STUDIES ON NORMAL AND EXPERIMENTALLY ALTERED CIRCADIAN
CORTISOL RHYTHMS IN PONIES AND CORTISOL LEVELS
IN NORMAL AND ADRENOPATHIC DOGS

by

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ACKNOWLEDGEMENTS

STUDY OF NORMAL AND EXPERIMENTALLY
ALTERED CIRCADIAN CORTISOL RHYTHMS IN PONIES

SUMMARY:

Serum cortisol levels were studied under normal and altered conditions of
light, exercise, ACTH stimulation and starvation in 3 ponies using a
radioimmunoassay specific for cortisol. A circadian rhythm for cortisol
was observed in 3 ponies with peak cortisol levels in the morning ($31.74 \pm$
5.7 ng/ml) between 0900 and 1100 hrs and lowest levels (19.6 ± 3.16 ng/ml)
between 2100 and 2300 hrs. Exposure to altered light conditions resulted
in a phase reversal of the circadian rhythm and 2 wks of continuous
illumination completely disrupted the circadian rhythm and depressed the
cortisol levels (12.8 ± 8.3 ng/ml) below the normal levels. One hundred
I.U. of ACTH in gelatin significantly increased the cortisol levels 3-4
fold 6-8 hrs following an I.M. injection and maintained a high level for
24 hrs without any indication of circadian sensitivity of the adrenals.
Exercise did not have significant effect on the cortisol levels. Starvation
for 8 days resulted in elevated levels of cortisol and disruption of the
circadian rhythm throughout the period of starvation. It is concluded that
while light plays a major role on the maintenance of adrenal rhythms,
physiological stress conditions such as starvation can alter the rhythms.

INTRODUCTION:

A circadian rhythm in adrenocortical activity has been demonstrated by
fluorometric method or competitive binding assay in several species,
including rhesus monkey (Migeon et al., 1955), cat (Krieger and Krieger,
1967), goldfish (Singley and Chavin, 1971), white-throated sparrow (Dusseau

and Meier, 1971), man (Asfeldt, 1971), and horses (Hoffsis et al., 1970 and Bottoms et al., 1972. Studies on rats indicate that the circadian adrenal cortical activity can be altered by environmental factors such as light (Scheving and Pauly, 1966), and water deprivation (Johnson and Levine, 1973). There have been no attempts to study the adrenocortical rhythms in equines under altered environmental conditions. This study is carried out using a specific radioimmunoassay to study the serum levels of cortisol in ponies under various conditions of light, exercise, ACTH stimulation and during and after a period of starvation.

EXPERIMENTAL PROCEDURE:

Animals and blood sampling:

Three mixed breed ovariectomized ponies were used for the experimental studies and another group of 6 horses were used to study the normal levels of serum cortisol. The ponies were housed in a room with controlled temperature of 20° C and lighting schedule. The jugular veins of the ponies were cannulated with PE 190 catheters which remained in place throughout the period of this study. The venous cannulae allowed frequent blood sampling without disturbing the animals. The ponies were fed alfalfa hay and oats twice a day and had access to water ad lib. A 2 week adjustment period was allowed before experimentation on the ponies. The light source consisted of overhead cool white florescent tubes which emitted a light with 125 ft. candle intensity.

Experiment I: Effect of light.

1). Normal light schedule: The ponies were housed under controlled conditions of 12 hrs light. The lights automatically switched on at 0600

hrs and off at 1800 hrs. Five ml of blood was drawn at intervals of 2 hrs

for 72 hrs. Care was taken in order not to disturb the animals and when

the lights were off, blood sampling was done using a pin-point source of

light on the catheter.

2). Reversed light: To study the effect of reversed light schedule on the

activity of adrenal gland, the ponies were kept for 2 weeks under altered

light schedule with lights on for 12 hrs between 1800 and 0600 hrs. At the

end of 2 weeks, 5 ml blood was collected at 2 hr intervals for 48 hrs.

3). Continuous illumination: To study the effect of continuous light on

adrenal cortical activity, the ponies were housed under continuous light

for a 2 week adjustment period and at the end of 2 weeks 5 ml blood was

drawn at 2 hr intervals for 2 consecutive days for the determination of

serum cortisol levels.

Experiment II: Effect of exogenous ACTH.

The response of the adrenals of the ponies to exogenous ACTH was

determined by administering 100 I.U. of ACTH gel[R] I.M. to the 3 ponies.

Blood was collected before injection, and at 2 hr intervals after injection

for a period of 24 hrs. Control consisted of the same ponies injected with

a solution consisting of 16% gelatin, 0.1% phenol, 0.1% cysteine, which

were the inert ingredients of the ACTH gel preparation. The controls were

run a week after ACTH stimulation test.

Experiment III: Effect of Exercise

Serum cortisol levels were studied in 2 ponies exercised for short

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ACTH gel[R] ADRENOMONE: Repository corticotropin injection, 40 I.U.

ACTH/ml. Armour-Baldwin laboratories, Omaha, Nebraska.

periods. The ponies were taken out-doors and were run in circles of 60' diameter for 15 minutes and blood was collected before, during and after exercise.

Experiment IV: Effect of Starvation.

Two ponies were starved for 8 days but water was provided ad lib and normal light schedule was maintained (light on between 0600 to 1800 hrs). Blood was drawn twice daily at 0900 and 2100 hrs during this period. On the 9th day, the ponies were given their usual feed at 1000 hrs and blood was sampled for a period of 24 hrs.

Radioimmunoassay of cortisol:

Serum was separated from all blood samples soon after collection and kept frozen at -20° C. At the time of assay, the sera were rapidly thawed and 0.1 ml of serum was extracted with 0.4 ml of absolute ethanol. Twenty μl of the ethanol extract was dissolved in 1 ml of 0.1 M phosphate buffer (pH 7) containing 0.1% gelatin (PBS-G) and allowed to equillibrate for at least 2 hrs before aliquoting 0.4 ml for duplicate assay. The radio-immunoassay method of Abraham (1974) as modified by our laboratory (Carter et al., 1976) was followed to quantitate the serum cortisol and is outlined briefly here. Antibody (S-162 #3) (professional staff Associate, Los Angeles, Calif.) was diluted with PBS-G to give a binding of 40-50%/0.1 ml. Tritiated hydrocortisone (New England Nuclear) diluted in PBS-G to give a 20,000 dpm for 100 ul was added to all tubes and incubated overnight at 4° C. Standards were run with each assay. After incubation, the unbound labelled steroid was separated from the bound labelled steroid by dextran coated charcoal (625 mg charcoal, 62.5 mg dextran T-70 in 100 ml PBS-G) and the supernatent was decanted into a vial containing 10 ml of scintillation

fluid (3030 ml toulene, 600 ml dioxane and 128 ml of liquiflour (New England Nuclear) and counted for 2 min in a Nuclear Chicago isocap 300 liquid scintillation counter.

