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MASSACHUSETTS INSTITUTE OF TECHNOLOGY

FINAL REPORT

CONTRACT NUMBER: NAS 9-15194

Title: "Potential Use of Nutritional Factors to Optimize Performance Under Stress"

Principal Investigator: Dr. H.N. Munro

(NASA-CR-151676) POTENTIAL USE OF NUTRITIONAL FACTORS TO OPTIMIZE PERFORMANCE UNDER STRESS Final Report, 1 Dec. 1976 - 30 Nov. 1977 (Massachusetts Inst. of Tech.) 74 p HC A04/MF A01 CSCL 06S G3/54

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FINAL SUMMARY REPORT

Contract Number: NAS 9-15194
Principal Investigator: Dr. H.N. Munro, Room 56-225, MIT
Reporting Date: 1/01/78
Title: Potential Use of Nutritional Factors to Optimize Performance under Stress.
Period of Contract: Dec. 1, 1976-Nov. 30, 1977.

Personnel:

1. Dr. H.N. Munro, Professor of Physiological Chemistry, M.I.T. (10% effort from Dec. 1-Nov. 30).
2. Dr. Marilyn C. Crim: Research Associate, M.I.T. (10% effort from Dec. 1-Sept. 30).
3. Dr. Frank Tomas: Visiting scientist, CSIRO, Adelaide, Australia (25% effort, April 1 - Sept. 30)
4. Richard Reimer, undergraduate, M.I.T. (10% effort, June 1-Sept. 1).

Objectives:

To examine the role of dietary protein and amino acids on responses to stress, with a view to optimizing performance under stress in flight.

General Description:

The work proposed arises from a study some years ago in which it was shown that the adrenal gland of the rat responds to intake of protein and individual amino acids in the diet. Specifically, the amino acids leucine and methionine were found to stimulate output of corticosteroid hormones from the gland for a period of several hours. Continuous administration of these amino acids lead to changes in adrenal steroid content. It was concluded that this change in adrenal function is probably affected through ACTH secretion from the pituitary gland, which in turn may be regulated by changes in hypothalamic secretion of corticotropin releasing factor (CRF) resulting from the administration of these amino acids. As part of the program, it has been possible to extend this to the question of how much the blood levels of corticosteroids need to rise in order to cause adverse metabolic changes.

From these observations, it was surmized that the judicious use of dietary protein, and specifically of certain amino acids, may allow the response of the adrenal cortex under stress to be controlled by appropriate dietary means.

Summary of Report:

The report falls into two parts. First, there is a study of the effects of amino acids on hypothalamo-pituitary capacity to secrete ACTH, and the response of the adrenal gland in terms of corticosterone level in the plasma. Second, the report includes a study of the response of protein metabolism various levels of corticosterone elevation in the plasma. We regard this second part as having considerable significance for space travel, since our data indicate a threshold level of plasma corticosteroids above which there is increased catabolism of muscle protein.

Part One: Effect of Amino Acid Intake by Rats on Levels of Corticosterone-Releasing Factor in Hypothalamus, ACTH in Pituitary, and Cortisol in Plasma.

Project 3.2 on p. 5 of original description of task described the objective of this section as follows: "The study will provide information on whether excess leucine will influence plasma corticosteroid levels through an action on hypothalamic function". The following data were obtained.

1. A study has been made of hypothalamic, pituitary and adrenal function in rats fed leucine or alanine up to 24 h before killing.
2. Procedures for ACTH measurements in isolated rat pituitary glands were developed. These were used for pituitaries alone or exposed to fluid from hypothalami (perifusion).
3. Perifusions using pituitaries from rats fed different diets tend to show that the basal rate that leucine pituitaries secrete ACTH is greater than the basal secretion of the alanine pituitaries. This is accompanied by sustained output of a fragment of ACTH called "CLIP", which is known to stimulate insulin output.
4. When hypothalamic extracts were used to stimulate ACTH secretion, there was increased secretion of ACTH when leucine was pre-fed.
5. At 4.5 hours after feeding, the plasma corticosterone levels from rats fed leucine showed a significant increase when compared with alanine fed rats. At later times measured, there was little or no difference. Feedback inhibition exerted over ACTH secretions by the corticosterone concentration could explain why there is no significant changes in ACTH and CRF at 18 hours after feeding.
6. It can be concluded that administered leucine increases the concentration of ACTH by acting on the pituitary, the hypothalamus or at some higher center at approximately 4 hours after feeding. The increased basal secretion of ACTH and the higher values of ACTH content found in the pituitaries of leucine treated rats 18 hours after leucine could be remnants of the earlier stimulation.
7. This work continues to open up the prospect of controlling stress by dietary means, through changes in capacity to secrete ACTH and corticosteroids.

Part Two: Effect of Glucocorticoids on Myofibrillar Protein Breakdown Measured by N^T-Methylhistidine Output.

N^T-Methylhistidine (3-MeHis) output was measured in the urine of young male adrenalectomized (adx) rats injected with 0, 0.2, 0.5, 1, 5 or 10 mg corticosterone/100 g body wt daily for 7 days. All rats were pair-fed with the untreated adx rats. Intact control rats and adx rats on doses between 0 and 1 mg steroid grew at similar rates, whereas growth immediately ceased on 5 and 10 mg doses. Output of 3-MeHis was unaffected by adrenalectomy or corticosterone at doses below 1 mg, but increased slightly on 1 mg and about 2-fold on 5 and 10 mg doses. At the two highest doses, the weight of the gastrocnemius muscle relative to final body weight was 25% less than in untreated adx animals. The soleus and extensor digitorum longus were unaffected, while liver wt. increased considerably. The changes in gastrocnemius and liver weights were not seen at lower doses. Treatment with steroids did not maintain plasma corticosterone levels above those of the intact controls until dosage reached 5 and 10 mg, when the plasma concentration was 2-3 fold greater at 5 hr after injection. These high doses also caused glycosuria and elevated plasma insulin levels. It is concluded that corticosteroids do not normally regulate myofibrillar protein breakdown rate, but that excessive levels in the plasma, equivalent to severe stress, can accelerate breakdown.

Conclusions and Recommended Actions

The above data emphasize that certain high plasma levels of corticosteroids are associated with increased muscle protein breakdown as a stress response. The role of certain amino acids in the diet in the production of such levels of steroids indicates dietary participation in the response to stress which could be manipulated.

Courses of Action:

The current contract has run out. It is suggested that the implications of steroid levels observed in astronauts in flight should be considered in relation to our findings and dietary contributions to such steroid levels evaluated.

Appendixes One and Two:

Detailed descriptions on the above work. See following pages.

Apprendix One: Effect of Amino Acid Intake by Rats on Levels of Corticosterone-Releasing Factor in Hypothalamus, ACTH in Pituitary, and Cortisol in Plasma.

I. BACKGROUND

A. ACTH

Corticotrophin (ACTH) is a 39 amino acid hormone with a molecular weight of about 4500. It is known to be synthesized and stored by the adenohypophysis. It is synthesized by the placenta and high concentrations of the hormone have been found in the hypothalamus and other CNS areas (Krieger et al 1977). The structure for ACTH has been determined for several species, and for the pig, sheep, beef and human the molecules are identical except for a variable region of the chain between amino acids 25 to 32 (figure 1). Removal of the C-terminal amino acids (25-39) results in no loss of biological activity, the active portion of the molecule being the NH_2-terminal 1-24 region.

1. Physiological actions.

ACTH is primarily synthesized and stored in the adenohypophysis. Its target organ is the adrenal cortex, which concentrates the ACTH. Its main effects on the adrenal cortex are:

i) It increases adrenal corticoid formation and content.

ii) It increases phosphate turnover and the hydrolysis of cholesterol esters.

iii) It decreases the adrenal concentration of cholesterol, ascorbic acid and lipids. (Schulster, 1974).

It is thought that ACTH acts via the second messenger adenosine 3' 5' - cyclic monophosphate (cAMP) (Grahame-Smith et al,1967). The cAMP induces a regulator protein to be synthesized and then this protein transfers cholesterol from the lipid

Figure 1. Comparison of structures[a] of adrenocorticotrophins from four different species.

ACTH H-Ser-Tyr-Ser-Met-Glu-His-Phe-Arg-Trp-Gly-Lys-Pro-Val-Gly-
1 2 3 4 5 6 7 8 9 10 11 12 13 14

Lys-Lys-Arg-Arg-Pro-Val-Lys-Val-Tyr-Val-(----)-Ala-Phe-Pro-
15 16 17 18 19 20 21 22 23 24 34 35 36

Leu-Glu-Phe-OH
37 38 39

25 26 27 28 29 30 31 32 33

Pig ACTH NH_2 | Asp- Gly- Ala-Glu-Asp-Glu-Leu-Ala-Glu

Sheep ACTH Ala-Gly-Glu-Asp-Asp-Glu-Ala-Ser-Glu-NH_2

Beef ACTH Asp-Gly-Glu-Ala-Glu-Asp-Ser-Ala-Glu-NH_2

Human ACTH NH_2 | Asp-Gly-Ala-Glu-Asp-Glu-Ser-Ala-Glu

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[a] From Schulster (1974)

droplets to the mitochondria (where the rate limiting steps for steroidogenesis take place). (Garren et al, 1971).

In the rat, the release of corticosterone has numerous effects including increasing gluconeogenesis, glycogen deposition, degrading albumin and accelerating uptake of amino acids by the liver (Liddle, 1974).

2. Synthesis, storage, release and transport of ACTH.

The adenohypophysis synthesizes, stores and releases ACTH in response to a dual control mechanism. It is under feedback control by the concentration of corticoids in the blood. It is also controlled by the hypothalamic releasing factor (CRF). The CRF responds by increasing output of ACTH as a function of the rats circadian rhythm (with a maximum release occuring just before waking), pain, pyrogens, hypoglycemia and as a response to increases in vasopressin concentration. (Gill,1972).

The rat pituitary contains approximately 400 ng ACTH per 100 grams body weight, (Scott et al, 1974). It is carried in the plasma as free ACTH. In the rat, the half-life of endogenous ACTH has been estimated as 1 minute (Snydor and Sayers,1953). About 20% of endogenous ACTH is taken up by the kidney (Schulster,1974). For the rat resting levels of ACTH are reported as around 23-63 pg/ml. After stress these levels climb to between 250-2000 pg/ml (Rees et al,1971)

3. Measurement of ACTH.

Due to the very low concentration of ACTH in the rat, the measurement of ACTH requires a very sensitive and precise assay.

At this time there are 2 generally accepted assays for the measurement of ACTH. One is a bioassay and the other is a radioimmunoassay.

The bioassay (Liotta and Krieger,1975) makes use of isolated adrenal cells from hypophysectomized animals (Sayers,1972). By using a silicic acid extraction of the plasma this bioassay is able to assay samples with concentrations of ACTH as low as 2 pg/ml.

The RIA (Rees et al,1971) uses the chloramine T oxidation of Greenwood and Hunter (1963) to label the ACTH. The antiserum was raised against the NH_2-terminal 1-24 portion of ACTH. Samples are extracted with glass, desorbed using acetone, the extract is mixed with antiserum and incubated. Separation of bound and free fractions is done with charcoal. The assay is sensitive to concentrations as low as 20 pg/ml. The RIA used in these experiments is based on Rees technique.

B. Radioimmunoassays; Theory and Practice.

The RIA is an example of a general type of technique called a competitive binding assay. As the name implies, there is competition, between an unknown and some type of labeled compound, for a limited number of binding sites on some molecule.

Usually the competition is between an unknown, or standard, and a radio-labeled molecule which has the same structure as the unknown. The binding molecule can be a cell membrane receptor, a transport protein, or an enzyme but it is usually an antibody.

A fixed amount of radio-labeled antigen is mixed with a

sample containing the unlabeled antigen. A limiting amount of antiserum is added to the mixture. After an incubation time in which the antigens equilibrate between the bound and free states, these fractions are separated and the radioactivity in the fractions are counted. (See figure 2).

The concentration of the unknown can be calculated by comparison with the displacement of the labeled antigen by a set of standards (figure 3). In this way one is able to evaluate the samples as a function of the percent of counts bound to the antiserum.

The technique, as first introduced by Berson and Yalow (1959) for the measurement of plasma insulin levels, is now used to measure many peptide and non-peptide hormones, drugs, serum proteins and other biological samples (Yalow, 1973). An RIA has 4 specific requirements which are:

1) An antigen, either synthetic or highly purified, for standards and radiolabeling.

2) A radio-labeled antigen which should be of a high specific activity. Most proteins are labeled using ^{125}Iodine. The ^{125}I attaches to the tyrosine residues. The use of chloramine T has enabled many peptides to be labeled.

