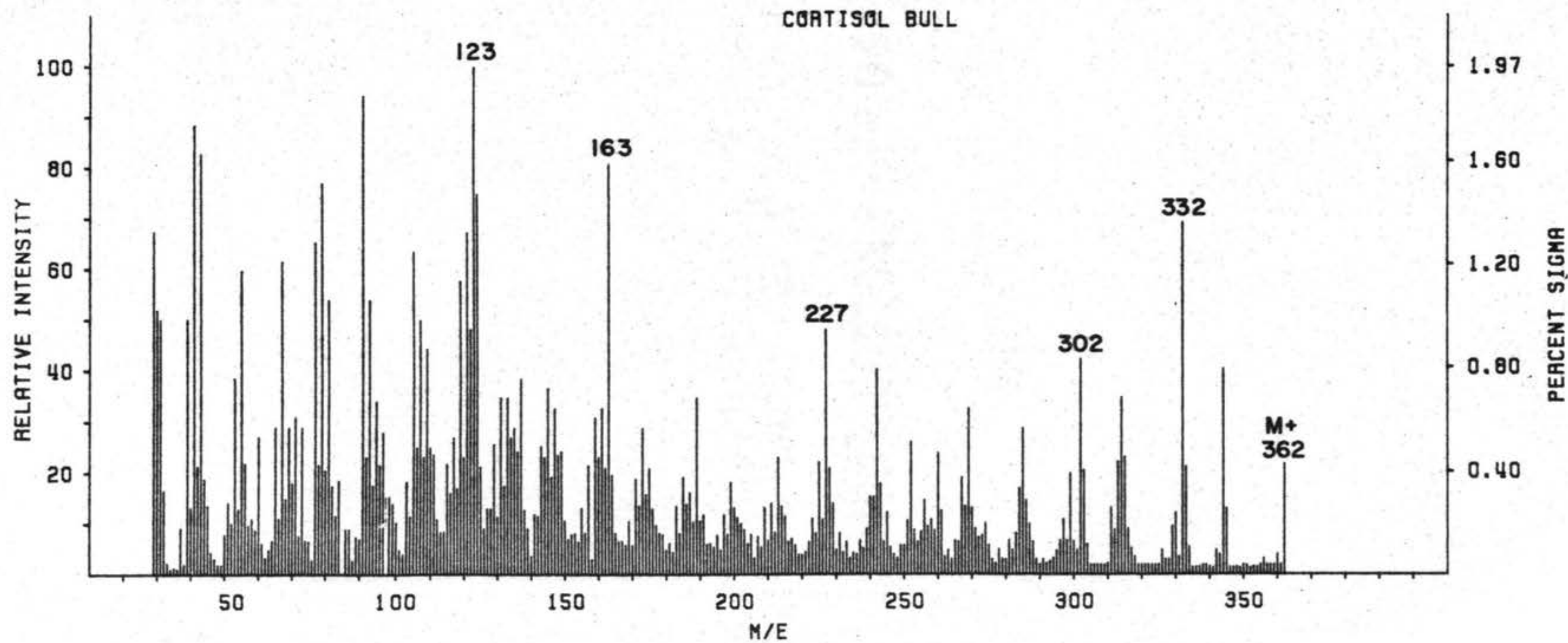


Figure 5. Mass Spectrum of Cortisol Isolated From the Peripheral Plasma of Hereford Bulls (MS 1971:9 - MS 1971:7).

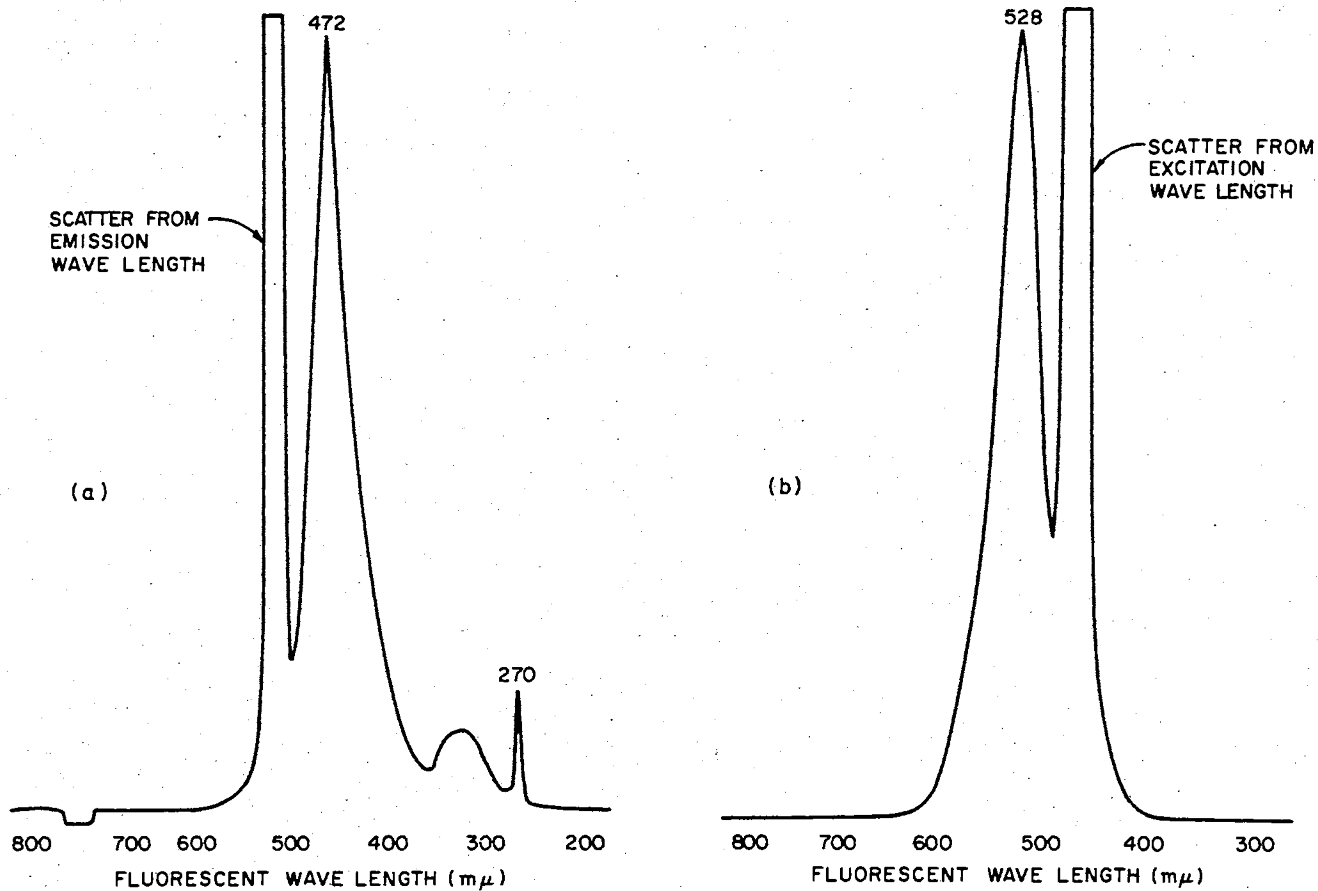


Figure 6. Spectrofluorometric Scan of Standard Corticosterone. a) Excitation wave length scan at a constant emission wave length of 528 mμ; b) Emission wave length scan at a constant excitation wave length of 472 mμ.

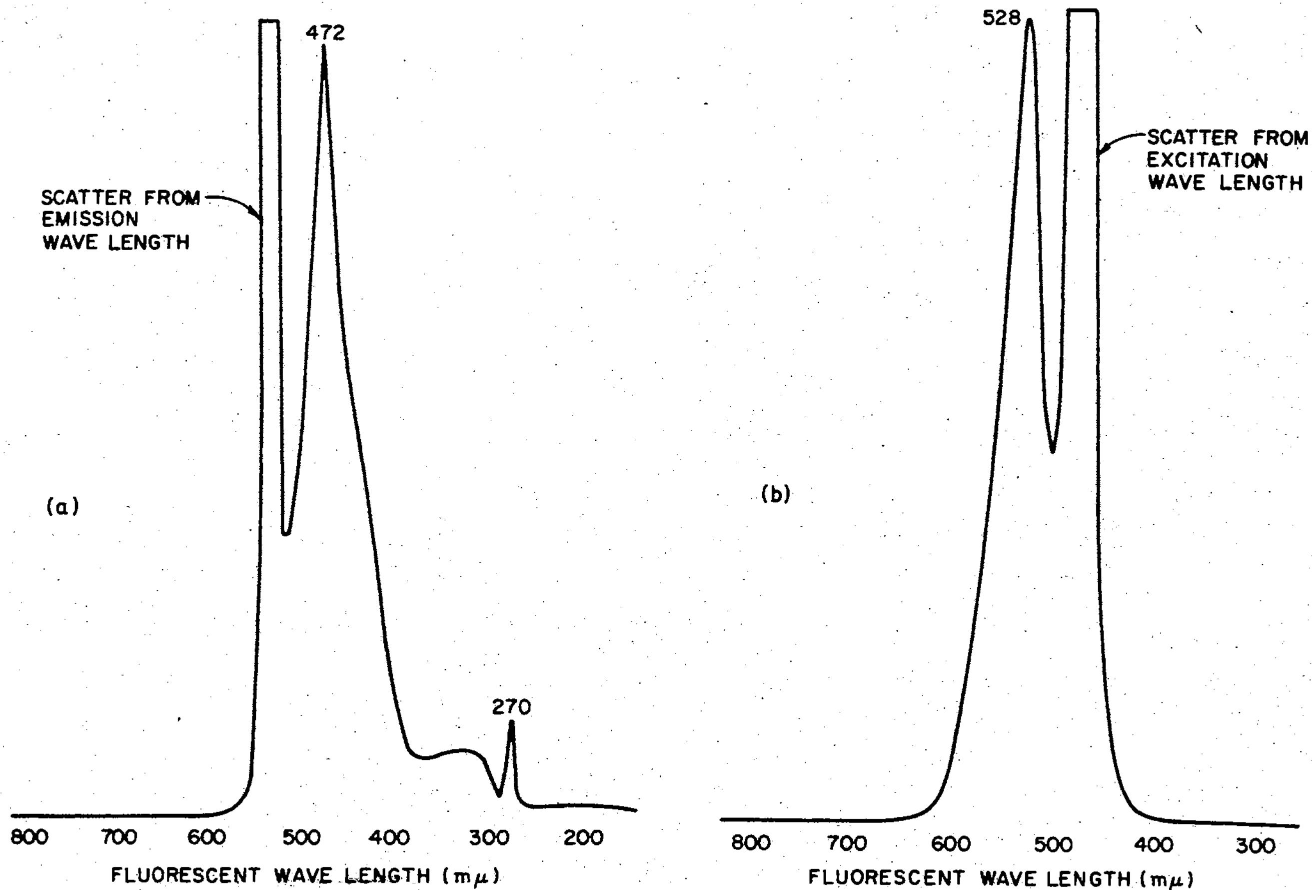


Figure 7. Spectrophotofluorometric Scan of Corticosterone Isolated From the Peripheral Plasma of Hereford Bulls. a) Excitation wave length scan at a constant emission wave length of 528 mμ; b) Emission wave length scan at a constant excitation wave length of 472 mμ.

Effects of High Ambient Temperature on Plasma Glucocorticoid Concentration

Cortisol and corticosterone have been isolated from bovine adrenal venous blood and are the major secretory products of the adult bovine adrenal cortex. A divserse group of factors are known to exert an influence on plasma glucocorticoids. Yates and Urquhart (1961) listed 17 different stimuli studied by various investigators which were shown to elevate plasma glucocorticoid concentration. These stimuli ranged from various environmental conditions to emotional distress. Thus, elevated plasma glucocorticoid concentrations have become one of the primary indices of physiological stress.