Statistics:

The sensitivity in measuring the unknown samples is defined as equal to 2 standard deviation of the mean of the blank, after correction is made for the blank (Abraham, 1974). The sensitivity S is expressed as $S = \dfrac{2 \times S.D}{R \times F} \times 100$

where R is the per cent recovery, F is the fraction of the recovered steroid used in the assay and the S.D. is the standard deviation of the mean of quadruplicate blank values. The hormone concentrations were calculated from the polynomial regression equation derived from the standard curve values. The equation used is $y = a^o + a_1 x + a_2 x^2 + a_3 x^3$ where y is the hormone concentration and x is the fraction of the percent of bound radioactivity. All the raw counts are collected from the counter on punched tape and processed by a Wang 600 programmable calculator connected to a Wang tape reader. T test was applied to test the significance of means.

Results

The sensitivity measured for cortisol was 10 pg and the recovery of cortisol with ethanol extract was 95%. The mean cortisol levels in the serum collected in the morning from 3 ponies and 6 horses was 41 ± 13.5 ng/ml. A circadian rhythm was observed in the serum cortisol levels in 3 ponies with highest cortisol levels (31.75 ± 5.7 ng/ml) at 0900 and 1100 hrs and gradually decreased to a minimum level (19.61 ± 3.16 ng/ml) at 2100 and

2300 hrs (Fig. 1). This pattern of adrenocortical activity was observed in all 3 days of blood sampling. There was a sharp rise in serum levels of cortisol 3 hours after transition of dark to light period.

Effect of altered light schedule on the circadian rhythm:

After 2 weeks exposure to altered light schedule, with lights on between 1800 and 0600 hrs, there was a complete phase reversal of the circadian rhythm. The serum levels of cortisol peaked at 2100 and 2300 hrs and were low at 0900 and 1100 hrs (Fig. 2). A sharp rise in serum levels of cortisol was observed at 3 hours after lights were turned on.

Effect of continuous illumination on the serum levels of cortisol:

Two weeks exposure of the ponies to continuous light resulted in a complete disruption of the circadian rhythm and the serum levels of cortisol showed an episodic rhythm with multiple peaks over a 24 hr period (Fig. 3). The serum levels of cortisol were generally low (12.8 ± 8.3 ng/ml) as compared to the values of serum levels of cortisol in ponies kept under normal light schedule (Fig. 1).

ACTH stimulation test:

One hundred I.U. of ACTH significantly increased ($P<0.025$) the serum levels of cortisol 3-4 fold (133.6 ± 18.0 ng/ml) 6-8 hrs following an I.M. injection (Fig. 4). The elevated levels of cortisol were maintained over a 24 hr period. The evoked cortisol production due to the exogenous ACTH was not circadian in nature.

Effect of exercise on serum levels of cortisol:

The mean cortisol values in the serum from the 2 ponies at the beginning of exercise was 44.2 ± 2 and 47.6 ± 10 ng/ml respectively at the end of 15 min exercise. The serum levels of cortisol 30 min post exercise was 45.0 ± 2 ng/ml.

<u>Effect of starvation on the serum levels of cortisol</u>:

Serum levels of cortisol were increased significantly (P<0.05) one day

after feed deprivation (62.4 ng/ml) and remained high throughout the 8 days

of starvation (66.0 $\pm$ 19 ng/ml). A circadian rhythm was absent, as the

evening levels of cortisol was elevated much above the normal levels and on

days 2 and 3, the evening serum levels of cortisol were much higher than the

morning levels. After the animals were fed at the end of 8 day starvation

period, the serum levels of cortisol rapidly decreased but did not approach

basal levels over a 24 hr period after feeding. The serum level of

cortisol at 2200 hrs during this feeding period was much lower than the

level of cortisol at 1000 hrs next day (Fig. 5).

DISCUSSION

The morning peak and the evening low levels of cortisol observed in this

study compare favorably with the values reported by Hoffsis <u>et al</u>.,

(1970) and Bottoms <u>et al</u>., (1972) who used a competitive protein binding

radioassay. The plasma levels of cortisol in horses (219-395.3 ug/100 ml)

reported by Zolovick <u>et al</u>., (1966), who used thin layer chromatography

and ultraviolet absorption and fluoresence, are very high and do not

compare well with the values obtained in the present study. An early

morning peak in the cortisol levels were similarly observed in man (Asfeldt,

1971) and pigs (Bottoms <u>et al</u>., 1972). On the other hand, the nocturnal

animals such as the rats have a nocturnal peak at 1800-2200 hrs when the

rats were kept under normal lighting (Zimmerman and Critchlow, 1967).

The complete phase reversal of the circadian rhythm secondary to

light alteration observed in this study is in accordance with the findings

in man (Perkoff et al., 1959) and in rats (Guillemin et al., 1959).
It is interesting to note that in man, a rapid synchronization of the adrenal
activity to reversed light occurs within 5 days time (Perkoff et al., 1959).
Orth and Island (1969) observed that the peak in plasma cortisol levels
occurs only when the lights were turned on and not when the subjects
awakened from sleep. Similarly, in ponies the sharp increase in the levels
of cortisol which take place at the time of dark to light transition
definitely points to light as a major environmental factor cueing the
circadian rhythm.

A general decrease in the serum levels of cortisol, disruption of the
circadian rhythm observed in the ponies kept under continuous illumination
is in general agreement with the findings in the rat (Schering and Pauly,
1966 and Cheifetz et al., 1968). Cheifetz et al., (1968) observed a
diminished level of plasma and pituitary corticotropin in rats exposed
to continuous light. Apparently, the continuous light has a suppressing
effect on the hypothalamo-hypophysial axis. On the other hand, it is
interesting to note that increased duration of light has a significant
stimulatory effect on the hypophysio-ovarian axis (Oxender and Noden, 1976)
in the mares.

The response to exogenous ACTH observed in the ponies in this study
is similar to the report by Hoffsis et al., (1970) in horses. The mouse
adrenal has been shown to have an endogenous rhythm of its own and a
circadian reacting rhythm of the mouse adrenal to exogenous ACTH was
demonstrated in vivo by Haus and Halberg (1962). Such a circadian
sensitivity to the exogenous ACTH could not be discerned in the present
study. Rather, the response appears to be proportional to the rate of

absorption from the site of injection. Since ACTH gel was used in this study, it is impossible to rule out the possible existance of a circadian sensitivity of the equine adrenals to exogenous ACTH.

Short periods of exercise does not seem to have appreciable effects on serum levels of cortisol in the ponies. It is known that the thoroughbred horses have a higher resting level of cortisol (Hoffsis et al., 1970) but no reference is found on the effect of short periods of exercise on the serum levels of cortisol in equines. Vernikos-danellis et al., (1971) in a study on human patients could not find any difference in the cortisol levels after short periods of exercise. It is possible that prolonged strenuous exercise might alter the cortisol levels while short periods of exercise have no significant effect on the serum levels of cortisol.

