PLASMA ADRENOCORTICOTROPIC AND GLUCOCORTICOID HORMONE LEVELS DURING AGING IN CATTLE

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John E Nellor

Major professor
John E. Nellor

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ABSTRACT

PLASMA ADRENOCORTICOTROPIC AND GLUCOCORTICOID HORMONE LEVELS DURING AGING IN CATTLE

by Gail Daniel Riegle

Pituitary-adrenal function was estimated in dairy and beef cattle ranging from three days to fourteen years in age, by biological assay of plasma adrenocorticotropic activity and chemical assay of glucocorticoid hormones. Levels of adrenocorticotropic activity were consistently low (less than 2.0 mU ACTH activity/100 ml plasma) and in most instances barely detectable in bulls from one to thirty months of age. Adrenocorticotropic activity was increased in the older cattle, reaching a peak value of 15.4 mU activity/100 ml plasma. Substantial amounts of plasma adrenocorticotropic activity were assayed in all animals above three years of age. Levels of plasma cortisol (6.0 to 9.0 μg/100 ml plasma) and corticosterone (6.6 to 13.9 μg/100 ml plasma) exhibited no consistent alterations related to increased adrenocorticotropic activity. These data are interpreted to reflect adrenocortical insufficiency or insensitiveness to ACTH during aging, with concomitant increased

adrenocorticotropic secretion in order to maintain blood levels of glucocorticoids within the normal range. This interpretation was supported by increased percentages of blood eosinophils, decreased percentages of plasma albumin, and increased percentages of plasma gamma globulins in the older bulls.

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By

GAIL DANIEL RIEGLE

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

INTRODUCTION

The field of adrenal endocrinology was conceived when Addison described the clinical picture of adrenocortical insufficiency in 1855. The following year Brown-SeQuard carried out the first adrenal experiments when he studied the effects of adrenalectomy.

The major impetus for research in pituitary-adrenal endocrinology followed the isolation and identification of the active constituents of the bovine adrenal cortex (Swingle and Pfiffner, 1930; Grollman and Firor, 1933, Kendall et al., 1937; and Wintersteiner and Pfiffner, 1936).

Macchi and Hechter (1954) demonstrated control of adrenocortical glucocorticoid hormone production depended almost entirely on hypophyseal adrenocorticotropic hormone. Kupperman el al., (1955) and many other workers have shown the inhibiting effect of adrenal glucocorticoids on hypophyseal-pituitary output of adrenocorticotropic hormone. Recently Dorfman (1957), Flink (1961) and many others have written excellent reviews covering many aspects of pituitary-adrenal physiology.

Gross and microscopic considerations of pituitary and adrenal glands associated with aging have been investigated by many workers. Wolfe (1943) reported a relative decrease in anterior pituitary eosinophils and an increased number

of pituitary chromophobes in aged female rats. There was also less mitotic activity in the pituitary gland cells from rats of advancing age. Wolfe interpreted these observations to be indicative of decreased functional activity of the anterior lobe of the pituitaries of aged rats.

The histological changes within the anterior pituitary of the fowl with increasing age were described by Payne (1949). He reported marked retrogression of all anterior pituitary cell types with age. Pycnotic nuclei and mitochondrial changes were observed in from 50 to 75 % of the basophils from older animals. There were also fewer acidophils possessing secretory granules in the pituitaries from the older birds. By 2 to 3 years of age cavities appeared in the posterior portions of the anterior lobe and with advancing age the cavities were present in all regions of the anterior pituitary. Spagnoli et al. (1955) observed increased vacuolation of the anterior pituitary associated with increasing age in the Golden Hamster. Male hamsters exhibited a decrease in acidophils and a corresponding increase in basophils with increasing age. Although the fore-mentioned workers reported histological evidence of anterior pituitary abnormalities associated with aging, Fortier (1958) reported no significant

alteration in the pituitary content of adrenocorticotropic hormone during aging in the rat.

Rats beyond 700 days of age had increased absolute adrenal gland weights (Yeake, 1944), however, their relative cortex volumes were decreased with increasing age. Payne (1949) observed a reduction in size of the adrenal glands of the fowl by 5 to 8 years of age. Dribben and Wolfe (1947) reported increased width of the adrenal capsule and a gradual transformation of reticular fibers to collagen fibers within the adrenal cortex from female rats of advancing age. The change to collagen fibers was especially noticeable in the zona reticularis. In mice beyond 1 year of age, Blumenthal (1955) observed a progressive reduction in adrenal gland mitotic activity. Blumenthal also found increased proliferation of adrenal connective tissue with senescence. Ninety percent of rats above 15 months of age had spotted and granular adrenal gland surfaces (Ingle, 1956). Jayne (1957) reported adrenal cortical degenerative changes in 10 of 20 rats 1 year of age and older. In the older rats there was a gradual decrease in size of the zona reticularis with a corresponding increase in the size of the fat laden zone. The adrenal cortex of the aging Golden Hamster was shown by Meyers and Charipper (1956) to contain

a progressive increase in collagenous connective tissue, a thickening of the capsule and degenerative changes involving cellular atrophy, necrosis and hemorrhage. Jayne (1953) observed histological alterations occurring in the adrenal gland of the aging rat similar to degenerative changes occurring after hypophysectomy.

Vezar (1960) studied differences with aging in the compensatory hypertrophy of the remaining adrenal gland when one was removed and reported significantly less ability to compensate by adrenal gland enlargement in the older rats. Adrenal glands from groups of 40 and 360 day old donor rats were transplanted into 40 day old rats (Geiringer, 1956) to determine if the glands from the older animals would supply the functional needs of the recipient animals as well as the transplants from the 40 day old rats. In these studies there were no differences in growth, reproductive capabilities, lactation or response to a water load test for cortical activity between the rats with adrenal transplants from the adult and 40 day old rats.

The possibility of utilizing changes in blood leucocyte concentrations as a measure of pituitary-adrenal function was recognized at an early date. Blood-cellular changes after adrenalectomy in the cat were investigated by Zwemer

and Lyons (1928) and Corey and Britton (1932). After adrenalectomy, Corey and Britton (1932) observed an increase in blood lymphocytes from 32 to 64% and a decrease in blood neutrophils from 63 to 30% of the total white blood cell counts. Replacement therapy with adrenal cortical extracts restored the differential counts to preoperative levels. Adrenalectomy reduced the total leucocyte count in the cat from 12,400 to 7,000/cmm (Corey and Britton, 1932). On the other hand, Shecket et al. (1935) studied leucocyte responses of the rat to adrenalectomy and found that before the rat died from adrenal insufficiency the total leucocyte count increased from 11,300 to 17,500/cmm of blood. In these studies lymphocytes were shown to increase from 81.2 to 88.4% of the total blood leucocytes. The increase in blood lymphocytes was correlated with a corresponding decrease in blood neutrophils.

Total red blood cell counts were increased after adrenalectomy in the rat (Dalton and Masson, 1940 and Dougherty and White, 1947) and in the cat (Corey and Britton, 1932). A slight decrease in red blood cells of the rat in response to ACTH or glucocorticoids was observed by Dougherty and White (1944).

Solomon and Shock (1950) demonstrated significantly smaller blood neutrophil increases after administration of ACTH in aged men compared to young men. These aged patients also exhibited smaller decreases in blood eosinophils and increases in blood neutrophils after injected epinephrine stress.

The influence of hormones on lymphoid tissue in rats and mice was investigated by Dougherty and White (1944). They found a significant decrease in blood lymphocyte counts following single injections of ACTH or glucocorticoid. Blood neutrophils were shown to increase after ACTH or glucocorticoid injection in rats, but this increase was of smaller magnitude than the corresponding lymphopenia. Reinhardt and Li (1945) demonstrated an ACTH and glucocorticoid effect on lymphatic tissue by the rapid decrease in thoracic lymph lymphocytes observed following injections of ACTH or glucocorticoids. Hormones of the adrenal cortex were shown to affect cellular suspensions of thymus or marrow cells (Schrek, 1949). Extracts of adrenal cortex, corticosterone and 17-hydroxycorticosterone all produced a cytotoxic effect in the cell suspensions leading to cellular disentegration. The cytotoxic effect was demonstrable at low concentrations of the hormones, but increased amounts of the adrenal

hormones did not augment the response. The cytotoxic response was specific for the glucocorticoids as no effect was observed when the cellular suspensions were mixed with ACTH, desoxycorticosterone or various male and female sex hormones. Desoxycorticosterone has also been shown to have no effect on peripheral lymphocyte counts (Dougherty and White, 1944).

Lymphocyte response after starvation, histamine and anaphylaxis stress in adrenalectomized mice was investigated by Dougherty and Kumagui (1951). Animals stressed 2 hours after adrenalectomy had significantly higher lymphocyte concentrations in the blood 2 hours after stressing and exhibited pronounced lymphocytosis 8 and 12 hours following stress. Lymphocyte concentration had returned to normal in these mice 24 hours following stress. These data were interpreted to indicate that stress not only augments the pituitary-adrenal system producing lymphopenia, but also stimulates lymphatic tissue growth in some manner.

Eosinopenia was associated with adrenal hormone production by Hills et al. (1948). Following administration of ACTH, human patients exhibited a decrease in blood eosinophils associated with the expected lymphopenia and increased neutrophil counts. Visccher and Halberg (1955) found that

blood eosinophil counts exhibited a diurnal variation very similar to plasma glucocorticoid concentrations. Best and Samter (1951) using injections of glucocorticoids or ACTH in man found the maximum eosinopenia occurred 4 hours after administration of the hormone. Forsham et al. (1948) investigated the hematological changes in man following administration of ACTH. A maximum response was obtained within 4 hours producing a 74% reduction in blood eosinophils, a less spectacular fall in lymphocytes and a non-consistent rise in neutrophils. When ACTH was administered to Addisonians there was only a 4% reduction in eosinophils. However, a subsequent fall in blood eosinophils in the Addisonians was produced by the administration of 20 mg of hydrocortisone. However, Best and Samter (1951) reported too many physiological variables effect blood eosinophil counts to consider eosinophil counts alone as a reliable measure of pituitary-adrenal physiology.

There is considerable evidence indicating a positive correlation between plasma adrenal glucocorticoid levels and relative and absolute amounts of plasma proteins. Although serum albumin was found to be depressed in Addisonians (McCullagh and Lewis, 1945) the relative concentrations of

all the globulin fractions tended to be increased. Jager et al. (1951) studying human patients exhibiting hypoalbuminemia found that injections of cortisone restored serum albumin levels to normal in 5 of 7 cases. The serum gamma-globulin concentration was reduced in patients following cortisone or ACTH therapy (Jager et al., 1951, and Vaughan et al., 1951).

White (1956) reported decreased amounts of protein were released from the liver and spleen of adrenalectomized rats compared to intact rats. The protein release was restored to normal by adrenal cortical extract injection in adrenalectomized rats and augmented by adrenal cortical extract or ACTH injections into intact animals.

Serum protein patterns from patients with Cushing's syndrome were investigated by Lewis and McCallagh (1947). Patients with Cushing's syndrome and normal subjects treated with cortisone exhibited decreased levels of gamma-globulin. Thorn et al. (1950) reported a 10.2 gm% to 4.8 gm% reduction in serum globulin of a multiple myeloma patient following ACTH injections.