The effects of hot environmental temperatures on plasma glucocorticoids of exposed animals has not been extensively investigated. The few data obtained are not conclusive in any species. There are wide discrepancies in the "normal" values reported by various workers with the bovine. Furthermore, some researchers have reported increases in plasma corticoids in response to heat, while others have reported decreases.

One of the primary purposes of this study was to determine how glucocorticoid levels in bulls were affected by hot environments. Another important objective was to relate adrenal cortical function to testicular androgen secretion. Cortisol and corticosterone were quantitated separately because it was felt that the ratio between the two might be as important as their absolute concentrations.

Plasma Cortisol Concentrations

Table IX presents mean values of plasma cortisol concentrations for the seven week control (70° F) period. The value for week zero was 31.90 ng/ml plasma and represents the first samples collected from the bulls while they were on pasture at Fort Reno. This very high cortisol concentration probably reflects extreme psychological distress when the bulls were initially subjected to restraint for bleeding. The average value for plasma cortisol throughout the final five weeks of the control period was 17.45 ng/ml (Table IX). All of the five weekly cortisol concentration values recorded during the control period were significantly ($P < .01$) lower than the zero week value.

During the first 24 hours of exposure to 96° F, plasma cortisol levels fell 33% (Table X). Table XI shows that cortisol values during heat treatment varied from 10.37 to 15.79 ng/ml. The average value for the hot period was 11.22 ng/ml plasma. This was lower ($P < .01$) than the 17.45 ng/ml average for the control period. Levels remained significantly depressed ($P < .01$) for six weeks.

Plasma Corticosterone Concentrations

Like cortisol, corticosterone levels were elevated when blood was collected from the bulls at Fort Reno. Table XII shows that this initial value was 6.47 ng/ml plasma. After the animals had adjusted to the environmental chamber and the bleeding routine, plasma corticosterone values were much lower ($P < .01$). They ranged from 2.67 to 3.92 ng/ml during the final five weeks of 70° F exposure.

Table XIII presents data collected during the first week of exposure to 96° F, and Table XIV shows corticosterone concentrations for

TABLE IX

PLASMA CORTISOL CONCENTRATION[1] OF EIGHT HEREFORD BULLS DURING EXPOSURE TO 70° F, 50% RH[2] ENVIRONMENT

Week of Exposure							
0	1	2	3	4	5	6	7
31.90[3]	-	-	16.45	19.75	20.5	17.2	13.15
± 1.72[4]	-	-	± 2.04	± 1.63	± 1.69	± 1.27	± 1.26
70° F Treatment Mean ± SE = 17.45 ± 0.90							

[1]Nanograms per ml.

[2]Relative humidity.

[3]Zero value represents mean of eight bulls. Plasma samples were collected while animals were on pasture (60° F, 50% RH) environment.

[4]± standard error of mean (SE).

TABLE X

PLASMA CORTISOL CONCENTRATION[1] OF EIGHT HEREFORD BULLS DURING INITIAL SEVEN DAYS OF EXPOSURE TO 96° F, 50% RH[2] ENVIRONMENT

Day of Exposure							
0	1	2	3	4	5	6	7
18.03[3]	11.93	9.83	10.74	10.05	11.33	9.39	11.19
± 1.83[4]	± 0.43	± 0.95	± 1.06	± 1.26	± 1.37	± 1.13	± 1.45

[1]Nanograms per ml.

[2]Relative humidity.

[3]Zero value represents mean of eight bulls (70° F, 50% RH). Plasma samples collected immediately prior to elevating ambient temperature.

[4]± standard error of mean (SE).

TABLE XI

PLASMA CORTISOL CONCENTRATION[1] OF EIGHT HEREFORD BULLS DURING EXPOSURE TO 96° F, 50% RH[2] ENVIRONMENT

Week of Exposure							
0	1	2	3	4	5	6	7
18.03[3]	11.19	11.04	12.60	11.11	10.37	10.45	15.79
± 1.83[4]	± 1.45	± 1.06	± 1.36	± 1.24	± 1.43	± 1.01	± 1.02
96° F Treatment Mean ± SE = 11.22 ± 0.75							

[1]Nanograms per ml.

[2]Relative humidity.

[3]Zero value represents mean of eight bulls (70° F, 50% RH). Plasma samples collected immediately prior to elevating ambient temperature.

[4]± standard error of mean (SE).

TABLE XII

PLASMA CORTICOSTERONE CONCENTRATION[1] OF EIGHT HEREFORD BULLS DURING EXPOSURE TO 70° F, 50% RH[2] ENVIRONMENT

Week of Exposure							
0	1	2	3	4	5	6	7
6.47[3]	-	-	2.83	3.34	3.26	3.92	2.67
± 0.65[4]	-	-	± 0.68	± 0.38	± 0.28	± 0.28	± 0.32
70° F Treatment Mean ± SE = 3.00 ± 0.20							

[1]Nanograms per ml.

[2]Relative humidity.

[3]Zero value represents mean of eight bulls. Plasma samples were collected while animals were on pasture (60° F, 50% RH) environment.

[4]± standard error of mean (SE).

TABLE XIII

PLASMA CORTICOSTERONE CONCENTRATION[1] OF EIGHT HEREFORD BULLS DURING INITIAL SEVEN DAYS OF EXPOSURE TO 96° F, 50% RH[2] ENVIRONMENT

Day of Exposure							
0	1	2	3	4	5	6	7
2.67[3]	2.52	2.41	2.55	3.24	2.66	3.29	2.45
± .32[4]	± .33	± .37	± .21	± .33	± .20	± .43	± .40

[1]Nanograms per ml.

[2]Relative humidity.

[3]Zero value represents mean of eight bulls (70° F, 50% RH). Plasma samples were collected immediately prior to elevating ambient temperature.

[4]± standard error of mean (SE).

TABLE XIV

PLASMA CORTICOSTERONE CONCENTRATION[1] OF EIGHT HEREFORD BULLS DURING EXPOSURE TO 96° F, 50% RH[2] ENVIRONMENT

Week of Exposure							
0	1	2	3	4	5	6	7
2.67[3]	2.45	2.69	3.29	3.04	3.37	2.19	2.20
± .32[4]	± .39	± .20	± .30	± .39	± .49	± .18	± .37
96° F Treatment Mean ± SE = 2.62 ± 0.15							

[1]Nanograms per ml.

[2]Relative humidity.

[3]Zero value represents mean of eight bulls. Plasma samples were collected immediately prior to elevating ambient temperature.

[4]± standard error of mean (SE).

each week of the seven weeks of heat exposure. Average corticosterone plasma concentrations during the hot period was 2.62 ng/ml (Table XIV). This was not significantly different ($P > .05$) from the average of 3.00 ng/ml found for the control period.

Measurement of Testosterone Concentration and Production During Exposure to Optimum and Hot Environments

Testosterone appears to be the principle androgenic hormone secreted by the testes of mature bulls (Lindner, 1959). The author quantitated peripheral plasma levels of testosterone and measured the half-life of circulatory testosterone in Hereford bulls. These observations were related to dynamic mathematical models which allowed evaluation of metabolic clearance rates and testosterone 24 hour production rates in conscious bulls.

Testosterone plasma concentration, biological half-life, metabolic clearance rate, and testosterone 24 hour production rate were evaluated under both natural (animals on pasture at 60° F) and artificial (animals in a psychrometric chamber at 70° and 96° F) conditions. These three periods were chosen because they permitted the best comparison of changes in testosterone levels with time of exposure to different environmental temperatures. The initial study, conducted under pasture conditions, served as a control for the 70° F psychrometric period. This allowed evaluation of possible effects incurred by confinement in the psychrometric chamber.

Testosterone Concentrations in Peripheral Plasma in Hereford Bulls

Mean plasma testosterone concentration was 3.81 ng/ml when the Hereford bulls were sampled at Fort Reno. Confinement in a

psychrometric chamber for seven weeks at 70° F did not produce any change ($P > .05$) in the average plasma testosterone concentration (3.32 ng/ml, Table XV).

During the first week of heat treatment, testosterone concentration declined irregularly from 3.24 to 1.62 ng/ml on day seven (Table XVI). Table XVII presents testosterone concentrations for each of the seven weeks of heat treatment and shows the mean concentration for the seven weeks to be 2.20 ng/ml. Analysis of variance showed a significant difference ($P < .01$) between the 70° F and 96° F periods. The Newman-Keuls Sequential Range Test indicated that weeks one, two, and five were lower ($P < .05$) than the control value, and that weeks three, four, six, and seven were similar to control levels. Mean plasma testosterone concentrations for weeks one, two, and five were 1.60, 1.40, and 1.98 ng/ml plasma, respectively. The initial dip in testosterone values, followed by a gradual increase to near-control levels, may be an indication that acclimation of testosterone secretion to heat treatment did ensue. This will be discussed more completely later.

Testosterone Production Rates

Figures 8, 9, and 10 present disappearance curves of radioactive testosterone in the plasma of Hereford bulls used in this study. Each figure represents the mean of the curves for eight bulls. These curves were determined at three different levels of environmental temperature: Figure 8 at 60° F when the animals were on pasture, Figure 9 at 70° F in the environmental chamber, and Figure 10 at 96° F in the chamber. It can be seen that the disappearance curve from bulls, maintained on pasture at 60° F, is essentially the same as that of bulls kept at 70° F

TABLE XV

PLASMA TESTOSTERONE CONCENTRATION[1] OF EIGHT HEREFORD BULLS DURING EXPOSURE TO 70° F, 50% RH[2] ENVIRONMENT

Week of Exposure							
0	1	2	3	4	5	6	7
3.81[3]	-	-	2.57	3.18	3.99	3.48	3.39
±1.08[4]	-	-	± .42	± .41	± .45	± .59	± .56
70° F Treatment Mean ± SE = 3.32 ± 0.23							

[1]Nanograms per ml.

[2]Relative humidity.

[3]Zero value represents mean of eight bulls. Plasma samples were collected while animals were on pasture (60° F, 50% RH) environment.

[4]± standard error of mean (SE).

TABLE XVI

PLASMA TESTOSTERONE CONCENTRATION[1] OF EIGHT HEREFORD BULLS DURING INITIAL SEVEN DAYS OF EXPOSURE TO 96° F, 50% RH[2] ENVIRONMENT

Day of Exposure							
0	1	2	3	4	5	6	7
3.24[3]	2.8	1.69	2.77	1.96	2.39	1.57	1.62
± .89[4]	± .58	± .19	± .64	± .30	± .40	± .36	± .28

[1]Nanograms per ml.

[2]Relative humidity.

[3]Zero value represents mean of eight bulls (70° F, 50% RH). Plasma samples were collected immediately prior to elevating ambient temperature.

[4]± standard error of mean (SE).

TABLE XVII

PLASMA TESTOSTERONE CONCENTRATION[1] OF EIGHT HEREFORD BULLS DURING EXPOSURE TO 96° F, 50% RH[2] ENVIRONMENT

Week of Exposure							
0	1	2	3	4	5	6	7
3.24[3]	1.62	1.40	2.22	2.76	1.98	2.27	2.74
± .89[4]	± .20	± .21	± .34	± .47	± .45	± .45	± .62
96° F Treatment Mean ± SE = 2.20 ± 0.21							

[1]Nanograms per ml.

[2]Relative humidity.

[3]Zero value represents mean of eight bulls (70° F, 50% RH). Plasma samples were collected immediately prior to elevating ambient temperature.

[4]± standard error of mean (SE).