3) A specific antiserum. This is usually raised in rabbits, goats or sheep. Most peptide hormones are immunogenic when emulsified in Freunds adjuvant (Yalow, 1973). When the peptide is too small to be antigenic, it can be rendered so by coupling

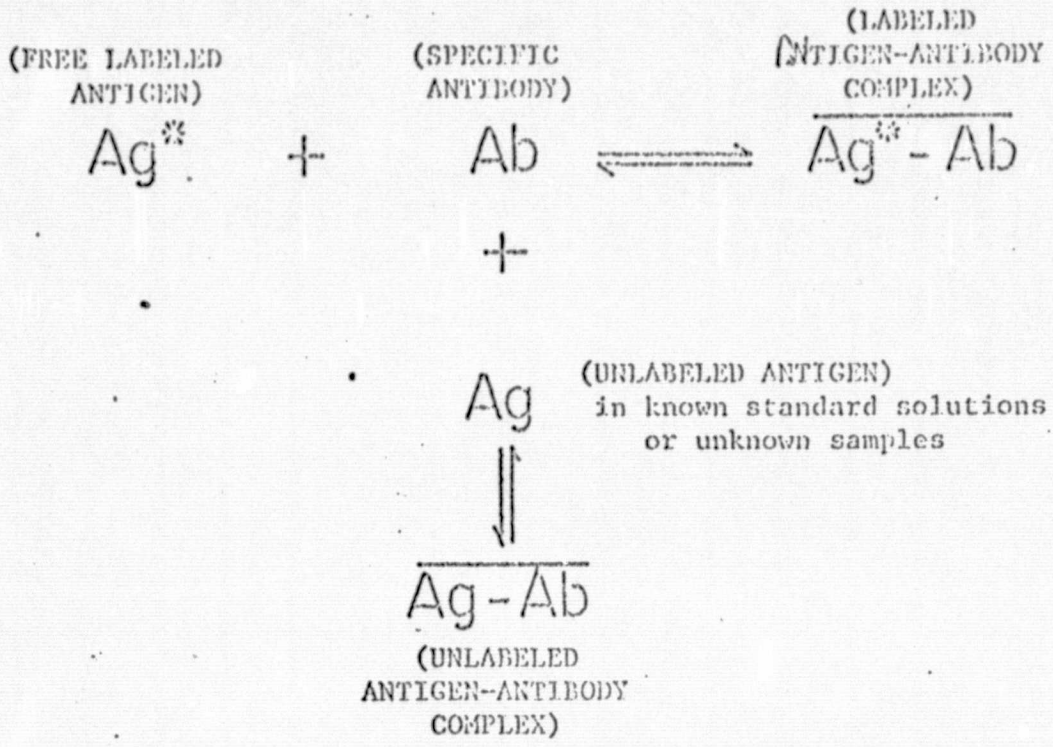


Figure 2. Competing reactions that form the basis of the RIA. (Yalow, 1973)

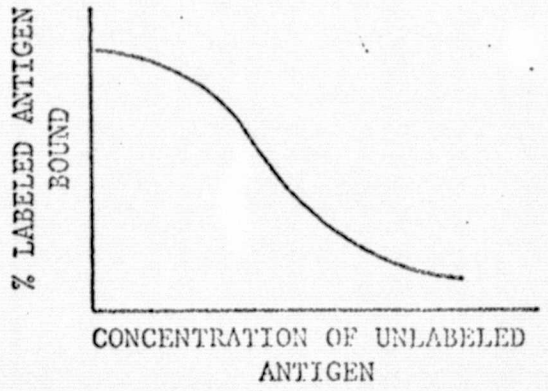


Figure 3. RIA standard curve.

to a larger protein.

4) A good method of separation. After incubation of all the components of the assay mixture, ther must be a method to separate the bound from the free fraction. There are several popular methods but they must be chosen to suit the needs of the assay system. The ideal technique should completely separate the free from the bound fraction without effecting the initial binding reaction. One can use adsorption of free antigen (to glass, charcoal), precipitation of the antibody-antigen complex (second antibody, salt solutions) or via adsorption of the antiserum (to glass or dextran). (Ratcliffe,1974).

Since the RIA is able to measure concentrations as small as 10^{-12}M., it is important that the assay should be precise (reproducible), and unbiased (not affected by unspecific factors). These unspecific factors (which must be carefully watched for) are pH, ionic environment, anticoagulants, bacteriostatic agents, temperature and hormonal cross reactivity.(Yalow,1973).

II. INTRODUCTION

In 1957, it was established that administration of casein to fasting rats caused an increase in incorporation of ^{32}P and (^{14}C)-glycine into liver RNA (Clark et al,1957). It was subsequently shown that the mechanism of response is sensitive to the deletion of one essential amino acid from a complete mixture of amino acids (Munro and Clark,1959). This suggests that the effect on liver RNA metabolism depends on the nutritional value of the protein. However the feeding of zein (which is deficient in 2 essential amino acids, tryptophan and lysine) led to the same stimulus on liver RNA metabolism as casein (Munro and Mukerji,1958). Since zein contains excessive amounts of leucine, it was decided to examine the effects of excessive levels of individual amino acids on liver RNA metabolism.

Young adult rats were fed a protein free meal to which individual amino acids had been added, ^{32}P was injected one hour later and the animals were killed 18 hours thereafter. The amino acids glycine, methionine and leucine increased ^{32}P incorporation greater than 65% when compare with control values. In exploring the actions of these three amino acids more extensively it was shown that they induce a significant increase in total liver RNA. Dose response curves demonstrated that the actions of leucine and methionine on ^{32}P uptake were linear from doses of 0.05g. upwards, whereas glycine required a threshold dose. It was also shown that a single large dose of leucine in rats produces an

increment in liver protein (Munro and Mukerji,1958). After giving cortisone, the amount of RNA and protein in the liver increases considerably. Another feature of adrenocortical activity is deposition of glycogen in the liver. After feeding leucine, methionine or glycine it was discovered there was an increase in the amount of glycogen in the liver. (Munro and Mukerji,1962). The same diet fed to adrenalectomized rats showed no increase in glycogen content. Measurements were also made of the effect of adrenalectomy on the response of liver RNA metabolism to these amino acids. Adrenalectomy prevented the action of methionine and leucine but only moderately diminished the effect of glycine. This suggests that large doses of methionine and leucine cause an increase in adrenocortical hormones. This was tested by measuring the corticosterone content in the plasma and adrenal glands of rats being fed individual amino acids. Both methionine and leucine caused a significant elevation of the plasma level and a tendency for the corticosterone content in the adrenal gland to be raised. (Munro et al,1963).

More evidence for the effects of leucine on the adrenal cortex comes from the studies by Hungarian workers (Goth et al,1955). They showed giving leucine to rats via stomach tube caused depletion of the ascorbic acid content of the adrenal. This action does not occur in hypophysectomized rats, suggesting that the primary site of action is the pituitary gland.

The evidence suggests that a single large dose of certain

amino acids causes increased secretion of corticosterone, presumably through release of ACTH. But the work was carried no further. The developement of a sensitive assay for ACTH and the existence of apparatus to study the response of organs to a flowing system (Edwardson and Bennett, 1974) have made continuation of this work possible.

The question of where the effect of these amino acids is located can be answered using the above techniques. Extraction of pituitaries from diet treated animals and the measurement of their ACTH content can tell if the effect is located in the pituitary. Perifusion of pituitaries which are stimulated with hypothalamic extracts from rats fed different diets can tell if the effect is localized in the hypothalamus.

By feeding rats high concentrations of leucine and a control amino acid diet, the location at which leucine acts can be discovered. This is the purpose of the studies undertaken for this thesis.

III. MATERIALS AND METHOD

A. Animals and Tissues.

Male Sprague-Dawley rats (Charles River Laboratories, Wilmington, Mass.) weighing between 150-250 g. were used for all experiments. They were housed individually and were exposed to light between 8 AM and 8 PM daily. They were fed lab chow and given water ad libitum for 3 to 5 days prior to dietary treatment. During this period rats were handled daily, to gentle and accustom them to human presence.

Diet treatments began by feeding rats 10 g. of control diet (table 1) at 4 PM on day 1 of the experiment. This feeding period was considered a training period in which the rats were taught to consume the powdered food in a single meal. At 4 PM on day 2 the rats were fed a 10% leucine or 10% alanine diet or placed back on the lab chow diet.

Animals were killed by decapitation from 11 AM to noon on day 3, and the trunk blood was collected in tubes, (for corticosterone assays to be performed at the Peter Bent Brigham Hospital, Boston, Mass.). Pituitaries and hypothalami were removed (no longer than 5 minutes after sacrifice). Pituitaries for perifusion were halved and placed in a 30° pool of KREBG buffer. Hypothalami that were to be extracted for measurement of CRF content were placed in 0.5 ml. of 0.1 N. HCl to denature any proteases (the same treatment was given to pituitaries when they were later extracted and assayed for ACTH content).

Table 1. Content of experimental diets

Protein Free Diet

Nutrient	grams/100 g. of diet
Glucose	46
Corn starch	40
Corn oil and d, l, ∝tocopherol	6.7
Mineral mix	5.3
Cod liver oil	1.3
Choline	0.4
Water soluble vitamins in sucrose	0.3

Control Diet
(25% Protein, 75% Protein free diet)

Protein free diet	7.5 g.
Casein	2.5 g.
	10.0 g.

Leucine Diet-10%

Protein free diet	7.5 g.
Starch-dextrose	1.5 g.
Leucine	1.0 g.
	10.0 g.

Alanine Diet-10%

Protein free diet	7.5 g.
Starch-dextrose	1.5 g.
Alanine	1.0 g.
	10.0 g.

Lab chow- Charles River Rat-Mouse-Hamster Maintenance Formula (Charles River Lab; Wilminton, Mass.)

Hypothalami were extracted in the 0.5 ml. of cold HCl using a Duell #22 homogenizer. The acid extract was poured into a plastic tube and the homogenizer was washed with 0.5 ml. of 0.1 N. HCl and the wash was mixed with the extract. Homogenates were spun at 12.000 G. for 10 minutes (at 4°C.). The supernatents were removed and brought up to 1 ml. volume, (this concetration is referred to as 1 hypothalamic equivalent (HE)), and was assayed for CRF via perifusion. In the case of frozen pituitaries, on the assay date, they received the same treatment.

B. Perifusions

Different experiments were performed by feeding rats leucine, alanine or regular lab chow. These rats were then sacrificed and pituitary halves (from the same or different dietary treatments) were perifused in the two chambers of the apparatus. The pituitaries were stimulated with different HE and fractions were collected, (waiting approximately 30 minutes between different HE were added). In this way the CRF could be measured (as a function of increase in ACTH output by the pituitary), and each pituitary could act as its own control.

C. Reagents

h-ACTH: synthetic corticotrophin (94 IU/mg.) provided by the NIAMDD.

^{125}I-ACTH: iodinated ACTH, prepared as described in the text.

ACTH Antiserum: Antiserum to ACTH prepared in rabbits against porcine ACTH, provided by NIAMDD.

RIA Buffer: 0.025 M. sodium phophate, 0.25% BSA pH 7.6
This buffer is used for all standard dilutions, 125-I-ACTH dilutions, antiserum dilutions and for final preparation of Thick Washed Norit.

BSA: Bovine serum albumin, ICN Pharmaceuticals Inc. Cleveland, Ohio.

Normal horse serum: Grand Island Biological Co. (605) Grand Island, N. Y..

Norit A: Activated charcoal prepared as follows:
20 g. charcoal to 200 ml. with 0.1 N HCl
Stir 20 minutes.
Transfer to a cylinder and let stand 20 minutes
Aspirate supernatent.
Wash with water 3 times by adding 250 ml de-ionized water, mix by inversion, let stand 10 minutes and aspirate supernatent.
Wash with 0.025 M. PO_4 buffer (pH 7.6) 2 times. Mix by inversion, let stand 20 minutes and aspirate supernatent.
Check pH (7.4-7.6). Store at 4°C.

Thick Washed Norit: 5 ml. Norit A
2 ml. 0.25 PO_4 buffer (pH 7.6)
15 ml. Normal horse serum
3 ml. De-ionized water

KRBG: Krebs Ringer Buffer with glucose, BSA, and O_2
118 mM sodium chloride, 25 mM sodium bicarbonate, 4.7 mM potassium phosphate, 2.52 mM calcium chloride, gas for 5 minutes with 95% O_2/5% CO_2 (v/v), 0.2% glucose, 0.25% BSA.

D. Preparation of ^{125}I-ACTH

Three types of ^{125}I-ACTH were used. The first was a gift from Dr. Franco Sanchez from Tufts University School of Medicine, Boston, Mass.. The second was iodinated in our lab and the third was from Amersham/Searle ACTH Immunoassay Kit IM.66 (Arlington Heights, Illinois.).

1. Iodination reaction.

^{125}I-ACTH of high specific activity was prepared by iodinating synthetic h-ACTH by first oxidizing it with chloramine T, neutralizing the mix, extracting with glass and then purifying it on a Sephadex G-25 column.

To a vial containing a basic solution of 0.5 mc. Na^{125}I (NEN #033L Boston, Mass.) the following were added:

10 ul. of synthetic h-ACTH (2 ug)
50 ul. of 0.5 M PO4 buffer (pH 7.6)
25 ul. of chloramine T (1 mg/ml. mixed immediatley prior to use and protected from direct light).

The contents of the vial are mixed from 15-20 seconds. No longer than 20 seconds after the addition of the chloramine T add:

25 ul. of sodium metabislfite (2 mg./ml., mixed immediatley prior to use)

The chloramine T is a strong oxidizing agent which may signifigantly damage the ACTH, sodium metabisulfite is added to neutralize the remainder. Then add:

50 ul. of BSA (5 g.%) and mix.

The total mixture is added to 10 mg. quso glass (G 32, Philadelphia Quartz Co., Phila., Penn), in a plastic tube and

vortexed.