Serum cortisol levels during starvation were much higher than the levels of cortisol observed in the post starvation period. Whether feed deprivation acts centrally on the hypothalamo-hypophysial function or at the level of the liver is not known. Feed deprivation definitely seems to depress the capacity of the liver to inactivate adrenocortical steroids (Herbst et al., 1960). It is possible that liver depression during starvation was an important factor contributing to the elevated cortisol levels observed during the period of feed deprivation. In conclusion, light periodicity and feed intake are the major factors affecting the circadian rhythm and basal cortisol levels in ponies.

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Figure 1. Circadian rhythm of serum cortisol levels
in 3 ponies kept under normal light
schedule with lights on between 0600 and
1800 hrs.

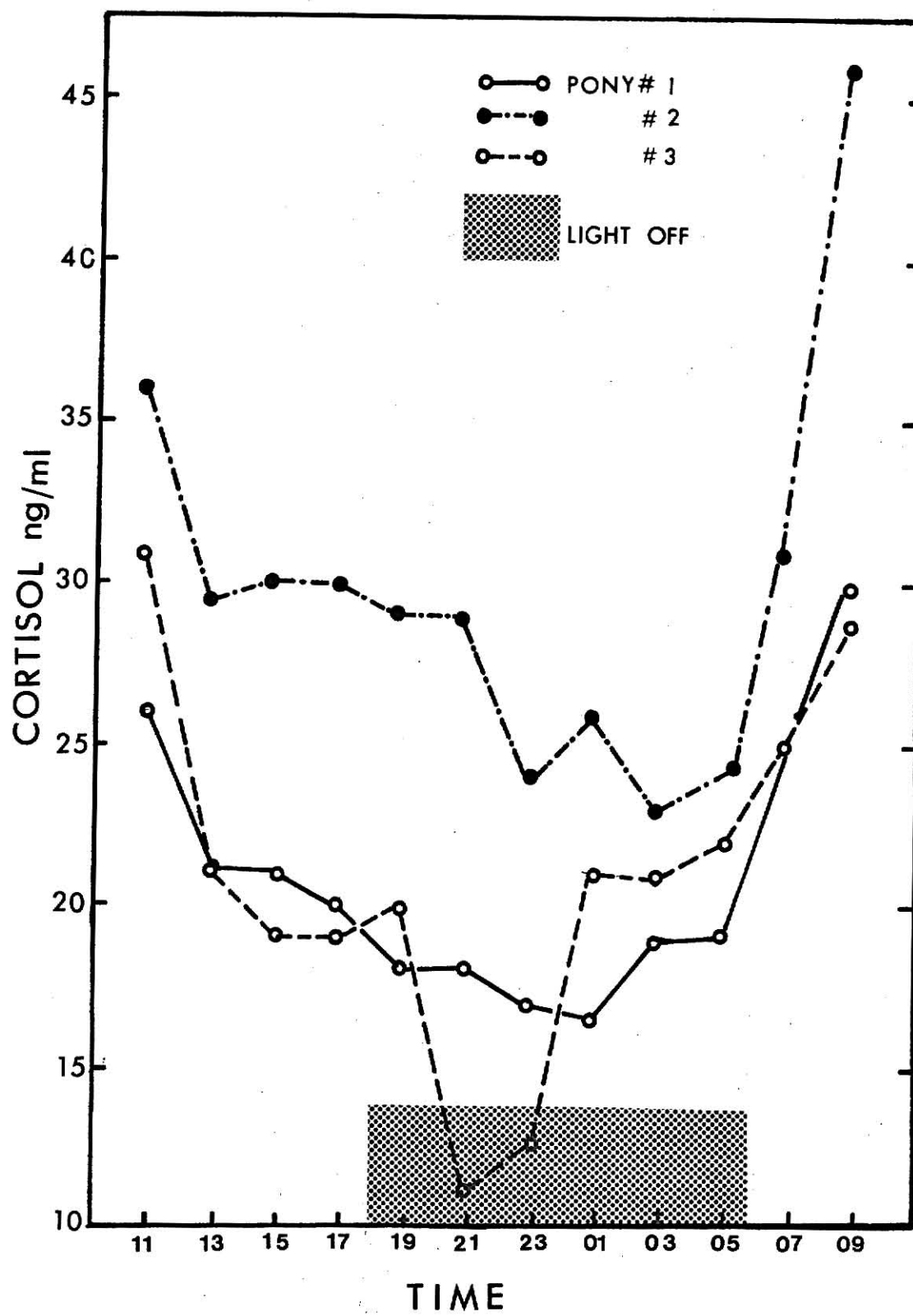CIRCADIAN RHYTHM: MEAN OF 3 DAYS SAMPLING

Figure 2. Phase reversal of the circadian rhythm of serum
cortisol levels in 2 ponies kept under altered
light schedule with lights on between 1800 and
0600 hrs.

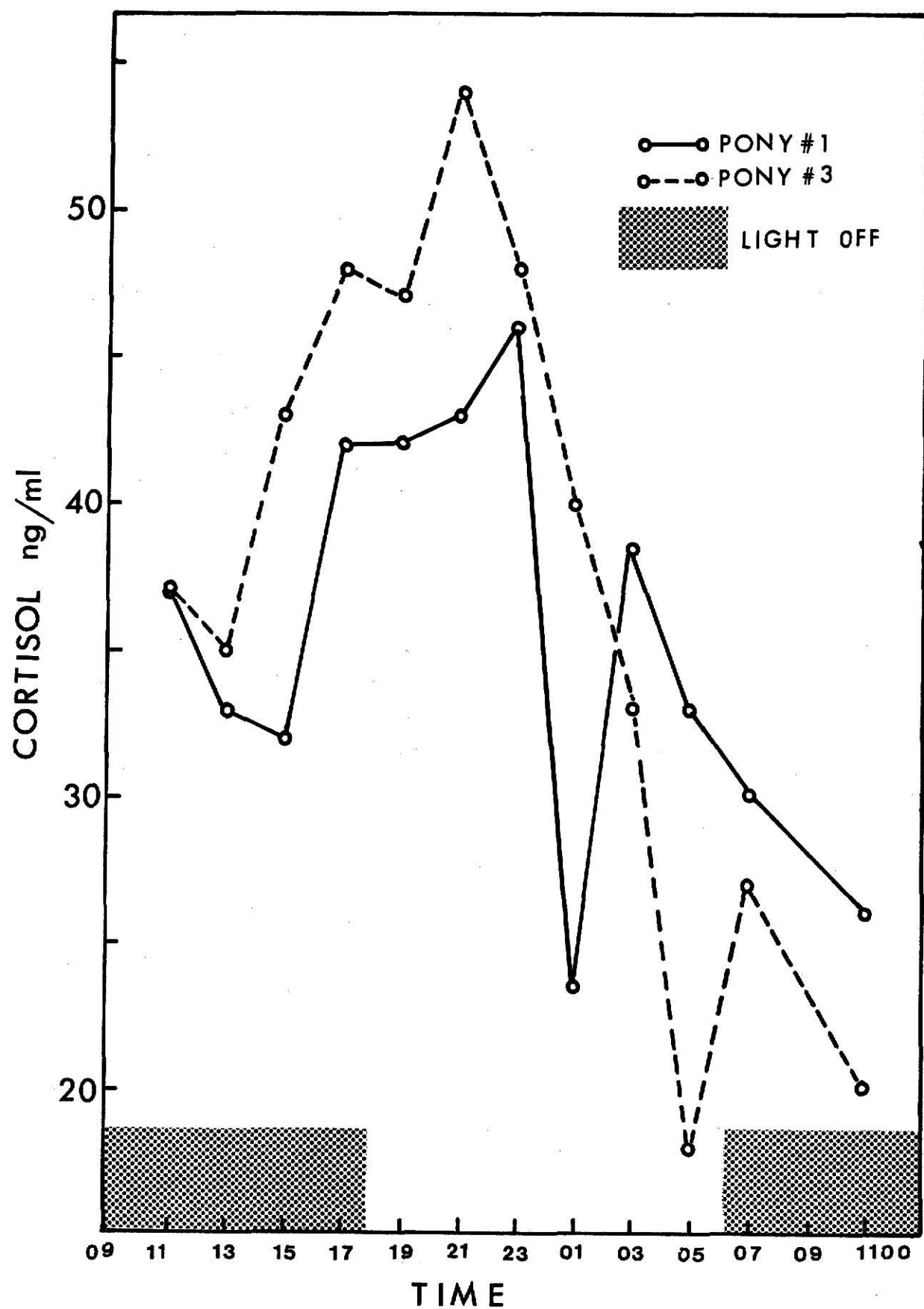PHASE REVERSAL: Values represent mean of 2 day-sampling

