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## Relationship of Circulating Eosinophils, Other Blood Cellular Components and Plasma Corticoids in Dairy Cattle Subjected to Nutritional Stress

Eugene W. Wisniewski  
*Utah State University*

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RELATIONSHIP OF CIRCULATING EOSINOPHILS, OTHER  
BLOOD CELLULAR COMPONENTS AND PLASMA  
CORTICOIDS IN DAIRY CATTLE SUBJECTED  
TO NUTRITIONAL STRESS

by

Eugene W. Wisniewski

A thesis submitted in partial fulfillment  
of the requirements for the degree

of

MASTER OF SCIENCE

in

Dairy Science

Approved:

UTAH STATE UNIVERSITY  
Logan, Utah

1975

## ACKNOWLEDGMENTS

I would like to express my sincere appreciation to my major professor, Dr. Clive Arave, for his many hours of unselfish guidance. I would also like to thank the other members of my committee, Dr. Joseph Blake and Dr. Robert Lamb for their critical review of this thesis and most of all for their limitless patience. I would also like to mention Dr. George Stoddard, Head of the Department of Dairy Science, in a very special way; thanks to his continual moral and financial support over the past years completion of this thesis was made possible.

In addition, I'd like to thank Dr. Ross Smart for his technical advice and the use of his laboratory, Art Svejda for performing the corticoid analysis, Mr. and Mrs. Robert Nell for their assistance in the statistical analysis, Pat French for her unselfish help in performing the blood analyses and Mrs. Floyd Israelsen for typing at all hours of the day and night in order to finish this thesis within the deadline. I'd like to thank my brother, Steve, for giving me the extra time to complete this thesis by doing my share of the work on our dairy farm.

Finally, I reserve my most sincere gratitude to my parents, Tad and Irene, without whom completion of this assignment would not have been possible.

*Eugene W. Wisniewski*  
Eugene W. Wisniewski

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## ABSTRACT

Relationship of Circulating Eosinophils, Other  
Blood Cellular Components and Plasma  
Corticoids in Dairy Cattle Subjected  
to Nutritional Stress

by

Eugene W. Wisniewski, Master of Science

Utah State University, 1975

Major Professor: Dr. Clive W. Arave  
Department: Dairy Science

Twenty-six first lactation dairy cattle were sampled biweekly for four months to determine the effect of five levels of nutrition on blood plasma corticoids, total and differential leucocytes, hemoglobin and packed cell volume. Cows completing their 305 day lactation during the four month sampling interval were removed from the experimental herd, but were sampled twice more.

It was hypothesized that cows on rations below NRC requirements for milk production would demonstrate stress symptoms of elevated plasma corticoids, elevated white blood count, neutrophilia and eosinopenia. It was also hypothesized that measuring the decrease in eosinophils would be a better method of quantifying nutritional stress than by

measuring corticoids, since circulating eosinophils are not subject to rapid increases from stresses of sampling, as are the corticoids.

Least squares analysis indicated a trend for increased corticoids with decreased level of nutrition, but the trend was not significant ( $p > .05$ ). Eosinophil level was significantly ( $p < .05$ ) correlated with ration indicating increased stress caused a rise in eosinophils instead of a decline as was expected. Eosinophils were negatively correlated with corticoids ( $-.12$ ) as expected but the relationship was not significant ( $p > .05$ ). Ration was positively correlated with milk yield, body weight, and number of circulating neutrophils. Ration was negatively correlated with total leucocyte count. No significant change in hemoglobin or packed cell volume was detected.

Significant ( $p < .01$ ) increases in leucocytes, neutrophils and hemoglobin were observed in the cows after completion of the 305 day lactation. Significant ( $p < .01$ ) decreases in corticoids and milk production were also observed in these cows.

(80 pages)

## INTRODUCTION

Extensive research has been conducted over the last 70 years to determine the physiological response of man and animals to various physical, psychological and social stresses. The primary response to stress is the stimulation of the anterior pituitary to secrete adrenocorticotrophic hormone (ACTH). ACTH stimulates the cortex of the adrenal gland to secrete a variety of adrenocorticosteroid hormones. Adrenocorticosteroids, also called corticosteroids, glucocorticoids, or simply corticoids, regulate the circulating concentrations of different cellular and non-cellular chemical components of the blood. Among the cellular components regulated by the pituitary-adrenal system are the circulating leucocytes, in particular the eosinophils.

Stress has been quantified by measuring increases in ACTH and corticoids or by measuring decreases in circulating eosinophils. Assaying ACTH and/or corticosteroids in dairy cattle is not always a valid indication of long term stress (such as nutritional stress) due to various technical problems. It is hypothesized that measuring the change in circulating eosinophils is a more accurate method of studying stress, because the eosinophils are less variable than the corticosteroids during short periods of time. This method has been used extensively in research with man, mice and rats but not in dairy cattle, especially in

the field of nutritional stress i. e. , stress induced by a diet below NRC requirements. (87).

The objectives of this study are to determine the effect of various dietary energy levels on the total and differential leucocyte count, the packed cell volume, hemoglobin concentration, and total plasma corticoid levels in first lactation dairy cattle. Furthermore, the relationships between circulating eosinophils (measured by two different methods) and plasma corticoids will be determined. If a significant relationship occurs, measuring the eosinophils could be a valuable quantitative indicator of nutritional, as well as other long term stresses.

It is recognized that many factors can alter the concentrations of circulating eosinophils and corticosteroids. Included among these factors are age, health of the animal, milk production, stage of lactation, stage of gestation, environmental as well as social and psychological conditions.

In order to analyze the effect of nutritional stress in this study, the effects of all these interfering factors will be minimized or eliminated from the experimental model wherever possible.

## REVIEW OF LITERATURE

Hypothalamic, Pituitary, Adrenal Response

Bajusz (4, p. 90) defined stress as "the emergency endocrine mobilization of the organism in response to intrinsic and extrinsic stimuli in order to achieve adaptation or to allow for struggle and defense." In other words, stress is a mechanism which allows the body to adjust to changes in the internal or external environment. When an animal is stressed the pituitary-adrenal axis is stimulated to secrete a variety of adrenocorticosteroid hormones in a well defined sequence of reactions. This response to non-specific stress has been termed the "General Adaptation Syndrome" by Selye (115). It has been suggested that the secretion of ACTH and large amounts of anti-inflammatory adrenal steroids during stress may be useful in enabling an organism to survive during an emergency by suppressing excessive inflammatory reactions and by adjusting other metabolic processes to cope with the emergency.

In addition to stimulating the pituitary-adrenal axis, stress influences the Central Nervous System (CNS) via the hypothalamus as evidenced by the work of Fortier and Selye (40). After the experiments by deGroot and Harris (26) showed that stimulation of certain areas in the hypothalamus can cause changes in pituitary-adrenocortical function,



over 300 papers on the regulation of the pituitary-adrenocortical system by higher brain centers were published (4).

It is apparent that the CNS and the endocrine system under the control of the pituitary are intimately integrated to provide coordination between the two systems. In separate laboratories Guilleman (50) and Saffran, Schally and Benfey (107), discovered a neurohormonal substance from the hypothalamus called corticotropin releasing factor (CRF) which had the ability to stimulate secretion of ACTH from the anterior pituitary. Bajusz (4) pointed out that even though the hypothalamus plays an important role in ACTH secretory control, it is not an autonomous primary center of regulation but merely coordinates and transmits impulses from higher brain regions to the pituitary by neurohormonal secretions.

This delicate balance between the hypothalamus, pituitary and adrenal cortex was exemplified by the research of Dallman and Jones (24). They observed that stress increased responsiveness in the CNS and the anterior pituitary so that subsequent secretions of ACTH provoked a greater than normal CRF induced release of ACTH, causing a period of over-responsiveness immediately after the initiation of stress. At the same time the feedback signal of rising corticosteroids, provoked by the ACTH secretion, provided an inhibitory affect on the pituitary. Consequently, the two effects canceled, leaving the adrenal cortical system continually responsive to repeated discrete stresses but not over-responsive.

The mechanism of adaptation is a complex process of inter-weaving controls and fine balances. The end result of the endocrine response to stress is activation of the adrenal glands to secrete corticosteroid hormones.

#### Adrenocortical Secretions

Almost 50 different steroids have been isolated from the mammalian adrenal cortex (130). This mixture contains mineral and glucocorticoids, androgens, progestogens, estrogens and inactive steroids which are precursors or metabolites of the active hormones. The structures of the more common adrenocortical steroids are illustrated in Appendix A.

The principal adrenocorticosteroids in jugular venous blood from dairy cattle under normal environmental conditions were positively identified as cortisol (11-B;17-a, 21-trihydroxy-4-pregnene-3, 20-dione) and corticosterone (11-B, 21-hydroxy-4-pregnene-3, 20-dione) by Ester-green and Venkateseshu (33).