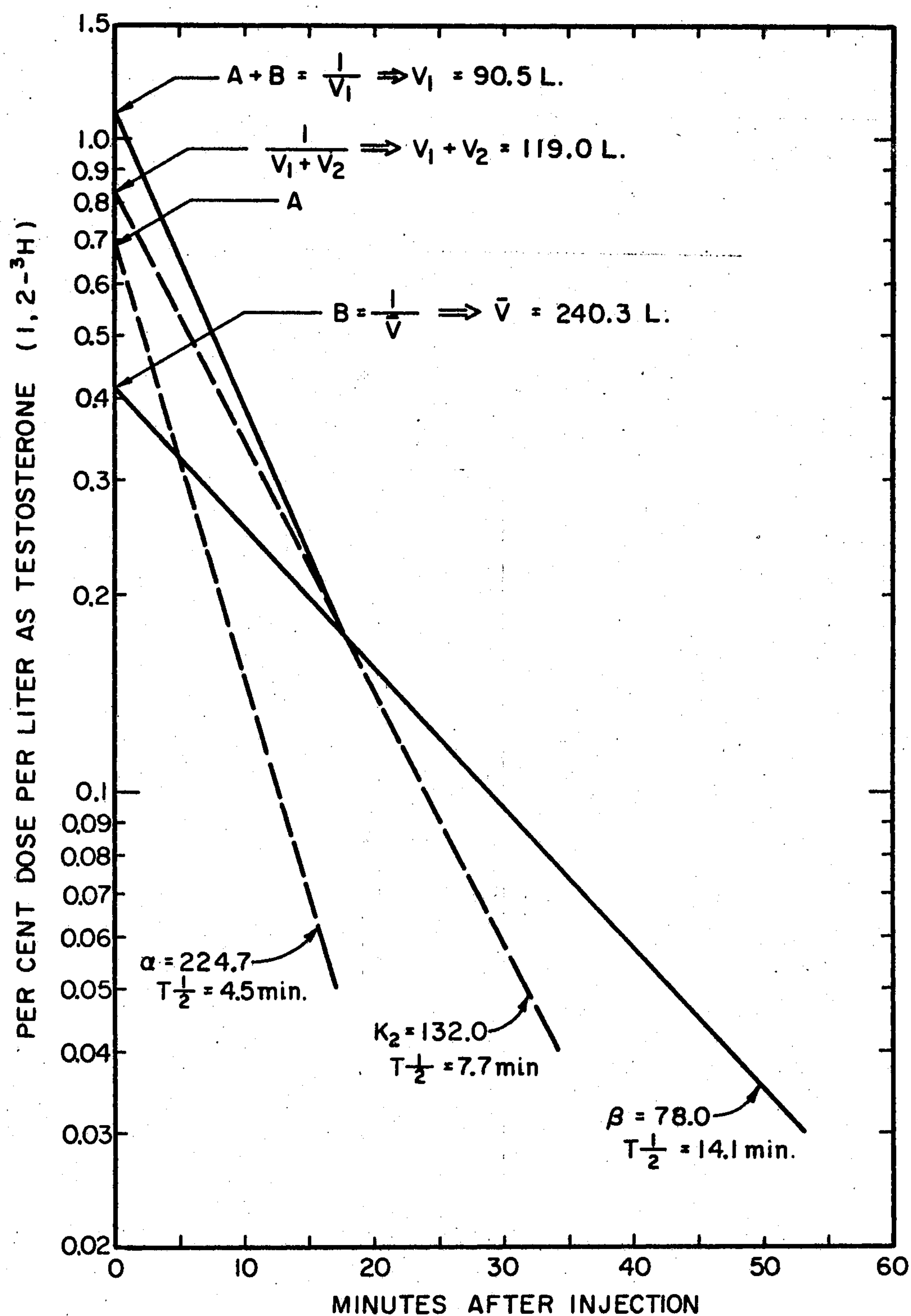


Figure 8. Disappearance of Testosterone-1,2-^{3}H in the Peripheral Plasma of Eight Hereford Bulls Following Exposure to a 60° F, 50% RH Pasture Environment

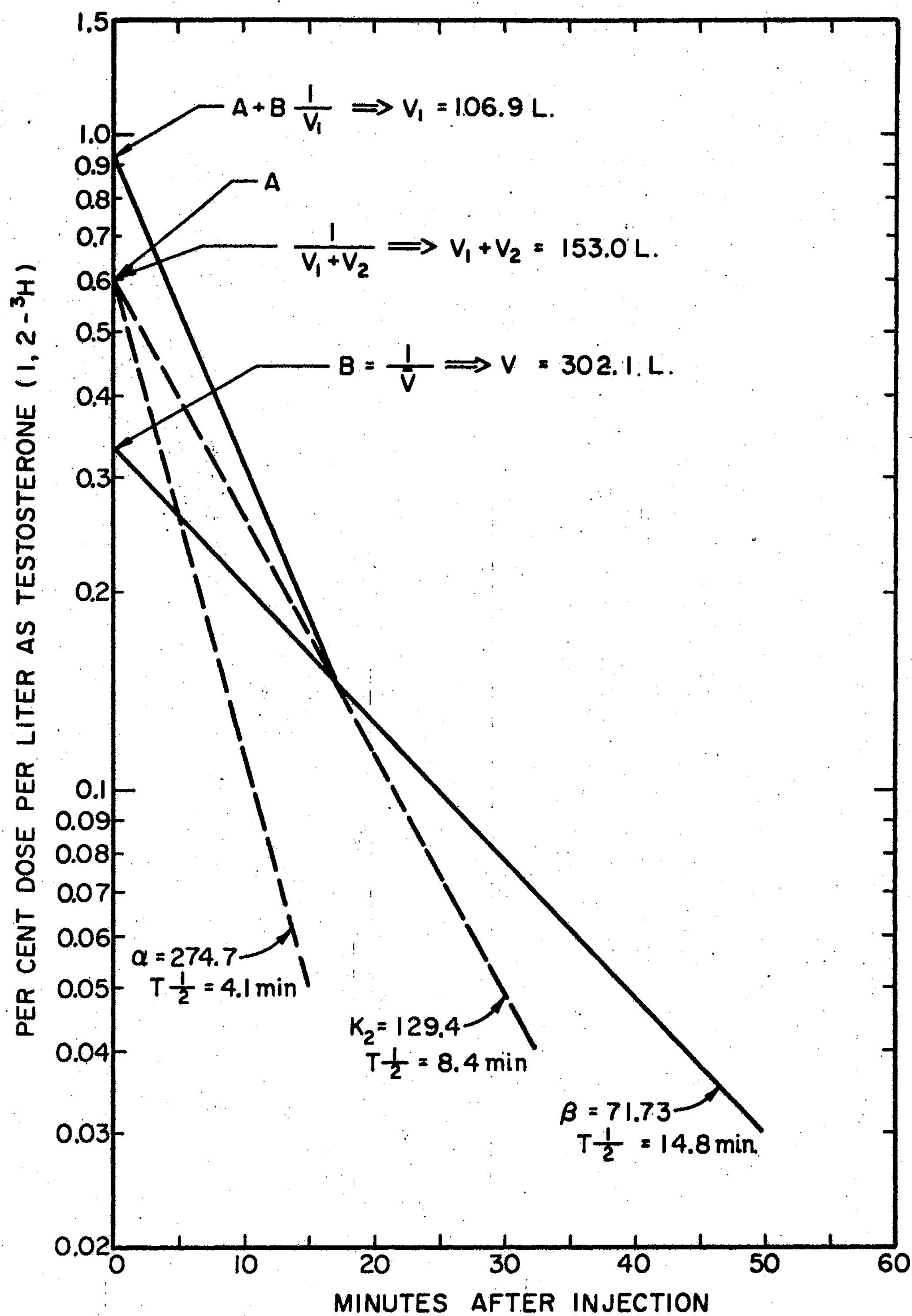


Figure 9. Disappearance of Testosterone-1,2-^{3}H in the Peripheral Plasma of Eight Hereford Bulls Following Exposure to a 70° F, 50% RH Environment for Seven Weeks

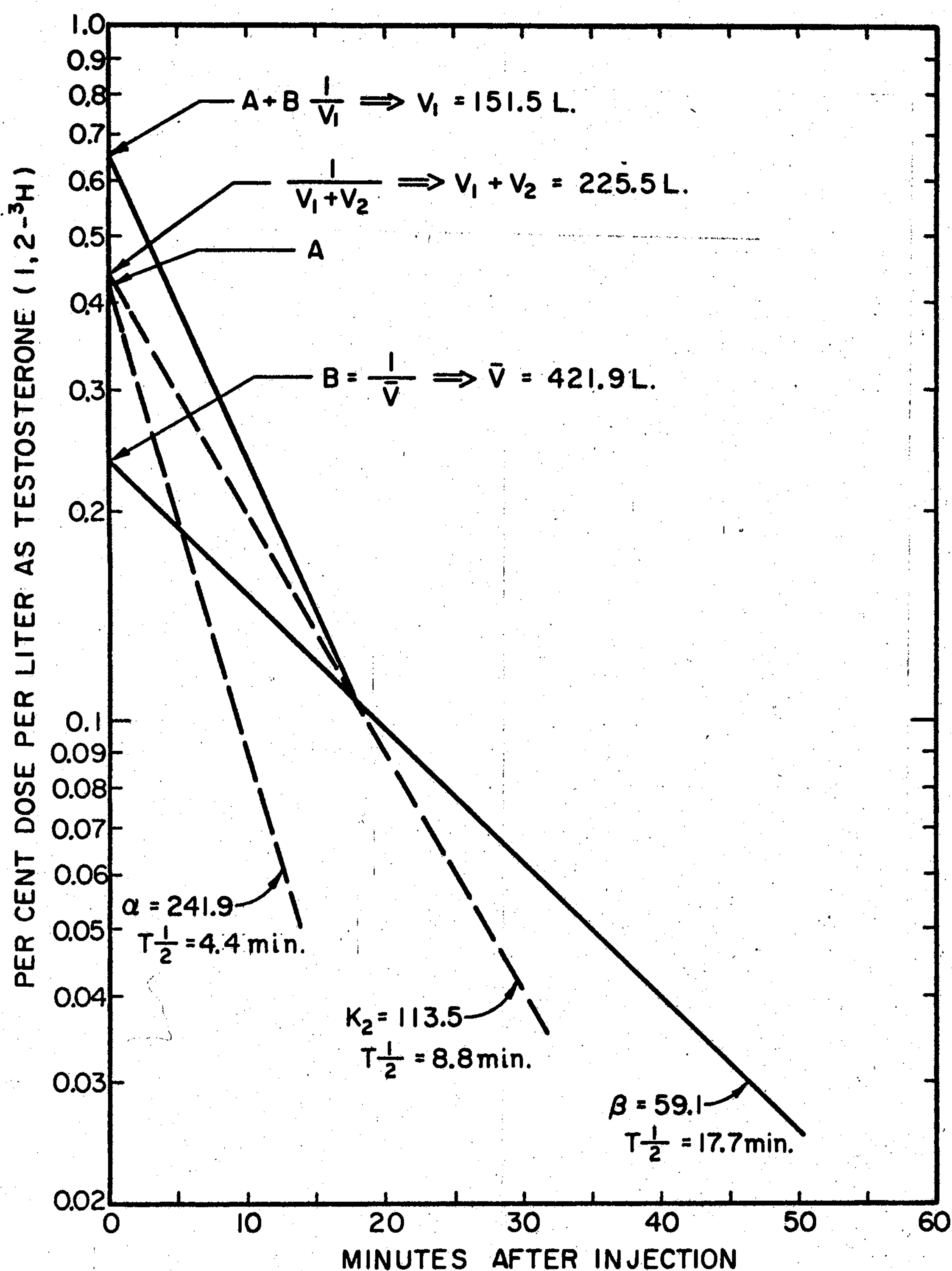


Figure 10. Disappearance of Testosterone-1,2-^{3}H in the Peripheral Plasma of Eight Hereford Bulls Following Exposure to a 96° F, 50% RH Environment for Seven Weeks

in the psychrometric chamber.