When quantitative chemical assays for biological quantities of steroid hormones were developed many different laboratories actively utilized these new tools to study

pituitary-adrenal function with aging. Pincus (1956) reported a very significant decrease in the output of urinary 17-ketosteroids in men and women with increasing age. In the span of from 20 to 80 years of age, urinary 17-ketosteroid excretion rates decreased from 8.5 mg/24 hours to 2 mg/24 hours among men and from 7.5 mg/24 hours to 1.0 mg/24 hours among women. However, the 11-oxy-17-ketosteroids were maintained relative to the 11-desoxy-17-ketosteroids indicating retention of adrenal function. Johnsen (1956) pointed out that while there was a decrease in urinary 17-ketosteroid output for both men and women, there was too much overlap between the sexes to determine if a sample was from a male or female subject. Relative concentration of urinary androsterone, the major metabolite of testosterone, was found to decrease at a much faster rate with increasing age than the other 17-ketosteroids (Johnsen, 1956, and Pearson and McGavack, 1955) indicating a more rapid loss of testicular androgen secretions than adrenal androgen. The decrease in urinary androsterone output was found to be directly correlated with decreased concentrations of testosterone in the human spermatic vein (Hollander and Hollander, 1958). Johnsen (1956) reported greater differences in biological androgenecity of urinary extracts between young and old men

than differences in total urinary 17-ketosteroids. Levels of urinary 17-ketosteroid metabolites after ACTH stimulus were 20% less in elderly patients compared to young subjects (Romanoff et al., 1957).

Diurnal variations in plasma 17-ketosteroids were studied by Migeon et al. (1957) and Duboff (1957). Plasma 17-ketosteroids varied in a diurnal fashion with peak values obtained at about 8 a.m. However, the diurnal pattern of plasma 17-ketosteroids was not nearly as uniform as the pattern for plasma 17-hydroxy-corticosteroids.

Borth et al. (1957) demonstrated 30% more 17-ketosteroid excretion in men than women of the same age. Urinary 17-ketosteroid values determined for eunuchs (Hamburger, 1948; Johnsen, 1956; Furman et al., 1958; and Hamilton et al., 1959) were found to be consistently lower than similarly aged intact men and usually closely paralleled urinary 17-ketosteroid production from women of like age. In all cases this decrease in urinary 17-ketosteroid output was as severe for the eunuchs as for intact men, as the aging process continued.

An intensive study of urinary steroids and aging from 617 subjects was carried out by Pincus et al. (1954). While urinary 17-ketosteroid output was found to decrease sharply

with advancing age, neutral reducing lipids, adrenal glucocorticoids and their metabolites, exhibited only a slight non-significant downward trend with increasing age. Romanoff et al. (1957) reported no change in relative occurrence of the characteristic urinary corticoids in young and elderly subjects after ACTH administration. In another study Romanoff et al. (1958) found that if the lesser amounts of corticoid metabolites present in the urine of elderly subjects were compared to urinary creatinine excretion from the various aged subjects, the quantitative differences in urinary corticoid metabolites related to age disappear.

The adrenal gland response to an intravenous infusion of ACTH was studied by Samuels (1957), Nugent et al. (1959) and Tyler et al. (1955). The older subjects achieved higher levels of plasma 17-hydroxycorticoids in response to 25 IU of exogenous ACTH than the young subjects. However, Samuels (1957) demonstrated that humans beyond 50 years of age have a smaller distribution volume for 17-hydroxycorticoids and the hormones have a longer half life in peripheral circulation. Because of these alterations, plasma 17-hydroxycorticoids require substantially more time to be lowered to the initial level present before ACTH stimulus in the

elderly subject. West et al. (1961) demonstrated a progressively slower removal rate of cortisol from the circulation with advancing age. When these alterations in cortisol utilization were considered, the older patients showed less responsiveness to exogenous ACTH. Adrenal 17-hydroxycorticoid production of men, 50 years of age or more, was shown by Moncloa et al. (1963) to be less for all levels of exogenous ACTH tested. This decreased response to exogenous ACTH was interpreted to indicate that adrenal gland response capacity and sensitivity to ACTH diminished with age.

Eik-Nes et al. (1955) tested the adrenal response to exogenous ACTH in clinical Addisonians by measuring plasma 17-hydroxycorticoids. None of the Addisonians exhibited a significant increase in 17-hydroxycorticoids from exogenous ACTH. Eik-Nes suggested that Addisonians with measureable 17-hydroxycorticoid levels showning no significant response to ACTH have adrenal gland remnants which are probably already maximally stimulated by endogenous ACTH.

Patterns of diurnal rhythmecity of 17-hydroxycorticoids have been established (Migeon et al., 1956). Pincus et al. (1954) reported delayed 17-hydroxycorticoid diurnal rhythmicity in senile subjects. The effect of exogenous ACTH on

diurnal 17-hydroxycorticoid patterns was studied by Nugent et al. (1959). Continuous intravenous infusion of ACTH at rates from 0.8 to 1.4 IU/24 hours maintained plasma 17-hydroxycorticoids above maximal levels obtained spontaneously during a diurnal variation. There were no diurnal 17-hyroxycorticoid variations when ACTH was infused at rates exceeding 0.8 IU/24 hours. In males, it was calculated that the maximal normal diurnal variation can be achieved by a secretion of ACTH of less than 4.0 IU/day for a period of less than 4 hours.

In cattle, studies of pituitary-adrenal function associated with aging have not been numerous. Perfused bovine adrenal glands were shown (Hechter, 1953; and Bush, 1953) to secrete nearly equal quantities of cortisol and corticosterone. Both cortisol and corticosterone were shown to increase markedly when ACTH was added to the perfusion media.

Adrenal gland abnormalities have been associated with many metabolic and reproductive disturbances in cattle. Cupps et al. (1954 and 1956) associated reproductive malfunctions in male and female dairy cattle with histological evidence of adrenal gland degeneration. Animals with reproductive troubles showed degranulations of cells in fascicular and reticular zones. These zones also contained

smaller cells which had many more pycnotic nuclei than had adrenal glands from normal cattle. The adrenal glands of ketotic cows are larger and exhibit degeneration of the epithelial cells (Shaw et al., 1948 and 1949). Saarinen and Shaw (1950) reported increased total fat in the adrenal glands of ketotic cows. Adrenal glands from these ketotic cows also exhibited a gross structural flabbiness that could not be explained on the basis of changes in water or fat content. Although adrenal glands from cows in early ketosis were enlarged, cows suffering from chronic ketosis had atrophied adrenals (Hatziolos and Shaw, 1958).

Grant (1960) reported higher ACTH content in bovine pituitaries than in pituitaries from other species. He described the bovine pituitary as "a stressed gland, full of compact cells." Shaw et al. (1948) found anterior pituitary abnormalities in ketotic cows. General cellular degeneration together with numerous vacuoles was evident in the anterior lobes from ketotic cows.

Although blood hemoglobin and hematocrits from ketotic cows were normal (Shaw et al., 1948), ketotic cows had increased concentrations of blood neutrophils and decreased concentrations of blood lymphocytes and eosinophils which could suggest an increased adrenal gland function in ketotic

cows. However, in another study Shaw et al. (1954) reported blood eosinophils were not decreased as much in a ketotic cow following epinephrine injection as in a normal cow injected with epinephrine. Shaw (1947) demonstrated a more marked hyperglycemic effect from ACTH in normal cows compared to ketotic cows.

On the basis of urinary glucocorticoids, Puntriano (1952) reported decreased adrenocortical activity in ketotic cows. Holcombe (1957) measured urinary "reducing corticoids" in cattle and sheep. While absolute amounts of urinary "reducing corticoids" were depressed with aging, similar to glucocorticoid excretion in older humans, the differences were not significant with increasing age. Urinary "reducing corticoids" in cattle also showed indications of a typical diurnal pattern.

Schalm (1961) studied changes in leucocytes in cattle with aging. The only significant alteration was in eosinophils which increased from 0.8% to 12.0% in dairy cattle from 4 months to 14 years of age.

Nellor (1958a) reported the quantitative detection of adrenocorticotropic activity in bovine plasma. Indications of increased adrenocorticotropic activity with increasing age in dairy bulls were observed by Nellor (1958b) and Sutton (1960).

At this point it becomes clear that there is strong evidence indicating alterations in pituitary-adrenal functions with aging. Pituitaries and adrenal glands from many species show degenerative changes with aging. Reductions in circulating blood leucocytes suggest adrenocortical hypofunction with advancing age. There is a definite decrease in adrenal 17-ketosteroid secretion in older humans, strong evidence indicating less glucocorticoid responsiveness to exogenous ACTH, and a tendency to lose diurnal patterns of plasma glucocorticoids with aging.

Since the symptoms indicating adrenocortical abnormalities are especially prevalent in cattle, the present study concerns the effects of increased age on the level of plasma adrenocorticotropic activity in cattle, along with simultaneous estimation of adrenal response to plasma adrenocorticotropic activity by analysis of plasma glucocorticoids.

MATERIALS AND METHODS

Pituitary-adrenal physiology was evaluated in the following cattle: fifteen Holstein Friesian bulls ranging in age from 3 to 14 years from the Michigan Artificial Breeders Co-op; five Holstein Friesian bulls ranging in age from 1 to 16 months and two Holstein Friesian cows 5 and 7 years of age from the Michigan State University Dairy Farm; one 3 year old Hereford cow, one 6 year old Angus bull, one 5 month old Hereford bull before and after castration and one Hereford calf 3 days old from the herd of the Endocrine Research Unit, Michigan State University. All the cattle were used to being handled and great care was taken to handle them in a routine manner before sampling was attempted to minimize possibilities for a stressful condition affecting their pituitary-adrenal systems.

Heparinized external jugular samples of approximately 500 mls were taken from 8:30 to 9:30 a.m. to obtain consistency in time of sampling and reduce errors due to diurnal hormonal patterns. All samplings were spaced at least 30 days apart. The blood samples were immediately placed in an ice bath until they could be returned to the laboratory for centrifugation which was completed as rapidly

as possible. Centrifugation of the plasma from the formed elements and all subsequent handling of the plasma prior to assay was carried out at 3°C.

Plasma adrenocorticotropic activity was assayed from plasma, plasma concentrated by lyophilization, and plasma separated into protein fractions by Cohn's cold ethanol No 6 method (Cohn et al., 1946), modified by using stepwise precipitation of proteins in the II + III fraction. Most of the information presented in this paper concerns assay of the 12-25% fraction of II + III and the IV-1 precipitate. Plasma to be used for adrenal glucocorticoid analysis was extracted immediately after centrifugation or stored at -10°C until it could be assayed.

Routine laboratory analysis of blood samples including red and white cell counts, white cell differential counts, total plasma proteins, plasma protein component concentrations after electrophoresis and hematocrits was conducted on as many samples as possible.

Plasma adrenocorticotropic activity was assayed by the in vitro technique of Saffran and Schally (1955). For each assay, eight Long-Evans rats of either sex weighing between 180 and 250 grams were sacrificed by decapitation.