○—○ PONY #1
○--○ PONY #3

LIGHT OFF

CORTISOL ng/ml

50

40

30

20

TIME

09 11 13 15 17 19 21 23 01 03 05 07 09 1100

Figure 3. Pony serum cortisol levels at the end of 2 weeks continuous illumination.

CONTINUOUS LIGHT

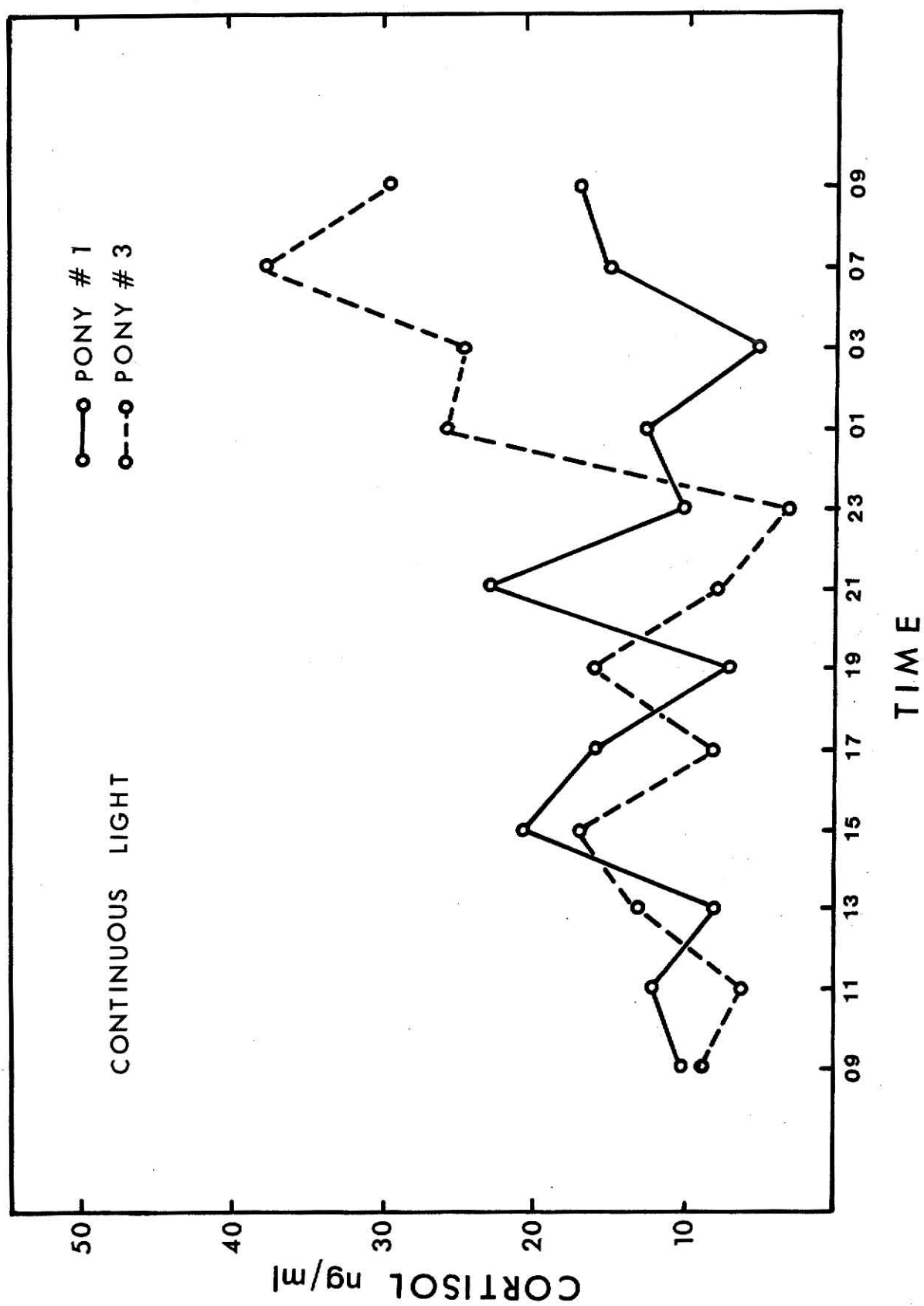PONY # 1
PONY # 3

CORTISOL ng/ml

TIME

Figure 4.   Effect of exogenous ACTH I.M. injection on serum
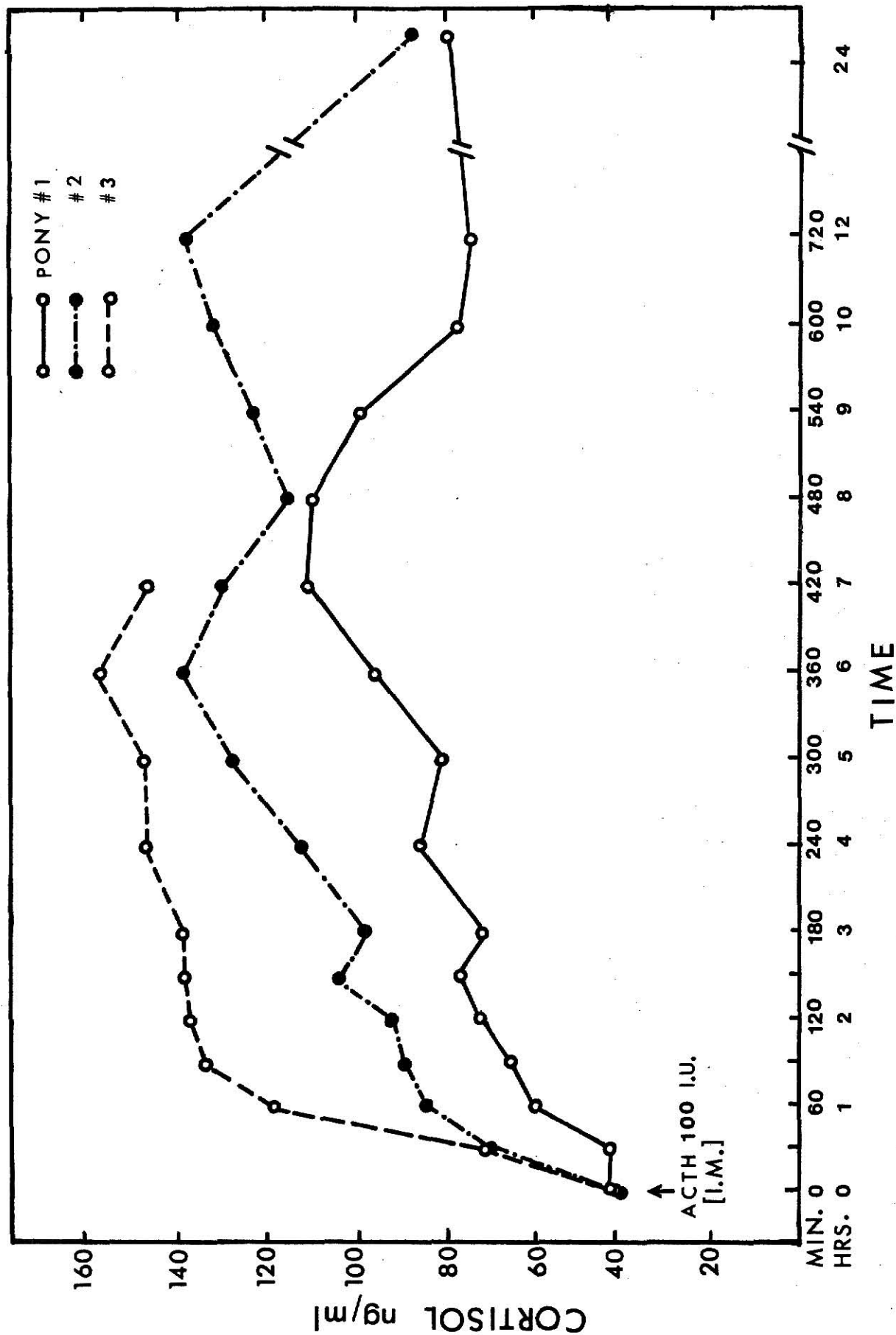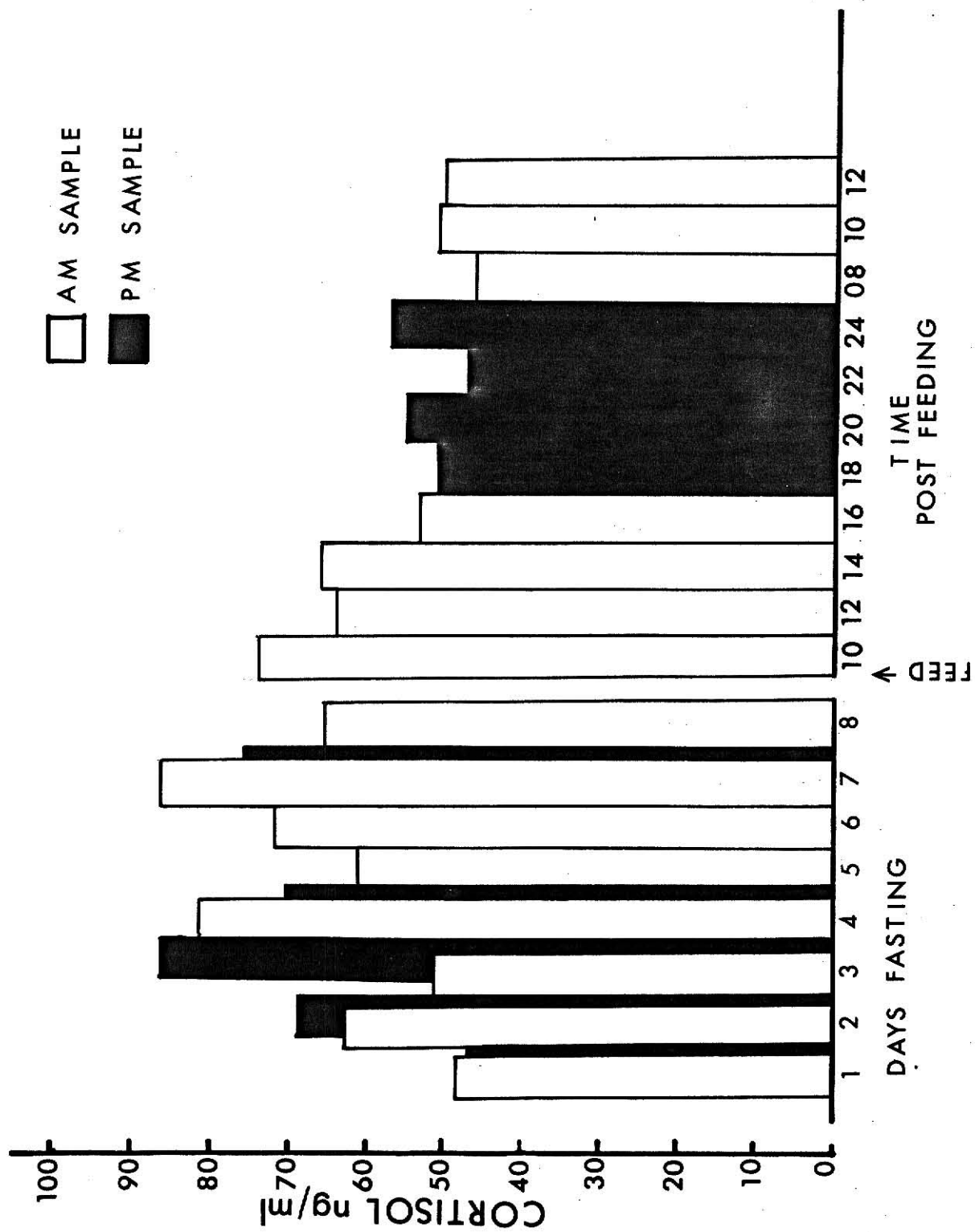   cortisol levels in ponies.