Values for transport (K_1) and metabolic rate (K_2) constants, and volumes of distribution (V_1, V_2, $\overline{V}$, $V_1 + V_2$) are given in Table XVIII. Calculated mean values for metabolic clearance rates (MCR) and plasma production rates (PR) are presented in Table XIX. Table XX shows the percent recovery from plasma, disappearance rate (α, β) and half-life (T 1/2 A, T 1/2 B) of intravenously injected tritium-labeled testosterone.

Each of the variables listed in Tables XVIII, XIX, and XX were used for comparison between the pasture period and the 70° F chamber period. There were no statistical differences ($P > .05$) found. Pasture bulls had a MCR of 12,038 liters/day, and 70° F bulls had a MCR of 13,782 liters/day. Testosterone production rates were 43.5 mg/day on pasture, and 41.25 mg/day at 70° F.

The bulls gained weight and grew during this study, making their weights different for each period (Table XIX). Weights at 70° F were higher ($P < .05$) than pasture weights, and weights at 96° F were higher ($P < .05$) than those of the other two periods. Consequently, MCR and pool sizes had to be adjusted according to body weight before meaningful comparisons could be effected.

The 96° F period values were higher ($P < .05$) than those of pasture bulls in the following parameters: volume of inner pool (V_1), volume of outer pool (V_2), and combined volume of pools ($V_1 + V_2$). In contrast the only significant difference found between 96° F and 70° F periods was an increase ($P < .01$) in combined pool volume ($V_1 + V_2$), whether expressed as total liters or as milliliter per pound of body weight. $V_1 + V_2$ for heat exposure was 228.5 liters, compared to 164.3

TABLE XVIII

CALCULATED TRANSPORT AND METABOLIC RATE CONSTANTS AND VOLUME OF DISTRIBUTION OF TESTOSTERONE IN EIGHT HEREFORD BULLS EXPOSED FOR SEVEN WEEKS TO VARIOUS AMBIENT THERMAL CONDITIONS

Ambient Temp ^{o}F	V_1	V_2	$\overline{V}$	V_1+V_2	V_1+V_2	K_1	K_2
	liters				ml/lb	units/day	
60	90.49[1] $\pm$ 5.77	28.50 $\pm$ 7.20	240.3 $\pm$69.1	119.0 $\pm$ 9.95	122.4 $\pm$ 6.5	34.51 $\pm$ 5.41	132.0 $\pm$ 7.1
70	106.9 $\pm$13.9	46.13 $\pm$10.97	302.1 $\pm$45.8	153.0 $\pm$22.1	150.9 $\pm$17.6	60.46 $\pm$16.89	129.4 $\pm$10.7
96	151.5 $\pm$ 8.6	74.00 $\pm$ 7.07	421.9 $\pm$33.4	225.5 $\pm$10.7	205.5 $\pm$14.9	61.06 $\pm$ 7.96	113.5 $\pm$ 7.1

[1] $\pm$ standard error of mean (SE).

TABLE XIX

CALCULATED TESTOSTERONE CONCENTRATION AND MCR[1] AND PRODUCTION RATE OF EIGHT HEREFORD BULLS EXPOSED FOR SEVEN WEEKS TO VARIOUS AMBIENT THERMAL CONDITIONS

Ambient Temp °F	Testosterone ng/ml	MCR L/Day	MCR L/#/Day	Production Rate mg/Day	Body Wt. Pounds
60	3.81	12,038	12.47	45.86	973
	± 1.08	± 572	±0.72	±11.90	± 33
70	3.24	13,782	13.59	44.65	1,014
	± .89	±2,079	±1.46	± 8.16	± 30
96	2.75	17.198	15.69	47.29	1,097
	± .62	± 971	±0.70	±10.94	± 36

[1]Metabolic clearance rate.

TABLE XX

CALCULATED CONCENTRATION AND DISAPPEARANCE RATE AND HALF-LIFE OF ^{3}H TESTOSTERONE IN EIGHT HEREFORD BULLS EXPOSED TO VARIOUS AMBIENT THERMAL CONDITIONS FOR SEVEN WEEKS

Ambient Temp °F	A	B	α	β	$t\frac{1}{2}A$	$t\frac{1}{2}B$
	% Dose/ℓ		Units/Day		Min	
60	0.687	0.416	224.7	78.00	4.45	14.06
	±.109	±.079	± 4.8	±8.29	±.10	±1.65
70	0.609	0.331	274.7	71.73	4.10	14.83
	±.122	±.047	±41.5	±5.61	±.49	±1.73
96	0.423	0.237	241.9	59.10	4.39	17.65
	±.032	±.017	±21.2	±4.68	±.48	±1.45

liters for the 70° F period and 121.3 liters for pasture bulls. When $V_1 + V_2$ was expressed in milliliter per pound of body weight, the values were 205.5, 150.9, and 122.4 milliliter per pound, respectively for the 96° F, 70° F, and pasture periods. When MCR was expressed in terms of liters per pound of body weight per day, the three periods were not significantly different at the 5% level. However, the increased volume cleared during the 96° F period was different from the other two periods at the 10% probability level and closely approached significance at the 5% level.

These data seem to indicate that prolonged exposure to 96° F temperature, 50% relative humidity has little demonstrable effect on the metabolism of testosterone. However, there appears to be a very definite expansion of pool volumes and a resultant nonsignificant, but obvious, increase in MCR.

CHAPTER V

DISCUSSION

Introduction

During the course of this experiment, physical and endocrine changes transpired in Hereford bulls exposed to high ambient temperatures. The physical changes were easily observed and confirmed. Rectal temperature, respiration rate, and semen characteristics are parameters which have been measured repeatedly by previous researchers. On the other hand, endocrine changes measured in this study represent unique observations not previously recorded. Thus, standard physical criteria have been established which can be used as valuable references for judging the import of less clearly understood endocrine changes.

Rectal Temperature and Respiration Rate

Rectal temperature and respiration rate data confirmed observations made by other workers under similar environmental conditions (Kibler, *et al.*, 1965; Johnston, *et al.*, 1963, Hewitt, 1921; Williams and Bell, 1963). Both rectal temperature and respiration rate are quite stable at 70° F, 50% RH, and this seems to be an ideal "normal" environment. Ninety-six degrees, F, 50% RH appears to be a suitable environment for "heat stress" experiments in bulls. The thermal load was sufficient to increase respiration rate two to three times, and rectal temperature two to 5° F without causing the bulls to reduce food intake. These

physical signs indicate that the animals were heat stressed but that no acclimation took place during the heat treatment since rectal temperature and respiration rate were elevated during the entire period.

Semen Characteristics and Testicular Morphology

Exposing Hereford bulls to high environmental temperature (96° F, 50% RH) produced deleterious changes in both semen characteristics and testicular morphology. The changes observed were generally the same as those reported by Steinberger, *et al.* (1959), Okomoto, *et al.* (1962), and Skinner and Louw (1966). When semen was collected from the bulls at the end of the 70° F control period, it exhibited characteristics which indicated that it would have been capable of fertilizing normal females. Examination of semen collected from the bulls, after seven weeks of exposure to 96° F, indicated the bulls were essentially sterile. Degeneration of the germinal epithelium was seen in histological preparations of the testes after heat exposure. Normal tubules were extremely scarce or absent. Damage to the tubules was generally severe, and in some areas, only Sertoli cells and a few spermatogonia were present. These lesions in the testes were severe enough to account for the changes in semen characteristics. The interstitial cells of Leydig appeared unchanged, and it was impossible to make any correlation between morphological and endocrinological changes which were observed. It was concluded that exposure to 96° F, 50% RH led to an intratesticular temperature, which was incompatible with spermatogenesis in Hereford bulls.

Cortisol and Corticosterone Plasma Concentration

Lindner (1964) reported plasma cortisol levels in the non-pregnant ox to be 26.0 ng/ml. Saba (1964) reported that cortisol concentration in pooled bovine plasma was 5.0 ng/ml. The results of this study (Table IX) indicate that under the 70° F control conditions imposed upon the bulls, plasma cortisol levels of Hereford bulls were intermediate between Lindner's and Saba's values. Over a five week period, plasma cortisol values in the eight Hereford bulls averaged 17.45 ng/ml. Venkataseshu (1965) reported plasma cortisol values in dairy cows varying from 65 to 95 ng/ml. For a number of technical reasons, those values must be regarded as inordinately high.

Generally, researchers have been unable to quantitate corticosterone in the peripheral plasma of the bovine, but a number of workers have noted that "low levels" or "traces" were present. Venkataseshu (1965) reported plasma concentrations in the dairy cow to be between 24.0 and 46.0 ng/ml. Plasma corticosterone concentration during the 70° F control period was found to average 3.0 ng/ml for the Hereford bulls in this study. Whipp, *et al*. (1967) reported that in bovine adrenal effluent blood the cortisol:corticosterone ratio was approximately 5:1. In this study the cortisol:corticosterone ratio in peripheral blood was found to be 6:1 at 70° F and 4:1 at 96° F.

Glucocorticoid Response to Excitement

The excitement and stress associated with penning and bleeding the bulls for the first time at Fort Reno Research Station seemed to stimulate a marked rise in plasma glucocorticoids. During this initial experience with bleeding procedures, all of the animals showed extreme

psychological distress. Plasma levels of cortisol and corticosterone were the highest recorded during the entire experiment (Tables IX and XII). After the bulls had been confined for three weeks, halter broken, and accustomed to being fastened in their individual stanchions, they were quite gentle and not the least apprehensive when bled. This psychological adjustment was apparently reflected by significantly lower ($P < .01$) plasma levels of both corticoids during the 70° F control period. Cortisol concentration declined to 55% of its level in the Fort Reno test, while corticosterone fell to 45% of its previous value.

These data tend to confirm the established fact that a variety of stressors can effect the release of ACTH from the anterior pituitary and bring about a rapid increase in plasma glucocorticoid concentrations. Perhaps differences in control conditions and bleeding methods could account for a part of the large variation in plasma cortisol values which have been reported by various workers with the bovine. These reports vary from low levels of 5 ng/ml (Saba, 1964) to high levels of 65 to 95 ng/ml (Venkataseshu, 1965). In this respect it would appear that it may be extremely difficult, perhaps impossible, to compare the data from different experiments, unless it was known that the studies were conducted with experienced animals under almost identical circumstances.

Effects of Heat on Cortisol Concentration

It was somewhat surprising to find that plasma cortisol levels in peripheral plasma dropped approximately 33% during the first 24 hours of heat treatment and remained near that level for the balance of the

heat exposure. It was anticipated that there might be some increase in cortisol concentration on initial exposure to 96° F temperature even though Bergman (1963) reported depressed levels of cortisol in Holstein cows exposed to 84° F for several weeks. Perhaps this level of heating (96° F) was not high enough to produce the type of acute stress which could result in increased cortisol levels, or else any cortisol increase could have been rapid and transient, thus not detected at 24 hours. Of course the possiblity also must be considered that no increase in peripheral cortisol concentration took place.

It would appear that the primary response by the bovine to heat stress is a lowering of plasma cortisol concentration. This hypothesis seems quite logical since cortisol is known to be calorigeneic. Johnston, et al. (1958, 1963) showed that metabolism was depressed in cattle exposed to heat. The bovine, not being a sweating animal, has difficulty in dissipating body heat when exposed to high ambient temperature; so there are a number of physiological mechanisms which act to decrease the heat load in hot environments. Lowering plasma cortisol concentration may be one of these mechanisms.

There are two major physiological adjustments by the body which could effect lowered levels of plasma cortisol. Increased metabolism is one; the other is decreased secretion by the adrenal cortex. Bergman (1963) conducted cortisol secretion rate studies in Holstein cows while exposing them to mild heat (84° F). Although the results were not absolutely conclusive, they did tend to show that adrenal cortical secretion of cortisol was depressed as a result of exposure to the hot environment.