The adrenal glands were rapidly removed and placed on a filter paper moistened with Krebs-Ringer bicarbonate glucose medium in a Petri dish which was kept cold by an ice bath. The adrenal gland pairs from each rat were kept separate on the filter paper. The adrenal glands were dissected free of fat and extraneous connective tissue leaving the adrenal capsule intact for easier handling. The adrenal glands were then carefully quartered with a small scissors on a filter paper saturated with cold Krebs-Ringer bicarbonate glucose medium. The eight quarters from each pair of glands were distributed onto eight sectors of a filter paper moistened with Krebs-Ringer bicarbonate glucose medium. The adrenal glands of all the rats in the assay groups were handled similarly so that each sector on the filter paper contained one quarter of an adrenal from each of the animals in the group. During all these preparatory steps the Petri dishes were kept over an ice bath. The quartered adrenal segments were weighed on a microtorsion balance to the nearest 0.1 mg and placed in 25 ml breakers containing 3.0 ml of Krebs-Ringer bicarbonate glucose medium. The flasks were placed in a Dubnoff metabolic incubator, flushed with a gas phase of 95% oxygen - 5% carbon

dioxide and incubated for 45 minutes. The Dubnoff apparatus was operated at 37°C, the agitating cam was set to operate at 120 agitations per minute and the 95% oxygen - 5% carbon dioxide flow was adjusted to 400 to 450 cc per minute. During this preincubation period the assay samples were prepared. Solutions of protein fractions were made up to a constant volume with Krebs-Ringer bicarbonate glucose medium. Plasma was assayed directly. After preincubation the flasks were removed from the Dubnoff apparatus and the preincubation media removed by aspiration and discarded. Duplicate flasks containing 5 mls of assay material were arranged in the following manner. Flasks No 1 and No 5 contained 5 mls of Krebs-Ringer bicarbonate glucose medium only and served as controls for the assay. Flasks No 2 and No 6 contained 5 mls of the protein solution being assayed and ACTH standards of 2 and 4 mU of oxycellulose purified bovine ACTH (Armour Lot 216-177-3). Flasks No 3 and No 7 and No 4 and No 8 were made up with 5 ml duplicates of the plasma-protein solutions being assayed. Appropriate blanks of assay medium and Krebs-Ringer bicarbonate glucose medium were also incubated with each assay. The flasks were then returned to the Dubnoff incubator, flushed with 95% oxygen - 5% carbon dioxide and incubated for 90 minutes

under the same environmental conditions as during preincubation.

After incubation the flasks were removed and two 2.0 ml aliquots of the incubation medium were pipetted into 12 ml glass stoppered centrifuge tubes containing 4.0 mls of methylene chloride purified according to the following scheme: methylene chloride was redistilled, washed with an equal volume of distilled water, dried by passing through anhydrous sodium sulfate, and decolorized by agitating 20 minutes with charcoal. The solvent was stored over sodium hydroxide pellets overnight, redistilled into foil-covered bottles and stored under refrigeration. Prepared in this manner the solvent remains usable after long periods of storage.

The medium was extracted with methylene chloride by shaking slowly for 2 minutes. After centrifugation, in a clinical centrifuge for 5 minutes, the aqueous phase was removed and discarded. The solvent was washed with 0.5 ml of ice cold 0.1 N sodium hydroxide by vigorous shaking for 30 seconds. After 3 minutes centrifugation, the aqueous phase was discarded and 3.0 mls of the methylene chloride were pipetted into clean test tubes. Care was taken not to transfer any of the aqueous phase into the clean tubes.

The methylene chloride was evaporated under a slow stream of air in a water bath at 40°C. The sides of the test tubes were washed down once with fresh methylene chloride to concentrate the residue in the bottom of the tubes.

Glucocorticoid production of the incubated rat adrenal gland quarters was quantitated by the Blue Tetrazolium method of Elliott el al. (1954). The methylene chloride residue was dissolved in 0.2 ml absolute ethanol (Gold Seal Alcohol USP). To aid in the dissolution of the residue the tubes were dipped several times into flasks of boiling water, ice cold water, and water at room temperature. After dissolution, 0.15 ml of blue tetrazolium solution (30 mgs blue tetrazolium in 20 mls absolute ethanol) was carefully pipetted into the test tubes. Following mixing, 0.15 ml of tetramethyl ammonium hydroxide solution (0.3 ml tetramethyl ammonium hydroxide and 9.7 mls absolute ethanol) was added. The tubes were mixed, stoppered and placed in a water bath at 30°C for 20 minutes to complete the color development. At the end of 20 minutes, 1.5 mls absolute ethanol was added, the tubes were agitated and their optical densities read on a Coleman Junior Spectrophotometer at 510 mμ. Total alpha-ketol content was quantitated by

comparison with optical densities of known amounts of corticosterone carried through the same assay procedure.

Adrenocorticotropic activity was expressed in terms of increased corticosterone production per 100 mgs incubated rat adrenal tissue. Adrenocorticotropic activity was quantitated by comparison of the increased corticosterone production by the plasma protein fraction to the increased corticosterone production in the standard ACTH incubation flasks.

The methods utilized for plasma glucocorticoid analysis were those used by Romanoff (1961) for plasma steroid hormone analysis. Twenty mls of fresh or freshly thawed plasma and about 0.01 μc of 4-C^{14}-hydrocortisone (less than .05 μg hydrocortisone) were pipetted into 250 ml erlenmeyer flasks. The plasma was extracted with 10 volumes of acetone:ethanol (1:1) with the aid of a magnetic stirrer. The solvent protein mixture was warmed in a water bath at 50°C for 6 minutes after which the precipitated protein was separated from the solvents by filtering through Whatman No 541 filter paper with the aid of vacuum. The acetone:ethanol extract was taken to dryness on a flash evaporator under reduced pressure. The residue was dissolved in 7 mls of 5% olive oil in n-hexane and transferred to a silica gel defatting

column (2 grams Davison silica gel, mesh size 100-200). The column was made up with a n-hexane slurry and conditioned by passing 15 mls n-hexane through it before the extract was added. The residue from the plasma extract was washed on the column with 4 mls n-hexane. The lipids and other low polar hydrocarbons were eluted from the column with 35 mls of 5% ether:benzene which were discarded. The corticosteroids were then eluted from the column with 30 mls of 4% ethanol:ethyl acetate. The ethanol:ethyl acetate was taken to dryness under reduced pressure and the residue dissolved in 60 mls n-hexane which was partitioned 4 times with 20 mls 70% methanol. Most of the remaining lipids (which were not removed from the column) and low polar C_{19} and C_{21} steroids remain in the n-hexane phase. The 70% methanol phases containing the glucocorticoids were combined and reduced to dryness on a flash evaporator. The residue was next dissolved in 40 mls of cold methylene chloride and washed with 10 mls of cold 0.1 N sodium hydroxide to remove estrogens and other acidic compounds. The methylene chloride was evaporated to dryness under a slow stream of air in a water bath at 40^{o}C. The residue was then concentrated in a conical centrifuge tube and transferred to Whatman No 1 filter paper prepared as follows:

(Eighteen cm strips of Whatman No 1 filter paper were divided into six 3 cm lanes by removing about a 2 to 3 mm strip from between the lanes. Twenty strips cut in this manner were washed by descending chromatography in a glass chromatography tank with the following solvents: 1000 mls ethanolic ammonia (60 ml conc. ammonium hydroxide); 500 mls water; 1000 mls 2 N acetic acid:water; 500 mls 50% ethanol: water; and 1000 mls ether. The washed strips were then dried and stored in aluminum foil until used). The paper strips with the separate lanes containing the plasma extract residues, blank strips and strips containing hydrocortisone and corticosterone standards were placed in glass chromatography tanks previously prepared with 75% methanol: toluene and allowed to equilibrate for about 2 hours. The chromatography tanks were contained in a chromatography cabinet maintained at an environmental temperature of 37°C. After equilibration, the toluene mobile phase was added and allowed to flow over the papers by the descending chromatographic technique for 2 to 3 hours. At the end of chromatography, the papers were removed and allowed to dry after marking the position of the solvent front. The hydrocortisone and corticosterone areas on the standard strips were located by dipping the dried strips into a blue tetrazolium

solution (2:1 solution of 0.2 gm blue tetrazolium in 15 mls methanol diluted to 100 mls with water and a 10% solution of sodium hydroxide in methanol:water (4:1)). The hydrocortisone and corticosterone areas of the standard strips were used to locate the respective zones on the plasma extract strips. The 3 to 5 cm portions of the paper containing the hormones were eluted with 6 mls absolute ethanol by allowing the ethanol to flow over the strips into clean test tubes from 22 gauge needles mounted on syringe barrels. The ethanol extract was reduced to dryness under a slow stream of air in a water bath at 40°C. The extract was washed to the bottom of the test tubes by washing the sides of the tubes with additional absolute ethanol.

Corticosterone was quantitated by the blue tetrazolium method of Elliott (1954) using the same procedure as was used in the adrenocorticotropic assay. Hydrocortisone was quantitated by dissolving the residue in 2 mls of phenylhydrazine hydrochloride ethanolic sulfuric acid solution (65 mgs phenylhydrazine hydrochloride dissolved in 100 mls 62% sulfuric acid:absolute ethanol (2:1)). The solution was allowed to stand overnight at room temperature and the optical densities were read at 410 mμ in a Colemen Junior Spectrophotometer. Amounts of hormone were quantitated by

comparison with the optical densities of known amounts of hydrocortisone and corticosterone carried through the assay procedures. Just before colorimetric assay of hydrocortisone, the residue was dissolved in absolute ethanol and 1/20 of its volume was planchetted and its radioactivity counted on a Nuclear Measurements Corporation gas-flow proportional counter. Recovery of hydrocortisone radioactivity was used to correct for loss of both hydrocortisone and corticosterone in the technique.

Solvents used in the glucocorticoid assay procedure were prepared as follows:

toluene -- redistilled, washed with concentrated sulfuric acid until the acid no longer becomes discolored, washed with water, dried by passing through anhydrous sodium sulfate and redistilled again.

methanol and ethanol used for plasma extraction -- redistilled from solution containing 0.5 gm 2-4-dinitrophenylhydrazine and 10 mls concentrated sulfuric acid per 2 liters.

absolute ethanol -- Gold Seal Alcohol USP (no preparation) ether -- Merck USP (no preparation)

N-hexane, ethyl acetate, benzene, acetone and methylene chloride -- freshly redistilled. (Except where previously noted.)

RESULTS

Plasma Adrenocorticotropic Activity

Attempts to assay adrenocorticotropic activity from 5 mls of plasma extracted with petroleum ether to remove plasma corticosteroids were not satisfactory. The increase in corticosterone production from the incubated rat adrenal sections was not of sufficient magnitude to give a clear increase over corticosterone secretion from the adrenal quarters incubated with Krebs-Ringer bicarbonate glucose medium alone.

The first attempt to increase the concentration of adrenocorticotropin in the assay solution was by lypholization of plasma and assay of a 5 ml solution containing 10 milliliter equivalents of lypholized plasma dissolved in Krebs-Ringer bicarbonate glucose medium. Again the increase in corticosterone secretion from the incubated rat adrenal quarters was not sufficient for reliable assay.

The next attempt to increase adrenocorticotropic activity in the assay medium was by cold ethanol fractionation of plasma. Plasma adrenocorticotropic activity was consistently found in fractions II + III and IV - I. Since fraction II + III contained considerable amounts of protein

(average about 4.1 grams/100 mls plasma), and increased protein concentrations reduce the effectiveness of ACTH, this fraction was subjected to stepwise addition of its 6 to 25% ethanol concentration in an attempt to eliminate some of the protein from the assay medium without losing adrenocorticotropic activity. After stepwise separation, the 12 to 25 % ethanol portion of fraction II + III contained the adrenocorticotropic activity and the protein concentration of the assay fraction was reduced from 4.1 to 2.1 grams/100 mls plasma. Fraction IV - I contained a protein concentration averaging about 0.8 grams/100 mls plasma. Thirty to fifty milliliter equivalents of the 12 to 25% ethanol portion of fraction II + III and fraction IV + I were used for assay of the plasma adrenocorticotropic activities presented in this study.

Since increased amounts of protein in the assay sample depressed the dose-response relationship expected from rat adrenal quaters incubated with exogenous ACTH, the ACTH standards for each assay were incubated in a portion of the protein solutions which were being assayed.

Corticosterone secretion by the assay rat adrenal tissue during ACTH stimulation exhibited a linear relationship for small amounts of ACTH (Figure 1). However, since the

increased corticosterone secretion stimulated by exogenous ACTH varied significantly between assays, it was necessary to include ACTH standards with each assay to accurately quantitate the adrenocorticotropic activity in the protein fraction being assayed.