2. Purification of ^{125}I-ACTH.

One ml. of de-ionized H_2O is added to the quso glass mixture and the mixture is centrifuged at 1000 G. for 5 minutes (at 4°C.). The supernatent is decanted and the glass is washed by adding 1 ml. of de-ionized H_2O vortexing and centrifuging at 1000 G. for 5 minutes. This wash is repeated 2 times. The ^{125}I-ACTH (now adsorbed to the glass) is desorbed using 5 ml. of a 40% acetone/1% acetic acid mix (v/v). The tube is vortexed and then centrifuged at 1000 G. for 10 minutes. The supernatent is aliquoted into 0.8 ml. fractions which are frozen at -20°C.

Prior to use, the aliquot is passed through a 50x2 cm. Sephadex G 25 (coarse) column, equilibrated with 0.1 N. acetic acid and 1.25 mg./ml. BSA (pH 3.6). The flow rate of the column is 1 ml. per 10 minutes. One ml. fractions are collected in plastic test tubes. The tubes are counted for 6 seconds to determine the radioactivity per tube. The counts are plotted and the ^{125}I will be found at the second peak of radioactivity. (Franco Sanchez, personal communication).

3. Iodination check.

The success of the attempted iodination is checked by mixing 50 ul. of appropriately diluted ^{125}I-ACTH (7000 cpm/50 ul.) with an excess of antiserum (50 ul. of antiserum diuted 1:250), and 100 ul. of RIA buffer. The samples are incubated 24-36 hours, counted, separated (using Thick Washed Norit) and the counts adsorbed to the charcoal are counted. Since there is an excess

of antiserum almost all of the counts should be bound to it (if the iodination is successful).

E. Preparation of the antiserum.

Three types of antiserum were used throughout the course of these experiments. The first antiserum was a gift from Dr. J. A. Edwardson, of the Department of Biochemistry, Imperial College of Science and Technology, London, England. He received it from the NIAMDD and the National Pituitary Agency of the University of Maryland. It was raised against the NH_2 terminal portion (1-24) of porcine ACTH and does not give signifigant cross reaction with αMSH. The second antiserum was a gift to our lab from the NIAMDD. The third type of antiserum was from the Amersham/Searle ACTH immunoassay kit.

F. Antiserum titration.

Antiserum was serially diluted 1×10^3, 2×10^3, 4×10^3, 8×10^3, 16×10^3, 32×10^3 and 64×10^3 using RIA buffer. To each tube was added 50 ul. of serially diluted antiserum, 50 ul. of the appropiatley diluted ^{125}I-ACTH (such that 50 ul. contained 7000 cpm), and 100 ul. of RIA buffer. They were incubated from 24-36 hours. After incubation, the tubes were counted and then mixed with 200 ul. of Thick Washed Norit, centrifuged and the supernatents removed. The charcoal residue was counted and the percentage of total ^{125}I-ACTH bound to the antiserum was calculated. The desired titer was 50% of the counts bound. Each time a new batch of ^{125}I-ACTH was used, an antiserum titration

was performed.

G. Assay procedure.

To each assay tube was added in the following order, 50 ul. of properly diluted ^{125}I-ACTH (7000 cpm), 100 ul. of standard, blank or unknown and 50 ul. of properly diluted antiserum (which binds 50% of ^{125}I-ACTH). The tubes were vortexed and then stored at 4°C. for 24-36 hours. After this incubation time tubes were counted. Then 200 ul. of Thick Washed Norit was added to each tube. The tubes were vortexed and then immediatley centrifuged (no more than 5 minutes from adding the charcoal) at 1000 G. for 5 minutes (at 4°C.). The charcoal adsorbs unbound ACTH, and by counting the ^{125}I-ACTH adsorbed to the charcoal, the counts bound to the antiserum can be calculated. The standard curve consists of serial dilution of synthetic h-ACTH (diluted with RIA buffer), using concentrations of 100,50, 20,10,5,2,1,0.5 ng./ml.. Along with the standard curve a tube containing 50 ul. of diluted ^{125}I-ACTH and 150 ul. of RIA buffer are added. This tube is treated like all other assay tubes. Since the charcoal is in a sufficiently high concentration to adsorb all of the ^{125}I-ACTH, any counts not adsorbing to the charcoal represents damaged ACTH. Therefore this tube gives an index of the ^{125}I-ACTH not specifically bound to the antiserum, and it is labeled NSB. All tubes were run in triplicate. The standard curves are plotted as the percent of counts bound to the antiserum. Therefore counting the counts adsorbed to the charcoal (free ^{125}I-ACTH) and

and subtracting this from the number of counts originally in the tube gives the number of counts bound to the antiserum. Dividing this by the number of counts originally in the tubes gives you the percent of counts bound to the antiserum.

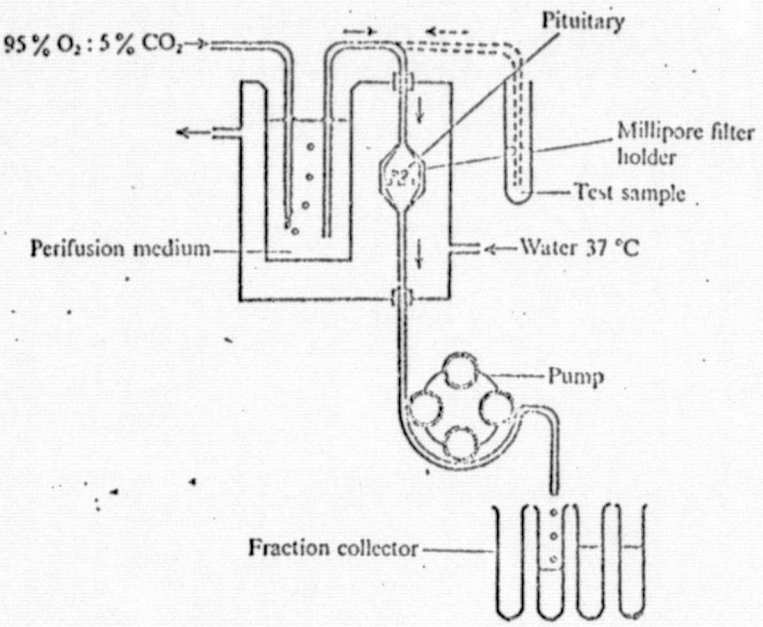
H. Perifusion apparatus.

The apparatus used for perifusion is shown in figure 4. According to experimental conditions, 2 or 3 rat hemi-pituitaries were placed in the millipore holder which served as the perifusion chamber. Care was taken to make sure that no air bubbles were in the chamber or any flow lines. The pituitaries were perifused using KREG buffer. All perifusions and buffers were at 37°C.. The flow rate was adjusted so 0.2 ml of buffer flowed per minute. Test hypothalamic extracts were kept on ice, warmed to 37°C. and then added via the three way valve at the top of the chamber. using the 2 chambered apparatus, 2 pituitaries from animals receiving different dietary treatments were able to be perifused at the same time, permitting testing and comparison of samples under equivalent conditions. All perifusion samples were collected as 1 ml. fractions (5 minutes) and were immediatley frozen. Samples were stored at -20°C. until analysis was performed.

I. Statistics.

Statistical signifigance between groups was determined by the Student's t-test.

Figure 4. The perifusion apparatus. (Edwardson and Bennett, 1974)



IV. RESULTS AND DISCUSSION

A. Antiserum titration.

Due to the 60 day half life of ^{125}I, antiserum titrations were run on each new batch of ^{125}I-ACTH. At the beginning of the course of experiments the Edwardson antiserum was used. After receipt of the NIAMDD antiserum, it was titrated. The NIAMDD advises a 1:120,000 dilution for 50% binding of ACTH. No signifi-gant binding was visible at antiserum concentrations as high as 1:1000. Therefore the Edwardson antiserum was used for all of these experiments (except when the Amersham/Searle kit was used). The Edwardson antiserum was usually diluted between 1:2000 and 1:8000. At this concentration it gave 45-50% binding of the total counts.

B. Iodination.

Iodination was attempted once during the course of these experiments and purification was attempted twice. Both times the G 25 eluate did not give the counting pattern desired (two distinct peaks). The only peak was eluted about 120 ml. after the sample was placed on the column. Samples of this peak were diluted to give approximately 7000 cpm/50 ul.. This sample was assayed with an excess of antiserum (1:250 dilution). The anti-serum (which was known to bind ACTH) did not bind any counts. The NSB was equal to 50% of the total counts. When antiserum was added to the sample, the percentage of counts adsorbed to the charcoal (which should have been signifigantly lower), did not change. Therefore no ACTH was labeled.

Iodination damage can have many causes. Manipulations of small quantities of a pure protein sample can reveal instabilities that are not seen when the protein is in a cruder form (when it is protected by contaminants or carrier proteins). The incorporation of ^{125}I into the tyrosine residues can result in a loss of affinity between the ACTH and the antiserum. Exposure to strong oxidizing agents like chloramine T, may result in signifigant damage to the antigen. (Hunter,1974). But since ACTH is iodinated routinely in labs using the same procedure, I feel these possible causes do not explain the unsuccessful iodination.

I think it is signifigant that the NIAMDD supplied both antiserum (which failed to bind ACTH) and the h-ACTH (which was not successfully iodinated.) Both were supplied in lypholized glass vials, and were stored at room temperature for 2 months prior to reconstitution and freezing. I feel that there is a chance that this storage might have led to degradation, which would explain why both the ACTH iodination and the antiserum titration failed.

C. Assays.

Initially the assay tubes had a large variation in radioactive counts (sometimes ranging from 50-200% of the mean). With increasing experience and standardization of tecniques, this variation was decreased. But as the manual techniques improved, the faults in the assay became apparent. The early standard curves showed a lack of repeatability. The cause of this imprecision

was investigated without ever really curing the problem. Times of incubation were varied from 12 to 60 hours but the results did not differ from the 24-36 hour incubation. The variability was found to be independent of order of addition of the reactants.

According to Ratcliffe (1974) charcoal in too high a concentration can strip the antiserum of the bound antigen. It was discovered that the same binding could be achieved with 50% of the charcoal, so this concentration was used.

The Amersham/Searle RIA kit was used for the most recent experiments. Unlike the earlier assays, the kit requires an adsorption of the sample to powdered glass and desorption with acetone. All duplicates were repeatable and the assay gave consistent results.

D. Studies on ACTH content and secretion by rat pituitary glands.

When animals were first handled they were quite nervous a response typified by their squirming and "barking". Since stress increases the release of ACTH, it was important to have the animals familiar with their handlers. By the third day of handling the rats were calmer and showed none of these nervous responses. On the first day of dietary treatments (control diets) the animals generally consumed all of their food. On the second day of dietary treatments, the group of animals was divided so that at least 2 animals were receiving the same diet. A time course study on the consumption of a 10% alanine versus a 10% leucine diet by the rats was performed,

(figure 5). At 4 hours after being fed, there was more leucine diet left in the bowls than alanine. By 8 hours this difference existed but was less marked. By 24 hours, the 2 diets had been equally consumed.

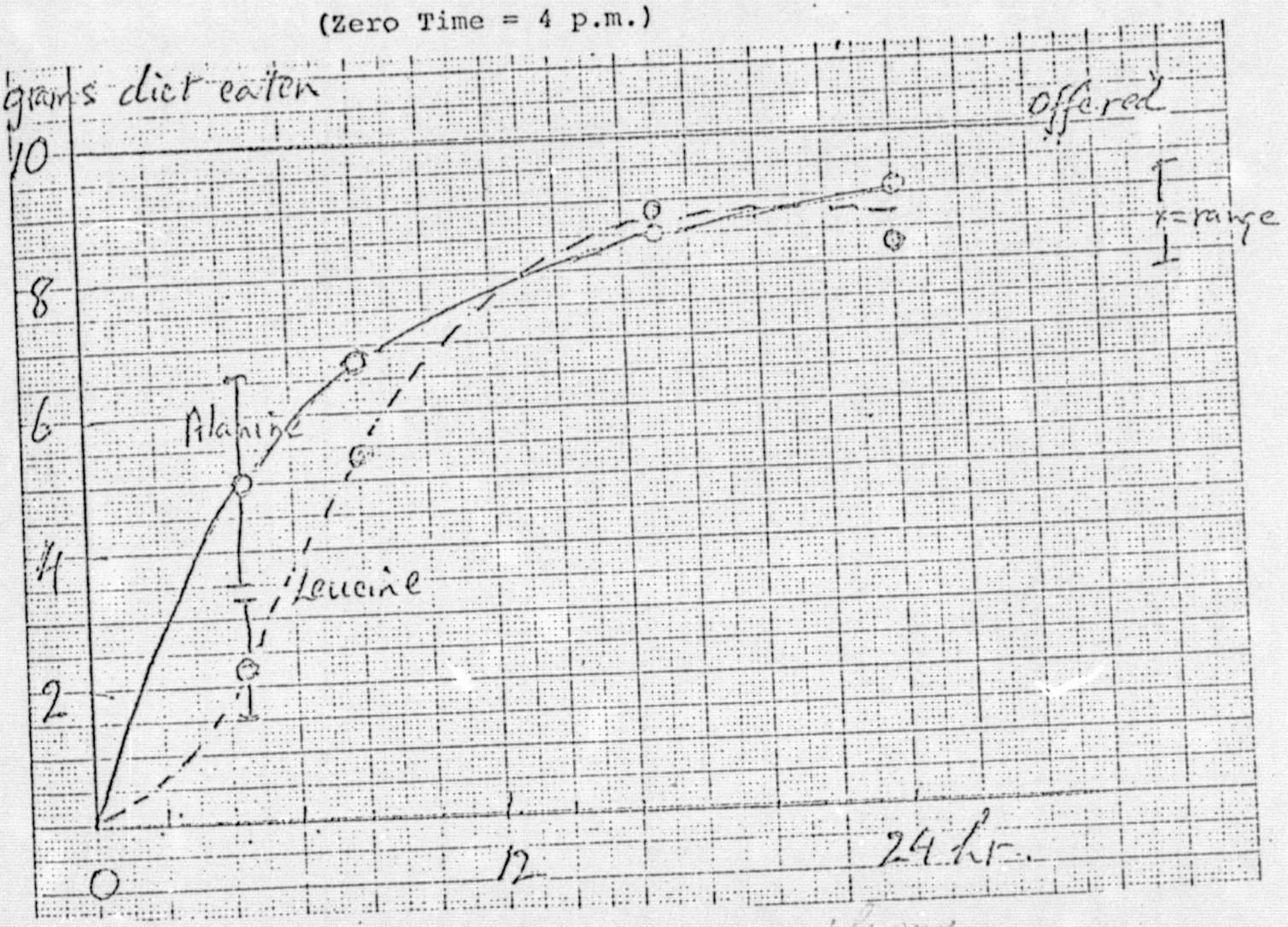
1. Perifusion experiments.