Suppression of cortisol secretion could come about as a result of

some direct effect of heat on the adrenal cortex, or secondarily, through depression of ACTH secretion or release from the anterior pituitary. It is the author's opinion that the latter possibility is more likely the case. This opinion is based on the fact that cortisol levels were elevated during the last week of the 96° F period in response to psychological stress associated with implanting jugular catheters for use in testosterone production rate studies (Table XI). The ability of the adrenal cortex to raise plasma cortisol concentration in these heat depressed bulls indicated that the cortex was potentially capable of producing increased amounts of cortisol when centrally stimulated.

If this last explanation can be accepted, it would appear that exposure to 96° F temperature results in inhibition of the synthesis or release of ACTH. In the future, heat stress experiments need to be conducted in which plasma levels of ACTH are measured. Then by blocking pituitary release of ACTH with exogenous corticoid, or by blocking cortisol production with metapirone, perhaps the action of heat on the hypothalamo-hypophyseal-adrenal axis could be elucidated.

Desjardins and Ewing (1971) have recently shown that adrenalectomy in male rats does not alter the capacity of the testes to produce testosterone *in vivo*. In view of this finding it seems unlikely that the heat induced depression of plasma cortisol played any significant role in the temporary lowering of plasma testosterone levels. A more reasonable hypothesis might suggest that depression of both cortisol and testosterone by heat were brought about through similar mechanisms.

Effects of Heat on Corticosterone Concentration

Plasma levels of corticosterone were not significantly ($P > .05$) altered during the seven weeks of heat treatments (Table XIV). Such constancy was unexpected in light of the dramatic depression which took place in plasma cortisol concentration. Any explanation of why the two corticoids behaved differently would be highly speculative.

It is likely that the low levels of corticosterone may not represent biologically important glucocorticoid secretion. Possibly enough corticosterone may "leak" from the adrenal gland to account for its low concentration in the blood. This explanation would be particularly appropriate in the bovine if corticosterone were simply an "intermediate compound" in adrenal steroid synthesis. Of course it is also possible that corticosterone functions in some capacity, other than as a glucocorticoid. In this case, its regulation may be different from that of cortisol and may not be affected by heat. In any case, this study confirms previous observations that cortisol is the predominant glucocorticoid secreted by the bovine. Additional experiments will be needed to elucidate the function and control of corticosterone in the bovine.

Plasma Testosterone Concentration

The bull testis has the distinction of being the source from which testosterone was first isolated. David, *et al.* (1935) prepared 10 mg of crystalline testosterone from 100 kg of bull testes, but it was almost 15 years before Lindner (1959) conclusively identified it in bovine testicular venous effluent. Savard, *et al.* (1961) confirmed Lindner's work and additionally reported the average value of testosterone in the peripheral plasma of the bull to be approximately 30.0

ng/ml. In the present experiment testosterone concentration in the peripheral plasma of Hereford bulls under control conditions (70° F, 50% RH) was found to average 3.26 ng/ml (Talbe XV). Researchers at Michigan State University recently measured testosterone concentration in the plasma of Holstein bulls and found that it varied from 4.0 to 6.0 ng/ml (Hafs, 1971). Peripheral testosterone concentrations found in other animals (rabbit, rat, dog, monkey, man) generally fall in the range from 3.0 to 10.0 ng/ml.

One significant observation made during this study concerned the extreme variability in testosterone concentration within and among bulls. It was not unusual to observe a five to ten-fold increase or decrease in an individual bull from one week to the next. By the same token an individual bull might have the lowest testosterone concentration in the group one week, but have the highest level the next week. In light of these observations it would seem that the popular concept of the testis being an organ which secretes its endocrine products at a constant rate will have to be reviewed. Experiments should be designed in the future to discover whether or not testosterone production shows any cyclicity, other than diurnal variation. These studies would ideally incorporate measurement of peripheral levels of LH in order to further elucidate the role of the pituitary in testicular endocrine function.

Effects of Heat on Testosterone Concentration

The overall mean value of plasma testosterone concentration was significantly ($P < .01$) depressed when bulls were exposed to 96° F heat treatment for seven weeks (Table XVII). Testosterone concentration

fell dramatically during the first few days of heat exposure, reached 50% of the control value, and remained at this level for approximately two weeks. Subsequently, testosterone levels rose erratically, so that by the end of the seventh week of heat exposure, they had returned to 80% of control values. This study appears to present the first conclusive evidence found in any species that environmental heat can lower plasma values of testosterone.

The import of this transient reduction is not readily apparent. However, since testosterone is known to be an anabolic steroid, reduction of its availability to peripheral tissues could theoretically lower heat production considerably. Whether there was enough reduction in testosterone to have any effect on spermatogenesis is not known.

The same questions must be asked about testosterone as were asked concerning cortisol. Were plasma levels of the steroid reduced because of increased peripheral utilization in the face of normal production by the testes; or was testicular production rate decreased? If there was a change in production rate, was it brought about by the direct effect of heat on the testis or indirect suppression through lowered pituitary gonadotrophin production, or both?

The answer to the first question appears to be that testicular testosterone production was reduced. A discussion of the reasons underlying this conclusion are presented in the next section of this paper under "Production Rate Studies".

The answer to the second question will have to await further investigation since the data from this study do not offer adequate information for a logical decision. MacFarlane (1957) presented indirect evidence of gonadotropic suppression by environmental heat. Numerous

other workers have shown that heat may directly lower androgen production in the cryptorchid testis. However, no matter how the reduction of testosterone levels was brought about, there is evidence that the mechanism was subject to acclimation. This is shown by the gradual return of testosterone concentration from low levels to near normal levels toward the end of the hot period (Table XVII).

Testosterone Production Rate

Distribution Spaces

The terms, compartment and pool, will be used frequently and interchangeably in the following discussion. They do not necessarily denote anatomical compartments. Rather, they are functional entities (distribution spaces), which in certain cases, coincide closely with some anatomical structure, such as the liver.

A pool, or compartment, may be defined as a space in which an injected specific substance mixes immediately and completely with all other molecules of the same species. If, for example, radioactive testosterone is injected intravenously, and the concentration of radioactivity (in the form of the labeled steroid) declines with time as a straight line, it can be assumed that the testosterone has been injected into a single functional compartment. If a second exponential is needed to describe the disappearance curve of testosterone, a second compartment is assumed.

Figures 8, 9, and 10 illustrate that the disappearance curve of testosterone from bull blood is, in fact, a double exponential function. This indicates that there must be two functional distribution spaces ($V_1 + V_2$). Seven weeks exposure to 96° F temperature brought about

significant increases ($P < .01$) in the volumes of all body pools (V_1, V_2, $V_1 + V_2$). This increase was evident whether the pool size was expressed as liters per animal or adjusted to body size by calculating milliter per pound of body weight. Such an increase in the total distribution space of testosterone would not be surprising, since it is an established fact that in hot environments there may be considerable expansion of the plasma volume, as well as total body water. In cattle this increase could be as much as 20 to 30% of the control value. Chronic peripheral vasodilation accompanies chronic exposure to heat; it is undoubtedly an important causitive factor in the expansion of plasma volumes.

Expansion of the total distribution space of testosterone could conceivably have been an important factor in the lowering of plasma testosterone values during the first two weeks of the hot period. If during these first two weeks of heat treatment, testosterone production and removal from the plasma remained constant, and there was a significant increase in distribution space, then there would have been a proportional drop in testosterone concentration. In contrast, if testosterone secretion rates had fallen, or hepatic removal increased even slightly, a large fall in plasma testosterone concentration might have occurred. However, it appears unlikely that any of these assumptions could explain the magnitude of change seen in plasma testosterone values.

Metabolic Clearance Rate (MCR)

Since there was a significant increase ($P < .05$) in the body weight of the eight Hereford bulls during the six months of this study, it was

necessary to express MCR in terms of liters per pound of body weight per day. This allowed evaluation of heat treatment effects on MCR. During the seven weeks of heat treatment MCR increased from 13.6 liters per pound per day to 15.7 liters per pound per day. This 15% increase was not significant at the critical five per cent level, but did closely approach it. Significance was seen at $P < .10$. The increase in MCR was directly related to expansion of the inner pool volume (V_1).

Metabolic clearance rate is related to volume of the inner pool in the following manner: $MCR = V_1 \times K_2$. The value of K_2 (metabolic constant for the inner pool) did not change significantly ($P > .05$) during heat treatment, and thus, was a constant value. Therefore, MCR was proportional to V_1, and the significant rise ($P < .01$) in V_1 should have been reflected in MCR values. However, MCR values were extremely variable between animals. This variation prevented expression of significance at the 5% level. Nevertheless, the trend was obviously toward a higher MCR at the end of the heat treatment period than was seen at the beginning.

Biological Half-Life of Testosterone

In the bull the biological half-life of testosterone appears to be quite stable, having an average duration of 4.31 minutes in the body compartment where rapid metabolism occurs. This value is remarkably close to the mean value of 4.98 minutes reported by Horton (1965) in five normal men. In fact, both man and bull yield testosterone production rate data which is quite similar in most parameters after corrections are made for differences in body weight. Seven weeks of exposure to 96° F temperature did not significantly alter ($P > .05$) the half-life

of testosterone in this study.

Twenty-Four Hour Production Rate

Twenty-four hour production rates were extremely variable and ranged between ten and 100 mg/day, with a mean value of 44.7 mg/day during the control period. This mean value, when adjusted for difference in body weight, is similar to the 8.0 mg/day reported for four normal men by Southren, et al. (1965). Heating the bulls for seven weeks at 96° F did not produce any significant change in testosterone production rate. Offsetting changes in MCR and plasma testosterone concentration probably accounted for the production rates being similar at the end of the control period and heat treatment period. There was approximately a 15% rise in MCR after heating, but a corresponding 15% fall in plasma testosterone concentration also occurred. Since PR = MCR x Conc., there was little change seen in PR.

The data from this study seems to indicate that 24 hour testosterone production rate in Hereford bulls is proportional to plasma testosterone concentration. All of the other factors analyzed were relatively stable when compared to testosterone concentration. However, secretion rate studies were only conducted at the end of the control period and at the end of the heating period. If acclimation was complete by the end of the heating period, then changes which took place during the early portion of heat exposure would not have been seen. This might have occurred in the study since there is some indication from plasma testosterone levels that acclimation did occur. Ideally, production rate studies should have been conducted during the first two weeks of heat stress when there was significant depression of

testosterone concentration. It would then have been possible to determine whether plasma concentration was reduced due to less testicular secretion or more rapid metabolism.

CHAPTER VI

SUMMARY AND CONCLUSIONS

Eight Hereford bulls, approximately 24 months of age, were confined in an environmental chamber for a 14 week experimental interval. This period was divided into two consecutive seven week treatment segments. Ambient temperature was the only experimental variable. Throughout the initial (control) period, temperature was maintained at 70° F, 50% RH; during the second (hot) treatment period, ambient temperature was kept at 96° F, 50% RH.

Rectal temperature and respiration rate of each animal was measured daily. One hundred ml jugular blood samples were collected at weekly intervals, except during the first week of heat treatment, when they were collected daily. Subsequently, each blood sample was quantitatively analyzed for testosterone, cortisol, and corticosterone. Testosterone half-life, metabolic clearance rate, and 24 hour production rate were evaluated at the beginning and end of each treatment segment. This was accomplished by monitoring the disappearance from plasma of a single intravenously injected dose of tritium-labeled testosterone. Following the final day of each treatment period, semen samples were collected and evaluated for spermatozoa motility, concentration, percent alive, and morphology. The experiment was terminated by removing the bulls from the environmental chamber and slaughtering them at a local abattoir. At that time, tissue samples from the reproductive tract and

adrenal glands were collected. These tissues were evaluated for histopathological changes.