Figure 5.   Serum cortisol levels during 8 days starvation
and one day post starvation period.

A STUDY OF SERUM CORTISOL LEVELS USING RADIOIMMUNOASSAY IN NORMAL AND
ADRENOPATHIC DOGS.

SUMMARY

Serum cortisol levels were measured in dogs using a specific radio-
immunoassay (RIA). The mean basal cortisol level for 56 clinically
healthy dogs was 17.8 $\pm$ 1.32 (SEM). No significant age, sex, body weight
or breed differences were observed in the cortisol levels. In 11 dogs
with definitive hyperadrenocorticism, the serum cortisol levels were
greater than 30 ng/ml and in 2 dogs with hypoadrenocorticism, the levels
were less than 5 ng/ml. The diagnosis of adrenopathies in dogs through
the estimation of serum cortisol levels by RIA is found to be simple and
precise.

INTRODUCTION

Hyperfunction of the adrenal cortex was first described in man[8]
and dog[26]. Recent reports indicate that hyperadrenocorticism[5,7,14,19,20,
22,23,24] and hypoadrenocorticism[2,9,10,13,15,16,21,25] are more common
than suspected. Attempts have been made to diagnose hypo or hyperadreno-
corticism by ACTH stimulation test and/or dexamethasone suppression test.

Estimation of the serum cortisol levels in the diagnosis of adreno-
pathy has been employed in humans and dogs[3,12]. The two most frequently
used assay methods for cortisol have been fluorometric analysis[4] and
competitive protein binding radioassay[12]. In man, radioimmunoassay (RIA)
was found to be very useful and specific for cortisol estimation[11]. No
attempts, however, have been made to quantitate cortisol levels in dogs
by RIA. This study is therefore undertaken to evaluate the serum
cortisol levels in clinically normal and adrenopathic dogs using RIA.

MATERIALS AND METHODS

Fifty-six clinically healthy dogs of either sex and belonging to different breed and age groups were used. A 5 ml blood volume was drawn from the cephalic vein between 0900 and 1000 hrs. The serum was separated immediately and stored at $-20^{o}C$ for estimation of cortisol by RIA. Eleven dogs with suspected hyperadrenocorticism and 2 dogs with suspected hypoadrenocorticism were obtained from the university veterinary clinic. The suspected hyperadrenocorticism dogs exhibited polydipsia, polyuria, bilateral alopecia, hyperpigmentation and were pot bellied. Lymphopenia and eosinopenia were consistently observed in these dogs. The suspected hypoadrenocorticism dogs showed anorexia, emesis and eosinophilia. Blood samples were collected from all these dogs and the serum was separated and stored for the estimation of cortisol as described earlier. In addition, ACTH stimulation tests were carried out using ACTH gel[a] (2 I.U./lb) on two dogs suspected for hyperadrenocorticism. Two normal dogs were given a similar treatment for a comparative evaluation of this test. Blood samples were collected before and 3 hrs after administration of ACTH gel.

Radioimmunoassay of cortisol:

The sera were rapidly thawed and 0.1 ml of the serum was extracted with 0.4 ml of absolute ethanol. Twenty microlitres of the ethanolic extract was mixed in 1 ml of phosphate gelatin buffer (0.1 M Phosphate buffer, pH 7, containing 0.1% gelatin). After equillibrating for at least 2 hrs, duplicate aliquots of 0.4 ml. volume of this mixture were

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[a]ACTH gel: Adrenomone: Repository corticotropin injection 40 I.U./ml. Armour-Baldwin Laboratories, Omaha, Nebraska.

drawn for cortisol assay. The radioimmunoassay method of Abraham[1] as modified by our laboratory[6], was followed to quantitate the serum cortisol. The sensitivity of this method was 10 picograms of cortisol and was calculated according to Abraham[1]. A standard curve of per cent bound radioactivity versus cortisol concentration was established with each assay. The hormone concentrations were then calculated from the polynomial regression equation derived from the standard curve. The equation used is: $Y = a^o + a_1 x + a_2 x^2 + a_3 x^3$ where Y is the hormone concentration and x is the per cent bound radioactivity. Student's 't' test was applied to test the statistical significance.

RESULTS

In clinically healthy dogs, the mean serum cortisol level was 17.8 $\pm$ 1.32 (SEM). Breed, sex, age and body weight differences in the cortisol levels were insignificant (Figs. 1 and 2; Table 1).

Serum levels of cortisol in the hyperadrenocorticism dogs were consistently above 30 ng/ml and ranged from 30 - 148 ng/ml. In 2 dogs with adrenal hyperfunction, the serum cortisol levels increased to 140 and 140.9 ng/ml following ACTH injection (base values were 49.3 and 64.6 ng/ml respectively). In normal dogs, on the other hand, the cortisol levels increased to 73.4 and 43.1 ng/ml (base values were 16.5 and 15.5 ng/ml respectively) upon ACTH injection (Fig. 3). Serum cortisol levels were less than 5 ng/ml in 2 dogs with suspected adrenal hypofunction.

DISCUSSION

The normal mean cortisol level observed in the present study is 17.8 $\pm$ 1.32 ng/ml (SEM). The values compare well with those reported

Table 1. Cortisol Levels in Different Age and Body Weight Groups

Groups	Number of dogs	Cortisol ng/ml $\overline{X}$+SEM	't' value
Age:			
6 months - 3 years	16	16.9 $\pm$ 1.92	0.712 (P>.05)
over 3 years	6	14.2 $\pm$ 3.59	
Body Weight:			
15 - 30 lbs	24	17.4 $\pm$ 1.87	0.775 (P>.05)
30 - 60 lbs	25	19.2 $\pm$ 1.39	

by previous workers employing competitive protein binding assay (CPB) for cortisol estimation[12]. The insignificant age, sex, body weight and breed differences in the cortisol levels of normal dogs suggest that these factors are of minor importance in evaluating the adrenal function in the dog.

The levels of cortisol in hyperadrenocorticism dogs in the present study (over 30 ng/ml) agree with those reported by Schechter et al.[22] for the Cushing's syndrome in dog. The exaggerated response of the hyperadrenocorticism dogs to exogenous ACTH is consistent with the observations in dogs[12]. Halliwell et al.[12] observed an overlap in the range of cortisol levels between the normal and hyperadrenocorticism dogs. Based on their observations, these authors suggested the inadequacy of a single basal cortisol determination in the diagnosis of adrenal function in dogs. In our study, the basal levels of cortisol in dogs with definitive adrenopathies were consistently outside the normal range. Our findings, therefore, suggest that estimation of the resting levels of cortisol in suspected adrenopathic dogs is of diagnostic value. Specific adrenal function tests such as the ACTH stimulation test and dexamethasone suppression test can be used in the confirmatory diagnosis of adrenopathies.