Range and mean values for corticosterone in non pregnant, lactating dairy cattle were 15 to 51 ng/ml and 30 ng/ml plasma respectively(33). The range and mean values for cortisol in the same cows were 1 to 131 ng/ml and 72 ng/ml of plasma, respectively. The ratio of cortisol to corticosterone was on the average 2.4:1. Bush (17) reported that the ratio of cortisol to corticosterone was constant in adults of the species studied. He reported ratios of 1:1 in the cow, 6:1 in the dog,

15 to 20:1 in the sheep and over 20:1 in the Rhesus monkey. Balfour, Comline and Short (5) reported a 1.4:1 ratio in calves 23 days old and a 3.9:1 ratio for calves 1 year old. Garverick et al. (42) observed a 2.1:1 ratio in cows and a 1.6:1 ratio in heifers during estrous. Randel et al. (94) found freemartins to have a 5.5:1 ratio while heifers at puberty had a 2.8:1 ratio. Pregnant cows were observed to have a 4.1:1 ratio by Randel and Erb (95). Venkateseshu and Estergreen (132) studied corticoid differences among the dairy breeds. Guernseys had an average ratio of 1.5:1 while Holsteins had a 4:1 ratio. However, it was noted that the ratio of corticosteroids secreted by the adrenals wasn't necessarily the same as the ratio in peripheral blood. Plasma circulation levels of corticosteroids depend on the distribution rate into body pools, utilization by different tissues and removal from circulation via degradation and elimination. Thus a different ratio might be due to differences in distribution, utilization, degradation and elimination, and not to breed differences.

Venkateseshu and Estergreen (132) observed that treatment with 200 I. U. of ACTH altered the cortisol to corticosterone ratio from 1:1 before treatment to 3:1, two hours after treatment. Kaass et al. (68) and Fevold (35) suggested that the 17-hydroxylating system of the adrenal gland is preferentially stimulated by ACTH and leads to the formation of (17-hydroxylated) cortisol while decreasing substrate availability for corticosterone (which lacks the 17-hydroxy group).

The cortisol to corticosterone ratio after the administration of exogenous ACTH or after a stress induced increase of endogenous ACTH is important if total corticoids are measured as an indication of adrenal function. Many of the methods used to measure total corticosteroids depend on various reactions with the 17-hydroxy group. Therefore, corticosterone, which lacks a 17-hydroxy group, would not be detected by these procedures.

Willet and Erb (142) measured total corticoids by competitive protein binding with progestins removed with n-hexane. Cortisol and corticosterone were separated by paper chromatography and then individually measured. They reported that the average sum of cortisol plus corticosterone equaled only about two thirds of the total binding corticoids. Plasma cortisone and deoxycorticosterone did not account for the differences observed and thus, not all of the protein bindable compounds were identified as corticosteroids. The correlations between total corticoids and cortisol was 0.85, between total corticoids and corticosterone was 0.46 and between cortisol and corticosterone 0.63. All three correlations were highly significant ( $p < .01$ ).

Gwazdauskas, Thatcher and Wilcox (53) suggested that since the ratio of cortisol to corticosterone was relatively stable, and since the concentration of corticosterone was low compared to cortisol, it was unnecessary to separate the two corticosteroids. In other words, quantifying total corticosteroids is an adequate measure of adrenocortical function.

Effect of ACTH on Adrenocorticosteroids

Guillemin et al. (51), Bliss, Nelson and Samuels (9), Eik-Nes et al. (32) and numerous other researchers, reported that intramuscular or intravenous injections of ACTH caused significant increases in circulating plasma corticosteroids in man. Migeon et al. (82) reported that ACTH injection doubled 17-hydroxy-corticosteroids (17-OHCS) within two hours after injection in the Rhesus monkey. Gwazdauskas, Thatcher and Wilcox (53), and Jones and Shannon (66) reported proportional increases in both cortisol and corticosterone, while Venkataseshu and Estergreen (132) observed a dramatic doubling in cortisol but no increase in corticosterone one hour after ACTH injection. Wagner, Strohbehn and Harris (138), Wagner and Oxenreider (137), Paape et al. (91), Robertson and Mixner (101), and Brush (16) reported significant increases in 17-OHCS within one half hour after ACTH injection with dosages ranging from 100 to 600 I.U. Brush (16) observed increases in 17-OHCS from 0 to 30 ng/ml plasma before ACTH treatment to as much as 200 ng/ml plasma after treatment. Most researchers reported increased 17-OHCS levels from one-half after treatment, followed by a rather sharp decline four hours later and a gradual return to normal preinjection levels in two to eighteen hours. Gwazdauskas, Thatcher and Wilcox (53) reported that ACTH injections also significantly increased plasma progesterone levels, indicating that the adrenal cortex also

secreted this hormone, since the adrenal cortex is the primary target organ of ACTH.

#### Methods of Measuring ACTH

The response of the adrenal cortex to ACTH is well documented. It seems that measuring the circulating levels of ACTH is a direct method of quantifying stress. Unfortunately, several complications prevent a practical, routine analysis of ACTH concentration in bovine plasma.

The ACTH molecule is a simple polypeptide composed of 39 amino acids with a molecular weight of 4567. Bovine and human ACTH have almost identical amino acid sequence differing only at positions 25 and 33. The structure, revised by Li (74), is illustrated in Appendix B. ACTH is a difficult molecule to assay. According to Felber and Aubert (34) it binds unspecifically to any surface even to glassware. In addition, ACTH is susceptible to degradation by proteolytic enzymes in the plasma. This degradation starts immediately following blood sampling.

Various biological assays have been used to measure ACTH concentrations. One method described by Sayers, Sayers and Woodbury (109) made use of the fact that ascorbic acid in the adrenal cortex was depleted by the presence of ACTH. Hypophysectomized rats were used in this assay. According to Royce and Sayers cited by Lipscombe and Nelson (75) only arginine vasopressin (a substituted neurohormone) can deplete ascorbic acid in the hypophysectomized rat in addition to ACTH.

This method had a low range of sensitivity or minimal effective dose (MED) of 0.25 milli unit (MU) of ACTH. Another biological assay depended on the ability of ACTH to stimulate corticosteroid secretion in vivo by the method of Nelson and Hume (88) or in vitro by the method of Saffran and Schally (106).

Lipscombe and Nelson (75) developed 2 additional methods of assaying ACTH. In the jugular technique, test material containing ACTH was injected into the jugular vein. Corticosteroids were measured in the adrenal venous blood 5 minutes after injection. The retrograde technique consisted of retrograde injection of ACTH (0.05 to 0.20 ml) in the adrenal vein, followed by sampling of adrenal venous blood 90 seconds later. The retrograde technique was less precise but provided greater adrenal sensitivity to minute doses of ACTH. Neither method was suited for routine analysis because a dissecting microscope must be used on hypophysectomized rats.

Less direct methods of assaying ACTH were based on maintenance of adrenal weight, depletion of cholesterol, and involution of the thymus in response to ACTH. Analytical problems stem from restrictions based on specificity, sensitivity, type and quantities of biologic fluid required.

Stress induced secretion of ACTH elevated plasma corticosteroids and according to Forsham et al. (37) and Spiers and Meyer (122) eosinopenia (reduction of circulating eosinophils) occurred. According to Rosemberg et al. (103) also in accordance with the findings of Spiers

and Meyer (122), eosinopenia was sufficiently precise to use in the bioassay of ACTH as a quantitative measurement of stress.

The only practical method of routinely analyzing ACTH levels in bovine plasma was the method of Yalow et al, (146) or this method modified by Demura et al. (27). This radioimmunoassay method was based on the specificity of an antibody to bind with ACTH. The need for a sensitive antibody was essential due to the extremely low concentration of ACTH. This method has been used to assay human ACTH but has not yet been used for the bovine hormone because of a lack of an available antibody specific for bovine ACTH. Since ACTH is a small peptide it is weakly antigenic. Also, ACTH tends to suppress antibody synthesis through its steroidogenic effects. Voight, Fehm and Pfeiffer (135) reported that ACTH contains no disulfide bridges and hence was a weak immunogen.

#### Response of the Adrenal Cortex to Stress

Since ACTH cannot be measured in dairy cattle for the aforementioned reasons, researchers have measured plasma levels of corticosteroids to quantify stress. According to Forsham cited by Williams (143), measurement of circulating 17-OHCS was the best method of assessing adrenocortical function. This method had the double advantage of focusing on the antiinflammatory function of the adrenocorticoids and quantitatively the 17-OHCS are the largest fraction of these adrenal steroids.



The stress of muscular exercise in humans was analyzed by measuring corticoid increases by Viru (133), Crábbe, Riondel and Mach (22), Ludvigsen (77) and Staehelin et al. (123). Similar increases in 17-OHCS were observed by Viru and Akke (134) in the guinea pig after strenuous exercise. Following ten minutes of strenuous exercise, Fortier, deGroot and Hartfield (39) observed an initial drop in corticoids, a period of rising levels, a peak within four minutes after the exercise, and then a subsequent decrease to values below the initial concentration. Numerous examples of elevated corticosteroid levels due to psychological or social stress have been reported by Bronson and Eleftheriou (14), Coover, Goldam and Levine (20), Coover, Ursin and Levine (21), and Lovely, Pagaro and Paolino (76). Barrett and Stockham (6), Fortier (38), Franksson and Gemzell (41), Mason, Harwood and Rosenthal (81), and Sandberg et al. (108) also observed elevated corticosteroid levels due to psychological stress. Extensive work relating social stress, increased corticosteroids and resistance to viral and bacterial infection in chickens has been done by Gross and Colmano (46-49).

Elevations in corticosteroids were reported by Reid (98) and Scharpiro, Marmorston and Sobel (112) due to low environmental temperatures while Khan, Dickson and Meyers (69) reported no increase in corticoids in newborn calves exposed to  $-4^{\circ}\text{C}$ . Bergman and Johnson (8) reported decreased plasma glucocorticoids in cattle subjected to high temperatures over long periods of time.

Robertson et al. (102) observed increases in 17-OHCS in dairy cattle due to the acute stress conditions of surgery, acute mastitis, milk fever, uterine prolapse, retained placenta and metritis.