All rat pituitaries were placed on the perifusion apparatus by 1 hour after sacrifice. Perifusion samples were collected 30 minutes after pituitaries were placed on the apparatus. The results from 2 typical perifusions are shown in figures 6 and 7. In figure 6, line A held 2 alanine pituitary halves, and line B held 2 leucine pituitary halves. It can be seen that the leucine pituitaries responded much more readily to the hypothalamic extracts than the alanine pituitaries. The leucine pituitaries respond equally well to the treatments, except for the first chow hypothalamic extract, which seems not to have stimulated an increased ACTH release at all. In line A, the alanine pituitaries seem not to respond to the chow hypothalamic extract either. The pituitaries seemed to respond to the leucine and alanine hypothalamic extracts equally. In this experiment the leucine pituitaries secreted a higher basal level of ACTH. There appears to be a smaller CRF content in the chow hypothalami when compared to the leucine and alanine hypothalami.

In figure 7, both chambers A and B contain 3 leucine pituitary halves. In chamber A the chow and leucine hypothalamic extracts give the biggest increase in ACTH release, whereas the

Figure 5 FOOD INTAKES OF RATS ON 10 GM. MEAL CONTAINING 1 GM. LEUCINE OR 1 GM ALANINE

(Zero Time = 4 p.m.)



(see table 4 for plasma corticosterone levels in these animals).

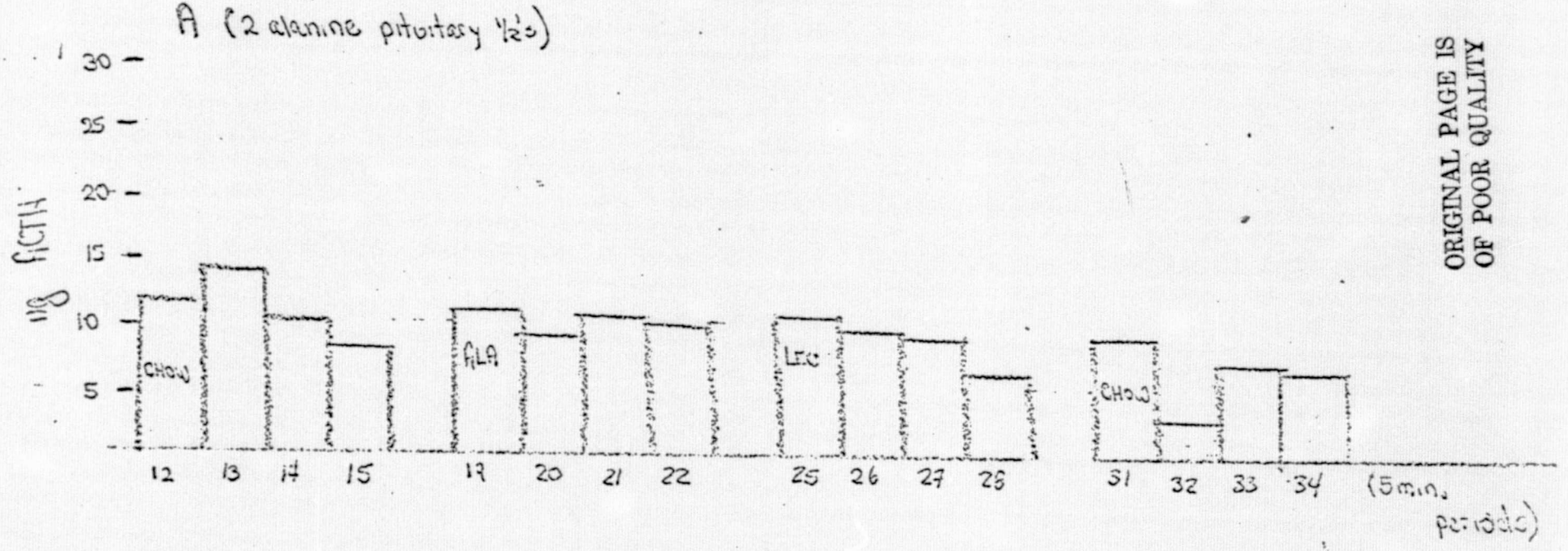
alanine hypothalamic extract gives only a small increase above the baseline. The final alanine stimulation was too small to give a value. In chamber B the chow, leucine and alanine hypothalamic extracts give equivalent increases in ACTH release. The small increase in release caused by the final leucine hypothalamic extract, parallels the small alanine stimulation in chamber A. This may be due to the exhaustion of the ACTH supply in the leucine pituitaries at the end of the perifusion.

At this time the perifusions have yielded mixed results. In some experiments leucine hypothalamic extract seems to stimulate more ACTH release than alanine hypothalamic extract (like figure 7, line A). In other experiments, the response appears to be the same, (as in figure 6). Results at this time support no general hyothesis, but the trend seems to show that leucine pituitaries seem to have a higher basal level of secretion when compared with alanine pituitaries.

2. Pituitary content of ACTH.

Pituitary extracts from both alanine and leucine treated rats are shown in table 2. Values seem to vary between assays but in the earlier assays, there is a higher concentration of ACTH in the leucine pituitaries when compared with the concentration in the alanine pituitaries. (But this difference fails to be signifigant at the 95% confidence level). In the most recent assays (done with the Amersham/Searle kit) the values seem to be very much lower for the ACTH content of the pituitaries. These pituitaries were frozen over 2 months

Figure. 6 The effects of CHOW (Chow treated Hypothalamic extract)
LEU (Leucine treated Hypothalamic extract)
ALA (Alanine treated Hypothalamic extract)
on perifused alanine and leucine pituitary halves



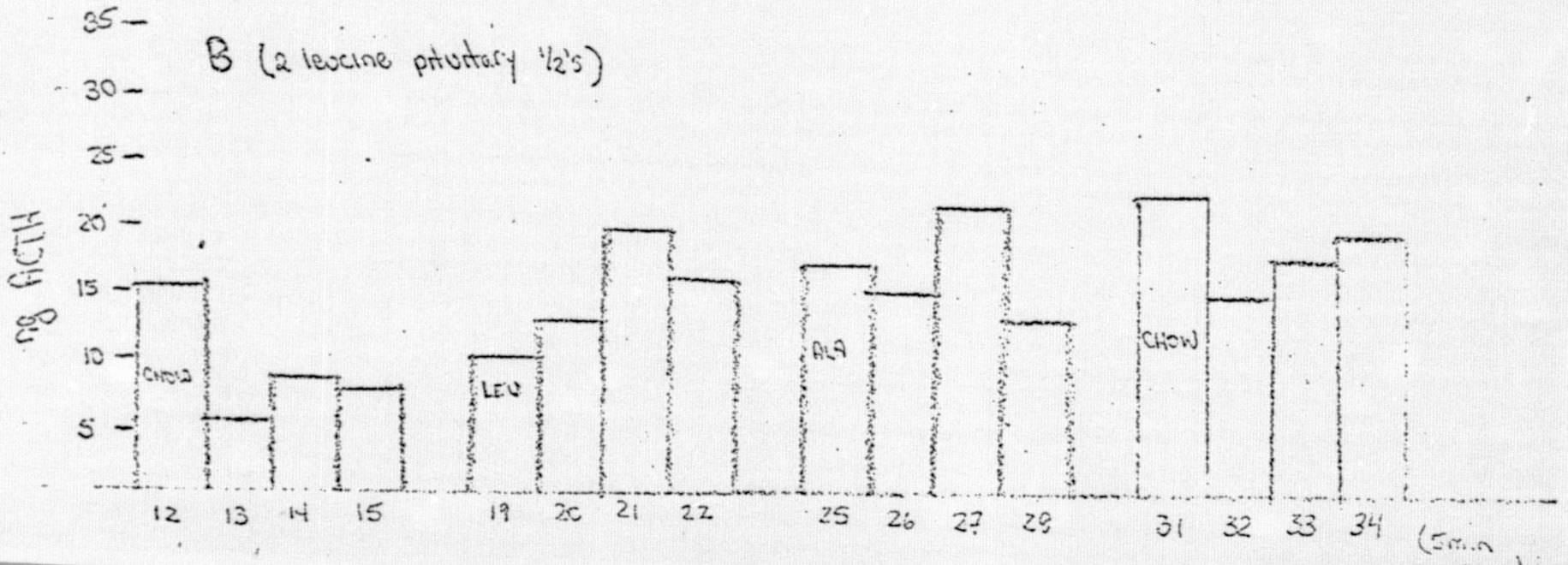
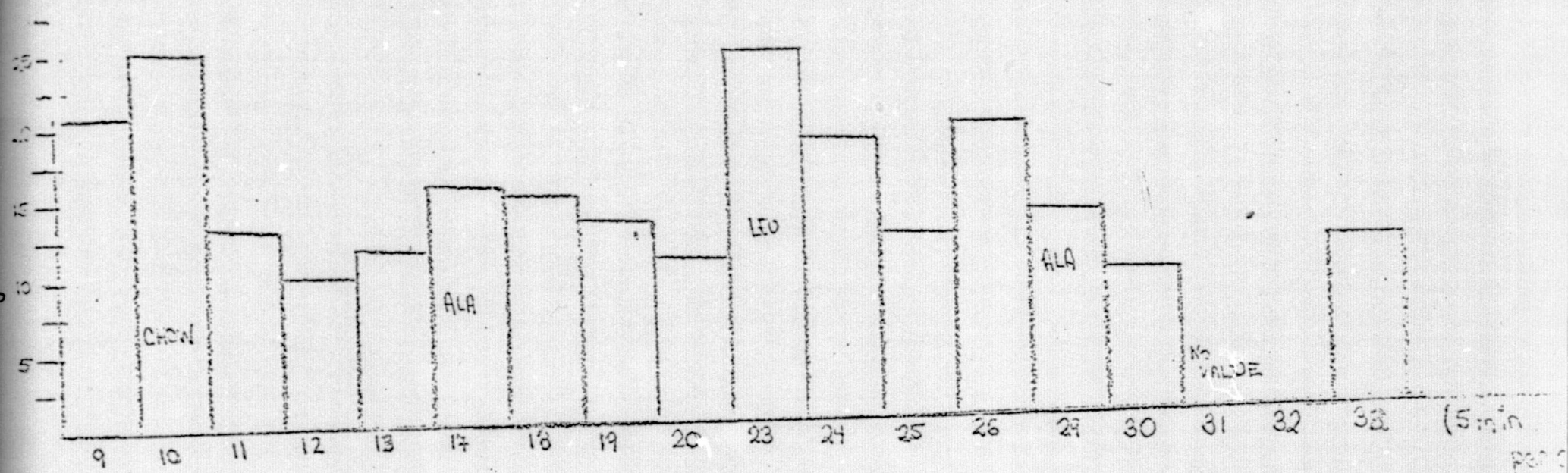


Figure. 7 The effects of CHOW (Chow treated Hypothalamic Extract)
LEU (Leucine treated Hypothalamic Extract)
ALA (Alanine treated Hypothalamic Extract)
on perifused leucine pituitary halves.