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Figure 1. Cortisol levels in various breeds of dogs. Standard
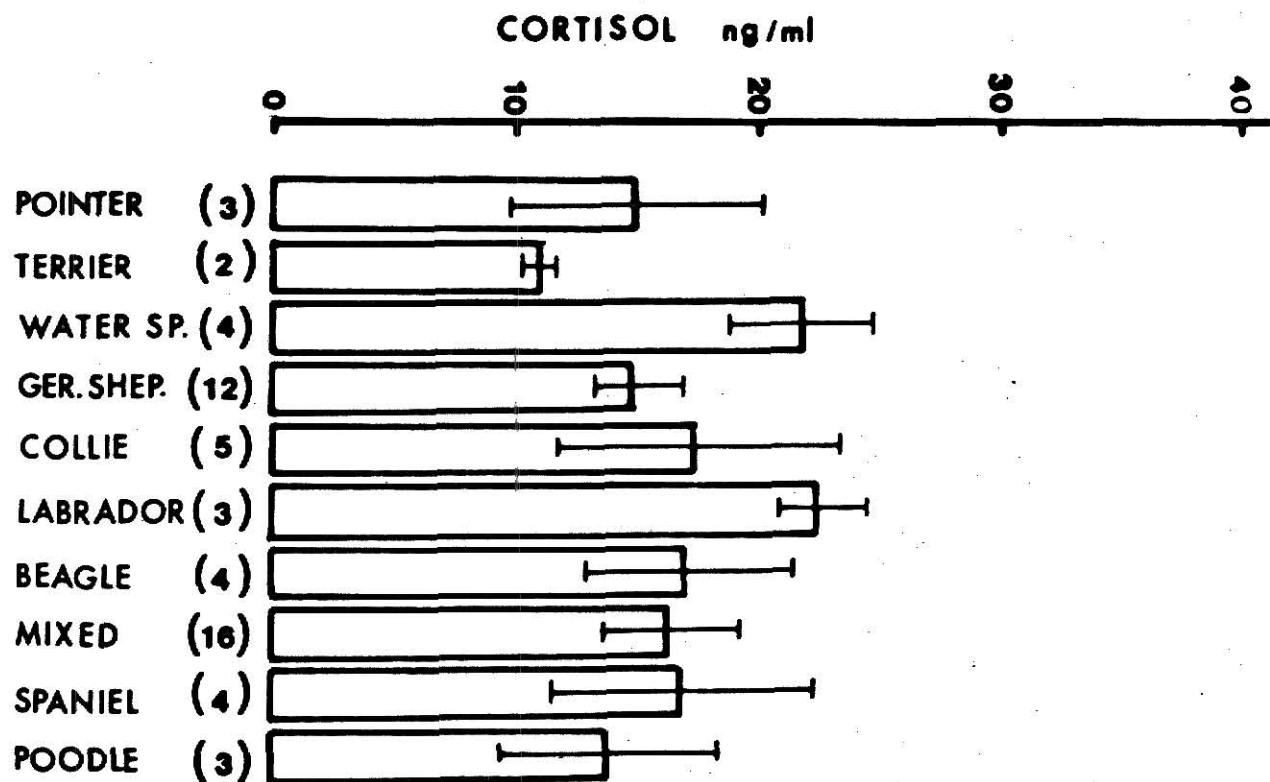error of the mean is represented by vertical lines.

CORTISOL ng/ml

Figure 2. Cortisol levels in clinically healthy males and females
as compared to hypoadrenocorticism and hyperadreno-
corticism dogs.