Levels of plasma adrenocorticotropic activity from the bulls utilized in this study are tabulated in Table 1. Average adrenocorticotropic activities from all bleedings for each bull are plotted in Figure 2. With the exception of instances when trouble was encountered in obtaining blood samples from obviously stressed animals, plasma adrenocorticotropic activity was low (below 2.0 mU/100 mls plasma) in all bulls under 2 years of age. Adrenocorticotrophic activity could not be detected in several blood samples. On the other hand, blood samples from all bulls above 3 years of age contained substantial amounts of adrenocorticotropic activity. The highest value recorded was 15.4 mU/100 mls plasma from Iowana, the 14 year old bull. It is apparent that (Figure 2) plasma adrenocorticotropic activity is higher in the older bulls. Data from the 6 year old Angus bull (6.9 mU adrenocorticotropic activity/100 mls plasma) and No 118-A, the one month old Hereford bull (1.0 mU adrenocorticotropic activity/100 mls

plasma at one month of age) were in agreement with data obtained from dairy bulls. Average levels of adrenocorticotropic activity from all bulls beyond 3 years of age were significantly higher ($P < 0.01$) than the plasma adrenocorticotropic activities from bulls under 2 years of age. The formula for the best straight line plotted from these data (Figure 2) was $Y' = 1.65 + 0.57x$ with a standard deviation of ± 2.0.

Adrenocorticotropic activity from No 118-A at 3 days of age (4.7 mU/100 mls plasma) was significantly higher than the activities from bulls 1 month to 2 years of age.

Although the accumulated values of plasma adrenocorticotropic activity from cows were not as voluminous or conclusive as the data from bulls, the activity from the 7 year old Holstein Friesian cow (9.6 mU/100 mls plasma) and the 3 year old Hereford cow (2.3 mU/100 mls plasma) were certainly within expected ranges if these data were included with those accumulated from work among the bulls.

Difficulties were encountered in collecting blood samples from the Hereford bull, No 209, (Table 1) both before and after castration. These difficulties must be considered in evaluating the rather high levels of plasma adrenocorticotropic activity from both samples (9.0 and 12.5

mU/100 mls plasma).

Although both fractions II + III and IV - I both contained substantial amounts of adrenocorticotropic activity in most plasma samples, it was apparent (Table 1) that the relative amounts of adrenocorticotropic activity in the respective fractions varied considerably from sample to sample. Since plasma adrenocorticotropic activity in human plasma is associated with both the gamma globulin and beta lipoprotein fractions and these proteins are distributed in both fractions II + III and IV - I in somewhat variable quantities, it is not surprising that the adrenocorticotropic activities of the respective fractions exhibit the observed alterations. These differences probably reflect slight alterations in fractionation procedure rather than real changes in protein assocation of adrenocorticotropin in plasma. Portions of all the protein fractions assayed for adrenocorticotropic activity were extracted to determine whether glucocorticoid hormones were associated with the protein. No glucocorticoid hormones were detected from any of the protein samples assayed indicating the plasma glucocorticoids remained in the supernatent after precipitation of fraction IV - I.

Plasma Glucocorticoids

Recoveries of 4-C^{14}-cortisol added to bull plasma ranged from 40 to 70%. Recoveries were substantially increased by the addition of 4% ethanol to the ethyl acetate used to elute the corticosteroids from the silica gel defatting column.

Plasma glucocorticoid values from the bulls are presented in tabular form in Table 3 and their averages are plotted in graphic form in Figure 3. Glucocorticoid values from the cows are included in Table 2. The plasma cortisol level from the bull calves No 118-A at 3 days of age (4.4 µg%) and the level from 703-B at 2 months of age (2.5 µg%) were considerably lower than average cortisol values from the older bulls. Above 2 months of age, plasma cortisol levels from the bulls ranged from 6 to 9 µg/100 mls plasma and did not demonstrate a consistent alteration with increasing age or differences in plasma adrenocorticotropic activity. Average corticosterone values, on the other hand, ranged from 6.6 to 13.9 µg/100 mls plasma and the levels were not as consistent for individual blood samples as were the cortisol data. Although there was an indication of decreased levels of corticosterone in the plasma from the older bulls, this difference was not significant. Levels

of glucocorticoids from the cow plasma (Table 2) were comparable to values for plasma glucocorticoids in the bulls.

Hematology:

Table 4 contains the average for the hematocrit, total red blood cell count and total and differential leucocyte count from each animal in the study. These data are presented graphically in Figures 4, 5, 6 and 7.

Red blood cell counts ranged from 11.64×10^6 cells per cubic millimeter in No 685-B, the 4 month old bull, to 6.84×10^6 cells per cubic millimeter in Iowana, the 14 year old bull (Figure 4). Bulls below 3 years of age had increased numbers of red blood cells when compared to the older animals.

Total leucocyte counts (Figure 5) also decreased with age and ranged from 14,550 cells per cubic millimeter in No 703-B, a two month old bull calf, to 3,150 cells per cubic millimeter in Iowana, the 14 year old bull. Total red blood cells and leucocyte counts from the cows were not different from the data collected from the bulls.

Uncorrected hematocrit values from the various aged bulls ranged from 38 to 51% and did not exhibit any consistent alterations among the various aged animals.

Percent blood eosinophils (Figure 6) from the bulls ranged from 0.0 to 11.3% of total blood leucocytes. Although changes in percent eosinophils were not directly related to aging, it was apparent the lower values predominently represent younger bulls while the higher percentages represent the older animals. Percent blood eosinophils from the cows (3% from No 118 and 5% from No 575) and the Hereford steer (1% for No 209) were not different from the data from bulls.

Figure 7 illustrates the average percent lymphocyte and neutrophil counts in the various aged bulls. Among bulls from 1 month to 3 years of age, blood neutrophils ranged from 15 to 39% of the total leucocytes, and in all instances were substantially lower than the percentages of blood lymphocytes, which ranged from 49 to 77% of the blood leucocytes. The ratios of lymphocytes to neutrophils were not consistent in bulls beyond 3 years of age. Seven of the bulls beyond 3 years of age had greater percentages of neutrophils than lymphocytes and 7 bulls had higher percentages of lymphocytes than neutrophils. The average percentage of blood neutrophils was 44% of the total blood leucocytes and the average percentage of blood lymphocytes was 46% of the total blood leucocytes, in the bulls 3 years

of age and older. From these data there were no apparent trends in percent concentration of these two cell types in the older bulls during aging.

Hematological alterations noted in No 118-A, the newborn Hereford bull, were very marked in the blood sample taken at 1 month of age compared to the sample taken at 3 days of age. At 3 days of age the packed cell volume was 26%, the lowest hematocrit recorded. At 1 month of age the hematocrit was 39%, a value within the range recorded from the older cattle. Total red blood cell count increased from 6.86×10^6 cells per cubic millimeter, from the 3 day old sample (which again was one of the lowest values recorded from any of the bulls), to 10.16×10^6 cells per cubic millimeter at 1 month of age, and the total leucocyte count increased from 5,111 to 12,700 cells per cubic millimeter of blood during the same time span. The ratio between lymphocytes and neutrophils was also markedly altered during the first month after birth. At 3 days of age the lymphocytes made up only 14% of the total leucocytes and by 1 month of age they had increased to 49% of the total white blood cells. The percentage of blood lymphocytes in the 3 day old animal was far lower than data recorded from any aged animal. On the other hand,

neutrophil percentage decreased from 77 to 39% of the total blood leucocytes during the first month of post-natal life.

One month after castration, a 5 month old Hereford bull, No 209, exhibited several hematological changes. The total blood leucocyte count increased from 8,700 to 13,200 cells per cubic millimeter. The percent concentrations of blood lymphocytes and neutrophils changed from 53% lymphocytes and 40% neutrophils before castration to 44% lymphocytes and 50% neutrophils following castration.

The hematological values from the cows did not exhibit significant differences when compared to comparable aged bulls.

Plasma Proteins

Total plasma proteins (Table 5 and Figure 8) ranged from 5.1 grams percent from No 118-A, the 3 day old bull calf, to 8.4 grams percent in the older bulls. Plasma protein increased with increasing age in bulls up to 3 years of age, but did not show consistent alterations between the various aged animals beyond 3 years of age. On the other hand, the percentages of plasma gamma globulins and albumins were different in bulls beyond 3 years of age (Table 6 and Figure 9). Plasma albumin ranged from 34.8%

of the total plasma protein from Iowana, the 14 year old bull to 59.5% from Climax, the 3 year old bull. As illustrated in Figure 9, plasma albumin tended to decrease with increasing age in the bulls. However, plasma gamma globulins, in general, increased with age of the bulls. Gamma globulin concentrations ranged from 18.0% of the total plasma protein from Climax to 46% of the total plasma protein from Madcap, a 13 year old bull. The formulas for the best straight lines for gamma globulins and albumin from these data were, $Y' = 20.5 + 1.17x$ with a standard deviation of 5.3 for plasma gamma globulins and $Y' = 57.35 - 1.23x$ with a standard deviation of 5.9 for plasma albumin.

After castration total plasma proteins from No 209 increased from 6.4 to 7.9 grams per 100 mls plasma.

TAB

PLASMA ADRENOCORTICOTR

Name	Age yr. mo.	1st sample II+III	1st sample IV-I	1st sample Total	mU/100 ml 2nd II+III
Iowana	14-0	3.0	12.4	15.4	0.4
Beauty	13-1	2.4	3.8	6.2	4.8
Madcap	12-10	3.2	6.4	9.6	5.6
Bell	12-6	3.0	0.0	3.0	8.8
Vic	11-6	7.8	4.8	12.6	7.4
Chieftain	10-9	2.8	2.6	5.4	0.8
Symbol	10-5	0.6	9.2	9.8	7.4
Duke	9-6	3.6	6.8	10.4	3.4
Birch	9-4	0.2	3.4	3.6	6.1
Ben	9-4	6.4	4.8	11.2	5.6
Roburke	6-2	1.6	4.2	5.8	3.9
Pure Gold	5-0	2.2	6.6	8.8	4.6
Explorer	4-7	2.0	5.6	7.6	5.0
Climax	2-10	0.0	4.0	4.0	0.0
Imperial	2-9	3.0	3.8	6.8	3.0
685-A	1-4	1.0	2.8	3.8	0.1
643	1-3	0.6	0.0	0.6	0.7
685-B	0-4	3.6	7.0	10.6*	0.0
703-A	0-2	0.0	1.4	1.4	0.0
703-B	0-2	0.0	0.0	0.0	0.0
Angus Bull	6-0	1.2	5.7	6.9	
118-A	3 da.	3.7	1.0	4.7	
	0-1	1.0	0.0	1.0	
209**	0.5	6.3	2.7	9.0*	6.7

*Difficulty encountered in collecting the blood sample
**Castrated 1 mo. before second sample

LE 1

OPIC ACTIVITY IN BULLS

s plasma sample IV-I	Total	II+III	IV-I	Total	Average
7.4	7.8				11.6
0.7	5.5				5.9
2.9	8.5				9.1
1.7	10.5				6.8
1.2	8.6	6.7	1.4	8.1	9.8
1.6	2.4				3.9
0.0	7.4	6.8	0.9	7.7	8.3
0.3	3.7				7.1
0.9	7.0				5.3
1.8	7.4				9.3
1.4	6.3				6.1
1.1	5.7				7.3
3.1	8.1	3.7	0.0	3.7	6.5
1.0	1.0	1.6	0.0	1.6	2.2
3.7	6.7				6.8
0.0	0.1				2.0
0.0	0.7	1.8	1.1	2.9*	0.7
1.4	1.4	0.4	0.3	0.7	1.1
0.0	0.0				0.7
0.0	0.0				0.0
					6.9
5.8	12.5*				