#### Methods of Measuring Corticosteroids

Methods used to determine plasma levels of corticosteroids include chemical analyses, biological assays and radioimmunoassays.

Brush (15) used paper chromatography to separate the corticosteroids and performed a blue-tetrazolium reaction to quantify the cortisol and corticosterone by measuring fluorescence. Porter and Silber (93) developed a method of measuring 17-OHCS by reacting large amounts of plasma extracts with sulfuric acid and phenylhydrazine and then measuring the concentration of 17-OHCS with a spectrophotometer. Robertson and Mixer (101) developed a micro modification of the Porter-Silber method which was also dependent on the formation of phenylhydrazones in acid solution specific for 17, 21-dehydroxy-20-ketones. Nelson and Samuels (89) used a modification of this same method in humans. Estergreen and Venkateseshu (33) used a variety of chemical means to identify bovine cortisol and corticosterone. Among these were paper chromatography, isotope dilution, spectrophotometric analysis, ultraviolet, sulfuric and infrared spectrum analysis. Talbot et al. (126) observed that many chemical assays were dependent on the reducing property of the side chain at carbon 17 i. e., the assays were specific only for 17-OHCS. Heitzman, Adams and Hunter (57) noted that many

chemical procedures (such as those employed in the Porter-Silber reaction and those used by Estergreen and Venkateseshu) required large amounts of blood, i. e. 100 to 2,000 ml. Higher values of cortisol were reported by these researchers due to the increased corticosteroid levels induced by the stress of blood collection.

Saba (105) introduced a method whereby cortisol and corticosterone from 50 ml of plasma could be purified and separated by solvent partition and differential adsorption on a floracil column followed by paper chromatography and measurement of soda fluorescence. Although this method proved to be more sensitive than the standard Porter-Silber method or the analysis using blue-tetrazolium, it was much too elaborate and time consuming for routine analysis of corticosteroids.

Glick, Von Redlich and Levine (44) reported a micro method of measuring unconjugated cortisol and corticosterone based on sulfuric acid fluorescence requiring as little as .02 ml of blood plasma.

In addition to the chemical measurements of plasma corticosteroids, several researchers have tried to assay corticosteroids through urine analysis. In contrast to other species of animals, Whipp and Lyon (141) reported that the chemical study of urinary excretion of adrenal steroids and their metabolites was not a valid index of adrenal function in ruminants because of the extremely low quantities of adrenocorticosteroids excreted into the urine and the presence of chemical compounds interfering with chemical analysis of 17-OHCS.

Holtz (62) called these interfering compounds ionone derivatives and discovered that they were conjugations of steroids with dietary carotenoids.

In order to circumvent many of the analytical problems of the chemical assays for corticosteroids, Thiessen and Nealey (127) developed a bioassay for total corticosteroids using plasma or urinary samples. The assay consisted of injecting plasma or urine into adrenalectomized mice and measuring the resulting eosinopenia. This method was advantageous because it measured the activity of conjugated urinary steroids in urine without preliminary hydrolysis. These conjugated urinary steroids cannot be measured by chemical means because these glucuronic derivatives are not extractable by organic solvents without preliminary hydrolysis. Thiessen and Nealey (127) pointed out that bioassay values for corticosteroids were characteristically lower than those measured by chemical methods not employing chromatographic purification, because chemical methods had a tendency to measure inactive as well as active steroids. Shaw, Dutta and Nichols (117) reported that only 10% of the urinary steroids were active biologically, the remainder consisting of the inactive dihydro and tetrahydro derivatives.

Several radioimmunoassay methods have been developed for the analysis of corticosteroids. Murphy (85) described a technique using the steroid binding properties of corticosteroid binding globulin (CBG or transcortin) for routine determination of corticoids. Sensitivity was observed to increase 100 times by using tritiated ( $^3\text{H}$ ) tracer steroids

instead of  $^{14}\text{C}$ -labeled steroids and by using adsorption onto insoluble substances (Florosil, Fuller's Earth, or Lloyd's Earth) instead of using dialysis or gel filtration to separate bound and unbound steroids. Depending on the substance specificity of the assay, several adsorbing agents were used to separate protein bound and protein unbound steroids and therefore this technique is commonly called the competitive protein binding method (CPB). Jones and Mason (65) used a similar CPB method using dialysis instead of adsorption onto an insoluble substance such as florosil to separate the bound and unbound proteins. A double isotope method of measuring cortisol was used by Bowman (11). Bowman and De Luna (12) compared this double isotope method with Murphy's CPB method and observed a correlation of .96 between the two methods for measuring cortisol. They concluded that CPB was the preferred method because of increased reliability, extreme sensitivity, simplicity, and economy. Much smaller quantities of plasma can be used since this method is more sensitive than the chemical assays previously described. Several modifications of Murphy's method are now used in the routine analysis of large numbers of samples including the modifications of Willet and Erb (142) and Randel et al. (95).

It appears that measuring plasma levels of adrenocorticosteroids would be a suitable method of quantifying stress in dairy cattle. However, problems inherent in techniques for collection of blood and assay of the corticoids in the plasma contribute to variations in corticosteroid levels measured. Rapid fluctuations in corticoid levels due to the

psychological stress of blood collection may mask the long range corticoid effect of other treatments i. e. nutritional stress.

Willett and Erb (142) noted a 400% increase in corticosteroid level by exciting dairy heifers during the blood collecting procedure. Ray et al. (97) observed a ten fold increase in corticoid level in Holstein steers after restraint in a chute for ten minutes. Bassett and Hinks (7) reported that successive venipuncture caused a significant rise in plasma corticosteroids. Trauma and manipulation of test animals during the bleeding procedure increased corticoids in cattle as evidenced by Fortier, de Groot and Hartfield (39). Glenister and Yates (43), Reid (98), Reid and Mills (99) and Yates et al. (147). Methods of blood collection in other species also influenced adrenocorticosteroid concentrations according to the research of Dunn and Scheving (29). Removal of psychological stress with anesthetics, analgesics or tranquilizers cannot be accomplished because they also elicit an adrenocorticosteroid response as indicated by Dunn and Scheving (29), Dunn, Scheving and Millet (30), Eik-Nes and Samuels (31) and Nakao et al. (86).

According to Shaw, Dutta and Nichols (117) there were serious limitations in using 17-OHCS concentration as the sole indicator of overall adrenal function. The adrenocorticosteroid secretion mixture is not constant in composition or concentration from day to day or from hour to hour. However, measurement of the 17-OHCS level as well as the eosinophils is still the most direct indication of overall adrenal function.

Stress, ACTH and Corticosteroid Effects

on Circulating Leucocytes

Paape, et al. (91) reported that intramuscular injection of 250 I.U. of ACTH in dairy cows resulted in significant leucocytosis after two hours, a peak within 10 hours, followed by a gradual decline to higher than basal values 24 hours after treatment. The actual number of neutrophils increased. The number of lymphocytes, monocytes and basophils was unaffected. The percent and concentration of eosinophils decreased but not until three to five hours after treatment. Wegner and Stott (139) reported similar effects of leucocytosis accompanied by neutrophilia. Convey, Miller and Tucker (19), Goetsch, McDonald and Odell (45), Schalm, Lasmanis and Carroll (111) using the synthetic corticoids, prednisolone, 9 $\alpha$ -fluorohydrocortisone and 9 $\alpha$ -flouroprednisolone reported varying degrees of leucocytosis, neutrophilia and eosinopenia. Lymphocytes and monocytes either remained unaffected or tended to decrease slightly. Leipold (73) observed slight leucocytosis, lymphocytosis and eosinopenia in syndactylous cattle stressed by high constant environmental temperature. Paterson (92) reported leucocytosis in cattle 2 weeks prior to parturition followed by a marked decline immediately post partum. Maximum eosinopenia was detected 2 weeks after parturition.

Contrary to the findings of most researchers, Daugherty and White (25) observed that administration of ACTH in man, rats or rabbits

was accompanied by a decrease in leucocytes and lymphocytes and an increase in the absolute number of polymorphonuclear cells (neutrophils, eosinophils and basophils). They hypothesized that pituitary ACTH was the primary factor in regulating blood leucocytes by its action on the adrenal glands.

Sturgis and Bethel (125) stated that variation in the leucocytes due to the influence of ACTH or adrenocorticosteroids can be explained by four factors. First, there was alteration in the rate of production of leucocytes; second, variation existed in the rate of destruction or elimination of leucocytes from circulation; third, changes resulted from differences in concentration of circulating blood plasma; fourth, redistribution of leucocytes occurred within the vascular channels. Craddock, Perry and Lawrence (23) stated that redistribution of leucocytes within the vascular compartments due to various hemodynamic alterations caused fluctuations in peripheral blood leucocyte concentration. However, through experiments with  $^{32}\text{P}$  labeled DNA in leucocytes, they determined that the chief source of mature leucocytes taking part in acute leucocytosis was the marrow reserve and not the vascular compartments.

Speirs (121) reported that as early as 1914, Emil Schwarz published a paper on eosinophils in which his review of literature included over 2,700 references to published papers dealing with eosinophils. Thousands of papers have been published since, on the same topic. Since eosinophils are stable in hypotonic solutions and the



granules have the ability to stain red in acid dyes, they are the easiest of the leucocytes to observe and quantify. For this reason the eosinophils have been the most intensively studied of all the leucocytes.

Thorn et al. (128) observed decreased numbers of eosinophils in man after injection with ACTH. Migeon et al. (83) reported similar results in the Rhesus monkey 4 hours after the administration of ACTH. Martin, Skillen and Deubler (80) reported eosinopenia in the dog after ACTH injection.