The results of this study show that prolonged exposure of bulls to an ambient temperature of 96° F at 50% RH produced significant effects on rectal temperature, respiration rate, plasma cortisol and testosterone concentration, and spermatogenesis. However, no effect was seen on plasma corticosterone concentration or testosterone production rate.

Both rectal temperature and respiration rate were increased significantly in response to the hot environment. This confirmed observations made by previous workers (Kibler, *et al.*, 1965; Johnston, *et al.*, 1963). Apparently no acclimation took place during the hot period since rectal temperature and respiration remained constantly elevated during the entire period.

Degenerative changes took place in the physical characteristics of ejaculated semen and testicular morphology, which conformed to changes reported by Okomoto, *et al.* (1962) and Skinner and Louw (1966) under similar circumstances. Histological preparations of the testes revealed severe damage to the germinal epithelium, and only spermatogonia remained in most tubules. Interstitial cells of Leydig appeared normal. The histopathological changes seen in the testes were sufficient to effect reduction in semen quality. It was concluded that 96° F exceeded the temperature at which bulls could maintain intratesticular temperature low enough to insure normal gametogenesis.

Cortisol was shown to be the predominant glucocorticoid in bulls. Seven weeks exposure to hot thermal conditions depressed bovine plasma cortisol concentrations. This was indicated by the significant decrease in plasma cortisol concentrations at 96° F after previous exposure to

70° F chamber temperature. The eight bulls had a mean plasma cortisol concentration of 17.45 ng/ml during the five weeks prior to the elevation in chamber temperature. In the first 24 hours following temperature increase, the mean value dropped to 11.93 ng/ml. Cortisol levels continued to be depressed during the high temperature treatment and showed no signs of acclimation.

This depression of plasma cortisol levels contrasts with the usual elevation of plasma corticoids seen in response to other stressful stimuli. However, the effects of "heat stress" on the adrenal cortex have not been extensively investigated in any species. The most logical conclusion to be reached from the foregoing observations is that the depression in plasma cortisol was a specific response by the bulls to a specifically applied heat stress. In view of the fact that Evans, et al. (1957) and Shida and Barker (1962) showed that cortisol promotes calorigenesis, it may be further concluded that the depression is a protective adjustment by the animals to lessen heat production.

There was no obvious effect of the hot environment on plasma levels of corticosterone, which averaged approximately 2.80 ng/ml over both seven week periods. This is quite different from the dramatic fall seen in cortisol levels. Perhaps there is some basic difference in the regulatory control or function of corticosterone. On the other hand, corticosterone may have little biological significance in the bovine. Corticosterone could be an intermediate compound in adrenal steroid synthesis which simply "leaks" from the gland, producing low plasma concentrations. Additional reserach will be necessary before a definite conclusion can be reached.

Plasma testosterone declined steadily from 3.24 ng/ml to 1.62 ng/ml

during the first week of heat exposure and remained low through the second week. However, values gradually rose to pre-treatment levels by the end of the hot period. This is a typical pattern of acclimation, indicating that the bulls were unable to maintain testosterone levels in peripheral plasma during the early part of heat exposure. Nevertheless, physiological adjustments occurred with time, resulting in restoration of testosterone to the control concentration. It would be necessary to obtain additional data, such as peripheral levels of luteinizing hormone, or testosterone production rates, before definite conclusions could be reached regarding the physiological mechanisms involved in these changes. These data support the conclusion that a hot environment tends to depress the concentration of testosterone in the peripheral plasma until the animal becomes acclimated.

Testosterone production rate studies were made at the beginning and end of both control and hot periods. No significant differences were noted in testosterone half-life, metabolic clearance rate, or 24 hour production rate. Prior to heating, production rate was 44.65 mg/day; after seven weeks, it was 47.29 mg/day. During both periods, production rate was proportional to plasma testosterone concentration. These observations tend to support the previous conclusion that the bulls were able to acclimate testicular endocrine function when exposed to 96° F for seven weeks. Future experiments should be designed to evaluate production rate during a period of depressed plasma testosterone concentration, thus elucidating the mechanisms which bring about acclimation.

The purpose of this study was to evaluate the effects of seven weeks exposure to high ambient temperatures (96° F, 50% RH) on adrenal

cortical and testicular endocrine function in the Hereford bull. The author's final conclusions are: 1) adrenal secretion of cortisol is rapidly and continuously depressed for at least seven weeks by high ambient temperature (96° F); 2) adrenal secretion of corticosterone is unaffected by heat exposure; and 3) testicular testosterone secretion can be depressed by 96° F ambient temperature. However, the effect is transient.

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APPENDIX A

TABLE I

COMPOSITION OF RATION

Ingredients		Pounds
Shelled Yellow Corn (Steam Rolled)		650
Oats (Steam Rolled)		200
Wheat Bran		200
Cotton Seed Meal (42%)		200
Dehydrated Alfalfa		200
Cotton Seed Hulls		440
Molasses (Liquid)		100
Salt		10
	TOTAL	2,000

APPENDIX B

TABLE XXI

ANALYSIS OF VARIANCE OF WEEKLY RECTAL TEMPERATURES OF EIGHT HEREFORD BULLS EXPOSED TO 96° F, 50% RH[1]

Source of Variance	Degrees of Freedom	Sum of Squares	Mean Square	F Ratio
Total	64	88	-	-
Replicates	1	2	2.00	2.67
Treatment	7	44	6.29	8.39**
Error	56	42	0.75	-

[1]Relative humidity.

**P < .01.

TABLE XXII

NEWMAN-KEULS SEQUENTIAL RANGE TEST[1] APPLIED TO WEEKLY RECTAL TEMPERATURES OF EIGHT HEREFORD BULLS EXPOSED TO 96° F, 50% RH[2]

Treatment (Weeks)	0	4	5	3	6	2	7	1
Mean $^{\circ}$F	100.0	101.9	102.3	102.4	102.8	102.9	103.3	103.7
Number of Means Degrees of Freedom = 56		2	3	4	5	6	7	8
Value of Q .05 S.E.M. = 0.31		2.85	3.41	3.75	3.99	4.17	4.32	4.45
Value of D		0.88	1.06	1.16	1.24	1.29	1.34	1.38

[1]Snedecor and Cochran (273).

[2]Relative humidity.

TABLE XXIII

ANALYSIS OF VARIANCE OF DAILY RECTAL TEMPERATURES OF EIGHT HEREFORD BULLS EXPOSED TO 96° F, 50% RH[1]

Source of Variance	Degrees of Freedom	Sum of Squares	Mean Square	F Ratio
Total	64	209	-	-
Replicates	1	24	24.0	18.5**
Treatment	7	116	16.6	12.8**
Error	56	69	1.3	-

[1]Relative humidity.

**P < .01.

TABLE XXIV

NEWMAN-KEULS SEQUENTIAL RANGE TEST[1] APPLIED TO DAILY RECTAL TEMPERATURES OF EIGHT HEREFORD BULLS EXPOSED TO 96° F, 50% RH[2]

Treatment (Days)	0	1	7	2	5	4	6	3
Mean °F	100.0	102.8	103.1	103.4	103.8	104.1	104.3	104.5
Number of Means Degrees of Freedom = 56		2	3	4	5	6	7	8
Value of Q .05 S.E.M. = 0.46		2.85	3.41	3.75	3.99	4.17	4.32	4.45
Value of D		1.30	1.55	1.71	1.82	1.90	1.97	2.03

[1]Snedecor and Cochran (273).

[2]Relative humidity.

TABLE XXV

ANALYSIS OF VARIANCE OF WEEKLY RESPIRATION RATE OF EIGHT HEREFORD BULLS EXPOSED TO 96° F, 50% RH

Source of Variance	Degrees of Freedom	Sum of Squares	Mean Square	F Ratio
Total	64	18,025	-	-
Replicates	1	20	20	0.01
Treatment	7	13,889	1984	27**
Error	56	4,116	74	-

[1]Relative humidity.

**P < .01.

TABLE XXVI

NEWMAN-KEULS SEQUENTIAL RANGE TEST[1] APPLIED TO WEEKLY RESPIRATION RATE OF EIGHT HEREFORD BULLS EXPOSED TO 96° F, 50% RH[2]

Treatment (Weeks)	0	4	5	7	6	3	1	2
Mean	33	59	61	63	67	73	81	82
Number of Means Degrees of Freedom = 56		2	3	4	5	6	7	8
Value of Q .05 S.E.M. = 3.04		2.85	3.41	3.75	3.99	4.17	4.32	4.45
Value of D		8.60	10.04	11.04	12.01	12.70	13.10	13.50

[1]Snedecor and Cochran (273).

[2]Relative humidity.

TABLE XXVII

ANALYSIS OF VARIANCE OF DAILY RESPIRATION RATE OF EIGHT HEREFORD BULLS EXPOSED TO 96° F, 50% RH[1]

Source of Variance	Degrees of Freedom	Sum of Squares	Mean Square	F Ratio
Total	64	25,145	-	-
Replicates	1	2,525	2,525	21**
Treatment	7	15,845	2,264	19**
Error	56	6,775	121	-

[1]Relative humidity.

**P < .01.

TABLE XXVIII

NEWMAN-KEULS SEQUENTIAL RANGE TEST[1] APPLIED TO DAILY RESPIRATION RATE OF EIGHT HEREFORD BULLS EXPOSED TO 96° F, 50% RH[2]

Treatment (Days)	0	1	4	2	5	7	3	6
Mean	33	73	73	76	78	81	83	84
Number of Means Degrees of Freedom = 56		2	3	4	5	6	7	8
Value of Q .05 S.E.M. = 4.56		2.85	3.41	3.75	3.99	4.17	4.32	4.45
Value of D		12.98	15.54	17.09	18.18	19.00	19.68	20.27

[1]Snedecor and Cochran (273).

[2]Relative humidity.

TABLE XXIX

ANALYSIS OF VARIANCE OF WEEKLY CORTISOL CONCENTRATION OF EIGHT HEREFORD BULLS EXPOSED TO 70° F, 50% RH[1]

Source of Variance	Degrees of Freedom	Sum of Squares	Mean Square	F Ratio
Total	48	2,525	-	-
Replicates	1	3	3	0.1
Treatment	5	1,614	269	12.0**
Error	42	908	22	-

[1]Relative humidity.

**P < .01.

TABLE XXX

NEWMAN-KEULS SEQUENTIAL RANGE TEST[1] APPLIED TO WEEKLY PLASMA CORTISOL CONCENTRATION OF EIGHT HEREFORD BULLS EXPOSED TO 70° F, 50% RH[2]

Treatment (Weeks)	7	3	6	4	5	0
Mean	13.15	16.45	17.2	19.75	20.50	31.90
Number of Means Degrees of Freedom = 42		2	3	4	5	6
Value of Q .05 S.E.M. = 1.65		2.86	3.44	3.79	4.04	4.23
Value of D		4.75	5.70	6.26	6.68	7.00

[1]Snedecor and Cochron (273).

[2]Relative humidity.

TABLE XXXI

ANALYSIS OF VARIANCE OF DAILY PLASMA CORTISOL CONCENTRATION OF EIGHT HEREFORD BULLS EXPOSED TO 96° F, 50% RH

Source of Variance	Degrees of Freedom	Sum of Squares	Mean Square	F Ratio
Total	64	1,116.0	-	-
Replicates	1	23.4	23.4	2.09
Treatment	7	466.8	66.7	5.97**
Error	56	625.8	11.2	-

[1]Relative humidity.