TABLE 2

PLASMA ADRENOCORTICOTROPIC ACTIVITY AND GLUCOCORTICOID HORMONE LEVELS IN COWS

Number	Age yr. mo.	mU/100 mls plasma adrenocorticotrophin II+III	IV-I	Total	µg/100 mls plasma glucocorticoids Cmp B	Cmp F
118	3-0	0.9	1.4	2.3	7.9	8.4
600	7-0	2.0	7.6	9.6		
575	9-0	0.0	0.0	0.0	13.4	8.6

TAB

PLASMA GLUCOCORT
(μg/100 mls

Name	Age yr. mo.	1st sample Cmp B	1st sample Cmp F	2n Cmp B
Iowana	14-0	14.0	6.3	6.8
Beauty	13-1	11.0	8.3	7.4
Madcap	12-10	8.0	6.6	10.8
Bell	12-6	12.5	4.9	9.4
Vic	11-6	10.3	5.4	10.5
Chieftain	10-9	13.5	6.2	11.5
Symbol	10-5	11.2	6.5	11.4
Duke	9-6	12.3	5.0	10.3
Birch	9-4	10.8	7.4	7.8
Ben	9-4	6.2	5.6	6.9
Roburka	6-2	15.6	9.0	12.1
Pure Gold	5-0	11.5	7.8	14.6
Explorer	4-7	7.0	6.9	4.6
Climax	2-10	14.7	6.9	7.4
Imperial	2-9	12.4	7.0	14.5
685-A	1-4	13.7	9.3	8.9
643	1-3	10.0	5.4	5.7
685-B	0-4		6.0	6.3
703-A	0-2	15.6	8.2	5.8
703-B	0-2	11.2	2.5	4.5
Angus Bull	6-0	8.4	6.1	
118-A	3 da.	8.5	4.4	
118-A	0-1	11.0	8.0	
209	after castration	7.9	10.8	

LE 3

ICOIDS IN BULLS
. plasma)

d sample		3rd sample		Averages	
	Cmp F	Cmp B	Cmp F	Cmp B	Cmp F
	9.4			10.4	7.9
	6.1			9.2	7.2
	8.9			9.4	7.8
	8.1			11.0	6.5
	10.2	13.5	7.7	11.4	7.8
	11.2			12.5	8.7
	6.4	13.5	5.2	12.0	6.0
	9.3			11.3	7.2
	10.1			9.3	8.8
	7.5			6.6	6.6
	7.4			13.9	8.2
	6.1			13.1	7.0
	6.8	13.5	7.8	8.4	7.2
	5.9	16.3	7.5	12.8	6.8
	8.4			13.5	7.7
	4.8			11.3	7.1
	9.4			7.9	7.4
	5.2	15.7	8.4	11.0	6.5
	5.5			10.7	6.9
	6.5			7.9	4.5
				8.4	6.1

TABL

HEMATOLOGY IN
Average

Name	Age yr. mo.	Hct.[1]	WBC[2]	RBC[3] x 10^6
Iowana	14-0		3,150	6.84
Beauty	13-1	42%	4,810	8.54
Madcap	12-10		5,700	9.38
Bell	12-6		5,750	8.21
Vic	11-6	41%	4,680	7.34
Chieftain	10-9		4,800	8.04
Symbol	10-5	50%	4,800	9.20
Duke	9-6	43%	6,720	6.98
Birch	9-4	38%	4,225	8.01
Ben	9-4	39%	6,150	7.02
Roburke	6-2	41%	6,270	7.55
Pure Gold	5-0	48%	6,400	7.86
Explorer	4-7		6,070	10.35
Climax	2-10		8,850	10.05
Imperial	2-9		6,225	9.20
685-A	1-4	51%	7,720	10.88
643	1-3	40%	8,850	7.58
685-B	0-4		10,775	11.64
703-A	0-2	46%	13,200	10.96
703-B	0-2	39%	14,550	11.53
Angus Bull	6-0	43%	7,200	9.50
118-A	3 da.	26%	5,111	6.86
	0-1	39%	12,700	10.32
209	0-5	47%	8,700	9.63
(after castration)			13,200	10.16
Cows				
118	3-0	45%	5,900	8.56
575	9-0		9,200	7.05

[1]Uncorrected packed cell volume

[2]Total leucocyte count per cubic millimeter blood

[3]Total red blood cell count per cubic millimeter blood

E 4

BULLS AND COWS
Values

Leucocyte Differential Count				
Mono.	Lymph.	Neut.	Eosin.	Baso.
5	42	47	6	0
4	42	52	2	0
4	34	60	2	0
7	48	41	4	0
1	59	24	5	0
6	51	40	3	1
6	52	38	4	0
1	40	47	11	1
3	50	43	5	0
7	38	41	9	0
3	50	2>	4	0
8	48	38	7	1
5	40	48	5	1
5	61	31	3	0
5	68	34	3	0
4	72	21	3	0
5	73	21	2	1
5	77	15	0	1
5	66	28	1	1
4	60	35	1	1
3	28	69	0	0
8	14	77	0	1
9	49	39	1	2
5	53	40	2	0
5	44	50	0	1
4	57	35	3	1
2	63	30	5	0

TABLE 5

TOTAL PLASMA PROTEINS IN BULLS AND COWS
grams%

Name	Age yr. mo.	1st sample	2nd sample	3rd sample	Average
Iowana	14-0	7.6	7.4		7.5
Beauty	13-1	8.2	7.7		8.0
Madcap	12-10	8.6	8.2		8.4
Bell	12-6	8.1	8.2		8.1
Vic	11-6	8.2	8.7	8.3	8.4
Chieftain	10-9	7.9	7.7		7.8
Symbol	10-5	8.5	8.4	8.3	8.4
Duke	9-6	7.2	7.7		7.5
Birch	9-4	6.9	7.6		7.2
Ben	9-4	8.2	8.1		8.1
Roburke	6-2	7.6	8.5		8.0
Pure Gold	5-0	7.4	7.9		7.6
Explorer	4-7	7.7	7.9		7.9
Climax	2-10	7.4	7.4	7.6	7.5
Imperial	2-9	7.2	8.0		7.6
685-A	1-4	6.8	6.4		6.6
643	1-3	6.0	6.6	6.9	6.5
685-B	0-4	5.7	6.0	6.2	6.0
703-A	0-2	6.4	6.2		6.3
703-B	0-2	5.7	6.2		6.0
Angus Bull	6-0	7.7			7.7
118-A	3 day	5.1			
118-A	0-1	6.2			6.2
209	0-5	6.4			
after castration		7.9			
Cows					
118	3-0	7.8			
600	7-0	7.5			
575	9-0	8.1			

TABLE 6

PLASMA PROTEIN COMPONENTS BY ELECTROPHORESIS[11/]

% of total plasma protein

Name	Age yr. mo.	Globulins			Ablumin
		Gamma	Beta	Alpha	
Iowana	14-0	36.1	15.7	13.4	34.8
Beauty	13-1	32.1	15.2	10.3	42.4
Madcap	12-10	46.0	11.0	7.0	36.0
Bell	12-6	33.7	10.9	9.7	45.7
Vic	11-6	35.0	15.0	9.6	40.4
Chieftain	10-9	30.8	10.4	10.0	48.8
Symbol	10-5	38.0	12.4	7.8	41.8
Duke	9-6	26.3	13.2	8.9	51.6
Birch	9-4	24.5	19.7	9.1	46.7
Ben	9-4	31.4	11.4	9.3	47.9
Roburke	6-2	26.4	11.1	7.4	55.1
Pure Gold	5-0	34.3	9.0	9.7	47.0
Explorer	4-7	22.0	12.0	9.0	57.0
Climax	2-10	18.0	14.5	8.0	59.5
Imperial	2-9	30.4	16.0	14.4	39.2