A (3 leucine pituitary 1/2's)



B (3 leucine pituitary 1/2's)

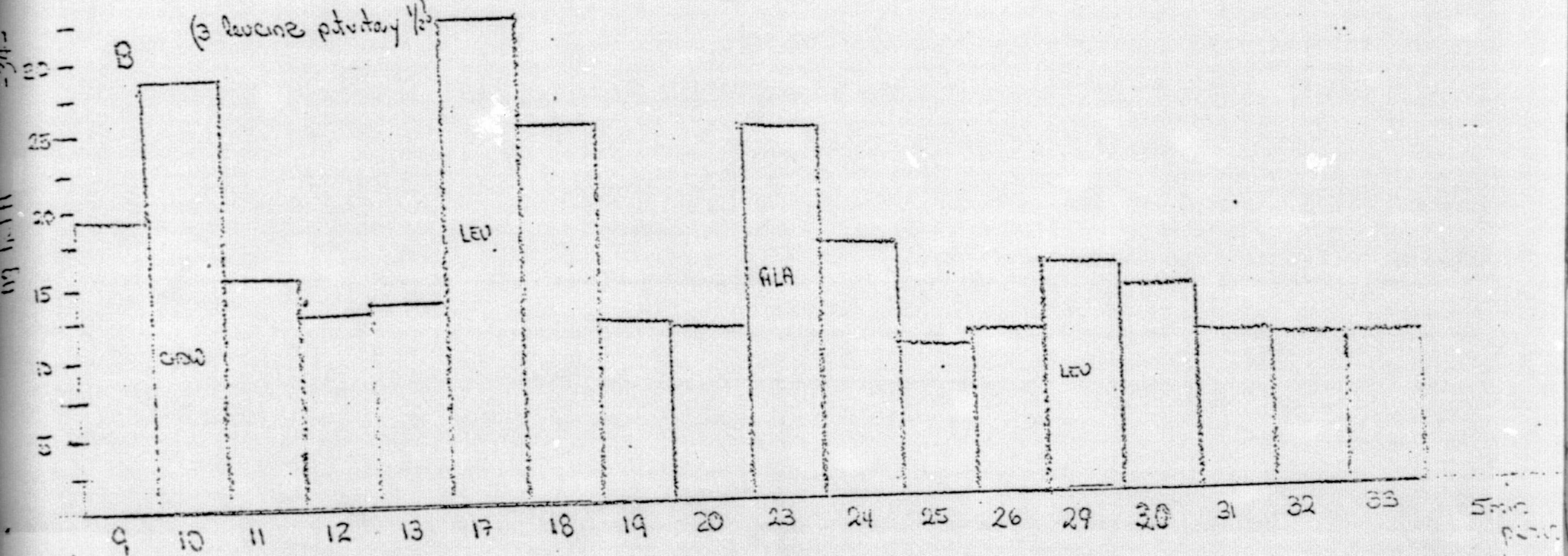


Table 2. Pituitary ACTH concentration / 100 gram body weight

Dietary treatment	Ng. pituitary ACTH / 100 g. body weight	Average ng. pituitary ACTH / 100 g. body wt.
Alanine	1520 1150 780 1010 550 1360 1040	1060 ± 320
Leucine	830 1310 1650 1000 1290 2370 2090 1340 1750	1520 ± 500
Alanine*	220 350	290 ± 90
Leucine*	240 280	260 ± 30

* measured with Amersham/Searle RIA kit.

Table 3. Literature value of pituitary ACTH conc./ 100 g. body wt.

Reference	Ng. pituitary ACTH / 100 g. body weight	Average ng. pituitary ACTH / 100 g. body wt.
Scott (1971)	435	
Scott (1974)	310	415 ± 100
Moriarty (1975)	500	

before assay (longer than any other pituitaries). Maybe the lower concentration of ACTH can be attributed to some loss in immunogenicity due to freezing and thawing.

In table 3 there is a comparison of values of ACTH content of pituitaries found by other investigators. The values found with this labs RIA are 2-3 times greater than those found in the literature. The values found using the RIA kit are lower than those found in the literature but they seem to be in closer agreement. The differences found between our assays values and the literature values might have many explanations. First we are working with different strains of rats, second the experimental conditions differ and third our standards may be lower than the value we assign them, therefore inflating the ACTH concetration in our unknowns.

3. Corticosterone levels.

In table 4, recent data on corticosterone levels of rat plasma are shown. It is apparent that the only time that there is a signifigant increase in corticosterone levels is 4.5 hours after the rats have been fed leucine. This result has important consequences for the rest of the experiment. Since the corticosterone level of the rat is controlled by the level of ACTH, when there is a change in corticosterone it most likely reflects a change in ACTH secretion (which in turn can reflect a change in CRF secretion). Since the only time that corticosterone levels are increased is 4.5 hours after it is

Table 4 Plasma Corticosterone Levels: (at time after meal)

	4.5 hr.	8 hr.	17 hr.	24 hr.
LEUCINE :	1. 63	7. 50	13. 6	18. 3
	2. 48	8. 6	14. 18	19A. 2
	3. 55	9. 12	15. 1	19B. 22
	4. 55	10. 33	16. 3	20A. 32
	5. 48	11. 28	17. 47	
	6. 55	12. 30		
Mean	54	27	15	15
ALANINE :	20B. 18	26. 10	32. 11	37. 23
	21. 7	27. 28	33. 29	38. 43
	22. 18	28. 32	34. 10	39. 9
	23. 39	29. 21	35. 10	40. 6
	24. 23	30. 36	36 ?	
	25. 51	31. 25		
Mean	26	25	15	20

Experimental study.
Blood taken from rats in
effect shown in Fig. 5. Data
given as µg Corticosterone/100 ml.

clear that this is the time to measure for changes in CRF and ACTH. Since animals were normally sacrificed 18 or more hours after feeding, and at this time there is no increase in corticosterone levels, increases in CRF and ACTH should not be expected.

5. Further studies of hypothalamic and pituitary responses to leucine.

With the cooperation of Dr. Edwardson, Dept. of Physiology, St. George's Hospital Medical School, London, hypothalami and pituitary glands from rats fed leucine or alanine were studied, using two antisera for ACTH, one of which recognizes the N-terminal portion of the peptide, the other the C-terminal portion. The latter can be removed from ACTH without loss of its potency for adrenocortical stimulation. There is some published evidence that the excised C-terminal portion (so-called CLIP) can cause release of insulin.

Rats were fed 0.25 gm leucine or 0.25 gm. alanine in 10 gm. of the non-protein diet and 24 hr. later their pituitaries were perifused. Fig. 8 shows that the leucine-fed animals released more ACTH into the perifusion medium over the first three 10 min. periods of perifusion, whereas the alanine-fed group remained at a low basal level. Second, a large amount of the C-terminal (CLIP) peptide was released from the pituitaries of those fed leucine, and this persisted throughout perifusion, but alanine feeding did not evoke this response. This implies that a reserve of ACTH and even more of CLIP exists in the pituitaries of leucine-fed rats.

This could be due to residual action of CRF in the pituitaries, and therefore a final experiment was performed to test for the CRF content of the hypothalamic of leucine-fed rats were fed 0.25 gm. leucine or alanine or casein in 10 gm. of non-protein diet. Twenty-four hours later, their hypothalami

Fig. 8.

Leu / Ala PERIFUSION DATA ~~1st SEPT.~~

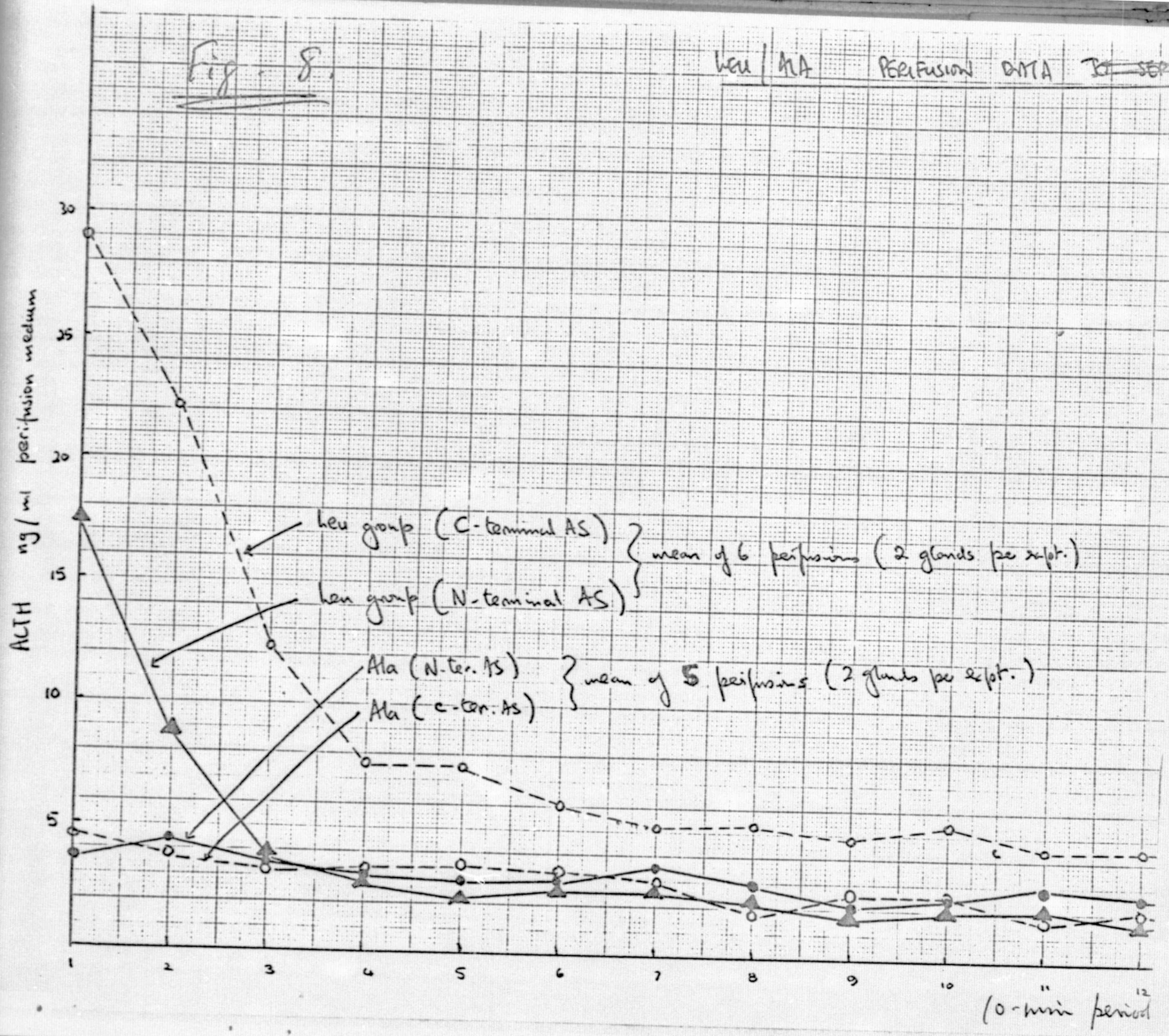


TABLE 5

Release of ACTH when Hypothalami are Incubated with the Pituitaries of Rats Fed on Stock Diets.

Expt. Number	ACTH release (ng./ml. medium)	
	Control animals*	Leucine-fed animals
I	20.8±4.3(Ala)	43.3±11.7
	24.2±6.1(Casein)	39.2± 8.4
II	33.5±4.1(Ala)	47.2± 9.4
	34.6±6.8(Casein)	43.8± 8.0

* Controls fed either alanine or casein, as indicated.

There were 5 studies in each group.

were removed and incubated with the pituitaries of rats fed on a stock diet. Table 5 shows two such experiments which agree in showing a greater release of ACTH from the pituitaries incubated in the presence of hypothalami from rats that received leucine. Note that casein feeding does not have this effect.

V. SUMMARY AND CONCLUSION

1. A study has been made of hypothalamic, pituitary and adrenal function in rats fed leucine or alanine up to 24 h before killing.

2. Procedures for ACTH measurements in isolated rat pituitary glands were developed. These were used for pituitaries alone or exposed to fluid from hypothalami (perifusion).

3. Perifusions using pituitaries from rats fed different diets tend to show that the basal rate that leucine pituitaries secrete ACTH is greater than the basal secretion of the alanine pituitaries. This is accompanied by subtained output of a fragment of ACTH called "CLIP", which is known to stimulate insulin output.

4. When hypothalamic extracts were used to stimulate ACTH secretion, there was increased secretion of ACTH when leucine was pre-fed.

5. At 4.5 hours after feeding, the plasma corticosterone levels from rats fed leucine showed a significant increase when compared with alanine fed rats. At later times measured there was little or no difference. Feedback inhibition exerted over

ACTH secretions by the corticosterone concentration could explain why there is no significant changes in ACTH and CRF at 18 hours after feeding.

6. It can be concluded that administered leucine increases the concentration of ACTH by acting on the pituitary, the hypothalamus or at some higher center at approximately 4 hours after feeding. The increased basal secretion of ACTH and the higher values of ACTH content found in the pituitaries of rats leucine treated 18 hours after leucine could be remnants of the earlier stimulation.

7. This work continues to open up the prospect of controlling stress by dietary means, through changes in capacity to secrete ACTH and corticosteroids.