Hopwood and Tibolla (63) observed a 66 to 93% decrease in eosinophil count in cows with a minimum value occurring 10 hours after treatment with ACTH. They suggested that the relative decrease in circulating eosinophils after the administration of ACTH may be a valuable screening test for adrenocortical function in cattle. Numerous investigators have been cited by Braunsteiner and Zucker-Franklin (13) reporting the inverse relationship of ACTH and adrenocorticosteroids with eosinophils in all species of animals. Paterson (92) observed the correlation of 17-OHCS to circulating eosinophils to range from + .38 to -.73 with a mean of -.29. Alexander (2) and Flux, Folley and Rowland (36) observed eosinopenia after intramuscular or intravenous injection of ACTH over a wide range of dosages. Significant decreases were measured 2 hours after injection and lasted between 24 and 48 hours, depending on dosage level.

In addition to ACTH induced eosinopenia, physical stresses such as cold environmental temperature, hypoxia and venesection were

observed to induce eosinopenia by Halberg, Kosital et al., Louch, and Speirs and Meir cited by Vandenberg (131). Southwick (120) observed eosinopenia due to short term behavioral disturbances in mice. Vandenberg (131) reported eosinopenia induced by social stress in mice. Döche and Warch, cited by Stott and Thomas (124) measured reductions in circulating eosinophils in undernourished heifers after the administration of ACTH to determine adrenal function.

It has been well established in the literature previously cited that physical, psychological, or social stress induced a pituitary-adrenocortical response which in turn affected the number of circulating eosinophils. The response to acute stress was an immediate increase in corticoids followed by a delayed eosinopenia. The mechanism of ACTH-corticosteroid induced eosinopenia is unknown. Archer (3) indicated there was no direct lytic effect of corticosteroids on eosinophils. He hypothesized that peripheral eosinopenia may be attributable to a decrease in circulating histamine which accompanied increased corticosteroids.

#### Stress Effect on Erythrocytes, Hemoglobin, and PCV

The effect of stress on the pituitary-adrenocortical system and its consequence on circulating leucocytes, especially the eosinophils, is well documented. However, information concerning the effect of stress on the packed cell volume (PCV) and hemoglobin concentration (Hb) has not been so abundant.

The stress of high constant environmental temperature was observed by Leipold et al. (73) to cause a slight drop in hemoglobin concentration and PCV. In acutely hyperthermic cows studied, an increase in hemoglobin and PCV was observed shortly before death, indicating hemoconcentration. Khan, Dickson and Meyers (69) observed a marked decrease in PCV in calves stressed by cold environmental temperatures. Daugherty and White (25) observed an initial increase in hemoglobin and circulating erythrocytes followed by a drop to subnormal levels in rabbits and man within 3 to 6 hours after injection of ACTH. Paape et al. (91) noted an increase in circulating erythrocytes in dairy cattle after treatment with ACTH.

Selye (115) reported that high levels of stress induced by muscular exercise, cold environmental temperature or intoxication with various drugs caused enlargement of the adrenal glands and lowering of the PCV.

#### Diurnal Variation of ACTH, Corticosteroids and Leucocytes

Research dealing with diurnal variation of ACTH, circulating adrenocorticosteroids and leucocytes is extensive, but controversial. Diurnal changes occur at the hypothalamic and pituitary level of control and are reflected in the secretions of the adrenal cortex. Adrenocorticosteroids caused changes in the circulating leucocytes, especially in the eosinophils.

Diurnal variation of corticosteroids was detected in man by Demura et al. (27), Donald, Espiner and Beaven (28), Krieger and

Krieger (70), Martin and Hellman(79) and Orth, Island and Liddle (90). Peak values were observed in the morning. Bottoms et al. (10), Hoffsis et al. (61) and Zolovick, Upson and Eleftheriou (148) detected diurnal variation of corticosteroids in the horse. Similar corticosteroid variations were observed by Bottoms et al. (10) in the pig and by Harwood and Mason (56) in the dog. Halberg and Visscher (54) detected a regular pattern of variation, i. e. circadian rhythm in mice while Dunn and Scheving (29) and Guillemin, Dear and Liebelt (52) reported the same in rats. Dunn and Scheving (29) noted that administration of pentobarbital can upset this circadian rhythm. Unlike man, dogs, horses and other animals active during the day, mice, rats and other nocturnal creatures demonstrated peak adrenal activity at night and minimum activity during the morning.

MacAdam and Ehbart (78) and Wagner and Oxereider (137) reported significant diurnal variations in corticosteroids in cattle while Abilay and Johnson (1), Paape et al. (91) and Shaw, Dutta and Nichols (117) reported no such variation. Paterson (92) observed a seasonal variation in corticosteroids with elevated corticoids in the summer months.

Diurnal variations in eosinophil levels in man were detected by Rud (104). Halberg et al. (55) detected diurnal variations of eosinophils in the dog while Halberg and Visscher (54) detected circadian rhythm in the mouse. Minimum number of eosinophils were measured during the period of peak corticosteroids (night for man, morning for mice).

Paape et al. (91) observed significant diurnal variation of leucocytes in the cow. These fluctuations were attributed to changes in the number of circulating neutrophils.

#### Miscellaneous Factors in Stress Response

Other factors must also be considered which effect or are affected by changes in corticosteroids and circulating leucocytes. Among these are age, genetic influences, milk yield, stage of lactation, estrus, stage of gestation, parturition and health.

Riegle and Nellor (100) observed no significant difference in plasma concentrations of cortisol or corticosterone with increasing age in cattle. Their research suggested, however, that as cattle grow older, the adrenal cortex becomes less responsive to ACTH and hence higher levels of circulating ACTH are needed to stimulate the adrenals to maintain adequate levels of corticoids. Shaw and Nichols (118) indicated that calves can secrete high levels of corticosteroids for relatively long periods of time but the response is slower and more limited than observed in mature cows.

Speirs and Meyer (122) and Thiesson and Nealey (127) reported significant genetic influence on the response of the adrenal cortex to stress in mice. Treiman, Fulker and Levine (129) observed a marked influence of genotype and maternal environment on the ability of mice to respond to stress from electric shock. Thiessen and Nealey (127),

Westberg, Bern and Barnawell (140) and Wragg and Speirs reported that genetically different strains of mice varied in adrenocortical response to stress induced by physical handling. Adrenal function was measured by relative eosinopenia and changes in adrenal weights.

Sebranek et al. (113) measured the ability of pigs to withstand environmental stress by measuring the adrenal response to exogenous ACTH. Stress-susceptible and stress-resistant strains of pigs were discovered, demonstrating the influence of genetics on adaptation mechanisms.

Paape et al. (91) reported that milk yield has a significant effect on the response to stress. It was shown that cows producing over 40 lbs milk/day responded to exogenous ACTH by secreting less corticosteroids than cows producing less than 40 lbs milk/day. The leucocytic response was also affected. Convey, Miller and Tucker (19) discovered that the artificial corticosteroid, 9 $\alpha$ -floroprednisolone acetate, significantly ( $p < .01$ ) reduced milk yield 10 to 24 hours after injection. Lee, Beatty and Roussel (72) reported a significant ( $p < .01$ ) correlation between circulating cortisol and milk yield.

Stage of lactation also influences the amount of corticoids secreted from the adrenal glands (137). However, no differences in corticoid levels or circulating levels of erythrocytes were observed in cattle at different stages of lactation as observed by Paape et al. (91). Total leucocytes and neutrophils decreased slightly while lymphocytes tended to increase, but not significantly, with increasing stage of

lactation. A significant decrease in adrenocortical response to treatment with exogenous ACTH was observed in cattle during advanced stages of lactation (91).

The effect of estrous on plasma levels of adrenocorticoids is unclear. Abilay and Johnson (1) reported a significant correlation between cortisol and progesterone ( $p < .01$ ). Since progesterone concentration changes during the estrous cycle, there was a corresponding change in corticosteroid concentration. Fluctuations in cortisol were observed from a low of  $5.5 \pm .7$  ng/ml plasma during estrus, to a high of  $21.3 \pm 6.3$  ng/ml plasma 10 days post estrus. Garverick et al. (42), on the other hand, observed fluctuations in plasma cortisol and corticosterone during the estrous cycle in dairy cattle but the differences were not significant.

Parturition and stage of gestation also altered the levels of circulating corticosteroids in dairy cattle. Average levels of cortisol, corticosterone and total corticoids did not vary significantly from 0 to 260 days pregnancy according to research conducted by Randel and Erb (96). However, the ratio of cortisol to corticosterone (4.1 to 4.2:1) was higher for pregnant than for non pregnant cows.

Paterson (92) reported increases in plasma corticosteroids over the prepartum period especially 10 days prior to parturition. Accompanying this increase in corticoids was an increase in circulating leucocytes. Marked decrease in plasma corticoids and circulating leucocytes

was observed immediately following parturition. Most of the changes in circulating leucocytes can be accounted for by changes in the number of neutrophils. A slight transient lymphopenia was occasionally observed, but most often, no change in lymphocytes was detected. No marked change in the eosinophil count was observed until immediately prepartum when a state of eosinopenia existed. The greatest decline in circulating eosinophils occurred immediately after parturition.

#### Evaluation of Nutritional Stress

The effect of sub-maintenance rations on reproduction in dairy cattle is well documented. Irregular estrous, anestrus and reduction of ovulation and conception has been reported by Highnett (60), Joubert (67), Leatham (71), Moustgaard (84) and Wiltbank et al. (144). According to Selye (115) inhibition of estrus associated with dietary deficiencies occurs as a result of the animal's effort to adapt itself to changing environmental conditions. This response to nutritional stress is an example of a "general adaptation syndrome" and elicits initial hypertrophy of the adrenal followed by a secondary hypotrophy as evidenced in experiments with rats.