[11/] Spinco, Durrum arrangement, veronal buffer, pH 8.6

FIGURE 1

RESPONSE OF INCUBATED RAT ADRENAL TISSUE TO ACTH

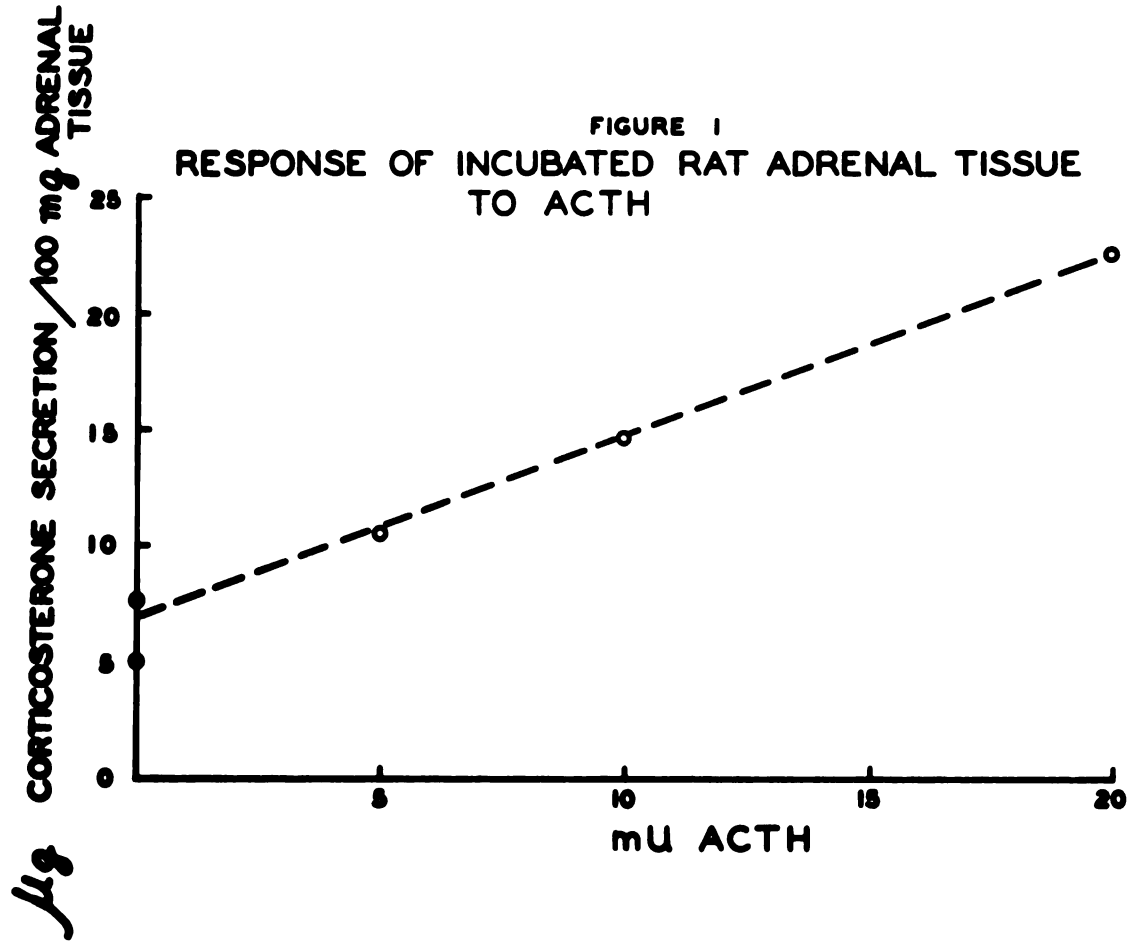


FIGURE 2

PLASMA ACTH WITH AGING

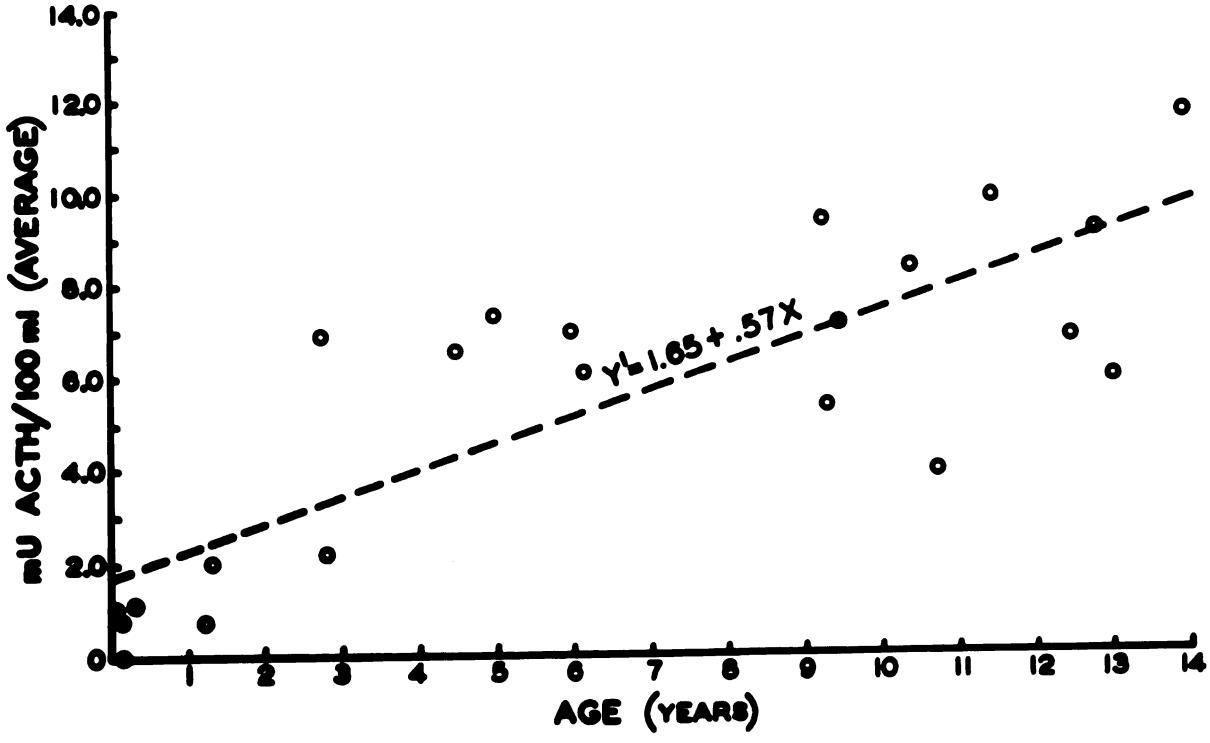


FIGURE 3

PLASMA CORTICOIDS WITH AGING

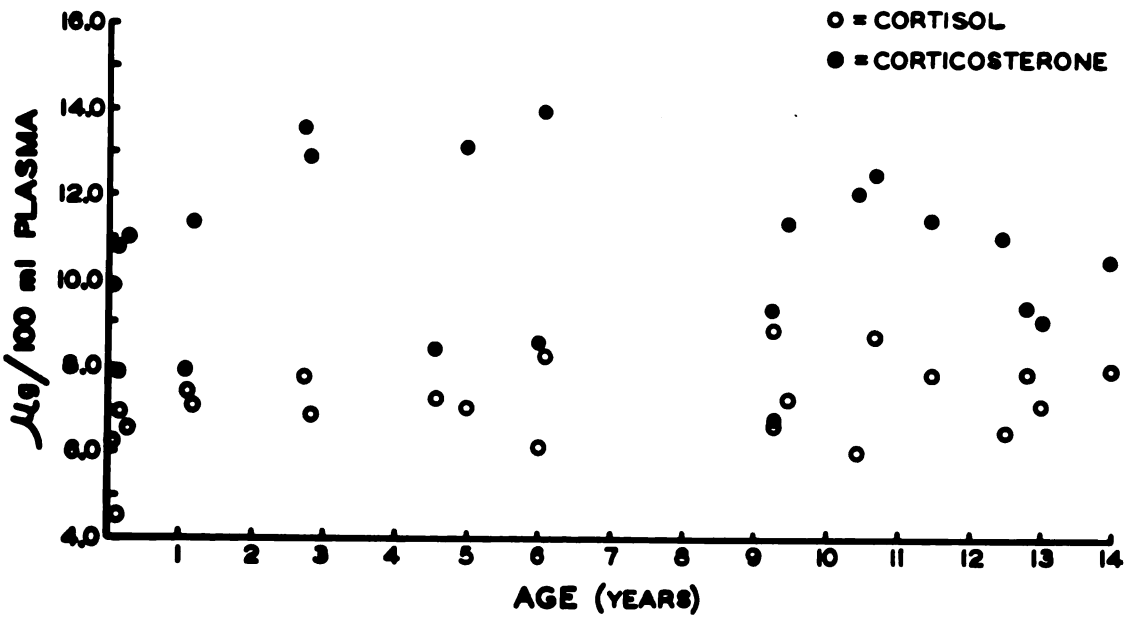


FIGURE 4

RED BLOOD CELLS WITH AGING

FIGURE 5

TOTAL BLOOD LEUCOCYTES WITH AGING

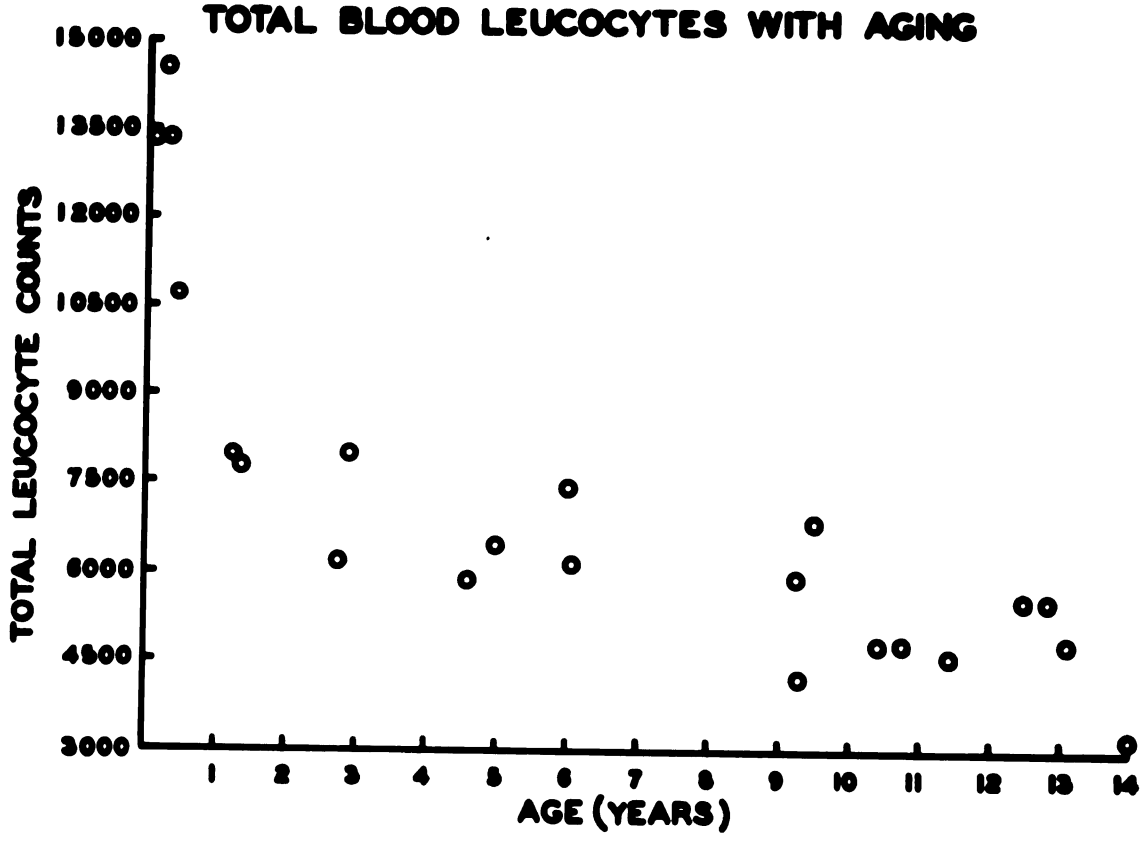


FIGURE 6
BLOOD EOSINOPHILS
WITH AGING

FIGURE 7

BLOOD LYMPHOCYTES AND NEUTROPHILS WITH AGING

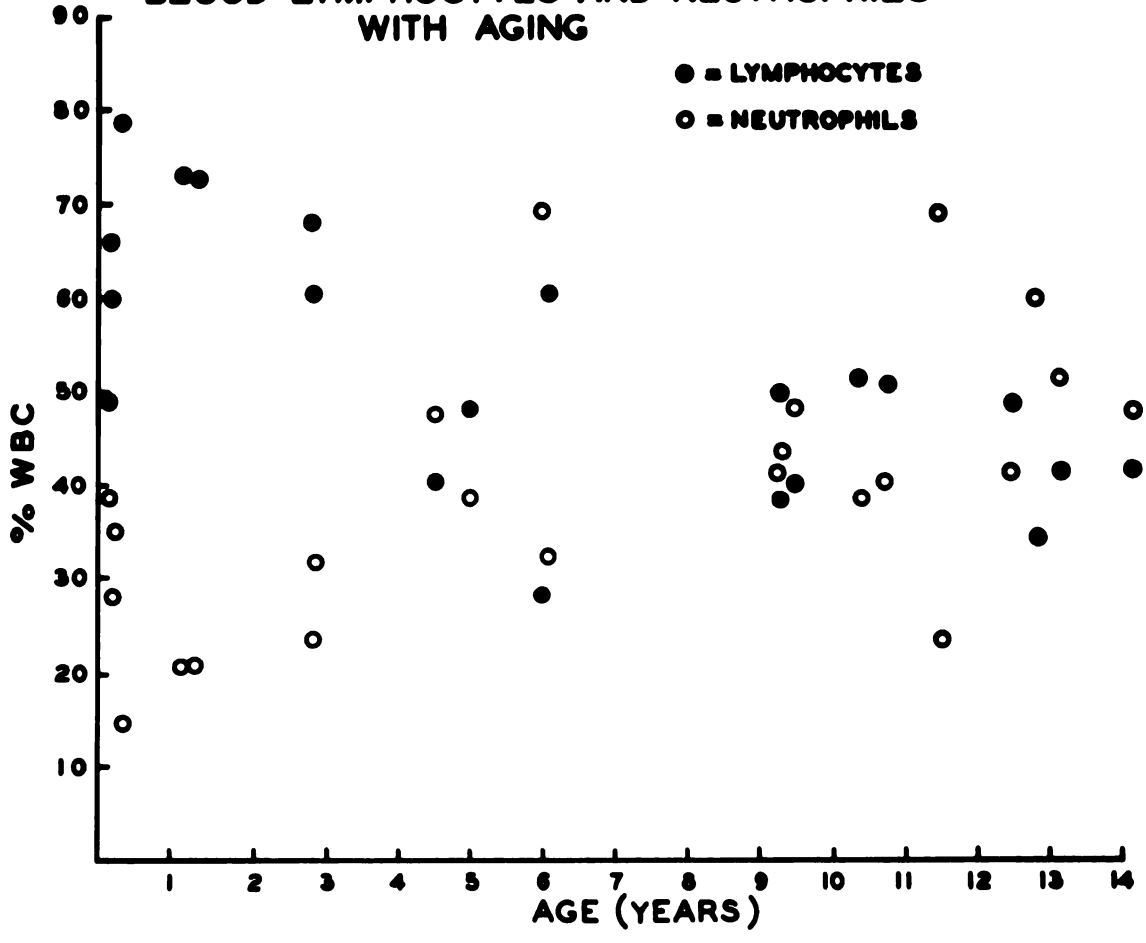


FIGURE 8

TOTAL PLASMA PROTEINS WITH AGING

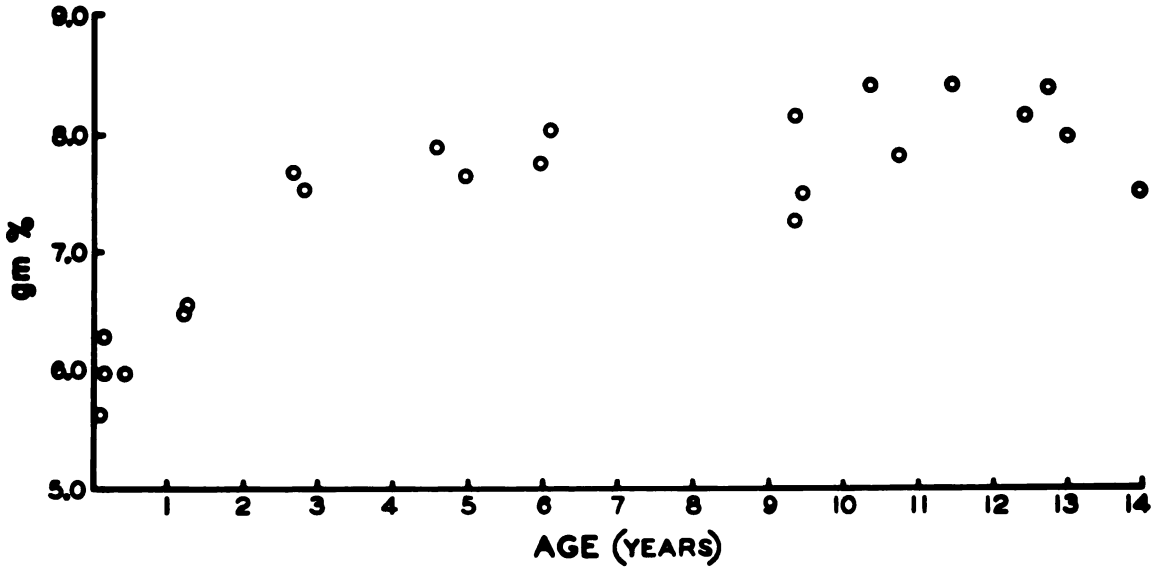
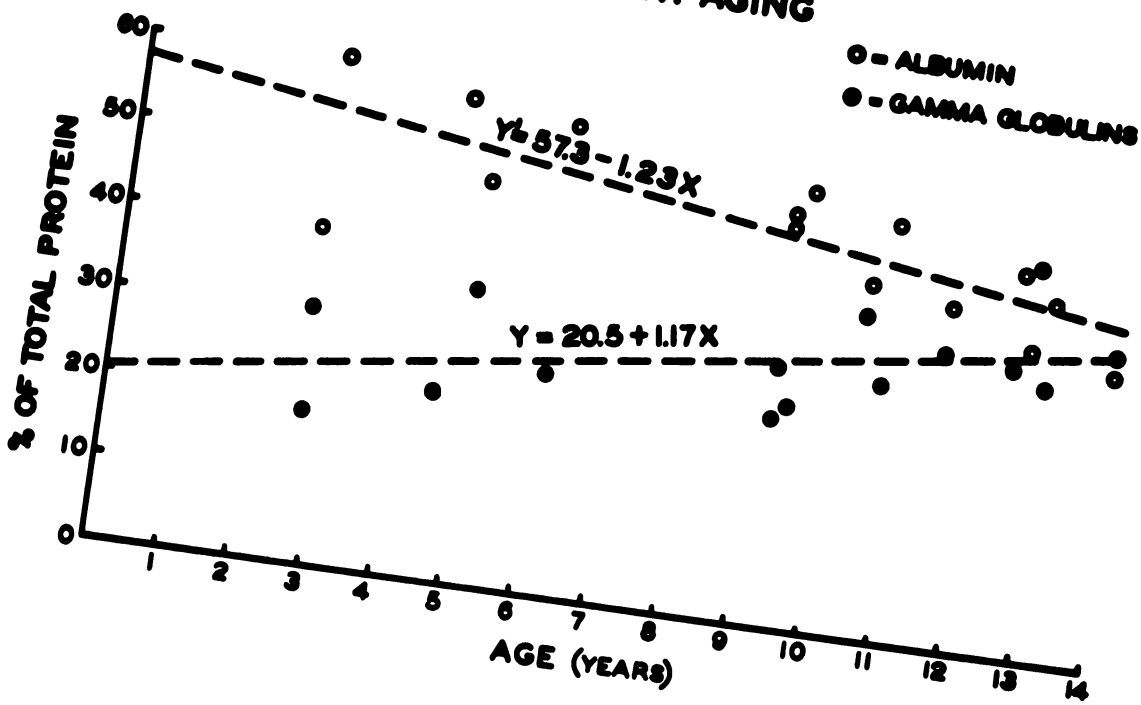


FIGURE 9

% PLASMA ALBUMIN AND GAMMA GLOBULINS WITH AGING



DISCUSSION

Clear indications of histologically detectable degenerative changes in the adrenal glands of many species of animals with increasing age have already been discussed. There is substantial indirect evidence implicating adrenocortical insufficiency in cattle. Cupps et al. (1954 and 1956) and Saarinen and Shaw (1950) reported gross and histological degenerative changes to be associated with ketosis in cows and reproductive difficulties in bulls. It has been clearly established that changes in adrenal physiology are related to these conditions. In another study Shaw (1947) noted the similarities between the clinical symptoms of bovine ketosis and Addison's disease in humans. In the same report Shaw noted a more marked hyperglycemic effect from injected ACTH in normal cows as compared to ketotic cows, further evidence that the adrenals of ketotic cows are not functionally normal. From this information, it would seem likely that cattle may be more prone to adrenal disorders than other mammalian species.

Sayers (1961) indicated that the normal titer of adrenocorticotropic hormone from normal human subjects was less than 0.5 mU/100 mls plasma. An extensive study of plasma

adrenocorticotropic hormone levels from normal subjects and patients with endocrine disorders was made by Miyake et al. (1962) using the corticosterone levels in rat adrenal venous plasma as a bioassay for ACTH activity (Lipscomb and Nelson, 1959). These workers could not detect adrenocorticotropic hormone in the plasma of normal subjects, patients with congenital adrenal hyperplasia, or Cushing's syndrome. On the other hand, patients suffering from Addison's disease had high titers of plasma adrenocorticotropic activity (greater than 40 mU/100 mls plasma).

The adrenal glands of senile humans were not as responsive to exogenous ACTH as the adrenals from younger subjects (Moncloa et al., 1963). The loss of diurnal variations of plasma adrenal corticoids in elderly human patients (Pincus et al., 1954) also indicates that the adrenal glands from these aged subjects may require increased amounts of plasma adrenocorticotropic hormone to achieve normal physiological secretory rates of adrenal corticoids.

Attempts to estimate adrenocorticotropic activity in bovine plasma are to this writer's knowledge original to this laboratory. Nellor (1958a) reported qualitative detection of gonadotropic hormone activities and quantitative detection of thyrotropic, growth promoting and

adrenocorticotropic activities in the plasma of young and aged bulls. Plasma adrenocorticotropic activity was estimated by induced lipid redistribution in the hypophysectomized rat adrenal gland (Simpson et al., 1943). Plasma adrenocorticotropic activities estimated by this method of assay (Nellor, 1958b) ranged from 1.0 to 56.0 IU ACTH activity/100 mls plasma in the young to aged animals. A subsequent stduy concerned with the separation of growth promoting and adrenocorticotropic activities in the plasma of a limited number of bulls (Sutton, 1960) supported the concept of increased plasma adrenocorticotropic activity with aging. Adrenocorticotropic activities in this study as estimated by Sayers (1948) adrenal ascorbic acid depletion in hypophysectomized rats, increased from less than 0.02 IU in a 2 year old bull to from 0.03 to 0.08 IU ACTH activity/100 mls plasma in aged bulls.