**$P < .01$.

TABLE XXXII

NEWMAN-KEULS SEQUENTIAL RANGE TEST[1] APPLIED TO DAILY PLASMA CORTISOL CONCENTRATION OF EIGHT HEREFORD BULLS EXPOSED TO 96° F, 50% RH[2]

Treatment (Days)	6	2	4	3	7	5	1	0
Mean	9.39	9.83	10.05	10.74	11.19	11.33	11.93	18.03
Number of Means Degrees of Freedom = 56		2	3	4	5	6	7	8
Value of Q .05 S.E.M. = 1.20		2.85	3.41	3.75	3.99	4.17	4.32	4.45
Value of D		3.42	4.09	4.50	4.79	5.00	5.18	5.34

[1]Snedecor and Cochran (273).

[2]Relative humidity.

TABLE XXXIII

ANALYSIS OF VARIANCE OF WEEKLY CORTISOL CONCENTRATION OF EIGHT HEREFORD BULLS EXPOSED TO 96° F, 50% RH[1]

Source of Variance	Degrees of Freedom	Sum of Squares	Mean Square	F Ratio
Total	64	1,244.0	-	-
Replicates	1	12.7	12.7	0.90
Treatment	7	448.3	64.0	4.57**
Error	56	783.0	14.0	-

[1]Relative humidity.

**P < .01.

TABLE XXXIV

NEWMAN-KEULS SEQUENTIAL RANGE TEST[1] APPLIED TO WEEKLY PLASMA CORTISOL CONCENTRATION OF EIGHT HEREFORD BULLS EXPOSED TO 96° F, 50% RH[2]

Treatment (Weeks)	5	6	2	4	1	3	7	0
Mean	10.37	10.45	11.04	11.11	11.19	12.60	15.79	18.03
Number of Means Degrees of Freedom = 56		2	3	4	5	6	7	8
Value of Q .05 S.E.M. = 1.33		2.85	3.41	3.75	3.99	4.17	4.32	4.45
Value of D		3.79	4.54	4.99	5.31	5.54	5.75	5.91

[1]Snedecor and Cochron (273).

[2]Relative humidity.

TABLE XXXV

ANALYSIS OF VARIANCE OF WEEKLY PLASMA CORTICOSTERONE CONCENTRATION OF EIGHT HEREFORD BULLS EXPOSED TO 70° F, 50% RH[1]

Source of Variance	Degrees of Freedom	Sum of Squares	Mean Square	F Ratio
Total	48	154.8	-	-
Replicates	1	0.9	0.9	0.50
Treatment	5	82.7	13.8	7.94**
Error	42	71.2	1.7	-

[1]Relative humidity.

**P < .01.

TABLE XXXVI

NEWMAN-KEULS SEQUENTIAL RANGE TEST[1] APPLIED TO WEEKLY PLASMA CONCENTRATION OF EIGHT HEREFORD BULLS EXPOSED TO 70° F, 50% RH[2]

Treatment (Weeks)	7	3	5	4	6	0
Mean	2.67	2.83	3.26	3.34	3.92	6.47
Number of Means Degrees of Freedom = 42		2	3	4	5	6
Value of Q .05 S.E.M. = 0.433		2.86	3.44	3.99	4.04	4.23
Value of D		1.24	1.49	1.73	1.75	1.83

[1]Snedecor and Cochron (273).

[2]Relative humidity.

TABLE XXXVII

ANALYSIS OF VARIANCE OF DAILY PLASMA CORTICOSTERONE CONCENTRATION OF EIGHT HEREFORD BULLS DURING FIRST WEEK OF EXPOSURE TO 96° F, 50% RH[1]

Source of Variance	Degrees of Freedom	Sum of Squares	Mean Square	F Ratio
Total	64	56.1	-	-
Replicates	1	0.0	-	-
Treatment	7	6.6	0.94	1.07
Error	56	49.5	0.88	-

[1]Relative humidity.

TABLE XXXVIII

ANALYSIS OF VARIANCE OF WEEKLY PLASMA CORTICOSTERONE CONCENTRATION OF EIGHT HEREFORD BULLS EXPOSED TO 96° F, 50% RH[1]

Source of Variance	Degrees of Freedom	Sum of Squares	Mean Square	F Ratio
Total	64	64.7	-	-
Replicates	1	0.7	0.70	0.78
Treatment	7	11.8	1.68	1.81
Error	56	52.2	0.93	-

[1]Relative humidity.

TABLE XXXIX

ANALYSIS OF VARIANCE OF WEEKLY PLASMA TESTOSTERONE CONCENTRATION OF EIGHT HEREFORD BULLS EXPOSED TO 96° F, 50% RH[1]

Source of Variance	Degrees of Freedom	Sum of Squares	Mean Square	F Ratio
Total	64	51.00	-	-
Replicates	1	1.83	1.83	3.21
Treatment	7	17.53	2.50	4.39**
Error	56	31.64	0.57	-

[1]Relative humidity.

**P < .01.

TABLE XL

NEWMAN-KEULS SEQUENTIAL RANGE TEST[1] APPLIED TO WEEKLY PLASMA TESTOSTERONE CONCENTRATION OF EIGHT HEREFORD BULLS EXPOSED TO 96° F, 50% RH[2]

Treatment (Weeks)	2	1	5	3	6	7	4	0
Mean	1.40	1.60	1.98	2.22	2.72	2.74	2.76	3.24
Number of Means Degrees of Freedom = 56		2	3	4	5	6	7	8
Value of Q .05 S.E.M. = .273		2.85	3.41	3.75	3.99	4.17	4.32	4.45
Value of D		0.78	0.93	1.02	1.09	1.14	1.18	1.21

[1]Snedecor and Cochron (273).

[2]Relative humidity.

TABLE XLI

ANALYSIS OF VARIANCE OF DAILY PLASMA TESTOSTERONE CONCENTRATION OF EIGHT HEREFORD BULLS EXPOSED TO 96° F, 50% RH[1]

Source of Variance	Degrees of Freedom	Sum of Squares	Mean Square	F Ratio
Total	64	135.94	-	-
Replicates	1	4.46	4.46	2.29
Treatment	7	22.45	3.21	1.65
Error	56	109.05	1.95	-

[1]Relative Humidity.

TABLE XLII

ANALYSIS OF VARIANCE OF 1,2-^{3}H-TESTOSTERONE HALF-LIFE (t $\frac{1}{2}$ A) IN THE PLASMA OF EIGHT HEREFORD BULLS EXPOSED TO VARIOUS LEVELS OF AMBIENT THERMAL CONDITIONS

Source of Variance	Degrees of Freedom	Sum of Squares	Mean Square	F Ratio
Total	24	1.848	-	-
Treatment	2	0.292	0.146	2.05
Error	22	1.556	0.071	-

TABLE XLIII

ANALYSIS OF VARIANCE OF 1,2-^{3}H-TESTOSTERONE HALF-LIFE (t $\frac{1}{2}$ B) IN THE PLASMA OF EIGHT HEREFORD BULLS EXPOSED TO VARIOUS LEVELS OF AMBIENT THERMAL CONDITIONS

Source of Variance	Degrees of Freedom	Sum of Squares	Mean Square	F Ratio
Total	24	496.0	-	-
Replicates	1	3.59	3.59	0.17
Treatment	2	57.6	28.80	1.39
Error	21	435.0	20.70	-

TABLE XLIV

ANALYSIS OF VARIANCE OF BODY WEIGHT OF EIGHT HEREFORD BULLS EXPOSED TO VARIOUS LEVELS OF AMBIENT THERMAL CONDITIONS

Source of Variance	Degrees of Freedom	Sum of Squares	Mean Square	F Ratio
Total	24	246,468	-	-
Treatment	2	63,608	31,804	3.8**
Error	22	182,860	8.312	-

**P < .05.

TABLE XLV

NEWMAN-KEULS SEQUENTIAL RANGE TEST[1] APPLIED TO BODY WEIGHTS OF EIGHT HEREFORD BULLS EXPOSED TO VARIOUS AMBIENT THERMAL CONDITIONS

Treatments	60° F, 50% RH[2]	70° F, 50% RH	96° F, 50% RH
Mean	973	1,014	1,097
Number of Means Degrees of Freedom = 22	1	2	3
Value of Q .05 S.E.M. = 32.2		2.95	3.58
Value of D		95.	115.

[1]Snedecor and Cochron (273).

[2]Relative humidity.

TABLE XLVI

ANALYSIS OF VARIANCE OF TESTOSTERONE MCR[1] OF EIGHT HEREFORD BULLS EXPOSED TO VARIOUS AMBIENT THERMAL CONDITIONS

Source of Variance	Degrees of Freedom	Sum of Squares	Mean Square	F Ratio
Total	24	344,990,948	-	-
Replicates	1	26,649,267	26,649,267	2.66
Treatment	2	107,960,056	53,980,028	5.39**
Error	21	210,381,625	10,018,172	-

[1]Metabolic clearance rate, liters/day.

**P < .05.

TABLE XLVII

NEWMAN-KEULS SEQUENTIAL RANGE TEST[1] APPLIED TO TESTOSTERONE MCR[2] OF EIGHT HEREFORD BULLS EXPOSED TO VARIOUS AMBIENT THERMAL CONDITIONS

Treatment	60° F, 50% RH	70° F, 50% RH	96° F, 50% RH
Mean	12,038	13,782	17,198
Number of Means Degrees of Freedom = 21	1	2	3
Value of Q .05 S.E.M. = 1190		2.96	3.57
Value of D		3,522	4,248

[1]Snedecor and Cochron (273).

[2]Metabolic clearance rate, liters/day.

TABLE XLVIII

ANALYSIS OF VARIANCE OF TESTOSTERONE MCR[1] OF EIGHT HEREFORD BULLS EXPOSED TO VARIOUS AMBIENT THERMAL CONDITIONS

Source of Variance	Degrees of Freedom	Sum of Squares	Mean Square	F Ratio
Total	24	203	-	-
Treatment	2	42	21	2.87**
Error	22	1,610	7.3	-

[1]Metabolic clearance rate, ml/pound body wt. per day.

**P < .10.

TABLE XLIX

ANALYSIS OF VARIANCE OF TESTOSTERONE PRODUCTION RATE OF EIGHT HEREFORD BULLS EXPOSED TO VARIOUS AMBIENT THERMAL CONDITIONS

Source of Variance	Degrees of Freedom	Sum of Squares	Mean Square	F Ratio
Total	24	17,467	-	-
Replicates	1	2,685	2,685	3.86
Treatment	2	174	87	0.13
Error	21	14,608	696	-

TABLE L

ANALYSIS OF VARIANCE OF THE TESTOSTERONE INNER POOL VOLUME (V_1) OF EIGHT HEREFORD BULLS EXPOSED TO VARIOUS AMBIENT THERMAL CONDITIONS

Source of Variance	Degrees of Freedom	Sum of Squares	Mean Square	F Ratio
Total	24	32,411	-	-
Treatment	2	16,066	8.033	10.8**
Error	22	16,345	743	-

**$P < .01$.

TABLE LI

NEWMAN-KEULS SEQUENTIAL RANGE TEST[1] APPLIED TO TESTOSTERONE INNER POOL VALUE (V_1) OF EIGHT HEREFORD BULLS EXPOSED TO VARIOUS AMBIENT THERMAL CONDITIONS

Treatment	60° F, 50% RH[2]	70° F, 50% RH	96° F, 50% RH
Mean	90.49	106.9	151.0
Number of Means Degrees of Freedom = 22	1	2	3
Value of Q .05 S.E.M. = 9.86		2.96	3.57
Value of D		29.20	35.20

[1]Snedecor and Cochron (273).

[2]Relative humidity.