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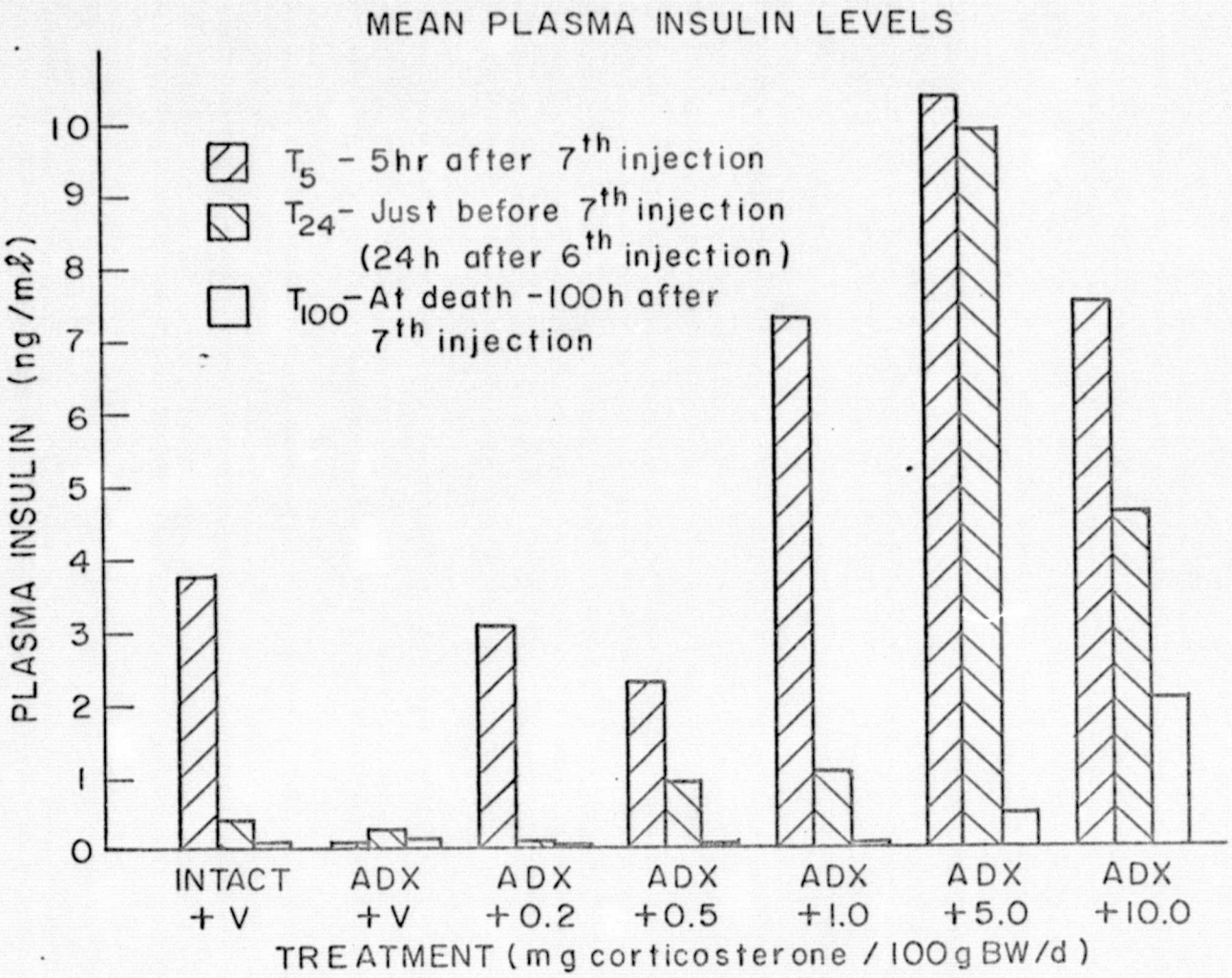
Appendix Two: Effect of Glucocorticoid Administration on the *In Vivo* Rate of Muscle Protein Breakdown in Rats, as Measured by Urinary Excretion of N^{τ}-Methylhistidine

SUMMARY

The role of glucocorticoids in regulating the rate of muscle protein breakdown was evaluated by measuring excretion of N^{τ}-methylhistidine (3-Mehis) during administration of various levels of corticosterone to adrenalectomized (ADX) rats. Groups of rats received daily subcutaneous injections of 0, 0.2, 0.5, 1.0, 5.0 or 10.0 mg corticosterone/100 g body weight/day for 7 days, followed by 3 days without hormone treatment, after which they were killed. A group with intact adrenal glands served as an additional control. All animals were pair-fed to the untreated ADX group. No significant differences were noted in growth rate or 3-Mehis excretion between the intact, ADX control, 0.2, 0.5 and 1.0 mg corticosterone groups, whereas growth ceased and 3-Mehis excretion rose markedly in the groups receiving 5 and 10 mg corticosterone. Following corticosterone doses up to 1 mg, the weights at death of the gastrocnemius, soleus and extensor digitorum longus muscles per 100 g final body weight were no less than those of the intact or ADX controls, and the weight of the liver was not affected. However, following treatment with 5 or 10 mg corticosterone daily, the weight of the gastrocnemius muscle per 100 g final body weight was about 25% less than that of the ADX controls; in contrast, the weights of the other two muscles examined, relative to final body weight,



did not differ from the controls. The livers of rats receiving 5 and 10 mg corticosterone weighed considerably more, both in absolute amounts and relative to final body weight, than those of controls. Plasma corticosterone levels, measured on the final day of injection and again at the time of killing, were reduced to near zero by adrenalectomy and were little raised by doses of 0.2 and 0.5 mg daily, but were increased to within the normal range by the 1 mg dose. At 5 and 10 mg doses, plasma corticosterone levels were sustained at 2-3 times those of intact rats, and thus in the range reported for rats exposed to severe stress. Rats given 5 and 10 mg doses of corticosterone had glycosuria, and showed considerably elevated levels of insulin in the plasma. It is concluded that plasma levels of glucocorticoids within the normal range do not regulate the rate of muscle protein breakdown, whereas excessive plasma levels of corticosteroids, equivalent to those seen in severe stress, can accelerate muscle protein breakdown.

The net catabolic effects of glucocorticoid hormones from endogenous or exogenous sources on skeletal muscle are widely recognized and well documented (1). Net loss of muscle protein occurs whenever breakdown rate exceeds synthesis rate. A depression in muscle protein synthesis rate following glucocorticoid administration has been established (1,2,3), but a specific increase in the breakdown rate of muscle proteins has not yet been unequivocally demonstrated, although it has been assumed to occur on the basis of increased proteolytic activity in the muscles of corticosteroid-treated animals (4,5). An increase in the *in vitro* breakdown rate of muscle protein following treatment of rats with high doses of glucocorticoids has been reported (6,7). More recently, however, Shoji and Pennington (8), using similar *in vitro* techniques, could not detect a change, a result which agrees with *in vivo* measurements by Millward *et al.* (9) of muscle protein breakdown in corticosteroid-treated animals. These various studies are difficult to interpret since they involve different muscles and differing types and levels of administered glucocorticoid. In addition, in some of these studies, the problem of reutilization of the labeled amino acid introduces methodological complications which cannot be readily minimized.

We have used the urinary excretion of N^{τ}-methylhistidine (3-methylhistidine; 3-Mehis), an amino acid present in

myofibrillar protein, as an *in vivo* index of muscle protein breakdown. We have previously shown that this amino acid is released upon breakdown of the myofibrillar proteins myosin and actin, and excreted quantitatively (10-13). This approach thus provides an integrated assessment of protein breakdown rates in the total skeletal musculature and is an elegant means of measuring changes in the rate of *in vivo* protein breakdown in muscle *per se* under the influence of various treatments. Here, we report the effects of corticosterone administration to rats on the rate of muscle protein breakdown, as determined by urinary 3-Mehis excretion; the results are correlated with plasma corticosterone levels. It is concluded that glucocorticoids do not influence the rate of muscle protein breakdown until plasma levels of the hormone rise to levels observed in states of severe stress.

EXPERIMENTAL PROCEDURE

Animals and Treatments: Adrenalectomized (ADX) and intact male Sprague-Dawley rats, about 120 g body weight (Charles River Breeding Laboratories, Wilmington, MA) were housed in individual metabolic cages. For one week after surgery, they were fed *ad libitum* a purified diet containing 18% lactalbumin (11), and the ADX rats were given 1% sodium chloride to drink. The ADX rats were then randomly assigned

to 6 treatment groups of 5 rats each. Treatments consisted of a daily subcutaneous injection of corticosterone (purchased from Calbiochem) for a 7-day period; each group received one of the following doses: 0.0, 0.2, 0.5, 1.0, 5.0 or 10.0 mg corticosterone/100 g body weight/day. A group of intact rats served as an additional control group. The steroid was injected in 0.5 ml vehicle, consisting of NaCl (0.8%), polysorbate 80 (0.4%), sodium carboxymethylcellulose (0.5%) and benzyl alcohol (0.9%), administered between 1100 and 1200 hrs each day, after the rats had been weighed. Both intact and ADX control groups received injections of the vehicle only. Intact controls and hormone-treated rats were pair-fed to the ADX control group; the daily ration was offered after completion of other experimental procedures, at about 1200 hrs.

Complete 24-hr urine collections were obtained from each rat, beginning 3 days before and ending 3 days after the 7-day hormone-treatment period; thymol was added as a preservative. On the final day of hormone treatment, blood samples were obtained from the tail of each rat immediately before (T_0) and 5 hr after (T_5) administration of the hormone. Three days later, upon completion of the urine collections, each rat was decapitated. Immediately, a blood sample was taken, the liver was excised and weighed, and the soleus, extensor digitorum longus and gastrocnemius muscles from the left rear leg were removed by careful dissection and individually weighed.

Determination of Urinary Constituents: Aliquots of the daily urine collections were pooled within each group for 3-Mehis and creatinine analysis. Concentrations of 3-Mehis were determined individually on a Beckman 121 amino acid analyzer, as previously described (13), after hydrolysis of the N-acetyl derivative with 2 N HCl in a boiling water bath for 1-2 hr, and subsequent desalting on a cation exchange column (Dowex AG50-X8), followed by stepwise elution with HCl of the acidic, neutral (2.0-2.5 N HCl) and basic (4.0-5.0 N HCl) amino acids. The acid eluate containing the basic amino acids was dried in a rotary evaporator and the sample was reconstituted with citrate buffer before application to the amino acid analyzer.

Urinary creatinine was determined by the method of Hare (14). Urinary glucose was monitored qualitatively with an enzymatic test strip ("Tes-Tape," Eli Lilly and Co., Indianapolis, IN).

Plasma Hormone Assays: Plasma corticosterone was determined by the direct radioimmunoassay method described by Gomez-Sanchez et al. (15). (The antibody was kindly provided by Dr. Gordon Williams, Peter Bent Brigham Hospital, Boston, MA. 1,2,6,7-^{3}H corticosterone [92 Ci/mmole] was obtained from New England Nuclear, Boston, MA.) Substantial blank values were obtained with this method, due to non-specific binding to the antibody

by materials which could not be entirely removed by charcoal treatment. A similar problem has been noted by others using this method (G. Williams, personal communication). Thus, it is likely that the values obtained for the ADX rats are in fact the true blank values.

Insulin assays on pooled plasma samples were kindly performed by radioimmunoassay by Dr. John Allsop, Shriner's Burns Institute, Boston, MA.

Statistical Procedures: Statistical evaluations were carried out by conventional one- and two-way analysis of variance. Least significant differences were calculated and are shown in Table 1.

RESULTS

Effect of corticosterone on body weight and organ weights: Figure 1 shows changes in body weight of the groups of rats during the experiments. The animals were given about 10 g diet daily, which was essentially fully consumed, even by the rats receiving the high corticosterone dosages. Since all rats were pair-fed to the ADX control group, the intact controls did not achieve the normal rate of weight gain for males of the Sprague-Dawley strain receiving an 18% lactalbumin diet ad libitum (about 6-7 g/day [11]).

During the 7-day period of hormone treatment, the intact controls and the ADX groups given 0.0, 0.2, 0.5 and 1 mg corticosterone/100 g body weight/day gained about 25 g; the rates of weight gain did not differ among these groups (Table 1 and Fig. 1). Rats receiving 5 and 10 mg corticosterone/100 g body weight/day either maintained weight or lost 5-10 g, with the major weight loss occurring on the first day or two of treatment. Although a similar initial weight loss was observed in rats of the group receiving 1 mg corticosterone, this group resumed a normal growth pattern from the second day of steroid treatment, as shown in figure 1A (insert), which indicates the percentage change in weight from the first day of hormone administration.

The weights of the livers and of the three skeletal muscles removed at the end of the experiment are shown in Table 2. Livers of animals receiving 5 and 10 mg corticosterone showed a considerable ($P < 0.01$) increase in both absolute weight and weight/100 g final body weight, in agreement with liver hypertrophy known to be induced by corticosteroid treatment (16). The three muscles, representing predominantly red (soleus), white (extensor digitorum longus; EDL) and mixed (gastrocnemius) fibers, showed differing responses. Relative to final body weight, the gastrocnemius weighed significantly ($P < 0.01$) less in rats given the two highest steroid levels, in comparison with the ADX controls (Table 2).

However, the soleus and EDL muscles did not differ from those in the ADX controls when the weights of these muscles were expressed per 100 g final body weight (Table 2).

Excretion of N^{τ}-methylhistidine and creatinine: Figure 2 shows the daily output of 3-Mehis/100 g body weight for all groups of rats. Intact and ADX controls and rats given 0.2, 0.5 and 1 mg corticosterone/day showed similar output patterns during the collection period. The initial output of about 1.3 µmoles/100 g body weight is similar to that observed by us previously (11) for 100 g rats, and, as the rats gained weight, the progressive reduction of about 25% in 3-Mehis output/100 g (Fig. 2) also agrees with our earlier observations on growing rats (11). Output of 3-Mehis per unit body weight or per unit creatinine excretion did not differ significantly between intact rats and ADX rats receiving up to 1 mg corticosterone. In contrast, administration of 5 mg corticosterone/100 g body weight/day rapidly increased daily 3-Mehis output over 3 days to 2.3 µmoles/100 g body weight, followed by a reduction to about 1.3 µmoles, while administration of 10 mg steroid caused a persistent elevation at about 1.9 µmoles 3-Mehis/100 g body weight. Output of 3-Mehis by both of these groups declined to levels below those of the other groups after steroid treatment was stopped (Fig. 2).