CORTISOL ng/ml

MALES FEMALES HYPER ADRENOCORTICISM HYPO. ADRENOCORTICISM
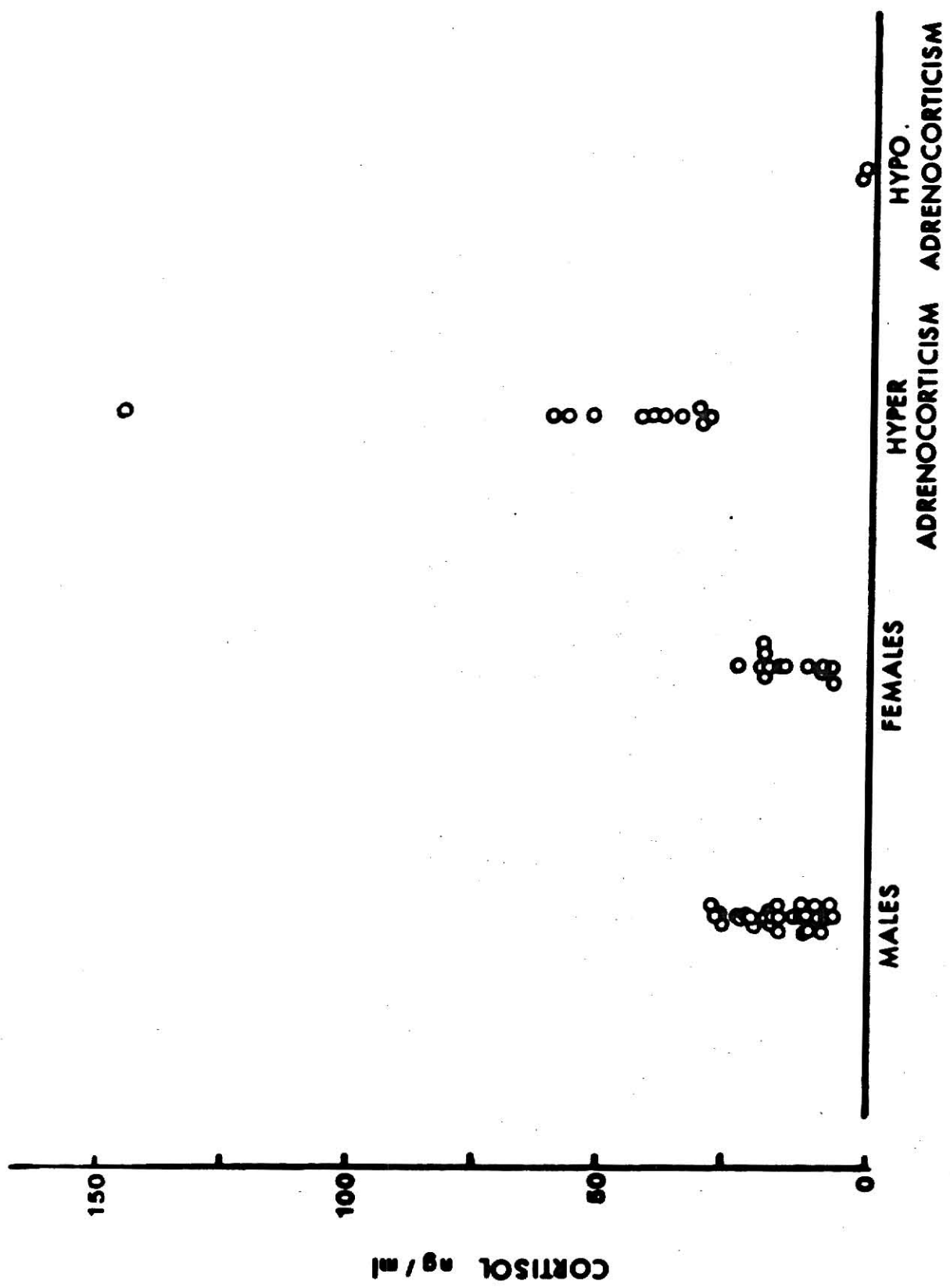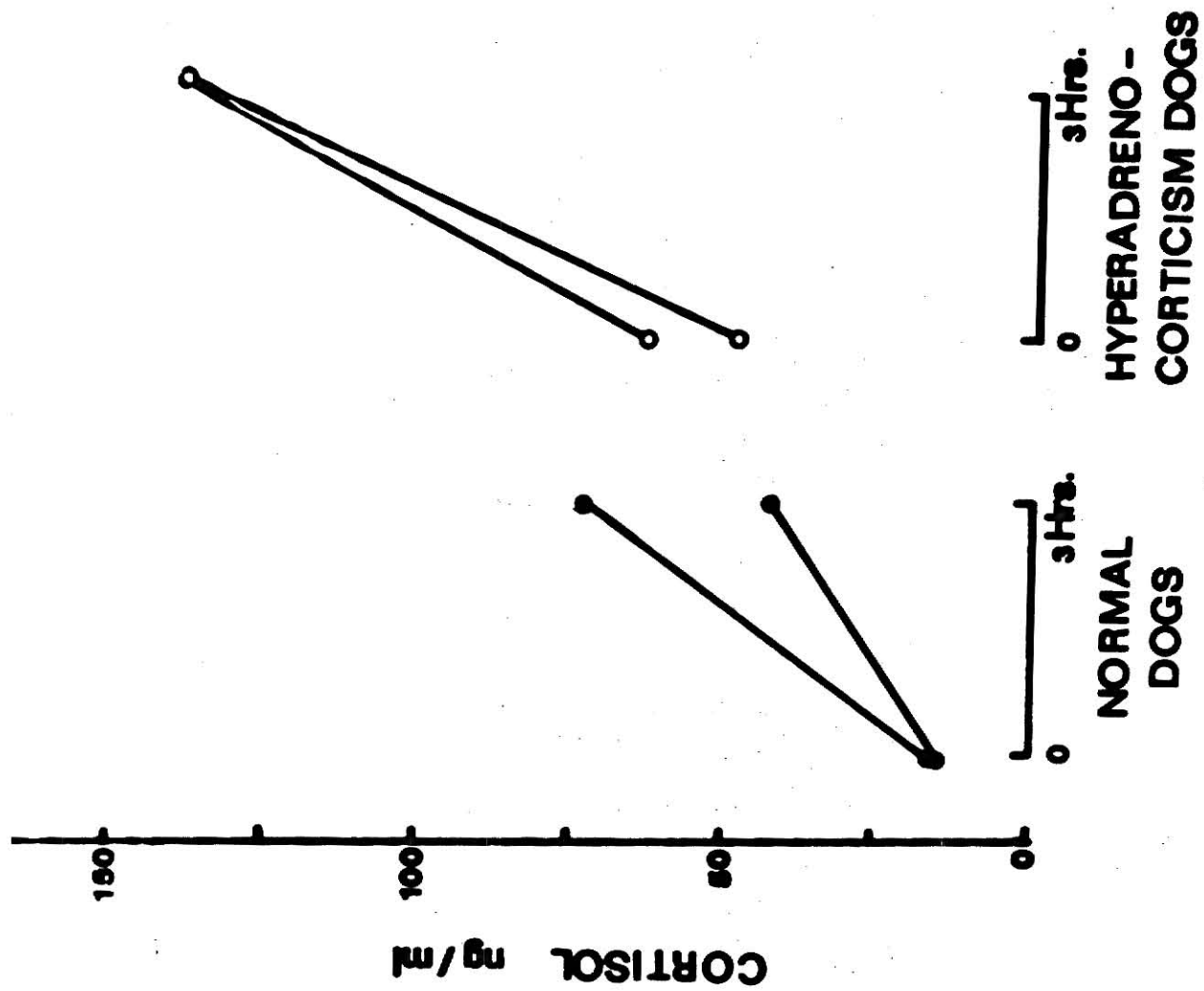
0 50 100 150

Figure 3.   ACTH response in normal and hyperadrenocorticism dogs.

STUDIES ON NORMAL AND EXPERIMENTALLY ALTERED CIRCADIAN
CORTISOL RHYTHMS IN PONIES AND CORTISOL LEVELS
IN NORMAL AND ADRENOPATHIC DOGS

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AN ABSTRACT OF A MASTER'S THESIS

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requirements for the degree

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Serum cortisol levels were studied by specific radioimmunoassay in ponies under various lighting schedules, starvation, ACTH stimulation and exercise and in normal and adrenopathic dogs. In the pony, a circadian rhythm was observed with peak cortisol levels (31.75 ± 3.16 ng/ml) in the morning at 0900 and 1100 hrs and lowest levels (19.6 ± 3.16 ng/ml) at 2100 and 2300 hrs. Exposure to reversed light regimen resulted in a complete phase reversal of the circadian rhythm. Exposure to 2 weeks of continuous light completely disrupted the circadian rhythm and decreased the cortisol levels (12.8 ± 8.3 ng/ml) in the ponies. An I.M. injection of 100 I.U. of ACTH in gelatin significantly increased the cortisol levels 3-4 fold and this treatment maintained a high level of cortisol throughout a 24 hr period. Serum cortisol levels were increased significantly one day after starvation (62.4 ng/ml) and were maintained for the entire period of 8 days of starvation. The circadian rhythm was disrupted during the period of starvation. Exercise did not have significant effect on the serum levels of cortisol in the ponies. In 56 clinically healthy dogs, serum cortisol levels of $17.8 + 9.9$ ng/ml were observed. No significant age, sex, breed or body weight differences were found in serum levels of cortisol in dogs. In 11 dogs with definitive hyperadrenocorticism, the serum cortisol levels were greater than 30 ng/ml and in 2 dogs with hypoadrenocorticism, serum cortisol levels were less than 5 ng/ml.

These results indicate that light plays a major role in maintaining the circadian adrenocortical activity and that physiological stress such as starvation can alter the rhythms in ponies. Further, the estimation of serum levels of cortisol by radioimmunoassay appears to be simple and precise in the diagnosis of adrenal function in the dogs.