TABLE LII

ANALYSIS OF VARIANCE OF THE TESTOSTERONE OUTER POOL VOLUME (V_2) OF EIGHT HEREFORD BULLS EXPOSED TO VARIOUS AMBIENT THERMAL CONDITIONS

Source of Variance	Degrees of Freedom	Sum of Squares	Mean Square	F Ratio
Total	24	26,950	-	-
Treatment	2	14.498	7,249	12.8**
Error	22	12,452	566	-

**P < .01.

TABLE LIII

NEWMAN-KEULS SEQUENTIAL RANGE TEST[1] APPLIED TO THE TESTOSTERONE OUTER POOL VOLUME (V_2) OF EIGHT HEREFORD BULLS EXPOSED TO VARIOUS AMBIENT THERMAL CONDITIONS

Treatment	60° F, 50% RH[2]	70° F, 50% RH	96° F, 50% RH
Mean	28.5	46.1	74.0
Number of Means Degrees of Freedom = 22	1	2	3
Value of Q .05 S.E.M. = 8.609		2.96	3.57
Value of D		25.48	30.73

(In the printed table, 28.5 and 46.1 are underscored by one line, and 46.1 and 74.0 by another.)

[1]Snedecor and Cochron (273).

[2]Relative humidity.

TABLE LIV

ANALYSIS OF VARIANCE OF THE COMBINED VOLUMES[1] OF THE INNER AND OUTER TESTOSTERONE POOL (V_1+V_2) OF EIGHT HEREFORD BULLS EXPOSED TO VARIOUS AMBIENT THERMAL CONDITIONS

Source of Variance	Degrees of Freedom	Sum of Squares	Mean Square	F Ratio
Total	24	85,890	-	-
Treatment	2	46,800	23,400	13.2**
Error	22	39,000	1,772	-

[1]Volume expressed as liters.

**P < .01.

TABLE LV

NEWMAN-KEULS SEQUENTIAL RANGE TEST[1] APPLIED TO COMBINED VOLUMES[2] OF THE INNER AND OUTER TESTOSTERONE POOLS (V_1+V_2) OF EIGHT HEREFORD BULLS EXPOSED TO VARIOUS AMBIENT THERMAL CONDITIONS

Treatment	60° F, 50% RH[3]	70° F, 50% RH	96° F, 50% RH
Mean	119.0	153.0	225.5
Number of Means Degrees of Freedom = 22	1	2	3
Value of Q .05 S.E.M. = 15.25		2.96	3.57
Value of D		45.15	54.00

(119.0 and 153.0 are joined by a single underline on the page.)

[1]Snedecor and Cochron (273).

[2]Volume expressed as liters.

[3]Relative humidity.

TABLE LVI

ANALYSIS OF VARIANCE OF THE COMBINED VOLUMES[1] OF THE INNER AND OUTER TESTOSTERONE POOLS (V_1+V_2) OF EIGHT HEREFORD BULLS EXPOSED TO VARIOUS AMBIENT THERMAL CONDITIONS

Source of Variance	Degrees of Freedom	Sum of Squares	Mean Square	F Ratio
Total	24	63,530	-	-
Treatment	2	31,444	15,722	10.8**
Error	22	32.086	1,458	-

[1]Volume expressed as ml/pound of body wt.

**P < .01.

TABLE LVII

NEWMAN-KEULS SEQUENTIAL RANGE TEST[1] APPLIED TO COMBINED VOLUMES[2] OF THE INNER AND OUTER TESTOSTERONE POOLS (V_1+V_2) OF EIGHT HEREFORD BULLS EXPOSED TO VARIOUS AMBIENT THERMAL CONDITIONS

Treatment	60° F, 50% RH[3]	70° F, 50% RH	96° F, 50% RH
Mean	122.4	150.9	205.5
Number of Means Degrees of Freedom = 22	1	2	3
Value of Q .05 S.E.M. = 13.82		2.96	3.57
Value of D		40.91	49.34

[1]Snedecor and Cochron (273).

[2]Volume expressed as ml/pound of body wt.

[3]Relative humidity.

TABLE LVIII

ANALYSIS OF VARIANCE OF THE VOLUME OF THE HYPOTHETICAL TESTOSTERONE POOL ($\overline{V}$) OF EIGHT HEREFORD BULLS EXPOSED TO VARIOUS AMBIENT THERMAL CONDITIONS

Source of Variance	Degrees of Freedom	Sum of Squares	Mean Square	F Ratio
Total	24	517,855	-	-
Treatment	2	70,512	35,256	1.77
Error	22	437,343	19,879	-

TABLE LIX

ANALYSIS OF VARIANCE OF THE TESTOSTERONE TRANSPORT CONSTANT (K_1) OF EIGHT HEREFORD BULLS EXPOSED TO VARIOUS AMBIENT THERMAL CONDITIONS

Source of Variance	Degrees of Freedom	Sum of Squares	Mean Square	F Ratio
Total	24	24,895	-	-
Treatment	2	3,668	1,834	1.9
Error	22	21,227	965	-

TABLE LX

ANALYSIS OF VARIANCE OF THE TESTOSTERONE METABOLISM RATE CONSTANT (K_2) OF EIGHT HEREFORD BULLS EXPOSED TO VARIOUS AMBIENT THERMAL CONDITIONS

Source of Variance	Degrees of Freedom	Sum of Squares	Mean Square	F Ratio
Total	24	13,612	-	-
Treatment	2	1,626	813	1.46
Error	22	11.986	555	-

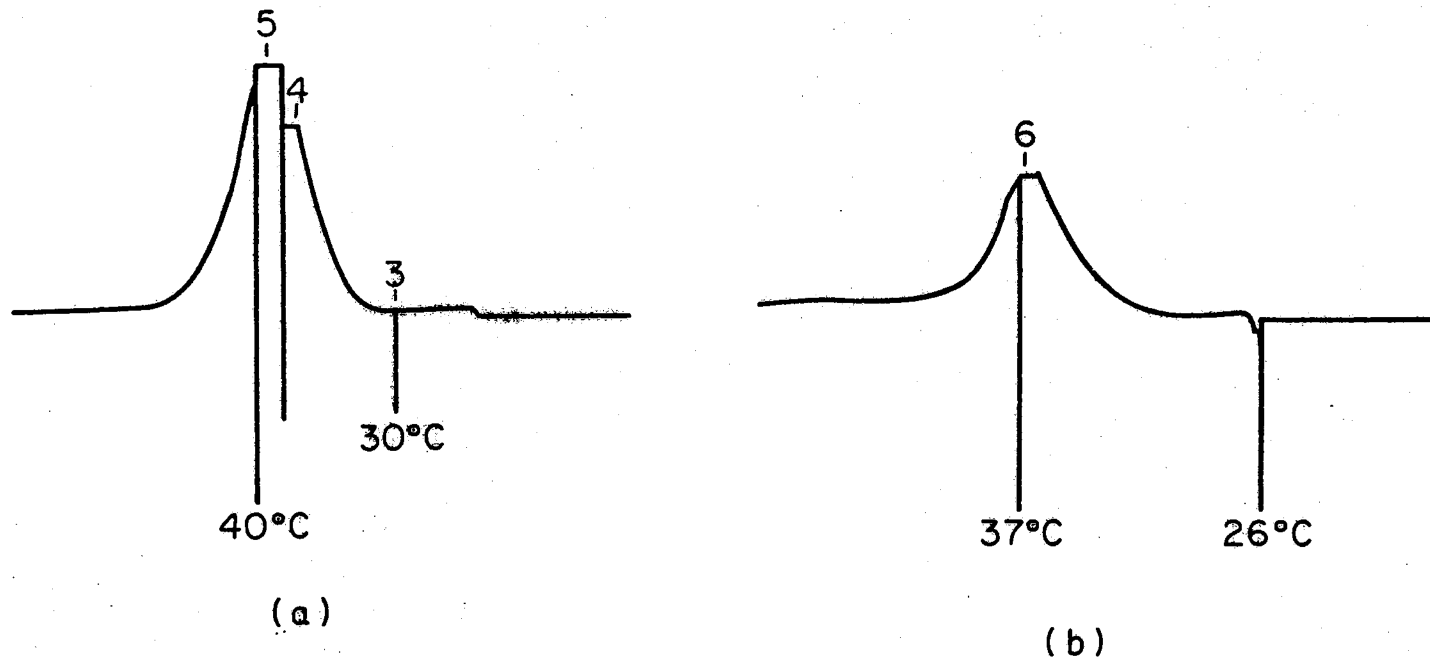

Figure 11. Total Ionization Curves for Mass Spectral Analysis of Testosterone Heptafluorobutyrate. Direct probe analysis at 70 electron volts, 65 μamps trap current, and ion source at 260° C. a) MS 1970, curve no. 2, testosterone heptafluorobutyrate standard. Numbers 3, 4, and 5 show points where individual mass spectra were recorded. b) MS 1970, curve no. 3, testosterone heptafluorobutyrate isolated as testosterone from hereford bull blood. Number 6 shows point where individual spectrum was recorded.

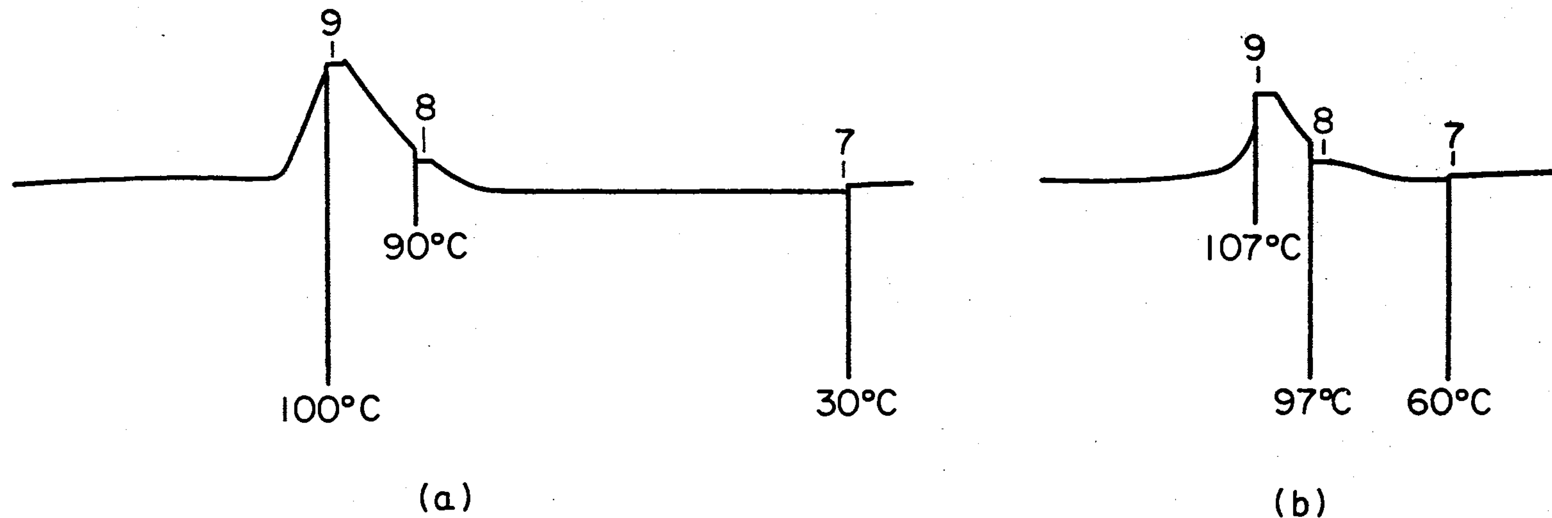

Figure 12. Total Ionization Curves for Mass Spectral Analysis of Cortisol. Direct probe analysis at 70 electron volts, 65 μamps trap current, and ion source at 260° C. a) MS 1970, curve no. 4, cortisol standard. Numbers 7, 8, and 9 indicate points where individual spectra were recorded. b) MS 1971, curve no. 3, cortisol isolated from hereford bull blood. Numbers 7, 8, and 9 indicate points where individual mass spectra were recorded.

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