In order to provide an index of muscle mass, and to reduce variability from daily urine excretion, creatinine output was measured. Figure 3 shows the ratio of 3-Mehis output to creatinine excretion, expressed as a percentage change from the mean ratio of each group during the 3-day period before hormone treatment. The ratio increased sharply throughout treatment for animals receiving 10 mg, and during the first half of treatment for those given 5 mg corticosterone; these elevated ratios persisted until after treatment was stopped. At the 1 mg dose level, there was a tendency for output of 3-Mehis/mg creatinine to increase on the first 3 days of dosage, whereas groups on lower doses showed no change in ratio as a result of treatment (Fig. 3).

Plasma Hormone Levels: Figure 4 shows corticosterone levels in plasma drawn from the tail just before and 5 hr after the last injection of corticosterone, and at the time of killing 3 days later. A certain amount of non-specific displacement of antibody-bound radioactivity by compounds in the plasma other than corticosterone was demonstrated following removal of authentic corticosterone by charcoal treatment. The data have therefore been interpreted in relation to this blank value. The values obtained for intact controls fall within the normal range reported by others (15,17). The untreated ADX control group still showed small amounts of

apparent corticosterone binding compared to charcoa-treated blank values (Fig. 4); this also is probably non-specific, and the values obtained for this group presumably represent the true blank values for the assay. Rats receiving 0.2 and 0.5 mg corticosterone showed no apparent increase in plasma levels of the steroid, whereas rats injected with 1, 5 or 10 mg corticosterone had increasingly elevated plasma levels 5 hr after treatment, which, in the case of the two higher dose levels, persisted into the post-injection period, in comparison with levels observed for the untreated ADX animals (Fig. 4).

Insulin content was measured on pooled plasma samples for each group. The levels within all groups except for the ADX controls were highest at 5 hr after corticosterone administration (Fig. 5). Corticosterone doses above 0.5 mg raised plasma insulin to levels exceeding those of control rats. At the 5 and 10 mg doses, insulin levels were still elevated 24 hr after corticosterone injection, and these groups also showed gross glycosuria.

DISCUSSION

As judged by changes in growth rate and urinary excretion of 3-Mehis, neither adrenalectomy nor replacement treatment with 0.2 or 0.5 mg corticosterone/100 g body weight/day had any effect when these groups were pair-fed to the ADX group

receiving the vehicle only. These two dose levels of corticosterone are similar to those considered by Steele (18), on the basis of various criteria, to be optimal for replacement therapy of ADX rats. Our data confirm this by showing that the plasma corticosterone level 5 hr after administration of 0.5 mg corticosterone was within the normal range in intact rats (Fig. 4). In contrast, growth ceased and 3-Mehis output rose sharply when rats received 5 or 10 mg corticosterone/100 g body weight/day. These doses caused persistent elevations of plasma corticosterone above the levels observed in intact controls, indicating that corticosterone increases the breakdown rate of myofibrillar protein only when excessive levels are present in the plasma. A critical dose level for catabolism is confirmed by the loss of weight of the gastrocnemius muscle only at the two higher dose levels (Table 2). Our data do not allow us to be precise about the minimum effective dose required to induce the catabolic response. At the 1 mg dose level of the steroid, non-significant minor effects on growth rate and 3-Mehis output were observed. These findings emphasize the need to establish the critical catabolic dose of corticosterone, preferably under conditions of administration reflecting the pattern of normal adrenal secretory activity.

The literature is contradictory regarding the action of corticosteroids on muscle protein breakdown. Millward *et al.* (9) treated rats for two days with triamcinolone acetonide

(0.5 mg/100 g body weight) and used the technique of continuous infusion of [^{14}C]-tyrosine to measure protein synthesis in individual muscles, from which they computed breakdown as the difference between the estimated rate of synthesis and the net change in muscle protein content. Although they found that synthesis was inhibited, they did not observe a significant increase in protein breakdown rate in rat gastrocnemius and quadriceps muscles. Shoji and Pennington (8) reported that *in vitro* release of tyrosine (taken to be an index of protein breakdown) from the extensor digitorum longus muscle of rats was not elevated following pretreatment with 10 mg cortisone acetate/100 g body weight for 3 days. On the other hand, Goldberg (6) injected [^{14}C]-leucine into rats and measured its *in vivo* release from plantaris and soleus muscles following administration of 10 mg cortisone acetate/100 g body weight/day for 7-10 days, and observed an increased rate of muscle protein breakdown in plantaris but not in soleus. Karl *et al.* (19) administered 20 mg cortisone acetate on 3 successive days to 120 g rats; 24 hr after the last dose, *in vitro* release of alanine by the epitrochlaris muscle was enhanced, implying a net protein loss from this muscle as a result of steroid treatment.

These studies, performed on single muscles, are contradictory and difficult to resolve due to the differences in the type of steroid administered, the level and period of dosage, the

age and sex of the rats, and the hormone responsiveness of the individual muscles. Our data (Table 2) and those of others (6, 20, 21) confirm that changes due to corticosteroid administration differ among muscle types. Measurement of urinary 3-Mehis excretion, as reported here, offers the advantage of an integrated estimate of average protein breakdown for the entire skeletal musculature at various dose levels of steroid.

The persistance of the catabolic response to corticosterone is also reflected in our studies. Since plasma corticosterone levels were assayed after 7 days of injections, it is possible that, due to adaptive enhancement of corticosterone removal, the levels induced by the two highest doses declined, after having been more elevated at the start of treatment. Nevertheless, Figure 4 indicates that the elevated levels persisted 3 days after the last injection of the hormone. Alternatively, the target organs may have become to some degree refractory to the hormone, as indicated by the decline in 3-Mehis output after 3-4 days of dosage with 5 mg/100 g body weight/day. Goldman and Frohman (22) noted that the body protein content of rats declined proportionately to body weight only after the first day of glucocorticoid treatment, and remained stable thereafter. Data reported by other workers also indicate an adaptive decline in such parameters as N excretion and plasma glucose and insulin levels in response to continued hormone therapy (18).

The dose levels of corticosterone that caused increased output of 3-Mehis in our studies also produced liver enlargement (Table 2). The latter response to a high dose of glucocorticoid agrees with other data in the literature (16) and is in accord with observations that steroid treatment can enhance the synthesis of RNA and protein by perfused liver (23). In intact rats, the critical dose level needed to produce this hypertrophy appears to be similar to that for skeletal muscle catabolism (Table 1). Although Loeb (24) found a reduction in DNA content of rat liver following low doses of hydrocortisone, we saw no evidence of changes in liver weight or body weight when our doses were less than the high levels we used to produce a catabolic response.

The low levels of insulin in the untreated ADX control rats agree with the finding by Sutter (25) of depressed pancreatic insulin secretion and lack of responsiveness to elevated plasma glucose levels in ADX rats. This attests to the completeness of adrenalectomy in our rats. With higher corticosterone doses, the concomitant elevation of insulin levels (Fig. 4) is consistent with the findings of others (18). The reason for the relatively low plasma insulin levels in the group receiving 10 mg, compared to that receiving 5 mg, corticosterone/100 g body weight/day is unclear. Both groups showed extensive glycosuria, indicative of corticosterone-related insulin resistance and consequent diabetes. Thus, our

data are in accord with the view that corticosteroids inhibit entry of glucose into muscle, with resulting elevation in plasma glucose levels and increased secretion of insulin caused by hyperglycemia. In addition, it is well known that corticosteroids increase gluconeogenesis, which would accentuate the hyperglycemia.

Our data do not permit any conclusions regarding the mechanism of the observed increase in the rate of muscle protein breakdown. Glucocorticoid treatment does not appear to activate or release muscle lysosomal hydrolases (26) but does lead to increased activity of several non-lysosomal proteases (4,5). Mayer et al. (4) have reported a two-fold increase in a myofibrillar protease following glucocorticoid treatment, consistent with our finding of increased myofibrillar degradation, as indicated by 3-Mehis excretion, following administration of corticosterone to rats in amounts sufficient to maintain elevated plasma corticoid levels.

Finally, it should be noted that the level of plasma corticosteroids is elevated by a number of pathological stress conditions. For example, rats with extensive burns can maintain corticosterone levels higher (50-55 µg/dl) than those noted by us in rats receiving 5 mg corticosterone/100 g body weight/day for several days (Allsop, unpublished results). Similar elevations in corticosterone levels in response to physical stress have been reported by others (27,28). Physiological

stress superimposed on physical stress can elevate plasma corticosterone levels in rats to 90 μg/dl for several days (17). On the basis of these data, our results suggest that an increased rate of muscle protein breakdown due to sustained increased steroid secretion is likely to be a feature of the response to severe stress, such as in burns with infection. Corticosterone dose not have a regulatory effect on myofibrillar protein breakdown until levels well above the normal range are present in the plasma.

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FOOTNOTES

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2. This study was conducted during Dr. Frank M. Tomas's leave of absence from the Commonwealth Scientific and Industrial Research Organsization, Division of Human Nutrition, Adelaide 5000, South Australia.

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TABLE 1

Summary of statistical analyses of body weight gain, N^{τ}-methylhistidine and creatinine excretion, liver and muscle weights and plasma corticosterone levels in intact and adrenalectomized rats receiving various doses of corticosterone.

Table or Figure		Significance (P) of Differences with Respect to: Hormone Treatment	Time	LSD*
Fig. 1	Body Weight Gain (g/day) during treatment	<0.01		1.56 g
Table 2	Organ Weights			
	Liver (g)	<0.01		0.86 g
	(g/100 g body wt)	<0.01		0.58 g
	Soleus (mg)	<0.05		17.2 mg
	(mg/100 g body wt)	NS**		
	Gastrocnemius (mg)	<0.01		216.0 mg
	(mg/100 g body wt)	<0.01		59.1 mg
	Extensor Digitorum Longus (mg)	<0.01		14.5 mg
	(mg/100 g body wt)	<0.05		7.75 mg
	Urinary Excretion during treatment			
	N^{τ}-methylhistidine			
	(µmoles/day)	<0.01	NS	0.37
Fig. 2	(µmoles/100 g body wt)	<0.01	>0.05<0.1	0.26
Fig. 3	(µmoles/g creatinine)	<0.01	0.01	320
	Creatinine (mg/day)	<0.01	0.01	0.55
	(mg/100 g body wt)	<0.01	0.025	0.39
Fig. 4	Plasma Corticosterone			
	(µg/dl) at 0 hr	<0.01		190.
	5 hr	<0.01		12.4
	100 hr	<0.01		9.0

*Least significant difference between group means for $P < 0.01$.

**Not significant

TABLE 2

Liver and muscle weights of intact and adrenalectomized (ADX) rats killed 3 days after the final day of treatment with graded doses of corticosterone for 7 days.[1]

Treatment (mg corticosterone/100 g body wt/day)	Liver		Gastrocnemius		Soleus		Extensor Digitorum Longus	
	g/100g body wt	% of ADX control	mg/100g body wt	% of ADX control	mg/100g body wt	% of ADX control	mg/100g body wt	% of ADX control
Intact + 0	3.50 ± 0.12	98	585 ± 20	105	37.4 ± 1.6	91	49.6 ± 2.6	107
ADX + 0	3.57 ± 0.10	100	559 ± 16	100	40.9 ± 1.5	100	46.2 ± 1.6	100
ADX + 0.2	2.89 ± 0.11	81	585 ± 15	105	37.4 ± 2.3	91	53.0 ± 2.2	115
ADX + 0.5	3.20 ± 0.06	90	580 ± 8	104	33.4 ± 4.0	82	55.4 ± 2.4	120
ADX + 1.0	3.28 ± 0.19	92	553 ± 6	99	38.4 ± 2.4	94	49.8 ± 1.5	108
ADX + 5.0	5.07 ± 0.27	142	442 ± 25	79	38.3 ± 1.3	94	49.0 ± 2.2	106
ADX + 10.0	5.96 ± 0.23	167	400 ± 11	72	38.8 ± 1.2	95	45.2 ± 0.4	98

[1]Values are means ± SEM for 4 or 5 rats.

FIGURE LEGENDS

Fig. 1: Daily body weight changes of rats before, during and after corticosterone treatment. Values are means of 5 rats in each of the following treatment groups: Intact + vehicle (x-x), adrenalectomized (ADX) + vehicle (0-0), ADX + 0.2 (mg corticosterone/100 g body weight/day) (●-●), ADX + 0.5 (▲-▲), ADX + 1.0 (▲-▲), ADX + 5.0 (■-■), and ADX + 10.0 (■-■). Injections were given subcutaneously for 7 days. All rats were pair-fed to the average intake of ADX controls.

Fig. 1A (inset): Daily body weight changes from commencement of corticosterone injections, expressed as a percentage change from initial weight. Legend is as given for Fig. 1.