Selye and Collip (116) reported that under the influence of damaging agents such as toxic agents, excessive exercise and dietary insufficiencies, a shift of hormone production of the pituitary was observed. The same agents causing hypotrophy of the sex glands elicit



enlargement of the adrenal cortex and inhibition of growth. In other words stress elicits production of ACTH at the expense of growth hormone (STH) and gonadotropic hormones (FSH and LH).

The research of Stott and Thomas (124) confirmed the findings of Selye and Collip. According to Stott and Thomas (124) the initiation of sub-maintenance diets elicited an adrenal response indicated by the rapid elevation in plasma corticosteroids. Elevated levels were sustained 20 to 30 days after initiation of the sub-maintenance diet. The corticoid level regressed to normal or subnormal levels, where they remained with limited variation until full feed was resumed. Döche and Warch cited by Stott and Thomas (124), also demonstrated that undernourished heifers suffered from hypotrophy of the adrenal cortices and were unresponsive to exogenous injections of ACTH.

## MATERIALS AND METHODS

### Materials

A total of 26 first lactation Holstein dairy cows were used in this experiment. The experimental design was molded around an already existing experiment involving the measurement of genetic ability, level of nutrition, and their interaction on consumption, milk production, feed efficiency, behavior and reproduction. Each cow in the experiment was initially fed alfalfa hay ad libitum, plus a grain supplement to meet its NRC (87) requirements for production and growth. Fifty days post partum each cow was assigned to a genetic group based on production and within each group the cows were randomly assigned to one of five rations. The rations consisted of 70, 85, 100, 115 and 130% of the NRC requirement for milk production and 100% of the requirement for maintenance and growth. The rations were recalculated every week and grain allowances were changed according to changes in milk production, body weights and hay consumption.

The data for the nutritional stress experiment were divided into two subsets. The first subset consisted of 20 cows from which blood was collected biweekly for four consecutive months. The other subset contained 9 cows, 3 of which were included in the first subset, which ended their 305 day lactation during the 4 month blood sampling interval, and

were removed from the experimental herd. Blood from each of these 9 cows was sampled twice after completion of the 305 day lactation.

Ideally, a random block design would provide the maximum amount of information with the least number of samples. However, due to the restrictions on availability of test animals the number of samples per cow and the number of cows per treatment were not equal. Consequently a completely randomized design was used.

#### Blood Collection and Analysis

Blood samples were taken biweekly for four months between 10:30 and 11:30 a. m. each sample day. Between 5 and 10 ml of blood were rapidly collected from each cow via tail vein puncture with a minimum amount of disturbance to the cow. The blood was carefully ejected from the syringe into heparinized tubes and immediately cooled to 0°C in an ice bath to prevent enzymatic degradation of the cells and corticosteroids. The needle was removed from the syringe during ejection of the blood to minimize physical lysing of the blood cells.

After all samples were collected for the day, approximately 2 ml of blood were removed from each heparinized tube and allowed to equilibrate to room temperature for at least 10 minutes before preparing blood smears for the differential leucocyte count. The remaining portion of blood was centrifuged<sup>a</sup> 15 to 20 minutes at 0°C in the original

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<sup>a</sup>International Clinical Centrifuge, Model CL was used at 3400 rpm, radius = 12.7cm.

heparinized tubes. The plasma was decanted into precooled tubes and immediately frozen at  $-23^{\circ}\text{C}$  for total corticoid analysis at a later date.

Duplicate blood smears were prepared from each sample of blood according to the procedure described by Miale (82). The blood smears were allowed to air dry and were then fixed for 10 minutes in methanol. The smears were air dried and were stained for 45 minutes to 1 hour. Extra slides were smeared and stained. At 45 minutes and at 5 minute intervals thereafter, one of these extra slides was examined microscopically to determine the proper staining time. When staining was complete, the slides were rinsed twice in distilled water and allowed to air dry. The dried slides were stored for later differential leucocyte determination. All smears were prepared within approximately one hour after collection. A minimum of one-hundred leucocytes was counted per slide and identified as lymphocytes, monocytes, neutrophils, eosinophils or basophils. The leucocytes were counted along the edge of the slide avoiding the beginning and end portions of the smear using the "battlement" pattern described by Sturgis and Bethel (125). The average of the two counts was recorded and used in the statistical analysis.

After preparation of the blood smears the white blood count was performed in duplicate (2 separate fields on the hemocytometer were counted). Turk's diluting fluid, consisting of water, glacial acetic acid and crystal violet, was used for the WBC. Four different 0.1 mm deep improved Neubauer Hemacytometers were used during the counting

procedure. The WBC was performed according to standard blood cell counting procedures as described by Coffin (18).

After completion of the WBC, the direct eosinophil count was performed using a propylene glycol, water, phloxine, sodium carbonate diluting fluid. The eosinophil count was carried out in quadruplicate according to the procedure of Miale (82). The same white blood diluting pipetes, and the same hemacytometers were used for the total leucocyte count and the direct eosinophil count.

After completion of all direct eosinophil counts, the PCV was determined using the microhematocrit method as described by Coffin (18). Dade "capilet" microhematocrit capillary tubes were centrifuged 5 minutes at 3,400 rpm and the PCV was read from a Sherwood "critocap" Micro-hematocrit tube reader.

Hemoglobin concentration was determined by the colorimetric reaction of 5 ml of Hycel-Cyanmethemoglobin reagent and .02 ml of blood. A colorimeter<sup>a</sup> was used at 530 nm to determine the concentration of hemoglobin in grams hemoglobin per 100 ml plasma.

The total corticoid analysis was performed by Murphy's competitive protein binding technique (85) using <sup>3</sup>H labelled cortisol bound to dog plasma. No attempt was made in this study to separate cortisol and corticosterone into different fractions. According to Gwazdauskas, Thatcher and Wilcox(53) there is a proportionate increase in cortisol and corticosterone due to stress, and therefore, it is not necessary to separate the two.

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<sup>a</sup> Braun-Knecht-Heimann, Lumetron, Model 401A.

A fecal examination for parasites was carried out for each cow using the sodium nitrate flotation method described by Coffin (18). Body weights were recorded every two weeks and the daily milk production was recorded for each day of sampling.

Initially, in the pretrial period of this experiment, red blood counts (RBC) were taken. The red blood counts took an inordinate amount of time and confidence in the values was low because of the large variations between duplicate samples of the same cow. Paape et al. (91) reported that the RBC frequently and randomly fluctuated throughout the day. Variations in erythrocyte level varied as much as  $0.7 \times 10^6$  cells/cmm during a single day. Because of this random variability and because of the time factor it was decided not to continue with the RBC.

As mentioned previously, the circulating eosinophils were measured by 2 different procedures. The first way (differential method) consisted of calculating the percent from a blood smear and the second way (direct method) consisted of counting the actual number on a hemacytometer from a given volume of blood. The phloxine dye was unavailable at the beginning of the experiment and hence, a direct eosinophil count was not made on all observations. Therefore, all data recorded in the tables and all correlations were calculated using the eosinophil count obtained from the differential method only.

The means of the differential and direct eosinophil counts (for those samples in which both methods were used) were compared using a standard t-test. No significant ( $p > .05$ ) difference was detected between

the values for percent and absolute eosinophils determined by either method. The correlation between the two methods for percent eosinophils was .60 and the correlation between the two methods for absolute eosinophils was .56.

#### Statistical Model

The data was analyzed using the MDCR & SMRR programs of Hurst (64) for multiple regression analysis. A complete randomly designed model was used, incorporating cow number, ration, date sampled, stage of gestation, stage of lactation, milk yield, body weight, minimum temperature of sample date and mean temperature of day preceding sample date as independent variables. Cows, date sampled and rations were included as qualitative variables. Interactions between milk production and body weight, milk production and stage of lactation, stage of lactation and body weight, stage of lactation and stage of gestation, stage of gestation and milk production, and minimum temperature and mean temperature were included in the model. Total leucocyte count, percent and absolute neutrophil count, percent and absolute lymphocyte count, percent and absolute eosinophil count, percent and absolute basophil count, hemoglobin concentration, PCV and plasma corticoid concentration were the dependent variables in the model.

## RESULTS AND DISCUSSION

The purpose of this experiment was to determine the effect of nutritional stress on various blood cellular components and to determine whether a relative decrease in eosinophil count could be used as a quantitative index of stress. It was hypothesized that measuring the eosinopenia resulting from increased corticoids would be a better indication of long term nutritional stress and would hence eliminate the problems of using corticoid level as a sole indicator of stress. It was pointed out previously that corticoids are subject to instantaneous elevation in concentration due to various blood collecting procedures, while a decrease in circulating eosinophils occurs over a relatively longer period of time and hence, would not be thus affected.

Most experiments involving sub-maintenance diets have been restricted to heifers, steers or other test animals. Lactating dairy cattle have seldom been subjected to deficient diets because of decreased milk production and subsequent economic loss. Cows used in this experiment were part of a long term genetic study involving over 300 first lactation dairy cows. These animals were used because they were already on controlled diets. However, fewer animals were on the 70 and 85% rations than the other 3 rations because a larger number of



these had already completed their 305 day lactation. Consequently, considerable unbalance was introduced into the subsequent analysis.