The results of the present study, utilizing in vitro corticosteroid production by the rat adrenal as an estimate of adrenocorticotropic activities confirm the concept of increased plasma adrenocorticotropic activities among older bulls. This increase has now been established by three methods of estimating adrenocorticotropin.

The plasma adrenocorticotropic activities recorded from non-stressed bulls from 1 month to 2 years of age were comparable with the low levels of activity reported from normal human subjects. On the other hand, the plasma adrenocorticotropic activities measured in bulls above 3 years of age were substantially higher than the accepted ranges for adrenocorticotropic activity from normal human plasma. It is clear that plasma adrenocorticotropic activity increases with increasing age in cattle. However, this increase may not occur in a strictly linear fashion. The data collected from the limited number of bulls from 3 to 9 years of age indicate that plasma adrenocorticotropic activity may increase rapidly from 3 to 6 years of age followed by a more moderate increase in subsequent years. The levels of adrenocorticotropic activity present in the plasma from the older bulls would be described clinically as Addisonian if they were found in humans.

The unusually high values of adrenocorticotropic activities detected by Nellor (1958b) and Sutton (1960) are not supported by those reported in this study. It must be pointed out that the lipid redistribution assay and the adrenal ascorbic acid depletion method as employed are

semi-quantitative. Both of these assay techniques are substantially less sensitive to exogenous ACTH than is the *in vitro* incubation of adrenal gland tissue method, used in the present study. The extremely variable dose-response relationship encountered with the ascorbic acid depletion assay as employed by Sutton (1960) made an accurate quantitation of adrenocorticotropic activity very difficult.

The Saffran and Schally (1955) assay used in this study is superior to the lipid redistribution and adrenal ascorbic acid depletion methods in that: the assay is based on adrenal hormone production which is accepted as the most important physiological action of adrenocorticotropin; the response is measured by a chemically determinable and point which is more easily quantitated; there is minimal animal preparation necessary for the assay; eight incubation flasks containing adrenal gland tissue of equal stimulatory potential are available for each assay which allows each assay to be standardized by blank and ACTH standard incubations; a linear dose-response curve is obtained at relatively low levels of ACTH stimulation; and the use of an *in vitro* system permits more environmental control during the assay period.

Levels of plasma cortisol recorded in this study were some what higher than values of 17-hydroxycorticosteroids in the plasma of cattle reported by Shaw et al. (1960) (non-stressed heifers and cows averaged from 3.09 to 4.68 μg 17-OHCS/100 mls plasma) and Robertson and Mixner (1956) (values from milking, non-pregnant cows averaged 4.58μg 17-OHCS/100 mls plasma). However, the higher values recorded in this study may in part be due to the correction for hormone loss during the preparative steps before final quantitation of the hormones by the addition of 4-C^{14}-cortisol to the plasma in the isotope dilution principle as an internal standard.

The relative biological importance of cortisol vs. corticosterone in cattle has not been ascertained. Therefore, it is important that both of these hormones be quantitated for an adequate understanding of bovine glucocorticoid physiology. It is of interest that the relative concentrations of corticosterone: cortisol reported in this study (about 1-1/2 times as much corticosterone as cortisol) are in agreement with the corticosterone: cortisol secretion ratio from perfused bovine adrenals (Hechter, 1953) before ACTH stimulation.