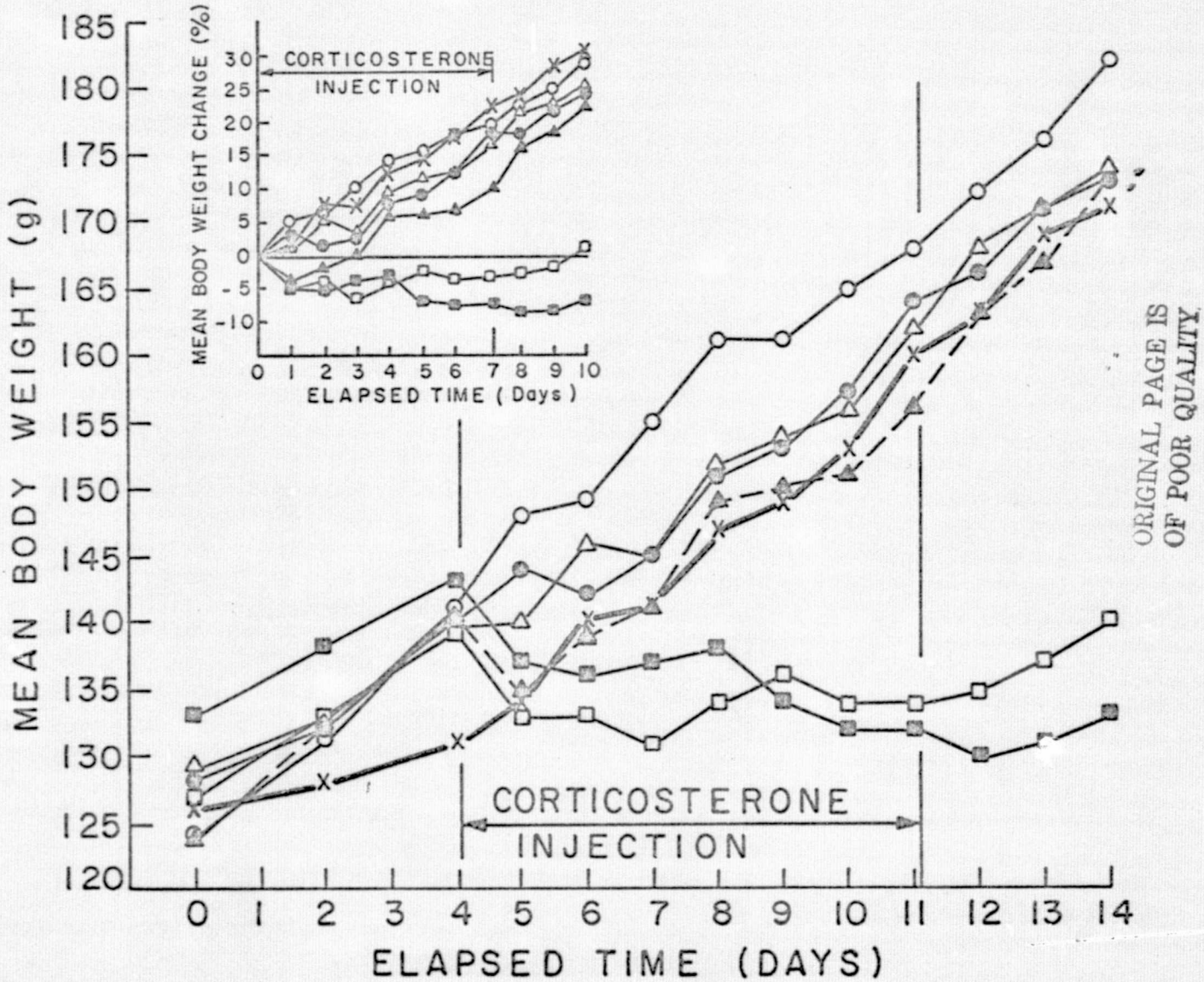
Fig. 2: Daily output of N^{τ}-methylhistidine/100 g body weight before, during and after corticosterone treatment. Each point is a pooled sample from 5 rats. Legend is as given for Fig. 1.

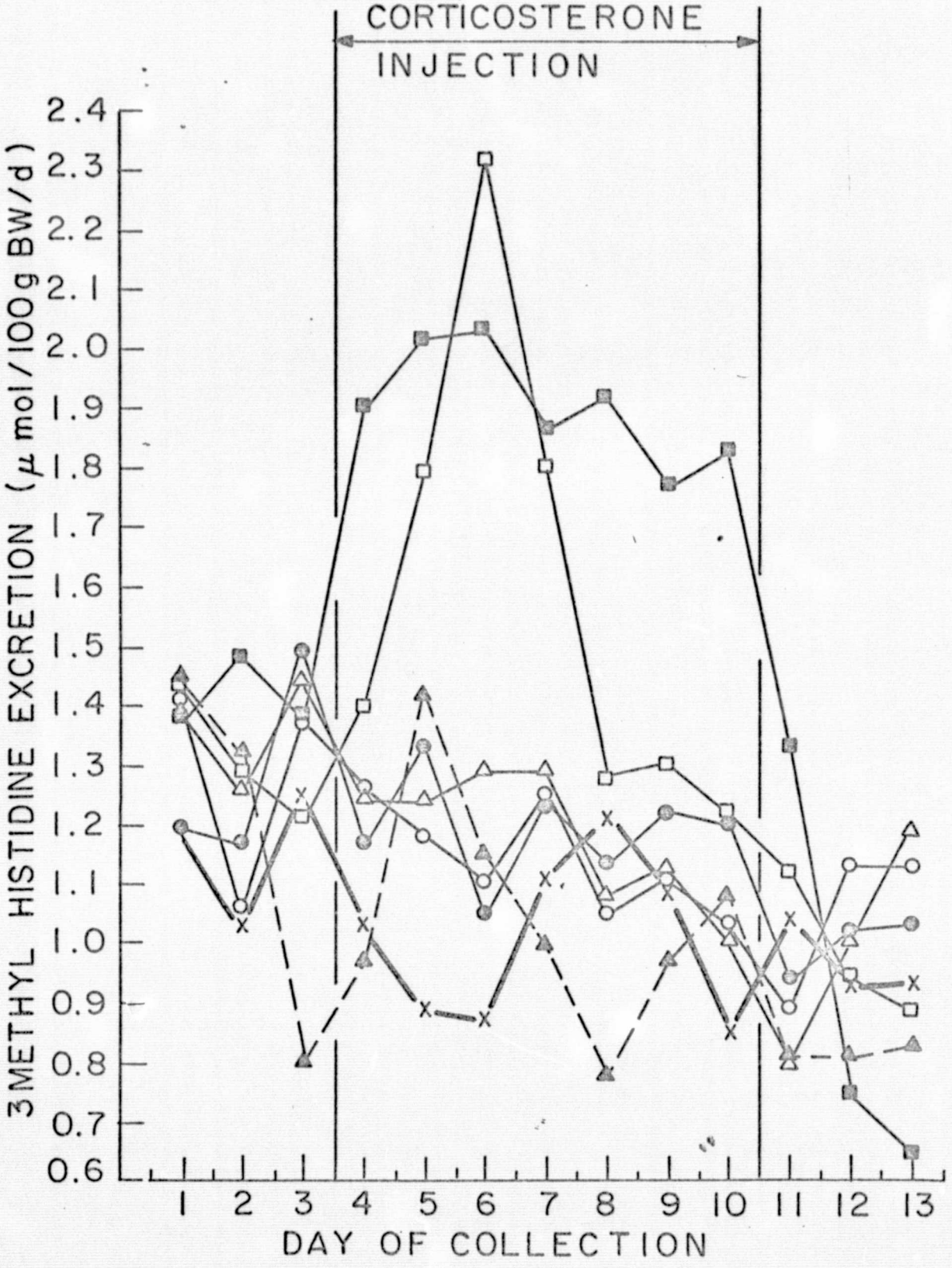
Fig. 3: Daily N^{τ}-methylhistidine:creatinine ratios for each treatment group from commencement of corticosterone treatment, expressed as the percentage change from the mean ratio of the preceding 3 days. Each point is a pooled sample from 5 rats. Legend is as given for Fig. 1.

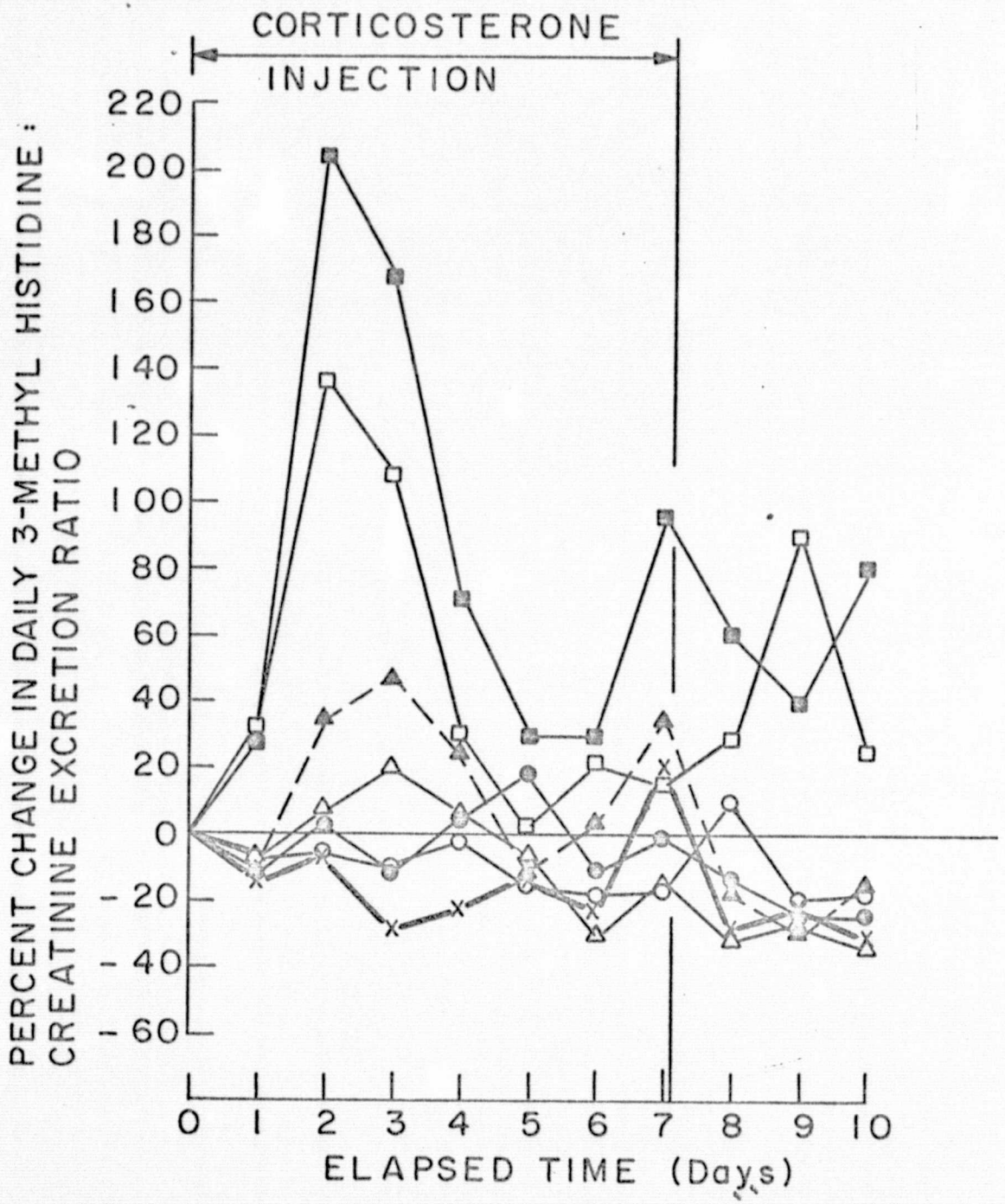
Fig. 4: Mean levels of corticosterone in plasma of rats sampled from the tail just before the 7th injection (24 hr after the 6th injection) (T_0, ▨), 5 hr after the 7th

daily injection (T_5, ▨), and at time of killing, about 100 hr after the 7th injection (T_{100}, ▥). Values are means and SEM for 4 or 5 animals. Charcoal-treated plasma (▥) is mean and SEM of 12 samples with range of 11.5 to 71.0 µg/dl before charcoal treatment.

Fig. 5: Levels of insulin in plasma samples of rats taken 0, 5, and 100 hr after corticosterone injection. Each value is for a pooled sample from 4 or 5 rats. Legend is as given for Fig. 4.




CORTICOSTERONE
INJECTION
3METHYL HISTIDINE EXCRETION (μ mol/100g BW/d)
2.4
2.3
2.2
2.1
2.0
1.9
1.8
1.7
1.6
1.5
1.4
1.3
1.2
1.1
1.0
0.9
0.8
0.7
0.6
1 2 3 4 5 6 7 8 9 10 11 12 13
DAY OF COLLECTION


CORTICOSTERONE
INJECTION
PERCENT CHANGE IN DAILY 3-METHYL HISTIDINE: CREATININE EXCRETION RATIO
220
200
180
160
140
120
100
80
60
40
20
0
- 20
- 40
- 60
0 1 2 3 4 5 6 7 8 9 10
ELAPSED TIME (Days)