Least squares analysis was used to eliminate some of the unbalance due to uneven sample size and to help eliminate the confounding effects of stage of lactation, milk production, stage of gestation and environmental temperature on corticoids and circulating leucocytes.

The cows were all sampled between 10:30 and 11:30 a.m. each sample date to eliminate diurnal changes in leucocyte and corticosteroid levels of the blood. The cows were tied at the manger at this time for individual feeding and could be approached quietly from the rear and bled with very little disturbance to the cow being sampled or to the other cows. Tail vein puncture was used instead of the frequently used indwelling jugular cannula, because the large number of animals tested made cannulization impractical. According to Shaw, Dutta and Nichols (117) the use of a cannula was unnecessary since no significant difference ( $p > .05$ ) was detected between sampling with a jugular cannula or tail vein bleeding as long as the test animal was not restrained over ten minutes. Basset and Hinks (7) recommended acclimating the animals to frequent handling before the experiment. Training the animals substantially reduced corticosteroid increases due to venipuncture. All animals in this experiment were accustomed to frequent handling since they were all tied at the manger 3 times daily for feeding. Any effect of age, sex or breed difference on corticoids was eliminated, since only first lactation Holstein cows were used. Environmental temperature,

stage of gestation, stage of lactation and milk production are all known to affect the circulating levels of corticoids and leucocytes.

Upon exposure to cold environmental temperature Selye (114) reported that the adrenal cortex hypertrophied. Héroux and Hart (58) reported a drop in circulating eosinophils which is another indication of increased secretion of adrenocorticosteroids. Héroux and Schönbaum (59) reported that after continuous exposure to cold, the corticoid secretions decreased and returned to normal. The eosinophil level also gradually returned to normal. It was concluded therefore, that corticoids increase only during the initial exposure to continuous low temperature.

In order to account for the variation of corticoids and eosinophils due to fluctuations in temperature, three variables were entered into the model. The first variable was the minimum temperature recorded for the sampling date. The second variable was the mean temperature observed for the day prior to sampling. The third variable was the interaction of the minimum and mean temperature. Significant correlations ( $p < .05$ ) with minimum temperature were observed in PCV, WBC, number of lymphocytes and number of monocytes. Corticoids were significantly ( $p < .01$ ) correlated with the interaction of minimum sample day temperature and mean temperature of the previous day.

Summary data from the genetic-environmental interaction project is listed in Table 1. The number of cows sampled, the average daily milk production, actual feed consumption, and weight changes are listed for each ration.

Table 1. Summary of production by ration for cows on genetic-environmental project. (a)

Nutrition level	No. cows	Avg. Milk/day		32 wk. trial	
		21-50 days	32 week trial	Feed consumption(b)	Weight change
70	58	50.05	26.55	77.02	-87
85	54	50.65	31.32	90.48	-54
100	54	50.92	33.75	105.86	+15
115	58	50.03	39.73	116.15	+87
130	52	51.54	42.08	124.79	+131

(a) Lamb, Robert C., Genetic by nutrition interaction in dairy cows, Project 178, Annual Project Report and Work Plan, Utah Agricultural Experiment Station. 1974. (unpublished) p. 3.

(b) Feed consumed as percent of requirements above maintenance.

The data in Table 1 clearly indicates that as the level of nutrition decreased, milk production and body weight decreased correspondingly. It was noted that feed consumption, i. e. the feed consumed as a percent of the requirements above maintenance, did not equal the planned nutritional level. Cows on the 70, 85, 100 and 115% ration were inadvertently

overfed because new rations were calculated only weekly. During the first part of the week the cow would be getting the proper amount of feed, but as milk production declined during the week her ration stayed the same and hence the cow was overfed. Cows on the lowest ration, i. e. 70%, were overfed most consistently since milk production in these cows declined most rapidly. Cows on the 130% ration were consuming at an average level of only 125%. Cows on this level were offered 130% of their milk production requirement but were unable to consume the total amount. Table 2 contains data from the first subset based on 130 observations on 20 cows. The range and the mean plus the standard error (SE) is listed for total corticoids, stage of lactation, body weight, daily milk production and stage of gestation.

Table 2. Observed values in subset 1 for corticoids stage of lactation, body weights, milk production and stage of lactation.

	Range	Mean $\pm$ S. E.
Corticoids (ng/ml plasma)	1.28 - 122.21	8.54 $\pm$ .48
Stage of lactation (days)	40 - 288	168 $\pm$ 5
Body weight (lbs.)	955 - 1353	1174 $\pm$ 9
Milk production (lbs/day)	0 - 59	34.3 $\pm$ 1.2
Stage of gestation (days)	0 - 215	56 $\pm$ 5

Bodyweight and milk production values in Table 2 are average for 1st lactation dairy cows. A wide range in stage of lactation and stage of gestation is noted.

The values obtained for total corticoids presented in Table 2 were consistent with the findings of Abilay and Johnson (1), Gwazdauskas, Thatcher and Wilcox (53), Heitzman, Adams and Hunter (57), MacAdam and Ebhart (78), Paape et al. (91), Randel et al. (95), Wagner (136) and Wagner and Oxenreider (137), all of whom used similar methods of competitive protein binding for measuring total corticosteroids. These values were considerably lower than the values obtained by Riegle and Nellor (100), Shaw, Dutta and Nichols (117) and Venkateseshu and Ester-green (132). The difference was attributed to the difference in assay method. It was well established that the colorometric methods which required large quantities of blood, resulted in stress. This additional stress caused an increase in corticoid concentration of the blood.

A comparison of normal blood values for mature, purebred Holstein cattle according to Schalm (110), was made with the values obtained in the first subset. Ranges and means were listed for hemoglobin (Hb), packed cell volume (PCV), white blood cell count (WBC) and differential leucocyte count, i. e. percent and actual numbers of neutrophils, lymphocytes, monocytes, eosinophils and basophils.

The data from Table 3 indicated that the mean value for total (41) leucocytes in the first subset was about 50% greater than the normal mean listed by Schalm (110). However, 12,410 cells/ccm was still

Table 3. Normal Hb, PCV, WBC and differential WBC ranges and means for mature purebred Holstein cows compared to cows in subset 1.

	Normal values (a)		Values from subset 1	
	Range	Mean	Range	Mean + SE
Hb (g/100ml plasma)	10.3-13.3	10.8	10.5-13.4	13.0 $\pm$ .2
PCV (%)	33-48	38	31-41	38.3 $\pm$ .6
WBC/cmm	4,750-12,700	7,840	5375-22300	12,419 $\pm$ 279
Neutrophils	8-46	32.5	12-44(912-9630) <sup>(b)</sup>	22.2 $\pm$ .6
Lymphocytes	36-71	54.0	51-84(5563-18403)	70.5 $\pm$ .6
Monocytes	3-8	5.7	0-7(0-927)	1.8 $\pm$ .1
Eosinophils	3-7	5.2	0-19(0-3300)	5.0 $\pm$ .4
Basophils	0-2	0.6	0-1(0-325)	0.4 $\pm$ .1

(a) Schalm, Oscar W. *Veterinary Hematology*. 1961. Lea & Febiger, Philadelphia, p. 155.

(b) Absolute number in ( ).

within the normal range. Since all WBC were counted by hand, the discrepancy was attributed to counting error and not to pathogenic infections in the cows. All cows were regularly examined for disease by a veterinarian. No major illnesses were observed during the experiment.

Sturgis and Bethel (125) identified 3 sources of error in counting WBC by hand. First, there were errors of mixing the cells with diluting fluid; second, errors occurred from filling the counting chamber by

capillary action; and third, there was the error from the settling of cells by chance on the ruled field of the counting chamber. This last error accounted for most of the variation of cell/cmm.

The counting error in this experiment probably involved counting leucocytic fragments as whole cells. Since the same relative error was consistent throughout the study it shouldn't affect the statistical analysis. The neutrophil count observed in the experiment was lower than the normal value reported while the lymphocyte count was higher than the normal mean. From Table 3 it was also noted that the hemoglobin values lay within the accepted range and the PCV was identical to the normal mean. Summary data for subset 1 according to the 5 rations is listed in Table 4. Mean values for corticosteroids, stage of lactation, body weight, daily milk production, Hb, PCV, WBC and the differential leucocyte count were compared at each treatment level with the pooled mean. Significant differences ( $p < .01$ ) among rations were observed for total leucocytes, percent eosinophils, absolute number of neutrophils and lymphocytes. The difference among rations for absolute eosinophil count was also significant ( $p < .05$ ).

Examination of the means for corticoids at the 5 levels of nutrition indicated a trend of increasing corticoids with decreasing feed levels. Regression analysis of corticoid level with ration confirmed the existence of this trend. Least square coefficients were 3.2, 1.0, -7, -1.2 and -26 for 70, 85, 100, 115 and 130% rations respectively. When these

Table 4. Summary data for subset 1 according to ration.