If the unchanged plasma levels of corticosterone and cortisol with increasing age are considered alone it could be concluded that adrenal function does not alter as cattle grow older. However, Gray et al. (1961) pointed out that in cases of adrenocortical insufficiency, the concentration of free glucocorticoids in the plasma depended upon the extent of destruction of the adrenal cortex. Therefore the levels of adrenocortical hormones in plasma from patients with adrenocortical insufficiency of Addison's disease may be extremely low or within the normal expected range. Remnants of adrenal cortex from a patient with adrenal insufficiency may be under continuous maximum ACTH stimulation and are thus able to maintain near normal levels of plasma glucocorticoids (Eik-Nes et al., 1955). However, these patients are not able to mobilize additional secretory activity during times of stress as evidenced by lack of increased glucocorticoid secretion during and after exogenous ACTH infusion. Under the principle of feedback control of pituitary-adrenal function, patients suffering from adrenocortical insufficiency would require additional amounts of plasma adrenocorticotropic hormone in their attempt to maintain physiologically normal amounts of circulating adrenal cortical hormones. From these data it would appear

logical that the increased levels of plasma adrenocorticotropic hormone may be required to maintain normal adrenal cortical secretory activity as cattle grow older.

If the adrenals in the cattle studied in this report were responding to increased plasma adrenocorticotropic activity with increased glucocorticoid hormone production, the increase should have been evident in increased levels of circulating adrenal corticoids. It is also possible that glucocorticoid hormone metabolism could increase simultaneously with increased adrenal synthesis producing no increase in levels of circulating glucocorticoids. However, Holcombe (1957) reported no increase in urinary "reducing corticoids" in older sheep and cattle of either sex. Additional evidence against increased adrenal cortical secretion in this study was the high level of plasma adrenocorticotropic activity. With the feedback mechanism of pituitary-adrenal control functioning normally, the increased plasma glucocorticoids would have been expected to effect a decrease in the levels of plasma adrenocorticotropic hormone similar to the undetected levels of ACTH activities reported from congenital adrenal hyperplasia and Cushing's syndrome patients (Miyake et al., 1962), both

of which involve high levels of circulating adrenal glucocorticoids.

There also seemed to be marked differences in the response to stress between young and old bulls in this study. When trouble was encountered in collecting blood samples from No 685-B, No 643 and No 209, high levels of plasma adrenocorticotropic hormone were encountered (10.6, 2.9, 9.0 and 12.5 mU/100 mls plasma). On the other hand when sampling difficulties were encountered among the older bulls, there was no apparent increase in plasma adrenocorticotropic hormone compared to ther samples taken from the same animals when no stresses from collecting the samples were involved.

On the basis of this evidence, the physiological alteration indicated by the increased levels of plasma adrenocorticotropic hormone and normal levels of circulating glucocorticoid hormones is interpreted to indicate either an adrenocortical insufficiency in older cattle or they are not as sensitive to adrenocorticotropic hormone. In view of the normal plasma glucocorticoid levels this adrenal insufficiency would be evident only in conditions of increased stress, would occur in disease, emotional or environmental changes, or a further increase in age. While

none of the bulls in this study can be classified as truly adrenocortically insufficient, it is probable that the condition would be evident in the terminal stages of this physiological condition.

The high level of plasma adrenocorticotropic hormone from No 118-A at 3 days of age (4.7 mU/100 mls plasma) is of considerable interest. Leeman (1963) studied the corticosterone secretion from newborn rats. She found no significant increase in plasma corticosterone after intravenous injection of ACTH in rats under 3 weeks of age. On this basis, the high level of plasma adrenocorticotropic activity from No 118-A at 3 days of age may indicate the adrenal gland or the newborn calf is either unable to respond normally to plasma ACTH, the relatively low level of adrenal glucocorticoids in the plasma of the newborn calf may not be of sufficient magnitude to trigger the feedback mechanism controlling pituitary ACTH synthesis and release, or the pituitary-adrenal regulatory systems of the newborn calf may not be functionally mature at this age.

A reduction in corticosterone secretion into adrenal venous blood from castrated male rats was demonstrated by Kitay (1963). Corticosterone secretion was reduced in spite of an increase in adrenal gland weights.

Administration of testosterone or high levels of ACTH restored corticosterone secretion rates. Nowell and Jones (1957) reported decreased pituitary content of ACTH after castration of rats. They hypothesize that the decrease in pituitary content of ACTH reflects increased secretion of ACTH by the pituitary. Although difficulty was encountered in collecting blood samples before and after castration from No 209, the higher levels of adrenocorticotropic activity encountered after castration may be due to adrenal secretory abnormalities as described by Kitay (1963).

Increased levels of red blood cells present in the bulls below 3 years of age are consistent with changes in red blood cell counts with age in cattle reported by Schalm (1961). Schalm also reported decreased amounts of all species of blood leucocytes except eosinophils in older cattle. Although total blood leucocyte counts did tend to decrease in the dairy cow from 2 to 14 years of age, the average value was 6,630 leucocytes per cubic millimeter of blood which is considerably above the average value of blood leucocytes from the bulls in this study of similar age (5,190 leucocytes per cubic millimeter of blood).

The low percentage of lymphocytes (14% total leucocytes) from No 118-A at 3 days of age was consistent with data reported by Schalm (1961) for newborn calves. Within the first weeks of life calves reach what are called "normal" levels of blood lymphocytes and neutrophils (lymphocytes 60 to 80% and neutrophils 8 to 35% of the total blood leucocytes). In contrast to the reduced percentages of lymphocytes and increased percentages of neutrophils from aged bulls in these studies, Schalm reported relatively constant concentrations of blood lymphocytes and slightly reduced percentages of blood neutrophils as cows increase in age from 2 to 14 years. Since these observations by Schalm are in agreement with data collected from the cows in this study (No 118, the 3 year old, had 57% lymphocytes and 35% neutrophils, and No 575, the 9 year old, had 63% lymphocytes and 30% neutrophils), there is an indication of sex induced variations in the relative percentages of leucocytes in aged cattle.

The percentage changes in blood neutrophils and lymphocytes from No 209 following castration are of great interest. Before castration the 5 month old Hereford bull had 53% lymphocytes and 40% of the blood leucocytes were neutrophils. However, 1 month after castration, the

leucocyte species had been reversed to 44% lymphocytes and 50% of the blood leucocytes were neutrophils. This observation may be correlated with the alterations in percentage of blood lymphocytes and neutrophils from the older bulls. The decrease in testicular humoral function that occurs with age (Hollander and Hollander, 1958), may be involved in the decrease in blood lymphocytes and increase in blood neutrophils observed in the older bulls.

Although the increase in blood neutrophils and decrease in blood lymphocytes could be related to increased adrenocortical hormone production (Daugherty and White, 1947), this is unlikely since these changes were not observed in like aged cows with similar levels of plasma adrenocorticotropic and glucocorticoid hormones and the other evidence presented in this study implicating adrenal insufficiency is too encompassing.

The increase in blood eosinophils with aging was not consistent. However the average eosinophil count from cattle beyond 3 years of age (5.1% of total blood leucocytes) was substantially higher than the average eosinophil count from cattle below 3 years of age (1.6% of the total blood leucocytes). This increase in blood eosinophils with age can be interpreted as indicative of decreased

adrenocortical function (Hills et al., 1948).

The reason for the increase in total plasma protein up to about 3 years of age was not apparent. However, since plasma protein levels show no further alteration beyond this age, the attainment of physical and/or reproductive maturity may be involved. If this assumption is correct the increased plasma protein could reflect an equilibrium in protein anabolism in the mature bull. This hypothesis of increased total plasma protein reflected from decreased protein anabolism was supported by the increase in plasma protein in No 209 from 6.4 grams percent before castration to 7.9 grams percent after castration.

The apparent changes in percentages of plasma albumin and gamma globulins in bulls beyond 3 years of age are consistent with the hypothesis of adrenal insufficiency in the older cattle. The increase in gamma globulins and decrease in albumin percentages are in agreement with observations from human subjects with adrenal insufficiency (McCullagh and Lewis, 1960).

There were no alterations in hematocrit, cell counts, or plasma protein concentrations that would indicate hemoconcentration with aging, a factor that would effect affective plasma distribution volume of the circulating hormones.

The changes in concentration of eosinophils, and relative alterations in percentages of plasma albumins and gamma globulins are considered to be additional evidence supporting the blood hormonal data implying adrenocortical insufficiency in aging cattle.

Cattle may be a near ideal biological subject to study pituitary-adrenal physiology, especially for those conditions involving adrenocortical insufficiency. Their size and ease of handling without obvious stress allow the investigator to collect large volumes of blood at frequent intervals, and to conduct experiments not physically feasible to consider in the common laboratory animals.

SUMMARY

Variations in pituitary-adrenal function in cattle, in relation to age, were investigated in 26 beef and dairy cattle ranging in age from 3 days to 14 years by the simultaneous biological assay of plasma adrenocorticotropic hormone and the chemical assay of the glucocorticoid hormones, cortisol and corticosterone.

Plasma to be used for adrenocorticotropic hormone assay was fractionated into its major protein components in order to increase the relative concentration of the adrenocorticotropic hormone in the protein sample being assayed. Adrenocorticotropic activity was estimated by the increase in corticostermone production of rat adrenal gland after incubation with the protein fractions containing the adrenocorticotropic activity. Levels of bovine plasma cortisol and corticosterone were chemically determined after several purification steps followed by isolation of the hormones by paper chromatography. The blood humoral data were supplemented by measurement of blood hematocrits, total red and white cell counts, leucocyte differential counts, total plasma protein levels, and relative amount of the plasma protein components following electrophoretic separation.

Adrenocorticotropic activity in bulls below 2 years of age was low (average values ranged from 0 to 2 mU ACTH activity/100 mls plasma) and in many cases undetectable. Beyond 2 years of age, plasma adrenocorticotropic activity was significantly increased. Cattle above 3 years of age had substantial amounts of plasma adrenocorticotropic activity (average values ranged from 3.9 to 11.6 mU ACTH activity/100 mls plasma).

Levels of plasma cortisol (6 to 9 μg/100 mls plasma) and corticosterone (6.6 to 13.9 μg/100 mls plasma) did not exhibit any consistent alterations related to the increased plasma adrenocorticotropic activity.

A newborn calf also exhibited a high level of plasma adrenocorticotropic activity with normal levels of circulating glucocorticoids.

Although total blood leucocytes decreased with increasing age, the percentages of blood eosinophils were higher in the older cattle. There were also increased percentages of blood lymphocytes in bulls beyond 3 years of age. Total red blood cell counts were higher in blood from bulls up to 3 years of age but there were no changes in red cell counts in bulls beyond 3 years of age.

Total plasma protein increased with increasing age in bulls up to 3 years of age. Although total plasma protein were not different in cattle beyond 3 years of age, the relative percentages of plasma albumin tended to decrease while plasma gamma globulins showed a slight increase with increasing age.

The increased levels of plasma adrenocorticotropic hormone with increasing age occurring without a simultaneous increase in levels of plasma glucocorticoids is interpreted as indicating that cattle undergo either a progressive adrenocortical insufficiency or become insensitive to ACTH with increasing age. The concomitant increases of adrenocorticotropin secretion are believed to be necessary in order to maintain blood levels of glucocorticoids within the normal ranges. This interpretation was supported by increased percentages of blood eosinophils, decreased percentages of plasma albumin and increased percentages of plasma gamma globulins from the older bulls.

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