	Pooled mean	Rations (%)				
		70	85	100	115	130
Corticosteroids (ng/ml plasma)	8.54	11.97	11.16	8.26	8.80	7.44
Stage of lactation (days)	168	220	182	165	201	126
Body weight (lbs)	1174	1118	1155	1143	1301	1205
Milk (lbs/day)	34.3	20.9	27.2	40.9	18.9	45.8
Hb(g/100 ml plasma)	13.0	12.9	12.5	12.7	13.1	12.5
PCV (%)	38.3	37.4	38.1	37.8	40.8	38.3
WBC/cmm	12,419	10,838	11,369	14,153	11,510	13,245
Neutrophils (%)	22.2	23.1	20.5	28.2	19.6	23.0
Neutrophils/cmm	2,757	2,487	2,358	3,239	2,211	2,603
Lymphocytes (%)	70.5	70.1	70.6	70.8	74.9	68.7
Lymphocytes/cmm	8,755	6,018	8,098	9,975	8,685	8,852
Monocytes (%)	1.8	2.0	1.7	2.0	1.2	3.1
Monocytes/cmm	224	217	238	282	125	202
Eosinophils (%)	5.0	4.8	6.9	4.2	4.0	4.0
Eosinophils/cmm	621	541	776	602	442	522
Basophils/cmm	.4	.4	.4	.5	.4	.4
Basophils/cmm	50	39	48	56	221	236



coefficients were plotted against ration in Figure 1, a linear response was indicated with a slight inflection of slope at the 115% level. This linear relationship however, was not significant ( $p > .05$ ).

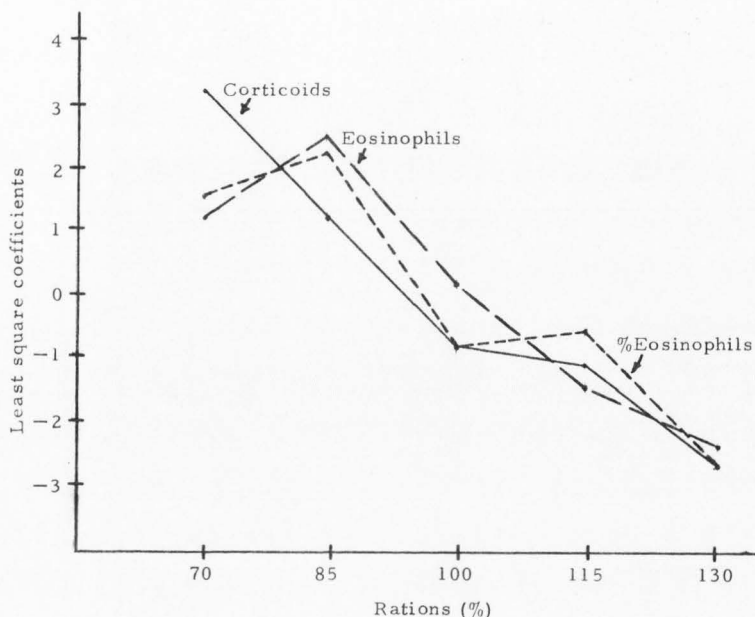


Figure 1. Least squares coefficients comparing ration with corticoids, percent and absolute number of eosinophils.

Significant differences among treatments were observed for percent and absolute number of eosinophils, but the trend was opposite to what was expected. When the least squares coefficients were plotted against ration in Figure 1, the trend indicated increasing eosinophils with decreasing levels of nutrition.

There was a negative relationship between percent eosinophils and corticoids as expected ( $r = -.15$ ) and between the actual number of circulating eosinophils ( $r = -.17$ ) but neither were significant. These findings corresponded to the conclusions of Migeon et al. (83) who stated that there was only a rough correlation between circulating eosinophil levels and plasma levels of corticosteroids. In individual cows Paterson (92) observed correlations between eosinophils and corticoids ranging from  $-.73$  to  $+.38$ . An overall pooled estimate based on 21 observations yielded  $r = -.29$ . He suggested that measuring adrenal function on the basis of one or two eosinophil counts might be subject to considerable error. He concluded that changes observed in leucocytes might form a reasonably reliable qualitative index of changed adrenal function but with no quantitative significance.

Differences in corticoid levels among sampled dates were highly significant ( $p < .01$ ). The difference between sample dates was much greater than the difference between treatments.

Differences in eosinophil level were detected among rations. Eosinophil level was not affected by date sampled.

This study of subset 1 failed to demonstrate an effect of nutritional stress on corticoids because perhaps, there was no measurable amount of stress due to the relatively mild treatments.

According to Stott and Thomas (124) initiation of a deficient diet induced an elevation in corticoids which lasted 20 to 30 days. As the

animal adjusted to the ration the corticoid concentration fell and then leveled off at normal or below normal values.

From Table 1 it was noted that the stage of lactation varied from 40 to 288 days. Therefore, some cows were sampled during a period of rising corticoids, others in a declining stage while others were in the stationary stage. Thus a cow in the middle segment of this curve may be reacting to nutritional stress with a decrease instead of an increase in corticoids. This may help to explain why no significant increase in corticoids was detected in this experiment.

Significant correlations among the variables in the first subset are tabulated in Table 5. Variables having no significant correlations have been excluded from the table.

Significant correlations observed in this study included the obvious positive correlation of ration on body weight ( $r = .29$ ) and milk yield ( $r = .47$ ). The reason for the negative correlations between milk production and stage of lactation ( $r = -.52$ ) and milk production with stage of gestation ( $r = -.64$ ) are self evident.

The relationship between stage of gestation and leucocytes was negative. Significant ( $p < .01$ ) negative correlations existed between stage of gestation and WBC, percent neutrophils, absolute number of lymphocytes and percent and absolute number of monocytes. Positive correlations between WBC and absolute number of neutrophils and lymphocytes were consistent with the findings of others. The highly significant ( $p < .01$ ) negative correlations between percent and absolute

Table 5. Significant correlations of variables in subset 1<sup>(a)</sup>

	body wt.	milk prod.	stage gest.	PCV	WBC	No Neut	% Lymp	No Lymp	% Mono	No Mono	% Eos	No Eos	No Bas
Ration	.29	.47				.27		.22				-.21	
Stage lact.		-.52	.60								-.21		
Body wt.						-.20							
Milk prod.			-.64										
Stage gest.					-.45	-.25		-.40	-.21	-.31			
Min. temp.				-.22	.24			.24	.40	.41			
Hb.				.61									
Cort.									.38	.36			
WBC						.58		.93					.32
% Neut.						.72	-.82	-.37			-.27		
No. Neut.							-.55	-.30			.25		
% Lymph.								.46			-.29	-.24	
No. Lymph.													.32
% Mono.										.96			
% Eos,												.95	
% Bas.													.93

(a)  $p < .05$   $r = .20$ ,  $p < .01$   $r = .25$

number of neutrophils with percent and absolute number of lymphocytes indicated that as neutrophils increased in concentration the lymphocytes decreased or remained constant. This was also consistent with the findings of other researchers.

Significant correlations existed between ration and percent eosinophils ( $p < .05$ ), absolute neutrophil count ( $p < .01$ ) and absolute lymphocyte count ( $p < .01$ ). Significant correlations ( $p < .01$ ) between ration and body weight, stage of gestation and milk production were also observed.

The fact that nothing was correlated with hemoglobin concentration and PCV except each other was not surprising. Hemoglobin concentration and PCV have been noted to change only in extreme cases of stress which usually terminated in death (73).

Summary data from the second subset comparing values observed during the 305 day lactation with values observed after the 305 day period are presented in Table 6. Mean values plus the standard error of the mean have been listed for corticoids, stage of lactation, milk production, Hb, PCV, WBC, and the differential leucocyte count based on 49 observations on 9 cows.

The results of the second subset comparing the blood analysis during and after the 305 day lactation illustrated the typical recovery from stress demonstrated by many researchers. After the 305 day lactation the cows were removed from the experimental herd. A majority

Table 6. Summary data from subset 2, during 305 day lactation and after.

	Means + S. E.					
	During 305 d. lact.			After 305 d. lact.		
Cort. (ng/ml plasma)	14.93	+	2.00	7.51	+	1.75
Stage of lactation (days)	245	±	6	354	±	9
Milk production (lbs/day)	21.1	±	2.6	7.8	±	2.7
Stage of gestation (days)	89	±	10	164	±	20
Hb (g/100 ml plasma)	12.6	±	.3	13.7	±	.3
PCV (%)	38.1	±	.7	38.5	±	.5
WBC/cmm	11,769	±	543	14,867	±	1,256
Neutrophils (%)	23.4	±	1.3	35.7	±	2.9
Neutrophils/cmm	2,340	±	289	5,705	±	1,001
Lymphocytes (%)	69.0	±	1.3	58.4	±	2.6
Lymphocytes/cmm	8,068	±	360	8,285	±	565
Monocytes (%)	2.3	±	.3	1.1	±	.2
Monocytes/cmm	264	±	40	181	±	27
Eosinophils (%)	4.8	±	1.0	4.5	±	.8
Eosinophils/cmm	542	±	109	645	±	128
Basophils (%)	.4	±	.1	.3	±	.1
Basophils/cmm	53	±	10	49	±	25

of these cows, especially the two animals which were on the 70% ration, showed a drop in corticoid level and an increase in absolute number of circulating eosinophils even though the percent eosinophils decreased slightly. The drop in corticoids was significant ( $p < .01$ ) but the increase in eosinophils was not significant ( $p > .05$ ). A significant ( $p < .01$ ) increase was observed in WBC, percent and absolute number of circulating neutrophils. This relationship of neutrophils and WBC was typical. Usually increased circulating leucocytes can be related to increased neutrophils. The percent lymphocytes dropped markedly but the actual number of circulating lymphocytes remained unchanged. Leucocytosis and neutrophilia along with a decrease in corticoids was contrary to the findings of most researchers, who reported leucocytosis and neutrophilia with increased corticosteroid levels.

It was hypothesized that this increase in leucocytes was due to increased stage of gestation even though the data from the first subset indicated a trend of decreased WBC, lymphocytes and neutrophils with increasing length of gestation. The dramatic increase in WBC may also be related to the decrease in milk production. Milk production for cows in subset 2 decreased from an average of 21.1 to 7.8 lbs/day. A majority of these 9 cows were dried after removal from the experimental herd. Decreased corticoids observed in the non-lactating cows was consistent with the findings of Wagner and Oxenreider (137).

These differences in WBC, corticoids, hemoglobin etc, may be due to variations in sampling.

Comparison of the means (119) for eosinophils measured by the differential and direct methods, indicated that the former method resulted in higher counts. This was consistent with the research of Migeon et al. (83). However, the difference between methods in this study was not significant ( $p > .05$ ).

The direct method of measuring eosinophils was more accurate because it eliminated the errors involved in preparing a blood smear and taking a differential count. According to Sturgis and Bethel (125), these errors can be enormous depending on the technique of the technician performing the test.

Fecal examination for internal parasites revealed that only 3 cows had fecal counts of one or less per low power field during microscopic examination. Haemonchus contortus was identified as the parasite in each of the 3 cows affected. The remaining 23 cows were negative for parasites.



## SUMMARY AND CONCLUSIONS

Summary

Twenty-six first lactation dairy cattle were assigned one of five rations based on 70, 85, 100, 115 and 130% of the NRC requirement for milk production above maintenance requirements. Tail-vein blood samples were obtained biweekly for 4 months to detect changes in total (WBC) and differential leucocyte composition, packed cell volume (PCV), hemoglobin (Hb) concentration and plasma corticosteroid levels.

Nine cows completed the 305 day lactation during the four month sampling interval and were removed from the experimental herd. These cows were sampled twice after completion of the 305 day record. Data from these cows were analyzed separately to determine changes in blood composition during and after completion of the 305 day lactation.

It was hypothesized that cows on the rations below NRC requirements would display stress symptoms of elevated plasma corticosteroids, elevated WBC, neutrophilia and eosinopena. Least squares analysis indicated a trend for increased corticosteroid level with decreased nutrient levels but the trend was not significant ( $p > .05$ ). Significant differences among rations for percent and absolute number of eosinophils, actual number of neutrophils and actual number of lymphocytes were detected but the differences were not linearly related to ration.

Eosinophil level was significantly ( $p < .05$ ) related to ration ( $r = .21$ ) indicating that increased stress caused an increase in eosinophils instead of a decrease as was expected. The eosinophil level was negatively correlated to corticoids ( $-.12$ ) but not significantly. Ration was significantly ( $p < .01$ ) correlated to body weight ( $r = .29$ ), daily milk production (.47), absolute number of neutrophils (.27), and WBC ( $-.33$ ). The WBC was significantly ( $p < .01$ ) related to actual numbers of neutrophils (.58), lymphocytes (.93) minimum temperature (.24) and negatively related to stage of gestation ( $-.40$ ).

Analysis of the second subset with a t-test indicated significant ( $p < .01$ ) leucocytosis, neutrophilia, increased hemoglobin concentration and decreased corticosteroids in the 9 cows removed from the experimental herd after completion of their 305 day lactation.

### Conclusions

Nutritional stress in lactating dairy cattle caused a decrease in milk production and body weight. Packed cell volume and hemoglobin concentration are not affected at the levels of nutrition studied.

WBC tended to increase with decreased nutrient intake but no change in corticoids was detected. Increased WBC can be attributed to increased number of circulating neutrophils. Although the percent of lymphocytes decreased, the actual number increased accompanying the leucocytosis. The number of circulating eosinophils increased with decreased levels of nutrition.

It was concluded that the combination of decreased milk production and advanced stage of gestation was accompanied by a significant ( $p < .01$ ) increase in WBC and decrease in corticoids. Although a negative correlation between corticoids and eosinophils exists it was insignificant and therefore cannot be used as a valid indicator of nutritional stress in this experiment.

Nutritional stress may have elicited an adrenocortical response which was masked in this experiment due to the large variations in corticoids within treatments and between sample dates. This experiment was designed to eliminate variations in corticoids due to stage of lactation, milk production, body weight, stage of gestation, environmental temperature, blood collection, diurnal variation and age. The experiment was not designed to eliminate variations within treatments and between sample dates due to limitations of suitable test animals. It was concluded that in order to determine the effect of nutritional stress, on corticosteroid and leucocytic composition of the blood a random block design or Latin Square design should be used to eliminate the variations encountered in this experiment.

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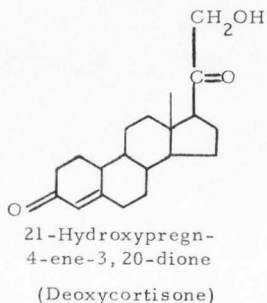
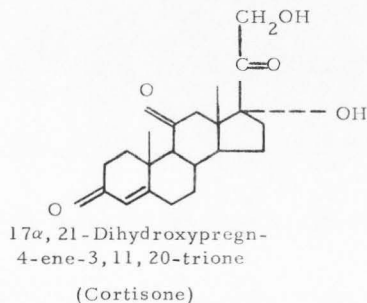
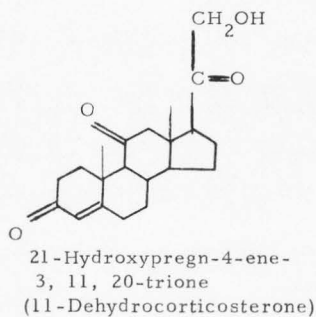
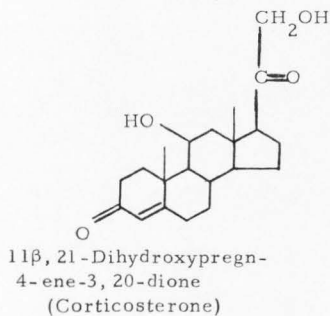
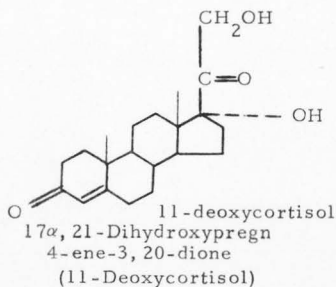
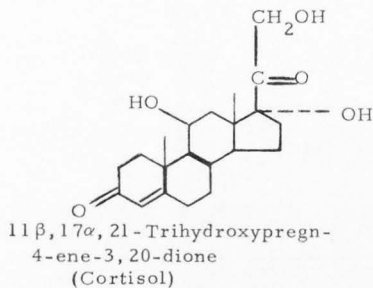
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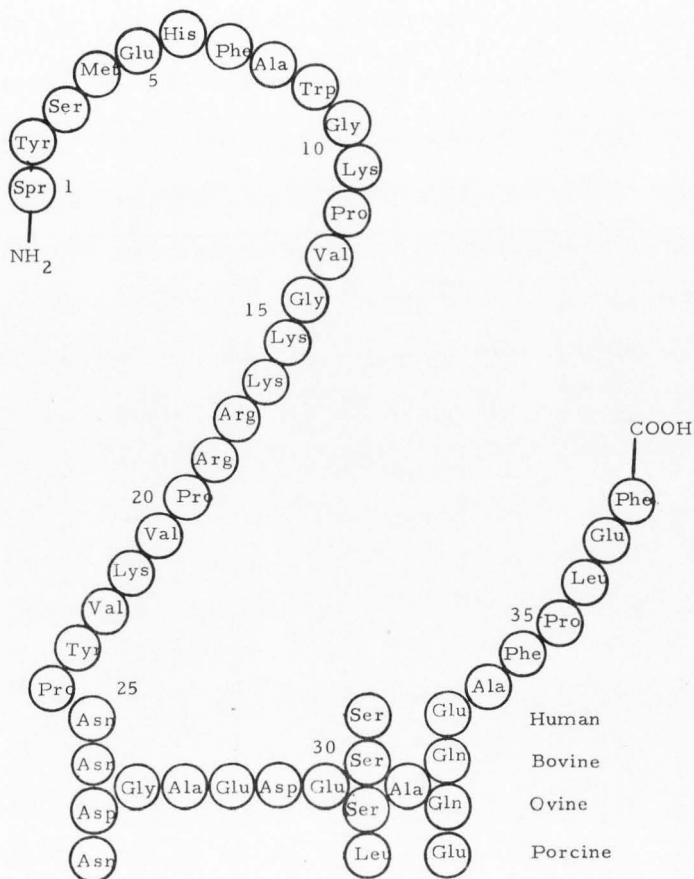
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## APPENDIXES

## Appendix A

Some Biologically Active Adrenocorticosteroids

## Appendix B

Amino Acid Sequence of Human, Bovine, Ovine and Porcine ACTH. (a)

(a) Li, C. H. Adrenocorticotropin 45, Revised amino acid sequences for sheep and bovine hormones. *Biochemical and Biophysical Research Communications* 49(3):835-839, 1972.

## VITA

Eugene W. Wisniewski

Candidate for the Degree of

Master of Science

Thesis: Relationship of Circulating Eosinophils, Other Blood Cellular Components and Plasma Corticoids in Dairy Cattle Subjected to Nutritional Stress

Major Field: Dairy Science

Biographical Information:

Personal Data: Born in Los Angeles, California, February 12, 1952, son of Tadeusz and Irene Wisniewski.

Education: Attended elementary school in Los Angeles, California; attended Don Bosco Technical Institute, Rosmead, California and graduated from Wilson High School in Los Angeles, California in 1970; attended California State College, Los Angeles during the summer 1970; received the Bachelor of Science degree from Utah State University, Logan, Utah with a major in Dairy Science in 1974; did graduate work at Utah State and completed requirements for the Master of Science degree in Dairy Science at Utah State University in 1975.